TITLE OF THE THESIS

# MicroRNA-9 Regulation of RUNX1/TGFβ Axis during Megakaryocyte Maturation: A study on Development Perspective

**Thesis submitted for the degree of**
**DOCTOR OF PHILOSOPHY (Ph.D.)**
**By**


**SANJEEV RAGHUWANSHI**
**(Regd. No: 14LBPH03)**

**Supervisor**
**Prof. Ravi Kumar Gutti**

**Department of Biochemistry**
**School of Life Sciences**
**University of Hyderabad**
**Hyderabad-500 046, INDIA**


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**University of Hyderabad**

**(A Central University established in 1974 by act of parliament)**

**Department of Biochemistry**

**School of Life Sciences**

**Hyderabad– 500 046,**

**INDIA**

## DECLARATION

**I, Sanjeev Raghuwanshi, hereby declare that this thesis entitled "MicroRNA-9 Regulation of RUNX1/TGFβ Axis during Megakaryocyte Maturation: A study on Development Perspective" submitted by me under the guidance and supervision of Prof. Ravi Kumar Gutti is a bonafide work.**

**I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.**

**Sanjeev Raghuwanshi**

**Regd. No: 14LBPH03**

**Department of Biochemistry**

**School of Life Sciences**

**University of Hyderabad**

**Hyderabad 500 046, INDIA.**

## CERTIFICATE

**This is to certify that the thesis entitled "MicroRNA-9 Regulation of RUNX1/TGFβ Axis during Megakaryocyte Maturation: A study on Development Perspective" submitted by Mr. Sanjeev Raghuwanshi bearing Reg. No. 14LBPH03 in partial fulfilment of the requirements for the award of Doctor of Philosophy in Biochemistry is a bonafide work carried out by him under my supervision and guidance.**

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**Prof. Ravi Kumar Gutti**
**Supervisor**

**Prof. Krishnaveni Mishra**
**Head**
**Department of Biochemistry**

**Prof. Dayananda Siddavattam**
**Dean**
**School of Life Science**

## CERTIFICATE

**(For Ph.D. Dissertations)**

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**Further, the student has the following publications(s) before submission of the thesis/monograph for adjudication and has produced evidence for the same in the form of reprint in the relevant area of his research.**

1. **Raghuwanshi S, Gutti U, Kandi R, Gutti RK. MicroRNA-9 promotes cell proliferation by regulating RUNX1 expression in human megakaryocyte development. Cell Prolif. 2018;51(1):e12414.**
2. **Raghuwanshi S, Sharma DS, Kandi R, et al. Pituitary adenylate cyclase-activating polypeptide (PACAP): Differential effects on neonatal vs adult megakaryocytopoiesis. Thromb Res. 2019;175:59-60.**
3. **Raghuwanshi S, Dahariya S, Sharma DS, Kovuru N, Sahu I, Gutti RK. RUNX1 and TGF-β signaling cross talk regulates Ca2+ ion channels expression and activity during megakaryocyte development [published online ahead of**

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4. **Stem Cell Society Singapore (SCSS) Symposium 2017 "Advances in Cell Therapy", Academia, SGSC, Singapore. PO-37. (International)**
5. **3rd RNA Biology Symposium-2017, NUS, Singapore. PO-60. (International)**
6. **10th Pan Pacific Symposium on Stem Cells and Cancer Research-2017 (PPSSC-2017), Hualien, Taiwan. PO-05. (International)**
7. **International Conference on Frontiers in Comparative Endocrinology and Neurobiology-2014 (IC-FCEN 2014), Hyderabad, India. PO-29. (International)**

**Further, the student has passed the following courses towards fulfillment of coursework requirement** for Ph.D.

| Course Code | Name | Credits | Pass/Fail |
|---|---|---|---|
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| BC802 | Research ethics, Data Analysis and Biostatistics | 3 | Passed |
| BC803 | Lab Seminar and Record | 5 | Passed |

**Supervisor** **Head of Department** **Dean of School**

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***GOD is my ultimate strength and power who bring best out of me***

*A PhD is not just a degree – it is an opportunity to develop the skills needed to deliver impact. For me, it was not just learning how to deliver quality research. I also know how to communicate clearly and how to take my research out of the lab and use it to positively impact the world. For me PhD was the ideal time for developing my research career, I feel I have grown to be more than just the title of my thesis. The lessons learnt no matter how big or small, will definitely continue to remain etched in my memory and provide me vital support in my scientific career ahead.*

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## LIST OF ABBREVIATIONS

**A**

Argonaut (Ago)

**B**

Bone Marrow (BM)

Burst-forming unit MKs (BFU-MKs)

**C**

Common myeloid progenitor (CMPs)

Common lymphoid progenitor (CLPs)

Congenital-amegakaryocytic thrombocytopenia (CAMT)

Colony-forming unit (CFU) MKs (CFU-MKs)

Calcium (Ca2+)

Cord blood (CB)

Collagen type1 (COL1)

**D**

Dichlorodihydrofluorescein diacetate (H2DCFDA)

**E**

Endoplasmic reticulum (ER)

Extracellular matrix (ECM)

**F**

Familial platelet disorder with propensity to acute myelogenous leukemia (FPD/AML)

Fetal bovine serum (FBS)

**G**

Glycophorin A (GYPA)

Glycophorin B (GYPB)

Granulocyte-Monocyte Progenitors (GMPs)

Granulocyte colony-stimulating factor (G-CSF)

GPIbα (CD42b)

**H**

Hematopoietic Stem Cells (HSCs)

Hematopoietic stem/progenitor cells (HSPCs)

**I**

Intra-ventricular hemorrhage (IVH)

Integrin aIIb (CD41)

Integrin b3 (platelet GP IIIa, CD61)

Intracellular Ca2+ [Ca2+]i

Intracellular free Ca2+ [Ca2+]i

**L**

Lymphoid-primed MMPs (LMPPs)

Long-term HSCs (LT-HSC)

**M**

Mitochondrial uncoupling protein 2 (UCP2)

MK/Platelet Glycoprotein (GP) IIb (CD41)

Megakaryocytes (MKs

Multipotent progenitors (MPPs)

Megakaryocyte-erythroid progenitors (MEPs),

MK progenitor (MKP)

MicroRNA-9 (miR-9)

Myeloid-derived suppressor cells (MDSCs)

**N**

Neonatal intensive care unit (NICU)

N-acetyl-L-cysteine (NAC)

Natural killer cells (NK)

Neuronal progenitor cells (NPCs)

**P**

Peripheral blood (PB)

Phorbol 12-myristate 13-acetate (PMA)

Phospho-SMAD2/3 (pSMAD2/3)

**R**

RISC (RNA inducing silencing complex)

Runt homology domain (RHD)

Reactive oxygen species (ROS)

Real-time quantitative-PCR (qRT-PCR)

**S**

Stem-like MKPs (SL-MKPs)

Stromal cell-derived factor 1 (SDF1)

Small non-coding RNA (microRNA/miRNA),

**T**

Trans-membrane potential ($\Delta\Psi m$)

Transient receptor potential (TRP)

Transforming growth factor beta (TGF-β)

TGF-β receptor type-II (TGF-βRII)

TGF-β receptor type-I (TGF-βRI)

Thrombopoietin (TPO)

TPO receptor (cMpl)

Transcription factors (TFs)

**U**

Untranslated regions (UTRs)

**v**

von Willebrand factor (vWF)

## 1.1. Hematopoiesis during human embryonic development

Hematopoietic stem cells (HSCs) within the human bone marrow (BM) are a rare adult cell population of primitive hematopoietic cells that possess the potential for both multi-potency and self-renewal, these abilities of HSCs allow to generate specialized blood cells and sustain blood production throughout life. During the third week of human embryonic development, the first wave of hematopoiesis (termed "primitive hematopoiesis") is initiated in the mesoderm of embryonic yolk sac [1]. During early embryonic development, the primitive hematopoiesis involves an erythroid progenitor that gives rise to nucleated erythrocytes which synthesize embryonic hemoglobin (ζε) [2,3], however, occasionally, the presence of macrophage population and primitive progenitors of megakaryocytes (MKs) has also been noticed [4]. The primary function of the primitive wave is to produce red blood cells that can facilitate tissue oxygen delivery (oxygenation) as the tissue begins to differentiate and undergo rapid proliferation during embryonic development [5]. The primitive wave is transient in nature because the progenitors do not have pluripotency and self-renewing ability [6], however the total disappearance of primitive hematopoiesis was commonly observed after 60$^{th}$ day of development (Figure 1.1).

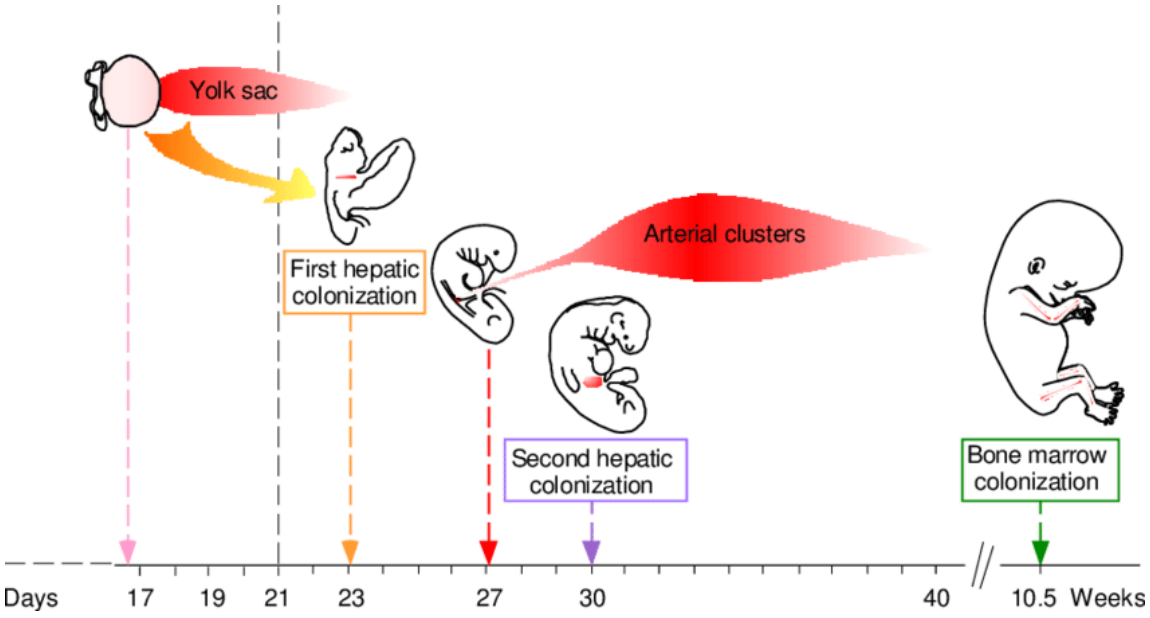



**Figure 1.1. Development of the hematopoietic system cells in the developing human embryo. Adapted from, Tavian and Péault [1].**

In later developmental stages, the generation of erythrocytes in the yolk during the first wave of hematopoiesis is replaced with the production of definitive erythrocytes in the liver (termed "definitive hematopoiesis") (Figure 1.1). After the initiation of the vascular connections between yolk sac and embryo, the beginning of cardiac beating (day 21) allow yolk-sac derived blood cells to colonize embryonic tissues. The first colonized organ is the liver (Figure 1.1), which remains the main site of blood cells production before the initiation of hematopoiesis in bone-marrow [1,7].

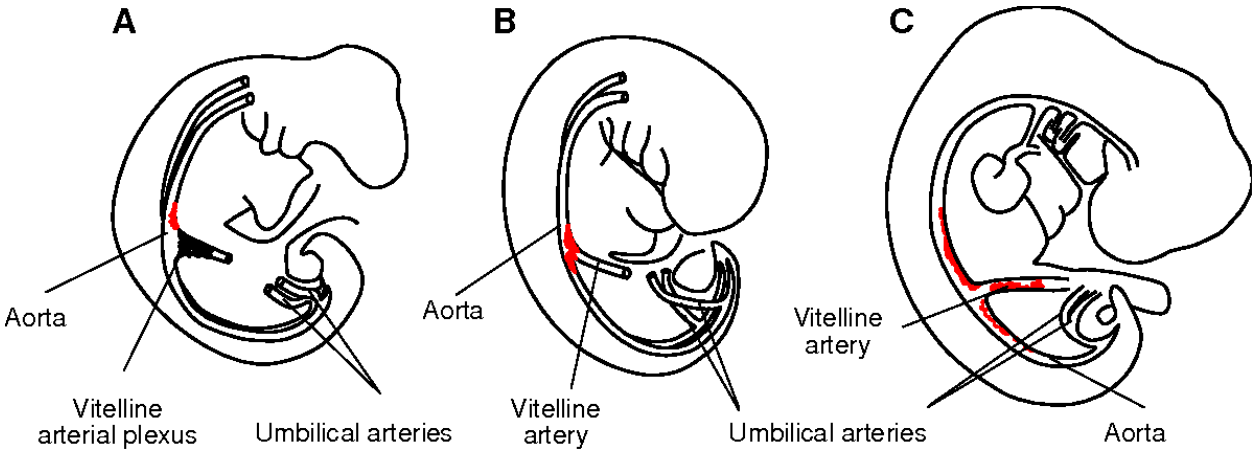



**Fig. 1.2. Sequence of HSC clusters emergence during embryonic development in the human. Adapted from, Tavian and Péault [1].**

In addition to the yolk sac, the capacity to produce definitive hematopoietic cells also arises in intraembryonic site, splanchnopleura, however the potential is expressed after 27 days with the emergence and further development of hematopoietic cells clusters within or near the ventral wall of the aorta and vitelline artery (Figure 1.2) [1]. The reports suggests that the re-differentiation of ventral aortic endothelial cells gives rise to hematopoietic cells at day 27 (Figure 1.3A) [8], however it was also speculated that hemangioblasts (hematopoietic and endothelial precursors), or even more primitive precursors (mesodermal stem cells) migrate through the peri-aortic connective tissues (mesenchyme) and colonize the floor of aorta, these cells at this stage generate two type of cells: blood-forming endothelial cells and hematopoietic progenitor cells (Figure 1.3B) [1]. With the advances in development the arterial clusters undergo gradual decrease, detected till day 40 (Figure 1.1). Yolk sac-derived primitive stem cells are limited in their ability, they can only

generate myelo-erythroid lineage (unilineage), however HSCs born in the embryo are the first multi-potent adult-stem cell progenitors that appear in the human ontogeny.

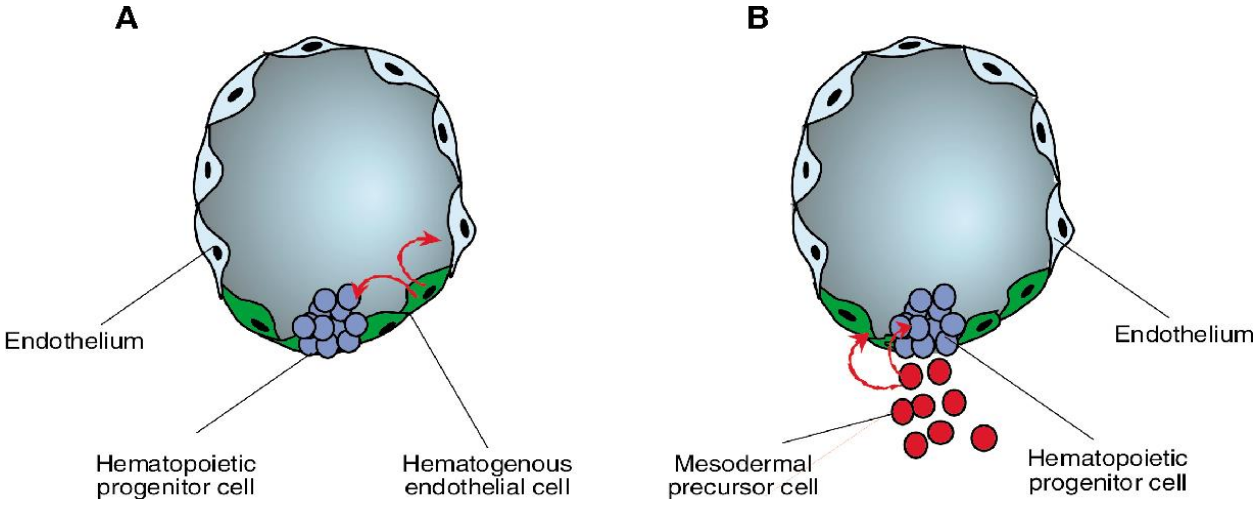



**Fig. 1.3. Models of HSCs emergence within the human embryo.** (A) Re-differentiation model. Begins in the ventral aspect of human intra-embryonic arteries at 27th day of embryonic development, where pre-existing hematogenous endothelial cells (green) starts differentiation locally into blood cell progenitors also called hematopoietic progenitors (blue). (B) Migration model. Starts from 27 days of gestation, where a dispersed population (red) of mesodermal precursor cells ($CD34^{-}CD45^{-}$ cell) colonize the ventral vascular wall of embryo and start differentiation into (blood-forming?) endothelial cells (green) and blood forming hematopoietic progenitor cells (blue). **Adapted from, Tavian and Péault [1].**

The transition of yolk sac to fetal liver hematopoiesis is defined by the change in globin synthesis program, the embryonic (ζε) to fetal globin switches (αγ), which reflects the transition from nucleated megaloblasts (primitive red cell precursors) to definitive and enucleated erythrocytes also called macrocytes [9,10]. The primitive nucleated megaloblast precursors are spotted in early hepatic rudiment tissues, these precursors start decreasing from week 4.5 to week 5 of embryonic development and at the same time they are replaced by definitive enucleated macrocytes. This hematopoietic development implies a monoclonal model, where a single cell pool which gives rise to primitive erythropoiesis in yolk sac, then migrate to fetal liver and generate definitive erythroblast [10]. This conclusion was also suggested by the analysis of in vitro colony-forming cell [11]. In contrast, recent work documents the appearance of hematopoietic cells ($CD34^{-ve}$ erythro-myeloid cells) inside the hepatic sinusoids at earlier stages of development (23 days),

suggesting the first hepatic colonization at this stage (Figure 1.1) [7]. However, the second hepatic colonization takes place from day 30 with the appearance of the first $CD34^+$ hematopoietic progenitors in the fetal liver anlage (Figure 1.1) [1,7].

The last blood forming tissue develops in ontogenesis is the BM, when the hematopoiesis is already extinct in the yolk sac, transiently proceeding in the fetal liver, thymus, spleen, placenta, and ultimately the BM space. The establishment of hematopoiesis within human medullary cavities was explored with the help of immuno-histochemical approach. Hematopoiesis begins in the BM during $11^{th}$ week of gestation, HSCs development in BM starts in specialized structures of mesodermal tissues and constituted by a loose network of mesenchymal cells surrounding a central artery [12]. The fetal BM as well as in the cord blood (CB), ACE expression with CD34 identifies bona fide HSC, that provides sustained multilineage hematopoietic cell engraftment [13,14]. During human ontogenesis, ACE marker is spotted in all blood-forming tissues, such as yolk-sac, para-splanchnopleura, aorta, fetal-liver, and the BM (Figure 1.4) [13,14]. In all sites, $ACE^+$ hematopoietic cells are found to be associated with the developing endothelium, suggesting that these two cell linages have direct ontogenic affiliation [15].

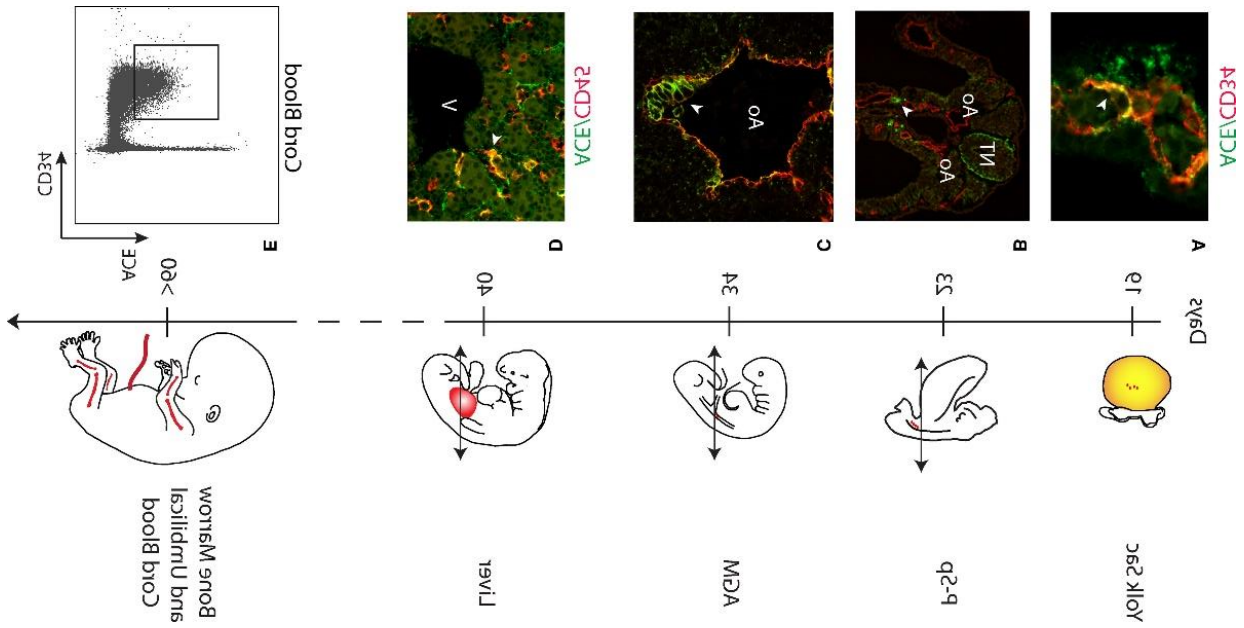



**Fig. 1.4. Ontology of appearance of ACE-positive cells though human embryo development. Adapted from, Julien E et al. [15].**

### 1.2. Hematopoiesis: A human perspective

Blood is a highly regenerative fluid form of connective tissue, with approximately one trillion ($10^{12}$) cells arising every day in adult human BM. Despite its complexity in producing different type of blood cells within lymphoid and myeloid blood lineages, blood is probably the best understood developmental system. The diversity of blood cells production was first explained by Maximow A. [16], he astutely postulated that hematopoiesis is a complex process organized as a cellular hierarchy derived from a common precursor, HSC. During the atomic era several evidences for the existence of HSCs came from different transplantation studies, however they could not resolve weather there were lineage specific stem cells, or weather a single multipotent HSC existed for all blood lineages [17]. During 1960s, the functional studies performed, such as clonal in vivo repopulation assays, confirmed the existence of multipotent HSCs [18,19]. Later, several other research groups developed the clonal in vitro assays that, combined with antibodies-based flow sorting, and generated a final view of the blood system developmental hierarchy with multipotent HSC population at the apex of hierarchy and terminally differentiated cells on the bottom (Figure 1.5). The lifelong blood production is maintained by a small pool of HSCs, which are uniquely defined by their potentiality in self-renew (generate daughter stem), and capacity to contribute the pool of differentiating cells throughout life. These differentiating cells (progenitor cell intermediates) undergo a gradual fate restriction to become a mature blood cell.

All mature blood cells, according to the classical model of hematopoiesis, belong to two fundamental branches of blood lineage: lymphoid and myeloid. The lymphoid branch of blood lineage consists of lymphocytes (T and B cells), and natural killer (NK) cells, which carryout immune functions (innate and adaptive immune responses). However, the myeloid branch includes various granulocytes (neutrophils, eosinophils, basophils, and mast cells), monocytes, erythrocytes, and platelets producing MKs. As it was discussed above, HSCs, the cells at the apex of hematopoietic system, undergo in a sequential and tightly regulated process of lineage commitment. The HSC through a complex roadmap of progenitor intermediates generates all the terminally differentiated blood cells, and this process is in a comprehensive view of different regulatory mechanisms (transcriptional and epigenetics) that sustain blood cells development.

The first comprehensive classical model of blood cells development (hematopoiesis) was formulated with the isolation of the cells of myeloid and lymphoid lineages (CMPs: common myeloid progenitor and CLPs: common lymphoid progenitor, respectively) using flow-cytometery [20-22]. According to this model, HSCs generate Multipotent Progenitors (MPPs) with transient repopulation capacity [23,24]. Further, these MPPs undergo bifurcations process and produce CLPs and CMPs [20,21]. Lately, each of these undergoes further progenitor commitment process. The CLPs generate precursors of B-lymphocytes, and earliest thymic progenitors committed to the T-lymphocyte and NK lineages. On the other side, CMPs give rise to Granulocyte-Monocyte Progenitors (GMPs), committed to the granulocyte and monocyte fate, and megakaryocyte-erythroid progenitors (MEPs), which only produce MKs and erythroid cells (Figure 1.5) [25,26]. Recent advances in the fluorescence-activated cell sorting analysis have provided more detailed developmental map downstream of HSCs. Notably, prior to the commitment of the CMP and CLP, HSCs appear to form myelo-erythroid vs myelo-lymphoid progenitors [27]. Though human and mouse hematopoiesis follow same path, there are considerable differences in distribution of surface markers (Figure 1.5).

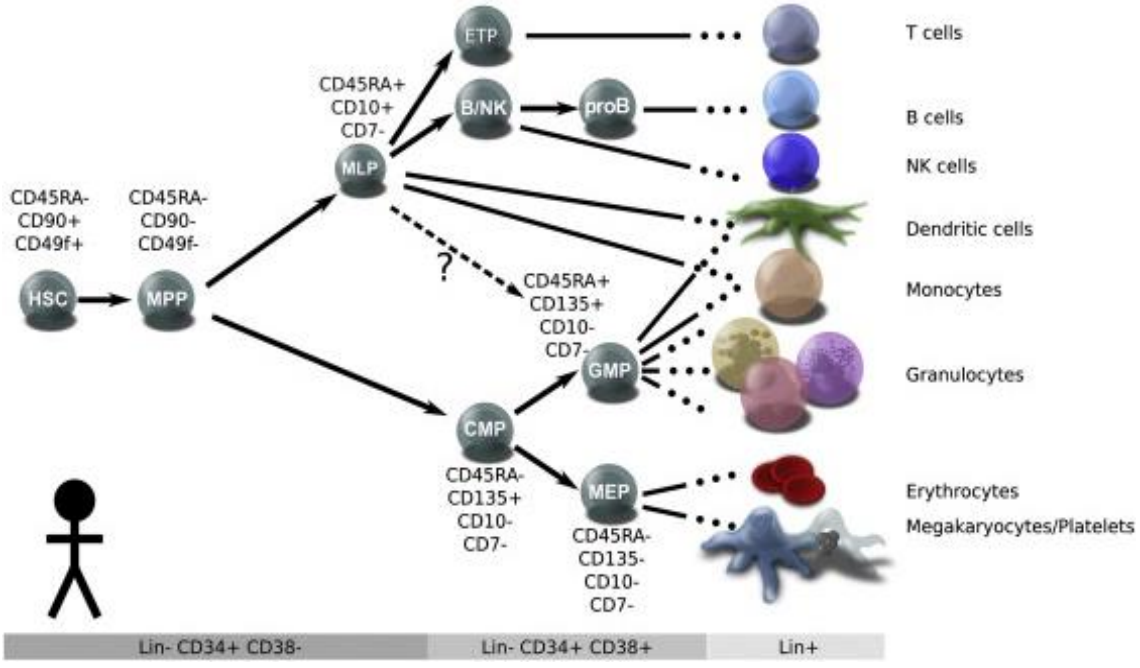



**Fig. 1.5. Human Hematopoietic Hierarchies. Adapted from, Doulatov S et al. [25].**

### 1.3. The molecular regulation of human HSCs

HSCs are able to both self-renew and differentiate throughout life to contribute the pool of blood cells; these cells are also able to repair damaged blood systems upon transplantation. Two basic models of cell division are discussed for the regulation of HSCs fate (Figure 1.6). The asymmetric model suggests that the HSC pool is maintained at the level of single cells, whereby each HSC generate a copy of itself (called self-renew) plus one differentiated cell [28-31]; whereas, the symmetric model: a proliferation division, generating two daughter stem cells, and a differentiation division, generating two differentiated cells [31-33] (Figure 1.7). The asymmetric division has advantage in keeping the HSC population level steady; the major disadvantage of this division is its inability to restore the stem cell pool in damaged blood systems. This problem is naturally solved by the symmetric division of stem cells; symmetric division model of stem cells maintains homeostatic balance at the population level, rather than at the level of individual blood cell. Differentiation/proliferation decisions are postulated to be governed by various signals initiating from the surrounding tissues and the stem cells themselves. In the literature, both type of divisions has been reported in stem cells of various tissues, moreover same stem cells are capable to generate daughter cells by both symmetric and asymmetric divisions [34]. The symmetric and asymmetric cell divisions are truly depending on the polarized organization of the dividing stem cell as well as the cell cycle length; this can also be influenced by asymmetric inheritance of cell-extrinsic factors (niche environment), or by the asymmetric inheritance of cell-intrinsic fate determinants [34].

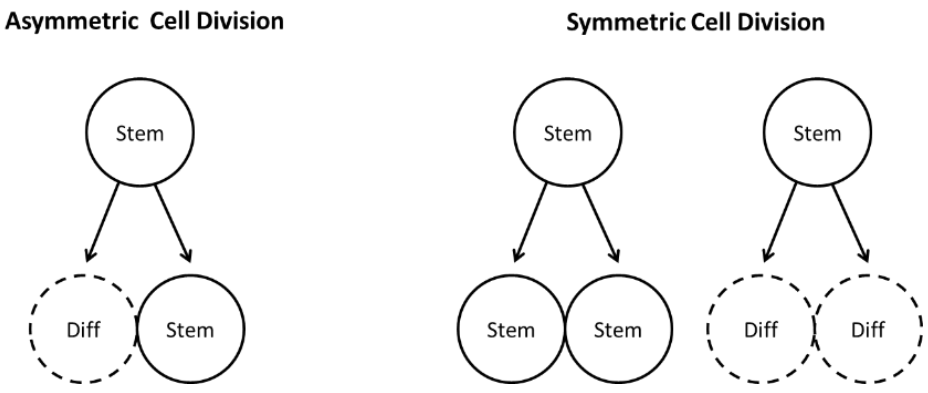



**Figure 1.6. Symmetric and asymmetric stem cell divisions. Adapted from, Shahriyari L and Komarova NL [34].**

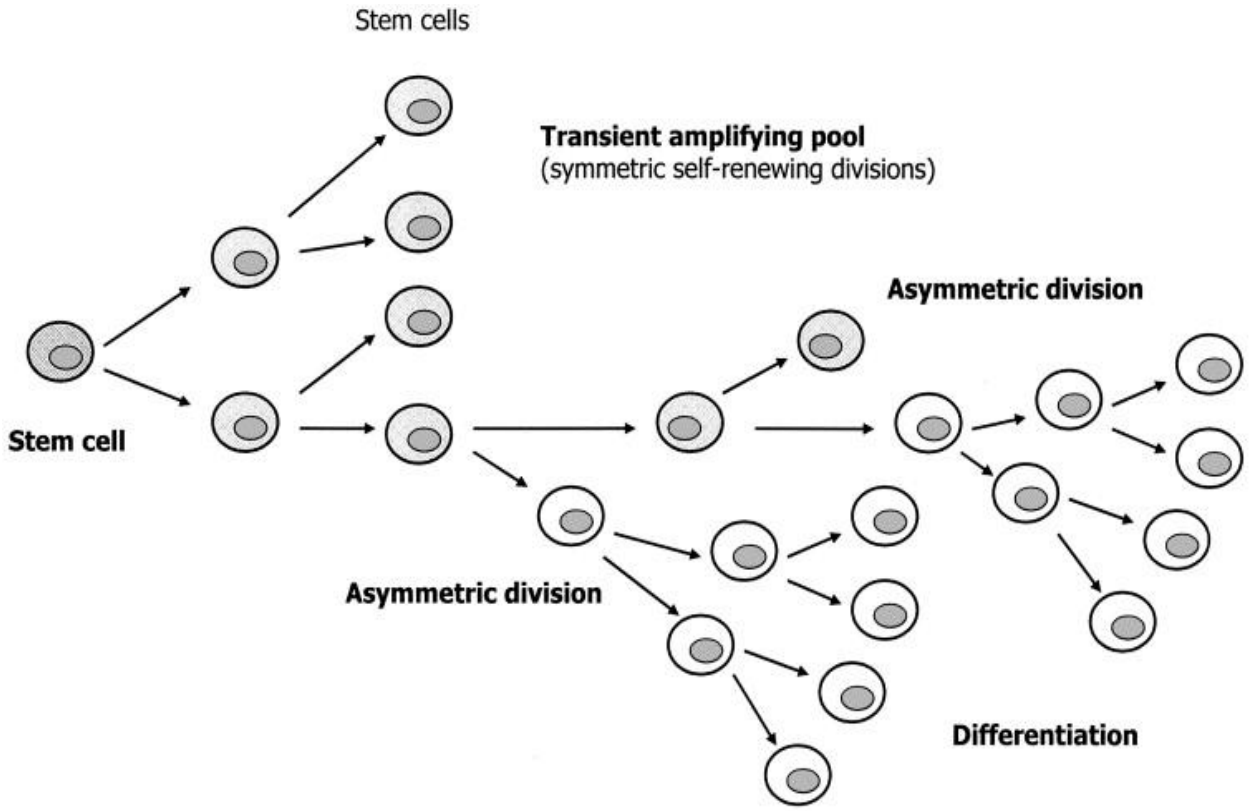



**Figure 1.7. Stem cells divide to generate either two daughter stem cells or to produce differentiating daughter cells. Adapted from, Ho AD [31].**

### 1.4. How human hematopoietic lineage commitment occurs

The multipotent HSCs, which maintain the sustain blood cells production throughout the life, dividing only in response to demand for blood cells [35]. Though, hematopoiesis is the well-studied system, the molecular mechanisms underlie the commitment of different lineages is yet a mystery in research. It has been well accepted that both multi-lineage and lineage-specific cytokines synergistically involved in the regulation of lineage commitment process, cell cycling, and differentiation. However, the major area of mystery and conjecture in the field of HSCs development is weather cytokines can "instruct" hematopoietic lineage commitment [36] or whether their functions are only "permissive" [37], allowing the survival and expansion of already committed lineage specific cells. While the current data do not rule out the possibility that growth factors or cytokine can influence the lineage choice of multipotent stem cells (instructive model)

(Figure 1.8A), data from different research groups suggest that growth factors may not be obligatory for the differentiation of multipotent stem cells; this data support the hypothesis that differentiation or lineage commitment is intrinsically determined and the cytokine or growth factors functions are permissive rather than instructive (Figure 1.8B) [38].

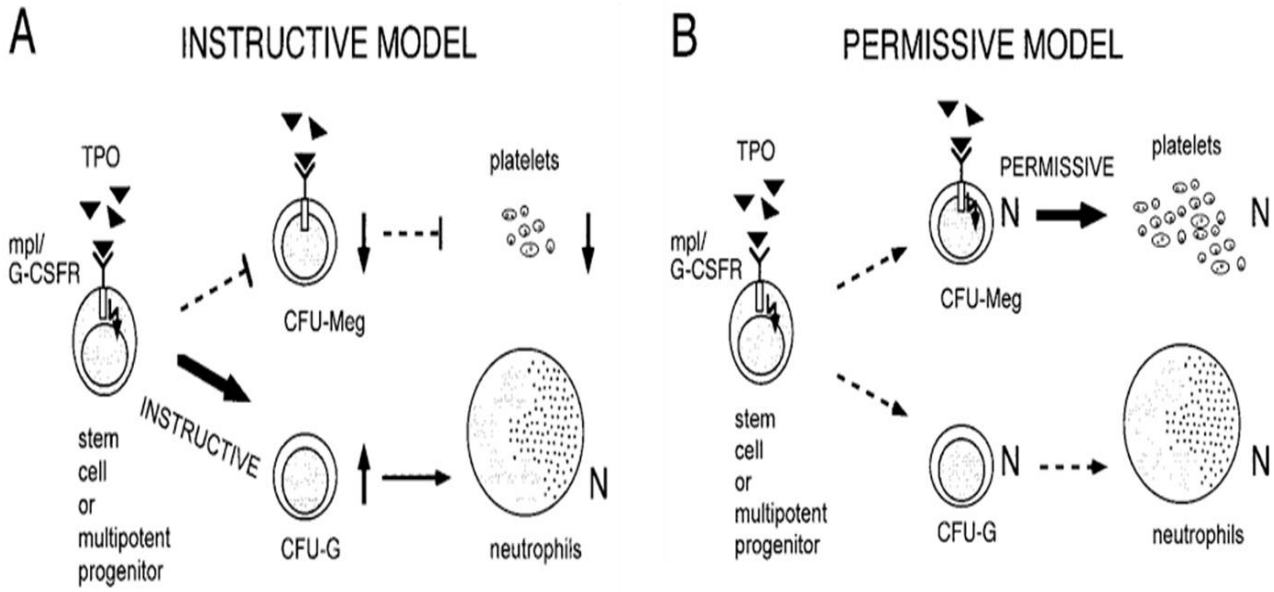



**Figure 1.8. Effects of the chimeric cMpl/G-CSFR on platelets and granulocyte production in ki/ki mice.** (A) Phenotype redistricted in the instructive model. (B) Predictions of the permissive model. **Adapted from, Stoffel R et al. [39].**

Later the permissive model received more attention after the discovery of a lineage dominant cytokine, Thrombopoietin (TPO); to prove the permissive hypothesis, TPO receptor (cMpl) was replaced with a chimeric receptor consists of the cytoplasmic domain of granulocyte colony-stimulating factor (G-CSF) receptor and extracellular domain of cMpl, which still resulted in normal platelet counts in mice model (Figure 1.8B) [39]. Moreover, recent studies showed that both the cell-autonomous transcription factors (TFs) [40] and extrinsic factors such as cytokine [41] instruct lineage choice, and the instructive action of cytokines has also been convincingly demonstrated in engineered cells [42,43] (Figure 1.8A).

### 1.5. Platelets and MKs

Platelets are small anucleate membrane-bound cell fragments of 1 to 3 µm size and discoid shape. Historically, for a long time, platelets were termed to as cellular dust, but they are now demonstrated as indispensable for different processes such as hemostasis (normal process of blood clotting), wound healing, angiogenesis, inflammation, and immunity. Platelets are released from the cytoplasm of matured MKs, their precursor cells, which reside predominantly within the BM [44]. MKs are among the largest (50–100 µm) and rarest cells (~0.01% of all nucleated cells) in the BM [45]. To assemble and release platelets, MK progenitor (MKP) undergo polyploidization through endomitosis (DNA replication without cell division), which leads to the formation of a giant cell, called the MKs; these cells subsequently undergo a maturation process in which entire cytoplasm of MK is rearranged into long, beaded extensions termed proplatelets and the nucleus is extruded. MKs may extend 10-20 long processes, called proplatelets; platelets are formed selectively at the tips of elongated branches of proplatelets and released into the bloodstream [46]. All platelets receive their granule and organelle content during their development from the precursor cell body [47]. Platelet development process can be explained broadly into two different phases: the first phase of development is the phase of MKP commitment and maturation which is under the control of different MK-specific growth factors and intrinsic regulators. During this phase, polyploidization and cytoplasmic maturation occurs to fulfill the demand of proteins, granules, and membrane for the platelet assembly process and their functions, this is the longest phase of platelet development process. The second phase is a rapid phase taking few hours and involves remodeling of the cytoplasm into long and rapidly growing membrane extensions called proplatelets, which further undergo fission events to generate discoid platelets (Figure 1.9) [48]. Human and rodents MKs, respectively, take about 5 and 3 days to complete the process of polyploidization, cytoplasmic maturation, and proplatelets release [49-51]. Once released into the blood, human and rodents' platelets survive 7-8 and 4-5 days, respectively [52-54].

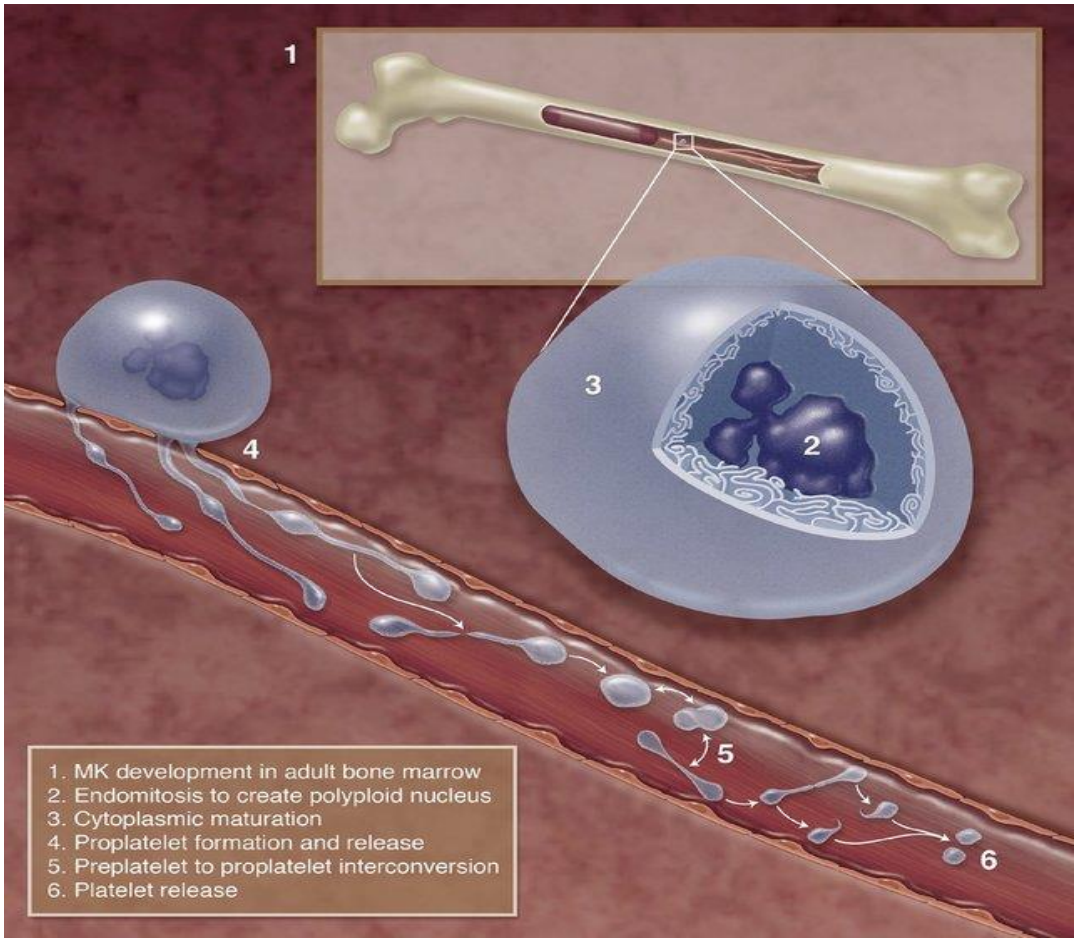



**Figure 1.9. Schematic of MK development process and platelet production. Adapted from, Machlus et al. [48].**

## 1.6.The Evolution of Mammalian Platelets

Polyploid MKs and platelets are found only in mammals, also they have similar function and structure in all mammals [55]; anucleate platelets aggregate to occlude the site of bleeding, to initiate thrombus (blood clot) formation and secrete different growth factors to repair blood vessels. However, in all other animal species different nucleated cells are involved in hemostasis and blood coagulation. For hemostasis, reptiles and birds primarily rely on the function of nucleated cells, thrombocytes [56], which are larger and less efficient than mammalian platelets [57,58]. Thrombocyte like cells are found in arthropods: coagulocytes in insects [59] and haemocytes in the limulus crab [60]. Thrombocytes of the first vertebrate (Fish) are functionally similar to mammalian platelets, however they are considered as the cells of primitive hemostasis.

The presence of anucleate platelets and their polyploid precursor cells (MKs) suggested to be beneficial for some traits of mammalian physiology and for the process of hemostasis in mammals. Recently, Martin and Wagner [61] proposed that hemochorial placentation required MK derived anucleate platelets for its evolution. They argued that the hemochorial placentation is limited to a clade of mammals, because they developed a unique and highly effective system of hemostasis, MKs/platelets. Another essential point is that the functional effectiveness of hemostasis is very important at the time of parturition where even minimally invasive placenta can hemorrhage. The fact is that neonates of mammals, regardless of their birth and development, rely on maternal lactation for their initial survival and development after birth, therefore, the survival of the mother is essential. Subsequently, the evolution of invasive placenta is most likely succeeded because of the highly effective hemostasis system developed before the origin of deep placentation (Figure 1.10).

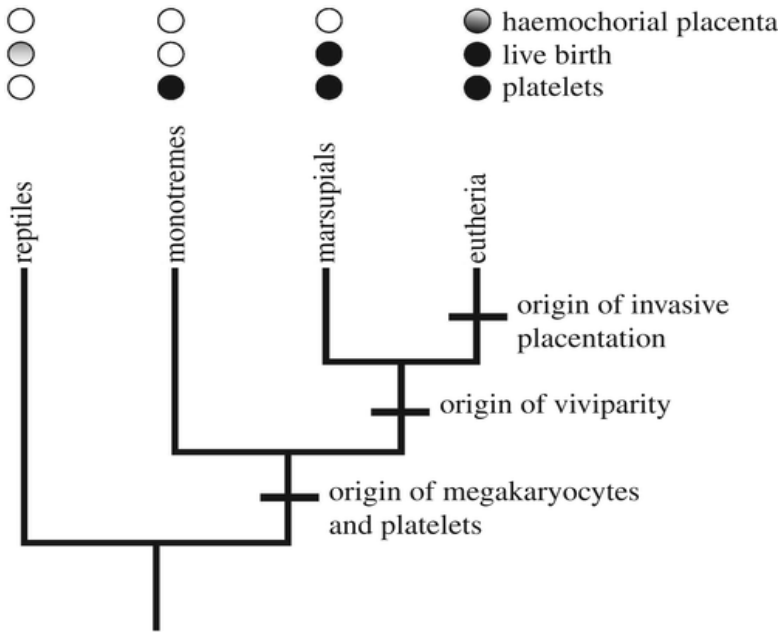



**Figure 1.10. Phylogenetic placement of the major mammalian clades and the taxonomic status of the hemostatic functions and reproductive characteristics. Adapted from, Martin and Wagner [61].**

Considering the evolutionary theory standpoint [62], platelets are exaptation for the evolution of the hemochorial placenta, i.e. a trait which is crucial, but that evolved for another purpose before taking on the role for the evolution of hemochorial placenta. Therefore, MKs/platelets could be

called a permissive exaptation, probably these features may have permitted the development of hemochorial placentation, instead of acquiring the new functions itself [61,63,64]. However, any other biological benefit gained from the development of anucleate platelets remains unidentified.

### 1.7. Shared features in HSCs and MKs

HSCs and MKs share many common biological and molecular features (Cell surface receptors, cellular signaling and expression profiles of different genes), as previously described (Figure 1.11) [65,66]. The TPO signaling is the crucial cytokine signaling pathway in both the HSCs and MKs. HSCs and MKs share the expression of cMpl and the dependence on TPO/cMpl signaling for the regulation of their maintenance and expansion [65]. TPO/cMpl signaling was originally recognized in MK/platelet biogenesis [65]. However, different studies with cMpl knockout mice demonstrated that cMpl-signaling pathways are also important for the maintenance of HSCs [67]. HSCs derived from cMpl knockout mice observed with reduced long-term repopulating capacity, suggesting that the function of TPO/cMpl signaling is essential for HSCs activity [67]. Different research groups have noticed that the signal transduction pathways triggered by TPO/cMpl are important in maintaining HSCs quiescence [68,69]. Loss-of-function mutations in cMpl results in severe low platelet condition in human, called Congenital Amegakaryocytic Thrombocytopenia (CAMT). Patients with CAMT are also commonly observed with bone-marrow failure, suggesting the functional role of cMpl signaling in the maintenance of the function of human HSCs [65]. These studies clearly indicate that TPO induced signaling might be a crucial regulatory bridge between HSCs and MKs. Moreover, many other signaling pathways such as CXCR4/CXCL12 [70,71], Notch [72-74], and Stem cell factor/c-Kit signaling [74] have also been identified as the shared signaling pathways between MKs and BM HSC population [65].

Furthermore, it has been observed that HSCs/HPCs and MKs co-express various cells surface antigens. In different studies, the markers/surface-antigens used for the identification and isolation process of MK/MKPs, such as MK/Platelet Glycoprotein (GP) IIb (CD41) [75], CD150 [76], and CD9 [77] are also expressed abundantly on HSCs/HPCs. The surface marker CD150 (also called SLAM1) is commonly used for the isolation of mouse HSCs [76], however it is also expressed on MKs, MKPs, and platelets [78]. A well know MK surface antigen CD41, which non-covalently

interacts with the Integrin b3 (platelet GP IIIa, CD61) to form a MK surface antigen complex aIIbb3 [65], plays crucial role in thrombus formation [79], however CD41 is also expressed on HPCs. It has been observed that the expression level of CD41 increases with aging and that enhances myeloid differentiation potential of mice HPCs [75,80]. These similarities in the dependence on same signaling pathways and the similar expression of different regulatory molecules arises the possibility of the direct commitment of MKP from HSCs.

Moreover, recently is has been observed that the von Willebrand factor (vWF), which is mainly released by endothelial cells (ECs), matured MKs, and platelets following tissue injury, is also present in a subpopulation of BM HSCs [81]. On the other hand, Nishikii H et al. [78] have also identified recently that the MKs and platelets membrane receptor GPIbα (CD42b, receptor for vWF) is also expressed by a population of HSCs ($CD34^{-}/CD41^{+}/CD150^{+}$ LSK) at the hematopoietic recovery phase [78]. These facts encouraging further investigation on the functional aspects of these common molecular features between MKs and HSCs. Furthermore, Whole transcriptome studies using single purified HSC identified that a subpopulation of BM HSCs expresses genes which are highly and exclusively expressed by MKs [82]. These reports show a close association between HSCs and MKs.

Another research group has demonstrated the mechanism which controls the expression of MK-related genes in HSCs [83]. They have reported that the phenotypic HSC compartment contains a population of Stem-like MKPs (SL-MKPs), which are characteristically similar to HSCs but retain MKP restricted differentiation potential. They reported that SL-MKPs are present in the $CD41^{+}$ subpopulation of HSCs and express the MK-specific mRNAs while the mRNA translation is suppressed in SL-MKPs in normal conditions. Different inflammation signals induced interferon signaling activates the translation of MK-specific mRNAs in SL-MKPs and trigger MK development and platelet release. And, the molecular mechanism defined by the authors is the FoxO3a mediated regulation of MK-specific mRNAs such as p27 and p57 (cell-cycle regulators). In steady state condition p27 and p57 are under strict control (suppressed) of FoxO3a [83]. Although furthers experimental evidences are required, the transcriptional/translational regulation of MK-specific genes may be crucial in MK lineage commitment from HSCs.

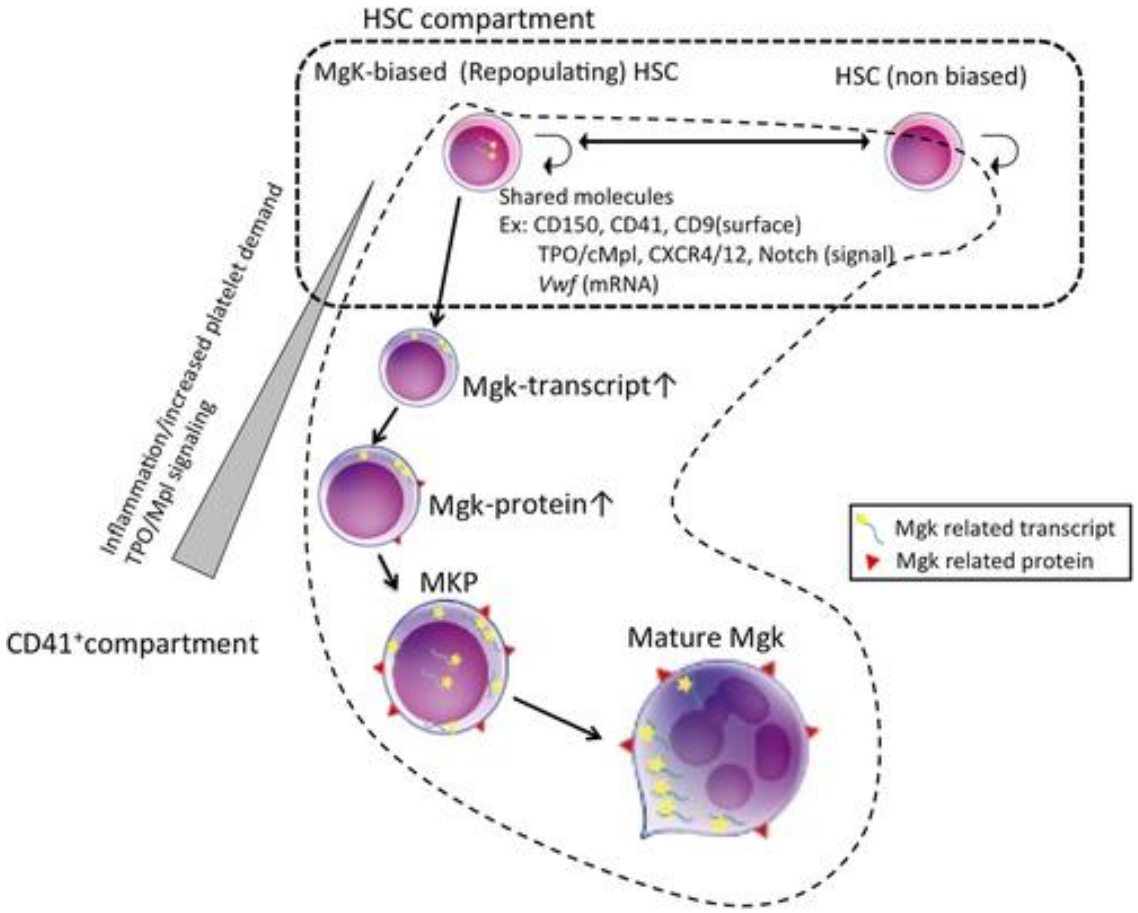



**Figure 1.11. Shared molecular features (receptors and signaling) in HSCs, MK-biased HSCs, and committed MKs. Adapted from, Nishikii, H et al. [66].**

## 1.8. Development of the MK lineage from HSCs

The heavily shared signaling pathways and molecular markers between HSC population and MKP/MKs have raised the possibilities of MK lineage commitment at the earliest stage of HSCs development/lineage selection hierarchy without following the postulated classical stepwise differentiation model [65]. If we discuss the hematopoietic system of mouse, the HSCs are mainly enriched in the population of either $CD34^{-/low}$ LSK [84] or $CD150^{+}/CD48^{-}$ LSK cells [76]. The immature myeloid progenitor cells population, in the classical model of HSC development are defines as CMPs. CMPs are derived from MPPs and differentiate into bipotent GMP and MEPs, eventually MEPs differentiate into MKPs while losing their erythroid lineage differentiation

capacity (Figure 1.12A) [20]. Furthermore, recently published results of RNA sequencing from single cell described that the phenotypic CMP population is highly heterogeneous, they found the expression of MK specific genes (particularly TFs) in a subpopulation of CMPs, however these genes were not detected in MEPs [85]. Indeed, Nishikii H et al., [78] confirmed that the CMPs subpopulation (CD42b+ CMPs) is the population of unipotent MKPs in BM [78]. Recently published reports commonly accept that the CMPs population could be a heterogeneous population of unipotent myeloid progenitors and MKPs, which support the notion that the MKP commitment occurs at the earliest stage of HSCs development hierarchy (Figure 1.12B).

There are multiple evidences which verify that the MK development pathway is bifurcated more earlier than assumed in the classical HSC development model (Figure 1.12B) [72,81, 86, 87]. Flt31LSKs are described as lymphoid-primed MMPs (LMPPs), which do not have MK/erythroid differentiation capacity, but retaining granulocyte/macrophage differentiation potential [86]. While in different other studies the MK/erythroid differentiation potential from LMPPs has been recognized, the actual megakaryopoietic capacity of LMPPs is significantly lower than that of HSCs of MPP [80,20,88]. Further, it has also observed in an in vitro study that Notch signaling pathway play an important functional role in MK lineage commitment from HSCs [72]. They suggested that the Notch signaling could promote a direct pathway of MKs development from BM HSCs, possibly bypassing the MEP stage.

More recently, many other reports, based on the finding from many novel single-cell functional analysis methods, suggested that the long-term HSCs (LT-HSC) population is functionally heterogeneous and includes the already committed MK lineage subpopulation within LT-HSC compartment (Figure 1.12C) [87]. Indeed, 10%–15% of the highly purified LT-HSCs, in single-cell in vitro culture conditions, can differentiate directly into MKs without cell division; the same population also includes cells which are highly proliferative in nature and capable of producing 100–1,000 single lineage MKs [78,87,89]. Moreover, the paired daughter assay results show the MK differentiation from HSCs even at the first division of HSC [87].

The in vitro assays may not always perfectly mimic the in vivo HSC development, thus these observations may not support the possibility of the differentiation of MK directly from HSCs, or

the presence of MK-biased/restricted subpopulation of HSCs. However, results from a single-cell transplantation study demonstrate that the highly purified HSC population ($CD34^{-ve}$ LSK) contains a heterogeneous population of several lineages, such as myeloid-biased, lymphoid-biased, and MK/erythroid-biased HSCs [87]. This study report that the lineage specific fate decision from multipotent HSCs might start earlier at the LT-HSC level and also suggest revising the conventional, stepwise, hierarchal, HSCs differentiation model. Their data also demonstrate that the subpopulation ($CD150^{+}/CD41^{+}/CD34^{-}$ LSK) of a mouse LT-HSC population contains MK-restricted repopulating cells (MKRPs), these cells are restricted to MK differentiation and show self-renewal capacity in vivo. Moreover, as discussed previously, SL-MKPs also reside as a subpopulation ($CD41^{med}/CD150^{+}/CD48^{-}$/c-Kit1/$Lin^{-}$) of the $CD41^{+}$ LT-HSC population [83]. Indeed, a single-cell culture study by Nishikii H et al., [78] revealed that the MK colonies derived from the $CD150^{+}/CD41^{+}$LSK population were higher in number than that derived from $CD41^{-}$ LSK population. They have also demonstrated that the continuous TPO/cMpl pathway activity, bypassing the bipotent stage (MEPs), is the key for MK differentiation from HSCs [78]. Further, more celerity comes from the studies that showed a subpopulation of vWF-positive HSCs; these cells reside at the top of the HSC hierarchy, possess MK-biased differentiation capacity, and expansion capacity during aging [78,81,82].

On the other hand, a group of Shin and colleagues showed that c-$Kit^{high+}$ HSC ($CD150^{+}/CD34^{-}$ LSK) population produces MK/Platelets more rapidly in vitro and in vivo with impaired self-renewal activity; however, c-$Kit^{low}$ HSCs were described as more primitive and quiescent than c-$Kit^{high+}$ HSCs [90]. The data of this study clearly define that the MK-biased HSCs subpopulation is not at the apex of the HSC development (hematopoiesis) hierarchy. To further confirm these finding, weather two distinct subsets of MK-biased HSCs exist at the apex and sub-apex of the HSC development hierarchy, more experimental evidences and further investigation is required. Also, further analysis would be required to understand platelet generation from the steady-state HSCs [66].

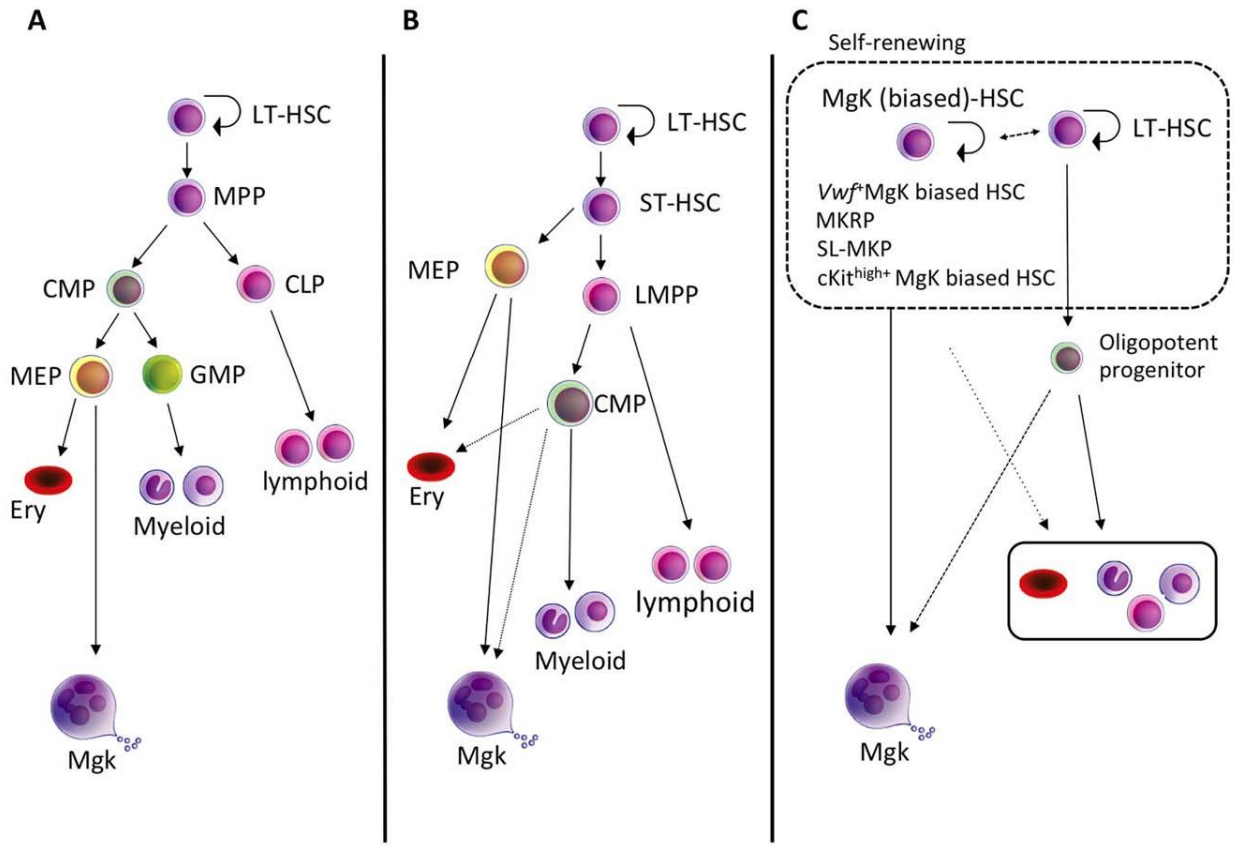



**Figure 1.12. The models of MKs differentiation from HSCs. Adapted from, Nishikii, H et al. [66].**

## 1.9. MK lineage commitment: A human perspective

The classical hematopoiesis hierarchies (HSCs differentiation models) are same in both the humans and mice and it has been widely accepted by most scientists of this field [91]. Similar to mice, an alternative MK development model was also proposed in human hematopoiesis and that reflects the mouse hematopoietic system [92,93]. In these studies, single-cell analysis, using human CB and BM cells, revealed that MK lineage fate choice occurs within the $CD34^{+}/CD38^{-}$ HSC-enriched compartment, and both the $CD34^{+}/CD38^{-}$ CMP and $CD34^{+}/CD38^{+}$ MEP populations are highly heterogeneous [94], retaining less megakaryocytic differentiation capacity [92]. They have also discussed the differences between adult hematopoiesis and fetal liver hematopoiesis; they demonstrated that the MK is generated from intermediate-oligopotent progenitors in fetal hematopoiesis, and this MK lineage differentiation system is different from

adult hematopoietic system [92]. However, further studies are needed to prove the MK biased hematopoiesis and the existence of MKs biased HSCs and progenitors in human BM. But, the functional analysis at the single-cell level in in vivo conditions is more challenging in humans as compared to mice model system [95]. Several recent studies have revealed the heterogeneity in some of the previously defined populations (MPPs, CMPs and MEPs) of hematopoietic system (Figure 1.13). Importantly, the commitment to the MK lineage occurs much earlier than previously expected in the classical model of hematopoiesis. The existence of MK and other lineages biased HSCs were also demonstrated. However, the physiological role of these lineage biased cells, especially in the human hematopoietic system, in steady state and in diseases conditions is not clearly demonstrated. However, these findings provide a new roadmap of human hematopoiesis [66, 92,96].

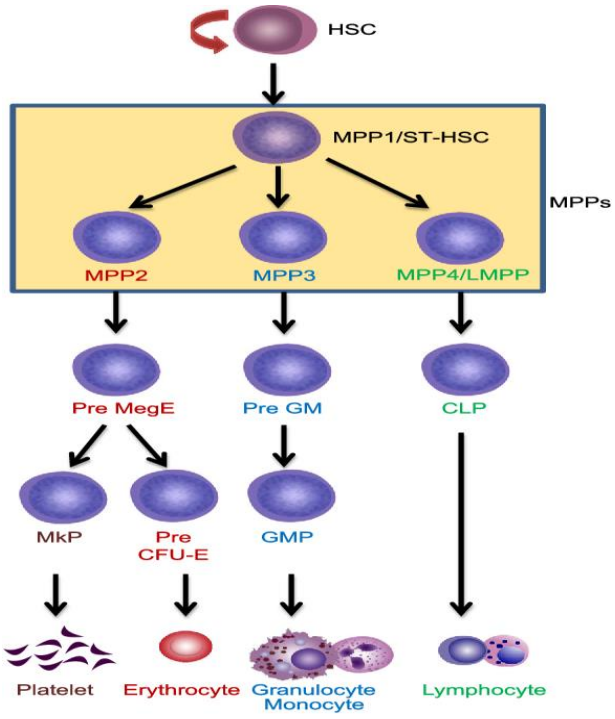



**Figure 1.13. A reconciled model for hematopoietic stem cell differentiation. Adapted from, Cheng H et al. [96].**

## 1.10. Overview of MK Differentiation and Maturation

MKs development from HSCs in the BM involves a number of differentiation events; In the megakaryocytic lineage, the first fully MK-lineage committed cell is colony-forming unit (CFU) MK (CFU-MKs), which is characterized by a unique cell surface phenotype. It is widely accepted that in BM CFU-MKs is derived from MKP [97-100]. However, in in vitro culture conditions CMPs and MEPs can also give rise to pure CFU-MKs [20]. There is one more class of human MKP cells: the burst-forming unit MKs (BFU-MKs), which is considered to be the most primitive progenitor cell committed to the MK lineage. The CFU-MKs is considered to be a more differentiated MKP and is thought to be ultimately a descendant of the BFU-MKs. The characteristics that allow us to differentiate human BM CFU-MKs and BFU-MKs are listed in Table 1.1 [101,102].

**Table 1.1. Characteristics of Human MKP cells. Adapted from, Hoffman R et al. [102].**

| | BFU-MK | CFU-MK |
|---|---|---|
| Time required for colonies to appear *in vitro* | 21 days | 12 days |
| Cells/colony | 108.6 ± 4.4 | 11.6 ± 1.2 |
| Foci/colony | 2.3 ± 0.4 | 1.2 ± 0.1 |
| Elutriation profile | 12–14 ml/minute | 18–20 ml/minute |
| Phenotype | $CD34^+DR^-$ | $CD34^+DR^+$ |
| Sensitivity to 5 Fluorouracil | Resistant | Sensitive |

Moreover, BM frequencies of CFU-MKs and BFU-MKs is respectively 36.7 +/- 2.5 and 7.3 +/- 0.7 per 10(5) total nucleated cells [103]. They have also reported that BFU-MKs, compared with CFU-MKs, require a strong stimulation for the differentiation; their invitro development is slow and show higher proliferation potential. From this information it can be concluded that the BFU-MKs are the early megakaryocytic progenitors of MK lineage that antedate the CFU-MKs as earlier studies predicted.

Further, MKPs enter in a series of complex biological processes [104] to become mature MKs, which can produce mature platelets (Table 1.2; Figure 1.14).

**Table 1.2. Events occurring during megakaryocytopoiesis. Adapted from, Hoffman R et al. [102].**

| |
|---|
| 1. Progenitor cell proliferation (BFU-MK, CFU-MK) |
| 2. Megakaryocyte polyploidization |
| 3. Cytoplasmic maturation (membrane and organelle development) of megakaryocytes |
| 4. Platelet release from megakaryocytes |

The development of the descendants of HSCs along the MK lineage (MKP) is first apparent by the expression of CD41/CD61 (GPIIb-IIIa), the markers of the MK differentiation. This is the most abundant platelet glycoprotein complex on the surface of terminally differentiated MKs and platelets, and form a calcium (Ca2+)-complex dependent functional heterodimer that functions as a receptor (integrin complex) for fibrinogen and vWF. The main function of this complex in platelets is to initiate the activation process. During maturation, MK expresses various other surface antigens which are also present on platelets, e.g., vWF receptor which is a complex of three chains: GPIbα, GPV, and GPIX. The CD42b is defined as the maturation marker of MKs. The expression of these surface antigens during megakaryocytopoiesis is presented schematically in Figure 1.14 [105]. It has been observed that the expression of these MK/platelet surface antigens consistently increases through MK development/maturation.

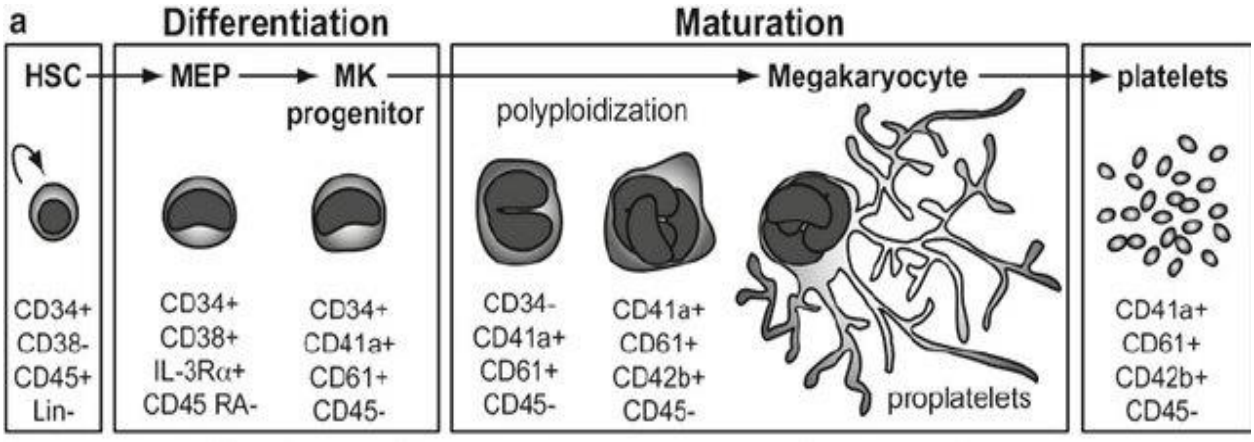



**Figure 1.14. An overview of MK development and platelet production. Adapted from, Robert et al. [105].**

## 1.11. Hallmarks of MK maturation

### 1.11.1. Polyploidization

Proplatelets are the small membrane protrusions from terminally differentiated/matured MKs that extend into BM vascular sinusoids, where platelets are sheared off from the proplatelet tips assisted by blood flow. Platelets are the end products of membrane protrusions from MKs. A single fully developed MK may generate 3-5 thousand of platelets before its membrane is fully exhausted, this process is followed by degradation of the remaining body of MK. To produce such a large number of platelets, MK need large amount of cytoplasm and membrane. For this reason, MK undergo through an extraordinary development process called polyploidization. The ploidy is increased by a phenomenon called Endomitosis; the high ploidy is not only required to increase the surface area-to-volume ratio of MKs but also necessary for the synthesis of a large amount of RNA and protein/enzymes required for the function of anucleate platelets in blood vascular system [106]. Polyploidization normally occurs as apart of MK development program, where cells increase their ploidy without cell division (Figure 1.14). Different cells use different mechanism to increase ploidy; Traka M et al. [107] showed that MKs use different unconventional cell cycles to increase ploidy and become polyploid without losing the normal function of cell in vivo. The large, multilobulated, polyploid nucleus is considered as one of the hallmarks of fully developed/matured MK (large cell of ~50–100 μm diameter) [108] (Figure 1.14). As we have discussed earlier that MK undergo an endomitotic cell cycle without going through anaphase or cytokinesis and replicate their DNA up to 256N/cell [109]. The mechanisms which regulates MKs polyploidization are not fully understood. However, previous reports suggested the functional role of cyclins, as a combined knockout of cyclin D1, D2, and D3 noticed with multilineage hematopoietic failure [110]. In the other hand, cyclin-E null mouse observed with defects in megakaryocytopoiesis, endomitosis of MKs was severely impaired in the absence of cyclin E [111]. Other research groups studied different chromosomal passenger proteins Aurora-B kinase, and other related proteins, such as surviving and inner centromere proteins, and showed normal distribution and function in MKs [112]. One report proposed that survivin and Aurora-B may be mis-localized or absent during an important phase of endomitosis [113] Others reports noticed that the degradation of cyclin B (a mitotic cyclin) and cdc2, a cyclin B dependent kinase, is involved in the switch to polyploidization

[114,115]. Moreover, some other reports observed that the defect in cleavage furrow (contractile ring) formation, that generate mechanical force for the separation of cell at late anaphase, resulted in the initiation of endomitosis [116]. The functional role of runt-related transcription factor 1 (RUNX1) has also been demonstrated in polyploidization, RUNX1 activate the switch from mitosis to endomitosis by silencing the expression of MYH10; the *MYH10* gene encode non-muscle-myosin heavy chain-IIB (NMMHC-IIB), which is involved in normal process of karyokinesis [117] (Figure 1.15). Rho-A, small GTPase, is known for the regulation of cytoskeleton contraction force at the time of cytokinesis. ECT2 activates and localizes Rho-A at the cleavage furrow [118,119]; the microtubule associated protein GEF-H1 also activates Rho-A. The reduced activity of GEF-H1 is required for the switch from 2N to 4N; to develop polyploidy the reduced activity of ECT2 is required, these reports suggests that there could be different mechanisms which are involved in the polyploidization of MKs [120,121]. The defect in cleavage furrow formation and chromosomal segregation, or different cell cycle regulators may involve in the process of polyploidization of MKs. The biological importance of endoreduplication was established from the facts showing direct relation between MK ploidy and the number of platelets.

### 1.11.2. Cytoplasmic and surface markers

Other than polyploid multilobulated nucleus, cellular maturation of MKs is also distinguished by accumulation of characteristic surface antigens (CD41, CD61, and CD42b; Figure 1.14) which are involved in platelets activation and platelet aggregates formation, a cytoplasmic demarcation membrane system which is believed to participate in platelet formation, and platelet organelles accumulation (dense granules and organelle granular proteins such as platelet factor 4 (PF4) and vWF [122]. Reports demonstrate that the polyploidization is not closely synchronized with the cytoplasmic maturation. Different levels of ploidy are observed in each stage of MK development; therefore, it is difficult to distinguish the early stage genes from late stage genes [123].

## 1.12. Megakaryocytopoiesis: A molecular perspective

In humans, the normal platelet counts within an approximately 3-fold range ($150 \times 10^3$ to $450 \times 10^3$ per cubic micrometer) are regulated by sustained hematopoiesis. However, in different disease

conditions MKs increase platelet production. Megakaryocytopoiesis is regulated at multiple levels by different growth factors/cytokines: GM-CSF, IL-3, IL-6, IL-11, IL-12, and erythropoietin (EPO) stimulate growth/proliferation of MKP [124]. Functions of other cytokines such as IL-1α and leukemia inhibitory factor (LIF) were also noticed as the modulators of MK maturation and platelet release [124,125]. Many of these cytokines observed with their differential effects on different hematopoietic lineages. Presently, for the treatment of thrombocytopenia, IL-11 (Neumega) is the only clinically approved drug [126]. TPO (cMpl ligand) was discovered and cloned more than 10 years ago in 1994 and identified as a potent and relatively more MK/platelet specific cytokine [127].

In different studies, it has been demonstrated that TPO promote megakaryocytopoiesis from HSCs precursors [128-135]. Most of the cells of hematopoietic system, such as HSCs, CMPs, and MEPs, express c-mpl, which may allow TPO induced MK differentiation from all these precursors [65]. TPO mediated regulation of megakaryocytopoiesis is the result of a feedback loop; TPO constitutively produced by the liver cells and its plasma levels are regulated by circulating platelets in a c-mpl–dependent manner (sequestered by circulating platelets). Reduction in blood platelet numbers in disease condition leads to increased levels of circulating TPO; Increased TPO eventually exert its stimulatory effects on HSCs in BM and increases megakaryocytopoiesis and blood platelets [136-138]. TPO/c-mpl complex initiate multiple signal transduction pathways, such as JAK2 (Janus Kinase), STAT3 (signal transducer and activator of transcription)/STAT5, MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinases), and PI3K (phosphoinositide 3-kinase)/AKT (protein kinase B) [139-142] (Figure 1.15). The TPO/c-mpl induced activation of JAK/STAT and other pathways results in the activation of MK specific TFs, eventually the expression of MK-specific genes. Different research groups with TPO and c-Mpl knockout mice observed a global decrease in HSCs, with a drastic reduction in MKs and platelets [67], however these knockouts are able to make platelets in sufficient number. On the other hand, humans with complete loss of function-cMpl observed with a median platelet counts, suggesting the functional role of TPO-independent pathways in megakaryocytopoiesis in patients without TPO functions or without cMpl mediated signaling [143]. Finally, though TPO/cMpl signaling is

crucial for megakaryocytopoiesis, it does not have essential role in the terminal stages of MK maturation and proplatelet formation and platelet release.

The stromal cell-derived factor 1 (SDF1), also called C-X-C motif chemokine 12 (CXCL12); in humans, SDF1 is encoded by the CXCL12 gene and have strong effects on megakaryocytopoiesis and homing HSCs to the BM during fetal development [144]. It has been reported that SDF1 stimulate megakaryocytopoiesis by enhancing the chemotactic activity of their progenitors via SDF1/CXCR4 receptor pathway [145,146]. Avecilla et al. [70] proposed that the chemotactic activity of SDF1 may be important for the movement of MKPs from osteoblastic niche (site of MKP proliferation) to the vascular niche (site of terminal maturation and thrombopoiesis) for platelet formation; interestingly, the infusion of SDF1 only can restore blood platelet in $TPO^{-/-}$ and in $cMpl^{-/-}$ mice. Previously, it has been proposed that the infusion of DF1 mimetic drugs may improve the platelets production in HIV induced thrombocytopenia by competing with the HIV virus for its MK receptor CXCR4 [147,148].

PF4 also called chemokine (C-X-C motif) ligand 4 (CXCL4), PF4 is a platelet α-granule protein which inhibits MK development and maturation in vitro [149]. Other closely related chemokines such as platelet basic protein (PBP; CXCL7), RANTES (CCL5) and ENA-78 (CXCL5) were also observed with their negative effects on megakaryocytopoiesis [150,151]. In diriment studies it has been observed that the discharge of chemokines during chemotherapy and radiation therapy may contribute to thrombocytopenia, and a strategy that inhibits this process can be applied to reduce the cases of chemotherapy and radiation therapy induced thrombocytopenia.

### 1.12.1. Transcription factors involved in megakaryocytopoiesis

HSC fate and MK differentiation are largely coordinated by the sequential expression of various TFs (Figure 1.15); HSCs and MKs also have similar expression of many TFs [For example, SCL, RUNX1, GATA2, ETS] involved in HSC fate decision, adult HSC maintenance, and MK differentiation [152-156]. TFs play important role in the process of lineage selection from HSC to MKs. In MK differentiation process, the myeloid commitment is the first step, where at the CMP juncture a balanced expression of antagonistic TFs, GATA1 and PU.1 influence CMP skewing

towards the MEP and GMP, respectively [27,157,158]. Further, MEP commitment to MK and erythrocyte is regulated by the coordinated expression and function of various TFs, they show their effects in time- and dose-dependent manner. Of these, nuclear factor erythroid-derived 2 (NF-E2), transcription factor stem cell leukemia (SCL), GFI1B (growth factor independent 1B transcription repressor), GATA-1, GATA-2, KLF1 (Kruppel-like factor 1), and ETV6 (ETS variant transcription factor 6), are all expressed in the MEP; on the other hand, TF Erythroid Krüppel-like factor (EKLF/KLF1) and c-Myb are expressed in the erythroid lineage, and FLI1 (Friend leukemia integration 1 transcription factor) and RUNX1 are exclusively expressed in MK lineage [159-162]. GATA2 and GATA1, along with cofactor FOG1, observed with an antagonistic expression in the MEP, GATA2 promotes megakaryocytopoiesis, whereas GATA1 promotes erythropoiesis [163-166]. But their functions are not exclusive; such as GATA1 functions are largely studied in erythropoiesis for the activation of globin gene expression, however it is also essential for megakaryocytopoiesis (for the transcription of MK/platelet receptor CD41) [164,167,168]. RUNX1 actively function as an inhibitor of erythroid differentiation program, however it is crucial for megakaryocytopoiesis; RUNX1 represses KLF1 (erythroid-specific transcription factor), thus allowing a shift in the ratio of increased MK-specific FLI1 to erythroid-KLF1 and promoting MK lineage differentiation [160-162]. During later stages of MK development, RUNX1 play important role in polyploidization and cytoskeleton reorganization for the proplatelet release by directly inhibiting and activating the expression of myosin heavy chain (MYH10) and myosin light chain (MYL9), respectively; both the polyploidization and cytoskeleton reorganization are the crucial events for MK maturation [117,169]. Further, FLI1 has also been reported for its functional role in megakaryocytopoiesis by regulating the expression of megakaryocytic receptors/surface antigens, GPIX (GPIX/CD42a) and CD42b [170,171]. Furthermore, NFE2 transcription factor has also been noticed for its functions in late stages of megakaryocytopoiesis. In different studies, mice lacking NFE2 observed with normal proliferation, but platelet production was affected [172,173]. Further, studies defined that this effect is owing to the absence of NFE2 regulated microtubule component β1-tubulin [174]. In conclusion, the functional importance of these TFs, including GATA1, RUNX1, FLI1, GFI1b, and ETV6, was realized in inherited thrombocytopenia instances in different mutant backgrounds [175-185]. Interestingly, most of these genes were originally

discovered in congenital thrombocytopenia, and functionally analyzed in MK and platelets for their role in megakaryocytopoiesis and thrombopoiesis.

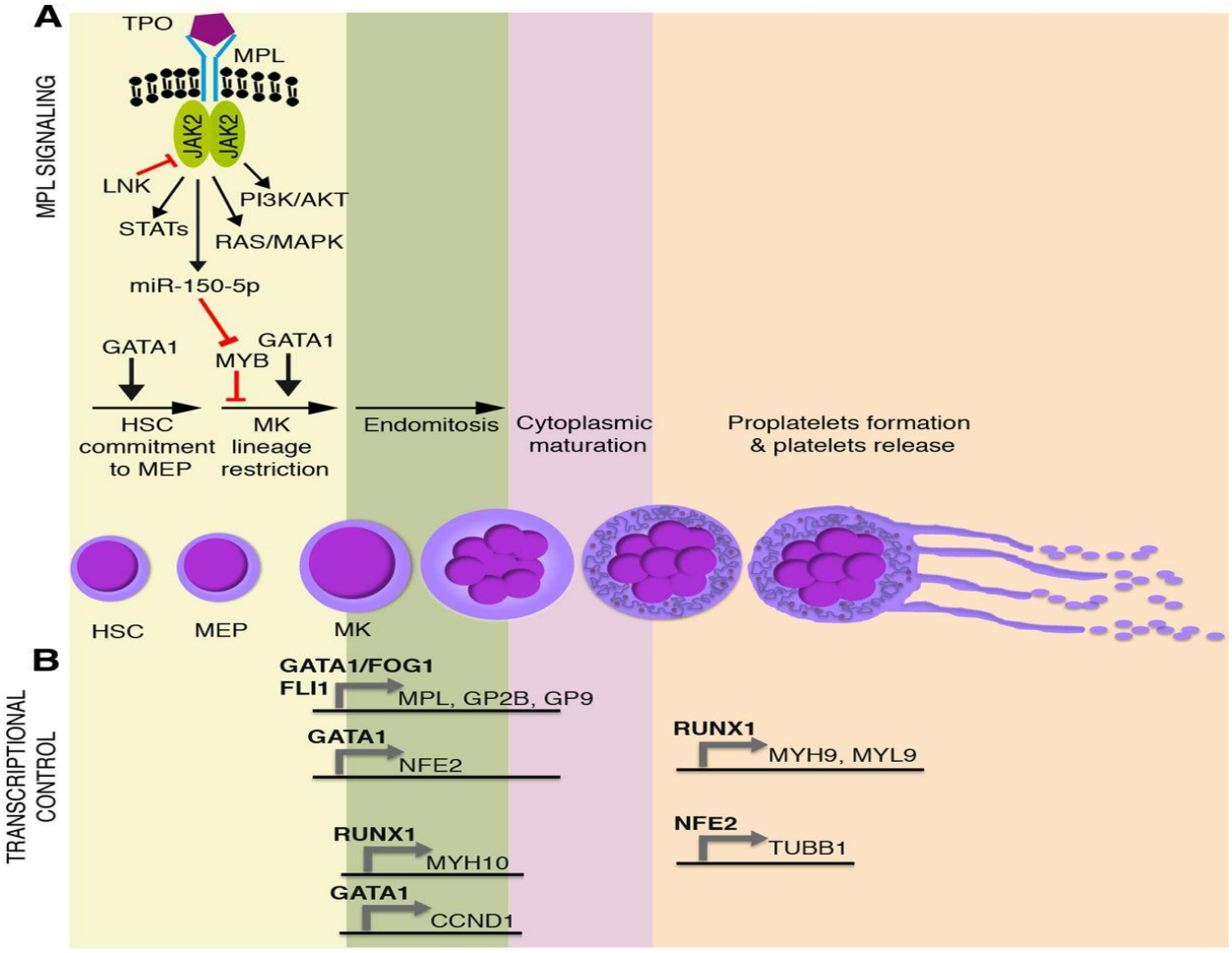



**Figure 1.15. Different extrinsic and intrinsic factors involved in megakaryocytopoiesis. Adapted from, Bianchi et al. [186]**.

## 1.13. An overview of microRNAs

After the first discovery of small non-coding RNA (microRNA, also abbreviated miRNA), a new era in molecular biology arose. There are now over 2694 mature miRNAs that have been annotated in the human genome and every year the number of identified human microRNAs is increasing (http://www.mirbase.org/). miRNAs are highly conserved small non-coding RNAs of approximately 22-nucleotide in length. It is believed that all miRNAs coercively regulate one third

of the genes in the human genome. Recent studies have uncovered the functional role of different miRNAs in normal hematopoiesis; moreover, miRNAs have also been liked to may hematologic diseases [187-189].

All miRNA families follow multiple processing steps that convert the immature miRNA (primary miRNA) transcript into the active, 20-23 nucleotide single stranded mature miRNA, different nuclear and cytoplasmic proteins are involved in this process.

### 1.13.1. Transcription

Genes encoding miRNA sequences are located throughout the human genome [190], many of them are non-coding genes whose transcriptional product generates only miRNA. In other cases, the miRNA encoding sequences are located within introns and untranslated regions (UTRs) of different protein coding genes [191,192]. The defining features of all genes generating miRNA is the stem-loop structure of their precursor RNA, where one or both the strands of the stem becomes functional mature miRNA [193-195]. For example, a three-miRNA precursor encoding cluster is located within intron region of the protein coding gene MCM7, this cluster generates three distinct miRNAs (miR-106b, miR-93, and miR-25) with a unique target set [196]. However, miRNAs coding sequences are not generally located within coding exons. Primary miRNA transcript (pri-miRNA) from the miRNA host gene is generated by RNA polymerase II [197,198]. Further, the pri-miRNA (immature miRNA) is spliced, capped, and polyadenylated, the process of the pri-miRN processing is similar to that of protein coding mRNAs [199].

### 1.13.2. Processing by Drosha and Dicer

The pri-miRNA transcript involves two endogenous processing events to becomes a biologically active/functional miRNA, (Figure 1.16) [200]. The first processing event occurs in nucleases, where the endogenous nuclease Drosha cleave the stem-loop region during or after the transcription of pri-miRNA transcript [201-204]. The RNA binding protein DGCR8, which is associated with Drosha, is required for the cleavage of the pri-miRNA transcript [205-207]. The stem-loop precursor generated by Drosha is exported out of the nucleus by Exportin5 in a Ran-GTPase dependent manner [208-210]. After the transport of precursor miRNA from nucleus, the

second processing event starts in cytoplasm, where the loop region of the precursor miRNA is cleaved by endonuclease Dicer [211-214] which is associated with an RNA binding protein TRBP [215-219], eventually the mature miRNA is released. Further, the product of Dicer, a duplex RNA of about 21 nucleotides is participate in RISC (RNA inducing silencing complex). Where one strand of the duplex is loaded into the RISC, and the other strand, which is called the star strand, is typically degraded (Figure 1.16).

the star strand is designated by an asterisk (star) to the miRNA name, e.g. miR-145*. On the other hand, in case of some miRNAs both the strands may participate in the formation of RISC with similar frequency. Here the strand from the 5' end of the stem-loop is termed miRNA- "5p" and the 3' strand is termed "3p". I fact, at the time of loading the RISC may favor the incorporation of one strand. Recently, with the help of next generation sequencing (NGS) methods it has been proved that of all miRNA families a small fraction of star strand is also loaded in RISC [220]. Furthermore, some miRNAs use different strand depending on cell type and biological state [221]; because of all these reasons, 5p/3p naming scheme is mostly used instead of the arbitrary mature/star nomenclature.

Although the nucleotide sites cleaved by Drosha and Dicer miRNA processing enzymes are narrowly defined, leading to produce mature and biologically active miRNAs with clearly demarcated terminal ends. Moreover, some of the miRNAs also have heterogenous cleavage sites, which results in the biogenesis of multiple "isomiRs" of the mature miRNA [222,223]. This heterogeneity allows to differential target repression and distinct biological functions. This target preference is cell type specific and may switch in different disease conditions [221,224,225]. Currently, the molecular mechanism involved in isomiR switching is unclear.

### 1.13.3. RISC loading and target repression

The miRNA after Dicer cleavage is incorporated into RISC (Figure 1.16). The exact composition of the RISC protein complex is not clearly defined, the protein of RISC complex which play the central role in the RNA silencing process is the Argonaut (Ago), of which four family members (Ago 1-4) have been identified and characterized in humans [226,227]. The miRNA duplex which

is loaded into RISC (miRISC) is further enter in a process where the star-strand is removed by different mechanisms. One mechanism of star-strand degradation is based on the miRNA duplex complementarity in the central region, if complementarity is there in the central region of duplex, the star-strand is cut by Ago2 and further completely degraded by the C3PO nuclease complex [219-230]. This is also the mechanism for siRNA RISC loading pathway. However, most of the miRNA duplexes lack complementarity in their central region, therefore, they cannot allow star-strand cleavage pathway. These miRNAs duplexes rely on the activity of several helicases for star-strand unwinding [231-235].

For the miRNA target interactions Ago play key role, it directly binds the mature miRNA sequence and seeks for the target mRNA that have nucleotide sequence complementarity to the sequence of mature miRNA. Specifically, 2-7 nucleotide sequence of mature miRNA (abbreviated “seed”) are involved in the interactions with target mRNA [236-238]. However, it has been observed that the 3’ end of the mature miRNA can also involve in the target recognition and interactions, and centrally matched targets have also been identified [239-242]. The interactions of miRNAs with centrally matched sequence of 9-11 nucleotide results in the cleavage of target via the endonuclease activity of Ago2 [243,244]. However, most of the miRNA targets in human cells lack this central sequence match, therefore Ago2 cannot cleave such targets. Rather, Ago-protein is recruited to a protein complex of GW182 family proteins (TNRC6A/B/C) within cytoplasmic foci termed P-bodies, leading to translational repression of target mRNA. After the miRISC binding the CCR4-NOT deadenylase complex is recruited to miRISC and start the removal of the Poly(A) tail, eventually the process of target mRNA degradation starts [245-249].

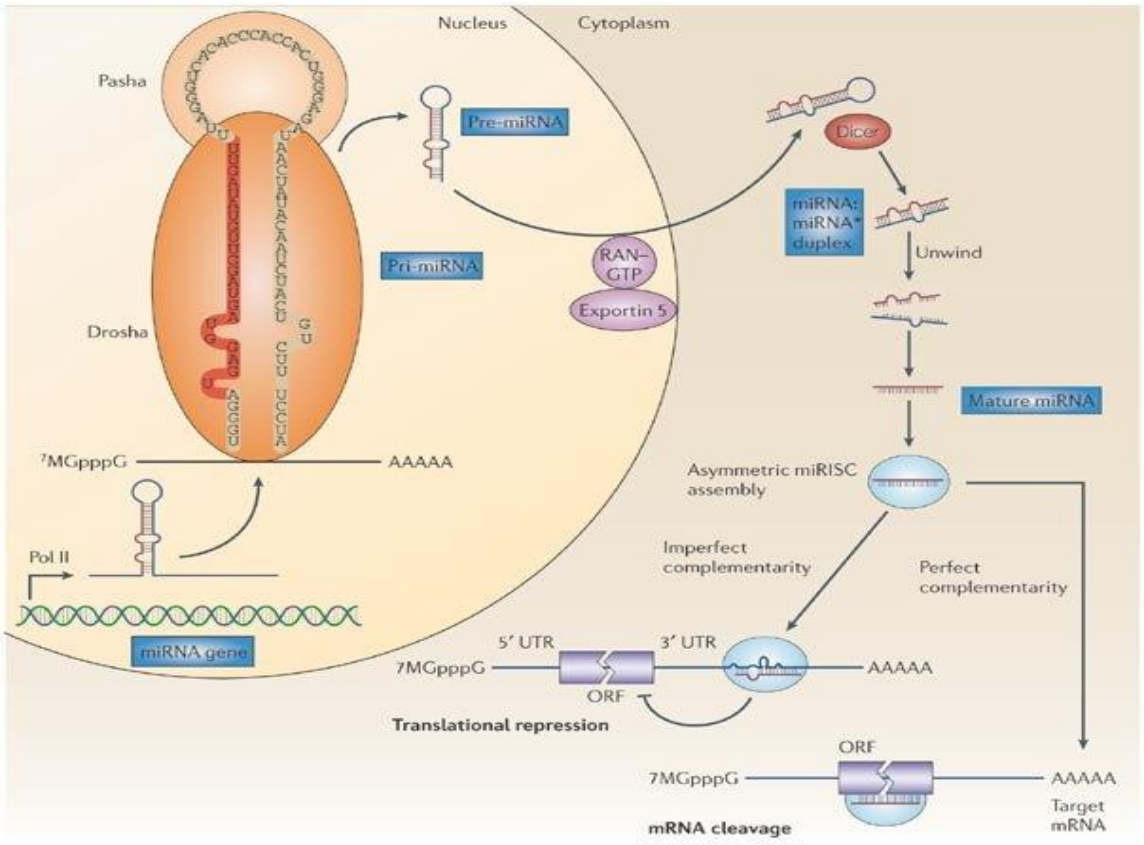



**Figure 1.16. The biogenesis of microRNAs. Adapted from, Esquela-Kerscher and Slack, [249].**

### 1.13.4. microRNAs involved in megakaryocytopoiesis

As we have discussed earlier that mostly human miRNAs bind to 3' untranslated regions (3'UTRs) of their target mRNA and inhibit the translation of target mRNA [250,251]. Actually, miRNAs are well known for their ability to fine-tune the protein levels, and this ability make them excellent candidate to regulate many complex biologically process. Indeed, recent advances in our knowledge of miRNA functions have established the notion that miRNAs are critically important regulators of many aspects of normal development and disease [252].

Usually, different studies in MK development biology are primarily focused on different cytokines and TFs involved in MK physiology and development biology, however over the last decade, researchers have also unveiled molecular functions of different miRNAs in MK physiology [253] and MK development Biology [187-189]. In several recent reports, the functional role of different

miRNAs in megakaryocytopoiesis has been established, the most abundant miRNAs in MKs are listed with their targets and functions in Table 1.3 and Table 1.4 [189].

**Table 1.3. miRNAs involved in megakaryocytopoiesis and developmental maturation of MKs. Adapted from, Raghuwanshi et al. [189].**

| | | |
|---|---|---|
| **MicroRNAs involved in earlier steps of megakaryocytopoiesis** | | |
| miR-150 | Favours MEPs commitment to the MKs lineage by regulating cMyb | [8] |
| miR-155 | Inhibits megakaryocytopoiesis by targeting Ets1 and Meis1 | [13] |
| miR-34a | miR-34a promotes megakaryocyte differentiation of CD34+ cells by regulating MYB expression | [21] |
| miR-223 | miR-223 expression increases during megakaryocytic differentiation of CB-CD34+ cells, it regulates LMO2 | [22] |
| miR-486-3p | Affects HPCs commitment by regulating MAF, it supports the erythropoiesis while restraining the megakaryocytopoiesis | [24] |
| **MicroRNAs involved in later steps of megakaryocytopoiesis** | | |
| miR-146a | Observed with down-regulation when human CB derived $CD34^+$ cells induced to differentiate into MKs, and forced over-expression resulted in reduced ploidy levels. | [25] |
| miR-142-3p | miR-142-3p promotes MK maturation and platelet release, it targets a battery of actin cytoskeleton regulators to facilitate proplatelet formation | [29] |
| **MicroRNAs involved in developmental megakaryocytopoiesis** | | |
| miR-9 | A constitutive reduced expression of miR-9 is observed through MK ontogeny. miR-9 maintain low expression levels of CXCR4 and RUNX1 in neonatal MKs. | [30] [31] [34] |
| Let-7b | Let-7b was observed with lower expression in neonatal MKs as compared to adult MKs. Lower expression levels of let-7b in neonatal MKs contribute to higher proliferative potential of neonatal MKs. | [41] |
| miR-99a | Observed with higher expression levels in CB CD34+ derived as compared to adult CD34+ derived MK. The high levels of miR-99a maintain the high cell proliferation potential of neonatal MKP by regulating CTDSPL. | [39] |

**Table 1.4. Studies on molecular targets & function of miRNAs in MK. Adapted from Raghuwanshi et al. [189].**

| miRNA | Targets | Function | |
|---|---|---|---|
| miR-150 | cMyb | Favours MEPs commitment to the MKs lineage | [8] |
| miR-155 | Ets1 and Meis1 | mR-155 inhibits megakaryocytopoiesis by targeting Ets1 and Meis1 | [13] |
| | | Sustained expression of miR-155 in HSCs causes MPD | [19] |
| | | Forced over-expression of miR-155 inhibites K562 differentiation into MKs | [12] |
| miR-146 | CXCR4 | miR-146a expression is down-regulated when human CB derived $CD34^+$ cells induced to differentiate into MKs, MiR-146a over-expression impaired megakaryocytopoiesis | [25] |
| | | miR-146a was up-reguled when human and murine HSCs were induced to differentiate into MKs, and forced over-expression observed with no significant effects on megakaryocytopoiesis | [26] |
| | | Knock-down of miR-146a in mouse HSPCs observed with increased platelets in the blood and MKs in the marrow. over-expression of miR-146a noticed with no significant effect on platelet and MKs, | [27] [28] |
| miR-9 | CXCR4 and RUNX1 | A constitutive reduced expression of miR-9 through MK ontogeny was noticed | [30] [31] [34] |
| | | Pre-miR-9 transfection in Meg01 and Dami cells observed with lower levels of megakaryocytic marker-CD61 and increased rate of cell proliferation | [34] |
| miR-34a | MYB | miR-34a is up-regulated during PMA induced megakaryocytic differentiation of K562 cells. Enforced expression of miR-34a promotes MK differentiation of CD34+ cells. | [21] |
| | MEK1 | miR-34a inhibits cell proliferation by targeting MEK1 during megakaryocytic differentiation of K562 cells. | [20] |
| miR-130a | MAFB | miR-130a is down regulated in differentiated MKs derived from CD34+ bone marrow cells, and targets MAFB. | [7] |
| miR-10a | HOXA1 | miR-10a is down regulated in CD34+ bone marrow progenitor derived MKs, and targets HOXA1. | [7] |
| miR-27a | RUNX1 | During megakaryocytic differentiation of K562 cells Runx1 and miR-27a are engaged in a regulatory interplay involving positive regulation of miR-27a expression by Runx1. | [44] |
| miR-181 | Lin28 | miR-181 targets Lin28, which is a negative regulator of Let-7 miRNAs. Over-expression of miR-181 during TPA induced megakaryocytic differentiation indirectly increased the Let-7 and enhanced differentiation. | [40] |

Our recently published articles have discussed different miRNAs, their targets, and their functional significance during megakaryocytopoiesis [187-189]. However, there are numerous aspects of miRNA function and development remain to be addressed in MKs. For instance, temporal regulation of miRNAs expression, role of evolutionary conserved miRNAs in the process of MKs development. Considering recent findings, we propose that the developmental stage specific expression of different miRNAs might regulate different cell-intrinsic factors and developmental differences between neonatal and adults MKs.

### 1.13.5. MicroRNA-9

MicroRNA-9 (miR-9) is one of the miRNAs with evolutionary conserved nucleotide sequence. The nucleotide sequence of matured miR-9 is recognized for high conservation though evolution. The sequence of mature miR-9 is identical in insects and humans (Figure 1.17) [252]. Out of 22 vertebrate species identified with the expression of miR-9, 17 have multiple copies of miR-9 encoding gene (http://www.mirbase.org/). It has been noticed that the mature sequences of all vertebrate miR-9 orthologs are identical except miR-9 sequence of Xenopus tropicalis. However, between invertebrates, the sequence of mature miR-9 is less stringently conserved. The mature miRNA-9 sequence of 30 invertebrates annotated in miRbase are identical to miRNA sequences in vertebrates (named as miR-9a). The sequence of miR-9 in 22 invertebrates differ in their mature sequence from human miR-9 sequence, however their precursors show similarities. Like invertebrates, vertebrate miR-9 precursors also generate a functional miR-9 strand (miR-9-5p) and a miR-9-star strand (named as miR-9*) that may function as a functional miRNA (miR-9-3p) (Figure 1.17). It has been observed that both the miR-9-5p and miR-9-3p strands may function in both the vertebrates and insects [254], however miR-9-5p has been well studied for its functions in human [252].

Despite the sequence conservation, diverse expression patterns of miR-9a have been observed in different model systems. At early embryonic development of Drosophila, the expression of miR-9a appears in most of the epithelial cells except those found in ventral ectoderm. However, at stage 12, the expression of miR-9a appears in the ectoderm but not in cells of the central nervous system

[255,256]. On the other hand, in vertebrates, the expression of miR-9 seems to be confined to the nervous system.

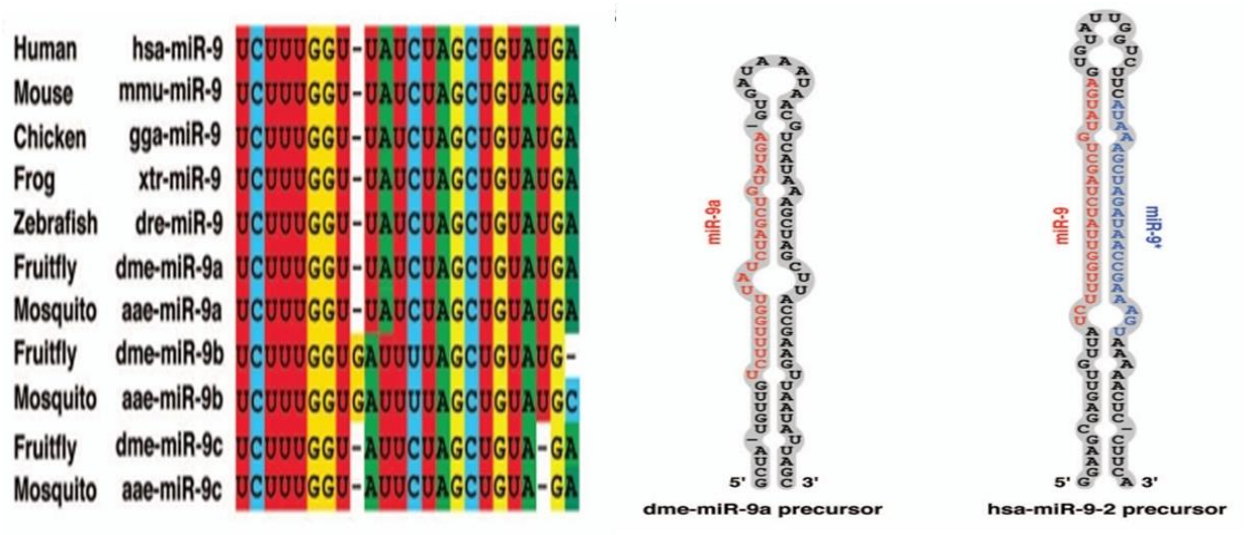



**Figure 1.17. Evolutionary conservation of miR-9 sequences and related miRNAs. Adapted from, Yuva-Aydemir Y et al. [252].**

In Drosophila miR-9 is involved in defining the neurogenic boundaries. In vertebrates, miR-9 is highly expressed in neuronal progenitor cells (NPCs), and its context-dependent functions in the regulation of proliferation, differentiation and apoptosis have been extensively reported by many research groups in different vertebrate model systems. It has been reported that miR-9 reduces the proliferation of adult-NPCs by down-regulating the orphan nuclear receptor TLX, which is involved in the regulation of neural stem cell self-renewal [257]. However, in other study, miR-9 has been reported to increases the proliferation of NPCs derived from cultured human-embryonic stem cells (hESCs) at the neurosphere stage [258]. In mammals, miR-9 is encoded by three genes. In earlier studies it has been confirmed that the development of miR-9-2 or miR-9-3 knockout mice is completely normal, however miR-9-2/3 double-knockout mice die after birth within a week [259]. These reports show the functional significance of miR-9 in the development process by regulating cell proliferation. On the other hand, the overexpression of miR-9 reported with the positive effects on neuronal differentiation in adult NPCs, and this function is mediated by the downregulation of TLX. miR-9 function in apoptotic cell death is a context-dependent feature.

Down regulation of miR-9 significantly increase the apoptosis in NPCs of the forebrain but not the hindbrain of stage 30 Xenopus embryos [260]. The recent report proposed that miR-9 specifically affect apoptosis in the forebrain likely though P53 pathway [260].

miR-9 is one of the most highly expressed miRNAs in brain tissues of vertebrates. However, current studies have observed the miR-9 expression in different other body organs and tissues; furthermore, miR-9 activity is highly versatile, exerting different and opposite functions in a species, tissues, and cellular-specific context, while maintains high conservation of sequence. The duplication of miR-9 sequence produces five different copies in Drosophila genome (miR-9a-b-c, miR-79, and miR-4), whereas in human genome only three copies (miR-9-1, miR-9-2, and miR-9-3) are present. The presence of multiple copies may be linked to the functional specificity between there copies. Chen X et al. [261] have observed that miR-9 is highly expressed in glioma specimens and cells, and enhance the rate of glioma cell proliferation, migration and invasion. They have also proposed that miR-9 could be secreted from glioma cells via exosomes and these miR-9 loaded exosomes observed by vascular endothelial cells, leading to an increase in the angiogenesis of glioma cells. In this study, MYC and OCT4 TFs were identify as the activators of miR-9 expression and COL18A1, THBS2, PTCH1 and PHD3 were verified as the targets of miR-9. On the other hand, in breast cancer cells, MiR-9 displays tumor-suppressor like activity by regulating its targets, including MTHFD2 which can recapitulate the anti-proliferative effect of miR-9 [262]. Further, in different blood cells also miR-9 has been reported with different functions. Bazzoni F et al. [263] reported that human monocytes and neutrophils exposed to proinflammatory signals (TLR4-activated NF-κB) rapidly increases the expression of miR-9 that initiate a feedback control on the NF-κB-dependent responses.

Interestingly, miR-9 has also been reported to play a critical role in the regulation of tumor-associated immune response via affecting the differentiation and function of Myeloid-derived suppressor cells (MDSCs) [264]. MDSCs are responsible for tumor-associated immunosuppression, thus affecting immunotherapies for cancer. They have reported that the forced inhibition of miR-9 promotes the differentiation process of MDSCs and reduces their immunosuppressive function; they have also reported that the down regulation of miR-9 in mice

reduces the growth of Lewis lung carcinoma tumor. Whereas, its overexpression enhanced the immunosuppressive function of MDSCs. Further, they identified that the miR-9 regulates MDSCs differentiation by targeting the Runx1, an essential TF in regulating the differentiation and function of MDSCs. To investigate whether Runx1 is a direct target of miR-9, they have performed 3′UTR luciferase reporter assays. Runx1 3'UTR have two seed sequences; this research has identified that both the seed sequences 3'UTR (1+2) contribute in the regulation of Runx1 by miR-9. Taken together, their research has identified miR-9 as a crucial player in regulating the differentiation and function of MDSCs via direct targeting Runx1 expression [264].

### 1.14. RUNX1

The RUNX1 belong to a family of heterodimeric TFs, The PEBP2/CBF TFs, which are well recognized for their functions in cell fate decisions and terminal differentiation of cells of different lineages [265,266]. The TFs of this family composed of two subunits: a DNA-binding α-subunit (RUNX1, RUNX2, and RUNX3) and a non-DNA binding β-subunit (Core-binding factor-beta, CBF-β), which stabilize α-subunit and increase its DNA binding ability [267]. The evolutionary conserved domain (Runt homology domain of 128-amino acid, RHD) of RUNX involved in DNA binding and heterodimerization with the β-subunit (Fig. 1.18A) [268]. RUNX also interact with several other proteins which are involved in the activation, or repression of the gene expression (Figure 1.18B) [269].

RUNX1 is the most studied member of this family, and crucial for the development of definitive HSCs and the establishment of hematopoietic system in vertebrates. This gene is also known for its high mutation frequency in various hematological malignancies (blood cancers). Germline mutations in RUNX1, such as loss of function RUNX1 mutations are more frequent in familial platelet disorder with propensity to acute myelogenous leukemia (or FPD/AML), it is characterized by thrombocytopenia, platelet disfunction and a 35% lifetime risk for developing myeloid malignancies. Moreover, somatic mutations in RUNX1 and rearrangements of chromosomes involving RUNX1 are more commonly observed in acute myeloid and lymphoid leukemia, myelodysplastic syndrome (MDS), and chronic myelomonocytic leukemia (CMML) [270].

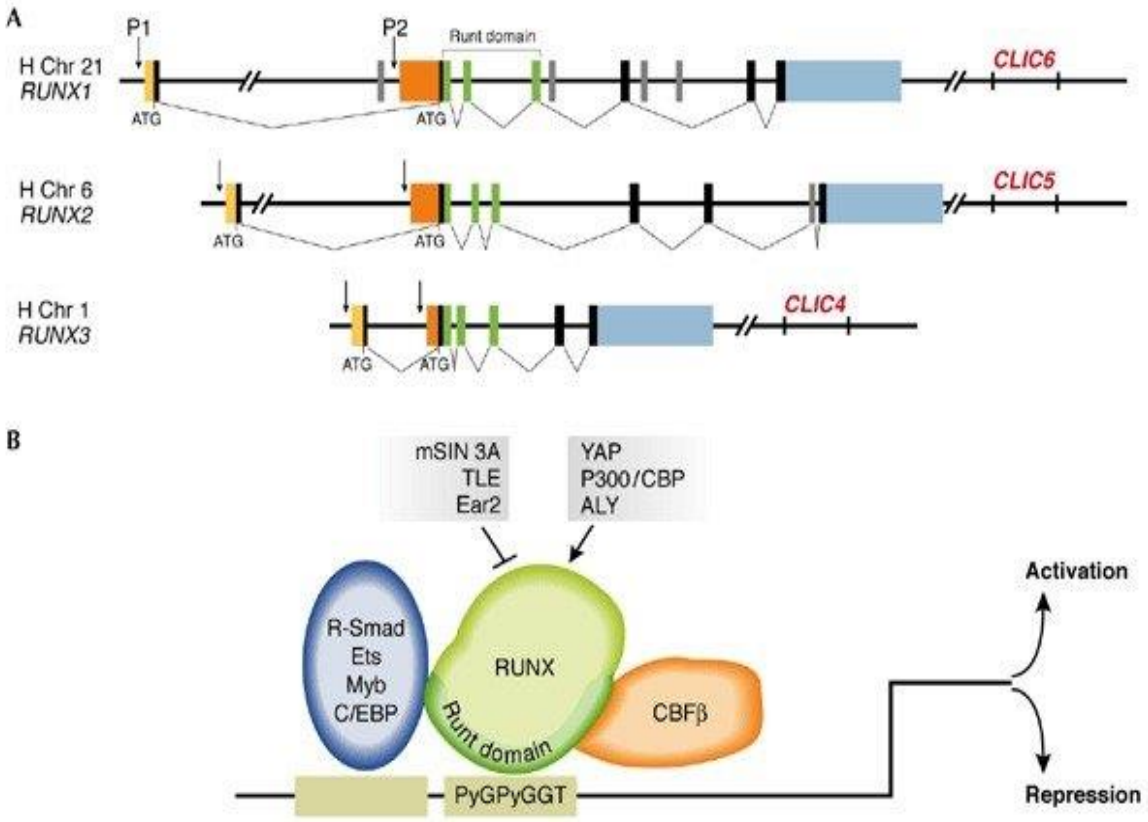



**Figure 1.18. The mammalian RUNX genes: structure and mode of function. (A). (B). Adapted from, Levanon et al. [269].**

The RUNX1 gene (~261 kb) is present on long arm of chromosome-21. Thee major isoforms (RUNX1A, RUNX1B, and RUNX1C) are transcribed by using two promoters and alternate splicing (Figure 1.19A). RUNX1A (250 amino acids) and RUNX1B (453 amino acids) isoforms are transcribed from the promoter 1 (P1, distal promoter) and the carboxyl-terminal of RUNX1A is smaller than RUNX1B (Figure 1.19A). whereas, RUNX1C (483 amino acids) isoform is transcribed from promoter 2 (P2, proximal promoter), and is identical to RUNX1B except 32 amino acids which are encoded by alternate exons at its amino terminus (Figure 1.19A). It has been commonly noticed that the expression of RUNX1 isoforms is mostly temporal and tissue specific, such as RUNX1C is expressed at the time of the emergence of definitive hematopoiesis, while RUNX1A and RUNX1B are expressed throughout the hematopoietic development [270,271].

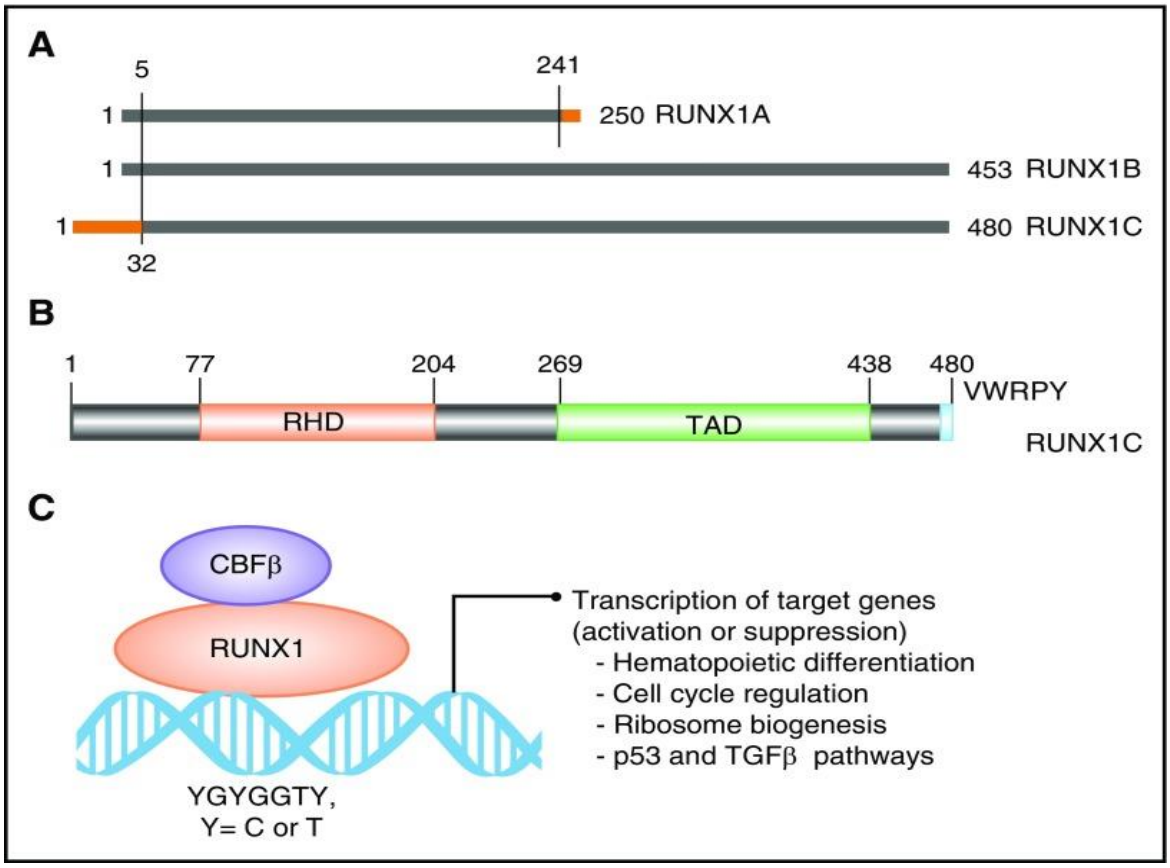



**Figure 1.19. Domain architect of RUNX1 protein and its role as a transcription factor.** (A) A schematic depicting the 3 major isoforms of RUNX1 (1A, 1B, and 1C). (B) Functional domain structure of RUNX1C (largest isoform) encoded protein: RHD and transactivation domain (TAD). (C) RUNX1 heterodimerization with its binding partner CBFβ; TUNX1 interactions with specific binding site (YGYGGTY, where Y is C or T) at target gene promoter. **Adapted from, Sood et al. [270]**.

All Three isoforms of RUNX1 share a conserved RHD of 128 amino acids (Figure 1.19B and C). On the other hand, RUNX1B and RUNX1C share another domain, termed TAD (transactivation domain), which consist of activating and inhibitory domains that interact with different activating and repressor proteins (Figure 1.19B). Also, the last 5 amino acids of RUNX1B and RUNX1C make a motif, termed VWRPY that interacts with TLE1 (Figure 1.19B) [272]. RUNX1 domains interact with different proteins and regulate the transcription of multiple genes involved in hematopoietic development and lineage selection, ribosome biogenesis, cell cycle control, and P53 and Transforming growth factor beta (TGF-β) signaling pathways [270,273,274].

## 1.15. TGF-β

The proTGF-β (LAP) and proLTBPs are folded and assembled in a complex in endoplasmic reticulum (ER). In this large latent complex (LLC) LTBP1 is associated with LAP via disulfide bonds. Further, TGFβ precursor (LAP) is cleaved inside the Trans-Golgi by furin proteases, but LAP and TGF-β remain bound with the help of non-covalent interactions. The TGF-β, LTBP, and LAP form the LLC, where LTBP may assist the proper folding of TGF-β. LTBPs originally, though to involve in maintaining TGF-β latency and its targeting to the extracellular matrix (ECM), but it has also been identified that LTBP1 participates in TGF-β activation with the help of integrins. LTBP1 may also function in the activation of TGF-β by other processes and factors in association with ECM. The active form of TGF-β released from latent complex can bind its receptors (Figure 1.20) [275].

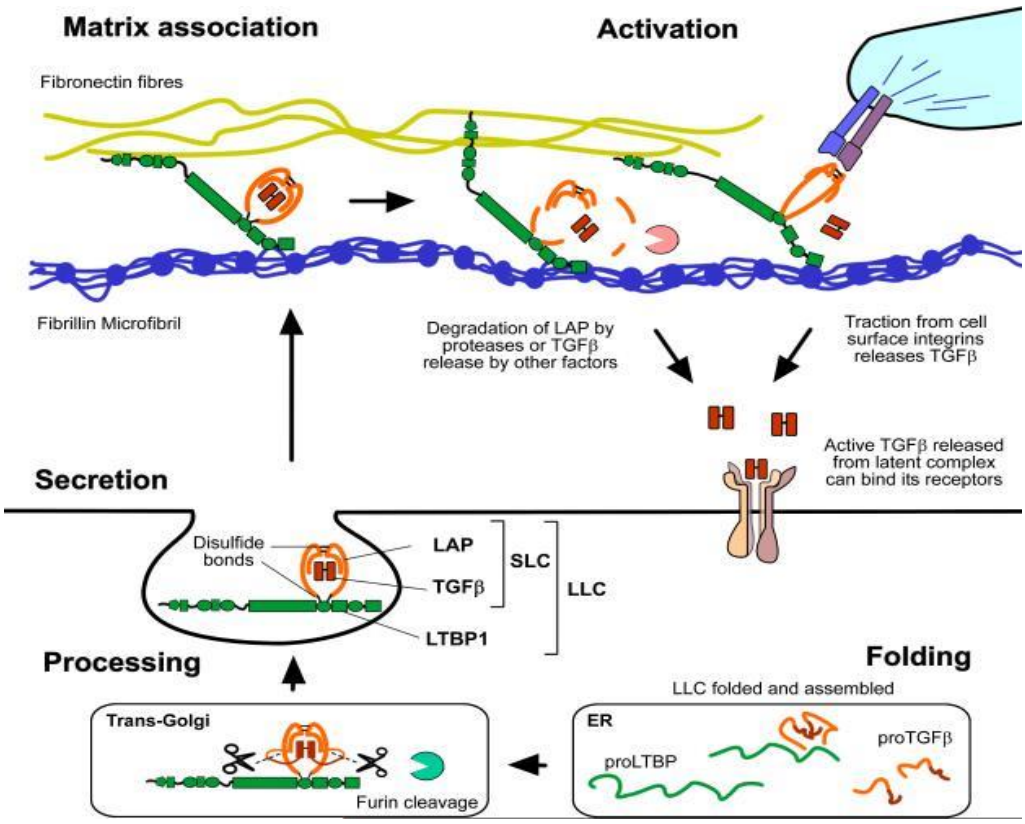



**Figure 1.20. TGF-β folding processing, secretion, and activation by integrins in association with ECM. Adapted from, Robertson et al. [275].**

### 1.15.1. TGF-β signaling pathways

TGF-β is a dimeric protein composed of two identical subunits of 112 amino acid. In humans, three separate genes encode three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3). TGF-β family members elicit diverse biological events, when the active TGF-β ligand interacts to their cognate cell surface receptors, followed by receptors activation as serine kinases, which then phosphorylate effectors or signal transducers. The canonical intracellular signaling is induced by TGF-β and mediated by receptor associated SMADs (R-Smads). Binding of TGF-β to its receptor type-II (TGF-βRII) in concert with the receptor type-I (TGF-βRI) leads to the formation of TGF-βRII/I complex and TGF-βRI phosphorylation. Subsequently, TGF-βRI phosphorylate SMAD2/SMAD3 (R-Smads) and sallow this complex to associate with SMAD4 (Co-Smad). Further, the complex of SMAD2/3 and SMAD4 move into the nucleus, where this complex associate with different other TFs and participate in the transcriptional control of gene expression (Figure 1.21) [276,277]. It has been observed that SMAD6/7 function as the inhibitory SMADs (I-Smads) [276,278]. Till now, about 50 TF, co-activators that interact with SMADs have been identified.

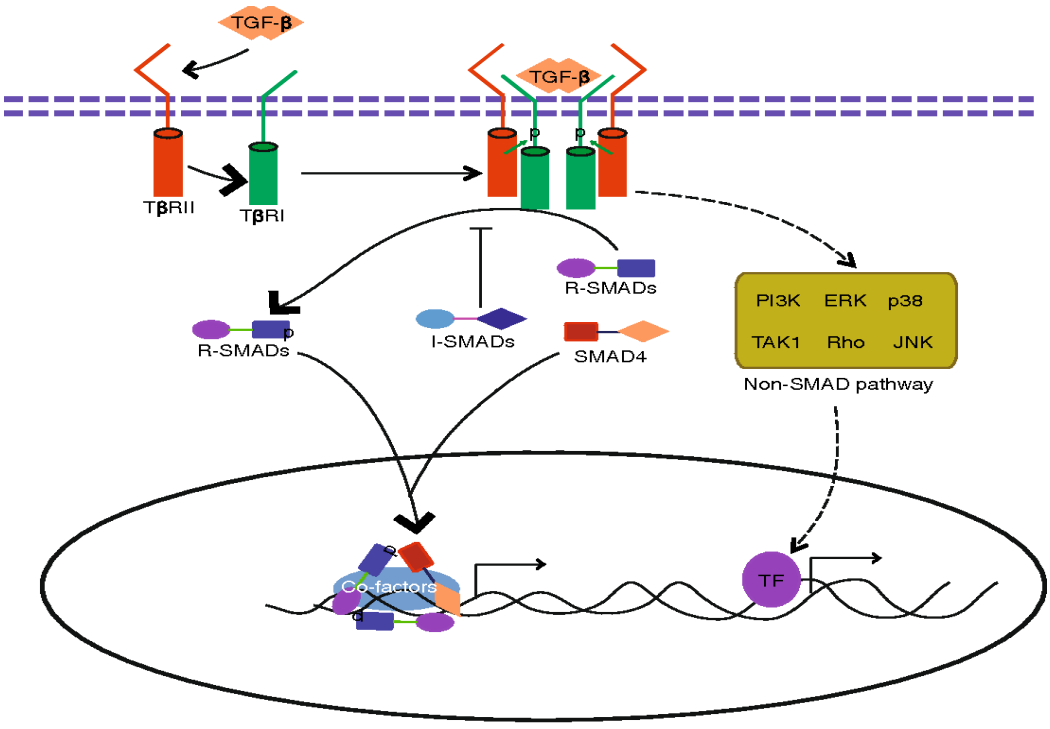



**Figure 1.21. A schematic representation of the TGF-β signaling pathways [Adapted from, Zhang J et al. [278].**

In addition to canonical TGF-βR\SMADs signaling pathway, there are various non-SMAD (called non canonical) pathways can also be activated by the TGF-βRs via direct interactions of pathway specific effector proteins with TGF-βRs or their phosphorylation. There are many non-SMAD pathways, e.g., mitogen activated protein kinases (MAPKs), nuclear factor κB (NF-κB), Rho-like GTPase, and phophoinositde 3-kinase (PI3K)/Akt pathways (Figure 1.21) [279-282].

### 1.15.2. Functions of TGF-β pathway

TGF-β/SMAD signaling pathway triggers diverse cellular responses such as, growth, differentiation, apoptosis, migration, and invasion/metastasis [283-289]. Also, the functional outcomes of TGF-β pathways are context-dependent and determined by cell, tissue, and cancer types [278].

It is well known that TGF-β is a pleiotropic cytokine that involved in the regulation of multiple cellular processes. Recent findings indicate that these effects may be due to activation of SMADs, or perhaps non-SMAD pathways that participate in crosstalk with SMADs. SMADs are well known for their regulatory effects on the transcription of different genes. TGF-β has been reported to increase intracellular free Ca2+ [Ca2+]i [290; 291]. Recently, Umbarkar et al. [292] reported that TGF-β canonical pathway is an important regulator of Ca2+ ion channels, in this study SMAD4 knock out in cardiomyocyte resulted in down regulation of several ion channels. Moreover, TGF-β has been shown to induce reactive oxygen species (ROS) production (superoxide anion and hydrogen peroxide) in a variety of cellular systems [293-298]. The TGF-β induced ROS generation is also linked with mobilization of Ca2+ [297-299]. Furthermore, the ROS generated by TGF-β regulated mechanisms has also been linked to the fibronectin expression and collagen mRNA accumulation [300,301]. These reports are suggesting that the regulation of the ECM production likely involve ROS upstream and/or downstream of the TGF-β action.

### 1.15.3. TGF-β signaling in the control of HSCs

TGF-β signaling has been identify as the critical mechanism in regulating HSC quiescence in BM. MKs secrete the bulk of latent TGFβ1 in the BM, TGF-β1 is a dominant signal emanating from MKs and non-myelinating Schwann cells that maintains HSC quiescence (Figure 1.22A)

[302,303]. In BM, TGF-β signaling regulates a wide spectrum of processes of the hematopoietic system. In HSC development process TGF-β has been identified with bidirectional effects such as, low concertation of TGF-β enhanced the proliferation of myeloid-biased HSCs (My-HSCs), while, on the contrary, the growth of lymphoid-biased HSCs (Ly-HSCs) is inhibited. On the other hand, the high concertation of TGF-β inhibits the proliferation of HSCs and induces HSCs quiescence, irrespective of HSC subtype. This regulation of HSCs development is regulated by SMAD2/3-SMAD4-dependent mechanisms (Figure 1.22B) [303].

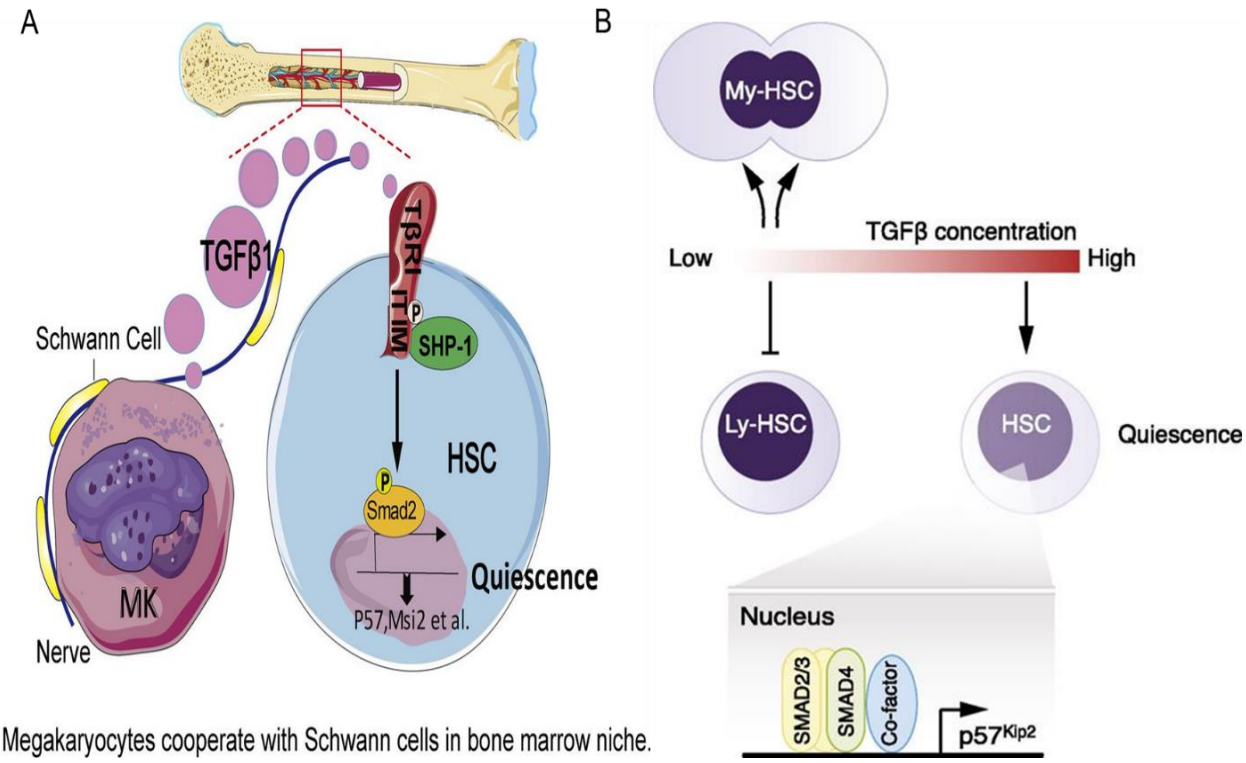



**Figure 1.22. (A) MKs and Schwan cells secrete the bulk of latent TGFβ1 in BM niche. Adapted from, Jiang et al. [302]. (B) Bidirectional effects of TGF-β. Adapted from, Blank and Karlsson, [303].**

### 1.15.4. TGF-β functions in MKs

It has been demonstrated in different reports that the TGF-β is majorly synthesized by MKs and stored in α-granules of MK and platelets [304]. Alitalo R et al. [305] also reported the enhanced expression of TGF-β during TPA induced megakaryocytic differentiation of K562 cells. The major role of TGF-β in platelets may be to regulate the activation of different components of the

connective tissue and well as the inflammatory and angiogenic factors during would healing after blood clotting. TGF-β1 has also been observed for its activity to enhance platelet aggregation and blood clot formation through its non-transcriptional effect on fibrinogen receptor [306]. A recent report published by Abbonante V et al. [307] shows that the synthesis and release of TGF-β1 is regulated by TPO induced pathways in adult CD34+ cells derived MKs. They have also identified that the released TGF-β1 may involve in the autocrine binding with its receptors and the activation of the downstream pathways. They have also noticed the functional effects of TGF-β1 in the production of ECM components such as fibronectin and collagen. Finally, they have demonstrated that the human MK produce and release ECM components, and their secretion is promoted by the autocrine activity of self-produced TGF-β1 under the control of TPO signaling (Figure 1.23).

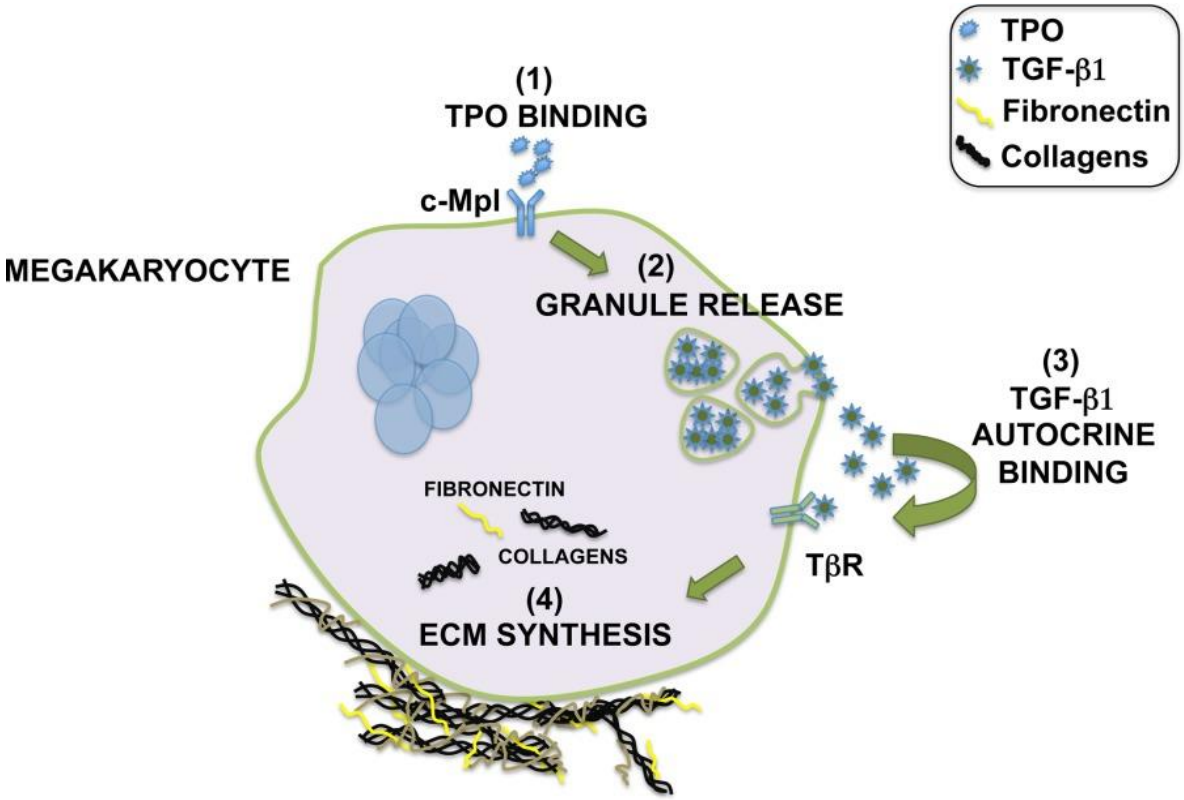



**Figure 1.23. TPO/TGF-β1 autocrine loop regulating ECM component synthesis in MKs. Adapted from, Abbonante et al. [307].**

There is one more recently published report by Wang Y et al. [308] shows the functional effects of SMAD4 knock out on platelets activities and blood clotting. They observed that the inactivation of TGF-β/SMAD pathway component SMAD4 impaired the platelet aggregation process, thrombus formation, and hemostasis. The platelets generated from the SMAD4 knock out MKs resulted in defective platelet activation, reduced platelet membrane spreading, and clot retraction. Finally, they have demonstrated that the DMAD4 knock in MKs reduces platelets aggregation by affecting integrin aIIbb3-mediated bidirectional signaling in platelets. However, to date, there are no reports which have discussed the existence of functional TGF-β pathway in MKs and its effect on the developmental maturation of MKs.

### 1.15.5. Functional relationship between RUNX and TGF-β signaling

It was reported that each of R-Smads interacts with RUNX1, RUNX2 and RUNX3. SMADs seems to interact with RUNX at different sites such as, C-terminal region, transcription activation domain, or Runt domain [277]. The molecular basis of the functional association between TGF-β receptors signaling and RUNX lies in the fact that SMADs and RUNX work synergistically to control the expression of target genes [277]. TGF-β regulates the expression of different genes including RUNX1 via TGF-βR/SMADs signaling [309,310]. On the other hand, RUNX1 is also involved in the regulation of TGF-β signaling in different cellular systems [277,311]. Specifically, in developmental hematopoiesis both the RUNX1 and TGF-β signaling are crucial regulators. RUNX1 is essential for the development of definitive hematopoiesis, definitive HSCs are not generated if RUNX1 function is lost [312,313]. In different studies, it has been observed that Runx1 knock results in massive hemorrhage in mice (at ~E12.5), and the hemorrhaging blood cells are nucleated red cells (primitive red cells), these primitive red cells are normally produced in the absence of Runx1 function [312,313]. It has been observed that the cause of this hemorrhage in Runx1 knock out mice is the angiogenic defect and abnormal development of blood cells, or lack of definitive HSCs that produce angiopoietin-1 [314]. On the other hand, TGF-β knock out mice were also observed with the defective hematopoiesis and vasculogenesis [315], which is similar to the RUNX1 knock out phenotype. However, a possible relationship between TGF-βR signaling and RUNX1 in hematopoiesis and megakaryocytopoiesis merits further examination.

## 1.16. Neonatal Thrombocytopenia

The platelets are generated from the cytoplasm of mature MKs, the key role of platelets is to prevent and stop bleeding (hemostasis) and promote both the processes of angiogenesis and wound healing [316,317]. During human pregnancy, platelets first appear in circulation at 5 weeks post-conception and increase in number during fetal development. The platelet count in circulation reaches a mean of $150\times10^9$/liter by the end of week 12 period of fetal development (the end of the first trimester of pregnancy), and the normal adult values (150 to $450\times10^9$/liter) by 22 weeks of pregnancy (the end of the second trimester). This means that even the smallest and immature infants generate platelets comparable to adults. More than 98% infants born at term to mothers with normal platelet count. Although, thrombocytopenia (platelet count $<150\times10^9$/liter) is found in up to 14% of premature infants (preterm infants), thrombocytopenia is also common among sick babies, It was recorded that more than 35% of babies admitted to the NICU (neonatal intensive care unit) are thrombocytopenic [318]. In 25% of cases, the platelet count reaches levels that are thought to significantly increase the risk of hemorrhage in neonates ($<50\times10^9$/liter). The majority are preterm babies (75–90%) born alive before 37 weeks of pregnancy, they develop early onset thrombocytopenia owing to the placental insufficiency/fetal hypoxia [319,320]. In fact, Preterm or low birth weight neonates have the highest incidence of intra-ventricular hemorrhage (IVH) of any age group [321].

Neonates are also affected by several disorders associated with platelet production process and their function, these disorders are predominantly or exclusively reported during this developmental age, including transient myeloproliferative disorder (TMD) [322], thrombocytopenia-absent radius (TAR) syndrome [323] and CAMT [324]. The exact reasons why these disorders selectively affect neonatal MK development and platelet biogenesis are unknown.

## 1.17. Developmental differences between neonatal and adult MKs

Although neonatal and adult MKs development process follow the same steps, there are substantial developmental differences between neonates and adults derived MKs. Specifically, neonatal MKs are smaller and of lower ploidy than adults MKs and produce fewer platelets compared to adult

MKs; in addition, neonatal MKPs are highly proliferative as compared to adults MKPs [325]. It has been confirmed that small MKs produce less platelets than larger MKs [325]. Based on these reports, it has been widely accepted that neonates maintain the normal count of circulatory platelets on the basis of the high proliferative potential of their MKPs.

It is also commonly observed that the adult MKs increase their mass in response to low blood platelet counts (or thrombocytopenia), but neonatal MKs show very weak response to low platelet conditions in neonates [318,326,327]. The adult BM MKP respond (within 3-5 days) to increased platelets demand by increasing size and ploidy followed by the increase in MK number [53,328]. This fast response by adult BM MKPs ultimately lead to a 2 to 8-fold increase in mass of matured MKs. However, different reports have demonstrated that thrombocytopenic neonates can increase the number of MKs, not the mass of matured MKs [326]. Different reports over the last two decades suggest that these Biological/developmental differences between neonatal and adult megakaryocytopoiesis likely contribute to the vulnerability of neonates to thrombocytopenia and associated disorders. Currently, the molecular mechanisms which regulates the developmental differences between neonatal and adults MKs are unclear, but neonatal vs adult hematopoietic progenitor cells transplantation into irradiated adult recipient (murine model) have demonstrated that both the BM micro-environmental regulators and cell-intrinsic factors contribute to the neonatal MK phenotype [329]. Of interest, the CB stem cells transplantation into adult BM have also recognized with significantly small BM MKs (for at least 3 months post-transplantation) than subjects transplanted with adult stem cells. Thus, these findings confirm that there is a strong contribution of cell-intrinsic differences in the regulation of MK mass and platelet production [330]. The molecular mechanisms underlying these cell-intrinsic differences are only beginning to be elucidated, however recent findings have proposed that the developmental stage specific expression of miRNAs might regulate some of these cell-intrinsic factors and development of MKs [331].

### 1.18. Management of thrombocytopenia in neonates

Platelet transfusions are frequently given to neonates with severe thrombocytopenia (platelet count $<150\times10^9$/liter) to reduces the risk of hemorrhage, but no study has demonstrated a beneficial

effect of platelet transfusions in thrombocytopenic neonates, or whether this is clinically appropriate. On the other hand, several reports have demonstrated that there is a strong association between the frequency of platelets transfusions and high morbidity and mortality among thrombocytopenic patients admitted to NICU with different disease/condition [332-334]. TPO may have a treatment option for thrombocytopenia, however the application of recombinant human TPO (rhTPO) as a treatment option seems likely to be limited because of the development of neutralizing autoantibodies and delayed response (6-7 days after administration of rhTPO before the platelet count rises) [335]. It means the lag time of rhTPO response is also long. IL11 have also studied as the potential treatment option for thrombocytopenia [336]. However, to date there are no reports of the use of recombinant human IL11 (rhIL11) for the treatment of neonatal thrombocytopenia.

### 1.19. Objectives of the Study

Thrombocytopenia, defined as a low platelet count <150 x $10^9$/L, is common among all low-birthweight, premature, and sick infants and patients undergoing CB transplantation. This plays a major role as co-factor in the frequency and severity of IVH in neonates [319], which is a main cause of poor neurological outcomes and high mortality in newborns [319,321,337]. Although the underlying cause for this predisposition is unclear, the developmental difference between neonatal and adult MKs is an increasingly recognized cause [327]. Specifically, neonatal MKPs are known for their high proliferation potential, and give rise to terminally differentiated MKs (small cell mass and ploidy) that generate a smaller number of platelets as compared to adult MKs [327,338]. Further, Bluteau et al. [338] has also revealed that target genes of different TFs, involved in MK ploidy development, cytoskeleton rearrangement, development of pro-platelet projections and platelet functions, were enriched during MK ontogeny. These findings suggest that MKs involve a development associated maturation process and functional specification leading to increase adult specific hemostatic function of MKs. All earlier studies suggest that these uncoupled mechanisms of neonatal megakaryocytopoiesis allow fetuses/neonates to populate their rapidly growing BM and intravascular spaces while maintaining the normal range of platelet counts (150 x $10^9$/L to 450 x $10^9$/L), but also set the stage for various disorders/conditions restricted to only fetal/neonatal

MKs/progenitors. Also, neonatal MKs are unable to increase their mass in response to increased platelet demand. The regulatory molecular mechanism of this uncoupled phenotype of neonatal MKs is not fully understood, and lack of such knowledge has significantly impaired our ability to understand and treat neonatal thrombocytopenia and low platelet conditions in CB recipients.

Although several TFs have been reported as the crucial regulators of gene expression during embryonic and adult hematopoiesis, RUNX1 is a key gene expression regulator in hematopoiesis, during development, lineage selection and terminal maturation of blood cells [162]. RUNX1 is a critically important TF in megakaryocytopoiesis, it regulates ploidy development and cytoskeleton rearrangement in the process of MK maturation and platelet release [117,339]. In humans, loss-of function RUNX1 mutations are common cause of FPD/AML, it is characterized by thrombocytopenia, platelet dysfunction, and a lifetime high risk for developing myeloid malignancies [340,341].

While the importance of RUNX1 in megakaryocytopoiesis is well-established [342,343] the information about the developmental regulation of RUNX1 in neonatal versus adult MKs and its crosstalk with differentially regulated signaling pathways is lacking in MKs. Therefore, it was important for us to understand the RUNX1 regulation in developmentally different neonatal and adult MKs. *In this study, we hypothesized that lower RUNX1 expression in neonatal MKs may lead to developmentally different, hyperproliferative and disease susceptible phenotype.*

To test the hypothesis, and thereby achieve the object of this study, we designed the following *Specific objectives:*

1. **To study the developmental differences and RUNX1 expression in neonatal versus adult MKs.**
2. **To investigate the RUNX1 regulatory mechanisms in neonatal versus adult MKs.**
3. **To study the RUNX1 regulation and its crosstalk with other signaling pathways**

## 2. Materials and Methods

### 2.1. Human- MK Cell Cultures

Human CB- and mobilized adult peripheral blood (PB)-CD34+ cells (HSPCs, hematopoietic stem/progenitor cells) from Lonza India Pvt. Ltd. (n=3 for each group) were cultured in serum free MK cell culture medium with TPO, 50 ng/mL (PeproTech Inc.) and maintained in humidified CO2 incubator following the method of Pastos et al. [344]. At day 14 of culture more than 90% of cells were MKs ($CD41^+$ cells) by flow cytometric analysis. Each experiment was repeated at least 3 times, using a different source of CB- and PB-HSPCs each time.

MK cell culture medium: Iscove's modified Dulbecco's medium (IMDM) supplemented with BSA (1%), transferrin (740 µg/ml) insulin (10 µg/ml) L-glutamin (2.0 mM) and LDL (40 µg/ml).

The K562 erythroleukemia cell line from NCCS (Pune, India) was majorly used in this study. Phorbol 12-myristate 13-acetate (PMA) is well known to induce megakaryocytic differentiation of K562 cells. Thus, PMA-induced megakaryocytic differentiated K562 cells were considered in this study as the classic model to expose the process of MK maturation.

K562 cells were cultured in RPMI-1640 (Invitrogen) media with 10% fetal bovine serum (FBS), 1% solution of antibiotic-antimycotic (Sigma) and maintained at 37 °C in humidified CO2 (5%) incubator. To induce megakaryocytic differentiation, exponentially growing K562 cells were treated with 50 nM PMA (Sigma-Aldrich); whereas, to induce Erythroid differentiation model, Hemin (50 µg/ml) was used (Sigma-Aldrich). Whereas, control cells received the same concentrations of DMSO alone. After 72 hrs of incubation cells were analyzed for megakaryocytic and erythroid marker expression. Each experiment was repeated at least 3 times.

The biological ligand, TGF-β1 was used to induce TGF-βR signaling in PMA induced megakaryocytic cells. As indicated, TGF-β1 ligand (10 ng/ml and 20 ng/ml) was added to the RPMI medium after 12 hours of PMA treatment; after additional 60 hours of incubation cell culture was collected and used to study the effects of TGF-β1 on MK maturation. The cellular phosphoproteins (pSMAD2/3) level of expression was detected upon TGF-β1 exposure for upto 60 min. Each experiment was repeated at least 3 times, with a different source of cells each time.

### 2.2. Transfection

Dami and Meg01 cells (megakaryoblastic cell-lines), respectively, were cultured in RPMI-1640 and DMEM (Invitrogen) media with 10% FBS, 1% solution of antibiotic-antimycotic (Sigma), and maintained in humidified CO2 (5%) incubator. Before transfection, exponentially growing cells at a concentration of $0.5 \times 10^6$/well of 24-well plate were seeded in 500 µl of medium (RPMI media containing serum and antibiotics) and incubated for a short time period (4-5 hrs) under normal growth conditions.

Lipofectamine-2000 transfection reagent (4 µl) was diluted with 50 µl of Opti-MEM I media (Invitrogen). The miRNA mimic (100 nM), inhibitor (200 nM), or the same concentration of scrambled miRNA (control) sequences were also diluted in 50 ul Opti-MEM I media and mixed separately with 50 ul of diluted transfection reagent (in a 1:1 ratio). After a pulse vortexing samples were incubation for 20 min at room temperature to allow the formation of lipoplexes (DNA–Lipid complexes). These complexes of miRNA mimic, inhibitor, or scrambled miRNA were added drop-wise to the wells of 24-well plate containing cells with serum and antibiotics free Opti-MEM I medium, mixed gently by rocking the plate to ensure uniform distribution of the transfection complexes and incubated for 6 hrs under normal growth conditions; medium was changed after 6 hrs with FBS and antibiotics containing RPMI medium. Cell proliferation and gene expression was monitored after 72 hrs of incubation. Each experiment was repeated at least 3 times with a different source of cells each time.

### 2.3. Trypan blue viability assay:

The Trypan Blue dye exclusion test is a useful assay to determine the number of viable cells present in a cell suspension. Dead cells take the stain and appear blue, while viable cells exclude the stain and appear clear.

The cell suspension and 0.4% trypan blue stain (Mediatech Inc.) were mixed in a 1:1 ratio and incubated for 3 min. After staining, live cells were counted via hemocytometer with the help of microscope. The number of viable cells in 1 ml suspension was calculated using the equation:

number of live cells counted × dilution factor × $10^4$ (correction factor). Each experiment was repeated at least 3 times with a different source of cells each time.

### 2.4. Flow cytometry analysis

After blocking with normal mouse serum diluted to 5% in PBS and 1% bovine serum albumin (BSA), cells ($1 \times 10^6$) were stained with Anti-Human CD41/FITC and anti-Human CD42/APC, Anti-Human CD61/PE (BD PharmingenTM), or isotype matched IgGs (negative controls). Cells were fixed with 1% paraformaldehyde (PFA) and analyzed by flow cytometry (BD FACS) for three independent samples. The data was analyzed using FlowJo Version 7.2.5 (TreeStar).

### 2.5. Immunofluorescence

Cells were allowed to attach to poly-L-lysine–coated glass-slides, followed by fixation with 4% PFA. After fixation the cells were permeabilized with 0.5% Triton X-100. After blocking with 5% normal goat serum, cells were stained with Anti-Human CD61/PE (BD PharmingenTM).

For the collagen type1 (COL1) immunofluorescence, cells were stained with Mouse Anti-Human COL1 (g-Biosciences), followed by incubation with the appropriate Alexa Fluor 593 (red)–conjugated secondary antibody.

After counterstaining with DAPI (Nuclear counterstain) cell images were analyzed in a fluorescent microscope. Each experiment was repeated at least 3 times with a different source of cells each time.

### 2.6. Benzidine staining

The benzidine-positive cells were quantified by counting triplicates of at least 300 cells under light microscope; harvesting of cells, benzidine staining, and analysis work was performed following the method described in [345].

### 2.7. Quantitative RT-PCR

Total RNA was extracted from cultured cells using the miRNeasy mini kit (Qiagen) following the manufacturer's protocol and instructions. Prior to start reverse transcription reaction, RNA samples

were treated with RNase-free DNase I (Qiagen) according to the manufacturer's instructions to remove the genomic DNA. All RNA samples were quantified by NanoDrop™ 2000/2000c Spectrophotometers (A260/280 nm; Thermo Fisher scientific), and 1 µg of total RNA input was reverse transcribed with EasyScriptTM cDNA Synthesis Kit using oligo(dT)20 primers and following the manufacturer's protocol (abm). The first-strand cDNA obtained by reverse transcription was amplified directly by using specific reverse and forward primers (Table 2.1) in ABI StepOne Plus system (real-time quantitative-PCR, qRT-PCR; Applied Biosystems) with SYBR Green FAST qPCR Master Mix (Kappa Biosystems) according to the manufacturer's protocol and instructions. Amplification was performed as follows: Cycling conditions, an initial denaturation at 52 °C for 2 min and 95 °C for 8 min, followed by 42 cycles of 95 °C for 15 sec, 56 °C for 20 sec, and 72 °C for 30 sec.

At the end of the PCR reaction, the specificity of qRT-PCR products was tested by melting curve examination to ensure the amplification of a single PCR product. The Ct values of the gene of interest were normalized by subtracting the Ct for the reference gene-GAPDH (Glyceraldehyd-3-phosphate-Dehydrogenase) from the Ct of the gene of interest; the relative quantification was done by comparative ΔΔCt method (2^(-ΔΔCt)). The results of this study are from three individual experiments (n=3), in which each sample was test in triplicate.

### 2.8. miScript miRNA PCR Arrays

miScript miRNA PCR Arrays, which contained pre-loaded miScript Primer Assays, are part of the miScript PCR System that allow sensitive and specific detection and quantification of miRNAs. The miScript Primer Assays utilized in this system (mature miRNA-specific forward primers) are arrayed in pathway-focused and disease-focused set of miRNAs, or whole miRNome panels.

Total RNA including miRNA was extracted from CB- and PB-MKs (n=3 for each group) using miRNeasy mini kit (Qiagen), according to the manufacturer's instructions. cDNA was prepared using the miScript II RT Kit with miScript HiSpec Buffer (Qiagen), following the manufacturers protocol. The cDNA was used as a template in qRT-PCR reaction performed with miScript miRNA PCR Array and the miScript SYBR Green PCR Kit. The reaction was amplified in

StepOnePlus™ PCR System (Applied Biosystems) following the cycling conditions of miScript miRNA PCR Arrays protocol (Qiagen). The Ct values were normalized to the level of endogenous reference small RNA, U6. The data was analyzed by comparative ΔΔCt method (2^(-ΔΔCt)). The results presented in current study are from three individual experiments (n=3), in which we test each sample in triplicate.

### 2.9. TR2 Profiler PCR Arrays

RT2 Profiler PCR Arrays are reliable tools for analyzing the expression levels of pathway focused panel of genes. Each plate of PCR array includes optimized primer assays for the amplification of the relevant, pathway- or disease-focused genes using qRT-PCR. The high-quality primer assays design for the amplification of different gene products in a 96-well plate enable the TR2 Profiler PCR Arrays to generate data for all the pathway focused genes products simultaneously under uniform cycling conditions. RT2 Profiler PCR assays have been optimized for RT2 SYBR Green master mix that make it sensitive enough for use with cDNA prepared from regular samples (0.1–5 µg RNA) and small samples (1–100 ng RNA).

The differential analysis of TGF-β-pathway gene expression was performed using Human TGF-β/BMP signaling pathway RT2 Profiler PCR Array (PAHS-035D, Qiagen), run in StepOnePlus™ Real-Time PCR System (Applied Biosystems) following the cycling conditions of RT2 Profiler PCR Arrays protocol (Qiagen). Experiments were carried out in biological triplicate (n=3), in which we test each sample in triplicate. Data was analyzed using Web-based software (Qiagen). Data analysis was based on the ΔΔCT method, and the raw data was normalized to housekeeping genes (ACTB, GAPDH, HPRT1, and RPLP0). For this array data, the level of statistical significance (p-value) were calculated based on t-test of the replicate 2^(- Delta Ct) values for each gene in the control group and treatment groups.

### 2.10. Western blots

Total protein for western blotting was extracted from cultured cells by cell lysis in ice cold radioimmunoprecipitation assay (RIPA) buffer (Sigma) in the presence of protease inhibitor cocktail (Sigma) and phosphatase arrest (g-Biosciences). Cell lysis and protein clarification was

performed following the manufacturer's (Sigma) protocol and instructions. Proteins (50 µg) samples were subjected for separation on 6%-12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose (NC) filter. Blots were blocked with 5% bovine serum albumin (BSA) for 1-2 hours at room temperature, followed by overnight incubation with primary antibodies at 4°C. The specific primary antibodies used in this study were directed against human RUNX1 (Santa Cruz), pSMAD2/3 (g-Biosciences), UCP2 (g-Biosciences), β-actin (Sigma). The detection of primary antibody interaction with specific protein was carried out with the help of appropriate peroxidase conjugated secondary antibodies. In this procedure after primary antibody incubation the blots were washed three times for 10 min with TBS-T buffer and then incubated with secondary antibody for one hour. After washing with TBS-T buffer protein blots were developed using chemiluminescent solution, Pierce™ ECL Substrate (Thermo Fisher Scientific) following manufacturer's instructions and visualized using Chemidoc Imaging System (BioRad). To quantify protein expression levels, we did densitometric analysis of protein blots with ImageJ program and the integrated density of the protein signal was normalized to actin levels.

### 2.11. DNA index analysis

The Muse™ Cell Cycle Assay (MCH100106; Millipore) is a proprietary formulation of DNA intercalating stain (propidium iodide, PI) and RNAse A. The Muse cell Analyzer based on PI fluorescence quantify the DNA content of each cell and discriminate cells at different phases of the cell cycle. In this assay RNAse increases the specificity of DNA staining. The Muse Cell Cycle Software Module display data in two plots: one is the dot plot of DNA content and cell size index, and second one is the histogram of DNA content and cell number index with markers to analyze particle number/cell number in each phase of the cycle.

The live cells ($1 \times 10^6$) were fixed, washed, and stained with Muse™ Cell Cycle Assay reagents following the Assay kit protocol (Millipore). DNA content/index was analyzed using Muse® Cell Analyzer (Merck Millipore, USA). The experiments were performed in triplicate and with a minimum number of 5000 events per run.

## 2.12. Analysis of Ca2+ levels (Fluo-3/AM assay)

Labeled Ca2+ indicators (Fluo-3/AM) are molecules that exhibit a rapid increase in fluorescence intensity upon binding Ca2+. Previously, Fluo-3/AM has been used in different studies to image the spatial dynamics of Ca2+ signaling [346]. In present study, Fluo-3 fluorescence was utilized to determine [Ca2+]i levels. In this study, cells of different treatment and control groups were incubated in fresh RPMI-1640 media without FBS at a density of 1 x $10^6$/ml. Cells were loaded with Fluo-3/AM Ester (3 µM, Biotium, Catalog # 50016) by adding the dissolved Ca2+ indicator directly to the culture dishes of different treatment and control groups. Loading was allowed to proceed for 60 min at 37°C temperature in a humidified incubator with 5% CO2. After incubation, cells of different treatment and control groups were washed 2-3 times in fresh FBS free RPMI-1640 media to remove all traces of extracellular Fluo-3/AM indicator and finally resuspended in PBS buffers that are free from Ca2+ and Mg2+ (Dulbecco's Phosphate Buffer Saline, DPBS; Gibco by life technologies).

Finally, to evaluate the change in fluorescence intensity in response to Ca2+ binding, the emitted fluorescence intensity of Fluo-3 on Ca2+ binding was recorded using a fluorescence-spectrometer at 25°C (excitation 505 nm, emission 526 nm, slit width 5 nm). Cells incubated without Fluo-3/AM were used to normalize the assay. Emission at 526 nm was used to determine the relative [Ca2+]i concentration in different treatment groups as compared to control group. The data presented in current study are from three individual experiments (n=3), in which each test was performed in triplicate.

Cells were also evaluated in the fluoresce microscope using excitation at 505 nm. The excitation wavelength and emission detection parameters were digitally controlled, and same settings were used for all the repeats of experiment. Further all the images captured with fluoresce microscope were quantitated using ImageJ software. For counting of total fluorescence, the Mean Gray Value and of images was invariably quantified in a visual filed selected. The bar graph of mean fluorescence intensity of 500 cells is displayed.

### 2.13. Determination of intracellular ROS generation (H2DCF-DA Assay)

Dichlorodihydrofluorescein diacetate (H2DCF-DA) is a cell permeable standard probe commonly used for detecting intracellular ROS [347-349]. H2DCF-DA is a non-fluorescent lipophilic probe that can easily diffuse/cross through the cell membrane. Inside the cell, H2DCF-DA is deacetylated by cytoplasmic esterase's. The acetyl group from H2DCF-DA is removed to form H2DCF, which is also a non-fluorescent probe, but it cannot cross/diffuse freely through the cell membrane. Finally, the DCFH2 reacts with ROS and upon oxidation gives the fluorescent DCF.

Intracellular ROS was measured in cells ($1 \times 10^6$) of different treatments and control group using H2DCF-DA Assay (Sigma). Briefly, cells were washed with PBS and incubated in dark for 30 min with H2DCF-DA dye (10 µM) at a density of $1 \times 10^6$/ml. After incubation, cells were washed 2-3 time with DPBS and transferred to microplate. The fluoresces of DCF was quantified using fluorescence detection spectroscopy (at 485 nm excitation and 535 nm emission wavelengths) and mean fluorescence intensity (MFI) represented as bar graphs. The results presented in current study are from three individual experiments (n=3), all samples were tested in triplicate.

### 2.14. MitoPotential Assay

The Muse™ MitoPotential Assay was used to analyze mitochondrial trans-membrane potential (ΔΨm). This assay utilizes a cationic and lipophilic dye to detect changes in mitochondrial membrane potential (ΔΨm) and 7-AAD as an indicator of cell death. Polarized inner membrane of intact mitochondrial (High membrane potential) allows the accumulation of MitoPotential dye resulting in high fluorescence. Whereas, reduced membrane potential or depolarized mitochondria demonstrate a decrease in fluorescence. This parameter is displayed in the MitoPotential axis of histogram.

Before staining, cultured cells from different treatments and control group were washed 2-3 time with PBS. Further, 100 µl cell ($1 \times 10^5$) suspension was loaded with 95 µl of MitoPotential Dye and 5 µl of 7-AAD following the Muse MitoPotential Assay Kit (Merck Millipore) protocol. The membrane potential was determined by the Flow cytometer (MuseTM Cell Analyzer, Merck Millipore, USA). All the experiments were repeated at least three times.

### 2.15. siRUNX1 transfection

RUNX1 siRNA (ON-TARGETplus siRNA reagent), and negative controls were synthesized by Dharmacon. Oligonucleotide transfection was performed with HiPerfact transfection regent (Qiagen) according to the manufacturer's instructions. After 48 hours of transfection the cells were used to observe the expression of RUNX1 and its targets. Each experiment was repeated at least 3 times with a different source of cells each time.

### 2.16. Statistical analysis

Data are provided as the mean (±SD) of three biological replicates, where, n represents the number of experiments. The statistical significance (p-value) of differences between individual groups was tested using Student t-test. The differences or changes were considered significant at a p-value of $<0.05$.

**Table 2.1. Primers used in current study.**

| QRT-PCR Primers | Primer Sequence (5'- 3') | |
|---|---|---|
| CD41 | F: GCATGGTTCAACGTGTCCTC | R: TTGAAGAAGCCGACCTTCCA |
| CD61 | F: ACCAGTAACCTGCGGATTGG | R: TCCGTGACACACTCTGCTTC |
| CD42b | F: TTCCCCACCAAAGCCCATAC | R: GGCTTGGGGTTGGTTCAGTA |
| RUNX1 | F: AACCTCGAAGACATCGGCAG | R: GGCTGAGGGTTAAAGGCAGT |
| TRPA1 | F: ACAGAAGACAAGTCCTGCCG | R: GAGGGCTGTAAGCGGTTCAT |
| TRPV1 | F: ACGGACAGAACACCACCATC | R: GGCCCTTGTAGTAGCTGTCC |
| TRPC3 | F: CAGCCAACACGTTATCAGCAG | R: TGCTTGGCTCTTGTCTTCCA |
| TGF-β1 | F: GCAACAATTCCTGGCGATAC | R: TAGTGAACCCGTTGATGTCC |
| TGF-βR1 | F: GTGACAGATGGGCTCTGCTT | R: GAGGGTGCACATACAAACGG |
| TGF-βR2 | F: AGACGTTGACTGAGTGCTGG | R: TTAGGGAGCCGTCTTCAGGA |
| TGF-β2 | F: TTGCCCTCCTACAGACTTGA | R: GTGGACGTAGGCAGCAATTA |
| SMAD1 | F: CAACAATCGTGTGGGTGAAG | R: TCCGGTTAACATTGGAGAGC |
| SMAD2 | F: GTCCATCTTGCCATTCACGC | R: CTGCCCATTCTGCTCTCCTC |
| SMAD3 | F: TAACTTGGACCTGCAGCCAG | R: CCTCTTCCGATGTGTCTCCG |
| SMAD4 | F: GATACGTGGACCCTTCTGGA | R: ACCTTTGCCTATGTGCAACC |
| GYP A | F: GGGGTGATGGCTGGTGTTAT | R: AAAGGCACGTCTGTGTCAGG |
| GYP B | F: GGCAATGCACACTTCAACCT | R: AGGAGCTGGTACAGTGAAACG |
| UCP2 | F: AAAGCACCGTCAATGCCTAC | R: CATAGGTCACCAGCTCAGCA |
| NOX4 | F: CAGAAGGTTCCAAGCAGGAG | R: GTTGAGGGCATTCACCAGAT |

## 3. Objective 1: To study the developmental differences and RUNX1 expression in neonatal versus adult MKs.

### 3.1. Introduction

Thrombocytopenia is a condition characterized by abnormally low levels of blood platelets (<150 × $10^9$/L), thrombocytopenia is a major clinical problem encountered in neonates admitted to NICU, it is common among all sick, preterm, and low birth weight neonates and in patients undergoing CB transplantation. Platelets help our body form clots to stop bleeding, Neonates with low platelet counts suffer from internal bleedings (hemorrhage). Thrombocytopenia is also a risk factor for IVH in neonates [319], which is a main cause of poor neurological outcomes and high mortality in newborns [319,321,337]. Currently, the primary cause of this predisposition is unclear, however in earlier studies is has demonstrated that neonatal-MKs are developmentally different from adult-MKs [327]. Neonatal MK development is characterized by an increased sensitivity to TPO [344,350], high proliferation potential of MKP [350-352], and terminally differentiated MKs with small cell size and ploidy [344; 312; 327]; Neonatal-MKs also generate a smaller number of platelets than adult-MKs [327,338]. A micro-array study by Bluteau et al. [338] has also revealed that target genes of different TFs, involved in MK ploidy development, cytoskeleton rearrangement, development of pro-platelet projections and platelet functions, were enriched during MK ontogeny. These findings suggest that MKs involve a development associated maturation process and functional specification leading to increase adult specific hemostatic function of MKs.

All earlier studies suggest that the uncoupled mechanisms of neonatal MK development allow them to populate their rapidly growing BM and intravascular spaces while maintaining the normal range of platelet counts (150 × $10^9$/L to 450 × $10^9$/L), but also set the stage for various disorders/conditions restricted to only fetal/neonatal MKs/progenitors. The regulatory molecular mechanism that underlies this uncoupled phenotype of neonatal MKs is not fully understood so far, and lack of such knowledge has significantly impaired our ability to understand and treat neonatal thrombocytopenia and low platelet conditions in CB recipients. Although several TFs have been reported as the crucial regulators of gene expression during embryonic and adult

hematopoiesis, RUNX1 is a key gene expression regulator in hematopoiesis, during development, lineage selection and terminal maturation of blood cells [162].

RUNX1 is a critically important TF in megakaryocytopoiesis, it regulates ploidy development and cytoskeleton rearrangement in the process of MK maturation and platelet release [339,117]. In humans, loss-of function RUNX1 mutations are common cause of FPD/AML, which is characterized by dysmegakaryocytopoiesis, platelet defects, and a lifetime high risk for developing myeloid malignancies [340,341].

While the importance of RUNX1 in megakaryocytopoiesis is well-established [342,343] the information about the developmental regulation of RUNX1 in neonatal versus adult MKs is lacking. we hypothesized that RUNX1 expression level will be significantly lower in MKs during fetal or neonatal life, compared to adults MKs. This hypothesis was supported by the finding that the adult phenotype of $RUNX1^{-/-}$ resembles normal fetal MKs. RUNX1 is required for hematopoietic stem cell emergence [353,354]. Mice that are deficient in either RUNX1 or core-binding factor-beta (Cbf-β) lack definitive hematopoiesis and die during midgestation, highlighting the functional role it plays in development [312,313,355]. In vitro studies and findings in human diseases, including leukemias [356,357], myelodysplastic syndromes [358] and FPD/AML further underscore the importance of RUNX1 in hematopoiesis and megakaryocytopoiesis [179].

## 3.2. Results

### 3.2.1. Establishment of the TPO-induced MK differentiation in CB- and PB-derived hematopoietic progenitors ($CD34^+$ cells).

To study the TPO-induced MK growth and differentiation and the developmental differences between neonatal- and adult-MKs, we cultured CB- and PB- derived $CD34^+$ cells in serum free MK cell culture medium in the presence of TPO (50 ng/mL). Cells were observed and collected at different time points to study the TPO-induced growth and differentiation of MKs in CB- and PB-derived $CD34^+$ progenitors. At each time point the total numbers of viable cells were counted (n=5; Figure 3.1A). In cell cultures derived from the CB-CD34+ cells, the TPO stimulated the greatest

expansion (~ 50-fold) of MKP population ($CD41^+$ cells) by day 14; however, we did not observe that much increase in the growth of MKP population derived from PB-$CD34^+$ cell (~ 4-fold by day 14). Further, in cell cultures derived from the CB- and PB-$CD34^+$ cells, we observed a consistently increased expression of MK markers (CD41 and CD61) by qRT-PCR during day point analysis of TPO-induced megakaryocytopoiesis (n = 3; $P < 0.02$; Figure 3.1C and D). However, CB-MKs were smaller in size than PB-MKs (n = 3; Figure 3.1B). In the present study, we have established the TPO-induced MK differentiation system in CB- and PB-derived $CD34^+$ cells.

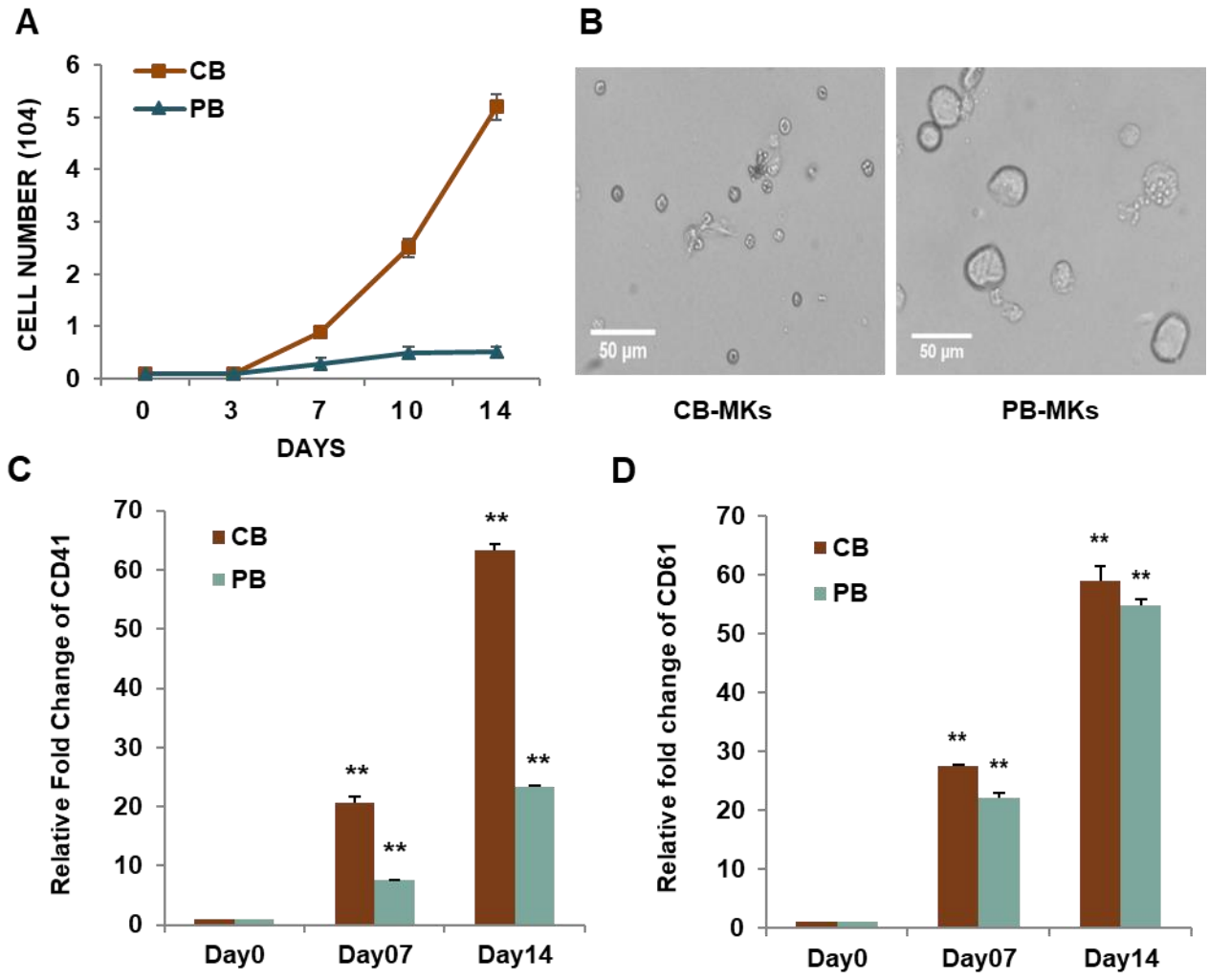



**Figure 3.1. TPO-induced MK differentiation in CB- and PB-derived hematopoietic progenitors ($CD34^+$ cells).** A. The graph demonstrates the cell number changed upon TPO stimulation in cell cultures derived from the CB- and PB-$CD34^+$ cells over a 14-day period (n = 5). B. Photomicrograph of CB- and PB-$CD34^+$ cells derived MKs (20x

objective; scale = 50 μm) at day 14, CB-MKs are smaller in size than PB-MKs (n = 3). C. qRT-PCR analysis of the expression of MK marker CD41 in cell cultures derived from the CB- and PB-CD34+ cells over a 14-day period (n = 3, **P < 0.02). D. MK marker CD61 mRNA expression analysis in cell cultures derived from the CB- and PB-CD34+ (n = 3, **P < 0.02) over a 14-day period. Bars represent the mean (±SD) from three independent experiments; all the data were tested using Student's t-test.

### 3.2.2. Developmental differences in CB vs PB CD34$^+$ cells derived MKs.

Several observations have shown the existence of substantial biological differences between neonatal and adult MK [325,326,350-352,359]. To assess the cytoplasmic maturation status of neonatal MKs ex vivo, we have checked the developmental differences in the expression of MK markers (CD41, CD61 and CD42b) in CB vs PB-CD34$^+$ cells derived MKs (Figure 3.2).

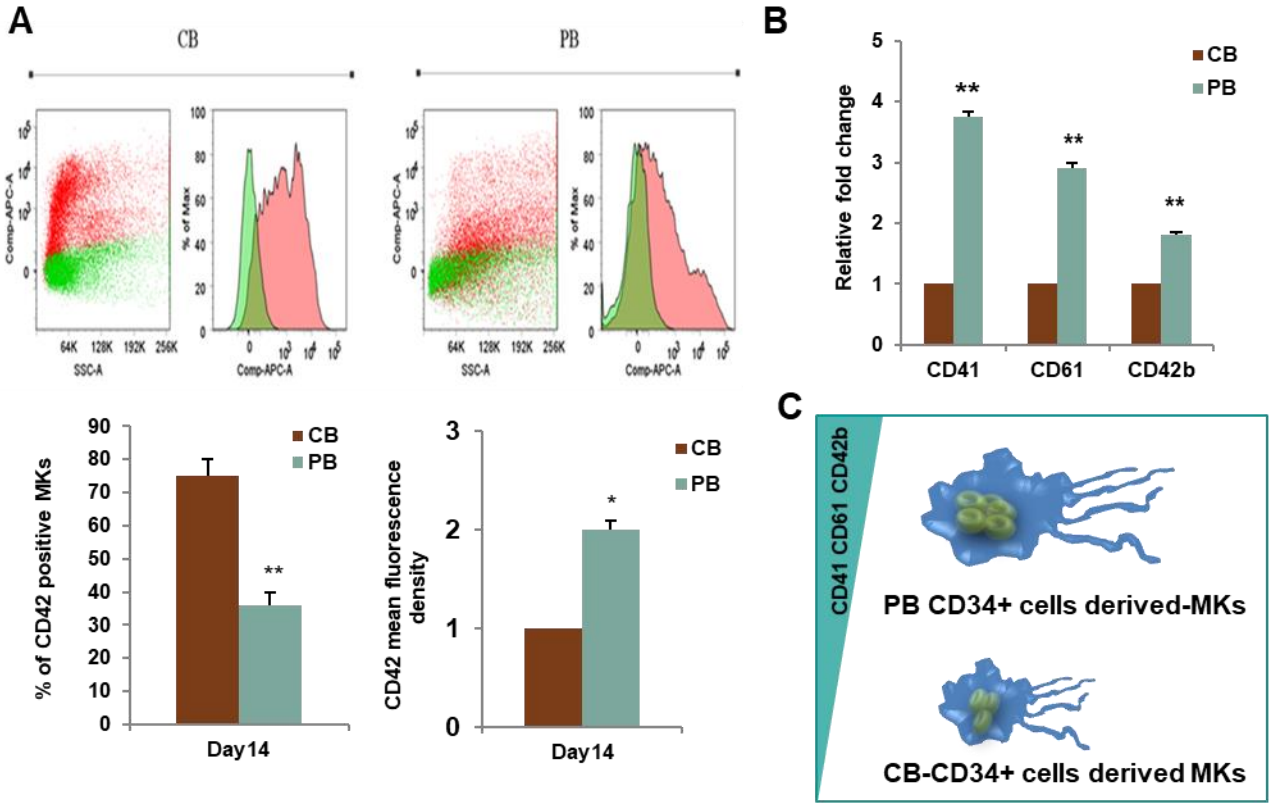



**Figure 3.2. Developmental differences in CB vs PB CD34$^+$ cells derived MKs.** A. FACS/Flow cytometric (FC) analysis of the expression of MK marker CD42b, evaluated after 14 days of CB- and PB-CD34$^+$ cell cultures in MK culture medium supplemented with 50 ng of TPO per ml (n=3; **p<0.02; *p<0.05). B. The MK markers (CD41,

CD61, and CD42) mRNA expression levels between CB- and PB derived MKs were analyzed by qRT-PCR at day 14 (n = 3; **P< 0.02). C. The graphical representation of the observed developmental differences between CB- and PB-CD34+ cells derived MKs. Bars represent the mean (±SD) from three independent experiments; all the data were tested using Student's t-test.

After 14 day of culture FACS/FC analysis was carried out using human anti-CD42/APC to monitor TPO stimulated MKs differentiation. After 14 days, ~ 93.3 ± 4.01% of CB-CD34+ cells and ~ 91.6 ± 3.61% of PB-$CD34^+$ cells were $CD41^+$ MKs. Further, we observed that over a period of 14 days $CD34^+$ cells of CB origin generated approximately 2-fold more $CD42^+$ MKs as compared to PB derived $CD34^+$ cells (n=3, $P < 0.02$; Figure 3.2A), however CD42 mean fluorescence density was significantly higher in PB-$CD34^+$ cells derived MKs when compared with CB-$CD34^+$ cells derived MKs on day 14 (n=3, $P < 0.05$; Figure 3.2A). Further we have analyzed the developmental differences in the mRNA expression levels of MK markers, the MK markers CD41, CD61, and CD42b were recorded with consistent higher expression in PB- as compared to CB-MKs through all biological repeats (n = 3; $P < 0.02$; Figure 3.2B).

### 3.2.3. Developmental differences in RUNX1-1 expression

While the importance of RUNX1 in megakaryocytopoiesis is well-established, however the information about its developmental regulation in neonatal versus adult MKs is lacking. Considering the RUNX-$1^{-/-}$ phenotype which resembles normal fetal MKs [341], we hypothesized that the RUNX1 expression is differentially regulated in neonatal versus adult MKs. To study the developmental differences in RUNX1 expression, we cultured CB- and PB- derived $CD34^+$ cells in serum free MK cell culture medium in the presence of TPO (50 ng/mL). As shown in Figure 3.3A, we have performed a time point analysis of RUNX1 mRNA levels in cell cultures derived from CB- and PB-$CD34^+$ cells. In cell cultures derived from PB-$CD34^+$ cells, upon stimulation with TPO RUNX1 mRNA expression levels were significantly reduced at day 7 as compared to day0 (n=3; $p<.05$), however at day 11 and day 14 RUNX1 mRNA expression levels were significantly high as compared to day 0 (n=3; $p<.05$). On the other hand, in cell cultures derived from CB-CD34+ cells, RUNX1 mRNA expression levels were significantly low at all time points (7, 11, and 14 day) as compared to day 0 (n=3; $p<0.05$). Given these findings, further we have explored the developmental differences in the RUNX1 protein expression levels. At day 14, as

shown in the Figure 3.3B, RUNX1 protein expression was significantly lower in CB-CD34$^{+}$ cells derived MKs as compared to adult PB-CD34$^{+}$ cells derived MK (n= 3; p<0.02). In the present study, the observed developmental differences in the expression levels of RUNX1 in CB-MK versus PB-MKs are consistent with the hypothesis.

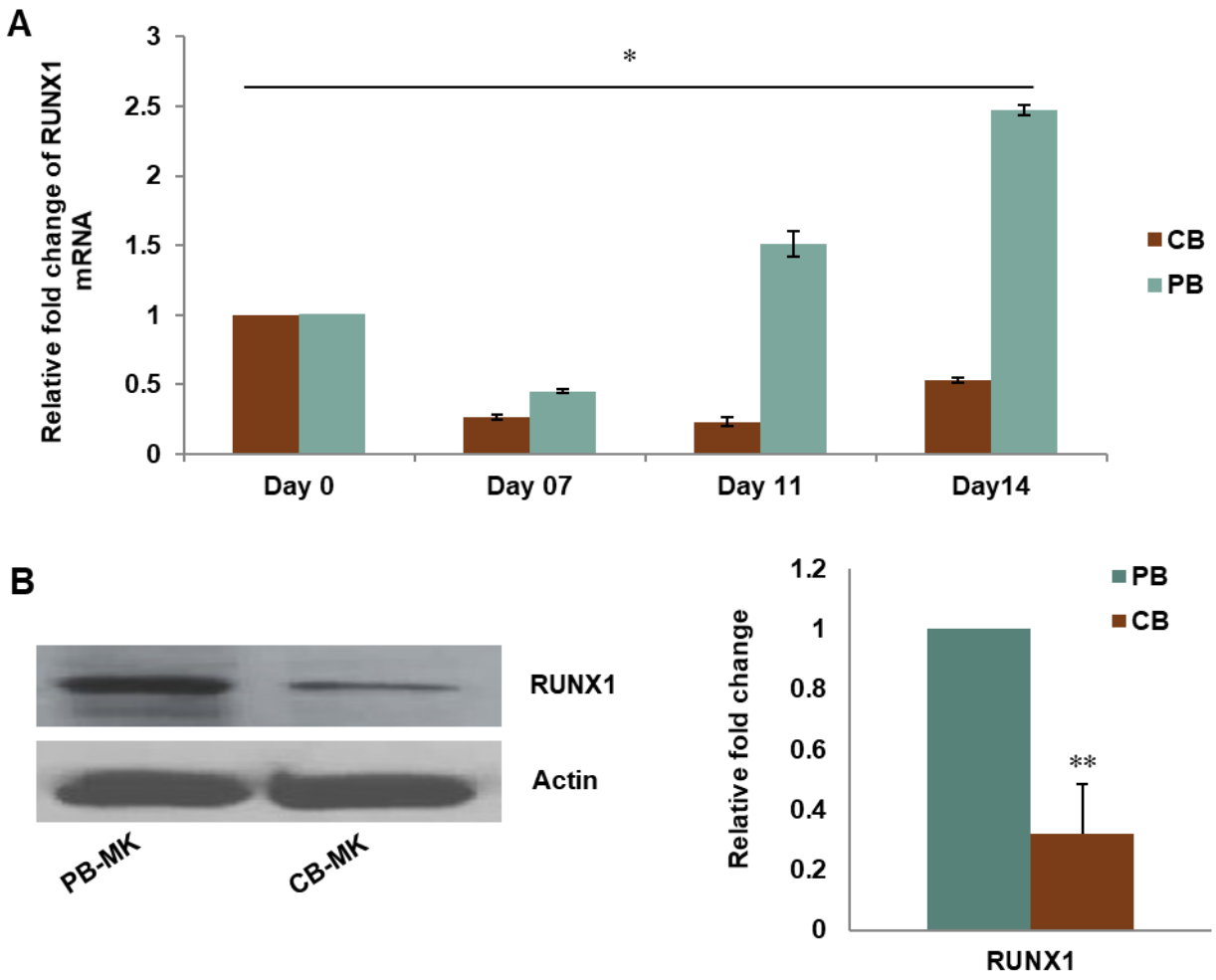



**Figure 3.3. RUNX1 levels in CB-MKs and PB-MKs.** A. qRT-PCR analysis of the expression of RUNX1 mRNA in cell cultures derived from the CB- and PB-CD34+ cells over a 14-day period (n = 3; *P < 0.05). B. RUNX1 protein levels expressed as a ratio to β-actin were significantly lower in CB- as compared to PB-CD34+ cells derived MKs (n = 3; **P <0 .02).

### 3.3. Discussion

Several observations support the existence of substantial molecular and cellular differences in neonatal and adult MKs [325,326,350-352,359]. Specifically, neonatal MKPs exhibit marked hyperproliferation, producing smaller and low ploidy MKs as compared to adults [327,360]. Some of them are directly related to the TPO/cMpl axis [344]. In present study, we cultured CB- and PB-derived $CD34^+$ cells in serum free defined media conditions. Our results based on analysis of CB- and PB- derived $CD34^+$ cells triggered to megakaryocytic differentiation by TPO show that TPO efficiently induces MK differentiation ex vivo, we observed that during TPO induced MK differentiation the MK markers are increasingly expressed [Figure 3.1 C and D]. We further confirm the TPO induced developmental differences in CB- and PB-$CD34^+$ cells derived MKs. We observed that CB-$CD34^+$ cells derived MKs/MKPs are hyperproliferative and generate MKs of small size [Figure 3.1A and B]. Furthermore, we have observed that the expressions of MK maturation markers were significantly low in CB-$CD34^+$ cells derived MKs as compared to PB-$CD34^+$ cells derived MKs [Figure 3.2A and B]. Our current study results conclusively demonstrates that cell proliferation and maturation processes are uncoupled during normal neonatal MK development, thus allowing for hyperproliferative progenitors to give rise to less mature MKs. These uncoupled mechanisms of neonatal MK development likely allow fetuses/neonates to populate their rapidly growing both the BM and intravascular spaces while maintaining the normal range of platelet counts ($150 \times 10^9$/L to $450 \times 10^9$/L), but also set the stage for various disorders restricted to only fetal/neonatal MKs/MKPs, and inability to increase MK mass in response to increased platelet demand. However, the molecular regulation of this developmental uncoupling in neonatal MKs have not been elucidated.

In this study, we have investigated the uncoupled phenotype of neonatal (CB) MKs/MKPs. This uncoupling is most likely owing to the developmental differences in the relative expression levels of RUNX1 in CB- and PB-MKs. In support of this hypothesis, conditional Runx1 deletion in mice exhibit an uncoupling of proliferation and maturation, phenotype characterized by abundant small low-ploidy MKs which closely resembles normal neonatal MKs [341]. In addition, RUNX1 is a key gene expression regulator in hematopoietic compartment [162], it is also demonstrated as a

critically important TF in megakaryocytopoiesis, RUNX1 regulates MK ploidy and cytoskeleton rearrangement in the process of MK maturation and pro-platelet release [339,117]. In humans, loss-of function RUNX1 mutations have been reported as a common cause of FPD/AML, which is characterized by dysmegakaryocytopoiesis, platelet defects, and propensity for progression to AML [340]. In present study, consistent with the hypothesis, RUNX1 expression levels were significantly low in CB-CD34+ cells derived MKs as compared to PB-CD34+ cells derived MKs (Figure 3.3A and B). Therefore, the lower RUNX1 expression could be responsible for the uncoupling of neonates MK development. However, the mechanisms of RUNX1 regulation in developmental megakaryocytopoiesis are not defined.

## 3. Objective 2: To investigate the RUNX1 regulatory mechanisms in neonatal versus adult MKs.

### 3.1. Introduction

In our study, we report that there are substantial developmental differences between neonatal and adult MKs in humans. However, the molecular mechanisms that govern these cell-intrinsic differences are unknown. While a developmental expression pattern of the critically important TF like RUNX1 might contribute to the different phenotypes of neonatal and adult MKs, a growing body of evidence suggests that a regulation at the post-transcriptional level by small double stranded non-coding micro RNAs (miRNAs) is crucially involved in the regulation of stem-cell differentiation in general and normal as well as malignant hematopoiesis in particular [361]. In order to better understand normal megakaryocytopoiesis at distinct developmental stages, we initiated a comparative study between neonatal and adult megakaryocytopoiesis; herein, we assess the functional role of miRNA's during MK development using human cultured CB- and PB-MKs. we observed that primary human neonatal CB- and adult PB-CD34+ cells derived MKs differ in their miRNA expression profiles. Ten microRNAs were found to be overexpressed in CB compared to adult PB derived MKs. Five of them were found to target the RUNX1 TF. Since miRNA acts by suppressing protein production, we hypothesized that RUNX1 expression level will be supressed by miRNAs in MKs during neonatal life, compared to adults MKs. This hypothesis was supported by the finding that multiple miRNA's upregulated in CB target RUNX1. We further investigated a mechanistic role of miR-9/RUNX1 pathway and its contribution on these developmental differences.

## 3.2. Results

### 3.2.1. miRNA profiling and identification of RUNX1 putative targets

In this study, we have identified the expression profile of miRNAs in CB-MKs (Group-A) and PB-MKs (Group-B). As discussed elsewhere, we evaluated the expression levels of 88 human miRNAs important in cell development and differentiation using a qRT-PCR-based array. The biological significance threshold level was set to a fold-change of ±2-fold, and the significance level was set to a p-value of <0.05. Heat map showing the differentially expressed miRNA genes between CB-MKs and PB-MKs (n=3; Figure 3.4 A). Further, the PCR Array data are presented in a type of scatterplot known as volcano plot which is showing the statistically significant differentially expressed genes in Group-B vs Group A (Figure 3.4 B). Of the 88 genes of miRNAs analysed in CB- and PB-MKs, 10 microRNAs (miR-9, miR-99a, miR-126, miR-129-5p, miR-134, miR-192, miR-210, miR-215, miR-370, miR-452) were found to be significantly overexpressed in CB-MKs as compared to PB-MKs (n=3; P<0.05; Figure 3.4 C).

We than identified the putative targets for these ten microRNAs using five different miRNAs target computational algorithms (miRANDA, TargetScan, PicTar, and RNAhybrid), interesting five of these ten overexpressed microRNAs had RUNX1 as a potential target, including the most differentially expressed miRNA miR-9 (Figure 3.5 A).

Interestingly, we have also noticed the evolutionary conservation in RUNX1 3' UTR that interact with miR-9 Seed-sequence (Figure 3.5 B) [http://www.targetscan.org/vert_72/]. Moreover, hsa-miR-9-5p and RUNX1 mRNA duplex showed mfe value of −17.6 kcal/mole using bioinformatics tool RNAhybrid (Figure 3.5 C). Further, we validated the expression of miRNA-9 by qRT-PCR using miScript Primer Assays kit, the data was normalized against internal control U6. These results confirmed that the hsa-miR-9-5p expression levels in CB-MKs were significantly higher as compared to PB-MKs, and the differences were consistently increasing through all the stages (0, 7, 11, 14 days) of megakaryocytopoiesis (n = 3, P<0.02; Figure 3.5 D).

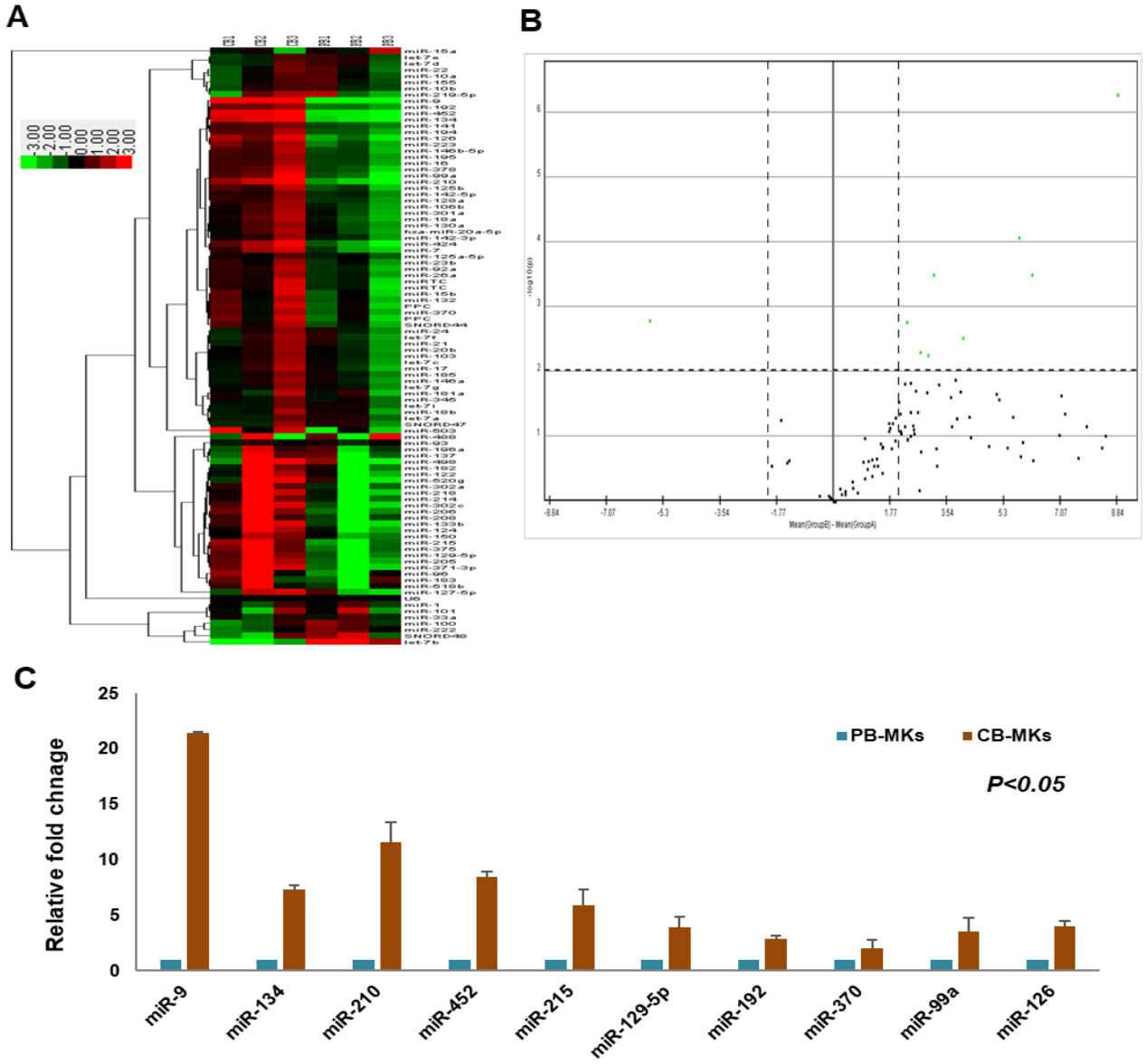
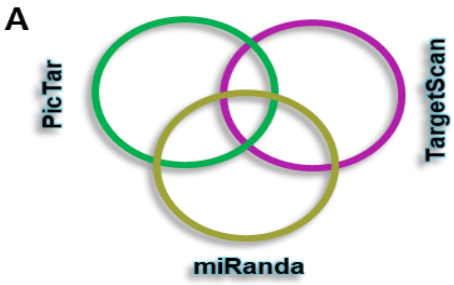
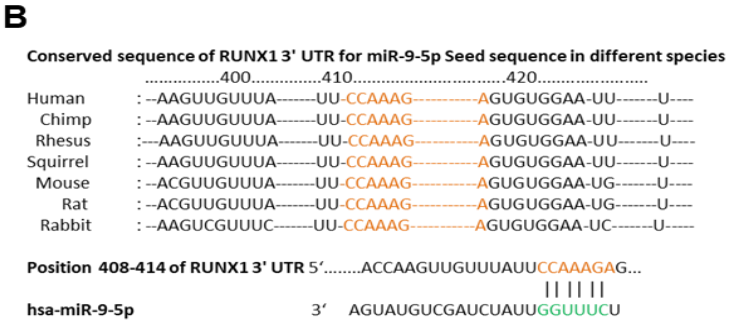
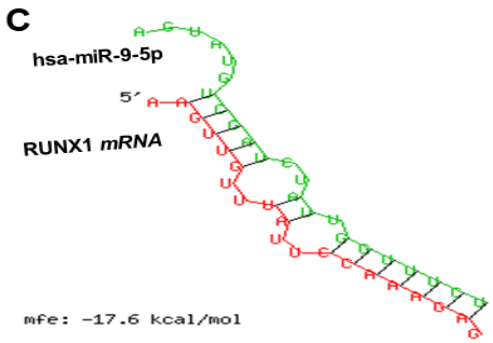



**Figure 3.4. miRNAs profiling and analysis of differentially expressed miRNAs in CB- versus PB-MKs.** A. Visualization of miRNAs expression in CB- versus PB-MKs using heat map (n = 3). B. The volcano plot y- and x-axes, respectively, displays p-value versus the fold change for each target in the data. This presentation enabling identification of statistically significant and differentially regulated miRNAs in group A (CB-MKs) versus group B (PB-MKs) data (n = 3; P < 0.05); green circles, represent the number of significantly overexpressed and down-regulated miRNA signatures in group A (CB-MKs). C. qRT-PCR showing top 10 most differentially expressed genes in CB- versus PB-MKs (n=3; *p<0.05). Bars represent mean ± SD of three independent experiments.




| miRNA ID | P-Value | CB/PB Expression | Putative Target |
|---|---|---|---|
| hsa-miR-370 | 0.05 | 2.03 | RUNX1 |
| hsa-miR-192 | 0.04 | 2.91 | RUNX1 |
| hsa-miR-129-5p | 0.05 | 3.89 | RUNX1 |
| hsa-miR-215 | 0.05 | 5.91 | RUNX1 |
| hsa-miR-9 | 0.03 | 21.4 | RUNX1 |



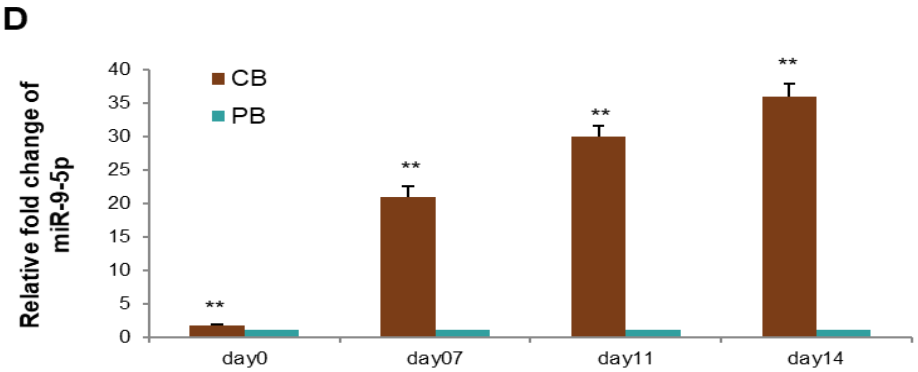




**Figure 3.5. Identification of RUNX 1 Putative targets. A.** RUNX1 was identified as a potential target for five of these ten upregulated miRNAs using five different miRNAs target computational algorithms (miRANDA, TargetScan, PicTar, and RNAhybrid). B. Sequences of RUNX1 3' UTR and conservation through evolution. (C) Minimum free energy (mfe) duplex of the hsa-miR-9-5p and RUNX1 3' UTR and mfe value for miRNA:mRNA duplex analysed by RNAhybrid. (D) hsa-miR-9-5p levels were measured by qRT-PCR in human CB- and adult PB-CD34+ cells derived MKs. miR-9 levels at different stages of CB-MK development (day 0, 7, 11, & 14) were expressed as relative to that in PB-MK development. hsa-miR-9 expression levels were significantly higher in CB- as compared to PB-MKs (n = 3, **P <0.02). Bars represent mean ± SD of three independent experiments.

### 3.2.2. miRNA-9 regulates developmental differences in RUNX1 expression.

Based on our finding that miR-9-5p expression is higher in neonatal CB-MKs as compared to adult PB-MKs (Figure 3.5 A and D), whereas its target RUNX1 expression levels are significantly low

in CB- and as compared to PB-MKs (Figure 3.3 A and B), we then explored the potential role of miR-9 in RUNX1 regulation in MKs. Given these findings, to prove the functional significance of hsa-miR-9 in RUNX1 regulation, we transfected human megakaryoblastic cell lines, MEG01 and Dami cells with pre-miR-9 or Cy3 labelled scrambled sequence (Control) using lipofectamine. After 72 hrs of transfection, we detected a significant increase in the miR-9 levels ($n = 3$, $P < .05$; Figure 3.6 A), along with increased cell proliferation ($n = 3$, $P < .05$; Figure 3.6 B) and reduced expression of MK marker CD61 ($n = 3$, $P = .02$; Figure 3.6 C). Further, we observed that the expression levels of RUNX1 protein were significantly reduced in pre-miR-9 transfected cells as compared to control ($n = 3$, $P < .001$; Figure 3.6 D). A study with MDSCs has also revealed the post-transcriptional regulation of Runx1 by miR-9 [264].

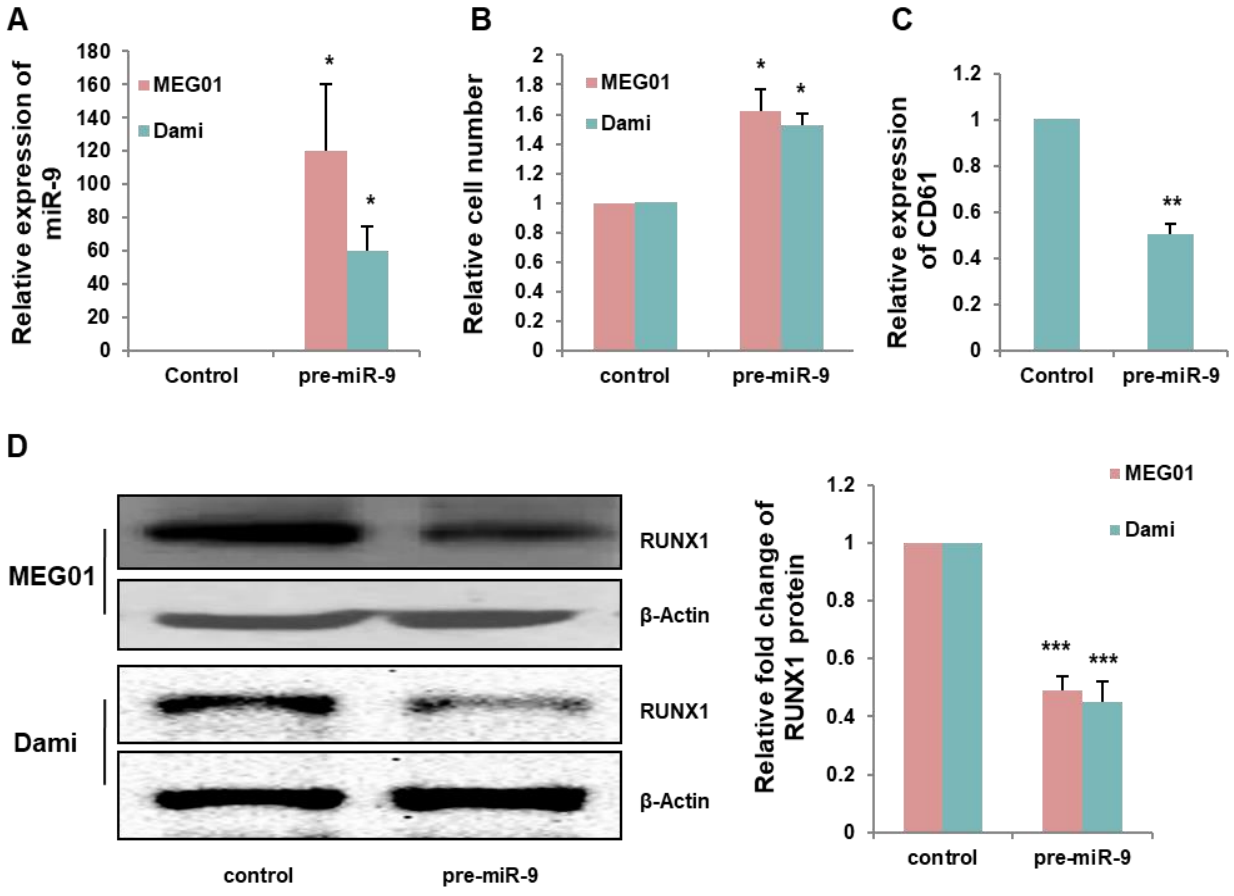



**Figure 3.6. miR-9 and RUNX-1 protein levels in hematopoietic cell lines.** A. hsa-miR-9 expression was ~60-fold higher in pre-mir-9 transfected cells as compared to control ($n = 3$, *P <0 .05). B. Results of Trypan blue assay, the

cell proliferation was increase (~1.6-fold) in pre-miR-9 transfected cells compared to control (n=3, *P <0 .05). C. qRT-PCR analysis of the MK marker CD61, expression was reduced after 72-hour of pre-miR-9 transfection (n=3, **P <0 .02). D. After 72 hours of transfection, RUNX1 protein levels were significantly reduced in pre-miR-9 transfected cells as compared to scrambled sequence transfected cells (n = 3, ***P <0 .001). Bars represent mean ± SD of three independent experiments.

Further, to confirm the functional role of miR-9 in RUNX1 regulation in MKs, we have performed miScript miR-9 Inhibitor (Qiagen) transfection study in Dami cells using HiPerFect transfection Reagent (Qiagen). miScript miRNA Inhibitors are designed to ensure efficient inhibition of endogenous miRNA function (Figure 3.7 A).

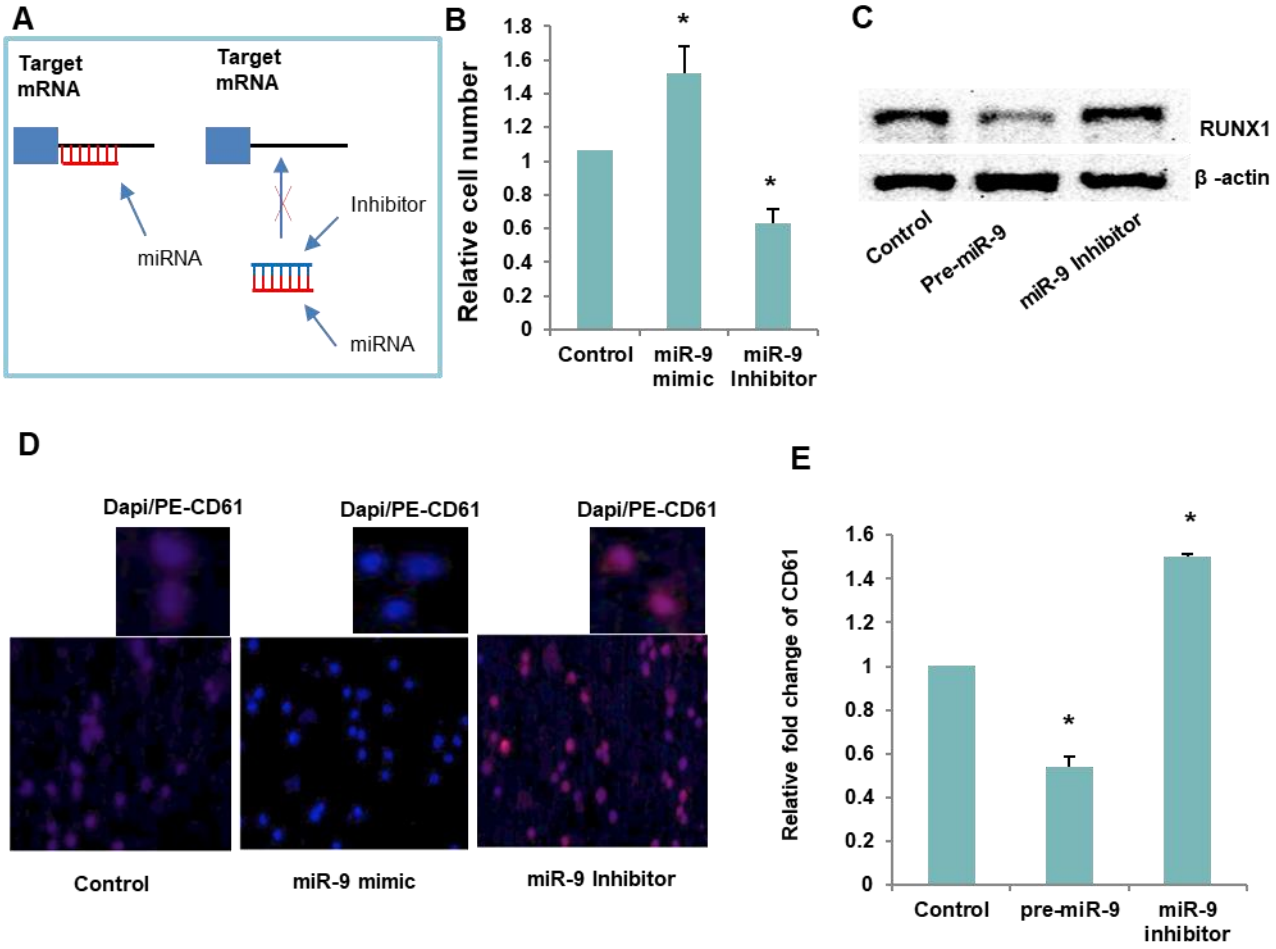



**Figure 3.7. miScript miRNA-9 Inhibitor counteracts miRNA-induced silencing.** A. Schematics showing the counteracting effects of miRNA Inhibitor on miRNA function. B. Results of Trypan blue assay, the cell proliferation was significantly reduced upon miR-9 Inhibitor transfection (n=3, *P <0 .05). C. Western blot analysis detected that

upon miR-9 Inhibitor transfection RUNX1 expression levels were restored (n=3; P<0.05). D. Fluorescence microscopy images (20x objective; scale = 50 μm) were observed with increased density of red florescence (PE-CD61 marker) upon miR-9 Inhibitor transfection as compared to control or Pre-miR-9 transfected cells (n = 3). E. qRT-PCR analysis shows the differential expression analysis of MK marker CD61 upon 72 hours of transfection with Pre-miR-9, miR-9 Inhibitor, or Control (n=3; *P<0.05). Bars represent mean ± SD of three independent experiments.

Cells were harvested 72 hours post-transfection for studies designed to analyze the expression of RUNX1, MK marker CD61, and cell proliferation. Interestingly, we observed that the cell proliferation was reduced post miR-9 Inhibitor transfection when compared with cell proliferation in control and pre-miR-9 transfection (n=3; P< 0.05; Figure 3.7 B). Western blot analysis detected that the decrease in RUNX1 protein in pre-miR-9 transfected cells was restored upon miR-9 Inhibitor transfection (n=3; P<0.05; Figure 3.7 C). Upon miR-9 inhibitor transfection, we have also observed increased expression of MK marker CD61 by fluorescence microscopy (n=3; Figure 3.7 D) and qRT-PCR (n=3; P<0.05; Figure 3.7 E).

### 3.3. Discussion

Herein we looked in the mechanism of developmental differences between neonatal and adult MKs in humans by illuminating the critical role of TF RUNX1 and miRNA's in regulating this developmental difference. Megakaryocytopoiesis mechanistic studies has been conventionally focused primary on TFs, over the last decade various laboratories have tried to address the role of miRNAs in hematopoiesis [362-364], but there only few studies about miRNA role in megakaryocytopoiesis are available [365]. However, given the mounting body of evidence supporting the existence of substantial biological differences between neonatal and adult MKs [326,350-352,366] our study is the first to address the molecular mechanism underlying these developmentally regulated biological differences by comparing MKs miRNA expression profiles of neonatal CB to the adult PB derived MKs. The finding that out of 10 microRNA's unregulated in CB compared to PB (Figure 3.4 C), 5 of those including miR-9 that was expressed 21-fold higher level had RUNX1 as a putative target [Figure 3.5 A, C]. miRNAs have been traditional viewed as miRNA-mediated translational repression mechanism; they regulate the expression of most (>60%) mammalian protein coding genes [367]. Recent work by Guo et al. [368] indicates that miRNA-controlled knock-down of protein expression is primarily via miRNA-RISC mediated

degradation of protein encoding transcript (mRNA), however miRNA-RISC mediated translation inhibition of protein synthesis represents only a minor mechanism. In different studies, miRNAs have been referred to as "rheostats" because their contribution in the regulation of gene function is generally to fine-tune but not abolish protein expression [369,370]. While the importance of RUNX1 in megakaryocytopoiesis is well-established [342,343], the information about the developmental regulation of RUNX1 in neonatal versus adult MKs is lacking. Our study highlighted miRNA's mediated regulation of RUNX1 in neonatal versus adult MKs and the critical role of RUNX1 in developmental megakaryocytopoiesis.

In vertebrates, recent studies offer compelling evidences on the development stage, cell type and context-dependent role of miR-9 in cell proliferation, migration and differentiation [252]. This study presents the developmental differences in the expression of miR-9 in neonatal versus adult MKs. (Figure 3.5 D), similar differences were also reported in embryonic stem cell derived MKs and adult stem cell derived MKs [338]. Interestingly, miR-9 and RUNX1 expressions were inversely related in both the neonatal and adult MKs (Figure 3.5 D; Figure 3.3 A and B). The down regulation of miR-9 during MK development and differentiation process may involve in developmental maturation of MKs by unblocking the TF RUNX1 expression. RUNX1 is a critical regulator of definitive hematopoiesis [371]. In the context of megakaryocytopoiesis and thrombocytopenia, the functional role of RUNX1 evidenced from loss of function mutations in RUNX1, which causes familial platelet disorder and thrombocytopenia [340]. Also, evidenced from the conditional Runx1 deletion phenotype, characterized by the large- number of small and low-ploidy MKs in mice BM, this phenotype of megakaryocytopoiesis is closely resembles normal neonatal megakaryocytopoiesis [341]. In this study, we report higher expression of RUNX1 targeting miRNA, miR-9 in neonatal MKs as compared to adult MKs (Figure 3.4 and Figure 3.5), whereas RUNX1 was significantly downregulated in neonatal MKs as compared with adult (Figure 3.3). We hypothesized that these developmental differences in the RUNX1 expression could be under the post-transcriptional control of miR-9. To prove the functional significance of miR-9 in RUNX1 regulation in MKs, we performed the transfection study in megakaryocytic cell lines, Dami and MEG01 (Figure 3.6). We observed significantly reduced expression of RUNX1 with pre-miR-9 (miR-9 mimic) transfection in Dami and MEG01 (Figure 3.6 D). Our observations

on miR-9/RUNX1 axis are consistent with the findings of Ben-Ami et al. [372] in HEK293 cells. Notably, the report of Tian et al [264] in MDSCs also described the functional role of miR-9 in translational attenuation of Runx1 in MDSC differentiation and function. Further, we have also observed that the miR-9 mediated down regulation of RUNX1 significantly affects cell proliferation and differentiation. Interesting, with RUNX1 downregulation the rate of cell proliferation was increased and MK marker CD61 expression was reduced in megakaryocytic cells (Figure 3.6 B and C). Furthermore, our miR-9 Inhibitor transfection study confirmed the role of MiR-9/RUNX1 axis in cell proliferation and MK differentiation (Figure 3.7).

In conclusion, our current findings indicating molecular differences between neonatal and adult MKs. The evidences of the present study suggest that the RUNX1 is regulated at the post-transcriptional level by a small non-coding RNA, hsa-miR-9-5p. Further, our study data suggest that the downregulation of RUNX1 in neonatal MKs is likely associated with higher expression of miR-9; miR-9/RUNX1 axis involved in the regulation of MKP proliferation and differentiation. This study provides the first evidence that miR-9 plays important role in human MK development via regulating RUNX1 expression, and for the first time reports a miRNA (miR-9-5p) as a molecular regulator of the developmental differences between neonatal- and adult-$CD34^{+}$ cells derived MKs. RUNX1, a known target of miR-9, is essential for definitive hematopoiesis and megakaryocytopoiesis, RUNX1 role is crucial for the regulation of MK specification and maturation [373]. Taken together, the results of this study showed the functional role of -hsa-miR-9-5p in the regulation of cell proliferation and differentiation by targeting RUNX1. High level of miR-9 expression observed in present study in neonatal MKs likely contribute to hyperproliferative and disease susceptible phenotype of neonatal MKs via targeting the expression of RUNX1; thus, it could be a potential target in neonatal thrombocytopenia and other platelet disorders.

## 3. Objective 3: To study the RUNX1 regulation and its crosstalk with other signaling pathways.

### 3.1. Introduction

In present study, we have observed that a key MK transcription factor RUNX1 is downregulated in neonatal-MKs when compared to adult-MKs. Moreover, we have observed that high cell proliferation rate and reduced MK-marker CD61 expression are associated with the miR-9 mediated down regulation of RUNX1 expression. RUNX1 belong to a family of metazoan TFs that functions as the master regulators of development; to regulate lineage specification and terminal maturation of cells RUNX1 communicate with major developmental pathways, e.g., TGF-β, WNT, Notch, receptor tyrosine kinases, etc. In a coordination with diverse signaling pathways and with various protein interacting partners RUNX1 play the role as a key regulator of diverse cellular processes/functions such as proliferation or cell cycling, cell differentiation, senescence, and apoptosis [270,273,274]. However, information about its crosstalk with developmentally regulated signaling pathways in MKs is lacking. The purpose of the present study is therefore to further ascertain the developmental differences between neonatal and adults MK and to study RUNX1 crosstalk with differentially regulated signaling pathways. In this study, we present the results of a microarray study performed in CB- versus PB-$CD34^+$ cells derived MKs. Notably, the most affected pathway detected in data analysis was TGF-β, we identified that most of the genes encoding TGF-β core pathway components and pathway effectors regulated components were down regulated in CB-MKs when compared with PB-MKs.

The molecular basis of the functional association between TGF-β receptors signaling and RUNX lies in the fact that SMADs and RUNX work synergistically to control the expression of target genes [277]. TGF-β regulates the expression of different genes including RUNX1 via TGF-βR/SMADs signaling [309,310]. On the other hand, RUNX1 is also involved in the regulation of TGF-β signaling in different cellular systems [277,311]. Specifically, in developmental hematopoiesis both the RUNX1 and TGF-β signaling are crucial regulators. RUNX1 is essential for the development of definitive hematopoiesis, definitive HSCs are not generated if RUNX1 function is lost [312,313]. In different studies, it has been observed that Runx1 knock out results

in massive hemorrhage in mice (at ~E12.5), and the hemorrhaging blood cells are nucleated red cells (primitive red cells), these primitive red cells are normally produced in the absence of Runx1 function [312,313]. It has been observed that the cause of this hemorrhage in Runx1 knock out mice is the angiogenic defect and abnormal development of blood cells, or lack of definitive HSCs that produce angiopoietin-1 [314]. On the other hand, TGF-β knock out mice were also observed with the defective hematopoiesis and vasculogenesis [315], which is similar to the RUNX1 knock out phenotype. However, a possible relationship between TGF-βR signaling and RUNX1 in hematopoiesis and megakaryocytopoiesis merits further examination.

In the present study, we confirmed the developmental differences in the expression of genes encoding components of the TGF-β pathway; majority of the differentially expressed genes encoding components of the TGF-β pathway were down regulated in CB-$CD34^+$ cells derived MKs as compared to PB-$CD34^+$ cells derived MKs. Moreover, our results suggest that the RUNX1/TGF-β axis augments terminal MK maturation. Present study data, report that during MK maturation TGF-β1 increase cellular $Ca^{2+}$ via ROS-mediated activation of TRP ion channels and TF RUNX1-mediated regulation of $Ca^{2+}$ ion channels expression. Thus, the lower expression of RUNX1 and TGF-β pathway components in neonatal (CB) MKs likely involved in the developmental uncoupling of neonatal megakaryocytopoiesis.

## 3.2. Results

### 3.2.1. TGF-β pathway in neonatal versus adult MKs

To determine developmentally controlled signaling pathways, we cultured CB- and PB-derived $CD34^+$ cell (HSPCs) in the presence of TPO; after 14 days, ~ 93% of CB-$CD34^+$ cells and ~ 91% of PB-$CD34^+$ cells were MKs. The CB- and PB-MKs after 14 days of growth were further used in a microarray study performed using Human U133 Plus 2.0 Array platform (Affymetrix, USA); the gene expression analysis was performed with ROSETTA RESOLVER data analysis system (Rosetta Inpharmatics LLC, USA). The microarray analysis comparing CB and PB MKs revealed substantial developmental differences in mRNA transcriptome: a total of 611 genes were identified with significantly differential expression ($n=6$; $P < 0.05$; data not showed).

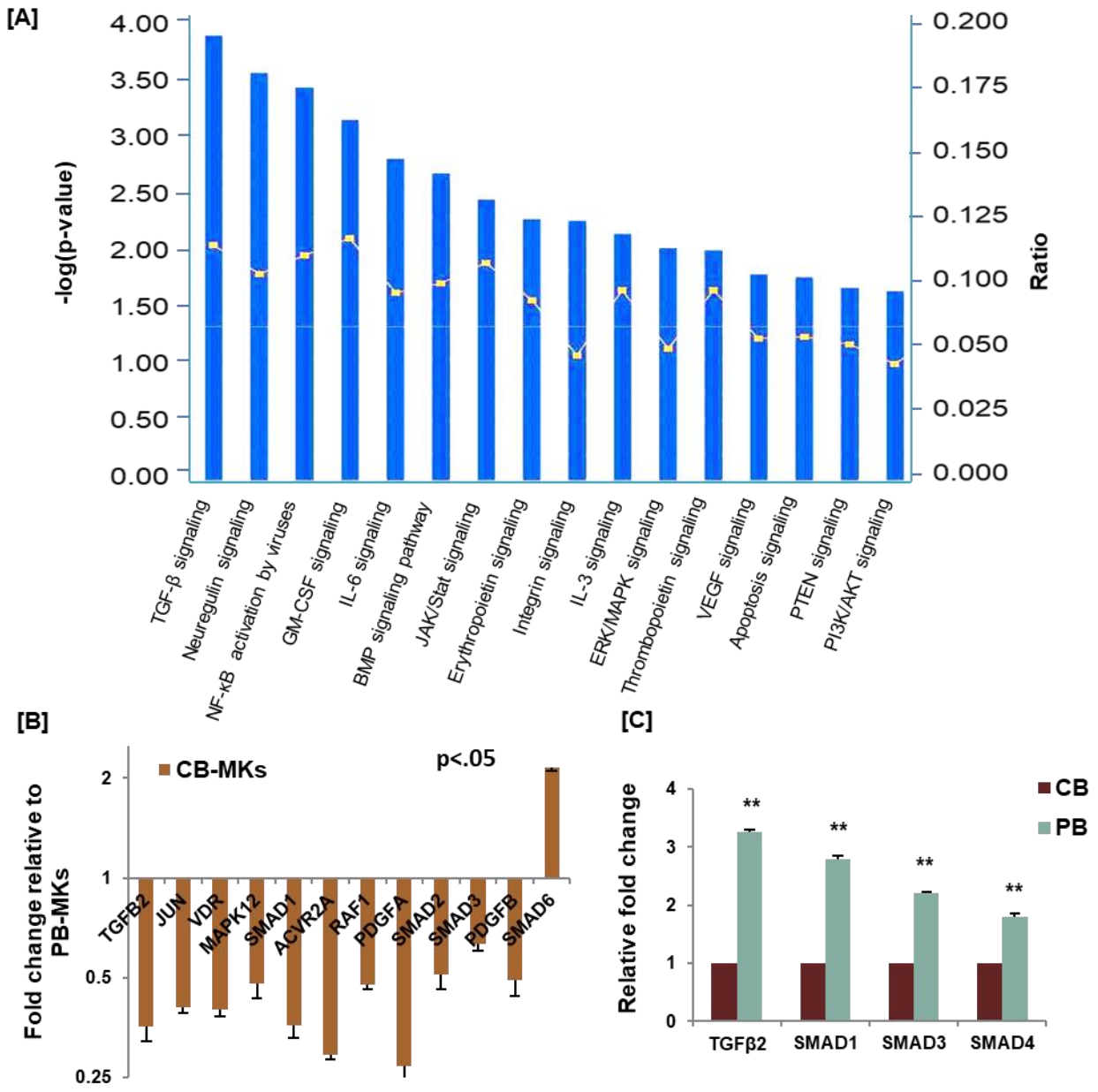



**Figure 3.8 Developmental differences in CB- and PB-CD34$^{+}$ cells derived MKs. (A)** The functional characterization of the differentially expressed genes with IPA INGENUITY showed the top affected pathways in CB-MKs. (B) The bar graph indicates the fold change of the TGF-β pathway signatures observed in the microarray study in CB-MKs as compared with PB-MKs (n = 3, P < 0.05). (C) qRT-PCR analysis of the differential expression of mRNAs encoding core TGF-β pathway components in CB- as compared with PB-CD34+ cells derived MKs (n = 3; **P < 0.02). Bars represent the mean ±SD from three independent experiments; all the data were assessed using Student's t-test.

The functional characterization and Ingenuity Pathway Analysis (Ingenuity Systems, Inc., USA) of the differentially expressed genes in the microarray data showed the top affected pathways (Figure 3.8 A). Notably, TGF-β signaling pathway was the most affected pathway among the differentially regulated genes; interestingly, in this array study, most of the TGF-β signaling genes were observed with down-regulation in CB- as compared to PB-MKs (n=6; $P<0.05$; Figure 3.8 B). Further, qRT-PCR based mRNA expression analysis of the selected TGF-β core pathway components in CB- versus PB-MKs confirmed the micro array findings (n = 3; $P<0.02$; Figure 3.8 C), the expressions of all the core TGF-β pathway components encoding genes were higher in PB as compared to CB-MKs (n = 3; $P<0.02$; Figure 3.8 C). The results of a previous study have also showed the increase in the synthesis and release of TGF-β during TPO induced megakaryocytopoiesis [307].

### 3.2.2. Erythroid and Megakaryocytic differentiation of K562 cells

In this study, we sought to investigate the existence of TGF-β pathway in PMA induced megakaryocytic differentiating K562 cells. In this study, we used PMA (50 nM) and Hemin (50 μM), respectively, to induce megakaryocytic and erythroid differentiation of K562 cells. Cells were analysed upon 72 hours of incubation to test the effect of Hemin and PMA on cell size, adherence, proliferation, and lineage specific markers expression. After Hemin treatment we observed only a marginal increase in cell size, however after PMA treatment a large increase in cell size and cell adherence was observed in K562 cells (n = 3; Figure 3.9 A). Further, we observed that the cell proliferation was significantly reduced over a 3-day period upon Hemin (~ 2-fold) or PMA (~ 4-fold) treatment (n = 3; $P < 0.02$; Figure 3.9 B) as compared to control.

Specifically, the erythroid differentiation was identified by testing the expression of erythroid markers (glycophorin A, GYPA and glycophorin B, GYPB) by qRT-PCR and haemoglobin by benzidine staining. We observed the increased expression of both the erythroid markers (n = 3; $P < 0.02$; Figure 3.9 C); moreover, over a 3-day period upon Hemin treatment, we observed that the percentage of benzidine-positive cells (about 60%) was significantly increased (n = 3; $P < 0.02$; Figure 3.9 D). We have also observed that the expression of MK markers (Figure 3.10 A and B)

and TF RUNX1 (Fig. 3.10 B and C) levels were significantly reduced in hemin induced erythroid differentiation as compared to control (n = 3; P< 0.05).

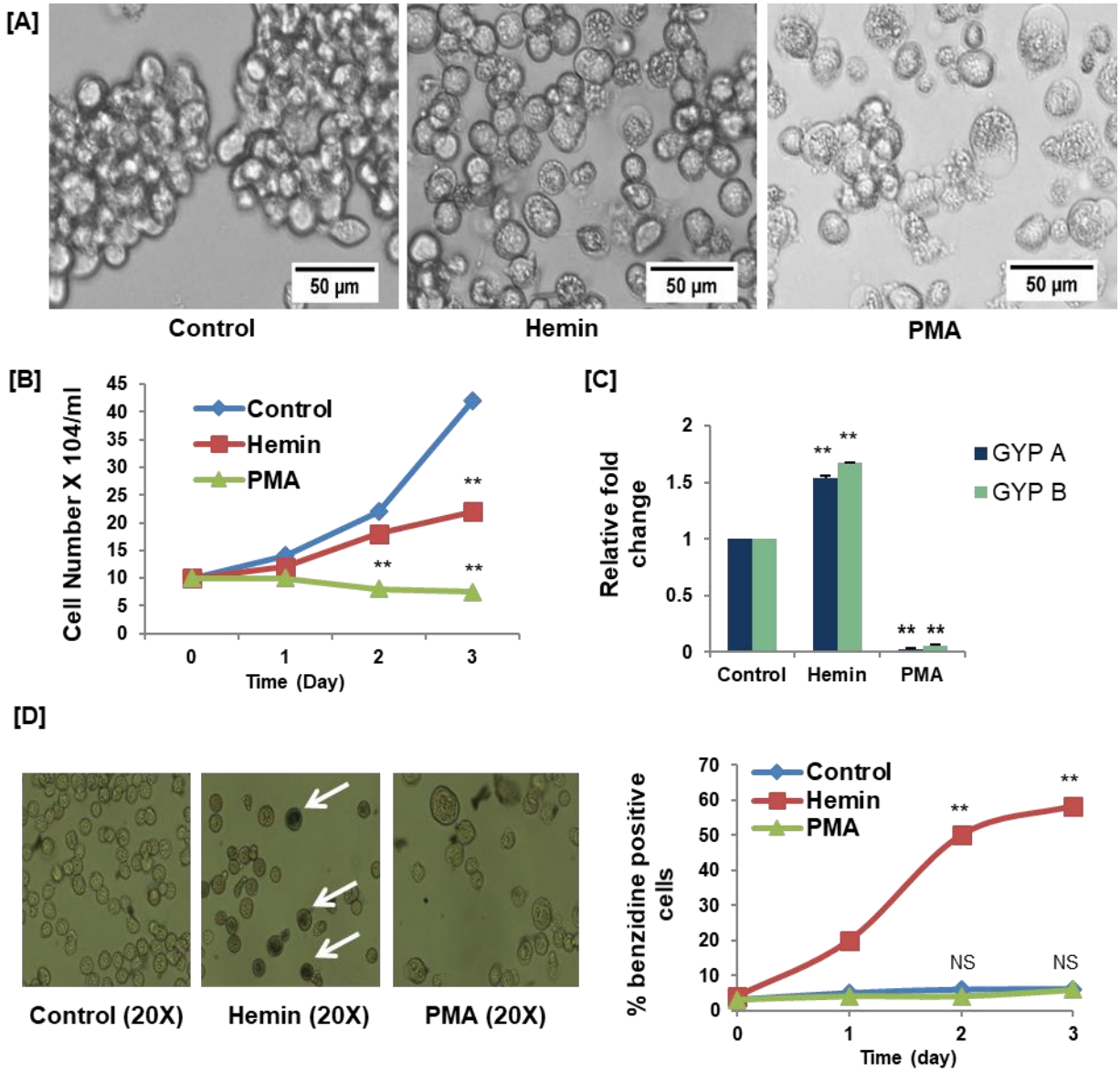



**Figure 3.9 Induction of erythroid differentiation of K562 by Hemin.** (A) Photomicrograph of cells, upon 72 hours of PMA and Hemin induction, showing morphological differences (cell size and adherence). (B) The bar graph demonstrates the number of cells changed over a 3-day period in Hemin and PMA groups as compared to control group (n = 3; **P < 0.02; nsP > 0.05). (C) qRT-PCR analysis of the differential expression of GYPA and GYPB upon PMA or Hemin treatment as compared to control (n = 3; **P < 0.02). (D) Photomicrograph of cells stained with benzidine after various treatments, white arrows indicate benzidine-positive cells (blue); bar graph represents the

statistics of the percentage of benzidine-positive cells increased over a 3-day period in different experimental conditions as compared to day 0 (n = 3; **P < 0.02; nsP > 0.05). Bars represent the mean ± SD from three independent experiments; the data were assessed using Student's t-test.

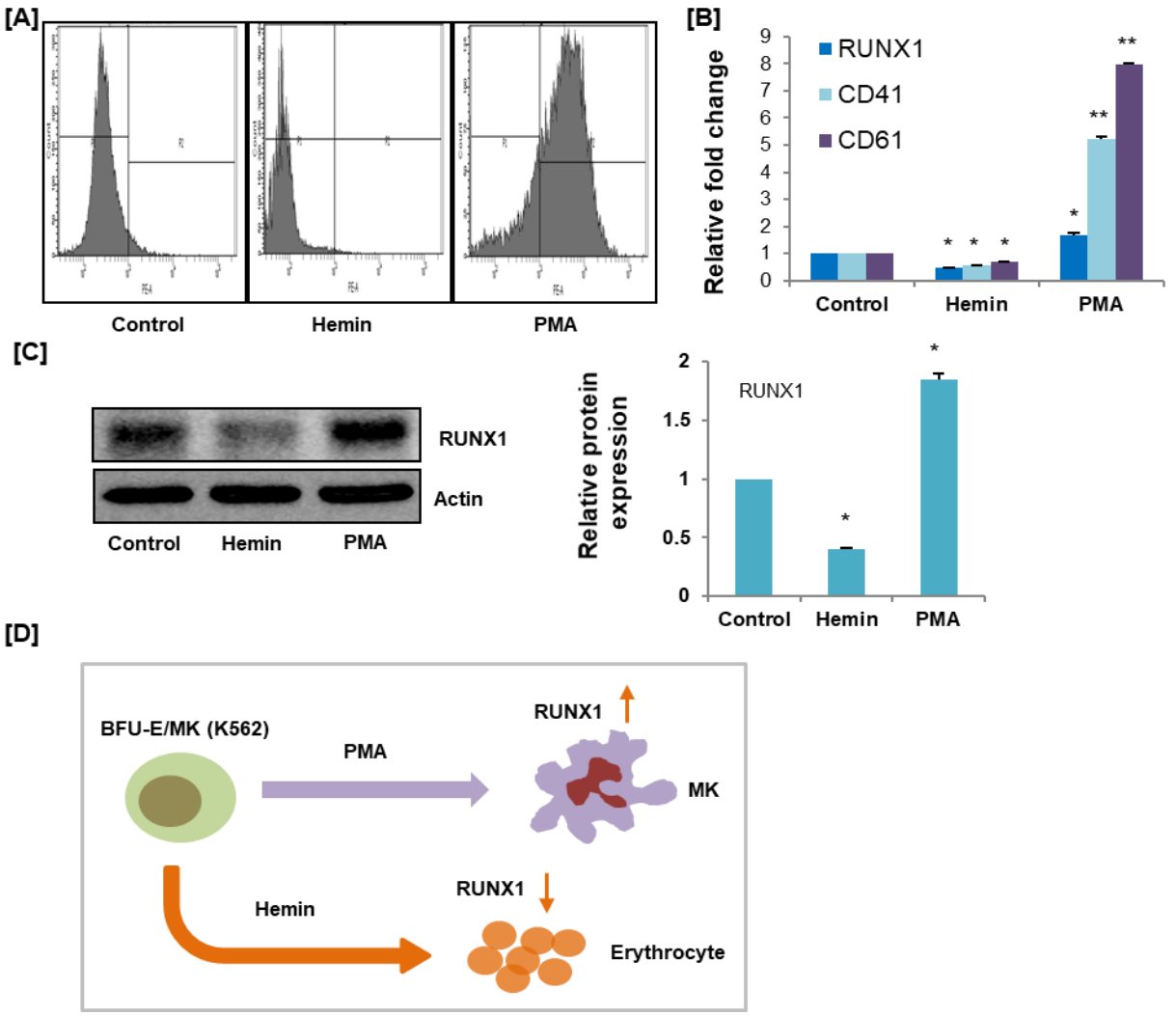



**Figure 3.10. Induction of megakaryocytic differentiation of K562 by PMA.** (A) Flow cytometry analysis of the MK marker CD61 expression in Hemin, PMA, or untreated control (n = 3; **P < 0.02). (B) qRT-PCR analysis of the differential expression of MK markers (CD41 and CD61) and TF RUNX1 mRNA after 72 hours of treatment with hemin or PMA as compared to control (n = 3; **P < 0.02; *P < 0.05). (C) RUNX1 expression levels in PMA or hemin treated cells were analysed by protein blot densitometry and compared to that in control cells (n = 3; *P < 0.05). (D) The graphical representation showing, respectively, PMA- and Hemin-induced differentiation of K562 toward MK and erythroid development. Bars represent the mean ±SD from three independent experiments; all the data were assessed using Student's t-test.

However, upon PMA treatment we observed a large change in cellular morphology, cells were observed with increased size, adherence, and membrane expansions upon microscopic examination (n=3; Figure 3.9 A). Further, analysis of MK surface antigen CD61 by flow cytometry identified a significant increase in the number of $CD61^+$ cells and the mean fluorescence intensity of CD61 in PMA-treated cells as compared to untreated control ($n = 3$; $P < 0.02$; Figure 3.10 A). The expression of MK markers CD41 and CD61 was further confirmed by qRT-PCR, resulted in a significant increase (> 4-fold) upon PMA treatment as compared to untreated control ($n = 3$; $P < 0.02$; Figure 3.10 B). Interestingly, the expression of megakaryocytic TF RUNX1, which is a key regulator of megakaryocytopoiesis [374], was upregulated in PMA induced K562 cells as compared to uninduced control ($n = 3$; $P < 0.05$; Figure 3.10 B and C). whereas, all the erythroid features and markers were significantly suppressed in PMA-induced megakaryocytic differentiating K562 cells ($n = 3$; $P < 0.02$; Figure 3.9 C and D).

### 3.2.3. Gene expression analysis of TGF-β pathway in PMA induced MKs

MKs secrete the bulk of latent TGFβ1 in the BM, TGF-β1 is a dominant signal emanating from MKs and play key role in the maintenance of HSC quiescence [302,303]. In BM, TGF-β signaling regulates a wide spectrum of processes of the hematopoietic system. However, the autocrine functions of TGF-β in MKs development is poorly understood. To study the existence of TGF-β autocrine functions in MKs we developed a PMA-induced megakaryocytic differentiation model system, as discussed elsewhere (Figure 3.10). In this model, the expression levels of TGF-β1, TGF-βR1, and TGF-βR2 were analyzed by qRT-PCR, the expressions were significantly high in PMA-induced MKs as compared to untreated K562 (n=3; $P < 0.02$; Figure 3.11 A). Our results are consistent with the observations of Alitalo et al. [305], reported a significantly enhanced expression of TGF-β1 in TPA-induced megakaryocytic differentiation of K562 cells. Using PMA-induced K562 cells, which partly recapitulate the MK development process, we have performed RT2 Profiler PCR Array (Qiagen, Inc., MD, USA) to extend our understanding about the existence of TGF-β pathway in MKs. In present study, 84 genes encoding TGF-β/BMP pathway components were evaluated in PMA induced MKs, as shown in Figure 3.11 B, C and Table 3.1. Most of the genes were showing a large change (>2-fold) in their mRNA expression when we compared the

normalised expressions levels between the group1 (PMA stimulated) and control group (DMSO) (n=3; Figure 3.11 B). As shown in Figure 3.11C, the volcano plot represented the statistically significant differentially expressed genes (Fold expression, ±2-fold; $p<0.05$; Figure 3.11 C and Table 1), Of the 84 genes analysed in RT2 profiler array (TGF-b/BMP pathway), only 35 genes showed significant difference in PMA induced MKs as compared to control group ($n = 3$; $P < 0.05$; FC ± 2.0; Figure 3.11 C and Table 1). In this RT2 Profiler array study, out of 35 differentially expressed genes, 33 (about 39%) were upregulated, and only 2 (about 2%) genes were downregulated (Figure 3.11 C and Table 3.1).

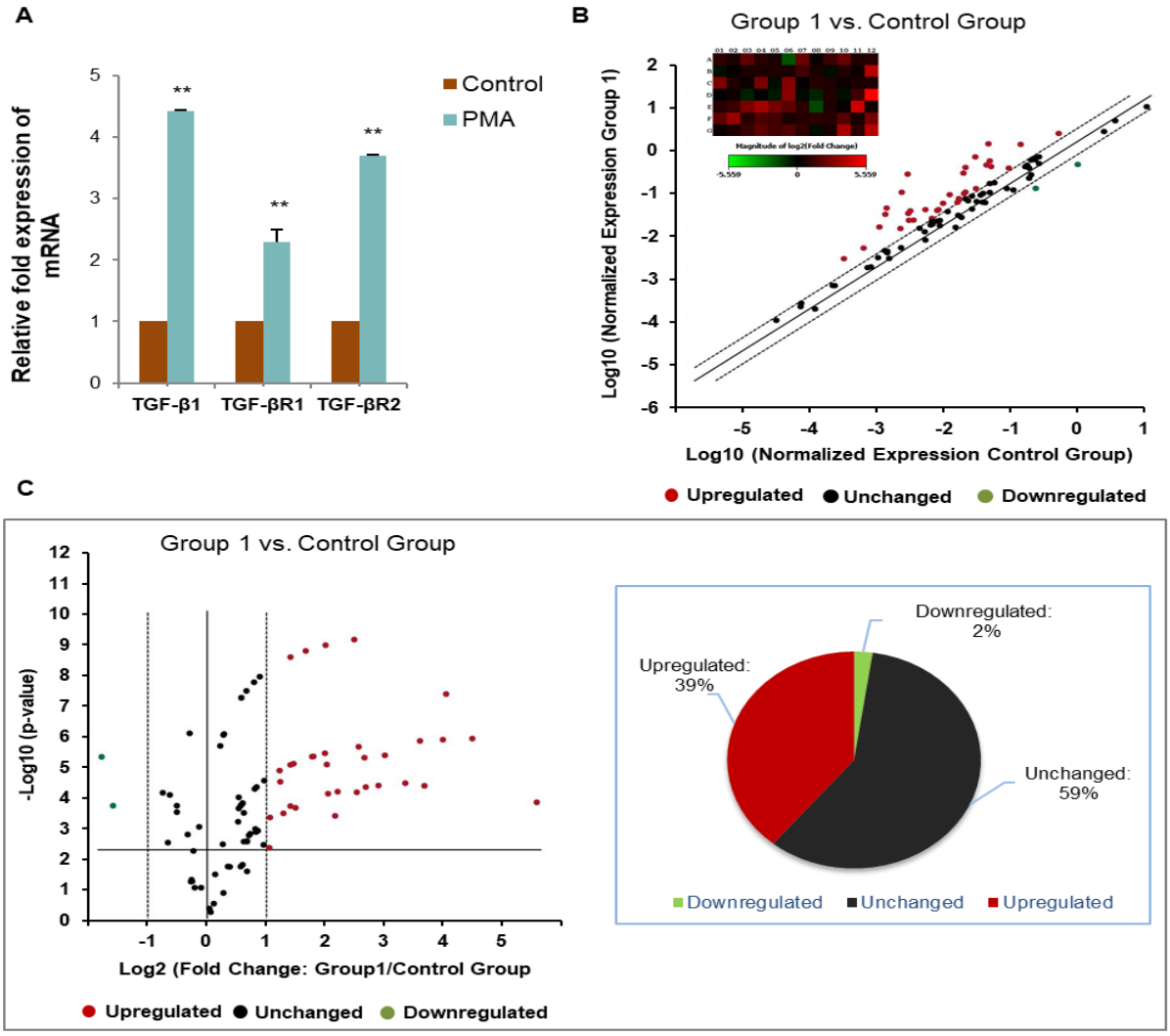



**Figure 3.11 RT2 Profiler™ PCR Array (TGF-β/BMP pathway).** (A) qRT-PCR analysis of expression levels of

TGF-β and TGF-βRs in PMA-induced MKs (n = 3; **P < 0.02). (B) Visualization of the normalised expression of genes using scatter plot (n = 3); lines above or under the mid-line denote the ±2 fold boundary of gene expression; red and green circles, respectively, represent the number of genes overexpressed and downregulated in group 1 (PMA-induced MKs). (C) The volcano plot shows statistical significance (P-value) versus magnitude of change (fold change) on the y- and x-axes, respectively; red and green circles represent only the differentially expressed genes at a statistically significant level. The red and green circles, respectively, represent the significantly up- and down-regulated genes in PMA-induced MKs (group 1) when compared with control group (n = 3; ±2 fold; P < 0.05). Pie chart is representing the percentage of genes in three different classes of expression changes in group 1. Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

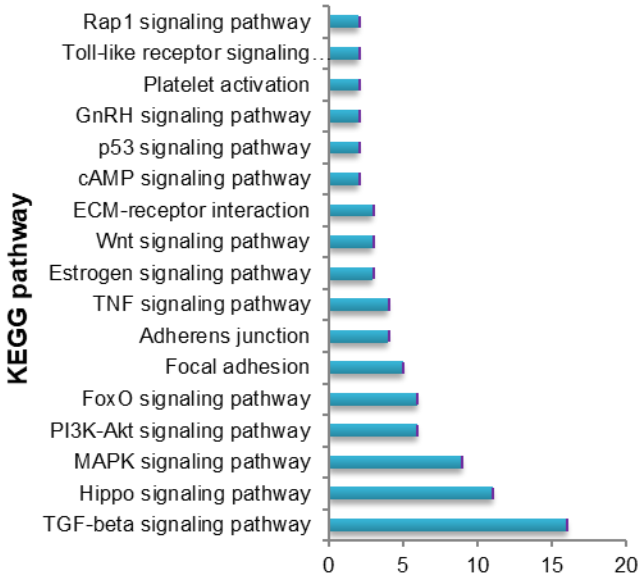
The functional characterization of the differentially expressed genes by using the KEGG PATHWAY online tool showed the top affected pathways (Figure 3.12 A). Notably, the most affected pathway was TGF-β signaling pathway, most of the differentially regulated genes are associated with the TGF-β pathway (Figure 3.12 A Table 3.2).

Further functional characterisation based on subcellular localization of the components by GO term-cellular component (GO-CC) classification of the DAVID database revealed that these components are mainly localized in extracellular region, ECM, platelet alpha granules lumen, cytosol, plasma membrane, nucleoplasm, and ER lumen (P< 0.02; Figure 3.12 B and Table 3.3). Notably, most of the components associated with extracellular region were upregulated in PMA-induced MKs and belong to TGF-β superfamily, TGF-β binding protein family, and ECM components (Figure 3.12 B), such as TGFB1, PDGFB, BMP1, THBS1, BMP2, SERPINE1, INHBA, TGFBR3, INHA, LTBP1, COL1A1, COL1A2, TGFBI, and TGFB3, were up-regulated in PMA-induced MKs (n = 3; P < 0.05; Table 3.1). The data presented in this study are consistent with the previous report that identified TPO induced synthesis and secretion of TGF-β by MKs, and its autocrine functions on MKs to regulate the synthesis of ECM components such as collagens and fibronectin which surrounds MKs in human BM [307].

In addition to extra-cellular region, GO-CC clustering have also detected enrichment of TGF-βR/SMAD pathway effectors regulated genes that encode TFs in the nucleoplasm (Figure 3.12 B), this group of genes encode TFs which are involved in myeloid development and megakaryocytopoiesis (Figure 3.13 A-D) [375]; RUNX1, SMAD2/3, AP-1 TFs (JUN, JUNB, and

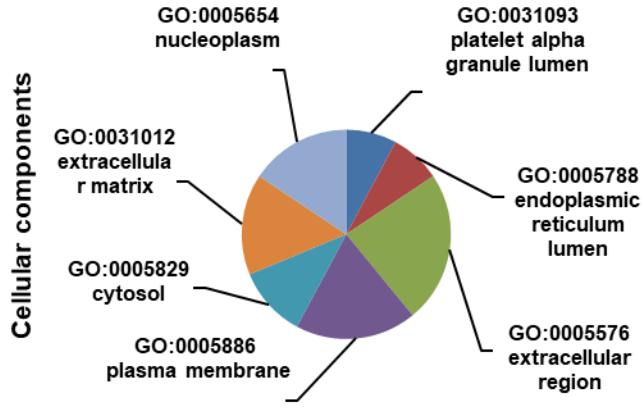
FOS) were upregulated in PMA-induced MKs as compared to control (P<0.05; Figure 3.13 C and D), whereas ATF4 and MYC TFs were observed with downregulation ((P<0.05; Figure 3.13 B).

[A]




| Category | Gene Up-Regulated | Fold Change (Group1/Control Group) |
|---|---|---|
| hsa04350:TGF-beta signaling pathway | INHBA | 47.14 |
| | LEFTY1 | 12.03 |
| | JUNB | 6.41 |
| | TGFB1 | 5.61 |
| | BAMBI | 4.62 |
| | BMP2 | 4.47 |
| | ACVRL1 | 4.12 |
| | TGFBR2 | 3.43 |
| | SMAD2 | 3.19 |
| | SMAD3 | 2.75 |
| | TGFBR1 | 2.66 |
| | BMPR2 | 2.65 |
| | BMP1 | 2.45 |
| | JUN | 2.36 |
| | TGFB3 | 2.34 |
| | ACVR1 | 2.09 |

[B]




| Category | Cellular components |
|---|---|
| GO:0031093 platelet alpha granule lumen | TGFB1, PDGFB, SERPINE1, THBS1, TGFB3 |
| GO:0005788 endoplasmic reticulum lumen | PDGFB, COL1A1, B2M, COL1A2, THBS1 |
| GO:0005576 extracellular region | PDGFB, BMP1, BMP2, INHA, SERPINE1, INHBA, TGFB1, COL1A1, TGFBR3, B2M, LTBP1, COL1A2, TGFBI, THBS1, TGFB3 |
| GO:0005886 plasma membrane | BAMBI, BMPR2, TGFB1, ACVRL1, SMAD3, EMP1, B2M, TGFBR2, TGFBR1, TGFBI, SERPINE1, TGFB3, |
| GO:0005829 cytosol | JUN, SMAD3, ID2, TGFBR2, FOS, MYC, SMAD2 |
| GO:0031012 extracellular matrix | TGFB1, PDGFB, COL1A1, LTBP2, COL1A2, LTBP1, TGFBI, SERPINE1, THBS1, TGFB3 |
| GO:0005654 nucleoplasm | JUN, RUNX1, SOX4, ATF4, SMAD3, ID2, FOS, MYC, JUNB, SMAD2, SMAD3 |

**Figure 3.12. PMA-induced megakaryocytic differentiation of K562 cells enhanced the expression of TGF-β pathway components.** (A) The functional characterization of the differentially expressed genes by using the KEGG PATHWAY online tool. (B) The functional characterisation based on subcellular localization of the components by GO term-cellular component (GO-CC) classification of the DAVID database.

**Table 3.1. Genes involved in TGF-*β*/BMP signaling pathway are fold up- or down- regulated in megakaryocyte differentiation of K562 cells upon PMA (50 nM) stimulation (n=3; p<0.05)**

| Gene Bank | Gene Symbol | Description | Fold Change Group 1/Control |
|---|---|---|---|
| NM_001105 | ACVR1 | Activin A receptor, type I | 2.09 |
| NM_000020 | ACVRL1 | Activin A receptor type II-like 1 | 4.12 |
| NM_001675 | ATF4 | Activating transcription factor 4 (tax-responsive enhancer element B67) | -3.41 |
| NM_012342 | BAMBI | BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) | 4.6 |
| NM_006129 | BMP1 | Bone morphogenetic protein 1 | 2.45 |
| NM_001200 | BMP2 | Bone morphogenetic protein 2 | 4.47 |
| NM_001204 | BMPR2 | Bone morphogenetic protein receptor, type II (serine/threonine kinase) | 2.65 |
| NM_000088 | COL1A1 | Collagen, type I, alpha 1 | 15.77 |
| NM_000089 | COL1A2 | Collagen, type I, alpha 2 | 6.32 |
| NM_001423 | EMP1 | Epithelial membrane protein 1 | 5.92 |
| NM_005252 | FOS | FBJ murine osteosarcoma viral oncogene homolog | 7.44 |
| NM_002166 | ID2 | Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein | 8.03 |
| NM_002191 | INHA | Inhibin, alpha | 2.66 |
| NM_002192 | INHBA | Inhibin, beta A | 47.14 |
| NM_002228 | JUN | Jun proto-oncogene | 2.36 |
| NM_002229 | JUNB | Jun B proto-oncogene | 6.4 |
| NM_020997 | LEFTY1 | Left-right determination factor 1 | 12.03 |
| NM_000627 | LTBP1 | Latent transforming growth factor beta binding protein 1 | 5.76 |
| NM_000428 | LTBP2 | Latent transforming growth factor beta binding protein 2 | 4.02 |
| NM_002467 | MYC | V-myc myelocytomatosis viral oncogene homolog (avian) | -2.99 |
| NM_002608 | PDGFB | Platelet-derived growth factor beta polypeptide | 22.23 |
| NM_001754 | RUNX1 | Runt-related transcription factor 1 | 4.06 |
| NM_000602 | SERPINE1 | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 10.14 |
| NM_005901 | SMAD2 | SMAD family member 2 | 3.19 |
| NM_005902 | SMAD3 | SMAD family member 3 | 2.75 |
| NM_003107 | SOX4 | SRY (sex determining region Y)-box 4 | 3.47 |
| NM_000660 | TGFB1 | Transforming growth factor, beta 1 | 5.61 |
| NM_003239 | TGFB3 | Transforming growth factor, beta 3 | 2.34 |
| NM_000358 | TGFBI | Transforming growth factor, beta-induced, 68kDa | 3.97 |
| NM_004612 | TGFBR1 | Transforming growth factor, beta receptor 1 | 2.66 |
| NM_003242 | TGFBR2 | Transforming growth factor, beta receptor II (70/80kDa) | 3.43 |
| NM_003243 | TGFBR3 | Transforming growth factor, beta receptor III | 2.07 |
| NM_003246 | THBS1 | Thrombospondin 1 | 12.67 |
| NM_006022 | TSC22D1 | TSC22 domain family, member 1 | 16.34 |
| NM_004048 | B2M | Beta-2-microglobulin | 2.81 |

**Table 3.2. The results of KEGG pathway analysis of 35 significantly changed genes in RT² Profiler™ PCR Array.**

| Category | Term | Count | % | PValue | ENTREZ_GENE_ ID |
|---|---|---|---|---|---|
| KEGG_PATHWAY | hsa04350: TGF-beta signaling pathway | 16 | 66.666 | 2.43E-24 | 659, 90, 3398, 650, 3624, 25805, 7040, 4088, 7048, 10637, 4052, 7046, 7057, 4609, 7043, 4087 |
| KEGG_PATHWAY | hsa04390: Hippo signaling pathway | 11 | 45.833 | 1.73E-11 | 659, 7040, 4088, 3398, 7048, 650, 7046, 4609, 5054, 7043, 4087 |
| KEGG_PATHWAY | hsa05166: HTLV-I infection | 11 | 45.833 | 2.96E-09 | 3725, 7040, 5155, 468, 4088, 7048, 2353, 7046, 4609, 7043, 4087 |
| KEGG_PATHWAY | hsa04010: MAPK signaling pathway | 9 | 37.5 | 9.10E-07 | 3725, 7040, 5155, 468, 7048, 2353, 7046, 4609, 7043 |
| KEGG_PATHWAY | hsa04068: FoxO signaling pathway | 6 | 25 | 6.60E-05 | 7040, 4088, 7048, 7046, 7043, 4087 |
| KEGG_PATHWAY | hsa04520: Adherens junction | 4 | 16.666 | 0.001 | 4088, 7048, 7046, 4087 |
| KEGG_PATHWAY | hsa04510: Focal adhesion | 5 | 20.833 | 0.004 | 3725, 5155, 1277, 1278, 7057 |
| KEGG_PATHWAY | hsa04151: PI3K-Akt signaling pathway | 6 | 25 | 0.004 | 5155, 468, 1277, 1278, 7057, 4609 |

**Table 3.3. The details of protein location, of 35 significantly changed genes in RT² Profiler™ PCR Array, identified by GOTERM_CC_DIRECT (DAVID 6.8 Tool).**

| Category | Term | Count | % | PValue | Genes ID |
|---|---|---|---|---|---|
| GOTERM_CC_DIRECT | GO:0031093 platelet alpha granule lumen | 5 | 14.285 | 1.15E-06 | 7040, 5155, 7057, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005788 endoplasmic reticulum lumen | 5 | 14.285 | 1.15E-06 | 5155, 1277, 567, 1278, 7057 |
| GOTERM_CC_DIRECT | GO:0005576 extracellular region | 15 | 42.857 | 2.36E-04 | 5155, 649, 650, 3623, 5054, 3624, 7040, 1277, 7049, 567, 4052, 1278, 7045, 7057, 7043 |
| GOTERM_CC_DIRECT | GO:0005886 plasma membrane | 12 | 34.285 | 4.96E-04 | 659, 25805, 7040, 94, 2012, 4088, 7048, 567, 7046, 7045, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005829 cytosol | 7 | 20 | 5.71E-04 | 3725, 4088, 3398, 7048, 2353, 4609, 4087 |
| GOTERM_CC_DIRECT | GO:0031012 extracellular matrix | 10 | 28.571 | 0.003 | 7040, 5155, 1277, 4053, 1278, 4052, 7045, 7057, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005654 nucleoplasm | 10 | 28.571 | 0.024 | 861, 3725, 468, 6659, 4088, 3398, 2353, 4609, 3726, 4087 |

Mostly, strong proteins interactions occur in cells whenever biological functions require. Further, in this study, to understand the protein interactions and associated functions, 35 differentially regulated gene-encoded proteins were analysed for interactions by STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) and the interactions were visualised with their expression levels by CYTOSCAPE 3.6 software (Figure 3.13 E).

[A]

| Positive cell differentiation | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| SOX4 | 3.47 |
| SMAD2 | 3.19 |
| SMAD3 | 2.75 |

[B]

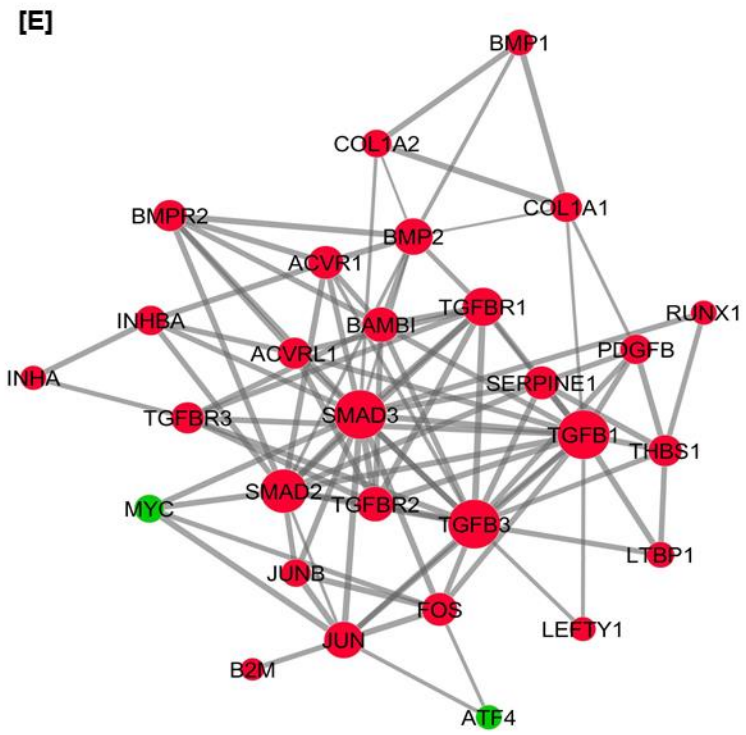
| Positive Regulation of Biosynthesis | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| SOX4 | 3.47 |
| ATF4 | -3.41 |
| MYC | -2.99 |

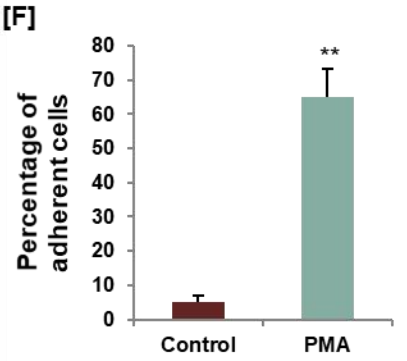
[C]

| Megakaryocytopoiesis | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |

[D]

| AP-1 Monomers | |
|---|---|
| **Gene** | **FC** |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| ATF4 | -3.41 |



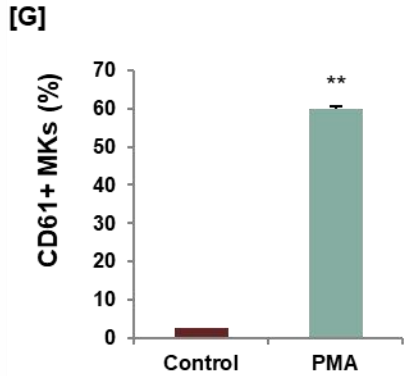




**Figure 3.13 Protein interaction network and associated functional aspects.** (A) cell differentiation (B) biogenesis, and (C) megakaryocytopoiesis regulators and (D) AP-1 monomers were identified with statistically significant

differential expression in PMA induced MKs as compared to uninduced control (n = 3; P < 0.005). (E) A protein interaction network, including 29 TGF-β pathway components, was extracted from STRING database. The size of node indicates the degree of interactions and line thickness indicates the strength of data support. The Red and Green colour of nodes, respectively, represents the up- or down-regulation of gene expression (visualized in CYTOSCAPE 3.6). (F) Graph shows the increased percentage of adherent cells (counted 20 fields under inverted microscope; n = 3, **P < 0.02) after 72 hours of PMA treatment as compared with that in unstimulated control. (G) Bar graph shows the increased percentage of MKs (CD61+ cells) upon PMA treatment as compared to unstimulated control (n = 3; P < 0.02). Bars represent the mean ± SD of three independent experiments; all the data were assessed using Student's t-test.

Of the 35 protein components, 29 are involved in the interaction network searched in STRING database. The size of node indicates the degree of interactions and the line thickness indicates the strength of data support; the active interaction sources considered in this interaction search are text mining, experiments, and databases. In this interaction network, the red and green colours of nodes, respectively, represent the upregulation and down regulation of genes from the present RT2 Profiler Array (Figure 3.13 E). Interestingly, most of the components identified with the differential expression in the present study were showing ingestions with TGF-β pathway core components and effectors such as TGF-βRs and SMADs (Figure 3.13 E), most of the components were upregulated (P < 0.02; Figure 3.11 C and Table 3.1) in PMA induced megakaryocytic differentiating K562 cells as compared to control (P < 0.02; Figure 3.13 F and G). The data presented in this study have shown the existence of TGF-β pathway in MK development.

### 3.2.4. Recombinant human TGF-β1 augments the maturation process of differentiating megakaryocytic cells

Based on our finding that TGF-β pathway components are downregulated in neonatal MKs as compared to developmentally matured adult MKs and upregulated during PMA-induced megakaryocytic differentiation of K562 cells, we next investigated the functional significance of the TGF-β pathway in MK maturation. To investigate the functional significance, PMA-induced MKs were treated with different concentrations of TGF-β1.

The change in cell shape, size, and membrane potential, and increase in membrane expansion and markers expressions are the commonly observed features or events of MK maturation. In present

study, we observed a large increase in cell size, adherence, and membrane expansions in PMA-induced MKs upon TGF-β1 treatment post-PMA stimulation as compared to that observed in PMA-stimulated model or control (n=3; Figure 3.14 A). Further, we checked the expression levels of TGF-β receptor and the effectors of the pathway SMAD2/3 which plays a fundamental role in mediating TGF-β response by regulating the expression of target genes. The results of this experiment showed the dose dependent increased expression of TGF-βR2 mRNA in PMA-induced MKs upon TGF-β1 treatment post-PMA stimulation (n=3; $P<0.05$; Figure 3.14 B); SMAD3 mRNA levels did not changed much upon TGF-β1 treatment (n=3; $P<0.05$; Figure 3.14 C), however cellular Phospho-SMAD2/3 (pSMAD2/3) levels were increased at the ligand concentration of up to 20 ng/ml (n=3; Figure 3.14 D). The phosphorylation state of the R-SMADs in cells thus serves as a marker for the activation of TGF-βRs signaling.

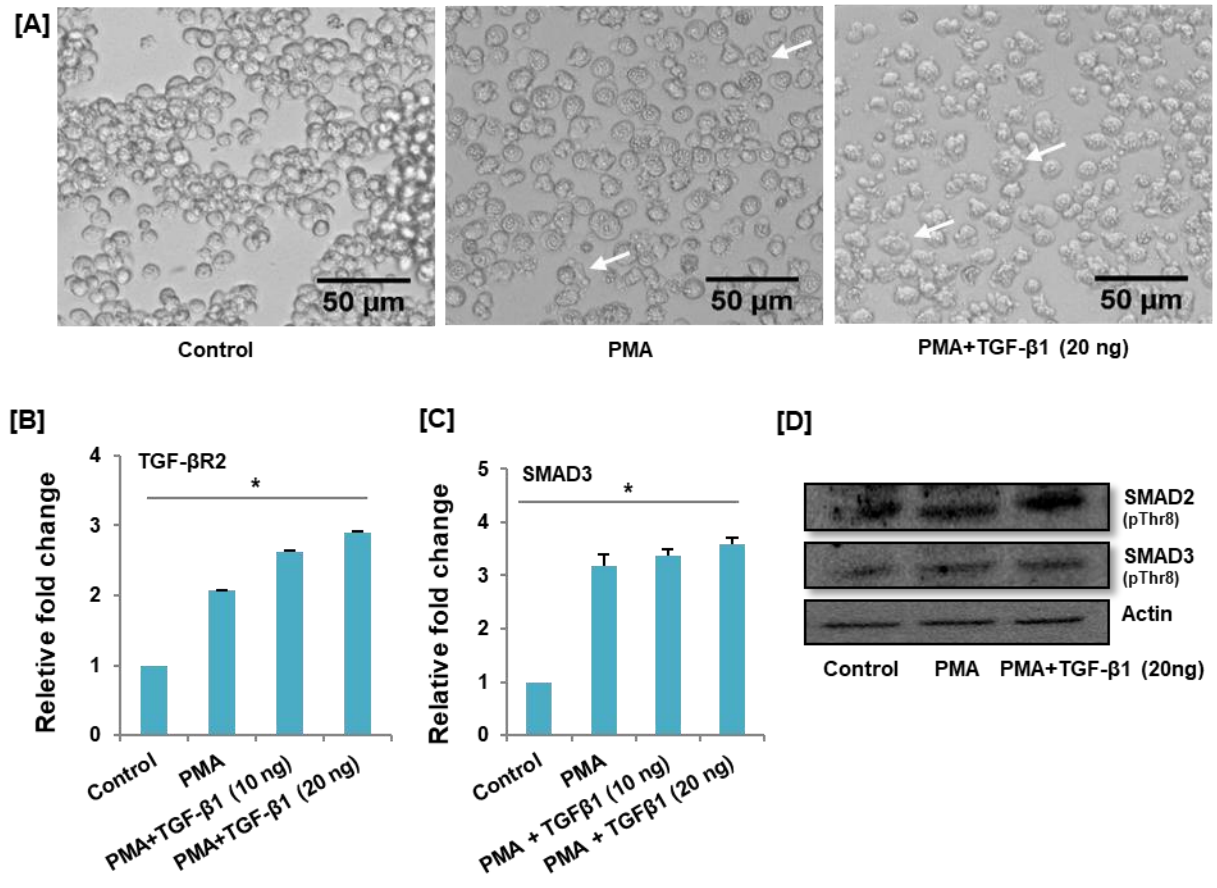



**Figure 3.14. Effects of TGF-β1 on PMA-induced megakaryocytic differentiation of K562.** (A) Photomicrograph

showing cells adherence and membrane expansion in different treatment conditions (n = 3). (B) qRT-PCR analysis shows TGF-βR2 expression in different treatment conditions as compared to control (n = 3; *P < 0.05). (C) qRT-PCR analysis shows DMAD3 mRNA expression levels in different treatment conditions as compared with control (n = 3; P < 0.05). (D) Protein expression analysis by western blot shows increased levels of pSMAD2/3 upon TGF-β1 treatment of PMA-induced megakaryocytic differentiating K562 cells (n = 3). Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

Given these findings, we then investigated whether TGF-β affect the expression of MK markers and TF RUNX1. We observed the dose dependent effect of the TGF-β1 on the expression levels of MK marker CD41 and TF RUNX1 in PMA-induced MKs, TGF-β1 significantly increased the expression levels of CD41 and RUNX1 in PMA induced MKs (n=3; P < 0.05; Figure 3.15 A). Further, we tested the expression and deposition of collagen upon TGF-β1 treatment of PMA-induced MKs; collagen is one of the ECM components which surround human MKs and observed with increased expression during megakaryocytopoiesis. We visualised the deposition of collagen using anti-type I collagen (COL1) antibody under fluorescence microscope, we observed the increased fluorescence intensity of COL1 in PMA-induced MKs upon TGF-β1 treatment post-PMA stimulation (n = 3; Figure 3.15 B), which confirmed the positive effect of TGF-β1 downstream effectors on collagen expression and deposition during MK maturation.

To further confirm the functional role of TGF-β pathway in MK maturation, MK surface antigen CD61 (Dapi/CD61-PE) expression was visualised under fluorescence microscope, we observed the increased fluorescence intensity of CD61 in TGF-β1 treated cells post-PMA stimulation as compared to control, or PMA alone (n = 3; Figure 3.15 C). In agreement with the results of the present study showing the augmenting effects of TGF-β1 pathway activation on the expressions of MK markers (CD41 and CD61), TF RUNX1, ECM component collagen, and the expansion of cell membrane, we then performed the cell cycle analysis (i.e., DNA ploidy analysis) using flow cytometry (Muse Cell Analyzer). The population profile, cell size distribution, depicted the increased cell size index with TGF-β1 treatment post-PMA stimulation and PMA alone as compared to control (n=3; Figure 3.15D). In this experiment further we observed that the PMA treatment, as well as TGF-β1 treatment post-PMA stimulation, generated more polyploid MKs (≥4N) as compared to control (n=3; Figure 3.15 D), However, TGF-β1 treatment of PMA-induced

MKs resulted in a marginal increase in the percentage of polyploid cells as compared to that generated after treatment with PMA alone (n = 3; Figure 3.15 D). The data of the present study shows the functional potential of TGF-β1 in augmenting the MK maturation with no striking change in DNA content.

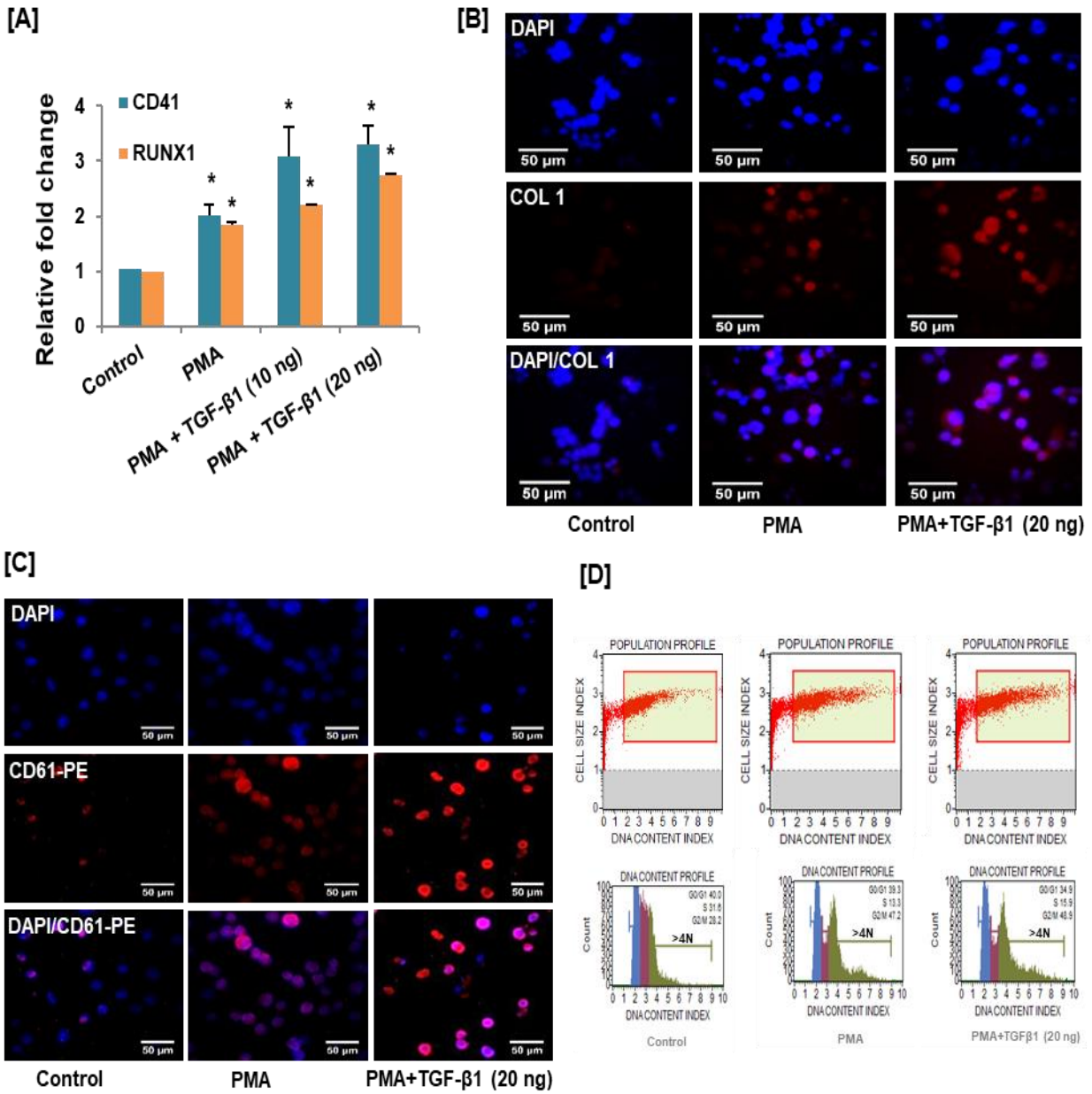



**Figure 3.15 TGF-β1 augments the maturation process in PMA-induced MKs.** (A) qRT-PCR analysis shows the increased expression of megakaryocytic marker (CD41) and TF RUNX1 upon TGF-β1 treatment of PMA-induced

MKs (n = 3; *P < 0.05). (B) Fluorescence microscopy images, deposition of collagen type I (COL1) was increased upon TGF-β1 treatment of PMA-induced MKs (n = 3). (C) Fluorescence microscopy images, florescence intensity of MK marker CD61-PE was increased upon TGF-β1 treatment of PMA-induced MKs (n = 3). (D) DNA index of cells analysed by Muse Analyzer, DNA content was increased in PMA-induced MKs as compared with control; however, it was not increased much upon TGF-β1 treatment of PMA-induced MKs (n = 3). Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

### 3.2.5. TGF-β increases cellular Ca2+ and TRP ion channel expression in PMA-induced MKs

As a major intracellular messenger Ca2+ is involved in various physiological activities, the functional role of Ca2+ has been well defined in the activation and function of platelets. TGF-β1 is the key regulator of [Ca2+]i in many cellular systems. Interestingly, a recent study has shown that TGF-β induces store-operated Ca2+ entry (SOCE) in MKs [376]. However, the functional role of TRP (Transient receptor potential) ion channels in TGF-β1 induced extracellular Ca2+ entry in MKs is currently unknown. To understand the effect of TGF-β1 autocrine signaling in cellular Ca2+ and TRP ion channels regulation, we have analysed the expression of TRP ion channels expression and cellular Ca2+ levels upon TGF-β1 treatment of PMA-induced MKs. As illustrated in Figure 3.16A, the expression of TRPV1 and TRPA1 was significantly high in PMA-induced MKs as compared with control (n 3; $P < 0.02$), however, only a marginal increase was observed in TRPC3 expression (n 3; $P > 0.05$). Interestingly, TGF-β1 treatment of PMA-induced MKs post PMA stimulation significantly enhanced the expression of TRPA, TRPV1, and TRPC3 in dose-dependent manner (n = 3; $P < 0.02$; Figure 3.16A).

Further, we have utilised Fluo-3 fluorescence to quantify $[Ca2^{+}]i$ activity in PMA-induced MKs upon TGF-β1 treatment. To stain intracellular Ca2+ we utilized fluorescent indicator, Fluo-3/AM. Using a fluorescence spectrometer, we observed a consistent increase in the activity of [Ca2+]i in dose-dependent manner upon TGF-β1 treatment of PMA induced MKs (n=3; $P<0.02$; Figure 3.16 B). I the same experimental conditions, we have also imaged cells after Fluo-3 staining, as shown in Figure 3.16C, the fluoresce microscope images with fluo-3 stain were quantified by IMAGEJ (Wayne Rasband at NIH, USA). Control cells exhibited weak green fluorescence, and quantified lowest [Ca2+]i among all the samples; however, the [Ca2+]i was increased in PMA-induced MKs, with increased green fluorescence intensity (n=3; $P < 0.02$; Figure 3.16 C). Interestingly, [Ca2+]i

levels detected in PMA-induced MKs were further augmented after TGF-β1 (20 ng/ml) treatment post-PMA stimulation (n=3; P < 0.02; Figure 3.16 C). The results of the present experiment suggest that the MK differentiation is associated with increase in [Ca2+]i activity and TRP ion channels expression. Moreover, TGF-β1 further enhance the expression of TRP ion channels and augment [Ca2+]i activity in PMA induced MKs, which attest the crucial role of TGF-β1 in MK maturation by increasing [Ca2+]i activity.

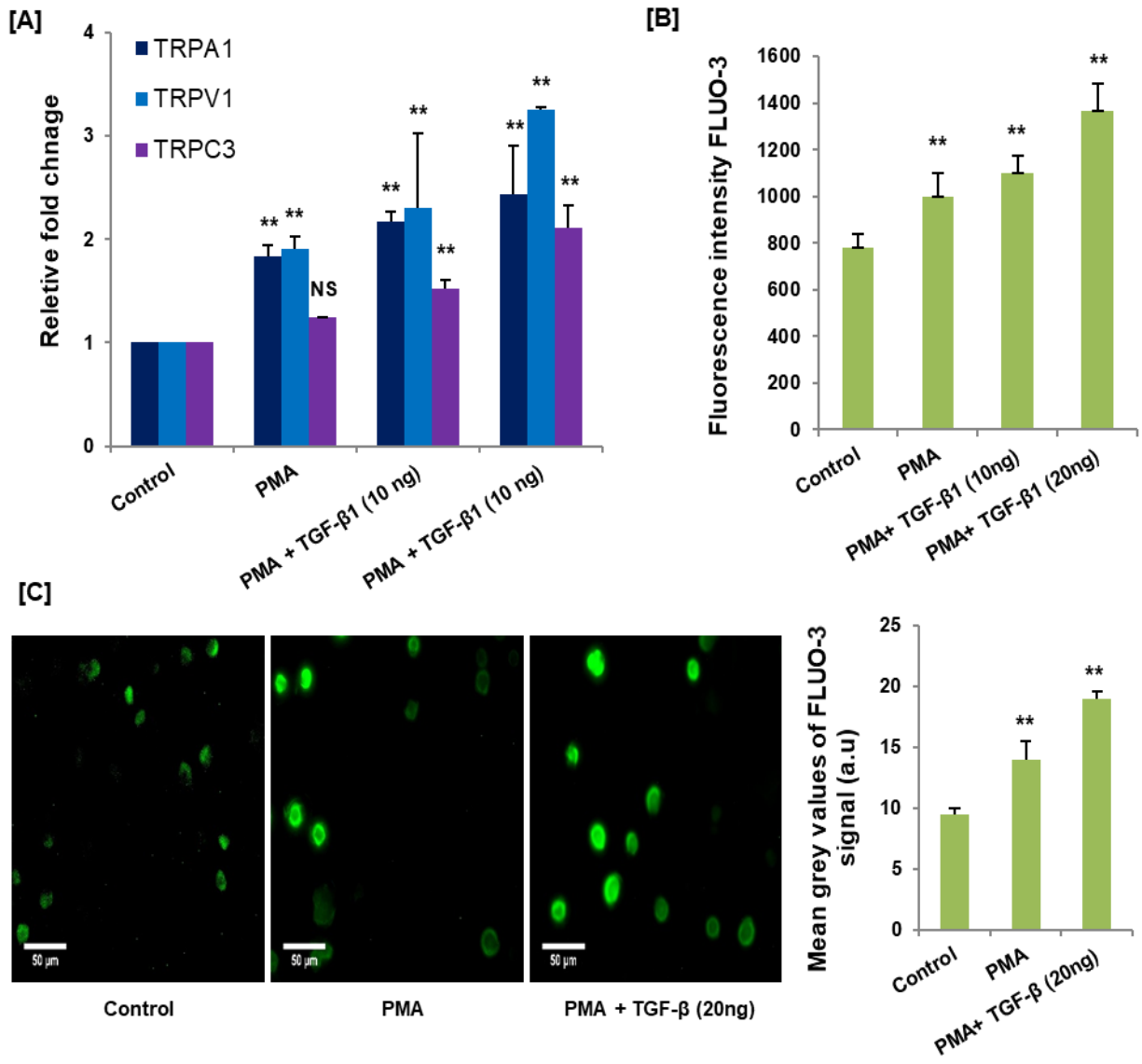



**Figure 3.16 TGF-β increases TRP ion channels expression and cellular Ca2+ during MK maturation.** (A) qRT-PCR results show the dose-dependent increased expression of TRP ion channels upon TGF-β1 treatment of PMA-

induced MKs (n = 3; **P < 0.02; nsP > 0.05). (B) Further, bar graph shows the mean fluorescence intensity of Fluo-3, the emission spectrum was recorded using fluorescence spectroscopy (excitation 505 nm, emission 526 nm, slit width 5 nm); (n = 3; **P < 0.02). (C) fluoresce microscope images of Fluo-3/AM (3 μM)-loaded cells, the histogram shows mean fluorescence intensity of 500 cells from each group (n = 3, **P < 0.02). Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

### 3.2.6. TGF-β1 increased cellular ROS during PMA-induced differentiation toward the MKs

It has been accepted widely that ROS function in microenvironment-dependent manner to influence the process of proplatelet release from mature MKs. Moreover, it has also been demonstrated that the ROS is necessary for PMA-induced differentiation of cells toward MKs [377-379]. To understand the effect of TGF-β1 on ROS production in PMA-induced MKs, we analysed the intracellular ROS production using fluorescence molecule H2DCFDA. In PMA-induced MKs, TGF-β1 treatment post PMA stimulation significantly enhanced the ROS production in dose dependent manner (n = 3; P < 0.05; Figure 3.17A). Mitochondria are the major source of ROS within most mammalian cells, and UCP-2 (uncoupling protein 2) is a well-known regulator of ROS production within mitochondria. Interestingly, TGF-β1 pathway effectors SMADs has been shown to maintain low UCP-2 levels in mitochondria by transcriptional regulation of UPC-2 expression [297]. In present study, we have also observed the significantly reduced expression of UCP-2 in PMA-induced MKs upon TGF-β1 treatment post PMA stimulation (n = 3; P < 0.02; Figure 3.17 B and C) when compared with PMA alone or control. On the other hand, we have also studied the expression of nonmitochondrial ROS contributor NOX4, was observed with consistent increased expression in PMA-induced MKs upon TGF-β1 treatment post-PMA stimulation (n=3; P<0.05; Figure 3.17 D). Further, to monitor mitochondrial membrane potential (ΔΨm), we analysed cells by flow cytometer using the Muse Mito Potential Kit, we counted the number of depolarised/live cells. The percentage of depolarised/live cells counted was lowest (23.71%) in control group, after PMA treatment >62.01% cells were depolarized/live, however further increase in the percentage of depolarised/live cells (68.87%) was recorded upon TGF-β1 treatment post-PMA stimulation (n=3; Figure 3.17 E). The increase in the percentage of depolarised/live cells upon PMA treatment or TGF-β1 treatment post-PMA stimulation is a sign of reduced ΔΨm during MK development. The results of the present study

show that TGF-βR/SMAD canonical pathway likely increases cellular ROS in MKs via transcriptional repression of mitochondrial ROS regulatory protein UCP2 (Mitochondrial uncoupling protein 2), that in turn results in reduced ΔΨm. However, the NOX4 in MK might also be involved in ROS production during MKs maturation and proplatelet release.

### 3.2.7. ROS regulates [Ca2+]i activity during PMA induced MK differentiation

Increasing evidences highlight the key role of ROS in the regulation of cellular Ca2+ via controlling the activity of Ca2+ ion channels. Previous studies have reported that ROS regulate the activity of Ca2+-permeable ion channel, such as TRPA1, TRPV1, and TRPC3 [380-382]. To further confirm the functional role of ROS in the regulation of cellular Ca2+, we carried out PMA-induced MK differentiation experiment using ROS quencher N-acetyl-L-cysteine (NAC). We observed that the NAC pre-treatment significantly reduced the TGF-β1 induced cellular ROS in PMA-induced MKs ($n = 3$; $P < 0.02$; Figure 3.17 F). In same experimental conditions, we observed significantly reduced levels of [Ca2+]i with NAC pre-treatment of MKs ($n = 3$; $P < 0.02$; Figure 3.17 G and H). Results of this experiment shows that NAC, a well-known antioxidant, reversed the effect of ROS on cellular CA2+ activity likely via affecting the ROS-dependent activity of TRP channels. The data of the present study support the notion that increase in [Ca2+]i is a crucial event in MK development and that, in turn regulated by TGF-β pathway-induced ROS in MKs.

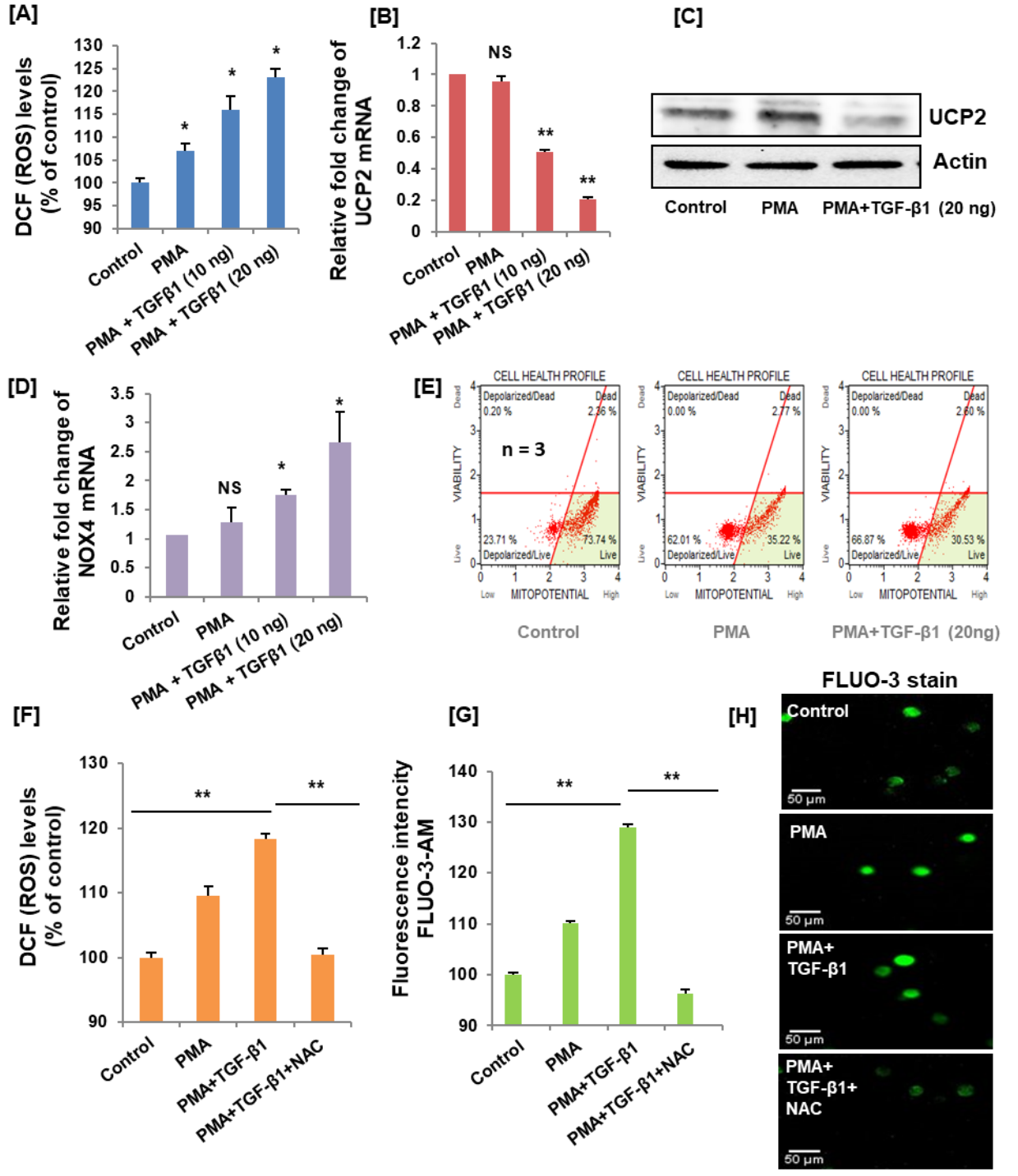



**Figure 3.17 TGF-β1 increases ROS production.** (A) Bar graph represent the DCF (ROS) levels, fluorescence of DCF was measured by fluorescence spectroscopy (excitation 485 nm and emission 535 nm). The level of ROS

increased in dose-dependent manure upon TGF-β1 treatment (n = 3; *P < 0.05). (B) qRT-PCR results showing that TGF-β1 affects UCP-2 mRNA expression in dose-dependent manner (n = 3; **P < 0.02). (C) Western blot of UCP2 protein, upon TGF-β1 (20 ng/ml) treatment of PMA-induced MKs UPC-2 protein levels were significantly reduced (n = 3; P < 0.05). (D) qRT-PCR analysis of the differential expression of NOX4 mRNA, NOX4 levels were observed with consistent increased expression in dose dependent manner upon TGF-β1 treatment (n = 3; *P < 0.05). (E) The percentage of depolarized/live cell was analysed by flow cytometer (Muse™ Cell Analyzer). Percentage of depolarized/live cell was increased upon TGF-β1 treatment of PMA-induced MKs (n = 3). (F) The DCF (ROS) levels, analysed by fluorescence spectrometer, were drastically reduced by ROS quencher NAC (n = 3; **P < 0.02). (G) The Fluo-3 fluorescence intensity was measured on a fluorescence spectrometer, mean fluorescence intensity was significantly reduced in NAC treated cells (n = 3; **P < 0.02). (H) Fluorescence microscopy images of Fluo-3/AM (3 μM)-loaded cells (n = 3). Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

### 3.2.8. TGF-β1-induced TRP ion channels expression is regulated by TF RUNX1

Based on our finding on RUNX1 and TRP ion channels expression in PMA-induced MKs and the augmenting effects of TGF-β1 their expression levels in PMA-induced MKs, we evaluated the functional effect of siRUNX1 on the expression of TRP ion channels in megakaryocytic cells. Because, findings from different studies in megakaryocytic models have proved that RUNX1 functions as the key gene expression regulator in MKs [342,343]. RUNX1 has also been demonstrated for its regulatory role in TRP ion channel expression in different cellular systems [383,384].

To test the functional effect of RUNX1 on TRP ion channels expression we performed a siRUNX1 transfection experiment in PMA-induced MKs. Interestingly, post siRUNX1 transfection, the expression levels of TRPA1, TRPV1, and TRPC3 were significantly reduced (n = 3; P < 0.05; Figure 18 A); post-siRUNX1 transfection, we have also observed cells with significantly lower levels of [Ca2+]i in PMA-induced MKs (n = 3; P < 0.02; Figure 18 B). The data is suggesting that RUNX1 might regulate TRP ion channels expression during MK development.

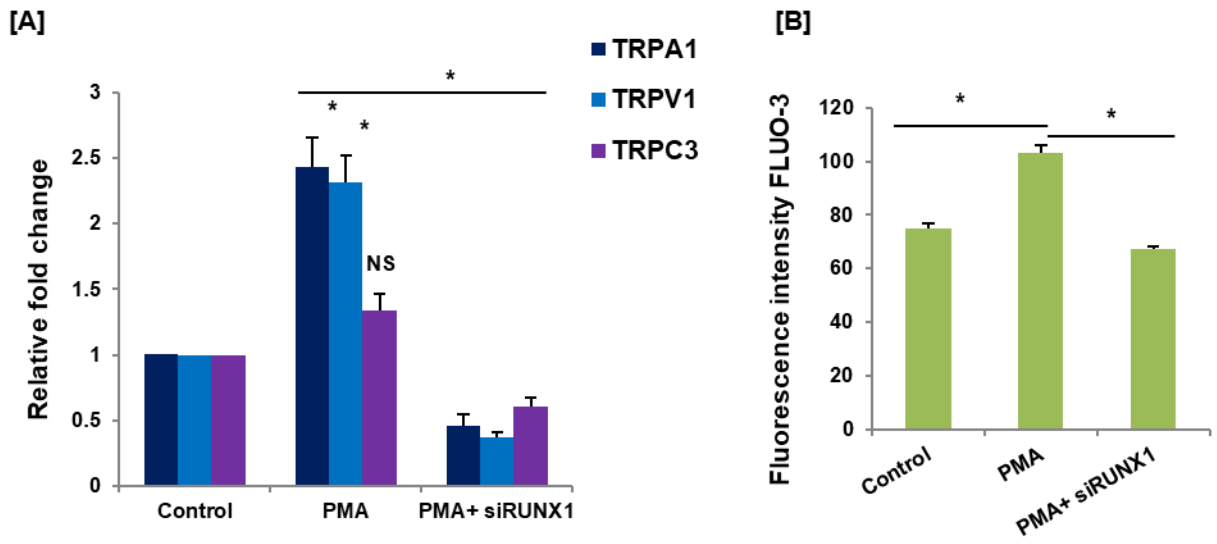



**Figure 3.18 siRUNX1 reduced the TRP ion channel expression in PMA-induced MKs.** (A) qRT-PCR analysis showing that the siRUNX1 transfection reduces the elevated levels of TRP ion channels in PMA-induced MKs (n = 3; *P < 0.05; nsP > 0.05). (B) The Fluo-3 fluorescence intensity was recorded on a fluorescence spectrometer, the mean fluorescence intensity of Fluo-3 was significantly low in siRUNX1 transfected MKs (n = 3; *P < 0.02). Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

### 3.2.9. TGF-β1 augments the MK marker CD41 and TF RUNX1 expression in TPO-induced CB-CD34+ cells derived MKs.

Finally, to confirm the functional role of TGF-β signaling in MKs development in vitro, we cultured CB derived CD34+ cells for 14 days in serum free MK culture medium supplemented with TPO (50 ng/ml), the effects of TGF-β1 on MK maturation were further demonstrated by treating developing MKs (at day 8 of culture) with 20 ng/ml concentration of TGF-β1 for 4 days (Figure 12A and B). interestingly, TGF-β1 treatment significantly increased the expression of MK marker CD41 and TF RUNX1 in PTO induced MKs as compared to control or TPO alone (n=3; P<0.02; Figure 3.19 A). Furthermore, in a comparative analysis, we observed that the costimulation at the late stage of MK development significantly enhanced the expression of MK marker CD41 (~ 3-fold; n = 3; P < 0.02; Figure 19 B), TF RUNX1 (~ 4-fold; n = 3; P < 0.02; Figure 19 B), and some of the TGF-β pathway core components (n = 3; P < 0.02; Figure 19 B) when compared with TPO alone. Our results are consistent with the previous report has shown the

TGF-β autocrine loop activation in MKs, their results showing the activation of TGF-βR downstream pathways upon TGF-β1 treatment of TPO stimulated MKs [307].

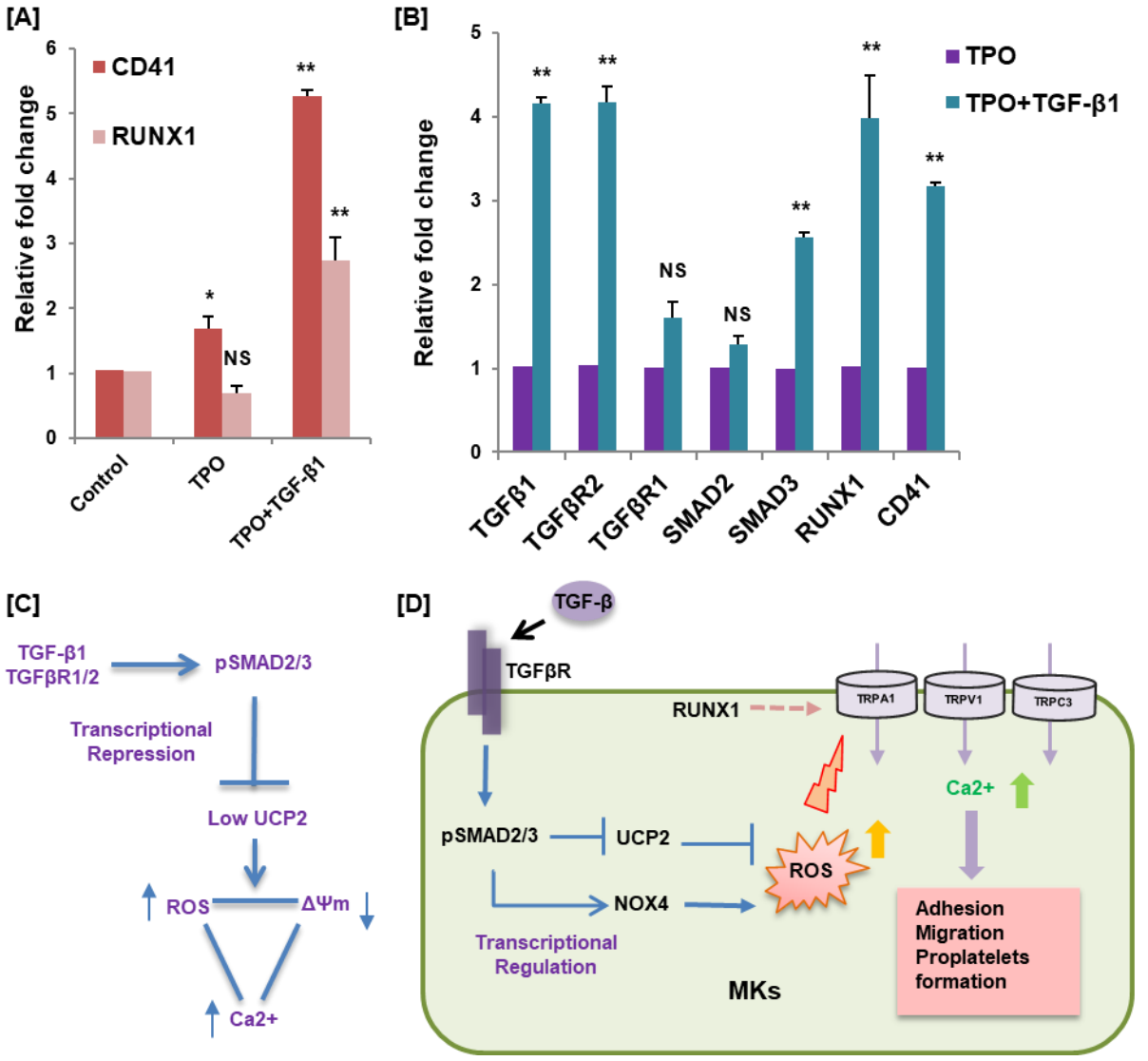



**Figure 3.19 TGF-β1 increases MK marker CD41 and TF RUNX1 expression in TPO-induced CB-CD34+ cells derived MKs.** (A) qRT-PCR analysis showing the differential expression of CD41 and RUNX1 in different experimental groups as compared to control (n = 3; **P < 0.02; *P < 0.05; nsP > 0.05). (B) qRT-PCR analysis showing the higher expression of CD41, RUNX1, and mRNAs of different TGF-β pathway components upon TGF-β1

treatment of TPO induced MKs as compared to TPO alone (n = 3; **P < 0.02; nsP > 0.05). (C) Graphics representing the ROS regulation, mediat3de by DMAD2/3 and UCP-2 axis. (D) Schematics showing the ROS and Ca2+ mediated functional effects of TGF-β1 and RUNX1 during terminal stage of MK maturation. Bars represent the mean ±SD of three independent experiments; all the data were assessed using Student's t-test.

### 3.3. Discussion

We have already discussed in earlier objectives on this study that there are several cellular and molecular differences in neonatal and adult MKs; in the present objective, we further confirmed that PTO induced CB- and PB-CD34+ cells derived MKs are developmentally different. Different other research groups also confirm the existence of developmental difference between neonatal and adult MKs [318,327,344,385], and it has been widely accepted that these developmental differences allow neonates to fulfil the demand of fast-growing BM and blood vascular system while maintain normal platelet counts, however contribute to the disorders of neonatal megakaryocytopoiesis and predisposition of neonates to develop different low platelet conditions. The functional role of both the cell-intrinsic and extrinsic factors has been suggested by previous reports in the regulation of the uncoupled phenotype of neonatal MKs. However, the molecular mechanism which governs this uncoupled phenotype is not fully understood.

In this study, we tried to explore the developmentally regulated signaling pathways in MKs. with this research endeavour, the have performed the microarray study in neonatal versus adult MKs and identified differentially regulated genes of different signaling pathway compounds. Interestingly, the functional analysis of these differentially regulated genes identified several affected pathways, the most affected pathway analysed was the TGF-β pathway (Figure 3.8 A). Several TGF-β pathway components were downregulated in neonatal MKs as compared with adult MKs. (Figure 3.8 B and C). Although there is no report on the developmental regulation of TGF-β pathway in MKs, TPO has been reported as a pivotal regulator of TGF-β release and its autocrine functions in MKs in both the in vitro and in vivo study [307]. The results of the present study, identified differential expression of TGF-β pathway components, suggesting that TGF-β may have functional role in MK development. Thus, in this study, we have elucidated the functional role of the TGF-β1 and its downstream pathways in megakaryocytic development.

With this perspective, we explored the TGF-β pathway in PMA-induced megakaryocytic differentiation of K562, model of in vitro megakaryocytopoiesis and very useful tool for molecular studies of megakaryocytic differentiation [162,386]. The PMA-induced megakaryocytic differentiation was confirmed by the expression analysis of the megakaryocytic features/markers in parallel comparison with the hemin-induced erythroid phenotype (Figure 3.10). Our results clearly demonstrate that PMA diminished erythroid phenotype of K562 and significantly established megakaryocytic features (Figure 3.9 and 3.10). Further, the megakaryocytic TF RUNX1 was also increased during PMA-induced megakaryocytic differentiation, whereas during hemin induced erythroid differentiation RUNX1 levels were reduced (Figure 3.10). RUNX1 is known to inhibit erythroid gene expression program and allow megakaryocytic differentiation, our findings are in accordance with the previous report by Kuvardina et al. [162].

Given these findings, we then explored the existence of TGF-β pathway in PMA-induced MKs in order to better define the existence and functional role of TGF-β pathway in MK development. The present study data report that PMA-induced megakaryocytic differentiation is associated with increased expressions of TGF-β and TGF-βR (Figure 3.11A). A report by Alitalo R et al. [305] also observed the increased expression of TGF-β during megakaryocytic differentiation of K562. The pathway focused gene expression profiling identified 35 differentially expressed genes, most of them were upregulated (Figure 3.11 B and C); interestingly, most of the differentially expressed genes encode the TGF-β core pathway components and the components of several established TGF-β pathway responsive genes (Figure 3.11 C Table 3.1).

Further the functional characterisation of the differentially expressed genes identified that most of the genes or their encoded component functionally involved in TGF-β signaling pathway (Figure 3.12A). They are mainly the legends (TGF-b, BMPs, and activins) and receptors (TGF-βR1, TGF-βR2, TGF-βR3, ACVR1, ACVRL1, and BMPR2,) of the TGF-β superfamily (Table 3.1 and Figure 3.12 A and B). The TGF-β superfamily receptors and ligands have been reported in a wide array of functions including development and differentiation [387,388]. Specifically, the TGF-β1 secreted by MKs in BM is the key player in the maintenance of HSC quiescence; however, low concentration of TGF-β1 allow HSC proliferation and myeloid differentiation specifically in My-

HSCs [303,389]. Previously, TGF-β1 has also been implicated in TGF-β receptor autocrine signaling and the activation of several downstream pathways and molecular events during megakaryocytopoiesis [307]. The present study with pathway focused gene expression analysis reports that most of the genes which encode TGF-β superfamily ligands, receptors and their downstream effectors are upregulated during PMA-induced megakaryocytic development; thus, these genes may be playing crucial role in MK maturation.

Furthermore, the existence of the functional TGF-β signaling in PMA-induced megakaryocytic differentiation of K562 was supported by the elevated expression levels of several genes regulated by the TGF-β effectors. These genes encode the ECM and platelet alpha granule components, such as PDGF, THBS1, SERPINE1, CPL1A1, COL1A2, TGFBI, LTBP1, and LTBP2 (Figure 3.12 B, Table 3.1). The PDGF, SERPINE1, THBS1, and TGFBI involved in megakaryocytopoiesis, platelets activation and normal blood clotting [390-397]. The COL1A1, COL1A2, and LTBPs are the important components of ECM and play important role in TGF-β activation by integrins [275,398]. All together the data of the resent study provide confirmation on the existence of the functional TGF-β pathway in megakaryocytic differentiation, it may be involved in the regulation of MK maturation and platelet activation.

In the pathway focused array profiling in PMA-induced MKs, several TFs, such as SMAD2/3, RUNX1, and AP-1 transcripts (JUN, JUNB, and FOS), were also recorded with increased mRNA expression levels (Figure 3.13 A–D). The SAMDs are known to regulate RUNX1 [399] and AP-1 [400] TFs in blood cells, and AP-1 TFs have been correlated with high MK polyploidisation in human MKs [375]. The AP-1 TFs [342,343] and SMADs [401-403] are the main cooperating partners of RUNX1 in megakaryocytopoiesis. Thus, the PMA induced megakaryocytic differentiation likely involve the integrations of RUNX1, SAMD2/3, and AP-1 TFs (Figure 3.13 E) for the maturation process (Figure 3.13 F and G). Moreover, we observed MYC and ATF4 TFs with downregulation in PMA-induced MKs, and these TFs are well known to be transcriptionally repressed by TGF-βR/SAMD2/3 signaling [404-406]. In a study, c-Myc-knockout mouse reported with positive effects on megakaryocytopoiesis, whereas the differentiation of erythrocytes was blocked at the stage of progenitor selection [407].

Consistently, the protein interaction network analysis identified different interaction clusters which play crucial role in cell biogenesis and differentiation, and these clusters have strong interactions with TGF-β and core pathway components such as TGF-βRs and SMADs (Figure 3.13 E). In this study most of the components were identified with elevated expression in PMA induced-MKs. in conclusion, this study reports with the refence of previous literature that TGF-β pathway and its effectors regulated genes might have functional role in MK maturation. Further, our study validates the functional role of TGF-β1 in MKs maturation.

The results of this study clearly define the augmenting effects of TGF-β1 on MK maturation. The TGF-β1 treatment post PMA stimulation resulted in the accumulation of pSMAD2/3, and increased cell adherence and membrane expansions (Figure 3.14). Furthermore, the treatment of TGF-β1 post PMA stimulation also enhanced the cytoplasmic maturation of PMA-induced MKs (Figure 3.15 A and C), and ECM deposition (Figure 3.15 B); however, the DNA content was not increased much upon TGF-β1 treatment of PMA induced MKs (Figure 3.15 D). TGF-β1 augmented the MK maturation likely by activating the TGF-β/SMAD canonical pathway, as it is clear from the accumulation of pSMAD2/3 after TGF-β1 treatment. And, the TGF-β effector SMADs likely playing functional role in the transcriptional regulation of different target genes, such as RUNX1, integrin's (CD41/61), and ECM components. Most of these targets are involved in MK development, such as Integrin's and ECM components are involved in different autocrine loops which work in MK maturation, platelet formation, and function [70,307,396,408,409].

TGF-β1 is a multifunctional cytokine that control cell proliferation, differentiation [387,388], and many other functions, such as Ca2+ influx [410], synthesis of ECM components, cell adhesion and migration [307] in most cells; interestingly, TGF-β1 has also been shown to be involved in the regulation of proplatelet release process [70,409]. Different studies have confirmed that Ca2+ downstream to the TGF-β pathways participate in several molecular and signaling events [411], stress fibers formation and cell adhesion [412,413], and functionally involved in microtubule assembly mechanism and proplatelet formation [70,409]. In this study, we report that TGF-β treatment augments the cellular Ca2+ levels (Figure 3.16 B and C) and the expression levels of TRP ion channels (Figure 3.16 A) in PMA-induced MKs. TRP ion channels including TRPA1,

TRPV1, and TRPC3 are permeable to Ca2+ [414]. In a report, TRPA1 was demonstrated as a strong inhibitor of erythropoiesis but enhance MK differentiation from K562 and HEL cells [415]. The TRP ion channels function as an important Ca2+ entry pathway, in MKs they may contribute to the fluctuations in the cellular Ca2+ and subsequent Ca2+ signaling [416,417].

In previous studies it has been observed that TRP ion channels are activated by various intracellular signals such as the increased cellular ROS, depletion of ER Ca2+ stores, and many other. In these studies, it has been reputed that TRPC ion channels are activated upon depletion of ER Ca2+ stores and called store operated Ca2+ (SOC) ion channels [418]; however, TRPA1 and TRPV1, well known to regulate [Ca2+]i, are activated by cellular ROS [419-421]. Recently, it has been reported that TGF-β is the main regulator of [Ca2+]i following ER store depletion in MKs [410]. However, in the present study we report that [Ca2+]i in MKs is regulated by TGF-β1 via ROS mediated activation of ROS-sensitive TRP ion channels. TGF-β1 is well known to generate ROS in various cell types; ROS is also necessary for MK development [377-379] and. Our study reports the consistent and dose dependent increase in cellular ROS production upon TGF-β1 treatment of PMA-induced MKs (Figure 3.17 A). Further, we report UCP2 downregulation upon TGF-β1 treatment of PMA-induced MKs (Figure 3.17 B and C), UCP2 is the main regulator of mitochondrial ROS production. The results of our study are in accordance with the previous reports, suggested a mechanism of mitochondrial ROS production via TGF-β/SMAD mediated down regulation of UCP2 (Figure 3.19 C) [297]. Furthermore, we noticed that during PMA-induced MK development and upon TGF-β1 treatment of PMA-induced MKs the mitochondrial membrane potential (ΔΨm) is decreased (Figure 3.17 E), the reduced ΔΨm is likely the effect of increased mitochondrial ROS. Although mitochondria are the main source of ROS, cellular oxidases such as NOX4 are also the contributors in cellular ROS production and known to be regulated by TGF-β in different cells [422]. Herein, we report the upregulation of NOX4 upon TGF-β1 treatment of PMA-induced MKs (Fig. 3.17 D). Thus, downstream to the TGF-β/SMAD pathway, NOX4 and UCP2 may be important regulators of ROS in MKs. Finally, we report that cellular Ca2+ in MKs is regulated by ROS, this was confirmed by an experiment using ROS quencher NAC (Figure 3.17 F, G, and H). Finally, considering all the results of the present study and previous lecture, it seems that TGF-βR/SMAD pathway induced ROS control the Ca2+ inflow

in MKs likely via activating the ROS-sensitive TRP channels (Figure 3.19 D). However, the regulation of TRP ion channels expression during MK development is not defined.

It is well defined that RUNX1 and SMADs work synergistically. Moreover, RUNX1 is the major regulator of gene expression during MK development [342,343]. In the present study, the siRUNX1 transfections verified that RUNX1 regulates the expression of TRP ion channels and thereby potentiate cellular Ca2+ inflow in MKs (Figure 3.18 A and B). Previous studies have also reported RUNX1 role in the regulation of various ion channels and receptors including TRP ion channels [383,384]. These results shows that TGF-β1 regulate the [Ca2+]i levels in MKs via ROS mediated activation of TRP ion channels and transcription factor RUNX1 controlled regulation of ion channels expression during MK maturation. In conclusion, RUNX1/TGF-β crosstalk through TRP ion channels functions as the important regulatory mechanism of [Ca2+]i during megakaryocytic development. Notably, Ca2+ has been implicated as the important regulator of MK functions such as cell adhesion, migrations and proplatelet formation via regulating the cytoskeletal reorganization [408]. And, the results of the present study clearly define that RUNX1 and TGF-β/SMAD pathway crosstalk play significant role in MK maturation by regulating the cellular ROS, Ca2+ inflow, and megakaryocytic gene expression profile. Thus, considering the functional role of RUNX1 and TGF-β crosstalk in MK maturation, we conclude that RUNX1 and the TGF-β pathway components down regulation in neonatal (CB) MKs likely involved in the developmental uncoupling of neonatal megakaryocytopoiesis.

To confirm the functional effect of TGF-β on neonatal megakaryocytopoiesis, we have investigated TPO-induced CB-CD34+ cells derived MKs following TGF-β1 treatment during the final stage of development. We present the results with significant enhanced expression of some of the TGF-β core pathway components upon TPO/TGF-β1 costimulation during late stages of MK development (Figure 3.19 B). More interestingly, TGF-β1 significantly enhanced the expression of megakaryocytic marker CD41 and TF RUNX1, a crucial TF for MK maturation (Figure 3.19 A and B). In brief, the effects of TGF-β1 observed on CB-CD34+ cells derived MKs are consistent with those observed PMA-induced MKs. Hence, it is clear from the present data that

TGF-β1 augment terminal MK maturation. And, the results are consistent with previous reports showing the functional effects of RUNX1 [117,162] and RGF-β1 [307] in MKs.

## 4. Summary

Thrombocytopenia is a condition characterized by abnormally low levels of blood platelets (<150 x $10^9$/L), thrombocytopenia is a major clinical problem encountered in neonates admitted to NICU, and it is common among all sick, preterm, and low birth weight neonates and patients undergoing CB transplantation. Platelets help our body form clots to stop bleeding, Neonates with low platelet counts suffer from internal bleedings (hemorrhage). Thrombocytopenia is also a risk factor for IVH in neonates [319], which is a main cause of poor neurological outcomes and high mortality in newborns [319,321,337]. Currently, the primary cause of this predisposition is unclear, however in earlier studies is has been demonstrated that neonatal-MKs are developmentally different from adult-MKs [327]. Neonatal MK development is characterized by an increased sensitivity to TPO [344,350], high proliferation potential of MKP [350-352], and terminally differentiated MKs with small cell size and ploidy [344; 312; 327]; Neonatal-MKs also generate a smaller number of platelets than adult-MKs [327,338]. A micro-array study by Bluteau et al. [338] has also revealed that target genes of different TFs, involved in MK ploidy development, cytoskeleton rearrangement, development of pro-platelet projections and platelet functions, were enriched during MK ontogeny. These findings suggest that MKs involve a development associated maturation process and functional specification leading to increase adult specific hemostatic function of MKs.

All earlier studies suggest that the uncoupled mechanisms of neonatal MK development allow them to populate their rapidly growing BM and intravascular spaces while maintaining the normal range of platelet counts (150 x $10^9$/L to 450 x $10^9$/L), but also set the stage for various disorders/conditions restricted to only fetal/neonatal MKs/progenitors. The regulatory molecular mechanism that underlies this uncoupled phenotype of neonatal MKs is not fully understood so far, and lack of such knowledge has significantly impaired our ability to understand and treat neonatal thrombocytopenia and low platelet conditions in CB recipients.

Although several TFs have been reported as the crucial regulators of gene expression during embryonic and adult hematopoiesis, RUNX1 is a key gene expression regulator in hematopoiesis, during development, lineage selection and terminal maturation of blood cells [162]. RUNX1 is a critically important TF in megakaryocytopoiesis, it regulates ploidy development and cytoskeleton

rearrangement in the process of MK maturation and platelet release [339,117]. In humans, loss-of function RUNX1 mutations are common cause of FPD/AML, which is characterized by dysmegakaryocytopoiesis, platelet defects, and a lifetime high risk for developing myeloid malignancies [340,341].

While the importance of RUNX1 in megakaryocytopoiesis is well-established [342,343], the information about its developmental regulation in neonatal versus adult MKs and its crosstalk with developmentally regulated signaling pathways in MKs is lacking. Therefore, it was important for us to understand the RUNX1 regulation in developmentally different neonatal and adult MKs. In this study, we hypothesized that lower RUNX1 expression in neonatal MKs may lead to developmentally different, hyperproliferative and disease susceptible phenotype. And, the research work carried out to understand the developmental differences in neonatal versus adults MKs is summarized as follows.

### 4.1. RUNX1 expression is differentially regulated in developmentally different neonatal and adult MKs

Human CB- and PB- $CD34^+$ cells (HSPCs) were obtained from Lonza India Pvt. Ltd., purified HSPCs ($4 \times 10^4$/ml) were cultured in humidified incubator (incubation parameters set to at 37°C temperature, approximately 95% humidity, and 5% CO2) with 50ng/ml concentration of TPO (PeproTech) in serum-free MK culture medium composed of IMDM supplemented with 1% of BSA; insulin, 10 μg/ml; transferrin, 740 μg/ml; L-glutamin, 2.0 mM; and LDL, 40 μg/ml. The cells were cultured following the MK differentiation culture method of Pastos et al [344]. Cells were collected at different time points and analyzed for MK specific marker (CD41, CD61, and CD42b) expression study. We observed that CB-derived $CD34^+$ cells generated more MKs than PB-derived $CD34^+$ cells (Figure 3.1). However, CB-MKs were significantly smaller and observed with low levels of megakaryocytic markers expression than PB-derived MKs (Figure 3.2). Interestingly, RUNX1, the master regulator of hematopoiesis [162] and known to regulate MK ploidy development and the rearrangement of cytoskeleton during terminal MK maturation and pro-platelet release [339, 117], was down regulated in CB-derived MKs (Figure 3.3). These results are consistent with our hypotheses. Therefore, the low RUNX1 expression could be responsible

for the uncoupling of neonates MK development. However, the mechanisms of RUNX1 regulation in developmental megakaryocytopoiesis are not defined.

### 4.2. microRNA-9 regulates RUNX1 expression in neonatal vs adult MKs

A growing body of evidence suggests that microRNAs are crucially involved in the post-transcriptional regulation of the expression of different genes and TFs playing role in the regulation of stem-cell differentiation as well as in malignant hematopoiesis [423]. miRNAs are 20-22 nucleotides in length and has been shown to be involved in RNA silencing and post transcriptional regulation of gene expression. Also, few recent reports have shown that miRNAs are playing a crucial role in MK development [253, 187-189]. Based on these facts, we hypothesized that post transcriptional regulators, particularly miRNA, could be a responsible mechanism for the regulation of the differential expression of RUNX1 between neonatal and adult MKs. Hence, this study was undertaken to understand the role of miRNAs in the regulation of RUNX1 during MK development.

In our PCR miRNA array study, out of 88 miRNAs involved in stem cell development and differentiation, 10 miRNAs were upregulated in neonatal- as compared to adult-MKs (Figure 3.4). Further, target prediction study of 10 upregulated miRNAs by different online miRNA-mRNA interaction tools (Pictar, miRanda, and TargetScan) identified five RUNX1 targeting miRNAs (Figure 3.5). In this study, five RUNX1 targeting miRNAs were identified with significantly higher expression levels in CB-MKs, although miR-9 was the most prominent up-regulated miRNA (Figure 3.4).

Moreover, hsa-miR-9-5p and RUNX1 mRNA duplex showed mfe of −17.6 kcal/mole using bioinformatics tool RNAhybrid (Figure 3.5). The expression levels of hsa-miR-9-5p were measured by qRT-PCR and recorded with significantly higher expression in CB-MK as compared to PB-MKs, and these differences were consistent through all the stages of megakaryocytopoiesis (Figure 3.5). To prove the functional significance of miR-9-5p in the regulation of RUNX1 expression, we transfected MEG01 and Dami cells with either pre-miR-9 or scrambled control (Cy3 labelled) using lipofectamine. After 72 hours of transfection, we detected a significant increase in the miR-9-5p levels, along with high cell proliferation, reduced MK marker CD61

expression, and lower RUNX1 protein levels (Figure 3.6). Further, we observed that the miR-9 inhibitor transfection in Dami cells restored the RUNX1 protein levels, enhanced MK marker CD61 expression, and reduced cell proliferation (Figure 3.7). Previous findings have also revealed the post-transcriptional regulation of Runx1 by miR-9 [264]. In line with the previous study [19], we found that RUNX1 expression in MKs is regulated by miR-9. Moreover, we understood that miR-9 regulates the cell proliferation and megakaryocytic differentiation via targeting RUNX1 expression. Thus, in conclusion, developmental differences between neonatal and adult MKs are likely under the control of miR-9 mediated posttranscriptional regulation of RUNX1.

### 4.3. RUNX1 regulation and its crosstalk with differentially regulated signaling pathways in MKs

In present study, we have identified that a key MK TF RUNX1 is downregulated in neonatal-MKs when compared with adult-MKs. Moreover, we have shown that the high cell proliferation and reduced MK-marker CD61 expression are associated with the miR-9 mediated down regulation of RUNX1 expression. Given these findings, we have evaluated the differentially regulated signaling pathways in neonatal versus adult MKs and RUNX1 crosstalk with differentially regulated signaling pathways. In this study, we present the results of a microarray study performed in CB- versus PB-$CD34^+$ cells derived MKs. Notably, the most affected pathway detected in data analysis was TGF-β, most of the differentially expressed annotated TGF-β pathway and its effectors regulated genes were detected with low expression in CB- as compared to PB-MKs (Figure 3.8). In order to confirm our findings, the expression levels of TGF-β core pathway components and effectors were assessed using qRT-PCR (Figure 3.8). Moreover, the existence of the functional TGF-β pathway in megakaryocytic development was confirmed in PMA induced megakaryocytic cells. In this study, RT2 profiler (TGF-β/BMP/Activin) PCR array resulted in significant up-regulation of most of the TGF-β pathway components during PMA-induced megakaryocytic differentiation of K562 cells (Figure 3.11 and Figure 3.12; Table 3.1). This data shows the existence of the functional TGF-β pathway in megakaryocytic differentiation.

Furthermore, we have shown that human recombinant TGF-β1 increases the accumulation TGF-βR signaling effectors p-SMAD2/3, along with megakaryocytic features (cell adherence and membrane expansion) and marker expression in PMA induced K562 cells (Figure 3.14 and Figure

3.15). These results define the functional role of TGF-β1 in augmenting the megakaryocytic maturation. We have also identified that the expression of many TGF-β pathway effectors regulated genes such as megakaryocytic TF RUNX1 and ECM components was increased (Figure 3.15); moreover, TGF-β1 significantly augmented the cellular Ca2+ in PMA-induced megakaryocytic cells (Figure 3.16). The functional effects of RUNX1, ECM components, and the Ca2+ has been well tested in MK development, pro-platelets release and platelet activation [117, 162, 307, 376-379]. Thus, the results of the present study demonstrate that the TGF-β pathway is an important regulator of megakaryocytic maturation.

Increasing evidence suggests a pivotal role of TGF-β induced ROS in the regulation of cellular Ca2+ [296]. Further, to see the functional effect of TGF-β1 on ROS mediated regulation of cellular Ca2+, we analyzed the intracellular ROS production by the fluorescence molecule DCFH-DA and observed increased ROS levels post TGF-β1 stimulation in PMA induced megakaryocytic cells (Figure 3.17). We have also observed that TGF-β1 treatment reduced the expression of UCP2, the inhibitor of mitochondrial ROS, and increases the expression of NOX4 which is a contributor of non-mitochondrial ROS (Figure 3.17). A typical target of ROS is Ca2+ channels. Evidence from different studies suggest that ROS is a key regulator of the TRP Ca2+-permeable ion channels activity [380-382]. To understand the functional role of ROS in the regulation of [Ca2+]i, cell differentiation experiments were performed using ROS quencher NAC. In differentiation experiments, NAC pre-treatment significantly reduced the cellular ROS that was augmented by TGF-β1 in PMA-induced MKs (Figure 3.17). Under same experimental conditions, we also found significantly reduced cellular Ca2+ levels (Figure 3.17). In previous studies, TF RUNX1 has been reported as the main regulator of TRP ion channels expression [383, 384]. Further, we designed siRUNX1 transfection experiment to test whether RUNX1 have role in the regulation of TRP ion channels expression. Interestingly, this study identified that the TRP ion channels (TRPA1, TRPV1 and TRPC3) expression and cellular Ca2+ levels were significantly affected (Figure 3.18) post siRUNX1 transfection. Therefore, we report that TGF-β1 likely augments [Ca2+]i by ROS mediated activation of TRP Ca2+ ion channels and RUNX1 transcription factor mediated control on the expression of TRP Ca2+ ion channels during MK development. Finally, the functional role of TGF-β1 was tested in the maturation of CB-CD34+ cells derived MKs. We found that

TPO/TGF-β1costimulation at late stages of MK development increase the expression of MK marker CD41, TF RUNX1, and TGF-β pathway components (Figure 3.19) and augment the maturation of MK.

### 4.4 Conclusion

In conclusion, our current findings are in accordance with previous reports showing cellular and molecular differences between neonatal and adult MKs. Herein, our study results in neonatal and adult MKs have clearly defined that miR-9-5p and its target RUNX1 are developmentally regulated. RUNX1 is crucial for the regulation of MK specification, maturation and thrombopoiesis. Further, our observations associate the reduced level of RUNX1 with higher expression of miR-9-5p in neonatal CB-MKs. These findings show the functional implications of hsa-miR-9-5p in the regulation of proliferation and differentiation by targeting RUNX1 expression in human MK development. In this study, further we have uncovered RUNX1 crosstalk with TGF-β signaling pathway. In our micro array analysis, TGF-β pathway was the most affected signaling pathway in neonatal versus adult MKs, most of the TGF-β pathway components were down regulated in neonatal- as compared to adult-MKs. In this study, first time we have not only confirmed the existence of TGF-β pathway in megakaryocytic differentiation but also its functional role in megakaryocytic maturation and RUNX1 expression in MKs. The TGF-β pathway effectors, ROS and Ca2+, were considered as the important regulators of terminal maturation of MKs. We report that TGF-β1 augments [Ca2+]i levels likely by ROS mediated activation of TRP ion channels and RUNX1 mediated regulation of their expression during MK development. The present observation defines that RUNX1 and TGF-β/SMAD pathway alliance involved in the regulation of megakaryocytic maturation process by regulating different effectors and target genes involved in MK maturation. Therefore, the Lower expression of RUNX1 and TGF-β pathway components observed in neonatal MKs may contribute to the developmentally different, hyperproliferative, and disease susceptible phenotype of neonatal MKs.

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**The list of publications**

**1. Principal Author:**

| S.No. | Authors | Title | Journal/Reference |
|---|---|---|---|
| 1. | **Raghuwanshi S**, Agarwal R, Raval R, Gutti RK. | Chitosan Nanoparticles and Their Applications in Drug Delivery, Hemostasis and Stem Cell Research. **Book Chapter 6:** In: Thangadurai D., Jayabalan S., Prasad R. (eds) Functional Bionanomaterials: From Biomolecules to Nanoparticles. | ***Nanotechnology in the Life Sciences. Springer Nature.*** *2020* DOI: 10.1007/978-3-030-41464-1. |
| 2. | **Raghuwanshi S,** Dahariya S, Sharma DS, Kovuru N, Sahu I, Gutti RK. | RUNX1 and TGF-β signaling Cross Talk Regulates Ca2+ Ion Channels Expression and Activity during Megakaryocyte Development | ***FEBS J.*** *2020. doi: 10.1111/febs.15329* . **IF: 4.739** |
| 3. | **Raghuwanshi S**, Sharma DS, Kandi R, Kovuru N, Dahariya S, Musvi SS, Venkatakrishnan AC, Pallepati A, Gutti RK. | Pituitary adenylate cyclase-activating polypeptide (PACAP): Differential effects on neonatal vs adult megakaryocytopoiesis. | ***Thromb Res. 2019;175:59-60.*** **IF: 3.266** |
| 4. | **Raghuwanshi S**, Dahariya S, Musvi SS, Gutti U, Kandi R, Undi RB, Sahu I, Gautam DK, Paddibhatla I, Gutti RK. | MicroRNA function in megakaryocytes. | ***Platelets.*** *2019;30(7):809-816.* **IF: 3.106** |
| 5. | **Raghuwanshi S**, Gutti U, Kandi R, Gutti RK. | MicroRNA-9 promotes cell proliferation by regulating RUNX1 expression in human megakaryocyte development. | ***Cell Prolif.*** *2018;51(1):e12414.* **IF: 5.039** |
| 6. | **Raghuwanshi S**, Dahariya S, Kandi R, Gutti U, Undi RB, Sharma DS, Sahu I, Kovuru N, Yarla NS, Saladi RGV, Gutti RK. | Epigenetic mechanisms: Role in hematopoietic stem cell lineage commitment and differentiation. | ***Curr Drug Targets.*** *2018;19(14):1683-1695.* **IF: 2.642** |
| 7. | **Raghuwanshi S**, Karnati HK, Sarvothaman S, Gutti U, | microRNAs: Key Players in Hematopoiesis. | ***Adv Exp Med Biol.*** *2015;887:171-211.* |

| | | | |
|---|---|---|---|
| | Saladi RG, Tummala PR, Gutti RK. | Book Chapter 10: In: Santulli G. (eds) microRNA: Basic Science. Advances in Experimental Medicine and Biology, vol 887. Springer, Cham | **IF: 1.953** |
| **8.** | **Raghuwanshi S**, Deswal D, Karp M, Kuhad RC. | Bioprocessing of enhanced cellulose production from a mutant of Trichoderma asperellum RCK2011 and its application in hydrolysis of Cellulose. | ***Fuel.*** *2014;124:183–189.* **IF: 3.406** |

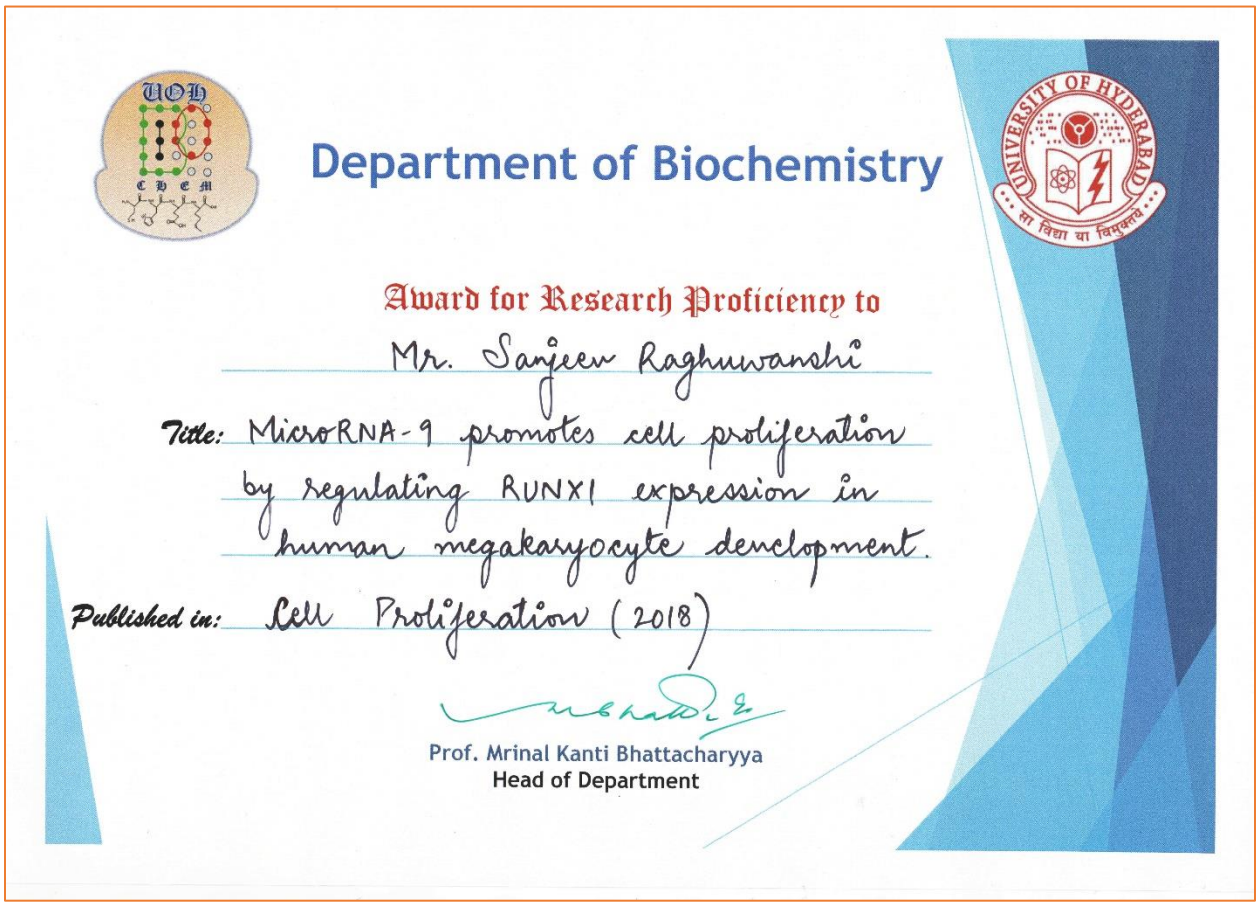
**2. Co-Author:**

| S.No. | Authors | Title | Journal/Reference |
|---|---|---|---|
| **1.** | Kovuru N, **Raghuwanshi S**, Sharma DS, Dahariya S, Pallepati A, Gutti RK. | Endoplasmic reticulum stress induced apoptosis and caspase activation is mediated through mitochondria during megakaryocyte differentiation. | ***Mitochondrion.*** *2020;50:115-120.* **IF: 3.449** |
| **2.** | Dahariya S, Pddibhatla I, Kumar S, **Raghuwanshi S**, Pallepati A, Gutti RK. | Long non-coding RNA: Classication, biogenesis and functions in blood cells. | ***Mol Immunol.*** *2019;112:82-92.* **IF: 3.064** |
| **3.** | Kovuru N, **Raghuwanshi S**, Gutti RK. | Exosome mediated differentiation of megakaryocytes: a study on TLR mediated effects. | ***J Thromb Thrombolysis.*** *2019;48(1):171-173.* **IF: 2.941** |
| **4.** | Bansal P, Dahate P, **Raghuwanshi S**, Sharma DS, Kovuru N, Gutti U, Yarla NS, Gutti RK. | Current Updates on Role of Lipids in Hematopoiesis. | ***Infect Disord Drug Targets.*** *2018;18(3):192-198.* |

**Awards & Distinctions**:

| S.No. | Name of the Award / Fellowship | Awards Given By (Organization) | Year |
|---|---|---|---|
| **1** | Award for Research proficiency | Department of Biochemistry of the University of Hyderabad (25th Foundation Day of the Department): National Seminar: "Biomolecular interactions in development and diseases" | 2019 |
| **2** | Travel award for best research work entitled ''MicroRNA-9: Role in The Developmental Differences of Human Megakaryocytes by Regulation of Runx1'' | International Congress of Bone Marrow Transplantation (BMT)-2019 Organizing Committee, Busan, Korea. | 2019 |
| **3** | Young Scientist Program fellowship for extraordinary Research in 2018 | International Union of Biochemistry and Molecular Biology (IUBMB), Young Scientist Program (YSP) of 24th IUBMB and 15th FAOBMB Congress, Seoul, Korea. | 2018 |
| **4** | Top stem cell research travel fellowship to attend the SCSS symposium-2017 | Stem Cell Society Singapore (SCSS), Singapore. | 2017 |
| **5** | Best paper entitled "MiR-9/RUNX1 Axis: Role in Developmental Megakaryocytopoiesis" and Travel Award | 10th Pan Pacific Symposium on Stem Cells and Cancer Research (PPSSC)-2017 Organizing Committee, Hualien, Taiwan. | 2017 |
| **7** | Junior Research Fellowship (NET) in Life Sciences | The University Grants Commission (UGC) of the Govt. of India. | 2014 to 2019 |

**2019 Award for Research proficiency: Department of Biochemistry, University of Hyderabad, India.**



Department of Biochemistry

Award for Research Proficiency to

Mr. Sanjeev Raghuwanshi

Title: MicroRNA-9 promotes cell proliferation by regulating RUNX1 expression in human megakaryocyte development.

Published in: Cell Proliferation (2018)

Prof. Mrinal Kanti Bhattacharyya
Head of Department

2019 Travel award in acknowledgement of the miRNA research work: APBMT & ICBMT, Busan, Korea.

APBMT & ICBMT 2019
- 24th Annual Congress of APBMT
- 24th Annual Congress of KSBMT

*For the best stem cell transplantation*

*Travel Award*

**Sanjeev Raghuwanshi**

*On behalf of the International Congress of BMT 2019 Organizing Committee, we present to you the Travel Award in acknowledgement of your achievements entitled "Microrna-9: Role in The Developmental Differences of Human Megakaryocytes by Regulation of Runx1"*

*August 30 (Fri) ~ September 1 (Sun), 2019*

Jong Wook Lee, MD, PhD.
Congress President, APBMT&ICBMT 2019
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Jong-Ho Won, MD, PhD.
Chairman of Organizing Committee, APBMT&ICBMT 2019
Chairman of Board Directors, KSBMT

2018 Young Scientist Program (YSP) Fellowship: IUBMB & FAOBMB Congress, Seoul, Korea.

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| Byung-Hoon LEE | Republic of Korea |
| Soyeon AHN | Republic of Korea |

2017 Stem cell research travel and student fellowship: SCSS symposium, Singapore.

2017 Travel Award for miRNA research work: PPSSC, Hualien, Taiwan.

The 10th PPSSC 2017 Hualien

Pan Pacific Symposium on
Stem Cells and Cancer Research

# *Travel Award*

The Organizing Committee are pleased to present the Travel Award to

**Sanjeev Raghuwanshi** 8

for the paper entitled "MiR-9/RUNX1 Axis: Role in Developmental Megakaryocytopoiesis"
held on April 15-17, 2017 in Hualien, Taiwan

**Professor Shinn-Zong Lin**
**Chairman,**
2017 the 10th Pan Pacific Symposium on Stem Cells and Cancer Research
**Superintendent,**
Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan
**President,**
The Buddhist Tzu Chi Bioinnovation Center, Hualien, Taiwan

15-17 April, 2017 • *Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation*

2013 Junior Research Fellowship (NET) in Life Sciences: The University Grants Commission (UGC) of the Govt. of India.

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**Sub: - Joint CSIR-UGC Test for Junior Research Fellowship and Eligibility for Lectureship (NET) held on 23-12-2012.**

Dear Candidate,

CSIR is pleased to inform you that you have been declared successful in the above examination for award of **JUNIOR RESEARCH FELLOWSHIP and secured 0067/0636 rank** in the subject **LIFE SCIENCES** under **UGC** Fellowship scheme. Further, you have also been declared **successful for Lectureship (NET)** in the above subject area, **subject to fulfilling the eligibility criteria laid down by the UGC**.

**The fellowship is tenable w.e.f 01.04. 2013. It will be governed by the terms and conditions for the UGC Junior Research Fellowship.** In order to accept this offer you may contact UGC at the address given overleaf.

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Kindly acknowledge the receipt.

Yours sincerely,

(P.K.SHARMA)
Deputy Secretary (Exam)

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# Reprint of publications

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# RUNX1 and TGF-β signaling cross talk regulates $Ca^{2+}$ ion channels expression and activity during megakaryocyte development

Sanjeev Raghuwanshi, Swati Dahariya, Durga Shankar Sharma, Narasaiah Kovuru, Itishri Sahu and Ravi Kumar Gutti

Department of Biochemistry, School of Life Sciences, University of Hyderabad, India

**Keywords**
megakaryocyte; thrombocytopenia; RUNX1; TGF-beta; platelets; calcium; TRP channel

**Correspondence**
R. Gutti, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, 500 046 Telangana, India
Tel: +914023134533
E-mail: guttiravi@gmail.com

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Thrombocytopenia is characterized by low platelet count and is typically observed among all preterm and low birthweight neonates admitted to the neonatal intensive care unit. Although the underlying cause for this predisposition is unclear, recent studies have proposed that the intrinsic inability of neonatal hematopoietic stem/progenitor cells to produce mature polyploid megakaryocytes (MKs) may result in delayed platelet engraftment. The developmental and molecular differences between neonatal and adult MKs are not yet fully understood. Previously, we had reported that the key MK transcription factor RUNX1, which is crucial for the regulation of MK specification and maturation, is down-regulated in neonatal MKs when compared with adult MKs. In humans, loss-of-function mutations in *RUNX1* cause familial platelet disorder, which is characterized by thrombocytopenia, indicating its crucial role in MK development. However, information about its cross talk with developmentally regulated signaling pathways in MKs is lacking. In this study, we performed a differential gene expression analysis in MKs derived from human cord blood (CB) and adult peripheral blood (PB) $CD34^+$ cells. Further, validation and correlation studies between RUNX1 and transforming growth factor beta (TGF-β) were performed in a differentiating megakaryocytic cell line model. The analysis revealed that TGF-β pathway was the main pathway affected between CB- and PB-MKs. RUNX1 is reported to be a modulator of TGF-β signaling in several studies. The correlation between RUNX1 and TGF-β pathway was analyzed in the PMA-induced megakaryocytic differentiating K562 cells, which exhibit mature megakaryocytic features. The $RT^2$ profiler PCR array analysis revealed that TGF-β pathway components were up-regulated in the PMA-induced megakaryocytic differentiating cells. Furthermore, our study indicated that human TGF-β1 promotes cytosolic calcium ($Ca^{2+}$) activity and MK maturation. We noticed that TGF-β1 increased intracellular free $Ca^{2+}$ ($[Ca^{2+}]i$) via reactive oxygen species-mediated activation of transient receptor potential (TRP) ion channels. Moreover, we observed that decreased cytosolic $Ca^{2+}$ activity in the siRUNX1-

**Abbreviations**
$[Ca^{2+}]i$, intracellular free $Ca^{2+}$; $Ca^{2+}$, calcium; CB, cord blood; CD41, platelet glycoprotein (GP) IIb; CD42b, GPIbα; CD61, platelet GP IIIa; ECM, extracellular matrix; GYPA, glycophorin A; GYPB, glycophorin B; H2DCFDA, 2, 7-dichlorodihydrofluoresceindiacetate; HS/PCs, hematopoietic stem/progenitor cells; HSCs, hematopoietic stem cells; MKs, megakaryocytes; NAC, *N*-acetyl-L-cysteine; PB, peripheral blood; PMA, phorbol 12-myristate 13-acetate; pSMAD2/3, phospho-SMAD2/3; ROS, reactive oxygen species; RUNX1, runt-related transcription factor 1; TFs, transcription factors; TGF-β, transforming growth factor beta; TGF-βR, TGF-β receptor; TPO, thrombopoietin; TRP, transient receptor potential; UCP2, mitochondrial uncoupling protein 2.

transfected cells was associated with down-regulation of TRP ion channel expression. Finally, we demonstrated that TGF-β/SMAD signaling augments the development of MKs derived from CB-$CD34^+$. Present data suggest that RUNX1/TGF-β pathway cross talk is crucial for MK maturation.

## Introduction

Thrombocytopenia, defined as a low platelet count $< 150 \times 10^9$ $L^{-1}$, is common among all sick, preterm, and low birthweight neonates. Thrombocytopenia plays a major role as a cofactor in the incidence and severity of neonatal intraventricular hemorrhages [1], which is the main cause of poor neurological outcomes and high mortality rate in sick neonates [1-3], although the underlying cause for this predisposition is unclear. In earlier studies, neonatal megakaryocytopoiesis increasingly recognized as different from adult megakaryocytopoiesis [4-6]. Specifically, neonatal megakaryocyte (MK) progenitors possess a high proliferative potential and give rise to MKs smaller and of low ploidy that generate fewer platelets compared to adult MKs [4,7]. Further, Bluteau *et al.* [7] have also revealed that target genes of transcription factors (TFs) involved in MK polyploidization, cytoskeleton rearrangement, proplatelet formation, and platelet functions were enriched during MK ontogeny that suggests development-associated MK maturation and specification leading to increase the adult-specific hemostatic function of MKs. All earlier studies proposed that these uncoupled mechanisms of neonatal megakaryocytopoiesis allow fetuses/neonates to populate their rapidly expanding bone marrow and intravascular spaces while maintaining normal platelet counts, but also set the stage for disorders restricted to fetal/neonatal MK progenitors and inability to increase MK mass in response to increased platelet demand. The molecular mechanisms underlying these developmental differences have not been elucidated, and lack of such knowledge has significantly impaired our ability to understand and treat neonatal thrombocytopenia and other low platelet conditions.

Though several TFs are known to control gene expression in embryonic and adult hematopoiesis, RUNX1 is a key gene expression regulator in hematopoiesis [8]. Additionally, RUNX1 is described as a critically important TF in MK development and regulates polyploidization and cytoskeleton rearrangement in the process of MK maturation and proplatelet release [9,10]. In humans, loss-of-function *RUNX1* mutations are a common cause of familial platelet disorder, which is characterized by thrombocytopenia and propensity for progression to AML [11,12]. Our previous report suggests that RUNX1, which is crucial for the regulation of MK specification and maturation, is down-regulated in neonatal as compared to adult MKs; moreover, we have also shown that RUNX1 significantly affects cell proliferation and megakaryocytic differentiation [5]. While the importance of RUNX1 in megakaryocytopoiesis is well established [5,13,14], the information about its cross talk with developmentally regulated signaling pathways in MKs is lacking.

TGF-β is a ubiquitously expressed cytokine that plays a central role in the regulation of a wide spectrum of biological processes. TGF-β initiates downstream signaling when it binds to the TGF-β receptors (TGF-βRs) and activates their kinase activity. In the canonical TGF-β pathway, activated TGF-βR phosphorylates intracellular SMAD2/3, which then complex with SMAD4 or many other TFs in the process of transcriptional gene regulation (reviewed in Ref. [15]). TGF-β is a multifunctional growth factor, involved in the development and differentiation [16,17], the regulation of cytoplasmic $Ca^{2+}$ influx [18], and the synthesis and release of extracellular matrix (ECM), cell adhesion, and migration [19] in different cellular systems. Large amounts of TGF-β are produced by MKs in the bone marrow. Hematopoietic stem cells (HSCs) in close proximity to MKs become exposed to TGF-β and exhibit activation of the TGF-β/SMAD2/3 canonical pathway, which play an important role in establishing quiescence of HSCs (reviewed in Ref. [20]). Recently, thrombopoietin (TPO) has been shown as a pivotal regulator of TGF-β synthesis and release in MKs, both *in vitro* and *in vivo* [19]. However, the developmental regulation of TGF-β pathway and its effects during megakaryocytic maturation needs further validation.

In the present study, we further confirmed that neonatal (CB)- and adult (PB)-derived $CD34^+$ cells show different biological response to TPO. Further, developmental differences were identified in the expression of genes encoding components of the TGF-β pathway; several genes encoding TGF-β pathway components were down-regulated in neonatal CB- as compared to adult PB-MKs. Moreover, our results suggest

that TGF-β core pathway components and its effectors are significantly up-regulated in differentiating megakaryocytic cell line model. Further, elevated phospho-SMAD2/3 (pSMAD2/3) levels confirmed the existence of the canonical TGF-β pathway during megakaryocytic maturation.

Furthermore, we show that recombinant human TGF-β1 augments the synthesis of many TGF-β pathway-regulated genes encoding megakaryocytic TF RUNX1 and ECM component collagen type I (COL1) in PMA-induced MKs; TGF-β1 can also increase cellular reactive oxygen species (ROS) and $Ca^{2+}$ in PMA-induced MKs. We have also tested the augmenting effects of TGF-β1 on MK marker and RUNX1 expression in CB $CD34^+$ cells-derived MKs. In previous studies, the megakaryocytic TF RUNX1, deposition of collagen, and the elevated ROS and $Ca^{2+}$ have been well tested for their role in megakaryocytopoiesis, platelet release, and activation [5,19,21-24]. In summary, findings of the present study suggest the functional significance of TGF-β pathway in megakaryocytic maturation, and we report that TGF-β1 increases cellular calcium likely by ROS-mediated activation of transient receptor potential (TRP) ion channels and RUNX1-mediated regulation of TRP ion channel expression during megakaryocytic development. Therefore, the down-regulation of RUNX1 and TGF-β pathway components in neonatal MKs could be involved in the developmental uncoupling of neonatal MK progenitor proliferation and maturation.

## Results

### TGF-β signaling pathway is differentially regulated in neonatal versus adult MKs

To determine the developmentally regulated signaling pathways, we cultured $CD34^+$ cell (HS/PCs) of neonatal (CB) and adult (PB) origin in the presence of TPO, which is well known to induce *in vitro* megakaryocytopoiesis [4]. After 14 days, about 93.3 ± 4.01% of CB-$CD34^+$ cells and about 91.6 ± 3.61% of PB-$CD34^+$ cells were MKs. We observed a consistently increased expression of MK maturation marker CD61 during time point analysis of TPO-induced megakaryocytopoiesis ($n = 3$; $P < 0.02$; Fig. 1A). However, CB-MKs were smaller in size than PB-MKs ($n = 3$; Fig. 1B), and the megakaryocytic markers [CD41: platelet glycoprotein (GP) IIb; CD61: platelet GP IIIa; and CD42b: GPIbα] were recorded with higher expression in PB- as compared to CB-MKs ($n = 3$; $P < 0.02$; Fig. 1C). Further, we performed a microarray study with Human U133 Plus 2.0 Array (Affymetrix, Inc., Santa Clara, CA, Rosetta Inpharmatics LLC., Seattle, WA, USA), and statistical analysis was performed with ROSETTA RESOLVER® (Rosetta Inpharmatics LLC). The microarray analysis comparing neonatal and adult MKs revealed substantial differences in mRNA transcriptome of neonatal and adult MKs, and a total of 611 signature genes were identified with differential regulation ($n = 6$ per group; $P < 0.05$; R. K. Gutti & S. Raghuwanshi, unpublished data). The functional characterization of the differentially expressed genes with IPA INGENUITY® (Ingenuity Systems, Inc., Redwood City, CA, USA) showed the top affected pathways, which are mostly well known to be involved in megakaryocytopoiesis. Notably, the most affected pathway was TGF-β and most of the differentially expressed annotated TGF-β signaling genes were down-regulated in CB- as compared to PB-$CD34^+$ cells-derived MKs ($n = 6$; $P < 0.05$; Fig. 1D). Further confirmation was done by quantifying the differentially expressed TGF-β canonical pathway genes in CB- and PB-$CD34^+$ cells-derived MKs by qRT-PCR. As shown in Fig. 1E, PB-MKs expressed higher levels of different TGF-β pathway components than CB-MKs at day 14 of TPO-induced megakaryocytopoiesis ($n = 3$; $P < 0.02$). The results of the present study, which are consistent with previous studies, showed that TGF-β synthesis and release during megakaryocytopoiesis are under the control of TPO [19].

### Hemin induces erythroid and PMA induces megakaryocytic differentiation of K562 cells

In order to study the TGF-β signaling pathway during MK development, we used PMA (50 nM)-induced K562 cells, a well-known model for the study of megakaryocytic development, whereas for erythroid differentiation, we used Hemin (50 μM). Control cells received the same concentrations of DMSO alone. K562 cells were seeded in 60-mm plates at a density of $1 \times 10^6$ cells in RPMI-1640-GlutaMAX™ Supplement media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific). The effect of Hemin and PMA on cell size, adherence, and proliferation was studied. The results clearly demonstrate that the rate of cell proliferation was reduced by both the Hemin (~ 2-fold of the control) and PMA (~ 4-fold of the control) over a 3-day period ($n = 3$; $P < 0.02$; Fig. 2B), and increased cell size and adherence were also noticed in PMA-stimulated K562 ($n = 3$; Fig. 2A).

Further, Hemin-induced erythroid differentiation of K562 cells was identified by two methods: analysis of glycophorin A (GYPA) and glycophorin B (GYPB)

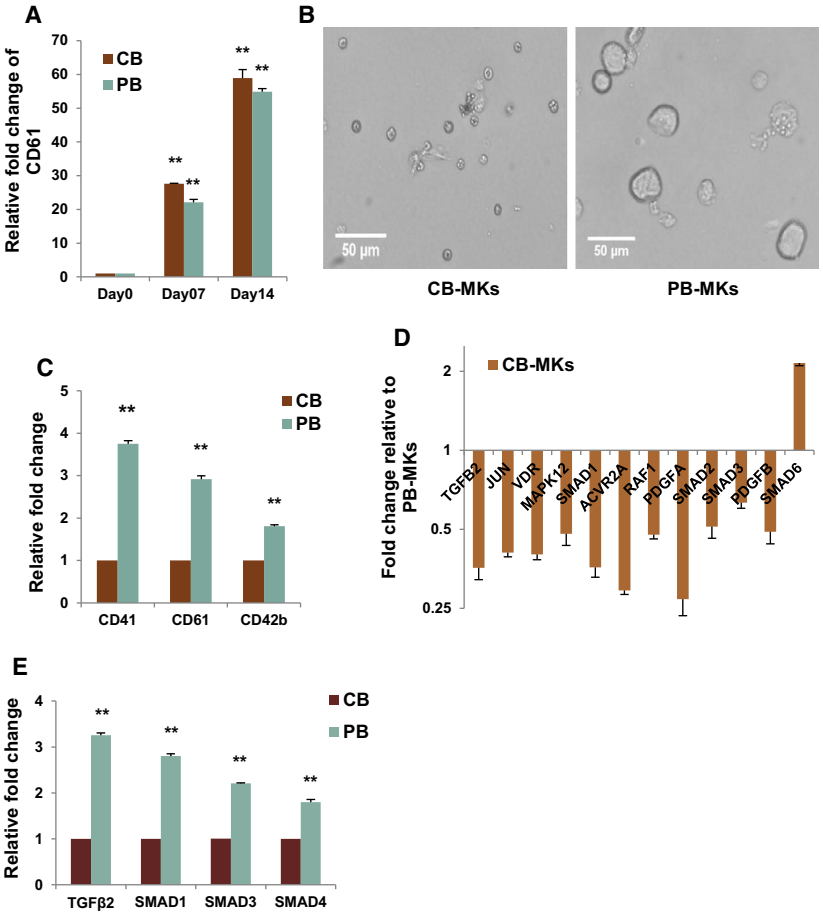



**Fig. 1.** Developmental differences in CB- and PB-derived megakaryocytes. (A) qRT-PCR analysis of the expression of megakaryocyte marker, CD61 in $CD41^+$ cells shorted from CB- and PB-derived $CD34^+$ cells at different day points of culture in a serum-free culture system with 50 $ng{\cdot}mL^{-1}$ of TPO as the only growth factor ($n = 3$, $^{**}P < 0.02$). (B) Both pictures of CB- and PB-derived MKs were taken on inverted microscope (20× objective; scale = 50 µm), and showing developmental differences between CB- and PB-$CD34^+$ cells-derived MKs, CB-MKs are smaller in size than PB-MKs ($n = 3$). (C) Relative expression levels of megakaryocyte markers (CD41, CD61, and $CD42^+$) were determined by qRT-PCR in CB- and PB-derived MKs at day 14 ($n = 3$, $^{**}P = 0.02$). (D) The graph indicates the fold change of mRNA, encoding components of TGF-β pathway in CB-MK as compared to PB-MKs, observed in the microarray study ($n = 3$, $^{**}P = 0.05$). (E) Further, qRT-PCR analysis confirmed the differential expression of mRNA encoding core TGF-β pathway components in CB- and PB-derived MKs ($n = 3$; $^{**}P < 0.02$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

expression, and benzidine staining. When K562 differentiates toward erythroid lineage, they became slightly larger ($n = 3$; Fig. 2A) and also increased expression of the early erythroid marker, GYPA and GYPB mRNA ($n = 3$; $P < 0.02$; Fig. 2C); moreover, over a 3-day period of Hemin treatment, the increased percentage of benzidine-positive cells (about 60%) was recorded ($n = 3$; $P < 0.02$; Fig. 2D). We have also observed that Hemin-induced erythroid differentiation reduced the expression levels of megakaryocytic markers ($n = 3$; $P < 0.05$; Fig. 3A,B) and, TF, RUNX1 ($n = 3$; $P < 0.05$; Fig. 3B,C). However, after PMA treatment a distinct change in morphology such as increased cell size and adherence ($n = 3$; Fig. 2A) was observed upon microscopic examination. Specifically, flow cytometry analysis was performed using human anti-CD61 PE to monitor the number of differentiated MKs after 72 h of PMA treatment. We observed a clear shift to $CD61^+$ MKs and a significant increase in mean fluorescence intensity values in PMA-treated K562 cells as compared to untreated control ($n = 3$; $P < 0.02$; Fig. 3A). Megakaryocytic markers, CD61 and CD41, were further confirmed by the mRNA expression analysis using qRT-PCR, resulted in an elevation of the mRNA expression levels (> 4-fold) upon PMA treatment as compared to control ($n = 3$; $P < 0.02$; Fig. 3B). Specifically, RUNX1, which is a key regulator of MK maturation and platelet production [25], was up-regulated at both the mRNA and protein levels in PMA-induced MKs as compared to control ($n = 3$; $P < 0.05$; Fig. 3B,C), whereas erythroid features were reduced upon PMA-stimulated megakaryocytic differentiation ($n = 3$; $P < 0.02$; Fig. 2C).

## PMA-induced megakaryocytic differentiation of K562 alters the expression of TGF-β signaling pathway genes

The mRNA expression levels of TGF-β and TGF-βRs were analyzed by qRT-PCR. As shown, the expression was significantly high (~ 2-fold) in PMA-induced megakaryocytic cells as compared to control ($n = 3$; $P < 0.02$; Fig. 4A). Previously, Alitalo *et al.* [26] have

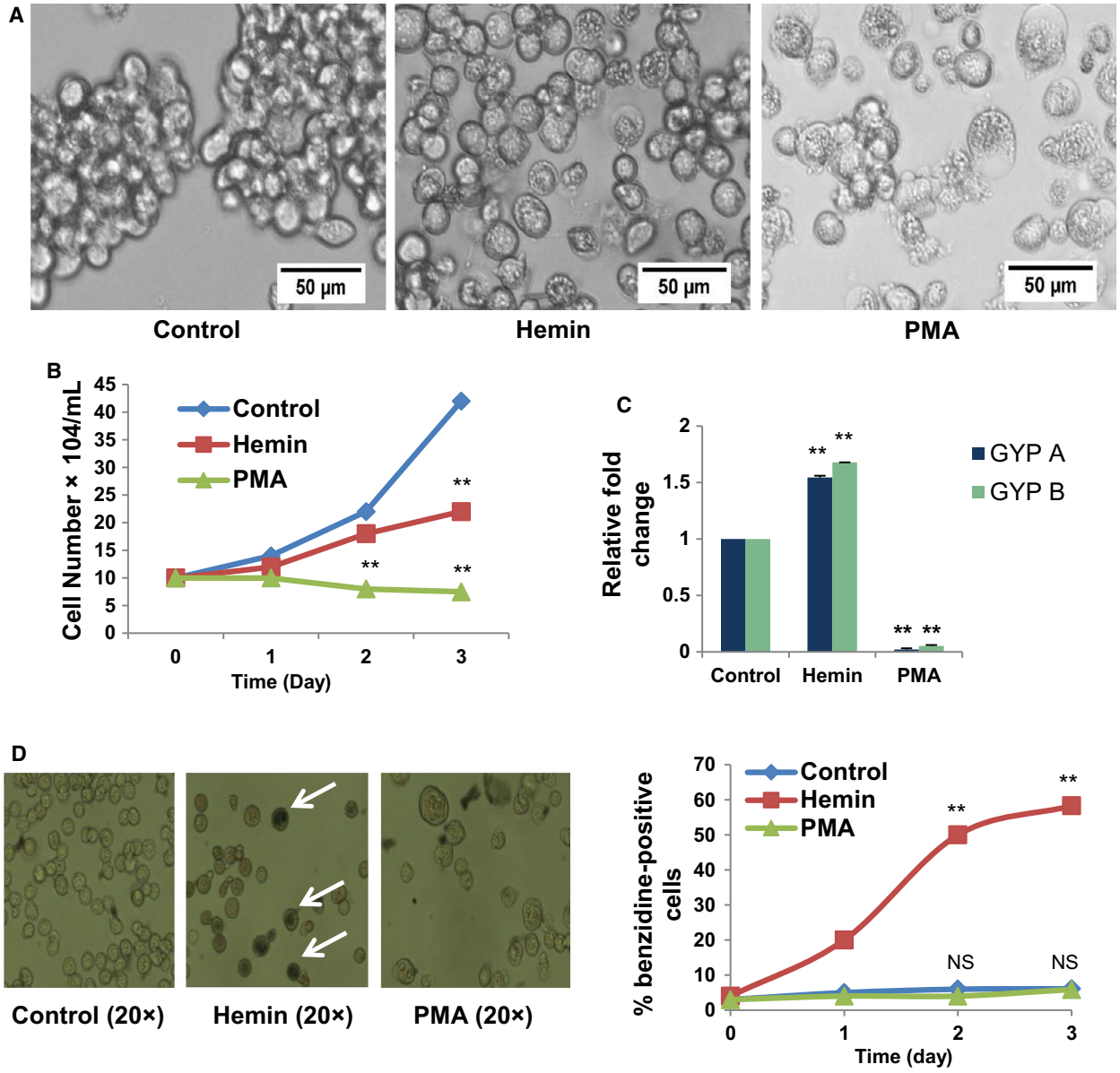



**Fig. 2.** Hemin induces erythroid differentiation of BFU-E/MKs (K562). (A) Photomicrograph of cells (20× objective; scale = 50 µm) showing morphological differences (cell size and adherence) in K562 cells upon 72 h of treatment with PMA or Hemin; cell size and adherence were increased upon Hemin or PMA stimulation as compared to control ($n = 3$). (B) The graph demonstrates the cell number changed in all groups over a 3-day period ($n = 3$; $^{**}P < 0.02$; $^{ns}P > 0.05$). (C) The bar graph represents the differential expression of GYPA and GYPB (early erythroid markers) upon PMA or Hemin treatment as compared to control over a 3-day period ($n = 3$; $^{**}P < 0.02$). (D) Representative microscopy images (20× objective) of the benzidine staining in K562 cells were shown after various tratments, white arrows indicate benzidine-positive cells (blue); graph shows the statistics of the increased percentage of benzidine-positive cells (blue) over a 3-day period of different treatments as compared to day 0 ($n = 3$; $^{**}P < 0.02$; $^{ns}P > 0.05$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

also shown the enhanced expression of TGF-β during TPA-induced differentiation of K562 cells. However, the regulatory role of the TGF-β pathway in megakaryocytic development is poorly understood. Using K562 cells, which could partly recapitulate the megakaryocytic development process in response to PMA, we extended our analysis using RT2 Profiler PCR Array (Qiagen, Inc., Germantown, MD, USA)

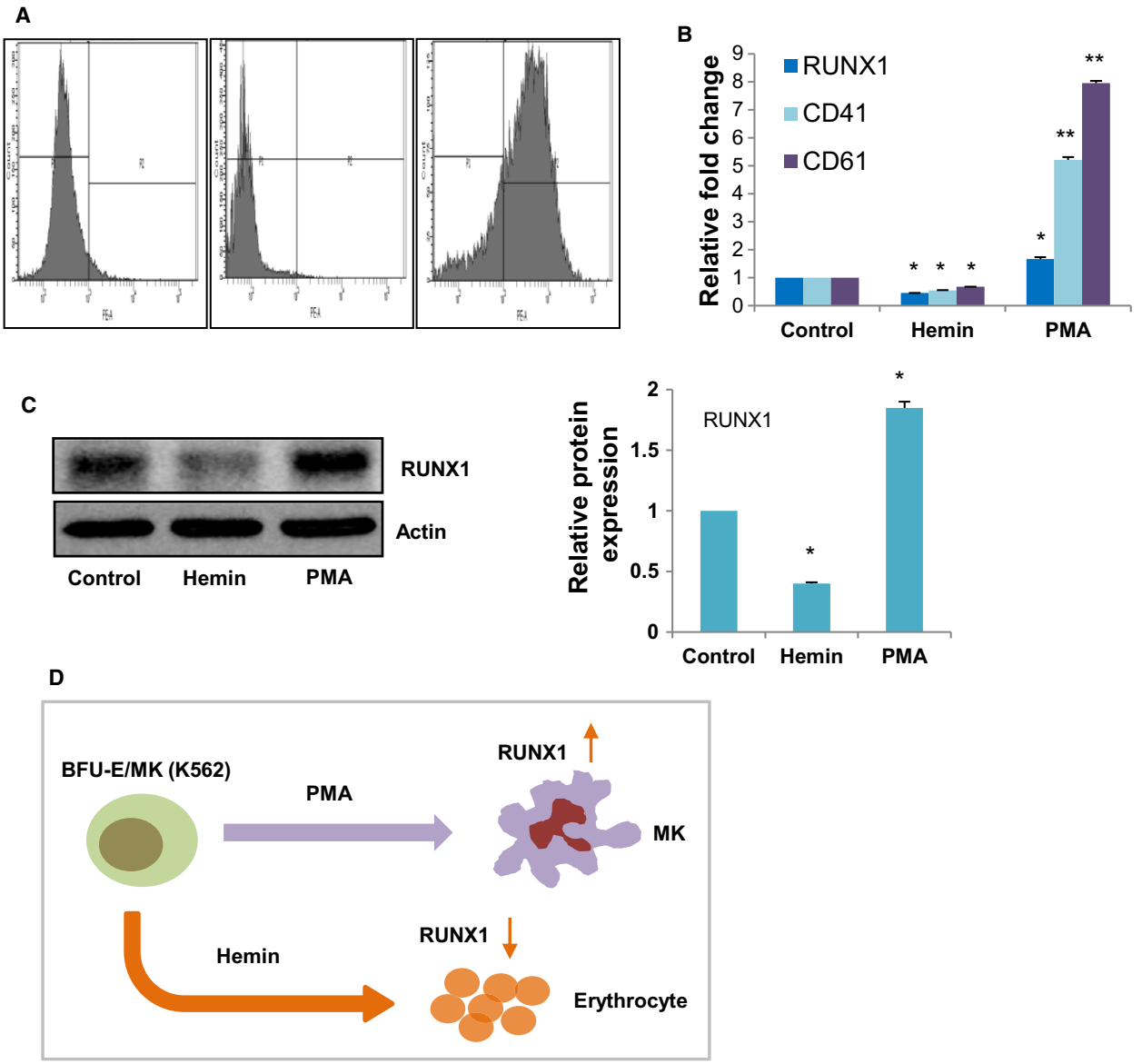



**Fig. 3.** PMA induces megakaryocytic differentiation of BFU-E/MKs (K562). (A) Flow cytometry analysis of the expression of megakaryocytic marker CD61 assessed after 72 h of treatment with Hemin, PMA, or untreated control ($n = 3$; $^{**}P < 0.02$). (B) Further, the differential expression of megakaryocytic markers (CD41 and CD61) and RUNX1 mRNA was confirmed by qRT-PCR after 72 h of treatment ($n = 3$; $^{**}P < 0.02$; $^{*}P < 0.05$). (C) Upon PMA or Hemin treatment, at 72 h, differential expression of RUNX1 protein was analyzed by blot densitometry ($n = 3$; $^{*}P < 0.05$). (D) Schematics showing, respectively, PMA- and Hemin-induced, megakaryocytic and erythroid differentiation of K562 cells with differential expression of megakaryocytic transcription factor RUNX1. Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

and explored the TGF-β pathway. We evaluated differences in the expression of genes encoding 84 TGF-β/BMP pathway components. Thus, K562 cells were cultured during a 72 h time period with PMA (50 nM) and control cells incubated with the same concentration of DMSO alone. Results from RT2 Profiler™ PCR Array are shown in Fig. 4B,C and Table 1. Most of the components of TGF-β pathway were showing a large change in gene expression (> 2-fold) when we compared the normalized expression of every gene on the array between the group 1 (PMA stimulation) and control group (DMSO) by plotting them against one another ($n = 3$; Fig. 4B). Further, the data are presented as volcano plot which shows statistically significant differentially expressed genes (Fig. 4C and Table 1). Of the 84 genes of TGF-β/BMP pathway

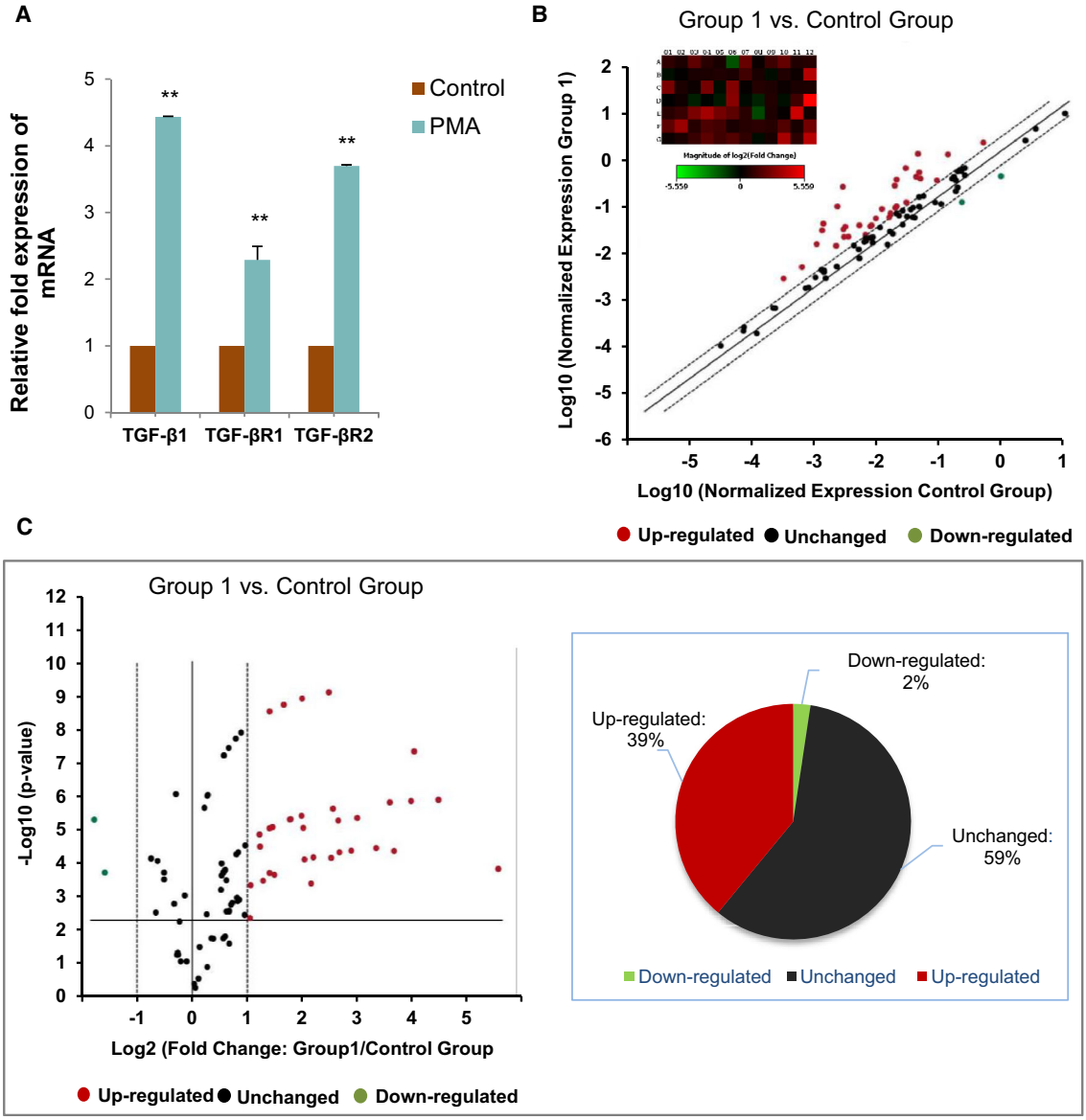



**Fig. 4.** RT2 Profiler™ PCR Array. (A) The mRNA expression levels of TGF-β and TGF-βRs were elevated in PMA-induced megakaryocytic model system ($n = 3$; $^{**}P < 0.02$). (B) Visualization of log2 (fold change) of genes using heat map and scatter plot ($n = 3$); lines above or under the mid-line denote the boundary of twofold regulation of gene expression; red and green circles represent the number of genes in group 1 (PMA-induced megakaryocytic cells) overexpressed and downregulated, respectively. (C) The volcano plot displays statistical significance versus fold change on the *y*- and *x*-axes, respectively. The volcano plot combines a *P*-value statistical test with the fold regulation change enabling identification of genes with both large and small expression changes (group 1, PMA-induced megakaryocytic cells vs control group, uninduced) that are statistically significant ($n = 3$; $P < 0.05$); red and green circles, respectively, represent the number of significantly overexpressed and down-regulated genes in group 1; pie chart shows the percentage of up-regulated, down-regulated, and unchanged genes in PMA-induced MKs (group 1). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

analyzed in PMA-induced megakaryocytic cells (group 1), only 35 genes showed statistically significant differences ($n = 3$; $P < 0.05$; FC ≥ 2.0; Fig. 4C and Table 1) with respect to control. Of these differentially regulated genes, 33 (39.28%) were up-regulated, whereas only two (2.38%) genes were down-regulated (Fig. 4C and Table 1).

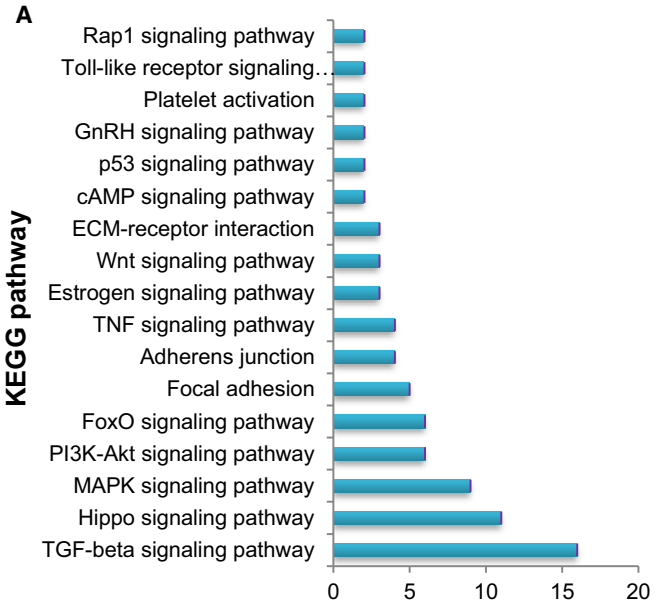
The pathways involving the statistically significantly changed genes were assessed by KEGG pathway. Though KEGG pathway analysis revealed that the

**Table 1.** Genes involved in TGF-β/BMP signaling pathway are folded up- or down-regulated in megakaryocyte differentiation of K562 cells upon PMA (50 nM) stimulation ($n = 3$; $P < 0.05$).

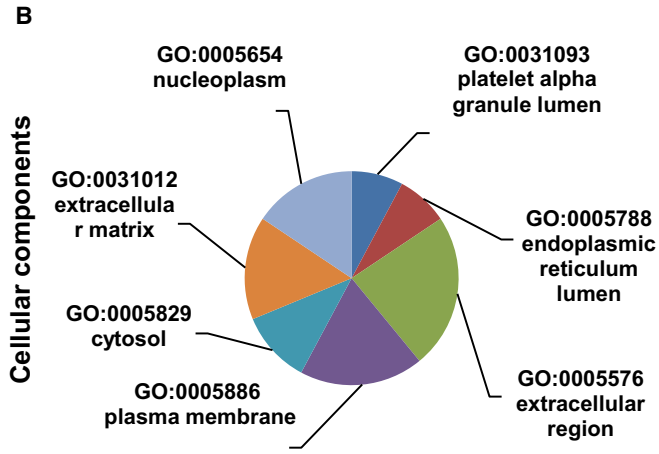
| GenBank | Gene symbol | Description | Fold change Group 1/Control |
|---|---|---|---|
| NM_001105 | ACVR1 | Activin A receptor, type I | 2.09 |
| NM_000020 | ACVRL1 | Activin A receptor type II-like 1 | 4.12 |
| NM_001675 | ATF4 | Activating transcription factor 4 (tax-responsive enhancer element B67) | −3.41 |
| NM_012342 | BAMBI | BMP and activin membrane-bound inhibitor homolog (Xenopus laevis) | 4.6 |
| NM_006129 | BMP1 | Bone morphogenetic protein 1 | 2.45 |
| NM_001200 | BMP2 | Bone morphogenetic protein 2 | 4.47 |
| NM_001204 | BMPR2 | Bone morphogenetic protein receptor, type II (serine/threonine kinase) | 2.65 |
| NM_000088 | COL1A1 | Collagen, type I, alpha 1 | 15.77 |
| NM_000089 | COL1A2 | Collagen, type I, alpha 2 | 6.32 |
| NM_001423 | EMP1 | Epithelial membrane protein 1 | 5.92 |
| NM_005252 | FOS | FBJ murine osteosarcoma viral oncogene homolog | 7.44 |
| NM_002166 | ID2 | Inhibitor of DNA binding 2, dominant-negative helix-loop-helix protein | 8.03 |
| NM_002191 | INHA | Inhibin, alpha | 2.66 |
| NM_002192 | INHBA | Inhibin, beta A | 47.14 |
| NM_002228 | JUN | Jun proto-oncogene | 2.36 |
| NM_002229 | JUNB | Jun B proto-oncogene | 6.4 |
| NM_020997 | LEFTY1 | Left–right determination factor 1 | 12.03 |
| NM_000627 | LTBP1 | Latent transforming growth factor beta binding protein 1 | 5.76 |
| NM_000428 | LTBP2 | Latent transforming growth factor beta binding protein 2 | 4.02 |
| NM_002467 | MYC | V-myc myelocytomatosis viral oncogene homolog (avian) | −2.99 |
| NM_002608 | PDGFB | Platelet-derived growth factor beta polypeptide | 22.23 |
| NM_001754 | RUNX1 | Runt-related transcription factor 1 | 4.06 |
| NM_000602 | SERPINE1 | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 10.14 |
| NM_005901 | SMAD2 | SMAD family member 2 | 3.19 |
| NM_005902 | SMAD3 | SMAD family member 3 | 2.75 |
| NM_003107 | SOX4 | SRY (sex-determining region Y)-box 4 | 3.47 |
| NM_000660 | TGFB1 | Transforming growth factor, beta 1 | 5.61 |
| NM_003239 | TGFB3 | Transforming growth factor, beta 3 | 2.34 |
| NM_000358 | TGFBI | Transforming growth factor, beta-induced, 68kDa | 3.97 |
| NM_004612 | TGFBR1 | Transforming growth factor, beta receptor 1 | 2.66 |
| NM_003242 | TGFBR2 | Transforming growth factor, beta receptor II (70/80 kDa) | 3.43 |
| NM_003243 | TGFBR3 | Transforming growth factor, beta receptor III | 2.07 |
| NM_003246 | THBS1 | Thrombospondin 1 | 12.67 |
| NM_006022 | TSC22D1 | TSC22 domain family, member 1 | 16.34 |
| NM_004048 | B2M | Beta-2-microglobulin | 2.81 |

statistically significantly changed genes were associated with several pathways, the pathways involved more affected components were TGF-beta, Hippo, MAPK, and PI3K-Akt signaling pathways. Interestingly, of the 35 significantly changed genes, 16 (45.7%) encode components of TGF-β signaling pathway ($P = 2.43E-24$; Fig. 5A and Table 2), such as ACVR1, BMP2, BMPR2, INHBA, SMAD3, BMP1, TGFBR1, ACVRL1, TGFB1, BAMBI, TGFB3, LEFTY1, SMAD2, JUN, JUNB, and TGFBR2 and were up-regulated (Fig. 5A and Table 1). The KEGG pathway's results suggest that the TGF-β signaling pathway is mainly associated with the differentially regulated genes observed during PMA-induced megakaryocytic differentiation of K562 cells.

Further, TGF-β pathway components and their regulated genes are classified according to subcellular localization by GO term-cellular component (GO-CC) classification of the DAVID database. They are mainly localized in the cytosol, nucleoplasm, plasma membrane, endoplasmic reticulum lumen, extracellular region, ECM,




| Category | Gene Up-Regulated | Fold Change (Group1/Control Group) |
|---|---|---|
| hsa04350:TGF-beta signaling pathway | INHBA | 47.14 |
| | LEFTY1 | 12.03 |
| | JUNB | 6.41 |
| | TGFB1 | 5.61 |
| | BAMBI | 4.62 |
| | BMP2 | 4.47 |
| | ACVRL1 | 4.12 |
| | TGFBR2 | 3.43 |
| | SMAD2 | 3.19 |
| | SMAD3 | 2.75 |
| | TGFBR1 | 2.66 |
| | BMPR2 | 2.65 |
| | BMP1 | 2.45 |
| | JUN | 2.36 |
| | TGFB3 | 2.34 |
| | ACVR1 | 2.09 |




| Category | Cellular components |
|---|---|
| GO:0031093 platelet alpha granule lumen | TGFB1, PDGFB, SERPINE1, THBS1, TGFB3 |
| GO:0005788 endoplasmic reticulum lumen | PDGFB, COL1A1, B2M, COL1A2, THBS1 |
| GO:0005576 extracellular region | PDGFB, BMP1, BMP2, INHA, SERPINE1, INHBA, TGFB1, COL1A1, TGFBR3, B2M, LTBP1, COL1A2, TGFBI, THBS1, TGFB3 |
| GO:0005886 plasma membrane | BAMBI, BMPR2, TGFB1, ACVRL1, SMAD3, EMP1, B2M, TGFBR2, TGFBR1, TGFBI, SERPINE1, TGFB3, |
| GO:0005829 cytosol | JUN, SMAD3, ID2, TGFBR2, FOS, MYC, SMAD2 |
| GO:0031012 extracellular matrix | TGFB1, PDGFB, COL1A1, LTBP2, COL1A2, LTBP1, TGFBI, SERPINE1, THBS1, TGFB3 |
| GO:0005654 nucleoplasm | JUN, RUNX1, SOX4, ATF4, SMAD3, ID2, FOS, MYC, JUNB, SMAD2, SMAD3 |

**Fig. 5.** PMA-induced megakaryocyte differentiation of K562 enhanced the expression of TGF-β signaling pathway components. (A) The results of KEGG pathway analysis of 35 significantly changed genes in RT2 Profiler™ PCR Array (*P*-value < 0.05); the column represents the enriched KEGG pathways using differentially expressed genes in PMA-induced megakaryocytic cells; the 'TGF-β signaling pathway' was identified as the most significantly enriched pathway in KEGG analysis. (B) The details of protein location, of 35 significantly changed genes in RT2 Profiler™ PCR Array, identified by GOTERM_CC_DIRECT (DAVID 6.8 Tool); (*P*-value < 0.02); chart showing the classification of significantly changed genes, in PMA-induced megakaryocytic cells, on the basis of their protein location.

and platelet alpha granule lumen (Fig. 5B). However, other locations, such as secretory granule and Golgi membrane ($P < 0.02$; Table 3), were also noticed. Notably, of the 35 components, 15 (40%) components are mainly situated in the extracellular region and belong to either TGF-β superfamily or TGF-β binding protein family, and ECM components (Fig. 5B), such as PDGFB, BMP1, BMP2, INHA, SERPINE1, INHBA, TGFB1, COL1A1, TGFBR3, B2M, LTBP1, COL1A2, TGFBI, THBS1, and TGFB3, were up-regulated ($n = 3$; $P < 0.05$; Table 1). These data are consistent with the previous observations, which states that TGF-β1 is acting through an autocrine loop on MKs in the regulation of downstream pathways and effector genes of ECM

**Table 2.** The results of KEGG pathway analysis of 35 significantly changed genes in $RT^2$ Profiler™ PCR array.

| Category | Term | Count | % | *P*-value | ENTREZ_GENE_ID |
|---|---|---|---|---|---|
| KEGG_PATHWAY | hsa04350: TGF-beta signaling pathway | 16 | 66.666 | 2.43E-24 | 659, 90, 3398, 650, 3624, 25805, 7040, 4088, 7048, 10637, 4052, 7046, 7057, 4609, 7043, 4087 |
| KEGG_PATHWAY | hsa04390: Hippo signaling pathway | 11 | 45.833 | 1.73E-11 | 659, 7040, 4088, 3398, 7048, 650, 7046, 4609, 5054, 7043, 4087 |
| KEGG_PATHWAY | hsa05166: HTLV-I infection | 11 | 45.833 | 2.96E-09 | 3725, 7040, 5155, 468, 4088, 7048, 2353, 7046, 4609, 7043, 4087 |
| KEGG_PATHWAY | hsa04010: MAPK signaling pathway | 9 | 37.5 | 9.10E-07 | 3725, 7040, 5155, 468, 7048, 2353, 7046, 4609, 7043 |
| KEGG_PATHWAY | hsa04068: FoxO signaling pathway | 6 | 25 | 6.60E-05 | 7040, 4088, 7048, 7046, 7043, 4087 |
| KEGG_PATHWAY | hsa04520: Adherens junction | 4 | 16.666 | 0.001 | 4088, 7048, 7046, 4087 |
| KEGG_PATHWAY | hsa04510: Focal adhesion | 5 | 20.833 | 0.004 | 3725, 5155, 1277, 1278, 7057 |
| KEGG_PATHWAY | hsa04151: PI3K-Akt signaling pathway | 6 | 25 | 0.004 | 5155, 468, 1277, 1278, 7057, 4609 |

components, such as collagens and fibronectin which surrounds human MKs in the bone marrow [19]. In addition, GO term-cellular components clustering also detected enrichment of TGF-β pathway effectors and their regulated genes (28.5%) in the nucleoplasm (Fig. 5B). This group of genes encodes TFs which play key functions during myeloid development and megakaryocytopoiesis (Fig. 6A–D) [27]. Herein, RUNX1, SMAD2/3, and activator protein 1 (AP-1) TFs, JUN, JUNB, and FOS were selectively up-regulated ($P < 0.05$; Fig. 6C,D), whereas MYC and ATF4 were down-regulated ($P < 0.05$; Fig. 6B).

Mostly, proteins interact to exert their biological functions in cells. To understand the protein–protein interactions and associated functional aspects, 35 significantly differentially regulated gene-encoded protein components were analyzed by searching the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. Further, these interacting genes/proteins were visualized in CYTOSCAPE 3.6 software (Fig. 6E). A protein–protein interaction network including 29 components was extracted from the STRING database. The size of node and line thickness, respectively, indicate the degree of interaction and the strength of data support; active interaction sources are text mining, experiments, and databases. The red and green colors of nodes, respectively, represent the up- or down-regulation of gene expression from the present TGF-β/BMP pathway array (Fig. 6E). Interestingly, most of the differentially regulated TGF-β effectors regulated gene-encoded components were showing interactions with TGF-β core pathway components, which are also mutually connected with each other in the protein interaction network (Fig. 6E). And, most of the network components were up-regulated ($P < 0.02$; Fig. 4C and Table 1) during PMA-induced megakaryocytic development ($P < 0.02$; Fig. 6F,G).

## Augmenting effects of recombinant human TGF-β1 on differentiating megakaryocytic cell line model

To investigate the functional effects of the TGF-β pathway in MK development, we treated PMA-induced megakaryocytic cells with TGF-β1. During differentiation and maturation of cells, the changes in cell's size, shape, membrane potential, and differential responsiveness to signals are commonly observed features or events. In the present study, cells were observed with increased cell size and adherence in both the PMA and TGF-β1 treatment post-PMA stimulation, as compared to control ($n = 3$; Fig. 7A). However, TGF-β1 treatment of PMA-stimulated megakaryocytic cells resulted in more membrane expansions as compared to that observed in PMA-stimulated megakaryocytic model ($n = 3$; Fig. 7A).

SMAD2/3 plays a fundamental role in mediating TGF-β responses, such as stimulation of different

**Table 3.** The details of protein location, of 35 significantly changed genes in $RT^2$ Profiler™ PCR Array, identified by GOTERM_CC_DIRECT (DAVID 6.8 Tool).

| Category | Term | Count | % | *P*-value | Gene ID |
|---|---|---|---|---|---|
| GOTERM_CC_DIRECT | GO:0031093 platelet alpha granule lumen | 5 | 14.285 | 1.15E-06 | 7040, 5155, 7057, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005788 endoplasmic reticulum lumen | 5 | 14.285 | 1.15E-06 | 5155, 1277, 567, 1278, 7057 |
| GOTERM_CC_DIRECT | GO:0005576 extracellular region | 15 | 42.857 | 2.36E-04 | 5155, 649, 650, 3623, 5054, 3624, 7040, 1277, 7049, 567, 4052, 1278, 7045, 7057, 7043 |
| GOTERM_CC_DIRECT | GO:0005886 plasma membrane | 12 | 34.285 | 4.96E-04 | 659, 25805, 7040, 94, 2012, 4088, 7048, 567, 7046, 7045, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005829 cytosol | 7 | 20 | 5.71E-04 | 3725, 4088, 3398, 7048, 2353, 4609, 4087 |
| GOTERM_CC_DIRECT | GO:0031012 extracellular matrix | 10 | 28.571 | 0.003 | 7040, 5155, 1277, 4053, 1278, 4052, 7045, 7057, 5054, 7043 |
| GOTERM_CC_DIRECT | GO:0005654 nucleoplasm | 10 | 28.571 | 0.024 | 861, 3725, 468, 6659, 4088, 3398, 2353, 4609, 3726, 4087 |

effector gene expression. To begin to elucidate the role of TGF-β in megakaryocytic maturation, we examined the expression of TGF-βR2 and SMAD3 upon TGF-β1 stimulation, resulted in a dose-dependent increased expression of TGF-βR in megakaryocytic cells ($n = 3$; $P < 0.05$; Fig. 7B). Upon TGF-β1 stimulation of PMA-induced megakaryocytic cells, SMAD3 mRNA expression levels were not increased further (Fig. 7C); however, cellular pSMAD2/3 levels were increased at TGF-β1 ligand concentrations of up to 20 $ng{\cdot}mL^{-1}$ ($n = 3$; Fig. 7D). The phosphorylation state of the cellular SMAD2/3 thus serves as a marker of the active TGF-β/SMAD signaling. With this observation, we determined the expression of megakaryocytic markers and TF RUNX1 upon TGF-β1 stimulation. We noticed that in PMA-induced megakaryocytic cells, TGF-β1 significantly increased the expression levels of a key megakaryocytic TF RUNX1 and megakaryocytic marker CD41 in dose-dependent manner ($n = 3$; $P < 0.05$; Fig. 8A).

On the other hand, collagen, a component of ECM, which surrounds human MKs in the bone marrow [19], was visualized under fluorescence microscope. We used anti-type I collagen antibody and observed the increased fluorescence intensity upon TGF-β1 treatment of PMA-stimulated megakaryocytic cells ($n = 3$; Fig. 8B), which shows that TGF-β downstream pathway activation increased the collagen deposition during MK maturation. To further determine the role of TGF-β in megakaryocytic maturation, MK surface antigen CD61 (Dapi/CD61-PE) expression was visualized under fluorescence microscope; we observed more number of CD61-PE-positive cells with increased intensity of fluorescence upon TGF-β1 treatment (20 $ng{\cdot}mL^{-1}$) of PMA-stimulated megakaryocytic cells as compared to control, or PMA alone ($n = 3$; Fig. 8C).

In agreement with the positive effects of TGF-β1 on megakaryocytic marker expression, RUNX1 expression, deposition of collagen, and cell membrane expansion, we performed the cell cycle analysis (i.e., DNA ploidy) using Muse Cell Analyzer, the population profile depicted increased cell size index with TGF-β1 treatment post-PMA stimulation, and PMA alone, as compared to control. PMA treatment, as well as TGF-β1 treatment post-PMA stimulation, resulted in a significant increase in the percentage of polyploidy cells as compared to control. However, TGF-β1 treatment of PMA-stimulated megakaryocytic cells resulted in a small increase in the percentage of ploidy cells (> 4N) as compared to PMA alone ($n = 3$; Fig. 8D). Data in the present study show that TGF-β1 has the potential to augment PMA-induced megakaryocytic maturation with no striking change in DNA content.

## TGF-β increases cellular calcium and TRP ion channel expression during megakaryocyte differentiation

As a major intracellular messenger, $[Ca^{2+}]i$ is involved in regulating the various physiological activities of different cells and tissues including differentiation. It is generally believed that $Ca^{2+}$ plays a key role in platelet activation and regulation of platelet function. TGF-β1 is well known to increase $[Ca^{2+}]i$ activity in many

**A**

| Positive cell differentiation | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| SOX4 | 3.47 |
| SMAD2 | 3.19 |
| SMAD3 | 2.75 |

**B**

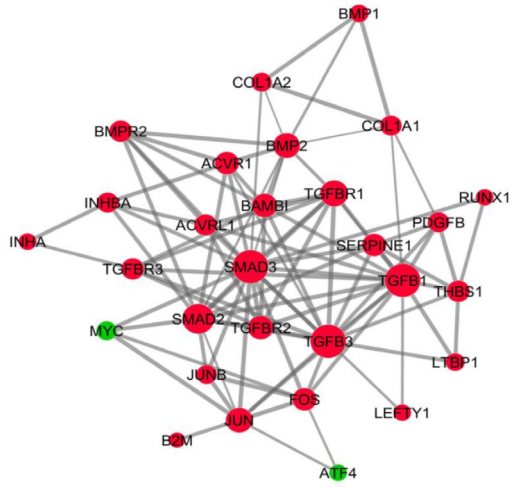
| Positive Regulation of Biosynthesis | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| SOX4 | 3.47 |
| ATF4 | −3.41 |
| MYC | −2.99 |

**C**

| Megakaryocytopoiesis | |
|---|---|
| **Gene** | **FC** |
| RUNX1 | 4.06 |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |

**D**

| AP-1 Monomers | |
|---|---|
| **Gene** | **FC** |
| JUNB | 6.4 |
| JUN | 2.36 |
| FOS | 7.44 |
| ATF4 | −3.41 |



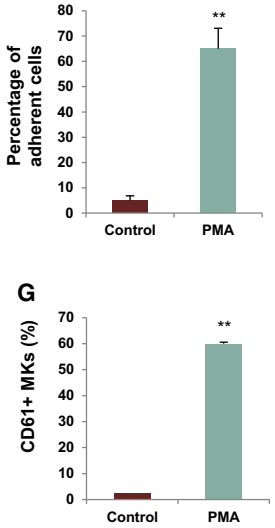




**Fig. 6.** The protein–protein interactions and associated functional aspects. (A) Positive cell differentiation regulators, (B) positive regulators of biogenesis, (C) positive regulators of megakaryocytopoiesis, and (D) AP-1 monomers were observed with statistically significant differential expression in PMA-induced megakaryocytic model system as compared to uninduced control ($n = 3$; $P < 0.005$). (E) A protein–protein interaction network including 29 components was extracted from the STRING database. The size of node and line thickness, respectively, indicate the degree of interaction and the strength of data support; active interaction sources are text mining, experiments, and databases, and the color of node represents the up- or down-regulation of gene expression (visualized in CYTOSCAPE 3.6 software, Cytoscape Consortium, Bethesda, MD, USA) from the present RT2 Profiler™ PCR Array (TGF-β/BMP pathway). (F) The bar graph shows the percentage of adherent cells counted under inverted microscope (20 fields; $n = 3$, $^{**}P < 0.02$) after 72 h upon PMA treatment. (G) Figure shows the increased percentage of MKs ($CD61^+$ cells) upon PMA treatment ($n = 3$; $P < 0.02$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

cellular systems. Recently, it has been reported that the time course of TGF-β1-induces store-operated $Ca^{2+}$ entry (SOCE) in MKs [24]. But, the functional role of TRP ion channels in extracellular $Ca^{2+}$ entry in MKs is currently unknown. In the first series of experiments, the expression of TRP ion channels was identified using qRT-PCR. Further, Fluo-3 fluorescence was utilized to determine $[Ca^{2+}]i$ activity. As illustrated in Fig. 9A, TRPA1 and TRPV1 were observed with statistically significantly increased expression in PMA-induced megakaryocytic cells as compared to control ($n = 3$; $P < 0.02$); however, TRPC3 did not show a significant increase ($n = 3$; $P > 0.05$). Interestingly, upon TGF-β1 treatment of PMA-stimulated cells, we observed a consistent increased expression of TRPA1, TRPV1, and TRPC3 in dose-dependent manner as compared to control ($n = 3$; $P < 0.02$; Fig. 9A); also, the expression levels were higher than that observed with PMA alone (Fig. 9A). Thus, this observation shows that TGF-β1 stimulates the transcription of several TRP ion channels in megakaryocytic cells.

In order to quantify TRP ion channel activity, $[Ca^{2+}]i$ concentration was determined utilizing fluorescent $Ca^{2+}$ indicator, Fluo-3/AM. Using a fluorescence spectrometer, as illustrated in Fig. 9B, it was determined from observations that the Fluo-3 fluorescence intensity of the cells had increased after PMA stimulation as compared to the normal value in the control ($n = 3$; $P < 0.02$); however, Fluo-3 fluorescence intensity was much higher in cells treated with TGF-β1 (10 and 20 $ng{\cdot}mL^{-1}$) post-PMA stimulation as compared to the normal value in the control ($n = 3$; $P < 0.02$), or PMA alone (Fig. 9B). For further confirmation, as shown in Fig. 9C, cells were imaged using a fluoresce microscope and the fluorescence image intensity was quantified by IMAGEJ (Developed by Wayne Rasband at NIH, Bethesda, MD, USA). The lowest $[Ca^{2+}]i$ was detected from the control sample, and cells exhibited weak green fluorescence. The $[Ca^{2+}]i$ level was increased in PMA-stimulated cells, with increased green fluorescence intensity. However, the $Ca^{2+}$ level detected in PMA-stimulated megakaryocytic model was further augmented after TGF-β1 (20 $ng{\cdot}mL^{-1}$) treatment post-PMA stimulation; many cells were observed with high level of green fluorescence intensity as compared to control, or PMA-stimulated megakaryocytic model ($P < 0.02$; Fig. 9C). These results suggest that TRP ion

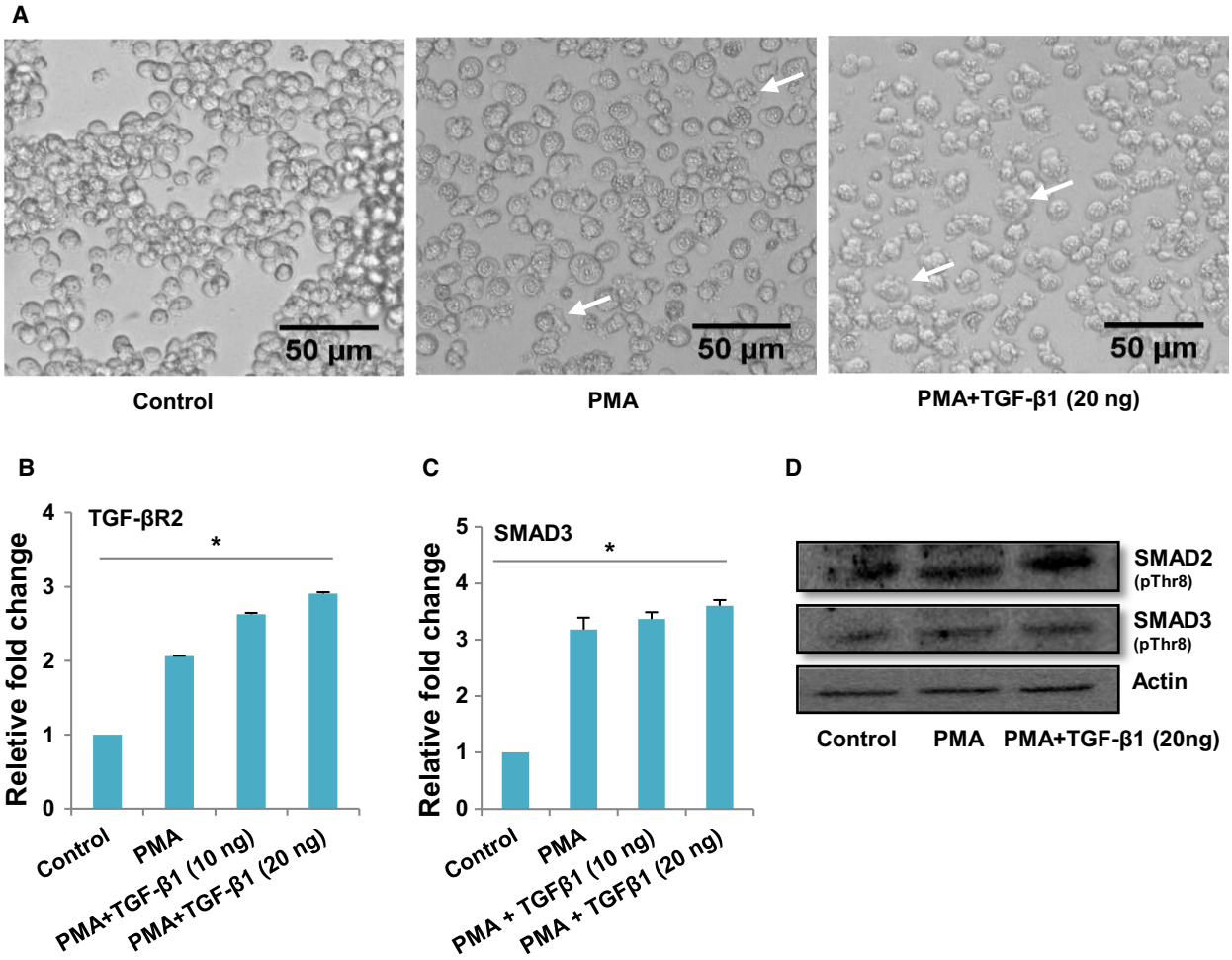



**Fig. 7.** Effects of recombinant human TGF-β1 on differentiating megakaryocyte cell line model. (A) Photomicrograph (20× objective; scale = 50 μm) showing cells with increased adherence and membrane expansion upon TGF-β1 treatment of PMA-induced megakaryocytic cells as compared to control, or PMA; the white arrows indicate the site of membrane expansion ($n = 3$). (B) qRT-PCR analysis results show a dose-dependent activation of TGF-βR2 expression upon TGF-β1 treatment of megakaryocytic cells ($n = 3$; $^*P < 0.05$). (C) SMAD3 mRNA levels were not changed much upon TGF-β1 treatment of PMA-induced megakaryocytic cells; however, the levels were more than control ($n = 3$; $P < 0.05$). (D) Increased pSMAD2/3 levels were observed in western blot analysis upon TGF-β1 treatment of differentiating megakaryocytic cells ($n = 3$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

channels and $[Ca^{2+}]i$ activity are involved in PMA-induced megakaryocytic development. Moreover, TGF-β1 treatment significantly increased the cellular $Ca^{2+}$ and TRP ion channel (TRPA1, TRPV1, and TRPC3) expression in PMA-induced megakaryocytic model, which attests to its crucial role in MK development by increasing $[Ca^{2+}]i$ levels.

## TGF-β1 increased cellular ROS levels in PMA-induced megakaryocytic differentiation

There is a common view that ROS can influence platelet production and their function in a microenvironment-dependent manner. In previous reports, ROS has been demonstrated necessary for PMA-induced megakaryocytic differentiation [21-23]. In order to understand the effect of TGF-β1 on intracellular ROS levels, we treated K562 cells separately with PMA, and TGF-β1 post-PMA stimulation and analyzed the intracellular ROS production by the fluorescence molecule H2DCFDA (2, 7-dichlorodihydrofluorescein diacetate). The ROS levels in PMA-stimulated megakaryocytic cells were consistently enhanced (~ 12%) upon TGF-β1 treatment at the concentrations up to 20 $ng{\cdot}mL^{-1}$ ($n = 3$; $P < 0.05$; Fig. 10A). Mitochondria are the major source of ROS in the cell. Mitochondrial uncoupling protein 2 (UCP2) is a well-known regulator of mitochondrial ROS production, and its transcriptional

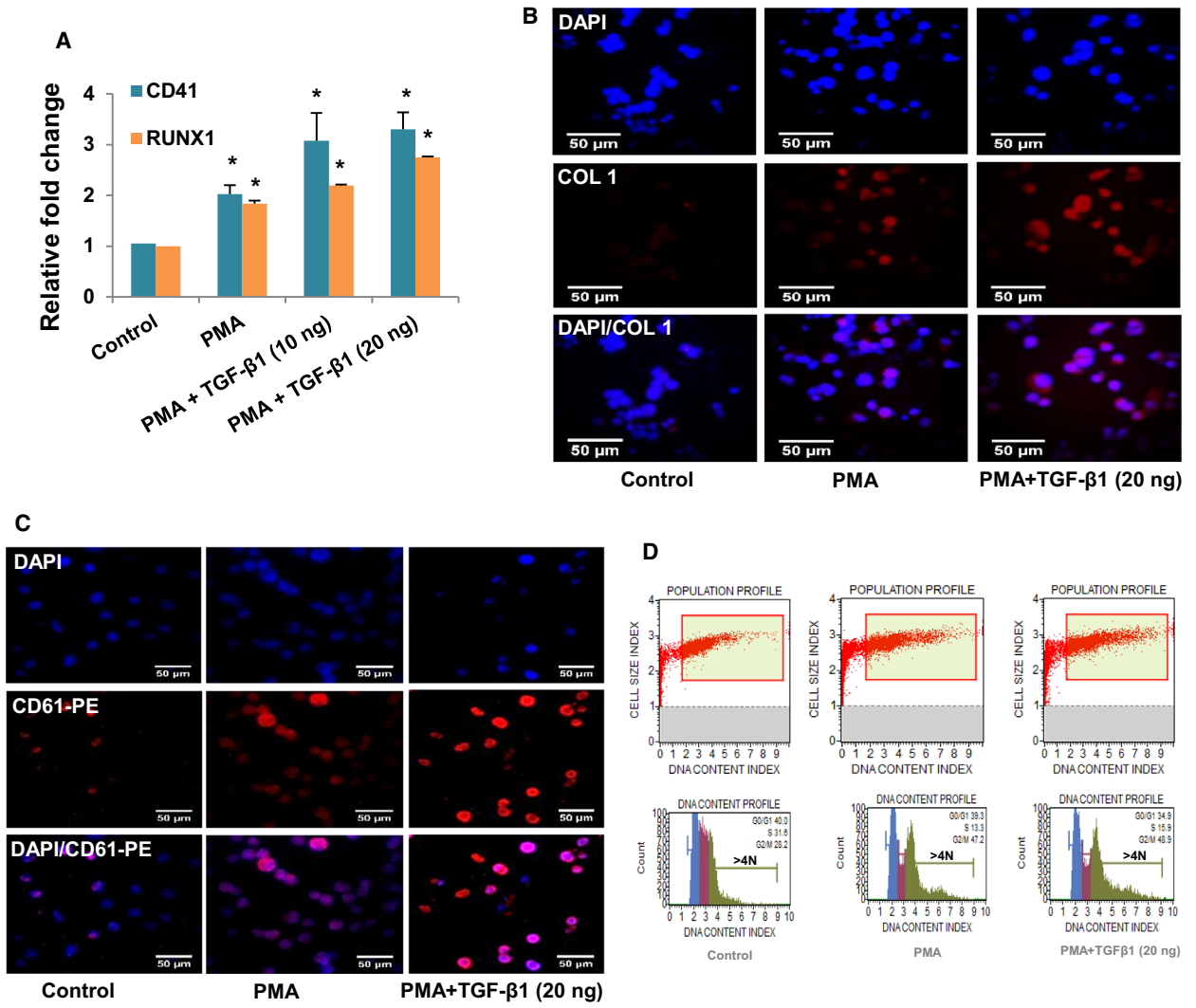



**Fig. 8.** Recombinant human TGF-β1 augments the maturation process in differentiating megakaryocyte cell line model. (A) qRT-PCR analysis results show a dose-dependent activation of megakaryocytic marker (CD41) and transcription factor (RUNX1) expression upon TGF-β1 treatment of differentiating megakaryocytic cells ($n = 3$; $^*P < 0.05$). (B) Fluorescence microscopy images (20× objective; scale = 50 μm) show the increased deposition of ECM component and collagen type I (COL1) upon TGF-β1 treatment of differentiating megakaryocytic cells ($n = 3$). (C) Fluorescence microscopy images (20× objective; scale = 50 μm) observed with increased florescence intensity of CD61-PE (megakaryocytic marker) upon TGF-β1 treatment of differentiating megakaryocytic cells ($n = 3$). (D) DNA content of K562, PMA-induced K562 (differentiating megakaryocytic cells), and upon TGF-β treatment of differentiating megakaryocytic cells (analyzed by Muse Analyzer), the DNA content was increased in differentiating megakaryocyte; however, it was not affected upon TGF-β1 treatment ($n = 3$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

repression is maintained by SMADs-mediated TGF-β signaling in various types of cells [28]. In the current study, we observed that UCP2 mRNA levels were not changed significantly during PMA-induced megakaryocytic differentiation of K562 as compared to control. However, upon TGF-β1 treatment of PMA-induced megakaryocytic cells, the UCP2 expression was significantly reduced in dose-dependent manner at concentrations up to 20 $ng{\cdot}mL^{-1}$ ($n = 3$; $P < 0.02$; Fig. 10B,C). On the other hand, the contributor of nonmitochondrial ROS, NOX4, was observed with a consistent increased expression upon TGF-β1 treatment of PMA-induced megakaryocytic cells in a dose-dependent manner ($n = 3$; $P < 0.05$; Fig. 10D). Further, to understand mitochondrial membrane potential (ΔΨm), the percentage of depolarized/live cells was determined by a flow cytometer using the Muse Mito Potential Kit. The lowest number of depolarized/live cells (23.71%) was detected from the normal control group ($n = 3$; Fig. 10E), whereas after PMA treatment, 62.01% cells

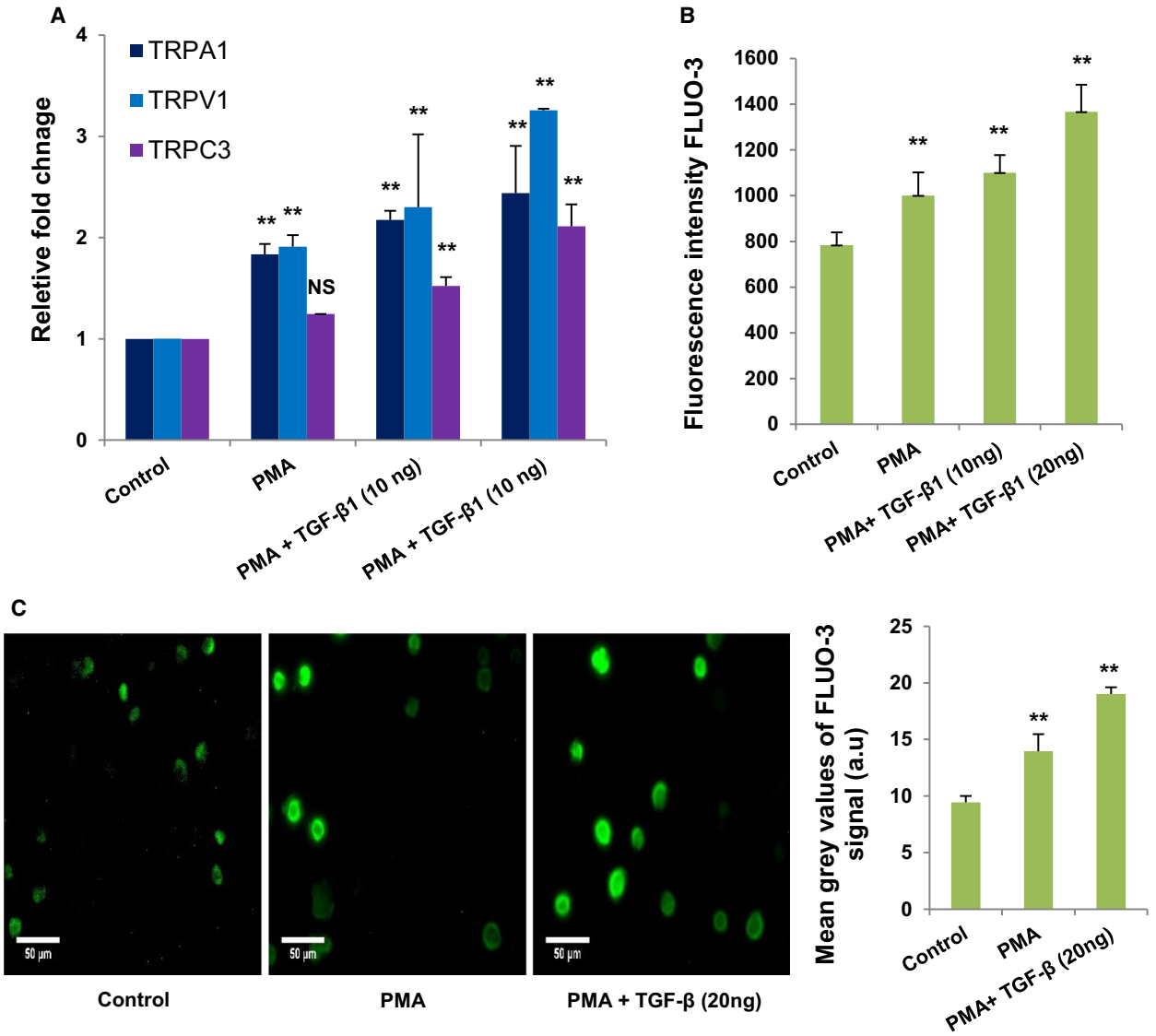



**Fig. 9.** TGF-β increases cellular calcium and TRP ion channel expression. (A) qRT-PCR results show the dose-dependent increased mRNA levels of TRP ion channels upon TGF-β1 treatment of differentiating megakaryocytic cells ($n = 3$; $^{**}P < 0.02$; $^{ns}P > 0.05$). (B) Further, Fluo-3 fluorescence was utilized to determine $[Ca^{2+}]i$ activity; the emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence spectrometer at 25 °C (excitation 505 nm, emission 526 nm, slit width 5 nm); ($n = 3$; $^{**}P < 0.02$). (C) Fluo-3/AM (3 µM)-loaded cells were imaged (20× objective; scale = 50 µm) for monitoring the fluorescence intensity using a fluoresce microscope; the image intensity was invariably quantified as the mean gray value in a visual filed selected; and the histogram of mean fluorescence intensity of 500 cells is displayed ($n = 3$, $^{**}P < 0.02$). The mean fluorescence intensity was increased upon TGF-β1 treatment of PMA-induced differentiating megakaryocytic cells. Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

were undergoing depolarized state (live) ($n = 3$; Fig. 10E). And, TGF-β1 treatment post-PMA stimulation further increased the percentage of depolarized/live cells (68.87%), ($n = 3$; Fig. 10E). The increased number of undergoing depolarized/live cells is a sign of reduced ΔΨm. These data show that TGF-β1 likely increases ROS by transcriptional repression of UCP2 and that in turn results in reduced ΔΨm. However, a portion of ROS might be generated by NOX4 in MKs during maturation and proplatelet release.

## ROS regulates cellular $Ca^{2+}$ activity during megakaryocytic differentiation

Increasing evidence suggests a pivotal role of ROS in the regulation of cellular $Ca^{2+}$. A typical target of

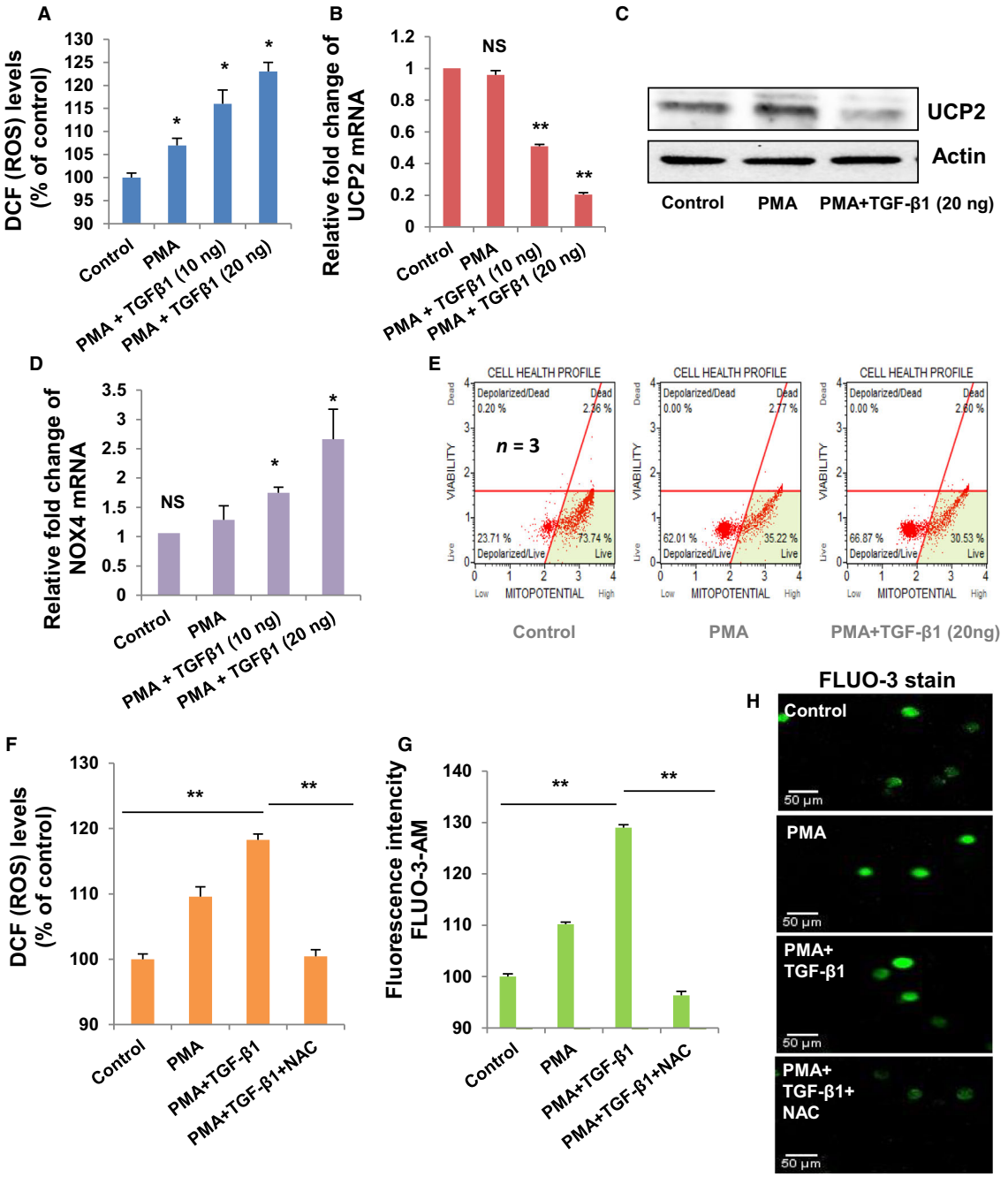



**Fig. 10.** TGF-β1 increases cellular ROS production. (A) ROS production was detected using H2DCFDA, which is a cell-permeable standard probe to detect ROS; fluorescence was measured by fluorescence spectroscopy at 485 nm excitation and 535 nm emission wavelengths. The ROS production was elevated in dose-dependent manure upon TGF-β1 treatment ($n = 3$; $^{*}P < 0.05$). (B) qRT-PCR results show the dose-dependent effects of TGF-β1 on UCP-2 mRNA levels ($n = 3$; $^{**}P < 0.02$). (C) UCP2 protein levels upon TGF-β1 stimulation of PMA-induced K562 ($n = 3$; $P < 0.05$). (D) qRT-PCR results show the dose-dependent effects of TGF-β1 on NOX4 mRNA levels ($n = 3$; $^{*}P < 0.05$). (E) The depolarized/live cell percentage was determined by a flow cytometer (Muse™ Cell Analyzer; Merck Millipore) and observed with increased percentage of depolarized/live cells upon TGF-β1 treatment ($n = 3$). (F) The ROS levels were significantly reduced by ROS quencher, NAC ($n = 3$; $^{**}P < 0.02$). (G) The emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence spectrometer; intensity was significantly reduced in cells treated with ROS quencher, NAC ($n = 3$; $^{**}P < 0.02$). (H) The Fluo-3/AM (3 µM)-loaded cells were imaged (20× objective; scale = 50 µm) for monitoring the fluorescence intensity using a fluorescence microscope ($n = 3$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

ROS is $Ca^{2+}$ channels. Evidence suggests key contribution of ROS in TRPA1, TRPV1, and TRPC3 [29-31], and $Ca^{2+}$-permeable ion channel activation. To understand the functional significance of ROS in the cellular $Ca^{2+}$ regulation, we have carried out cell differentiation experiments using ROS quencher *N*-acetyl-L-cysteine (NAC), which interacts directly with ROS and reduces intracellular ROS levels. In differentiation experiments, NAC pretreatment significantly reduced the cellular ROS increased by TGF-β1 in PMA-induced megakaryocytic model ($n = 3$; $P < 0.02$; Fig. 10F). Under the same experimental conditions, we also found significantly reduced cellular $Ca^{2+}$ levels ($n = 3$; $P < 0.02$; Fig. 10G,H). This evidence shows that NAC, a well-known inhibitor of ROS, reversed the positive effect of ROS on the cellular $Ca^{2+}$ likely via reducing the ROS-dependent activity of TRP ion channels. Taken together, these data support the notion that increased cellular $Ca^{2+}$ is a key event in megakaryocytic development and that, in turn, regulated by TGF-β1-induced ROS production.

### TGF-β-induced TRP ion channel expression is likely regulated through TF RUNX1

As we have demonstrated in our results that TRP ion channels are up-regulated in PMA-induced megakaryocytic cells and the expression levels were further augmented upon TGF-β1 treatment (Fig. 9A), the findings of previous studies suggest that in differentiating megakaryocytic cell line models, RUNX1 cooperates with different TFs and functions as a key gene expression regulator during megakaryocytic differentiation [13,14]. Therefore, we designed a siRUNX1 transfection experiment to test whether or not RUNX1 has a role in TRP ion channel expression. Interestingly, we observed significantly reduced levels of TRP ion channel (TRPA1, TRPV1, and TRPC3) expression ($n = 3$; $P < 0.05$; Fig. 11A) and cellular $Ca^{2+}$ ($n = 3$; $P < 0.02$; Fig. 11B) post-siRUNX1 transfection. Previously, RUNX1 has been reported for its regulatory role on TRP ion channel expression [32,33].

### TGF-β augments the MK marker CD41 and RUNX1 expression in TPO-induced MKs

To better define the functional role of TGF-β on *in vitro* MK maturation, we cultured CB-$CD34^+$ hematopoietic progenitors for 14 days in serum-free medium in the presence of TPO (50 ng·$mL^{-1}$). The effect of TGF-β1 was further demonstrated by treating MKs at day 8 of culture with TPO (50 ng·$mL^{-1}$) in the presence or not of 20 ng·$mL^{-1}$ recombinant human TGF-β1 for 4 days ($n = 3$; $P < 0.02$; Fig. 12A, B). Interestingly, as compared with TPO alone, upon costimulation the expression of megakaryocytic TF RUNX1 (~ 4-fold; $n = 3$; $P < 0.02$; Fig. 12B) and MK marker CD41 (~ 3-fold; $n = 3$; $P < 0.02$; Fig. 12B) was enhanced with increased expression of some of the TGF-β core pathway components ($n = 3$; $P < 0.02$; Fig. 12B). In the previous study also, it has been noticed TGF-β acting through an autocrine loop on MKs and their results showing the activation of different TGF-βR downstream pathways upon TGF-β1 and TPO costimulation as compared with TPO alone [19].

## Discussion

In the previous studies, we have reported developmental differences between neonatal and adult MKs [5,6];

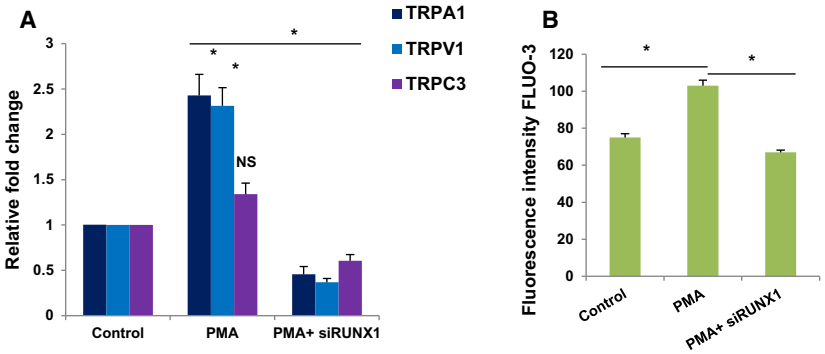



**Fig. 11.** siRUNX1 reduced the mRNA expression levels of TRP ion channel. (A) qRT-PCR results show that siRUNX1 transfection reduced the elevated levels of TRP ion channels observed during PMA-induced megakaryocytic differentiation ($n = 3$; $^{*}P < 0.05$; $^{ns}P > 0.05$). (B) Interestingly, the emitted fluorescence intensity of Fluo-3 was significantly reduced in PMA-induced megakaryocytic cells upon siRUNX1 transfection ($n = 3$; $^{**}P < 0.02$). Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

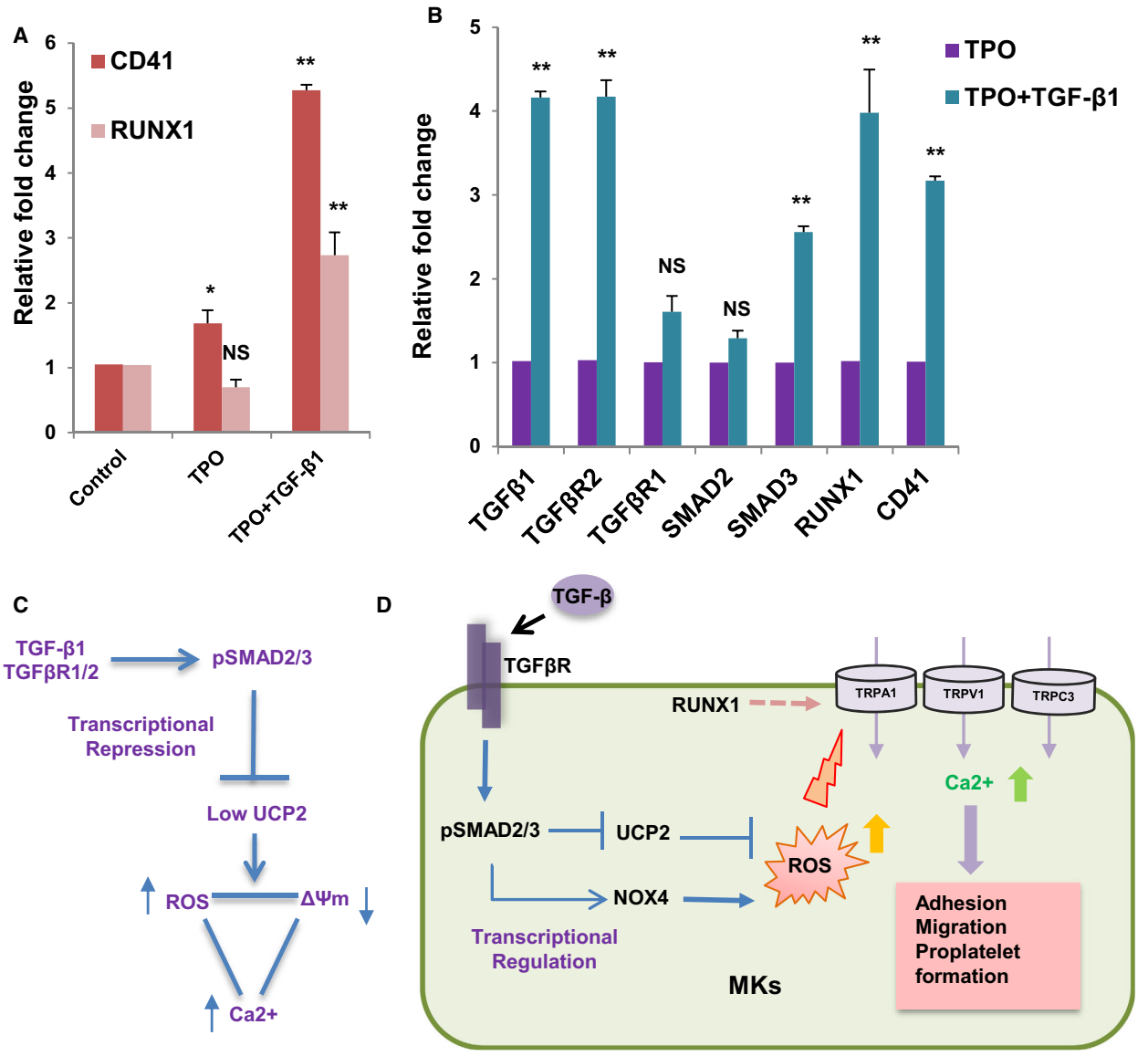



**Fig. 12.** TGF-β1 augments the MK marker and RUNX1 expression in TPO-induced CB-MKs. (A) qRT-PCR results show the differential expression levels of MK marker CD41 and RUNX1 in TPO alone or TPO and TGF-β1 costimulation as compared to control ($n = 3$; $^{**}P < 0.02$; $^{*}P < 0.05$). (B) qRT-PCR results show the high expression levels of MK marker CD41, RUNX1, and different TGF-β pathway components upon TGF-β1 treatment of MKs as compared with TPO alone ($n = 3$; $^{**}P < 0.02$). (C) The graphical representation of the UCP2 regulated mechanism. (D) Schematics showing the effects of TGF-β/SMAD pathway and RUNX1 on MK maturation, mediated by the regulation of ROS and $Ca^{2+}$. Bars represent mean ± SD of three independent experiments; all the data were tested using Student's *t*-test.

in the present study, we further confirmed that neonatal (CB)- and adult (PB)-derived $CD34^{+}$ cells show different biological response to TPO. MKs derived from CB-$CD34^{+}$ cells were smaller in size and show lower expression of megakaryocytic markers as compared to PB-$CD34^{+}$ cells-derived MKs (Fig. 1B,C). The observations from several other research groups also support the existence of developmental differences between neonatal and adult megakaryocytopoiesis in regard to MK progenitor proliferation and their maturation to terminally differentiated MK [4,34-36]. And, it is widely accepted that differentially regulated mechanisms allow newborns to maintain normal platelet counts but contribute to their predisposition for low platelets and disorders of neonatal megakaryocytopoiesis.

Previous reports suggest the functional role of both the extrinsic and cell-intrinsic factors in the regulation

of these developmental differences. However, the molecular mechanisms governing these differences are not fully understood. In this research endeavor, the gene expression profiling of MKs derived from CB- and PB-$CD34^+$ cells identified differentially regulated genes encoding components of different signaling pathways (unpublished data). Interestingly, in functional analysis, the most affected pathway analyzed from the differential gene expression profile in CB- vs PB-MKs was the TGF-β pathway. Several genes encoding TGF-β pathway components were down-regulated in CB- as compared to PB-MKs (Fig. 1D,E). Previously, TPO has been reported as a pivotal regulator of TGF-β release and consequent activation of the downstream pathways in MKs, both *in vitro* and *in vivo* [19]. Specifically, the present study defines that TGF-β signaling pathway components are differentially regulated in neonatal versus adult MKs, suggesting its crucial role in MK development. However, a further experimental effort was required to elucidate the functional role of the TGF-β signaling pathway in megakaryocytic development.

Subsequently, with that perspective, we explored the TGF-β signaling pathway in megakaryocytic maturation using K562 cells as an *in vitro* model of megakaryocytopoiesis. K562 cells have been proven to be a useful tool for molecular studies of megakaryocytic as well as erythroid differentiation [8,37]. A number of previous observations suggest that K562 cells exhibit clear erythroid features comparable to immature erythroblasts [38,39]. In our study, Hemin exhibited a significant increase in erythroid features (as indicated by benzidine staining for hemoglobin and surface marker analysis) (Fig. 2C,D), which is in accordance with previous findings [37], whereas PMA diminished the erythroid phenotype (Fig. 2C) and significantly established the megakaryocytic features – increased cell size and adherence with reduced cell proliferation (Fig. 2A,B) and increase in the expression of megakaryocytic markers (Fig. 3A,B). In this study, we have also observed differential expression of RUNX1, reduced (mRNA and protein) during Hemin-induced erythroid differentiation and increased during PMA-induced megakaryocytic differentiation (Fig. 3B,C). These observations are in accordance with findings reported by Kuvardina *et al.* [8]; RUNX1 inhibits erythroid gene expression program during megakaryocytic differentiation of primary human $CD34^+$ progenitor cells and works as a central player during lineage decision at the MEP branching point. These results clearly indicate that Hemin induces erythroid differentiation, whereas PMA diminished the erythroid phenotype and allowed K562 cell differentiation into megakaryocytic lineage (Fig. 3D). The PMA-stimulated K562 cells seem to provide a classic model for the molecular studies of megakaryocytic differentiation and maturation because in the present study, megakaryocytic markers and TFs are increased in the differentiated cells, and the results are consistent with the previous observations. Further, our results confirm that the PMA-stimulated K562 cells lose erythroid features such as the expression of GYPA and GYPB in parallel with the increased expression of the megakaryocytic TF RUNX1.

We used this model of megakaryocytic differentiation in order to better define the involvement of the TGF-β pathway in MK development. We report that PMA-induced megakaryocytic differentiation of K562 cells is associated with statistically significant up-regulation of TGF-β and TGF-βR mRNA expression (Fig. 4A). In this study, the increased expression of TGF-β shown in PMA-induced megakaryocytic differentiation further confirms previous observations on TGF-β expression in PMA-stimulated K562 cells [26].

In an effort to better define TGF-β signaling in MK development, the current study examined differential expression of genes encoding components of the TGF-β signaling pathway as well as TGF-β effectors during PMA-induced megakaryocytic differentiation of K562. Using pathway-focused gene expression profiling (Fig. 4B,C) and targeted protein analysis, the observations from this study revealed transcriptional activation of several established TGF-β pathway responsive genes that coincided with the up-regulation of several key components of the classical TGF-β signaling pathway (Table 1 and Fig. 5A,B). Several genes encoding TGF-β superfamily ligands such as TGF-β, BMPs, and activins were up-regulated along with the TGF-β superfamily receptors, TGF-βR1, TGF-βR2, TGF-βR3, BMPR2, ACVR1, and ACVRL1 (Table 1 and Fig. 5A,B). These TGF-β superfamily ligands have been implicated in a wide array of functions including development and differentiation [16,17]. Specifically, the higher concentration of TGF-β1 has been implicated in the maintenance of the quiescent state of HSCs; however, low concentration of TGF-β1 promotes proliferation and myeloid differentiation specifically in My-HSCs [20,40]. Moreover, MKs are the main source of TGF-β in bone marrow; also, it has been shown that TPO induces TGF-β release and the activation of downstream pathways in MKs [19]. As identified, the TGF-β superfamily ligands produced during megakaryocytic differentiation may be crucial in megakaryocytic maturation through receptors-activated SMADs (R-SMADs).

Evidence of the functional TGF-β signaling during megakaryocytic maturation supported by the present

study findings showed elevated expression levels of several downstream TGF-β pathway effectors-regulated genes encoding components of ECM and platelet alpha granules, such as PDGF, SERPINE1, THBS1, CPL1A1, COL1A2, LTBP1, LTBP2, and TGFBI (Fig. 5A). Platelet-derived growth factor (PDGF), stored in alpha granules of platelets (Fig. 5B) and released from the activated platelets during the clot formation, was elevated greater than 22-fold (Table 1). The PDGF deficiency in mice resulted in hematopoietic defects, such as microvessel leakage, hemorrhage, and thrombocytopenia [41]. Apart from its crucial role in thrombosis and hemostasis, PDGF is also known for its function in the megakaryocytic lineage determination [42,43]. Plasminogen activator inhibitor-1 [PAI-1; serine protease inhibitor clade E member 1 (SERPINE1)], stored in alpha granules of platelets (Fig. 5B), was about 10-fold up-regulated (Table 1). PAI-1 is involved in normal blood clotting by inhibiting the action of other proteins called plasminogen activators, which are involved in the activation of matrix metalloproteinases, cleavage of fibrin, and degradation of ECM components by activating plasminogen [44,45]. The THBS1 (thrombospondin-1) was observed with a 12.67-fold increase (Table 1) and is an adhesive glycoprotein, which associates with the ECM (Fig. 5B) and mediates cell-to-cell and cell-to-matrix interactions. Thrombospondin-1 is stored in platelet alpha granules (Fig. 5B) and released during platelet activation, and it is known to be involved in platelet aggregate stabilization (reviewed in Ref. [46]). Additionally, expression of the ECM components, such as type I fibrillar collagens (Fig. 5B), was elevated significantly (COL1A1, 15.77-fold; COL1A2, 6.32-fold; Table 1). Moreover, the LTBPs (latent TGF-β binding proteins), important components of the ECM (Fig. 5B), were also resulted in a statistically significant increase (LTBP1, 5.76-fold; LTBP2, 4.02-fold; Table 1). LTBPs play a crucial role in targeting the latent TGF-β to the ECM, but it has also been shown that LTBP-1 participates in TGFβ activation by integrins [47,48]. TGFBI (TGF-β-induced, 68 kDa), a secreted ECM protein (Fig. 5B), was identified with a critical role in platelet activation and function [49]. Recently, it has also been reported that TGFBI gene overexpression enhanced megakaryocytic differentiation [50]. Herein, the TGFBI gene expression resulted in a 3.97-fold increase (Table 1). Together, these results provide confirmation that the classical TGF-β pathway is intact in megakaryocytic differentiation and may be involved in the regulation of genes crucial for the MK maturation and platelet activation, such as components of platelet alpha granules and ECM. Present data with the previous observations show that the TGF-β pathway plays a functional role in MK maturation.

Several transcriptional regulators also displayed increased mRNA expression in megakaryocytic differentiation of K562 cells (Fig. 6A–D). Transcription factors such as SMAD2/3, RUNX1, and AP-1 transcripts such as JUN, JUNB, and FOS were significantly up-regulated (> 2.0-fold). The SMADs are well known to regulate the expression of RUNX1 in hematopoietic cells [51]. The expression of some of the AP-1 TFs is also regulated by TGF-β1-activated SMADs [52]; previously, the high AP-1 expression was correlated with greater DNA synthesis and high MK ploidy in human $CD34^+$ cells-derived MKs [27]. The AP-1 TFs are the main cooperating partners of RUNX1 in early megakaryocytopoiesis to drive the expression of genes involved in early megakaryocytic differentiation by recruiting RUNX1 to *de novo* binding regions of chromatin lacking RUNX1 binding motif [13,14]. SMADs are also well known to activate AP-1 sites through direct physical interaction with DNA-bound AP-1 proteins [53-55]. Herein, we observed increased cell adherence and cytoplasmic maturation of cells (Fig. 6F,G) upon PMA stimulation, and this may be correlated with the increased expression of SMAD2/3, RUNX1, and AP-1 TFs (Fig. 6A–D) and their interactions (Fig. 6E). Further, MYC and ATF4 transcript levels were observed with significant down-regulation, and TGF-β is well known for transcriptional repression of c-myc through SMAD3 [56-58]. Previously, the effects of c-Myc-knockout mice reported in lineage selection: Differentiation of erythrocyte was blocked at the stage of erythroid progenitor, whereas megakaryocytopoiesis was significantly increased [59]. Consistently, STRING analysis detected different interconnected gene clusters, depicting individual interactions with TGF-β pathway core components (e.g., SMAD2, SMAD3, TGFBR1, TGFBR2, TGFB1, and TGFB3; Fig. 6E). The interaction network of the core TGF-β signaling pathway components and the up-regulation of the TGF-β-regulated genes (Table 1) and their individual interactions with each other (Fig. 6E) show the functional significance of the TGF-β pathway in MK development. Overall, this study of TGF-β pathway components with the reference of previous literature shows that TGF-β pathway might have regulatory role in megakaryocytic maturation. However, further experiments are needed to validate TGF-β pathway effects on MK maturation.

To study the effects of TGF-β1 on MK maturation, we cultured PMA-induced megakaryocytic cells in the presence of recombinant human TGF-β1. The results

of this study clearly define the augmenting effects of TGF-β1 on the accumulation of pSMAD2/3 (Fig. 7D), cytoplasmic maturation of PMA-induced megakaryocytic cells (Figs 7A and 8A,C), and ECM component deposition (Fig. 8B); however, we did not observe any striking change in DNA content (Fig. 8D). In the present study, the augmenting effect of TGF-β1 noticed on cell adherence, membrane expansion, and cytoplasmic maturation (Figs 7A and 8A,C) is likely the effect of TGF-β downstream pathways (Fig. 7B,D) on integrin's (CD41/61) expression and ECM production (Fig. 8A–C). Integrin's and ECM components play an important role in different autocrine loops working as regulators of MK development, proplatelet formation, and platelet function [19,49,60-62].

Transforming growth factor beta is a multifunctional growth factor and is involved in the development and differentiation [16,17], the regulation of cytoplasmic $Ca^{2+}$ influx [18], the synthesis and release of ECM, cell adhesion and migration [19], proplatelet formation and release [60,62], and more. And, downstream to the TGF-β pathway, $Ca^{2+}$ plays an important role in the activation of different signaling pathways [63], stress fibers formation and cell adhesion [64,65], and microtubule assembly and proplatelet formation [60,62]. In the present study, we disclose the augmenting effect of TGF-β1 on TRP ion channel (TRPA1, TRPV1, and TRPC3) expression (Fig. 9A) and the cellular $Ca^{2+}$ levels (Fig. 9B,C) in differentiating megakaryocytic cells. Previously, TRPA1 has been reported for its ability to suppress erythroid differentiation but enhances megakaryocytic differentiation in K562 and HEL cells [66]. Most TRP ion channels including TRPA1 are permeable to $Ca^{2+}$ [67]. Activation of TRP ion channels serves as an important $Ca^{2+}$ entry pathway which further contributes to the fluctuations in $[Ca^{2+}]i$ and subsequent $Ca^{2+}$ signaling [68,69].

Transient receptor potential ion channels are activated by various intracellular signals such as the depletion of the endoplasmic reticulum (ER) $Ca^{2+}$ stores, increased cellular ROS levels, and many more. TRPC functions as store-operated $Ca^{2+}$ (SOC) channels, and these channels are responsible for $Ca^{2+}$ influx activated upon depletion of ER $Ca^{2+}$ stores [70]. However, cellular ROS has also been shown to activate TRPA1 and TRPV1 ion channels which regulate $[Ca^{2+}]i$ [71-73]. Previously, it has been reported that TGF-β augments the cellular calcium following store depletion in MKs [18]. However, the novel contribution of the present work is to demonstrate that TGF-β1-induced ROS regulate cellular $Ca^{2+}$ in MKs, possibly by the activation of ROS-sensitive TRP ion channels. TGF-β1 generates ROS in various types of cells. In the present study, we found a consistent and dose-dependent increase in ROS upon TGF-β1 treatment of megakaryocytic cells (Fig. 10A). It has also been demonstrated that ROS is necessary for megakaryocytic differentiation [21-23]. Mitochondria are the main source of ROS in most cell types, and ROS production in mitochondria is mainly regulated by UCP2. Interestingly, in the present study, UCP2 was observed with down-regulation upon TGF-β1 treatment of PMA-induced megakaryocytic cells (Fig. 10B,C). Our findings are consistent with the previous report, suggesting an underlying mechanism of ROS production upon transcriptional repression of UCP2 by TGF-β-induced SMADs (Fig. 12C) [28]. In the same line of experiments, we have also noticed the decreased $\Delta\Psi m$ upon TGF-β1 treatment of PMA-induced megakaryocytic cells and that could be because of increased mitochondrial ROS production (Fig. 10E). Apart from the mitochondria, cellular oxidases, such as NOX4, are also important sources of ROS. Also, it has been shown that TGF-β up-regulates NOX4 expression in a variety of cells by the SMAD2/3 pathway (reviewed in Ref. [74]). Herein, we report the positive effects of TGF-β1 on NOX4 expression (Fig. 10D). Thus, NOX4 NADPH oxidase may be an important downstream effector in mediating TGF-β-induced ROS production. Subsequently, the functional significance of ROS in cellular $Ca^{2+}$ regulation was confirmed in an experiment using ROS quencher, NAC (Fig. 10F–H). From the results of the present study, it seems that TGF-β1-induced ROS regulate cellular $Ca^{2+}$ inflow in MKs, possibly by the activation of ROS-sensitive TRP ion channels (Fig. 12D).

Moreover, the regulation of TRP ion channel expression during megakaryocytic maturation needed further validation. RUNX1 is the major gene expression regulator in maturing MKs [13,14]. Herein, siRUNX1 transfection study verified that RUNX1 is involved in the regulation of TRP ion channel expression and that, in turn, affects cellular $Ca^{2+}$ inflow (Fig. 11A,B). Our siRUNX1 transfection study results are consistent with previous findings shown that RUNX1 regulates the expression of many ion channels and receptors including TRP ion channels [32,33]. In the present study, increased cellular calcium observed during megakaryocytic maturation shows the effect of TGF-β1 on extracellular $Ca^{2+}$ inflow possibly by ROS-mediated activation of TRP ion channels; on the other hand, RUNX1 regulates the $Ca^{2+}$ inflow possibly by regulating the expression of TRP ion channels during MK maturation. These results show that RUNX1/TGF-β signaling loop through TRP ion channels

augments the cellular calcium during megakaryocytic development. Notably, in MKs $Ca^{2+}$ plays an important role in the regulation of cytoskeletal reorganization responsible for cellular adhesion and migration and final proplatelet formation on ECM components [61]. Hence, present observations in the PMA-induced megakaryocytic cell line model defined that RUNX1 and TGF-β/SMAD pathway not only involved in the regulation of target gene expression but also augment cellular $Ca^{2+}$ inflow during MK maturation. However, in our initial findings, the TGF-β pathway was majorly affected and RUNX1 was down-regulated in neonatal MKs. Therefore, based on the findings, TGF-β pathway and RUNX1 cross talk could be the regulatory mechanism of the developmental differences between neonatal and adult MKs.

Furthermore, to confirm the functional role of TGF-β1 on MK maturation, we have investigated the effect of TGF-β on TPO-induced megakaryocytic development from neonatal CB-derived $CD34^+$ cells. In the present study, we observed that TPO/TGF-β1 costimulation significantly enhanced the expression of some of the core TGF-β pathway components. More importantly, the TF RUNX1, which is crucial for MK maturation, was increased upon costimulation as compared to control or TPO alone (Fig. 12A,B). Another essential point is that the expression of MK marker CD41 was also enhanced significantly (Fig. 12A,B) by TGF-β1. In brief, effects of TGF-β1 observed on RUNX1 and CD41 expression in CB-derived MKs are consistent with our results in PMA-induced megakaryocytic cell line model upon TGF-β1 treatment. Hence, consistent with previous reports [5,19], it is clear from present findings that RUNX1 and TGF-β signaling plays an important role in terminal MK maturation; thus, they could be potential targets in neonatal thrombocytopenia.

## Conclusion

In the present study, TGF-β pathway components are differentially regulated in neonatal and adult MKs, observed with significantly low expression levels in CB $CD34^+$ cells-derived MKs as compared to adult $CD34^+$ cells-derived MKs. Further, to better define TGF-β pathway in megakaryocytic maturation we used the megakaryocytic differentiating cell line model. In this report, we show that PMA-induced megakaryocytic differentiation of human BFU-E/MK (K562) is associated with enhanced expression of megakaryocytic markers (CD41 and CD61), TF RUNX1, and TGF-β. Furthermore, to better define the TGF-β pathway and the expression of the components regulated by TGF-β downstream effectors during megakaryocytic differentiation, we performed TGF-β/BMP array; interestingly, most of the TGF-β core pathway components (TGF-β, TGF-βRs, and SMADs) and pathway-regulated components (TFs involved in megakaryocytopoiesis, components of platelet alpha granules, and ECM) were up-regulated in differentiated megakaryocytic cells. These results demonstrate that the TGF-β pathway is intact in megakaryocytic differentiation and likely involved in the regulation of different target genes, which are crucial for MK maturation and platelet function. Further, we report the functional role of recombinant human TGF-β1 in the regulation of cellular ROS and $Ca^{2+}$ with the augmenting effect on megakaryocytic marker CD41 and TF RUNX1 expression. Further, siRUNX1 affected TRP ion channel expression in megakaryocytic cells. In conclusion, these results show that the RUNX1/TGF-β signaling loop through TRP ion channels augments the cellular calcium during megakaryocytic development (Fig. 12D). Notably, in MK $Ca^{2+}$ plays an important role in the regulation of cytoskeletal reorganization responsible for cellular adhesion and migration and final proplatelet formation on ECM components [61]. Hence, present observations define that RUNX1 and TGF-β/SMAD pathway not only involved in the regulation of target gene expression but also augment cellular $Ca^{2+}$ inflow during MK maturation. In addition to the results presented in the PMA-induced megakaryocytic cell line model, TGF-β1 was also observed with augmenting effects on TPO-induced MK development from neonatal CB-derived $CD34^+$ cells. In conclusion, our present findings are in line with previous reports indicating developmental differences between neonatal and adult MKs. This study clearly reveals the developmental difference in the expression of RUNX1 as well as different TGF-β signaling pathway components. Further, our results associate the megakaryocytic maturation and platelet biogenesis with RUNX1 and TGF-β signaling and conclude that the down-regulation of RUNX1 and TGF-β signaling pathway components may contribute to the developmentally different and disease-susceptible phenotype of neonatal MKs. Perhaps, the down-regulation of TGF-β pathway and RUNX1 observed during neonatal MK development could be a mechanism to allow developmental uncoupling of proliferation and terminal differentiation of MKs in the fast-growing neonatal bone marrow but also set the stage for disorders restricted to neonatal MK progenitors and inability to increase MK mass, in response to increased platelet demand.

# Methods

## Cell cultures

Human umbilical CB- and mobilized PB-$CD34^+$ hematopoietic stem/progenitor cells (HS/PCs) were obtained from Lonza India Pvt. Ltd. To avoid interference of serum, purified HS/PCs ($4 \times 10^4$ $mL^{-1}$) were cultured in humidified $CO_2$ incubator with 50 $ng{\cdot}mL^{-1}$ concentration of TPO (PeproTech, Inc., Rocky Hill, NJ, USA) in serum-free MK culture medium: Iscove's modified Dulbecco's medium (IMDM) supplemented with 1% of BSA; insulin, 10 $\mu g{\cdot}mL^{-1}$; transferrin, 740 $\mu g{\cdot}mL^{-1}$; L-glutamin, 2.0 mM; and LDL, 40 $\mu g{\cdot}mL^{-1}$. HS/PCs were cultured following the MK differentiation culture method of Pastos *et al.* [34]. Cells were collected at different time points (0, 7, 14 days) and analyzed for MK-specific marker (CD41 and CD61) expression study.

PMA-induced K562 provides a model to study the process of MK maturation. These cells were grown in RPMI-1640-GlutaMAX™ Supplement media (Gibco; Thermo Fisher Scientific) with 10% FBS (Gibco; Thermo Fisher Scientific) and 1% antibiotic–antimycotic solution (Sigma-Aldrich, Inc., St. Louis, MO, USA), and maintained in humidified $CO_2$ incubator. To induce megakaryocytic differentiation of K562, 50 nM PMA (Sigma) was used, whereas erythroid differentiation of K562 cells was induced by Hemin (50 $\mu g{\cdot}mL^{-1}$). After 72 h of incubation, cells were counted via hemocytometer using trypan blue stain (Mediatech Inc., Manassas, VA, USA) and used for the analysis of PMA-induced megakaryocytic and Hemin-induced erythroid features and their comparative study.

As indicated, TGF-β1 (10 and 20 $ng{\cdot}mL^{-1}$) was added to the medium after 12 h of PMA treatment; after additional 60 h of incubation, cells were collected and used to study effects of TGF-β1 on PMA-induced megakaryocytic differentiation. The cellular pSMAD2/3 levels were detected upon TGF-β1 exposure for up to 60 min.

## Benzidine staining

The number of benzidine-positive cells was quantitated by counting triplicates of at least 300 cells under light microscope; harvesting, benzidine staining, and analysis work were performed as described in Ref. [75].

## Quantitative RT-PCR

Total RNA was isolated from cultured cells (at different time points) using the miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Prior to start of reverse transcription reaction, RNA samples were treated with RNase-free DNase I (Qiagen). RNA input (1 μg) was reverse-transcribed by using EasyScript™ cDNA Synthesis Kit (Applied Biological Materials, Inc., Richmond, BC, Canada) using oligo(dT)20 primers. The first-strand cDNA obtained by reverse transcription was amplified using specific primers in quantitative real-time PCR (qRT-PCR; Applied Biosystems; Thermo Fisher Scientific) with KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) according to the manufacturer's protocol. Cycling conditions were as follows: an initial denaturation at 52 °C for 2 min and 95 °C for 8 min, followed by 42 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 30 s.

The specificity of qRT-PCR products was tested by melting curve examination and further verified on agarose gel. The results presented in this study are from three individual experiments ($n = 3$), in which we test each sample in triplicate. The $C_t$ values of the gene of interest were normalized to the $C_t$ values obtained from the amplification of the housekeeping gene, GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), and the relative quantification was done by comparative $\Delta\Delta C_t$ method ($2^{(-\Delta\Delta C_t)}$). Specific primers used for qRT-PCR are listed in Table 4.

Quantitative RT-PCR analysis of TGF-β-related genes was performed using human TGF-β/BMP signaling pathway (PAHS-035D; Qiagen) RT2 Profiler PCR Array and run on ABI StepOnePlus (Applied Biosystems). Experiments were carried out in biological triplicate ($n = 3$), and data analysis was done using the manufacturer's integrated web-based software package with cycle threshold ($C_t$)-based fold-change calculations. Samples were assigned to controls and treatment groups (group 1). $C_t$ values were normalized based on a manual selection of reference genes (ACTB, GAPDH, HPRT1, and RPLP0). For the RT2 Profiler PCR Arrays data, the *P*-values are calculated based on a Student's *t*-test of the replicate $2^{-\Delta C_t}$ values for each gene in the control group and treatment groups.

## Flow cytometry analysis

Viable cells were counted via hemocytometer using the trypan blue (Mediatech Inc., USA) exclusion test. After counting, the viable cells ($1 \times 10^6$) were fixed with 1% paraformaldehyde (PFA) and labeled with Mouse Anti-Human CD61 PE (BD Pharmingen™ Inc., San Diego, CA, USA). The labeled cells were analyzed by flow cytometry for three independent samples; the same staining protocol was used to visualize anti-CD61 PE and collagen under fluorescence microscope.

## Western blots

For western blotting, the cells were harvested, washed twice with ice-cold PBS, and lysed in ice-cold RIPA buffer (Sigma) containing protease inhibitor cocktail (Sigma-Aldrich) and phosphatase arrest (G-Biosciences; Geno Technology Inc., Saint Louis, MO, USA) for 30 min with constant shaking at 4 °C. The samples were then centrifuged at 12 000 ***g*** for 15 min at 4 °C to remove insoluble material. The protein content in each sample was estimated

**Table 4.** Primers used in the current study.

| QRT-PCR primers | Primer sequence (5′–3′) | |
|---|---|---|
| CD41 | F: GCATGGTTCAACGTGTCCTC | R: TTGAAGAAGCCGACCTTCCA |
| CD61 | F: ACCAGTAACCTGCGGATTGG | R: TCCGTGACACACTCTGCTTC |
| CD42b | F: TTCCCCACCAAAGCCCATAC | R: GGCTTGGGGTTGGTTCAGTA |
| RUNX1 | F: AACCTCGAAGACATCGGCAG | R: GGCTGAGGGTTAAAGGCAGT |
| TRPA1 | F: ACAGAAGACAAGTCCTGCCG | R: GAGGGCTGTAAGCGGTTCAT |
| TRPV1 | F: ACGGACAGAACACCACCATC | R: GGCCCTTGTAGTAGCTGTCC |
| TRPC3 | F: CAGCCAACACGTTATCAGCAG | R: TGCTTGGCTCTTGTCTTCCA |
| TGF-β1 | F: GCAACAATTCCTGGCGATAC | R: TAGTGAACCCGTTGATGTCC |
| TGF-βR1 | F: GTGACAGATGGGCTCTGCTT | R: GAGGGTGCACATACAAACGG |
| TGF-βR2 | F: AGACGTTGACTGAGTGCTGG | R: TTAGGGAGCCGTCTTCAGGA |
| TGF-β2 | F: TTGCCCTCCTACAGACTTGA | R: GTGGACGTAGGCAGCAATTA |
| SMAD1 | F: CAACAATCGTGTGGGTGAAG | R: TCCGGTTAACATTGGAGAGC |
| SMAD2 | F: GTCCATCTTGCCATTCACGC | R: CTGCCCATTCTGCTCTCCTC |
| SMAD3 | F: TAACTTGGACCTGCAGCCAG | R: CCTCTTCCGATGTGTCTCCG |
| SMAD4 | F: GATACGTGGACCCTTCTGGA | R: ACCTTTGCCTATGTGCAACC |
| GYP A | F: GGGGTGATGGCTGGTGTTAT | R: AAAGGCACGTCTGTGTCAGG |
| GYP B | F: GGCAATGCACACTTCAACCT | R: AGGAGCTGGTACAGTGAAACG |
| UCP2 | F: AAAGCACCGTCAATGCCTAC | R: CATAGGTCACCAGCTCAGCA |
| NOX4 | F: CAGAAGGTTCCAAGCAGGAG | R: GTTGAGGGCATTCACCAGAT |

and adjusted. Proteins (50 μg) were separated on 6–12% SDS/PAGE and transferred to nitrocellulose (NC) membranes. Membranes were blocked with 5% BSA and incubated overnight with specific primary antibodies (RUNX1, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; pSMAD2/3, G-Biosciences; UCP2, G-Biosciences; β-actin, Sigma; collagen type I, G-Biosciences], followed by further incubation for 1 h with the appropriate peroxidase-conjugated secondary antibodies. Protein bands on NC membranes were visualized by chemiluminescent solution, Pierce™ ECL Substrate (Thermo Fisher Scientific), and ChemiDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. Relative protein levels were quantified by densitometry of western blot bands using the IMAGEJ system and normalized with β-actin.

## DNA index analysis

The cells were harvested and stained ($1 \times 10^6$) with reagents of Muse Cell Cycle Assay Kit (Merck Millipore, USA) according to the manufacturer's protocol. DNA content index was analyzed using a flow cytometer (Muse® Cell Analyzer; Merck Millipore, Germany). The experiments were performed in triplicate and contained a minimum of 5000 events per run.

## Analysis of calcium levels

Fluo-3 fluorescence was utilized to determine $[Ca^{2+}]i$ levels [76]. The cells were spun at 1000 r.p.m. for 3 min and resuspended at a density of $1 \times 10^6$ $mL^{-1}$ in fresh RPMI-1640. Loading of Fluo-3/AM Ester (3 μM; Biotium, Inc., Fremont, CA, USA; Catalog # 50016) was allowed to proceed for 60 min with occasional gentle agitation of the cell suspension at 37 °C in a humidified incubator with 5% $CO_2$. After 60-min incubation, cells were washed three times in fresh serum-free RPMI-1640 to remove all traces of extracellular Fluo-3/AM and finally resuspended in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline (DPBS; Gibco; Thermo Fisher Scientific). The emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence spectrometer at 25 °C (excitation 505 nm, emission 526 nm, slit width 5 nm). The fluorescence intensity at 526 nm was used to determine the relative $[Ca^{2+}]i$ concentration. The experiments were performed in triplicate.

Further, Fluo-3/AM (3 μM)-loaded cells were also imaged for monitoring the fluorescence intensity using a fluoresce microscope. Fluorescence images of cells with Fluo-3 were collected using 505 nm excitation wavelength. The excitation wavelength and emission detection parameters were digitally controlled to keep the same settings throughout the experiments. Images were analyzed using IMAGEJ software; for quantitative analysis of Fluo-3 fluorescence, images were invariably quantified as the mean gray value in a visual filed selected. The histogram of mean fluorescence intensity of 500 cells is displayed.

## Determination of intracellular ROS generation

H2DCFDA (Sigma) is a cell-permeable standard probe to detect ROS, commonly used to monitor the intracellular ROS levels. Intracellular ROS was measured in cells ($1 \times 10^6$) from different experimental conditions. Briefly,

cells were washed with PBS and incubated with H2DCFDA dye (10 μM) in PBS for 30 min in dark at room temperature. Further, cells were washed twice with PBS and transferred to microplate and detected using fluorescence spectroscopy at 485 nm excitation and 535 nm emission wavelengths. The experiments were performed in triplicate.

### Analysis of the mitochondrial membrane potential

The cells were collected and washed twice with PBS, and according to the manufacturer's protocol, $1 \times 10^5$ cells from different experimental conditions were prepared following the Muse MitoPotential Assay Kit (Merck Millipore) protocol. The depolarized/live cell percentage was determined by the flow cytometer (Muse™ Cell Analyzer; Merck Millipore). All the experiments were repeated at least three times.

### siRUNX1 transfection

The PMA-induced K562 cells were transfected with human RUNX1 siRNA, ON-TARGETplus siRNA reagent (GE Healthcare Dharmacon, Inc., Lafayette, CO, USA), and negative controls (Cy3-labeled scramble sequence) using the HiPerFect Transfection Regent (Qiagen), following the manufacturer's instructions. After 48 h of transfection, the cells were detached using 0.25% trypsin in 0.02% EDTA and counted using trypan blue assay and used for later experiments.

### Statistical analysis

Data are provided as means ± SD of three independent replicates; *n* represents the number of experiments. Statistical significance for all the data was tested using Student's *t*-test. Results with $*P < 0.05$ or $**P < 0.02$ were considered statistically significant.

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## Conflicts of interest

The authors declare no conflict of interest.

## Author contributions

SR designed and performed experiments, analyzed data, and prepared the manuscript. SD, DSS, NK, and IS contributed reagents and contributed to review and editing. RG designed and supervised the study.

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LETTER TO THE EDITOR

WILEY Cell Proliferation

# MicroRNA-9 promotes cell proliferation by regulating RUNX1 expression in human megakaryocyte development

To the editor:

We read with interest the article by Tian et al,[1] describing the role of MicroRNA-9 (miR-9) in the regulation of myeloid-derived suppressor cell (MDSC) differentiation and function by targeting the runt-related transcription factor 1 (Runx1). This finding has revealed post-transcriptional regulation of Runx1 by miR-9. RUNX1, the master regulator of hematopoiesis,[2] is known to regulate megakaryocyte (MK) polyploidization and cytoskeleton rearrangement in the process of MK maturation and pro-platelet formation.[3,4] Specifically, RUNX1 sequence is conserved for miR-9 among vertebrates and miR-9 was also identified with decreasing intensity of expression during human MK ontogeny,[5] which shows the functional significance of miR-9/RUNX1 axis during evolution. Thus, miR-9 could be a potential therapeutic target in neonatal thrombocytopenia and other platelet disorders.

Thrombocytopenia, the deficiency of platelets in the blood, is a major clinical problem encountered among several conditions and is common in all sick and preterm neonates admitted to NICU; the primary outcome of thrombocytopenia in infants is the incidence and severity of intra-ventricular haemorrhages (IVH), which is a leading cause of poor neurological outcome and mortality in sick neonates.[6,7] It is believed that developmental differences between neonatal and adult MKs contribute to this vulnerability.[8] Specifically, neonatal MK progenitors possess a high proliferative potential and give rise to MKs smaller and of low ploidy that generate fewer platelets compared with adult MKs. The regulatory mechanisms underlying these developmental differences are unknown, but we have revealed a critical role of small non-coding RNAs (miRNAs) in the regulation of MK development.[9] Further, RUNX1 emerged as a putative target of miR-9 by several bioinformatic databases such as TargetScan, miRbase and RNAhybrid. In our miRNA array study of neonatal vs adult MKs, four other RUNX1 targeting miRNAs were identified with higher levels of expression in neonatal MKs, although miR-9 was the highest up-regulated miRNA (Table 1, $P < .05$). Hsa-miR-9 and RUNX1 mRNA duplex showed minimum free energy of −17.6 kcal/mole using bioinformatic tool RNAhybrid (Figure 1A).

To test the role of miR-9 in RUNX1 regulation in megakaryocytes, we cultured human CB- and adult PB-CD34$^+$ cells (n = 3 for each group) in the presence of serum free medium with TPO (50 ng/mL), as previously described[10]; after 14 days of culture, >90% of cells were MKs (CD41$^+$). The expression levels of hsa-miR-9 and RUNX1 mRNA were measured by qRT-PCR (n = 3) and were normalized against internal control U6 and β-actin, respectively.[10,11] Initially, we confirmed the hsa-miR-9 expression levels and found that CB levels were significantly higher compared with PB-derived MKs, and these differences were consistent through all the stages (0, 7, 11, 14 days) of megakaryocytopoiesis (n = 3, $P = .02$; Figure 1B), whereas its target RUNX1 mRNA was observed with significant lower expression in CB-derived MKs compared with PB-derived MKs (n = 3, $P < .05$; Figure 1C). RUNX1 protein levels were determined by western blot using anti-RUNX1 (Santa Cruz, CA, USA) and anti-β-actin (Santa Cruz) antibodies. RUNX1 protein levels were quantified by densitometry using the ImageJ system and normalized with β-actin. We further confirmed the developmental differences between CB-derived MKs and PB-derived MKs in RUNX1 protein expression (n = 3, $P = .02$; Figure 1D). To prove the functional significance of hsa-miR-9 in RUNX1 regulation, we transfected MEG01 (human megakaryoblastic leukaemia cell line) and Dami (human megakaryocytic leukaemia cell line) cells with either pre-miR-9 or scrambled control (Cy3 labelled) using lipofectamine Hi-perfect (Qiagen, Hilden, Germany). MEG01 and Dami cells, respectively, were maintained in DMEM and RPMI with 10% FBS. After 72 hours of transfection, we observed a significant increase in the miR-9 levels (n = 3, $P < .05$; Figure 1E), along with high cell proliferation rate (n = 3, $P < .05$; Figure 1F) and reduced MK marker, CD61 expression (n = 3, $P = .02$; Figure 1G). Further, lower RUNX1 protein levels (n = 3, $P < .001$; Figure 1H) were noticed using western blot analysis.

Over the past many years, multiple reports have identified the essential role of miRNAs in the regulation of lineage-selective TFs.[10] In vertebrates, recent studies evidenced the development stage and cellular context dependent role of miR-9 on cell proliferation, migration and differentiation.[12] In this study, we observed developmental differences in the expression levels of hsa-miR-9 in neonatal vs adult MKs (Figure 1B), and similar developmental differences were also observed in embryonic stem cell MKs vs adult MKs.[5] Hypothetically, down-regulated miRNAs during MK development and differentiation

**TABLE 1** RUNX1 targeting miRNAs expression in CB- and PB-derived MKs. miR-9 exhibited the largest developmental difference, being expressed at levels 20-fold higher in neonatal compared with adult MKs

| miRNA ID | *P*-value | CB/PB expression | Putative target |
|---|---|---|---|
| hsa-miR-370 | .05 | 2.03 | RUNX1 |
| hsa-miR-192 | .04 | 2.91 | RUNX1 |
| hsa-miR-129-5p | .05 | 3.89 | RUNX1 |
| hsa-miR-215 | .05 | 5.91 | RUNX1 |
| hsa-miR-9 | .03 | 21.4 | RUNX1 |


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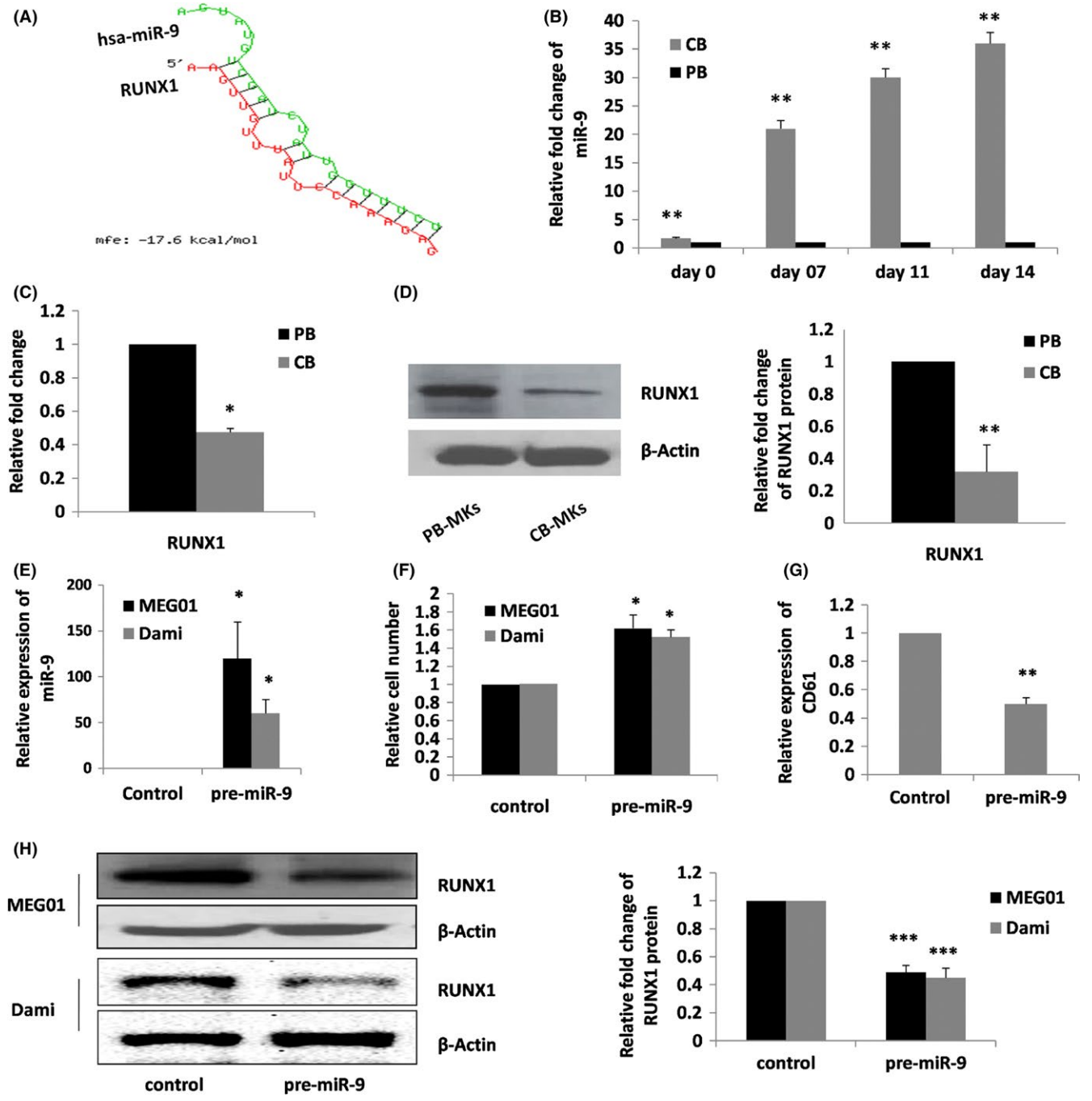


**FIGURE 1** hsa-miR-9 and RUNX1 expression is inversely related in neonatal vs adult MKs. (A) Minimum free energy (mfe) duplex of the hsa-miR-9 and RUNX1 analysed by RNAhybrid. (B) hsa-miR-9 levels were measured in human cord blood (CB)- and adult peripheral blood (PB)-$CD34^+$ progenitor-derived MKs. miR-9 values were normalized against U6 (internal control), and miR-9 levels in CB at different stages (0 day, 7 days, 11 days, 14 days) of MK differentiation were expressed as relative to miR-9 levels in PB cells at the same stage of MK differentiation. hsa-miR-9 levels were significantly higher in CB- compared with PB-derived mature MKs (n = 3, $^{**}P = .02$). (C) RUNX1 mRNA levels were measured in human CB- and adult PB-$CD34^+$ progenitor-derived MKs. RUNX1 mRNA levels in MKs derived from CB expressed as relative to RUNX1 levels in PB-derived MKs at day 14 (n = 3, $^{*}P < .05$). (D) RUNX1 protein levels expressed as a ratio to β-actin were significantly lower in CB- compared with adult PB-derived MKs (n = 3, $^{**}P = .02$). (E) hsa-miR-9 expression was >60-fold higher in pre-mir-9 transfected cells than cells transfected with the control (n = 3, $P < .05$). (F) The rate of cell proliferation was also affected with ~1.6-fold increase in pre-miR-9 transfected cells compared with control (n = 3, $^{*}P = .05$). (G) The megakaryocyte marker, CD61 expression was also reduced after 72-hour post-transfection (n = 3, $^{**}P = .02$). (H) After 72 hours of transfection, RUNX1 protein level was significantly reduced in pre-miR-9 transfected cells compared with non-targeting sequence transfected cells (n = 3, $^{***}P < .001$). Bars represent mean ± SD of three independent experiments

unblock mRNAs involved in developmental maturation and differentiation. Interestingly in our study, hsa-miR-9 and its putative target RUNX1 were inversely related in neonatal vs adult MKs (Figure 1B-D).

RUNX1 is a critical regulator of embryonic and adult definitive hematopoiesis.[13] In human, loss of function mutations in RUNX1 causes familial platelet disorder with predisposition to thrombocytopenia.[14] In adult mice, conditional Runx1 deletion generates a phenotype characterized by abundant small low ploidy MKs in the bone marrow, which closely resembles normal neonatal megakaryocytopoiesis.[15] In this study, we found a significant lower expression of RUNX1 in CB-derived MKs compared with PB-derived MKs (Figure 1C,D), and these developmental differences in the expression levels of RUNX1 could be under the post-transcriptional control of hsa-miR-9. Our miR-9 transfection study in MEG01 and Dami cells showed a significantly reduced expression of RUNX1 (Figure 1H). Interestingly, we found increased rate of cell proliferation and reduced level of MK-specific marker, CD61 in miR-9 mimic transfected cells as compared with control (Figure 1F,G). Our findings are consistent with those of Ben-Ami et al[16] observed in HEK293 cells. Notably, Tian et al[1] also described the role of miR-9 for translational attenuation of Runx1 in MDSC differentiation and function.

In conclusion, our current findings are in line with previous observation indicating molecular differences in neonatal vs adult MKs. This study clearly reveals the developmental differences in the expression of hsa-miR-9 and its target RUNX1. Further our observations associate the reduced level of RUNX1 with higher expression of miR-9 in neonatal MKs. Our data provide the first evidence of an important role of hsa-miR-9 in human megakaryocyte development, and for the first time identify a miRNA as a molecular regulator of the differences between neonatal and adult MKs. A known target of miR-9 is the key MK transcription factor RUNX1, which is crucial for the regulation of MK specification, maturation and thrombopoiesis.[17] Taken together, this study shows the functional significance of hsa-miR-9 in the regulation of cell proliferation by targeting RUNX1 in human MK development. The higher expression of miR-9 may contribute to the developmentally different and disease susceptible phenotype of neonatal MKs via regulating the RUNX1 expression; thus, it could be a potential target in neonatal thrombocytopenia and other platelet disorders.

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## CONFLICT OF INTEREST

The authors have no conflict of interest.

## ORCID

*Ravi Kumar Gutti* http://orcid.org/0000-0002-0912-5796

Sanjeev Raghuwanshi[1]
Usha Gutti[2]
Ravinder Kandi[1]
Ravi Kumar Gutti[1]

[1]*Stem Cells and Haematological Disorders Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, India*
[2]*Department of Biotechnology, GITAM Institute of Science, GITAM University, Visakhapatnam, Andhra Pradesh, India*

**Correspondence**
*Ravi Kumar Gutti, Ph.D.,*
*Department of Biochemistry, S59 School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, India.*
*Email: guttiravi@gmail.com*

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Letter to the Editors-in-Chief

# Pituitary adenylate cyclase-activating polypeptide (PACAP): Differential effects on neonatal vs adult megakaryocytopoiesis

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuroendocrine hormone of the secretin peptide superfamily, is mainly produced in hypothalamus. PACAP shows diverse effects on CNS, peripheral organs, and some hematopoietic cells by VPAC1 and 2 receptor signaling [1]. Megakaryocytes (MKs) express the stimulatory G protein (Gs)-coupled PACAP/VIP-indifferent VPAC1 receptor. Previously, Peeters et al. [2] reported the inhibitory effects of PACAP receptor on in vitro megakaryocyte differentiation. Recently, it was observed that PACAP contribute to the proliferation of hematopoietic progenitor cells in murine bone marrow by PACAP specific receptor [3]. In our study on human MK development, we found that PACAP receptor-VPAC1 is differentially regulated in MKs through development. Considering differential expression of PACAP receptor-VPAC1 in human MK development, we hypothesized that PACAP may have different effects on MK differentiation from neonatal (CB)- and adults (PB)-derived CD34+ hematopoietic progenitors.

Thrombocytopenia, the deficiency of platelets (platelet count $< 150 \times 10^9$/l) in the blood, is a major clinical problem encountered in infants admitted to Neonatal Intensive Care Unit (NICU); this hemostatic abnormality is more common in all sick, preterm and low birth weight neonates, and play a major role as co-factor in the incidence and severity of neonatal intra-ventricular hemorrhages (IVH) [4], which is a leading cause of poor neurological outcome and mortality in sick neonates [4–6]. The cause for this predisposition is unclear, however we and many other research groups have observed that neonatal MKs are developmentally different from adult's MKs. Specifically, neonatal MK progenitors (MP) are hyper-proliferative and produce smaller and low ploidy MKs as compared to adults. These developmental differences may contribute to this predisposition in neonatal MKs [7–9].

To test the developmental differences in the expression levels of PACAP receptor-VPAC1 in neonatal vs adult's MKs, we cultured human CB- and PB-CD34+ cells ($n = 3$ for each group) in serum-free medium with thrombopoietin (50 ng/mL), as previously described [10]. After 14 day of culture Flow cytometry analysis was performed using human anti-CD42/APC to monitor thrombopoietin stimulated MKs differentiation. We observed that CD34+ cells from CB generated approximately 2-fold more CD42 positive MKs as compared to PB-CD34+ cells ($n = 3$, $P < .02$; Fig. 1A), however the change in CD42 mean fluorescence density was higher in PB-MKs as compared to CB-MKs on day 14 ($n = 3$, $P < .05$; Fig. 1A). Further, we observed the PACAP receptor-VPAC1 mRNA expression in neonatal- and adult-CD34+ progenitor-derived MKs and resulted in approximately 6 fold higher expression levels in CB- as compared to PB-MKs ($n = 3$, $P < .02$; Fig. 1B). However, because of sample limitations, the protein content of PACAP receptor VPAC1 was not measured in current study. Finally, to study the effect of PACAP on in vitro MK differentiation from CB- and PB-CD34+ hematopoietic progenitors, we cultured CB- and PB-CD34+ hematopoietic progenitors for 14 days with or without PACAP (1 μM) (TOCRIS, Cat # 1186) in the presence of TPO (50 ng/mL) in serum-free medium ($n = 3$ for each group). After 14 days, Flow Cytometry analysis was performed using human anti-CD41/FITC and anti-CD42/APC to monitor the number of differentiated MKs. The CD41 and CD42 positive CB- and PB-MK numbers in presence of PACAP were expressed as relative values to their controls (without PACAP) at the same stage of culture. The number of CB-CD41 positive MKs was significantly reduced in the presence of PACAP compared to their control ($n = 3$, $P < .05$; Fig. 1C), whereas PB-CD41 positive MKs were not affected significantly ($n = 3$, $P > .05$; Fig. 1C). Similarly, we also observed about 2 fold decrease in the number of CB-CD42+ MKs in presence of PAPAC as compared to control (n = 3, $P < .05$; Fig. 1D), whereas the decrease in the number of PB-CD42+ MKs was not significant (n = 3, $P > .05$; Fig. 1D).

Over the past two decades, observations from different research groups have proven that human neonatal- and adult-MKs are developmentally different [7–10]. In the same line, we have also observed developmental differences in neonatal vs adult MKs. Current study results are also suggesting that human CB and PB MKs have substantially different biological responses to PACAP. In current study, we observed that PACAP is a negative regulator of MK differentiation, as previously reported [2]. However, the negative effects of PACAP were more drastic on MK differentiation process from CB-CD34+ hematopoietic progenitors as compared to MK differentiation from PB-CD34+ hematopoietic progenitors (Fig. 1C and D). Further, we have also observed developmental differences in the expression levels of PACAP receptor-VPAC1 in neonatal vs adults MKs and found about 6 fold higher expressions in neonatal MKs as compared to adults. Hypothetically, the higher expression levels of PACAP receptor-VPAC1 in neonatal MKs might contribute more to the inhibitory effects of PACAP on neonatal MK differentiation, as we have observed in current study.

In conclusion, our results demonstrate that PACAP is an inhibitor of MK differentiation however the negative effects are more drastic on neonatal MK differentiation as compared to adults. Higher expression of the PACAP receptor-VPAC1 in neonatal MKs possibly contributes to the predisposition of neonatal MKs via PACAP/VPAC1 signaling mechanism.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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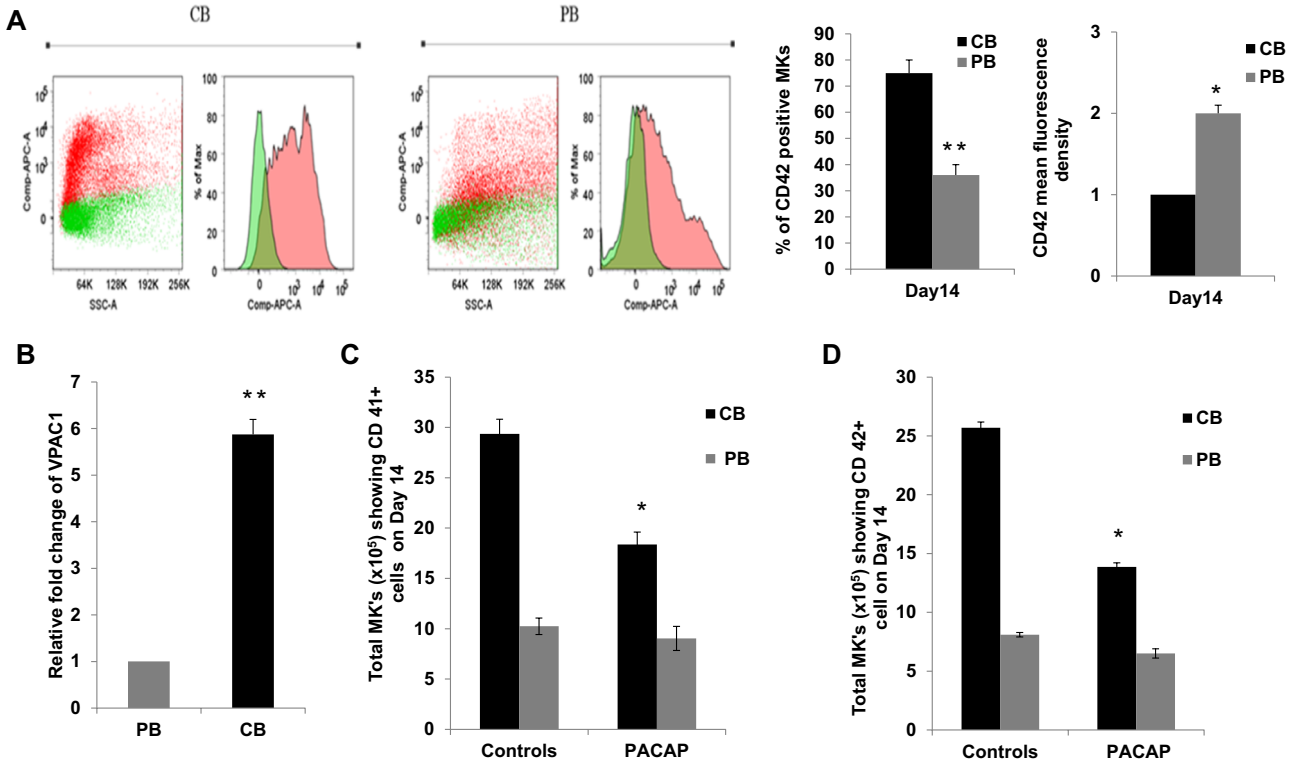


**Fig. 1.** Effects of PACAP on in vitro megakaryocytopoiesis. (A) Flow cytometry analysis of the expression of megakaryocytic marker-CD42 assessed after 14 days of culture of the CB- and PB-CD34+ hematopoietic progenitors in medium containing 50 ng of thrombopoietin per ml ($n = 3$, $^{**}p = .02$; $^{*}p = .05$). (B) VPAC1 mRNA levels were determined by QRT-PCR in CB and PB derived MKs. VPAC1 levels in MKs derived from CB expressed as relative to VPAC1 levels in PB derived MKs at day 14 (n = 3, $^{**}p = .02$). (C and D) The inhibitory effect of PACAP was observed on thrombopoietin-induced megakaryocytopoiesis by Flow cytometry. The number of (C) CD41+ and (D) CD42+ MKs, derived from CB- and PB-CD34+ progenitor, in presence of PACAP, was expressed as relative to their control (without PACAP) (n = 3, $^{*}p < .05$). Bars represent mean ± SD of three independent experiments.

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Sanjeev Raghuwanshi, Durga Shankar Sharma, Ravinder Kandi, Narasaiah Kovuru, Swati Dahariya, Syed Shahid Musvi, Anuradha Chimata Venkatakrishnan, Adithya Pallepati, Ravi Kumar Gutti*
*Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad, 500046, TS, India*
*E-mail address:* guttiravi@uohyd.ac.in (R.K. Gutti).

* Corresponding author.

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# MicroRNA function in megakaryocytes

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# MicroRNA function in megakaryocytes

Sanjeev Raghuwanshi[1], Swati Dahariya[1], Syed Shahid Musvi[1], Usha Gutti[2], Ravinder Kandi [1], Ram Babu Undi[1], Itishri Sahu [1], Dushyant Kumar Gautam[1], Indira Paddibhatla[1], & Ravi Kumar Gutti [1]

[1]*Stem Cells and Haematological Disorders Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad, TS, India and* [2]*Department of Biotechnology, GITAM Institute of Science, GITAM University, Visakhapatnam, AP, India*

**Abstract**

Megakaryocytes (MKs), the largest cells in the bone marrow, are generated from hematopoietic stem cells (HSCs) in a sequential process called megakaryocytopoiesis in which HSCs undergo MK-progenitor (MP) commitment and maturation to terminally differentiated MK. Megakaryocytopoiesis is controlled by a complex network of bone marrow niche factors. Traditionally, the studies on megakaryocytopoiesis were focused on different cytokines, growth factors and transcription factors as the regulators of megakaryocytopoiesis. Over the past two decades many research groups have uncovered the key role of microRNAs (miRNAs) in megakaryocytopoiesis. miRNAs are a class of small length non-coding RNAs which play key regulatory role in cellular processes such as proliferation, differentiation and development and are also known to be involved in disease development. This review summarizes the current state of knowledge of miRNAs which have changed expression during megakaryocytopoiesis, also focuses on miRNAs which are differentially regulated during developmental maturation of MKs. Further, we aimed to discuss potential mechanisms of miRNAs-mediated regulation underlying megakaryocytopoiesis and developmental maturation of MKs.

**Keywords**

Megakaryocyte, miRNA

**History**

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## Introduction

Among the multiple classes of non-coding RNAs (ncRNAs), the most studied ncRNAs are miRNAs. MicroRNAs are approximately 22-nucleotide in length and highly conserved through evolution. They are potent negative regulators of gene expression by degrading mRNA or repressing mRNA translation [1]. The biogenesis of miRNAs has been well reviewed by Krol et al. [2]. To summarize the biogenesis, the pri-miRNA, transcribed from DNA by RNA polymerase II, is processed to pre-miRNA in nucleus by Drosha (type III RNase). The pre-miRNA is exported to cytoplasm and further cleaved by Dicer (another type III RNase) to generate a short, partially double-stranded RNA. One strand of this molecule in the presence of argonaute proteins is loaded into the RNA-induced silencing complex (RISC), the RISC then target mRNA. So far, more than 2694 human miRNAs have been identified and this number is increasing (http://www.mirbase.org/). Recent studies have demonstrated a critical role of miRNAs in normal human hematopoiesis, and deregulated miRNAs have been associated with numerous hematologic diseases (reviewed in [3]). Some of them have been consistently identified in MKs and, together with their targets, they are implicated in MKs development.

Megakaryocytes, the precursors of platelets (thrombocytes), are the largest cells with a polyploidy multi-lobed nucleus in the bone marrow. Megakaryocytes arise from hematopoietic stem cells (HSCs) in a sequential process of development called megakaryocytopoiesis, in which HSCs undergo lineage commitment to become MK-progenitors (MKPs). Further, MKPs generate immature MKs, these immature MKs undergo in the process of endomitosis, cytoplasmic maturation and platelet production [4]. Traditionally, mechanistic studies on megakaryocyte physiology and development biology were primarily focused on identifying functional role of different cytokines and TFs. Over the last decade, multiple reports from different research groups have unveiled molecular and functional roles of miRNAs in megakaryocyte physiology [5].

The essential role of Dicer1 [6] and miRNAs [7] in megakaryocytopoiesis has been established. Recently, Rowley JW et al. [6] revealed that murine MK-specific knockdown of Dicer1 reduces the expression levels of the majority of miRNAs in platelets, that results in altered platelet mRNA expression profiles and mild thrombocytopenia. Many other studies have also addressed various aspects of miRNAs in megakaryocytopoiesis using MKs derived from in vitro cultured CD34+ HSCs or transformed cell lines with megakaryocytic properties, the most abundant miRNAs in megakaryocytes are listed in Table I.

In this review, we bring together most of the miRNAs studies, which were particularly focused on megakaryocytopoiesis and associated miRNAs. From the literature to date, we discuss many miRNAs reported in last two decades in MKs and their functional significance during megakaryocytopoiesis and developmental maturation of MKs.

## miRNAs in megakaryocytopoiesis

Megakaryocytopoiesis is a sequential process of MK-lineage commitment, progenitor proliferation and its maturation to

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Correspondence: Ravi Kumar Gutti guttiravi@gmail.com; guttiravi@uohyd.ac.in Department of Biochemistry, S59, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, 500046 TS, India

Table I. miRNAs involved in megakaryocytopoiesis and developmental maturation of MKs.

| | | |
|---|---|---|
| MicroRNAs involved in earlier steps of megakaryocytopoiesis | | |
| miR-150 | Favors MEPs commitment to the MKs lineage by regulating cMyb | [8] |
| miR-155 | Inhibits megakaryocytopoiesis by targeting Ets1 and Meis1 | [9] |
| miR-34a | miR-34a promotes megakaryocyte differentiation of CD34+ cells by regulating MYB expression | [10] |
| miR-223 | miR-223 expression increases during megakaryocytic differentiation of CB-CD34 + cells, it regulates LMO2 | [11] |
| miR-486-3p | Affects HPCs commitment by regulating MAF, it supports the erythropoiesis while restraining the megakaryocytopoiesis | [12] |
| MicroRNAs involved in later steps of megakaryocytopoiesis | | |
| miR-146a | Observed with down-regulation when human CB derived $CD34^+$ cells induced to differentiate into MKs, and forced over-expression resulted in reduced ploidy levels | [13] |
| miR-142-3p | miR-142-3p promotes MK maturation and platelet release, it targets a battery of actin cytoskeleton regulators to facilitate proplatelet formation | [14] |
| MicroRNAs involved in developmental megakaryocytopoiesis | | |
| miR-9 | A constitutive reduced expression of miR-9 is observed through MK ontogeny. miR-9 maintain low expression levels of CXCR4 and RUNX1 in neonatal MKs | [15 16 17] |
| Let-7b | Let-7b was observed with lower expression in neonatal MKs as compared to adult MKs. Lower expression levels of let-7b in neonatal MKs contribute to higher proliferative potential of neonatal MKs | [18] |
| miR-99a | Observed with higher expression levels in CB CD34+ derived as compared to adult CD34 + derived MK. The high levels of miR-99a maintain the high cell proliferation potential of neonatal MKP by regulating CTDSPL | [19] |

terminally differentiated MK. The miRNAs can be broadly classified in three classes: (i) miRNAs involved in earlier steps of megakaryocytopoiesis (ii) miRNAs involved in later steps of megakaryocytopoiesis (iii) miRNAs in developmental maturation of MKs (Table I).

## miRNAs involved in earlier steps of megakaryocytopoiesis

### *miR-150 in lineage commitment*

Lu et al. [8] first discussed miR-150 as a critical factor in lineage selection of megakaryocyte-erythrocyte progenitors (MEPs): miRNA expression profiles in MEP, erythroid and megakaryocytic primary cells derived from human umbilical cord blood showed that miR-150 is preferentially expressed in megakaryocytic lineage, further gain- and loss-of-function analysis confirmed that miR-150 drives MEP differentiation towards MKs by regulating *MYB*, a critical target of miR-150. Various *in vitro* and *in vivo* experiments confirmed that miR-150 functionally interacts with the 3′-UTR of *MYB* and down-regulates *MYB* expression, which favors MEPs commitment to the MKs lineage, these observations are consistent with the data showing that low c-Myb levels support megakaryocytopoiesis [20]. Further, Barroga et al. [21] observed that thrombopoietin increased the expression of miR-150, which reduced the c-Myb expression and thereby enhanced thrombopoiesis. In a recent study, miR-150 was identified as an inhibitor of blood count recovery after 5-fluorouracil (5-FU)-induced injury in mice [22]. The forced expression of miR-150 led to negative effects on multiple bone marrow progenitor populations. Whereas, miR-150 knockout mice model observed with normal steady state hematopoiesis, only peripheral platelet counts and spleen megakaryocyte frequency trended lower in the knockouts. However, after 5-FU treatment the number of blood cells was increased in miR-150 knockouts as compared to wild type. In conclusion, the inhibitory effects of miR-150 on murine blood cells recovery after bone marrow damage could be due by its effect on early progenitors. The lower trend of MKs and platelets in miR-150 knockouts shows functional significance of miR-150 in late-stage megakaryocytopoiesis. Taken together, these studies suggest the functional significance of miR-150 in late-stage megakaryocytopoiesis and thrombopoiesis (Figure 1).

### *miR-155 represses MK differentiation*

According to recent study miR-155 is a potent inhibitor of $CD34^+$ hematopoietic stem-progenitor cells (HSPCs) differentiation [23]. In this study, Georgantas et al. [23] performed both the miRNA and mRNA array profiling and the *in silico* prediction from the Transcriptome Interaction Database showed miRNAs that target mRNAs encoding TFs associated with hematopoietic differentiation. Further, functional characterization pointed that miR-155 control on hematopoiesis by regulating expression of multiple hematopoietic differentiation-associated genes, including *C/EBPβ, CREBBP, JUN MEIS1, PU.1, AGTR1, AGTR2*, and *FOS*. MiR-155 is abundantly expressed in early HSPCs and a constitutive reduced expression pattern is observed through MKs differentiation [9,23]. Further, miRNA-mRNA interaction validation studies confirmed the key role of mR-155 in megakaryocytopoiesis by regulating Ets1 and Meis1 [9], which are well known TFs in MK-specific gene promoter regulation. Many MK-specific genes such as *c-MPL* (TPO receptor), play crucial role in megakaryocytopoiesis, the human platelet glycoprotein IIb-IIIa (CD41/CD61) receptor that participates in calcium dependent cell adhesion and normal platelet aggregation, and the platelet glycoprotein IX (CD42a), subunit of the receptor (GPIb/IX/V) for VWF, important for initiating platelet adhesion to damaged blood vessels, are positively controlled by co-operative action of Fli-1, Ets-1 and GATA-1 [24]. The GATA-1 and Ets-1 [25] in combination with Meis-1/Pbx complex [26] transactivate the platelet factor 4 (PF4) gene, MK-lineage specific marker appear in late stage of MK differentiation; MEIS1 is also an essential factor in MK development, MEIS1 deficient embryos reported with extensive bleeding and MK/Platelet deficiency [27]. MiR-155 was also reported for its role in the regulation of chromatin remodeling protein JARID2 [28]. JARID2, the chromatin remodeling protein, is also down regulated by miR-155, which leads to the increased rate of $CD41^+$ MK-precursor formation [28]. Forced over expression of miR-155 inhibits K562 differentiation into MKs and inhibits the CD34+ HSC-derived myeloid and erythroid cell differentiation *in vitro* [23]. Further, O' Connell et al. [29] confirmed that the sustained expression of miR-155 in HSCs cause myeloproliferative disorder (MPD). All together, these data suggest that the miR-155 control megakaryocytopoiesis in a complex manner by regulating gene and TFs having critical role in MK development. MiR-155 with its identified oncogenic potential inhibits megakaryocytopoiesis and its sustained expression cause MPD (Figure 2).

### *miR-34a in MK differentiation*

miR-34a is up-regulated in K562 cells during phorbol ester–induced megakaryocyte differentiation [10,30]. In K562 cells enforced expression of miR-34a induced cell cycle arrest in G1

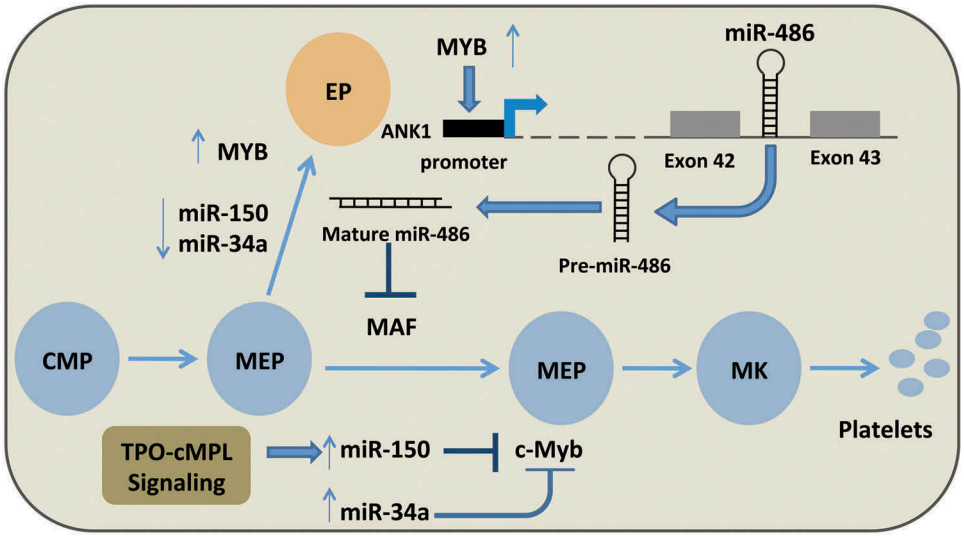


Figure 1. Schematic illustration of the mechanism through which the miRNAs regulate erythroid versus megakaryocyte lineage fate decision. The increased expression levels of MYB favors MEPs commitment to the erythroblast. MYB mediated transactivation of ANK1 up-regulates the expression of miR-486-3p, which in turn targets MAF mRNA and down-regulates its expression. Further, down-regulation of MAF favors the MEP commitment to the erythroid lineage and restrains the MK commitment. Whereas, increased expression levels of miR-150 and miR-34a in earlier steps of megakaryocytopoiesis down-regulates MYB expression, which favors MEP commitment to MK.

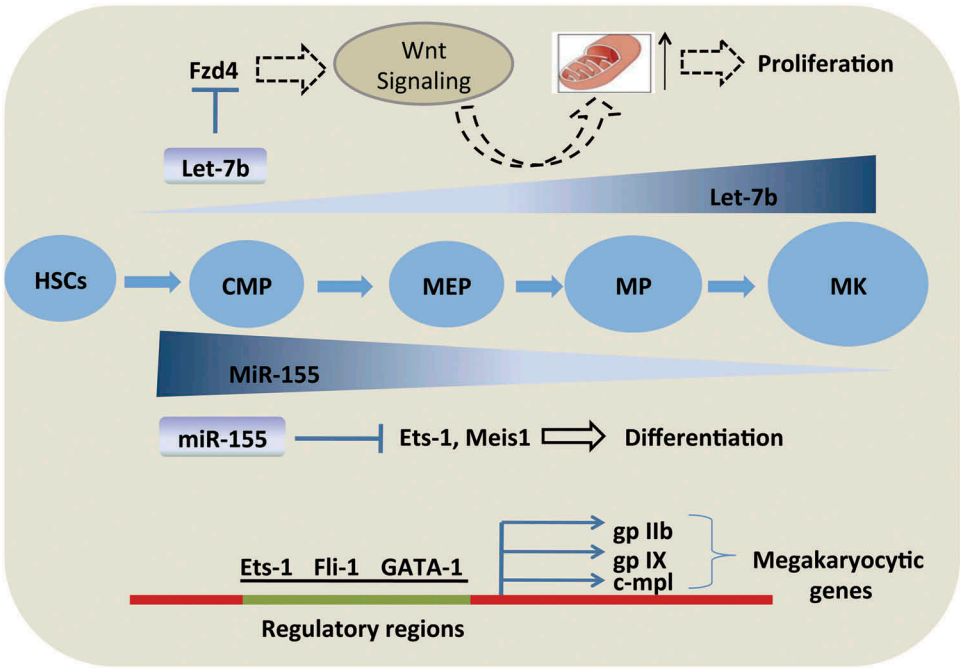


Figure 2. Regulatory role of miR-155 and Let-7b in megakaryocytopoiesis.
Schematic representation of the hematopoietic stem cell lineage commitment to megakaryocyte progenitor and its further maturation to terminally differentiated MKs. The height of triangle indicates the levels of miRNA through HSPCs lineage commitment and differentiation. MiR-155 works as a potent inhibitor of megakaryocytopoiesis by suppressing the Ets-mediated transcription of megakaryocytic gene, whereas Let-7b observed in the regulation of canonical wnt signaling and mitochondrial biogenesis by regulating wnt family protein, Fzd4.

phase and promoted MKs differentiation, further miR-34a was observed with increased rate of megakaryocyte colony formation in TPO-induced differentiation of CD34+ cells, in these cells miR-34a contributes to megakaryocyte differentiation via maintaining the lower levels of MYB (a negative regulator of megakaryocytopoiesis), as well as CDK4 and CDK6 (regulators of the cell cycle) [10]. Another study by Ichimura et al. [30] observed that MEK-ERK pathway in response to PMA strongly activated the transcription of miR-34a, and mitogen-activated protein kinase 1 (MEK1) was identified as a direct target of miR-34a. These studies uncover the functional role of miR-34a in *in vitro* megakaryocyte differentiation. Further, *in vivo* studies need to confirm the role of miR-34a in megakaryocyte physiology (Figure 1).

### *miR-223 in MK differentiation*

miR-223, a fine-tuner of granulocyte differentiation, was observed with a constitutive increased expression during megakaryocytic

Table II. Studies on molecular targets and function of different miRNAs in MK.

| miRNA | Targets | Function | |
|---|---|---|---|
| miR-150 | cMyb | Favors MEPs commitment to the MKs lineage | [8] |
| miR-155 | Ets1 and Meis1 | mR-155 inhibits megakaryocytopoiesis by targeting Ets1 and Meis1 | [9] |
| | | Sustained expression of miR-155 in HSCs causes MPD | [29] |
| | | Forced over-expression of miR-155 inhibits K562 differentiation into MKs | [23] |
| miR-146 | CXCR4 | miR-146a expression is down-regulated when human CB derived $CD34^+$ cells induced to differentiate into MKs,<br>MiR-146a over-expression impaired megakaryocytopoiesis | [13] |
| | | miR-146a was up-regulated when human and murine HSCs were induced to differentiate into MKs, and forced over-expression observed with no significant effects on megakaryocytopoiesis | [32] |
| | | Knock-down of miR-146a in mouse HSPCs observed with increased platelets in the blood and MKs in the marrow<br>over-expression of miR-146a noticed with no significant effect on platelet and MKs | [33,34] |
| miR-9 | CXCR4 and RUNX1 | A constitutive reduced expression of miR-9 through MK ontogeny was noticed | [15 16 17] |
| | | Pre-miR-9 transfection in Meg01 and Dami cells observed with lower levels of megakaryocytic marker-CD61 and increased rate of cell proliferation | [17] |
| miR-34a | MYB | miR-34a is up-regulated during PMA induced megakaryocytic differentiation of K562 cells<br>Enforced expression of miR-34a promotes MK differentiation of CD34+ cells | [10] |
| | MEK1 | miR-34a inhibits cell proliferation by targeting MEK1 during megakaryocytic differentiation of K562 cells | [30] |
| miR-130a | MAFB | miR-130a is down regulated in differentiated MKs derived from CD34+ bone marrow cells, and targets MAFB | [7] |
| miR-10a | HOXA1 | miR-10a is down regulated in CD34+ bone marrow progenitor derived MKs, and targets HOXA1 | [7] |
| miR-27a | RUNX1 | During megakaryocytic differentiation of K562 cells Runx1 and miR-27a are engaged in a regulatory interplay involving positive regulation of miR-27a expression by Runx1 | [35] |
| miR-181 | Lin28 | miR-181 targets Lin28, which is a negative regulator of Let-7 miRNAs. Over-expression of miR-181 during TPA induced megakaryocytic differentiation indirectly increased the Let-7 and enhanced differentiation | [36] |
| miR-223 | LMO2 | miR-223 expression increases during megakaryocytic differentiation of CB-CD34+ cells, it regulates LMO2 | [11] |
| | | Over-expression of miR-223 during PMA induced megakaryocytic differentiation reduces LMO2 levels and increases megakaryocytic differentiation | [31] |
| Let-7b | Fzl4 | Differentially regulated though development, observed with lower expression levels in neonatal as compared with adults MKs. Play role in developmental maturation of MKs | [18] |
| miR-28a | MPL, E2F6 And MAPK1 | Expression of miR-28 in CD34+ derived MKs inhibited terminal differentiation<br>Increased expression levels of miR-28a were noticed in BCR-ABL negative myeloproliferative neoplasms (MPN; polycythemia vera, essential thrombocythemia, primary myelofibrosis) patients | [37] |
| miR-486-3p | MAF | miR-486-3p regulates MEP commitment, it supports the erythropoiesis while restraining the megakaryocytopoiesis | [12] |
| miR-142 | Wasl, Cfl2, Twf1, Itgav or Grlf1 | It supports MK polyploidization, maturation and proplatelet release. It targets a group of actin cytoskeleton regulators and maintains cytoskeleton dynamics | [14] |
| miR-99a | CTDSPL | miR-99a maintain the high cell proliferation potential of neonatal MKP by regulating CTDSPL<br>miR-99a expression was also reported in megakaryoblastic leukemic cell lines | [19 38] |
| miR-145 | TIRAP | miR145 knockdown in mouse HSPCs results in abnormal megakaryocytopoiesis | [30] |
| miR-125b | | miR-125b is down regulated during megakaryocytopoiesis miR-125b proposed for its function in cell proliferation and survival of neonatal megakaryocytes | [39] |

differentiation of CB-CD34+ cells [11]. Further, experimental evidences confirmed that miR-223 directly interacts with the LMO2 3′UTR. Moreover, functional studies showed that the over-expression of miR-223 reduced the LMO2 protein levels and impaired erythroid differentiation. Another study by Yuan JY et al. [31] showed that LMO2 expression sharply reduces during PMA induced megakaryocytic differentiation of K562 cells, and the over-expression of miR-223 reduces the protein levels of LMO2 and increases megakaryocytic differentiation. Thus it is clear that miR-223 may control the MEP commitment process by regulating LMO2 expression (Table II).

### *miR-486-3p and ankyring (ANK1)*

Recent study evidenced the role of transcription factor MYB in erythroid versus MK lineage fate decision through the miR-486-3p mediated down-regulation of MAF, It was demonstrated that MYB regulates the expression of miR-486-3p through the transactivation of its host gene, ANK1, over-expression and down-regulation experiments confirmed that MYB supports the erythropoiesis while restraining the megakaryocytopoiesis through the MYB/miR-486-3p/ANK1 axis [12] (Figure1).

## MiRNAs involved in later steps of megakaryocytopoiesis

### *miR-146a with complex effects on megakaryocytopoiesis*

Different research groups demonstrated that miR-146a dramatically changes its expression when HSCs differentiate into MKs, but there is conflicting evidence about the direction of change and the function of miR-146a in megakaryocytopoiesis. Labbaye et al. [13] observed that miR-146a expression is down-regulated when human CB derived $CD34^+$ cells are induced to differentiate into MKs and the forced over-expression of miR-146a significantly reduced the ploidy levels in CB derived MKs. Further, miR-146a down-regulation experiments confirmed that the normal phenotype of CB-derived MKs correlates with the phenotype observed after down-regulation of miR-146a. In normal megakaryocytopoiesis, they identified PLZF/miR-146a/CXCR4 pathway: in MK differentiation from

CB-derived megakaryocytic progenitor, PLZF (promyelocytic leukemia zinc-finger) suppresses miR-146a transcription and thereby activates CXCR4 translation. The CXCR4 was identified as a target negatively regulated by miR-146a that, in turn, under the transcriptional negative control of PLZF [13]. In contrast, Opalinska et al. [32] found the up-regulation of miR-146a when human and murine HSCs were induced to differentiate into megakaryocytes. Further, forced over-expression of miR-146a was observed with no significant effects on megakaryocytopoiesis in both the *in vitro* and *in vivo* studies, also the over expressed miR-146a does not alter circulating platelet number and activation. In addition, two recent studies by Starczynowski et al. [33,34] recognized the expression and function of miR-146a in mouse hematopoietic stem/progenitor cells, but the conflicts were not clarified, as compared with HSPCs, miR-146a expression was lower in MEP, and the knockdown of miR-146a in HSPCs observed with increased platelets in the blood and MKs in the marrow, this observation is consistent with previous findings by Labbaye et al. [13],, whereas the over-expression of miR-146a did not have significant effect on platelet and MKs, similar to observations by Opalinska et al. [32]. All together, the data from different studies suggests the regulatory role of miR-146a in megakaryocytopoiesis, but the effects are complex and vary with different conditions and developmental stage (Table II).

### *miR-142 in MK maturation*

miR-142 is a broadly expressed miRNA in adult mouse hematopoietic lineage, moreover a critically important miRNA for MK maturation and proplatelet release. Loss-of-function mutation of miR-142 was observed in mouse with impaired megakaryocyte maturation, inhibition of polyploidization, abnormal thrombopoiesis, and thrombocytopenia. Further, it was confirmed that miR-142-3p targets a group of actin cytoskeleton regulators (Wasl, Cfl2, Twf1, Itgav or Grlf1) during megakaryocytopoiesis to maintain cytoskeletal dynamics and normal hemostasis [14] (Table II).

## miRNAs in developmental maturation of MKs

### *miR-9 in developmental regulation of RUNX1 and CXCR4*

miR-9 has been reported as important regulator of cell fate in hematopoietic development; Bluteau et al. [15] in a miRNA microarray study first noticed the differential expression of miR-9 during MK ontogeny, they observed a constitutive reduced expression of miR-9 through MK ontogeny, whereas the *CXCR4*, miR-9 putative target, was noticed with increased expression through MK ontogeny. The same results were observed by Ferrer-Marin et al. [16], they carried out miR-9 transfection studies in MEG01 cells to investigate whether miR-9 regulates CXCR-4 protein expression and observed significantly lower CXCR-4 protein levels in pre-miR-9 transfected cells. CXCR4, a receptor for SDF-1, is expressed on many hematopoietic cells including MKs. SDF-1 has been shown to stimulate maturation of human-MKs in vitro [40] and murine-MKs in vivo [41]. Interestingly, the administration of SDF-1 in conjunction with fibroblast growth factor-4 (FGF-4), restored thrombopoiesis in Thpo(-/-) and Mpl(-/-) mice [41]. Maturational effects of SDF-1/CXCR-4 axis in MK were thought to be mediated by stimulating the MKP interactions with sinusoidal bone marrow endothelial cells (BMECs), allowing the progenitors to relocate to a microenvironment that is permissive for MK maturation. Recently, in a miRNA-microarray study, we observed that miR-9 is the highest up-regulated miRNA in CB-derived MKs compared with PB-derived MKs. Interestingly these differences are consistent through all the stages of megakaryocytopoiesis [17]. Further, RUNX1 emerged as putative target of miR-9 by several miRNA databases; hypothetically the down-regulated miRNAs through development and differentiation unblock the expression of genes involved in developmental maturation and differentiation of cell. More interestingly, we observed that hsa-miR-9 and RUNX1 expressions levels are inversely related in neonatal versus adult MKs [17]. To confirm the functional significance of miR-9 in RUNX1 regulation in human MKs, we carried out pre-miR-9 transfection studies in Meg01 and Dami cell lines, and observed significant lower expression of RUNX1 protein and its regulated gene CD61 mRNA with increased rate of cell proliferation. RUNX1 is a critically important TF in MKs development, in human, loss of function RUNX1 mutations are common in familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML), which is characterized by thrombocytopenia and propensity for progression to AML [42]. Moreover, Runx1 conditional knock-out mice phenotype characterized with abundant number of small low ploidy-MKs, which closely resembles normal neonatal megakaryocytopoiesis [43]. Jacob et al. [44] observed impaired niche interactions and increased rate of stem/progenitor cells proliferation with down regulation of *Cxcr4* in *Runx1*$^{-/-}$ mice. The increased hematopoietic stem/progenitor cell fraction with the down-regulation of *Cxcr4* expression in *Runx1*$^{-/-}$ mice indicates the transcriptional regulation of *Cxcr4* expression and niche interaction by Runx1. All together, miR-9 noticed to regulate developmental differences in CXCR4 and RUNX1 expression in neonatal versus adult MKs, also from previous finding it appears that miR-9 may influence CXCR4 expression indirectly via regulating the RUNX1 expression. The differential expression of miR-9 in neonatal versus adult MKs implies its functional role in the developmental differences of neonatal and adult MKs whereas the RUNX1 and CXCR4 could be the downstream factors playing role in niche interaction and maturation of MKs (Figure 3).

### *miR-99a promotes cell proliferation*

Garzon et al. [7] compared the miRNAs expression profile of megakaryoblastic leukemic cell lines with that of CD34+ progenitors derived MKs, data demarcate the up-regulation of miR-99a, miR-101, miR-126, miR-135, and miR-20 in megakaryoblastic leukemic cell lines. Other studies also showed the oncogenic potential of miR-99a [38]. Further, Kandi et al. [19] did a comparative study between neonatal (CB)- and adult (PB)- CD34 + progenitor derived MKs and observed up-regulation of miR-99a in neonatal MKs, whereas miR-99a target, CTDSPL, a phosphatase-like tumor suppressor, was down regulated in neonatal MKs, which in turn led to the phosphorylation of RB and accumulation of E2F1. The E2F1 promotes cell cycle progression by increasing cyclins (CyclinD1, cyclin D2 and Cyclin D3) expression. All together data suggests that the high levels of miR-99a maintain the high cell proliferation potential of neonatal MKP (Figure 4).

### *Let-7b in developmental maturation of MK*

Li X et al. [36] observed an increased expression of Let-7b and other let-7 miRNAs during MK differentiation, Let-7b was also observed as a differentially regulated miRNA through development [18]. Multiple observations support the existence of developmental differences in megakaryocytopoiesis; neonatal MK progenitors are hyperproliferative and give rise to MKs smaller and of lower ploidy than adult MKs. Let-7b was observed with lower expression in neonatal MKs as compared to adult MKs.

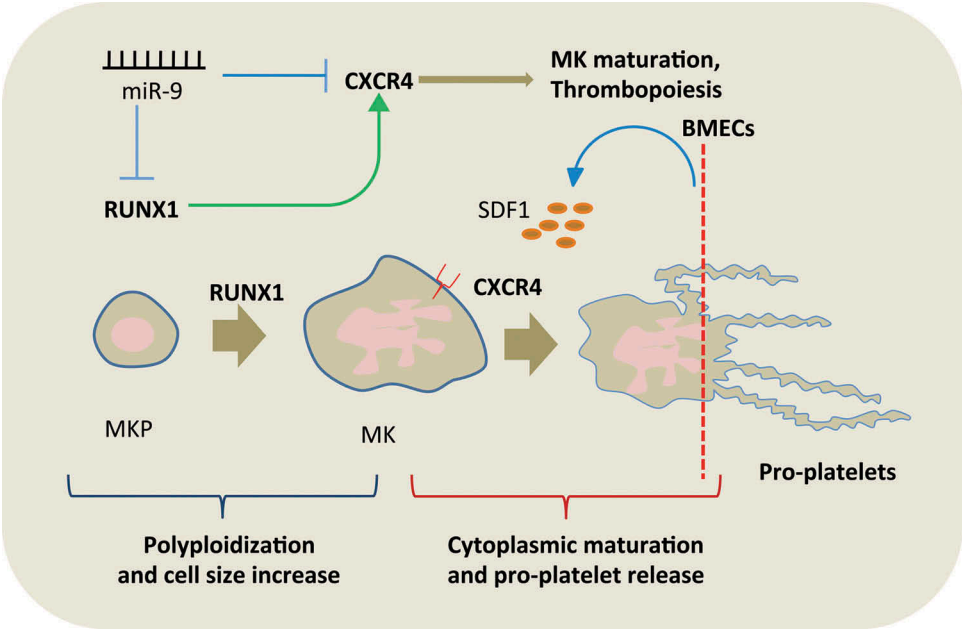


Figure 3. Regulatory role of miR-9 in megakaryocytopoiesis.
The cartoon summarizes key steps, endomitosis and cytoplasmic maturation in megakaryocytopoiesis and platelet production. Extracellular signaling pathway, SDF-1/CXCR-4, is involved in the maturational effects of MK and these effects thought to be mediated by stimulating the MKP interactions with sinusoidal bone marrow endothelial cells (BMECs), allowing the progenitors to relocate to a microenvironment that is permissive for MK maturation and proplatelet release. MiR-9 is a potent inhibitor of MK development. MiR-9 directly targets the expression of RUNX1, having role in MK endomitosis, and CXCR4, SDF-1 receptor involved in MK maturation and proplatelet release.

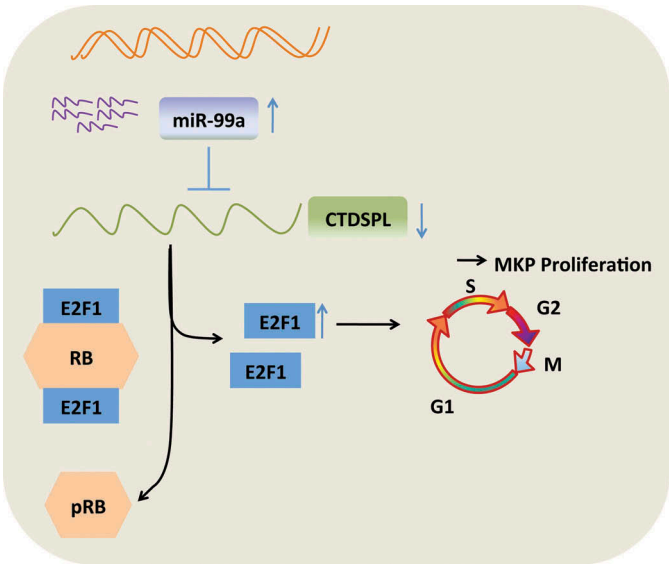


Figure 4. Schematic representation of the pathway modulated by miR-99a in MK.
miR-99a targets CTDSPL, which modulates E2F1, a cell cycle effector protein. miR-99a over-expression induces progression through the G1/S transition and promotes S-phase entry. miR-99a down-regulates the target gene CTDSPL, which in turn results in the phosphorylation of RB and accumulation of E2F1.

Further, Introduction of let-7b oligos in CB-MK progenitors resulted in the reduction of progenitor proliferation [18]. From the above observations it appears that lower expression levels of let-7b in neonatal MKs contribute for higher proliferative potential of neonatal MKs compared to adult-MKs. Further, Let-7b over-expression was observed with down-regulation of Fzd4 (Frizzled-4), a wnt family receptor protein and lower expression levels of wnt signaling molecules. Also Let-7b down regulation observed with induced wnt signaling and mitochondrial biogenesis and its markers PGC-1α and NRF1 [18]. These observations suggest inhibitory effects of Let-7b on Fzd4 expression, which could be a possible regulatory mechanism on wnt signaling pathway and mitochondrial biogenesis. Further experimental evidences are needed to confirm the regulatory role of Let-7b on wnt signaling and mitochondrial biogenesis (Figure 2).

### *Other miRNAs in megakaryocytes*

In addition to those discussed above, many other miRNAs were also noticed in MKs but less well studied in megakaryocytopoiesis. Many research groups observed that most of the miRNAs are down-regulated during MK differentiation [7,32] and development [17,38], hypothetically the down regulation of miRNAs unblocks the expression of target genes involved in MK differentiation and development. Example: in a miRNA profiling study, miR-130a and miR10a were down regulated in *in vitro*-differentiated MKs derived from $CD34^+$ cells, further experimental evidences confirmed that miR-130a targets the *MAFB*, which is involved in the promoter activation of GPIIB, a crucial protein in platelet biology, further *HOXA1* was also observed as direct target of miR-10a [7] (Table II). Ichimura et al. [30] proposed that miR145 can directly target 3′UTR of TIRAP (Toll–interleukin-1 receptor domain– containing adaptor protein), and miR145 Knockdown in mouse HSPCs results in thrombocytosis, neutropenia and megakaryocytic dysplasia (Table II). miR-125b was observed with down regulation during megakaryocytopoiesis, further it was proposed that miR-125b can control cell proliferation and survival of neonatal megakaryocytes [39] (Table II). Thrombopoietin (Tpo) through its receptor, MPL, activates many signaling pathways and can regulate all phases of MK development, interestingly the Tpo receptor is regulated by many miRNA. Girardot et al [37] reported that miR-28a targets the 3′UTR of MPL, inhibiting its translation, as well as other proteins involved in MK differentiation, such as E2F6 and

MAPK1. Expression of miR-28 in CD34+ derived MKs inhibited terminal differentiation (Table II).

Several microRNAs were also observed with increased expression levels during differentiation and development of MKs. For Example, TPA induced megakaryocytic differentiation of K562 cells was observed with increased expression levels of miR-181. miR-181 over-expression affects the stability of Lin28 in K562 cells. In this study a reciprocal regulatory loop between the let-7 miRNAs and Lin28 was observed. The over-expression of miR-181 during TPA induced megakaryocytic differentiation interrupts the Lin28–let-7 reciprocal regulatory loop by targeting Lin28, which resulted in the greater levels of mature Let-7 and enhanced differentiation [36] (Table II). TPA induced megakaryocytic differentiation of K562 cells noticed with the binding of RUNX1 to miR-27a regulatory region and increased expression of miR-27a, that in turn attenuates Runx1 expression. Thus, during megakaryocytopoiesis, Runx1 and miR-27a appear to work in a feedback loop involving positive regulation of miR-27a by Runx1 [35] (Table II).

## Conclusion

Several miRNA expression profiles from different research groups in last decade have increased the number of MK derived miRNAs, though all are not functionally characterized for their target selection and regulatory role in MK physiology. Hundreds of miRNAs have been identified yet in MKs but still new miRNAs are emerging. Here we reviewed the expression pattern of different miRNAs and their targets in megakaryocytopoiesis. Moreover, we have reviewed their positive and negative functions during megakaryocytopoiesis and their role in developmental megakaryocytopoiesis. We discussed the current literature on the functions of miRNAs in MKs. A special emphasis was given to miRNAs that have demonstrated a potential in several *in vitro* and *in vivo* studies in MKs. The data from different studies discussed in this review suggests that miRNAs are important regulators of megakaryocytopoiesis, they involved in the regulation of many molecular mechanisms via creating positive and negative feedback loops. Few miRNAs were also observed with more complex effects which vary with different conditions and developmental stage. Also, there are conflicting evidences on the expression and function of some of the miRNAs reported in megakaryocytopoiesis.

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## Disclosure of conflicts of interest

The authors declare that they have no conflict of interest.

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## ORCID

Ravinder Kandi http://orcid.org/0000-0002-2875-8046
Itishri Sahu http://orcid.org/0000-0002-6069-656X
Ravi Kumar Gutti http://orcid.org/0000-0002-0912-5796

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REVIEW ARTICLE

# Epigenetic Mechanisms: Role in Hematopoietic Stem Cell Lineage Commitment and Differentiation

Sanjeev Raghuwanshi[1], Swati Dahariya[1], Ravinder Kandi[1], Usha Gutti[2], Ram Babu Undi[1], Durga Shankar Sharma[1], Itishri Sahu[1], Narasaiah Kovuru[1], Nagendra Sastry Yarla[2], Raja Gopal Venakata Saladi[2] and Ravi Kumar Gutti[1,*]

[1]*Stem Cells and Haematological Disorders Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad-500046 (TS), India;* [2]*GITAM Institute of Science, GITAM University, Visakhapatnam-530 045 (AP), India*

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**Abstract:** Major breakthroughs in the last several decades have contributed to our knowledge of the genetic regulation in development. Although epigenetics is not a new concept, unfortunately, the role of epigenetics has not come to fruition in the past. But the field of epigenetics has exploded within the past decade. Now, growing evidences show a complex network of epigenetic regulation in development. The epigenetic makeup of a cell, tissue or individual is much more complex than their genetic complement. Epigenetic modifications are more important for normal development by maintaining the gene expression pattern in tissue- and context-specific manner. Deregulation of epigenetic mechanism can lead to altered gene expression and its function, which result in altered tissue specific function of cells and malignant transformation. Epigenetic modifications directly shape Hematopoietic Stem Cell (HSC) developmental cascades, including their maintenance of self-renewal and multilineage potential, lineage commitment, and aging. Hence, there is a growing admiration for epigenetic players and their regulatory function in haematopoiesis. Epigenetic mechanisms underlying these modifications in mammalian genome are still not completely understood. This review mainly explains 3 key epigenetics mechanisms including DNA methylation, histone modifications and non-coding RNAs inference in hematopoietic lineage commitment and differentiation.

**Keywords:** Epigenetics, hematopoietic stem cell (HSC), DNA methylation, histones, miRNA, acetyltransferases (HATs), DNA methyltransferases.

## 1. INTRODUCTION

Blood is a highly regenerative fluid form of connective tissue, with an enormous number of cells produced every day in adult human bone marrow (BM). Hematopoiesis is a complex process of all blood cells formation from HSCs. HSCs are self renewing and can differentiate into multipotent progenitors, which in turn produce all the mature blood cells. During hematopoiesis, HSCs respond to several extracellular signals and differentiate into cells of different lineages. HSCs also respond to different stress conditions, such as blood loss, bacterial and viral infections or cytotoxicity, *via* increasing the population of HSCs and different progenitors to preserve blood homeostasis [1, 2]. HSC functions, including self renewal and differentiation are regulated by cell – intrinsic and –extrinsic factors such as transcription factors (TFs), signaling pathways and niche factors [3]. But, to date, very limited information is available about epigenetic regulatory mechanism and its impact on the regulation of hematopoiesis and blood homeostasis.

Epigenetic mechanisms involve heritable (mitotically and/or meiotically) changes in gene function that are not associated with any alterations in the nucleotide sequence of DNA [4]. Epigenetic regulation of gene function comprises DNA methylation, histones modifications and small non-coding RNA mediated interference [5]. Change in the DNA methylation pattern and histone modification can transform chromatin structure and serve as sites for the recruitment of different regulatory proteins and enzymes [6, 7]. Recent studies have also shown the role of small non-coding RNAs in post-transcriptional fine-tuning of gene expression during hematopoiesis [8, 9].

The chromatin structure and its accessibility are regulated by different chromatin-modifying enzymes which include DNA modifying enzymes, such as DNA methyltransferases (DNMTs) and ten-eleven translocation methylcytosine dioxygenases (TETs); and histones modifying enzymes such as histone acetyltransferases (HATs), histone methyltransferases (HMTs), histone deacetylases (HDACs) and histone demethy-

*Address correspondence to this author at the Department of Biochemistry, S59 School of Life Sciences, University of Hyderabad, Gachibowli Hyderabad- 500046 (TS) India; Tel: +91-9052665597; Fax: +914023010120; E-mail: guttiravi@gmail.com

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lases (HDMs) [10, 11]. These epigenetic regulators play a vital role in normal development *via* regulating gene expression pattern in mammalian cells, also maintain tissue homeostasis *via* balancing the self-renewal and differentiation of HSCs. Epigenetic modifications are crucial for self-renewal of HSCs and also increase the proliferation rate of leukemia stem cells (LSCs) [12]. Alteration in epigenetic marks can result in aberrant gene expression and dysregulation of signalling pathways, leading to diseases [13].

In this review, we first discuss possible mechanisms underlying DNA methylation and its regulatory role in hematopoiesis. We focus on the DNMTs mediated maintenance and de novo DNA methylation, and TETs mediated DNA demethylation process. In this way, we also described few aspects of histone modification and their role in the regulation of gene expression. Further, we tried to cover the role of different miRNAs in lineage commitment and differentiation of blood cells. This review focuses mostly on different DNMTs, TETs family of proteins, histone modifying enzymes and miRNAs in lineage commitment and differentiation of blood cells.

## 2. HEMATOPOIESIS

All blood cells originate from a type of cell called HSC. The HSCs are able to differentiate in all types of blood cells and they can maintain their number by cell division. In humans, the hematopoiesis, the formation of all the blood cells and their development start in embryonic yolk sac during the first week of development. After the third month of gestation fetal liver and spleen are the major sites of hematopoiesis. After that, bone marrow becomes the major site of hematopoiesis, which, after birth, throughout life, occurs primarily within the bone marrow [14]. HSCs are self-renewing and can differentiate into different progenitor cells. These cells mainly follow two pathways, namely, lymphoid and myeloid pathway. In the lymphoid pathway, the common lymphoid progenitor cells differentiate into immune cells such as T – lymphocytes, B-lymphocytes and Natural killer cells, and in myeloid pathway, the progenitor cells differentiate into granulocytes, monocytes, erythrocytes and platelets (Fig. **1**) [15]. Self-renewal and lineage commitment of HSCs are tightly regulated by both cell intrinsic and extrinsic factors, including TFs, signaling pathways, and niche factors. In addition to these environmental factors that regulate hematopoiesis, a number of epigenetic events are critical for development and hematopoiesis. The differentiation of HSCs in lineage specific progenitors is based on differential gene expression which is controlled at transcription as well as post transcription level. The transcriptional control of gene expression is regulated through chromatin remodelling and DNA accessibility and this, in turn, is regulated by various epigenetic factors such as DNA methylation and histone modifications, whereas small non-coding RNAs are involved in post transactional regulation.

## 3. DNA METHYLATION

DNA methylation is a crucial epigenetic modification of the genome that influences gene expression in tissue- and context-specific manner. There is a significant amount of variation in DNA methylation signatures during hematopoietic lineage commitment and differentiation. DNMTs are the key regulators of the DNA methylation process, whereas active DNA demethylation is catalyzed by TETs family of proteins [16, 17].

### 3.1. DNA Methylation and Demethylation Mechanisms

DNA methylation and demethylation are major epigenetic mechanisms used by eukaryotes in chromatin organiza-

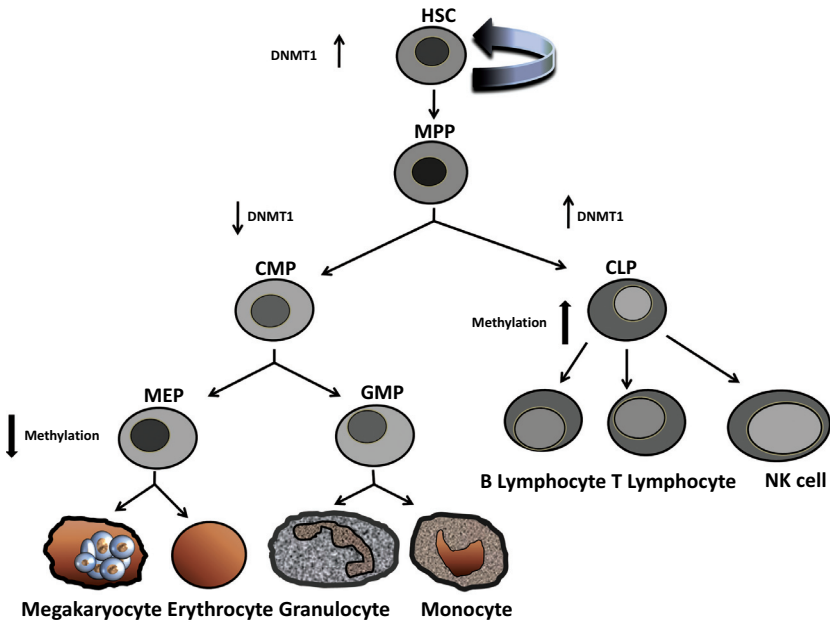


**Fig. (1). Overview of the hematopoietic lineage commitment process and genomic DNA methylation during lineage commitment and differentiation process.** HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor. Direction of arrows represents the increases and decreases in DNMT1 expression and DNA methylation.

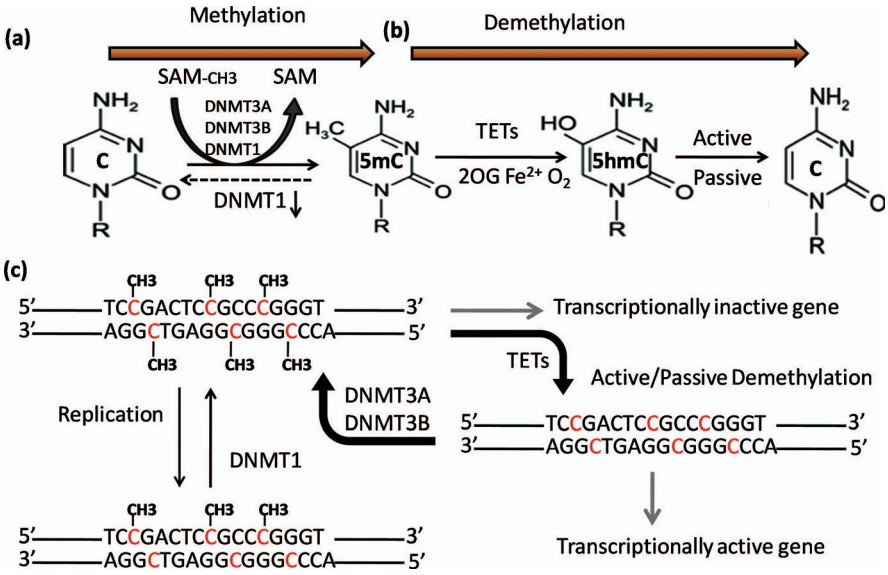


**Fig. (2). Schematic representation of DNA methylation and demethylation and its impact on the transcriptional activity of a gene.** (**a**) DNA methylation is a process of –CH3 group transfer from SAM to the carbo-5 of cytosine to yield 5mC, the methylation initiated de novo by the DNMT3A and B and after replication maintained by the DNMT1. Demethylation is thought to occur globally by reduction of DNMT1 activity. (**b**) Oxidative demethylation in mammals. TET proteins consecutively oxidize –CH3 group of cytosine in –OH (5hmC) and further oxidized derivatives, TET mediated oxidation process is dependent on 2OG, $Fe^{2+}$ and $O_2$. TET enzymes promote both active and passive cytosine demethylation. (**c**) CpG-methylation and gene regulation by the interplay between DNMTs and TETs. Methylated CpG sites inactivate gene expression; when demethylated, gene is potentially active. Replication results in hemi-methylated form of CpG sites, DNMT1 restore fully methylated CpG sites. DNA methylation is dynamic but tightly regulated by DNMTs and TETs.

tion and gene regulation [16]. In mammals, three distinct DNMTs, DNMT1, DNMT3a and DNMT3b, are identified with DNA methyltransferase activity. The C-terminal of DNMTs is a highly conserved region, but they show no sequence similarity in their N-terminal regions. The highly conserved C-5 motifs at the C-terminal of the Dnmt1 and Dnmt3 families have catalytic activity, which transfer methyl group ($-CH_3$) from the methyl donor (*S*-adenosyle-$_L$-methionine) to the carbon-5 of the cytosine (Fig. **2a**) [18, 19]. Whereas, the active DNA demethylation involves the TET family of proteins (TET1/2/3), these members initially found to be chromosomal translocation partners in leukaemia, subsequently TET protein family, were found to have a role in DNA demethylation (Fig. **2b**). DNA methylation and hydroxymethylation status of the mammalian genome are dynamically regulated by the interplay between DNMTs and TETs (Fig. **2c**) [20, 21]. The TET enzyme family members are 2-oxoglutarate (2-OG) and $Fe^{2+}$ dependent dioxygenases that have a highly conserved C-terminal catalytic domain [68, 69] which has the capacity to change methylation status of DNA by catalyzing consecutive oxidation of the methyl group of 5mC to form 5hmC and further oxidised derivatives *in vitro* as well as *in vivo* (Fig. **2b**) [22-25]. The C-terminal catalytic domain is highly conserved and contains binding sites for 2-OG and $Fe^{2+}$, the catalytic region alone is capable to oxidise 5mCs in DNA [22, 26]. The evolutionary conserved N-terminal CXXC-type domain is present in TET1/3 which preferably recognizes unmethylated CpG compared to methylated CpG [27]. Whereas, the CXXC domain is not present in TET2, studies on the ontogeny of *TET* genes considered that during vertebrate evolution, the CXXC-type domain of *TET2* separated from C-terminal catalytic domain and becomes a separate CXXC domain containing gene [28].

### 3.2. DNMTs: Targeting Mechanisms

#### *3.2.1. Mechanism of DNMT1*

DNMT1 is the principal enzyme responsible for the maintenance of cytosine methylation at CpG sites in mammals [29], and has a strong preference for hemi-methylated DNA sequence compared to the unmethylated DNA sequence (Fig. **2c**) [30]. Dnmt1 is needed in S phase to copy methylation patterns of parental DNA strand onto the newly synthesized strand after each round of DNA replication, which provides a way of passing epigenetic information between cell generations [18, 31]. Dnmt1 possesses an intrinsic affinity for hemimethylated DNA substrate [32-34], but the molecular mechanisms for correct loading of Dnmt1 onto the newly replicated daughter DNA strand are not fully understood.

Dnmt1 copy methylation pattern of parental strand of DNA onto the daughter strand, provably with the help of Np95 (also known as Uhrf1 in mouse and ICBP90 in human) protein and the localization of Np95 to the replication foci, is hemi-methylated heterochromatin dependent [31]. Np95 binds methylated CpG dinucleotides *via* its SET and RING finger-associated (SRA) domain [35]. The proliferating cell nuclear antigen (PCNA) also interacts with DNMT1 and promotes its localization to the replication foci [36], but PCNA is not absolutely required in this process [37]. During replication DNMT1 also directly interacts with G9a at the replication foci, which results in posttranslational modification of newly deposited histones [38]. In summary, NP95 form complex with DNMT1 and PCNA at heterochromatin sites of replication foci, where the hemi-methylated DNA is converted to full-methylated DNA (Fig. **3**).

### *3.2.2. Mechanism of DNMT3*

Dnmt3 is a family of two related genes, *Dnmt3a* and *Dnmt3b* [39]. Human *DNMT3A* and *DNMT3B* gene encoded proteins show 98% and 94% amino acid identity with their murine homologues [40]. Dnmt3a and Dnmt3b both function as de novo methyltransferases (Fig. **2c**) [41]. The N-terminal region of Dnmt3a and Dnmt3b has Csy-rich PHD domain and PWWP domain, the PWWP domain plays a role in the targeting of enzyme to pericentric heterochromatin [18]. Whereas, PHD domain of Dnmt3a and Dnmt3b like DNMT1 plays a role in methylation independent transcriptional gene silencing by interacting with other proteins like transcriptional repressor RP58, silencer heterochromatin HP1β, HDAC1 and histone methyltransferase SUV39H1 [42-44]. Dnmt3A and Dnmt3B can recognize unmethylated DNA as well as hemi-methylated DNA as substrate and catalyze de novo methylation [39, 45]. The third member of DNMT3 family is the Dnmt3L [46], which has a N-terminal PHD domain like Dnmt3a and Dnmt3b, but the PWWP domain is absent. Moreover, the C- terminal does not have any catalytic activity [47]. The Dnmt3L does not guide Dnmt3a and Dnmt3b to the specific DNA sequence but the *in vitro* results suggest that C-terminal part of Dnmt3L directly binds with Dnmt3a and Dnmt3b and stimulates the catalytic activity [48]. Subsequent study showed that Dnmt3L makes conformational changes in Dnmt3a that facilitates the binding of Dnmt3a with DNA and *S*-adenosyle-$_L$-methionin (AdoMat), after that the Dnmt3L is dissociated from Dnmt3a, therefore the Dnmt3L functions as a substrate exchange factor for Dnmt3a and Dnmt3b (Fig. **2b**) [47]. Recent report revealed that the DNMT3L can modulate the de novo DNA methylation pattern formation by DNMT3A and DNMT3B [49].

The current studies suggest three possible mechanisms of Dnmt3A and Dnmt3B recruitment to the targeted loci first, the specific domains of Dnmt3a and 3b might have intrinsic ability to recognize DNA; second, some transcriptional repressor proteins or other factors might involve in the loading process of Dnmt3 to the targeted DNA or chromatin structures; third, the RNA interference (RNAi) process might recruit the Dnmt3 to the targeted DNA sequence. There are reports from different research groups supporting the above possible targeting mechanisms.

To date, the exact mechanism of the specific sequence targeting by Dnmt3 is not fully understood. Chen *et al.* [18] reports that the PWWP domain of Dnmt3a and Dnmt3b is required to localize these enzymes at pericentric heterochromatin. Further, the mutagenesis analysis indicates that the mammalian de novo Dnmt's are associated with chromatin *in vivo* through the PWWP domain containing region [50], but this interaction is in a sequence independent fashion [51]. The PWWP domain of Dnmt3b has positive electrostatic surface, which might be involved in the interaction process of the enzyme with negatively charged DNA (Fig. **4a**) [50].

The protein-protein interaction between Dnmt3 and transcription repressor or other protein has also been reported in targeted loading of Dnmt3 [52-54]. The PML-RAR is a leukemia-promoting fusion protein, which can recruit the Dnmt's to target promoters and cause hypermethylation and gene silencing [52]. In another report, it has been shown that the Dnmt3a activity is associated with the Myc, the direct interaction between Myc and corepressor Dnmt3a is required for the recruitment of corepressor Dnmt3a onto the *p21Cip1* promoter region, leading to de novo methylation and silencing of *p21Cip1* gene [53]. These findings indicate that the Dnmt3 enzymes function as corepressor for different TFs and cause gene silencing by de novo DNA methylation (Fig. **4b**).

Small interfering RNA (siRNA) and miRNA are well known to silence genes at the transcriptional, post-transcriptional and translational level. In addition to these mechanisms, the siRNAs can also induce CpG methylation in some systems. The recruitment of DNA methyltransferases and de novo DNA methylation of CpG dinucleotides in the small interfering RNA (siRNA) targeted gene is well known in plants (Fig. **2b**) [55]. Kanellopoulou *et al.* [56] reported that the disruption of dicer-1 expression in mouse embryonic stem (ES) cells results in complete loss of micro RNAs and epigenetic marks of centromeric repeat sequences, and re-expression of Dicer in knockout cells re-

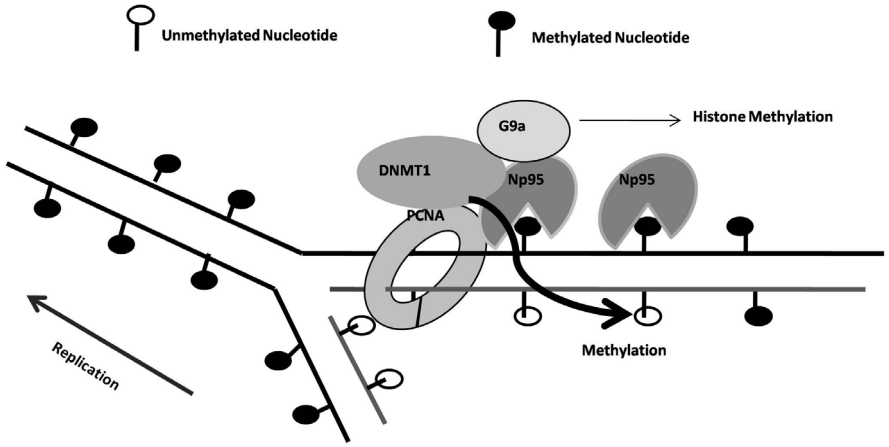


**Fig. (3). Recruitment of Dnmt1 for maintenance DNA methylation.** The schematic representation of Dnmt1 loading on the replication foci of DNA during S phase of the cell cycle. The Dnmt1 recruited by the Np95 complex at the hemi-methylated replicating heterochromatin. Dnmt1 associates with PCNA, tracks with the DNA replication complex. DNMT1 also interacts with G9a and other proteins and participate in methylation independent transcriptional gene silencing.

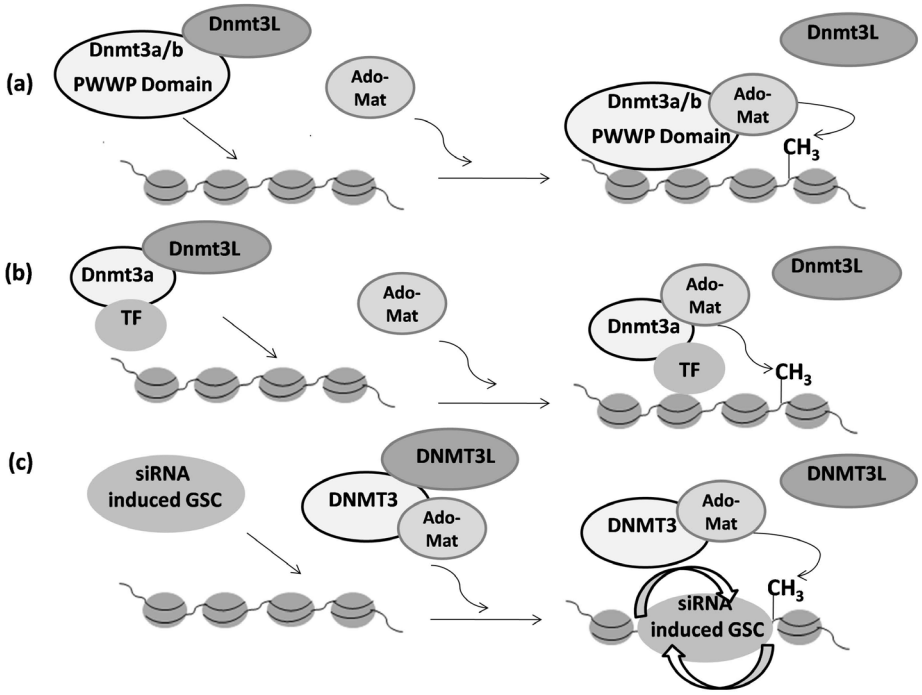


**Fig. (4). Recruitment of Dnmt3 for de novo DNA methylation.** (**a**) The PWWP of Dnmt3a and Dnmt3b is required to localize these enzymes at pericentric heterochromatin. The PWWP domain is function as a chromatin targeting domain. (**b**) TFs have the intrinsic property to interact with Dnmt's and to recruit Dnmt's activity to shutdown targeted gene expression. (**c**) The de novo Dnmt's might be recruited by small interference RNA (siRNA) induced gene silencing complex (GSC). The molecular mechanisms regulating these pathways are still unknown.

sulted in normal phenotypes. But, other study found no significant changes in DNA methylation pattern in dicer-null ES cells [57]. In human cells, Morris *et al.* [58] reports siRNA mediated transcriptional silencing of elongation factor 1 alpha (*EF1A*), and silencing is associated with DNA methylation of siRNA targeted sequence. Another study with HeLa cells demonstrates that shRNAs perfect complementary to the *RASSF1A* promoter sequences can direct partial gene silencing and DNA methylation, whereas RNAi-induced methylation was not detected in incomplete complementary base pair interactions with *RASSF1A* promoter [59]. Research of Suzuki *et al.* [60] also supports the previous findings on RNAi-induced DNA methylation. Their findings indicate that HIV-1 can be silenced by siRNA mediated mechanism that interferes with transcription and induces DNA methylation, which results in prolonged silencing of HIV-1 infection. These findings verify the existence of siRNAs mediated induction of de novo DNA methylation in mammals. But the mechanism by which RNAi triggers DNA methylation in targeted sequence is not fully understood (Fig. **4c**).

### 3.3. Regulatory Role of DNA Methylation in HSC Lineage Commitment

Hematopoiesis is a complex process under the tight regulation of different extrinsic and intrinsic factors. DNA methylation is one of the key regulatory mechanisms of normal hematopoiesis during development and differentiation. Additionally, abnormal DNA methylation pattern is a common characteristic in acute myeloid leukemia (AML) and other hematologic malignancies. The overall DNA methylation during stem cell differentiation and lineage commitment is a more complex process in which a general pattern of decreased methylation is observed in gene-regulatory elements of the chosen lineage, whereas the gene-regulatory regions associated with other lineages are increasingly methylated [61]. This indicates that methylation and de-methylation both have an essential role in proper lineage progression.

Current findings reveal that DNA methylation is critical for hematopoietic differentiation, a genome-wide DNA methylation analysis in primary mouse HSCs reports that globally DNA methylation is most abundant in HSCs with a reduction of 40% in CMPs and a 67% in erythroblasts, respectively [62]. Other reports are also demonstrating genome wide decline in DNA methylation during erythropoiesis [63, 64]. The DNA methylation increases in fully differentiated lymphoid cells when compared to early progenitor [65, 66]. DNA methylation analysis report of Block *et al.* [64] shows that erythroid differentiation is associated with genome-wide loss of DNA methylation and lymphoid differentiation is associated with the global acquisition of DNA methylation, whereas granulocytes and monocytes differentiation follow default DNA methylation when compared to MMP (Fig. **1**).

Further reports viewed the striking epigenetic plasticity during the lymphoid and myeloid commitment. Most of the differentially methylation regions (DMRs) distinguishing $MPP^{FL+}$ cells from CLP loss DNA methylation during early lymphoid commitment, whereas during subsequent transition

to DN1 (thymocyte progenitor) 15 fold more DMRs gain methylation. Similarly, during the early myeloid commitment from $MPP^{FL+}$ cells observed with more hypermethylated DMRs, but nearly all DMRs observed with the loss of methylation during transition from CMP to GMP [66]. Other genome wide DNA methylation analysis report also identified roughly twice as many genomic regions with higher methylation in CLP than regions with higher methylation in CMP [64]. This clearly suggests that DNA methylation is high in lymphoid lineage compared to the myeloid lineage.

DNMTs are the main regulators of DNA methylation during lineage commitment and final differentiation process. The impaired function of DNMTs disrupts hematopoietic homeostasis [67, 68]; consistently, loss of DNMT1 phenotype is reported with defective differentiation and unbalanced lineage commitment process [69, 70]. Moreover, reduced expression of DNMT1 in hematopoietic stem and progenitor cells is associated with reduced lymphoid differentiation and skews progenitor toward the myeloid state [69]. Recently, Trowbridge *et al.* [70] found different functional and molecular defects in DNMT1 deficient HSCs, defects in HSCs self-renewal, niche retention, and lineage commitment were observed. Taking together information from different studies, we found that Dnmt1 is essential for self-renewal of HSCs and lymphoid commitment and its down regulation is required to skew commitment process toward myeloid lineage (Fig. **1**) [69, 70].

Dnmt3a and Dnmt3b are essential for HSCs self-renewal and loss of both impairs self-renewal of HSCs but loss of Dnmt3a or Dnmt3b alone does not show any effect [71], the increased expression of Dnmt3b was observed during CLP to DN1 differentiation and it is down regulated during myeloid commitment which helps to maintain hypo-methylation in myeloid committed cells [66]. Dnmt3a deficient HSCs show aberrant methylation which results in the up-regulation of multipotency specific genes and down-regulation of differentiation specific genes [72]. *DNMT3B* expression is higher in CD34+ cells compared to differentiated cells; also the expression level of *DNMT3B* is high in AML compared with the normal bone marrow cells [73]. These findings show the role of DNMT3B in epigenetic regulation during HSCs differentiation and its aberrant expression results in leukaemia development.

As we have discussed earlier that DNA methylation is one of the key regulatory mechanisms of HSC lineage commitment and differentiation which in turn is regulated by the interplay between DNMTs and TETs. TET family proteins are well-known to modify DNA by hydroxylating 5mC. Further, oxidation of 5hmC gives rise to unmodified cytosine. *TET2* is a close relative of *TET1* and is also considered as a key epigenetic regulator of normal and malignant hematopoiesis. *TET2* mutations are common in myelodysplastic syndromes (MDS) [74] and other myeloproliferative disorders [75]. But, *TET1* and *TET3* mutations are rarely identified in myeloproliferative neoplasms. Another group reported impaired hydroxylation of 5mC in myeloid carcinomas, patients with *TET2* mutations carrying low levels of 5hmC in genomic DNA compared to healthy individuals [23]. Most of the mutations in TET2 coding region are heterozygous and disrupt the catalytic activity of TET2 protein [76]. These reports suggest that TET2 plays an important role in the regulation of normal hematopoiesis. Ko *et al.* [23] reports that the shRNA-mediated depletion of TET2 skewed the differentiation of mouse hematopoietic precursors towards monocyte/macrophage lineages *in vitro*. Another group reports an animal model of conditional TET2 loss in the hematopoietic compartment which leads to increase stem cell self renewal and hematopoietic homeostasis imbalance [77]. Fig.ueroa *et al.* [78] also found that the loss of TET2 in $CD34^+$ cells increases the expression of stem cell markers and impair myeloid differentiation. These studies suggest that TET2 limits the function of HSCs and maintain homeostasis. A microarray profiling study of normal hematopoietic cells proposed that the committed $CD33^+$ myeloid progenitor cells exhibit significantly higher *TET2* and *TET3* expression, but a lower level of *TET1* expression compared to the $CD34^+$ cells. The *TET* genes expression level in MNC was similar to that of $CD34^+$ cells, but significantly higher (*TET1*) and lower (*TET2* and *3*) than that observed in $CD33^+$ cells [79]. Thus, this study suggests that *TET1* is down regulated and *TET2/3* are upregulated during hematopoietic lineage commitment. TET1 expression is also significantly higher in MLL-rearranged AML relative to normal controls, but *TET2/3* do not show any significant dysregulation in MLL-rearranged AML compared to normal controls [79]. *TET1* functions as a tumor suppressor in solid tumors [80, 81], but in leukemogenesis, it works as an oncogene [79], recent report on *TET1* proposed its function as a tumor suppressor of hematopoietic malignancy, more specifically B-cell lymphoma [82]. Taking together these observations *TET1* seems to have distinct role depending on the type of cancer. *TET3* expression level is dynamically regulated during hematopoietic differentiation in murine model [77, 23]; loss of TET3 results in merely a moderate imbalance in the level of 5hmC, however the overall hematopoiesis takes place normally [83]. TET2 and TET3 seem to have overlap compensation functions and further studies are required to resolve overlapping functions of TET2 and TET3 in hematopoiesis.

## 4. HISTONE MODIFICATIONS IN HSC LINEAGE COMMITMENT AND DIFFERENTIATION

Histones play a major role in epigenetic processes and maintain chromatin structure [84]. The fundamental unit of chromatin is nucleosome which is made up of ~146 base pairs of DNA wrapped around an octamer of four core histone proteins including H2A, H2B, H3, and H4. Histone H1 acts as a linker that binds to DNA entering and exiting from the nucleosome, thus providing further structural stability [5, 85, 86]. The surface of nucleosome consists of several modifications such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation [11] (Fig. **5a**). The epigenetic marks on histones regulate the chromatin accessibility and thereby the transcriptional activation or repression, the pattern of these epigenetic marks is regulated by several catalytic enzymes. The epigenetic marks of histones are categorised into two groups such as activation marks (H3R2me2s, H3K4me2, H3K4me3, H3K9ac, H3K14ac, K3K27ac, and H4K16ac) and repression marks (H3K9me3, H3K27me1, H3K27me3, and H4K20me3) [86]. Histone H3R2me2s, H3K4me2,3, H3K9ac, H3K14ac, K3K27ac, and H4K16ac

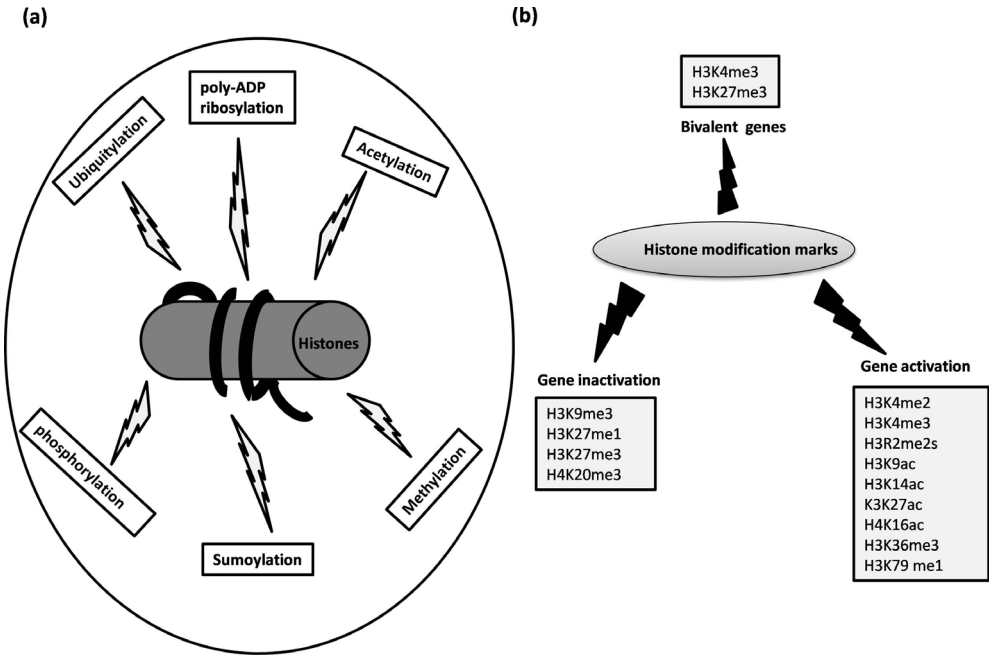


**Fig. (5). Histone epigenetic modifications.** (**a**) Posttranslational regulators of histones. (**b**) Different types of histone epigenetic markers related to transcriptional activation and inactivation.

marks are associated with euchromatin regions particularly in transcriptional enhancer and promoter regions [87, 88]. Whereas, H3K27me1, H3K27me3, H3K9me3, and H4K20me3 marks are involved in the formation of constitutive heterochromatin and related to the transcriptionally silenced genomic regions [86, 89] (Fig. **5b**).

Histone methylation is a post-translational modification: the methylation marks are established by histone methyltransferases and removed by histone demethylating enzymes. Three methylation sites, H3K4, H3K36 and H3K79 are implicated in transcriptional activation. Whereas, methylation sites, H3K9, H3K27 and H4K20 are connected to transcriptional repression. Transcriptionally active chromatin regions are commonly recognized by H3K4me2 and H3K4me3 (activation marks) methylation marks. Whereas, heterochromatin or silenced DNA associated genes are associated with H3K9 and H3K27 (repressive marks) methylation marks. H3K4me3 and H3K27me3 are found in the undifferentiated cells and involved in the hematopoietic stem cell fate determination [90, 91]. The coexistence of H3K4me3 and H3K27me3 on promoter region fine-tunes the gene expression during development and allows either rapid activation or stable silencing upon differentiation of HSCs [92]. K3K4 methylation is generated by the SET [Su(var)3-9, Enhancer of zeste, Trithorax] and mixed lineage leukemia (MLL) family of HMTs [93, 94] and erased by HDMs, lysine-specific demethylase 1 (LSD1) [95] and the Jumonji AT-rich interactive domain 1 (JARID1) family of proteins [96, 97]. Whereas, H3K27 methylation is generated by the enhancer of zeste homolog 2 (EZH2), and removed by UTX and Jmjd3 [98-100].

A recent study showed that lymphoid-affiliated genes are associated with histones H3 and H4 acetylation and H3K4-dimethylation (activation marks) in HSCs and this accessible conformation allows lymphoid lineage commitment and further histone modifications like H3K4me3 [101]. Whereas, on commitment of HSCs in other lineages, the histone marks present in HSCs are replaced by repressive H3K9/27-methylation marks [101]. Ectopic expression of Ezh2 reported a significant increase in HSC numbers and myeloid lineage cells [102]. These results suggest that active histone modification marks are already present in lymphoid specific loci in HSCs. Upon ectopic expression of Ezh2, active marks are replaced by repressive marks which result in an increase in HSC numbers and myeloid lineage cells. Conditional ablation of LSD1 in murine HSCs observed with increased activating H3K4me1 and H3K4me2 marks at enhancers and promoters of stem- and progenitor cell-specific genes in differentiating cells. These results suggest that upon differentiation, LSD1 silences hematopoietic stem and progenitor cell-specific genes by removing the activating H3K4 methylation marks from enhancer and promoter regions to promote differentiation [103].

Histone acetylation is one of the most studied translational modifications, which is catalyzed by the HATs such as p300, MORF, MOZ, MOF, HBO and TIP60 [17]. Acetylation is more conserved at lysine residues of histone H3 and H4, but is less conserved at H2A and H2B. H3 acetylation is restricted to K9, K14, K18, K23, whereas, K5, K8, K12, K16 acetylation occurs at H4 [104, 105]. In vertebrates, H2A is acetylated at K5 and K9, whereas K5, K12, K15 and K20 acetylation is known with H2B [105]. Histone modifications change transcription factor binding and thereby regulate chromatin structure and function. Acetylation is generally associated with transcriptional activation and maintains the transition of heterochromatin to euchromatin and facilitates the gene expression. The H3 and H4 acetylation marks are associated with lymphoid lineage specific genes [101].

Histone acetylation marks can be removed by HDACs. In humans, 18 HDACs are documented, which are divided into

four classes such as Class I, II, III and IV. Class I (HDAC1, HDAC2, HDAC3 and HDAC8) are ubiquitously expressed and regulate cell proliferation [106]. Class II HDACs can shuttle between nucleus and cytoplasm and express in a tissue-specific manner; they are subdivided into Class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10). Class III HDACs are NAD dependent and include 7 members of sirtuin family (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7). Class IV includes only one member, HDAC11 [107]. HDACs are essential for the maintenance of HSCs development and differentiation. One study on HDACs expression has shown that HDACs expression is very low in HSCs, and increased expression is observed in differentiated progenitors and erythroid precursors but mature myeloid cells particularly granulocytes are noticed with decreased levels of HDACs; they also report that HDAC1 expression is suppressed by CAAT/enhancer binding protein and activated by GATA-1 in myeloid and erythro-megakaryocytic differentiation, respectively [108]. These observations suggest that HDACs also play an important role in HSCs fate decision. The levels of HDAC1 and HDAC2 are essential for erythrocyte-megakaryocyte differentiation, double knock out of HDAC1 and HDAC2 leads to apoptosis in megakaryocytes (MKs) and thrombocytopenia [109]. Toscano *et al.* [110] report that valproic acid (VPA), HDAC inhibitor modulates differentiation process of hESC and increases both the number of MKs and platelets. Many studies report that HDACs mediated repression of gene expression can increase the rate of cell proliferation [111]. HDAC1/2 are well known to down regulate the expression of *P21/27/57* by hypoacetylation and also affect the activity of P53; HDAC1 and P53 both antagonistically regulate the *P21* expression with the help of SP1 [112]. This data shows that first class HDACs have a strong role in cell cycle progression in proliferating cells.

## 5. MicroRNAs MEDIATED REGULATION OF GENE EXPRESSION

MicroRNAs (miRNAs) are small and highly conserved class of non-coding RNAs (ncRNAs) involved in many cellular processes like cell cycle, apoptosis and differentiation by regulating target gene expression [113]. Mature miRNAs are short, about ~22 nucleotides in length, generated from pre-miRNAs by Dicer mediated mechanism [114]. These Pre-miRNAs in turn generated by Drosha mediated processing of pri-miRNAs in nucleus [115, 116]. The Pre-miRNAs are transported from nucleus to cytoplasm by Ran-GTP and karyopherin exportin complexes [117, 118]. Mature miRNAs are recognized by different proteins of Argonaute family and form miRNA-induced silencing complex (miRISC) with target RNA [119]. The degradation of RNA and repression of mRNA translation are two commonly known miRNA mediated silencing mechanisms (Fig. **6**).

### 5.1. MicroRNAs in Lymphoid Pathway

Previous studies observed functional significance of miRNAs in HSC differentiation and lineage commitment. Over the past decade, many studies have confirmed the role of miRNA in lymphopoiesis. One of the miRNA studies confirmed the role of miR-181 in lymphopoiesis; miR-181 expression is high in thymus and other lymphoid organs which contain T-lymphocytes and also its expression is high in B-lymphocytes compared to undifferentiated bone marrow cells. It acts as a positive regulator of B-lymphocyte differentiation [120]. The increased expression of miR-181 in mature T-lymphocytes enhances the T-cell receptor sensitivity for antigens, whereas lower expression decreases the sensitivity to antigen and alters both the positive and negative selection [121]. Neilson *et al.* [122] report the increased expression of miR-181a in double positive thymocytes. They found that miR-181a can reduce the expression of CD69 Bcl-2 and the T-cell receptor, all of which have a coordinating role in positive selection. miR-142 expression was found to be low in T-lymphoid lineages and high in B-lymphoid lineages. The ectopic expression of miR-142 had no significant effect on the B-lymphoid lineage, but it altered lineage differentiation with a 30–40% increase in T-lymphoid lineage [120, 123]. One of the recent studies on miRNA found high expression of seven miRNAs including miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-7f in T-cell subsets. Among these, miR-142-3p and miR-142-5p were found to play an important role in T-cell development and also their expression changed dynamically during T cell differentiation and maturation [124]. Zhou *et al.* [125] showed an miRNA, miR-150, is mainly expressed in spleen, thymus and lymph nodes and is up-regulated during the development of mature B and T cells; indeed, prematurely expressed miR-150 impairs B-cell development with moderately enhanced T cell development. miR-150 overexpression in HSC does not affect the B cell lineage commitment, but overexpression blocks the process of pro-B to Pre-B cell differentiation. Stage specific expression of miR-150 regulates B-cell differentiation by targeting the transcription factor c-myb which is involved in the normal lymphocyte development, and ectopic expression of miR-150 impairs the process of lymphocyte development [126] (Fig. **7**).

### 5.2. MicroRNAs in Myeloid Lineage Commitment and Differentiation

Many studies have reported the regulatory role of miRNAs in myeloid lineage, one such differential expression profile of murine miRNAs found up-regulation of many miRNAs such as miR-125b, miR-196a, miR-196b, miR-130a, let-7d, miR-148b, and miR-351 in stem and progenitor cells compared to differentiated cells, whereas other miRNAs such as miR-140, miR-200c, miR-484, miR-331, and miR-324–5p were down regulated in stem and progenitor cells compared to differentiated cells [127]. A comparative study of myeloid- and lymphoid-selective miRNAs found up-regulation of miR-223 in myeloid cells, whereas it was down-regulated in lymphoid cells, and in myeloid cells its expression was higher in GMP compared to MEP [127]. miR-223 is also noticed with up-regulation in granulocytes as compared to monocytes [128]. One study showed that miR-155, miR-222, miR-424 and miR-503 partially promotes monocytes differentiation and the combination of these four miRNAs shows more positive effects on monocytes differentiation compared to their individual activity [129]. During monocytes differentiation, Wang *et al.* [130] observed positive correlation between miRNAs-mRNAs like miR17/C13orf25, miR93/MCM7 and miR22/MGC14376. Another miRNA, miR-155 was found to play a role in macrophage differentiation from monocytes [131].

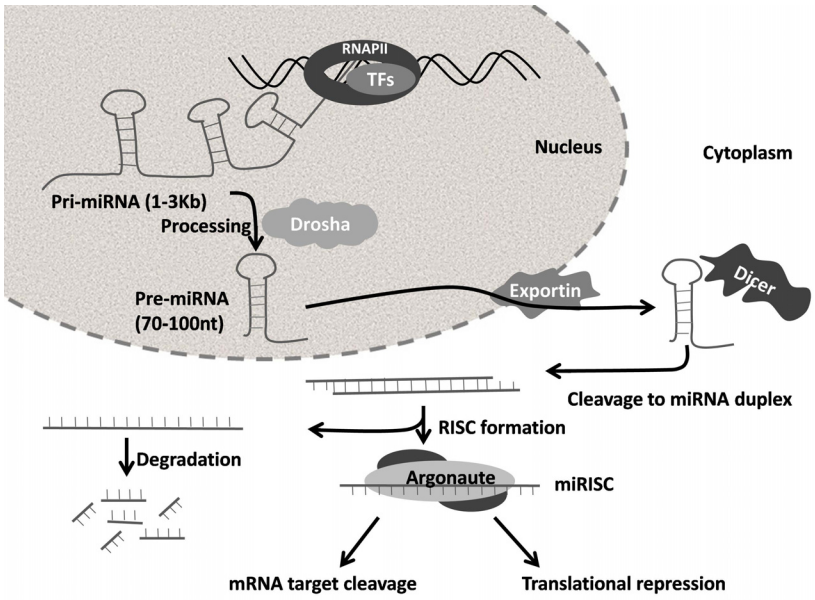


**Fig. (6).** General overview of the biogenesis of miRNAs, RISC formation and silencing mechanism.

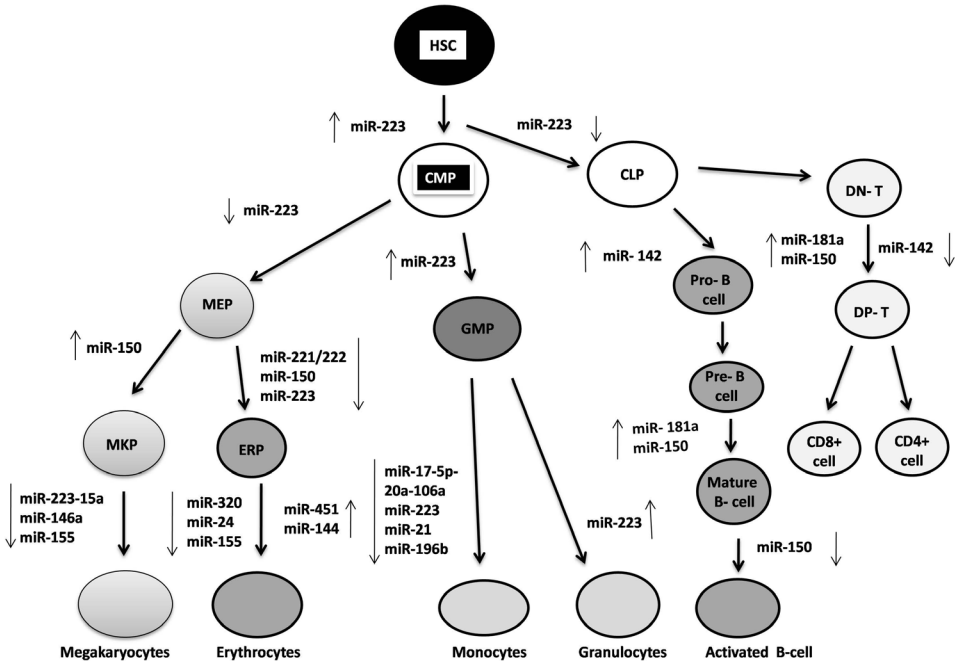


**Fig. (7). MiRNA regulation in lineage selection and differentiation process of myeloid and lymphoid cells.** HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; MKP, megakaryocyte progenitor; ERP, erythrocyte progenitor; Pro-B cell, progenitor B cell; Pre-B cell, precursor B cell; DN-T, double negative T lymphocyte; DP-T, double positive T lymphocyte; $CD8^+$ cell, TC cells; $CD4^+$ cell, TH cell. Direction of arrows represents the increased and decreased levels of miRNA.

Fontana *et al*. [132] reports down-regulation of miR-17-5p-20a-106a during monocyte differentiation, whereas miR-17-5p-20a-106a transfection results in enhanced blast proliferation and inhibition of monocytes differentiation and maturation by targeting AML1 protein expression, leading to macrophage colony-stimulating factor (M-CSF) receptor down regulation. miR-21 and miR-196b are inhibited by growth factor independent-1 (Gfi1) which regulates the normal granulocyte differentiation [133]. A recent study shows that miR-142-3p is a negative regulator of colony-stimulating factor-1 (CSF1) induced macrophage differentiation by targeting Egr2. CSF1 enhances the expression of Early Growth Response 2 (Egr2), which in turn down regulates mir-142-3p and promotes monocyte differentiation in association with Spi-1/PU1by increasing the expression of *cFMS* gene [134].

The functional significance of certain miRNAs has also been explored in erythropoiesis during the last 10 years research work. miR-221 and miR-222 are down-regulated during the early stages of erythropoiesis, thereby unblocking the expression of c-kit, a receptor of SCF, which has an important role in early erythropoiesis to regulate proliferation and survival of erythroblast [135, 136]. Another miRNA, miR-223 is also down-regulated during erythroid differentiation, which targets LIM-only protein 2 (LMO2), an essential factor for erythroblast differentiation [137]. Mittal *et al.* [138] reports that miR-320 is down-regulated during erythroid differentiation and it targets 3'-UTR region of SMAR1 (scaffold/matrix attach-ment region-binding protein 1). They found that SMAR1 regulates erythroid differentiation by targeting miR-221 and miR-222. miR-24 is highly expressed in hematopoietic progenitors cells (HPCs) and down-regulated during erythroid differentiation. It interferes with activin signaling by down-regulating ALK4 (Anaplastic lymphoma kinase-4) expression and reduced the erythroid burst-forming units and colony-forming units [139]. miR-144 along with miR-451 is found to have a role in erythroid development. They showed increased expression during erythropoiesis and their expression is regulated by GATA-1 transcription factor [119-122]. Human CD34+ HSPCs shows high expression of miR-155 and it is down-regulated during myeloid differentiation [140]. Two individual studies also found inhibitory effects of miR-155 on erythroid differentiation [140] and megakaryocyte differentiation [141]. miR-150 is moderately expressed in MEP and progressively down-regulated during the erythropoiesis, whereas increased expression is observed during megakaryocytopoiesis [142]. miR-150 regulates erythroblast-megakaryoblast fate decision by targeting c-myb [142], Charlene *et al.* [143] reports that thrombopoietin is a regulator of megakaryocytopoiesis which controls c-myb level by increasing the expression of miR-150. One study on miRNA expression in megakaryocytopoiesis reports that the expression of miR-223, miR-15a and miR-16-1 cluster is down-regulated during megakaryocytopoiesis, but after 14 days of culture, these expressions are back to comparable with that of CD34+ cells, whereas miRNAs like miR-17, miR-20, miR- 106, miR-155,10a 126, 10b and miR-181b are down-regulated during megakaryocytopoiesis [144]. CXCR4-SDF-1 signaling plays an important role in the MKs differentiation, whereas miR-126 inhibits this process by targeting CXCR4 mRNA. PLZF-miR-146a-CXCR4 forms a positive feedback loop required for megakaryocytopoiesis. PLZF inhibits the expression of miR-146 and, in turn, promotes the expression of CXCR4 [145] (Fig. **7**). This information strongly suggests the role of miRNAs in HSCs self-renewal, lineage commitment and differentiation. But, further study needs to utilize these miRNAs as a new therapeutic intervention in different haematological disorders.

## CONCLUSION

Studies have evidenced the role of different epigenetic regulators in the hematopoietic lineage commitment and differentiation process of different blood cells. There are many questions to be addressed in epigenetics and we are now only beginning to understand the mechanism of DNA methylation and histone modifications and miRNA mediated interference. But increasing evidences have shown the role of different DNMTs and genomic DNA methylation in the hematopoietic lineage commitment and differentiation of different blood cells. Different histone modification marks are also known to play a role in HSCs differentiation process by regulating the process of transcription of different genes playing a role in hematopoiesis. The mode of action of several miRNAs is not yet understood, but mostly they regulate gene expression by mRNA target cleavage and translational repression. Several studies in the last decade have shown the role of miRNAs in the regulation of hematopoietic lineage commitment, progenitor proliferation and their differentiation into respective blood cells. In this review, we tried to cover the DNA methylation and demethylation mechanism and also the role of different epigenetic regulators in hematopoiesis.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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# Chapter 10
# microRNAs: Key Players in Hematopoiesis

**Sanjeev Raghuwanshi, Hanuma Kumar Karnati, Shilpa Sarvothaman, Usha Gutti, Raja Gopal Venakata Saladi, Prathibha Reddy Tummala, and Ravi Kumar Gutti**

## Abbreviations

| | |
|---|---|
| CLP | Common lymphoid progenitor |
| CMP | Common myeloid progenitor |
| CTCL | Cutaneous T-cell lymphoma |
| DLBCL | Diffuse large B-cell lymphoma |
| GMP | Granulocyte-monocyte progenitor |
| HL | Hodgkin lymphoma |
| HSC | Hematopoietic stem cell |
| HSPC | Hematopoietic stem and progenitor cells |
| LT-HSCs | Long-term reconstituting HSCs |
| MALT | Mucosa-associated lymphoid tissue lymphoma |
| MCL | Mantle cell lymphoma |
| MEP | Megakaryocyte-erythrocyte progenitor |
| MM | Multiple myeloma |
| ST-HSCs | Short-term reconstituting HSCs |
| SzS | Sézary syndrome |
| Tregs | T regulatory cells |

S. Raghuwanshi • H.K. Karnati • S. Sarvothaman • R.K. Gutti (✉)
Stem Cells and Haematological Disorders Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad, Telangana 500 046, India
e-mail: guttiravi@gmail.com

U. Gutti • R.G.V. Saladi
Department of Biotechnology, GITAM Institute of Science, Gandhi Institute of Technology and Management (GITAM) University, Visakhapatnam, Andhra Pradesh 530 045, India

P.R. Tummala
Fernandez Hospital Pvt. Ltd., Bogulkunta, Hyderabad, Telangana 500 001, India

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## Introduction

microRNAs (miRNAs) are one of the most studied noncoding RNA molecules found in animals, plants, and DNA viruses. They regulate gene expression at post-transcriptional level by RNA silencing or destabilizing target transcripts. Mature miRNAs are small, about 20–25 nucleotides in length, generated from pre-miRNA that underwent the slicing mechanism by Dicer [1]. These pre-miRNAs are in turn generated from the pri-miRNAs that undergo splicing mechanism by Drosha in nucleolus [2, 3], which then transported to the cytoplasm with the help of Ran-GTP and karyopherin exportin complexes [4–6]. Mature miRNA in the presence of Argonaute family proteins binds to the target mRNA in the RNA-induced silencing complex (RISC) [7]. This leads to gene silencing either by the degradation of the mRNA or translation repression (Fig. 10.1).

Recent studies have evidenced an increased role of miRNA in the regulation of normal hematopoiesis, the formation of blood components from hematopoietic stem cells (HSC). The differentiation of HSC into different lineages involves complex network of transcription factors, growth factors, and cytokines, signaling path-

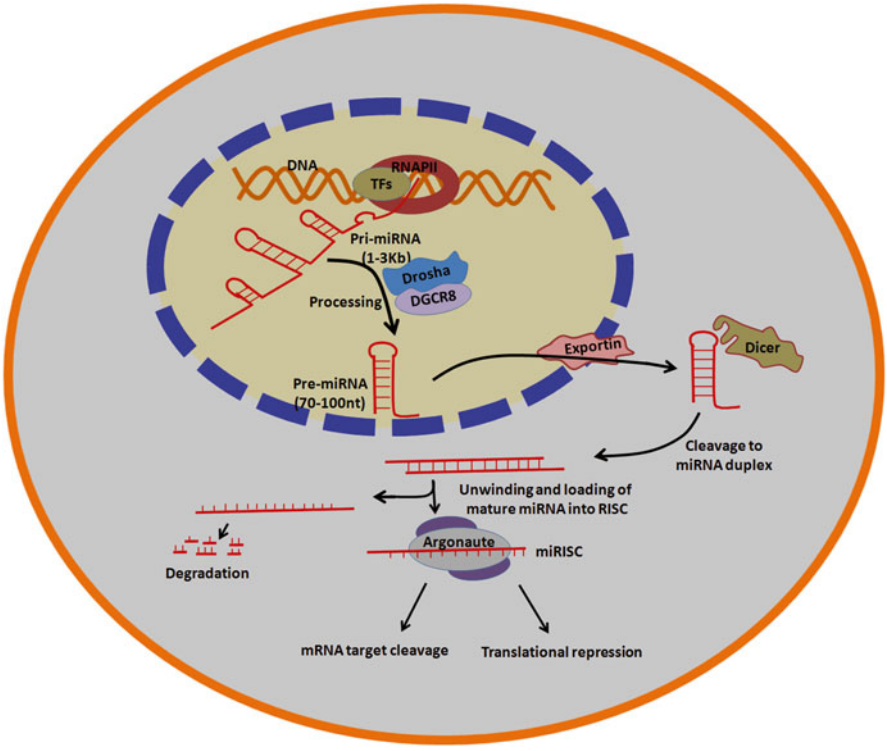


**Fig. 10.1** Biogenesis pathway of miRNA, RISC, and silencing mechanism

ways, and interconnected molecular networks that requires proper regulation. Even though several transcription factors have been found to be regulating the process of hematopoiesis, evidence to establish the role of miRNAs in hematopoiesis has been improved significantly in recent years (Table 10.1). Here, we focus on the role of miRNA in hematopoiesis and hematological disorders.

## Hematopoiesis

Hematopoiesis is the formation of blood cells from HSC. Hematopoiesis is highly regulated and operates throughout the life of an organism. During embryonic development, the first wave of blood cell production (termed "primitive hematopoiesis") comes from yolk sac. The primitive wave of hematopoiesis is transient and rapidly replaced by the adult type hematopoiesis (termed "definitive hematopoiesis") in fetal liver, which finally form niche in bone marrow [8, 9]. The hematopoietic stem and progenitor cells (HSPC) reside primarily in the bone marrow during adult life, though several studies have shown their presence in the blood and lymph during homeostasis and stress [10–13].

During hematopoiesis, HSC responds to several extracellular signals and differentiate into different cells of the myeloid and lymphoid lineages (Fig. 10.2). Overall, the entire hematopoietic system is organized as a tree where the developmental potential is restricted in each branch point: each step is tightly regulated by signals from microenvironmental and macroenvironment, specific transcription factors, signaling molecules, as well as miRNAs [14].

## Hematopoietic Differentiation

Several reports have shown the key roles of miRNAs in normal hematopoiesis [15, 17]. After the first groundbreaking discovery of miRNA Lin 4 which downregulates Lin14 transcript, several studies have shown significant role of miRNA in hematopoietic system markedly increased to more than 500 as listed in miRBase (www.mirbase.org) [18]. The role of miR-125a is known when deletion studies of Dicer in HSCs led to enhanced apoptosis and reduction in hematopoietic ability [19]. Studies have shown that several miRNAs which are expressed at early progenitor stage of CD34+ HSCs of bone marrow or peripheral blood compartments are similar. Functional validation studies have shown that miR-17, miR-24, miR-146, miR-155, miR-128, and miR-181 prevent the differentiation of early stage progenitor cells. And few miRNAs such as miR-16, miR-103, and miR-107 act later on, and miR-221, miR-222, and miR-223 regulate the terminal stages of hematopoietic development [20].

**Table 10.1** miRNAs role in hematopoiesis

| miRNA | Expression | Targets | Function | Regulated by | References |
|---|---|---|---|---|---|
| *Granulopoiesis and Monopoiesis* | | | | | |
| miR-223 | Up G | NFI-A/E2F1 MEF2C | Inhibits granulocytic proliferation and activity | CEBPα, NFI-A, E2F1 | Fazi et al. [89], Johnnidis et al. [91], Pullikan et al. [179], Garzon et al. [180] |
| miR-21 miR-196a | Down GMP | Unknown | Regulation between CMP-GMP transition | GFI-1 | Velu et al. [93] |
| miR-424 | Up Mo | NFI-A | Induces Mo differentiation and proliferation | PU.1 | Rosa et al. [95], Forrest et al. [96] |
| miR-17-92 | Down Mo | AML1 | Inhibits Monopoiesis in vitro but no effects in vivo | AML1 | Fontana et al. [94], Ventura et al. [191] |
| *Megakaryopoiesis* | | | | | |
| miR-130a | Down | MAFB | Unknown | Unknown | Garzon et al. [58] |
| miR-10a | Down | HOXA1 | Unknown | Unknown | Garzon et al. [58] |
| miR-150 | Up | c-Myb | Drives MK differentiation | Unknown | Lu et al. [56], Barroga et al. [59] |
| miR-155 | Down | Ets-1/Meis1 | Inhibits MK differentiation and proliferation | AP-1, JNK | Kanellopoulou et al. [181], O'Connell et al. [182], Rodriguez et al. [183], Romania et al. [62] |
| miR-146a | Down | CXCR4 | Inhibits MK differentiation and proliferation | PLZF | Navarro et al. [63] |
| miR-125b-2 | Down | DICER1/St18 | Increase MK proliferation | Unknown | Klusmann et al. [184] |
| *Erythropoiesis* | | | | | |
| miR-221 miR-222 | Down | c-KIT | Inhibits erythroid proliferation and perinatal Hb switching | Unknown | Felli et al. [76], Bruchova et al. [78], Zhan et al. [80], Gabbianelli et al. [77] |

(continued)

**Table 10.1** (continued)

| miRNA | Expression | Targets | Function | Regulated by | References |
|---|---|---|---|---|---|
| miR-451<br>miR-144 | Up | RAB14,<br>14-3-3Zeta | Regulates oxidative stress, positive regulation of terminal erythroid differentiation and in zebrafish hemoglobin synthesis (miR-144) | GATA-1 | Bruchova et al. [78], Zhan et al. [80], Rasmussen et al. [185], Kim et al. [85], patrick et al. [83], Yu et al. [84] |
| miR-24 | Bi-phasic | hALK4 | Modulates negatively erythropoiesis | Unknown | Wang et al. [86] |
| miR-155 | Down | PU-1/ETS-1<br>CEBPb/SHIP1 | Inhibits erythropoiesis | AP-1,<br>JNK | Escobar et al. [22], Ghisi et al. [23], Wu et al. [30], Lu et al. [31], Bruchova et al. [78], Masaki et al. [186], O'Connell et al. [182], Rodriguez et al. [183] |
| miR-223 | Down | LMO2 | Inhibits erythropoiesis | CEBPa,<br>NF1A,<br>E2F1 | Felli et al. [72] |
| *Lymphopoiesis* | | | | | |
| miR-150 | Down prog. | c-Myb | Inhibits the transition from pro-B to pre-B cells | Unknown | Xiao et al. [187], Zhou et al. [188] |
| | Up mature B/T cells | | Block DN3 to DN4 T-cell transition | | |
| miR-181a | Up prog. and in DP T cells<br>Down in mature B/T cells | BCL2/CD69<br>TCR/DUSP5<br>DUSP6/SHP2<br>PTPN22 | Increases CD19+ cells/Modulate late thymic T-cell development, TCR sensitivities and strength | Unknown | Neilson et al. [189], Li et al. [190] |
| miR-17-92 | Up in B/T precursors<br>Down in mature B/T cells | Bim PTEN | Modulates positively the transition from pro-B to pre-B cells | c-Myc,<br>E2F1 | Ventura et al. [191] |

(continued)

**Table 10.1** (continued)

| miRNA | Expression | Targets | Function | Regulated by | References |
|---|---|---|---|---|---|
| miR-155 | Up in Tregs and in activated T/B cells | SOCS1 SHIP-1 CEBPb AID | Enhanced Tregs proliferation Maintain competitive Treg fitness Regulates GC-B cells responses, innate immunity, T-cell dependent antibody responses, negative regulator of somatic hypermutation | Foxp3 | Rodriguez et al. [183], Thai et al. [192], Vigorito et al. [193], Stahl et al. [194], Zheng et al. [195], Teng et al. [196], Dorsett et al. [197] |

*MK* megakaryopoiesis, *Mo* monopoiesis, *GC* germinal center, *DN3* double negative stage 3, *DN4* double negative stage 4 *Prog* progenitors

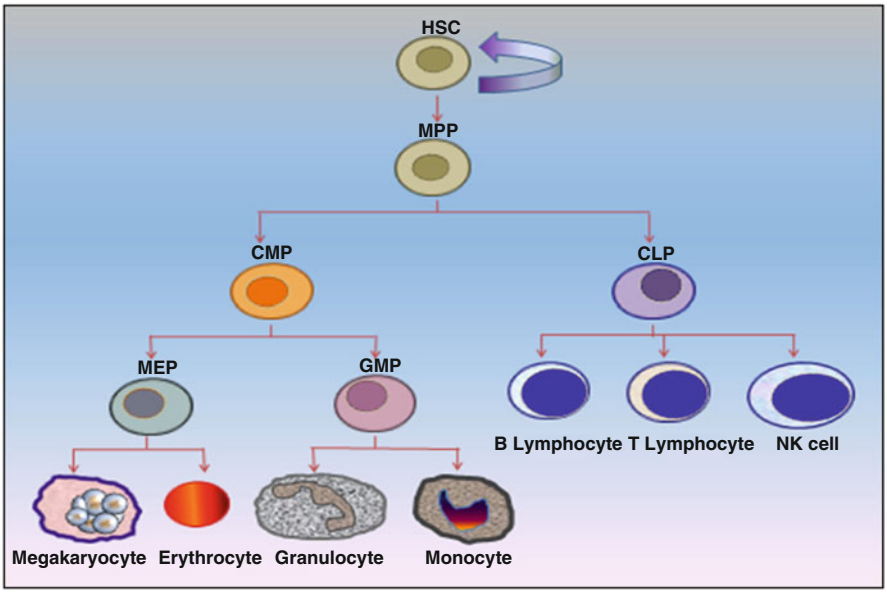


**Fig. 10.2** A schematic representation of hematopoietic development depicting the specific points in differentiation

## Lymphopoiesis

Lymphopoiesis refers to a developmental process involving distinct stages of commitment and differentiation, finally resulting in the generation of immune cells that mediate adaptive immunity, namely—T cells, B cells, and NK cells. These cells develop from a common lymphoid progenitor which is developed from the pluripotent HSC. The process ends up in the generation of a vast and highly diverse repertoire of T, B, and NK cell which efficiently fights against pathogenic organisms. Evidences suggest that these short noncoding RNA extends their influence in almost every aspect of lymphocyte development.

Expression pattern of miRNA in HSCs have been studied extensively. miR-125b is a highly expressed class of miRNA in HSCs and its expression decreases in committed progenitors. Mature peripheral blood cells derived from the miR-125b-overexpressing HSC increase early B-progenitor cells in the spleen and induce the expansion and enrichment of the lymphoid-balanced and lymphoid-biased HSC subset through an antiapoptotic mechanism, reducing the mRNA expression levels of two proapoptotic targets, Bmf and KLF13, which can have drastic effect on the phenotype of an organism [21]. miR-155-deficient CD4+ T cells in vitro and in vivo have shown defective Th17 cell cytokine and Jarid2 is found to be more expressed which is required to recruit Polycomb Repressive Complex 2 to chromatin in Th 17 and T regulatory (Treg) cells [22].

### *T Cell Development and Differentiation*

T cells are immune cells that are the critical controllers of adaptive immune response in organisms. T cells develop from the common lymphoid progenitor cells through several critically regulated developmental and differentiation steps.

T lymphocyte has several subtypes that are classified on the basis of certain surface marker expression, type of cytokines they release, and maturation status. Cell surface markers such as CD4, CD8, and CD3 define the developmental stages of a particular T lymphocyte in the thymus. On the basis of expression of these markers T cells are grouped as double negative (DN), double positive (DP), and single positive (SP) CD4+ or CD8+ cells. Mature T cells that get released from the thymus forms another class of T cells—naïve T cells, effector T cells, and memory cells T cells each with corresponding cell surface components.

The miRNA miR-155 can be induced by signals that activate T cells, B cells, and antigen-presenting cells, and regulate immune response of these cells such as the differentiation of T regulatory (Treg) cells and CD4+ T helper (Th) cells. They are extensively studied class of miRNA as it influences several immune cells. miR-181 family member, miR-181a, has a profound role in T cell biology as it is evident from distinct studies carried out by several independent research groups.

miRNA profiling study of three different maturation stages of T lymphocytes, DP (CD4+ CD8+), SP CD4 (CD4+ CD8−), and SP CD8 (CD4− CD8+), has identified the role of miRNAs in regulation of T cell maturation. miR-150 was highly expressed in this population of cells during maturation and that its target was identified to be NOTCH3, a key player in T cell development. The studies have identified an indirect relation between miR-150 and NOTCH-3p by upregulation of miRNA 150 during T cell maturation [23]. They are highly expressed in double negative (DN) and double positive (DP) population and downregulated to undetectable levels in mature T lymphocytes [24].

## *CD4 and CD8 T Cell Biology*

CD4 T cells are lymphoid derived immune cells which plays a central role in adaptive immunity against )pathogens. These cells not )only help B cells to make antigen specific antibodies but also involved in recruitment of neutrophils, eosinophils, and basophils to the site of activation, enhancing microbicidal activity of macrophages and synthesis of their own cytokines and chemokines.

Majority of the studies conducted to identify the expression pattern of miRNAs reported that their expression is higher in the primitive forms when compared to the differentiated form. This can be mainly attributed to the genetic switch from idealness of a progenitor cell or the hematopoietic stem cell itself to a more metabolically active developed cell. The number of miRNAs involved in different stages of T cell development has been listed in Table 10.2. For instance, miR-125b belonging to miR-125 family was reported to be highly expressed in HSCs but as it progresses into a more differentiated progenitors, the expression level gradually decreased [21]. AHR or aryl hydrocarbon receptor is often involved in the differentiation process of TH 17 cells. Study has shown TH17 activated AHR upregulates miR-132/212 clusters but not Treg cells and has also shown AHR activated TH17 differentiation was not happened in miR-132/212 cluster deficient cells. Mice deficient in the miR-132/212 cluster have shown more resistance to the development of experimental autoimmune encephalomyelitis [25].

T cells express mir-21 expression and activated αCD3/CD28 upregulates mir-21 expression. Inhibition studies of mir-21 functions in activated naive and memory T-cells have shown the regulatory role of this miRNA in different aspects of T-cell biology. Inhibition of miR-21 function in activated memory T-cells led to apoptosis, whereas in activated naive T-cells led to induction of CCR7 protein expression, hence proving multiple roles for single miRNA in two subsets of CD4+ T cells [26]. As a result of T cell specific Dicer ablation resulted in increased level of effector CD4 T cells committing to Th1 (T-helper 1) responses [27] with reduction in the development to T regulatory cells [28]. miRNA also plays a regulatory role in maintaining T cell numbers. This was reported in a study conducted by Boldin et al. The study reported miR-146a as a negative regulator of inflammation. Mice lacking miR-146 were found to be more responsive to bacterial challenges and produced more pro-inflammatory cytokines [29].

**Table 10.2** miRNAs in the different stages of T cell development

| miRNAs | Target | Function | References |
|---|---|---|---|
| miR-146 | *STAT1* | Regulation of the Treg suppressor function through the targeting of *STAT1* promotion of differentiation into Th1 cells rather than Th2 cells | Neilson et al. [189], Monticelli et al. [198], Lu et al. [199] |
| miR-17-92 | *CREB1*<br>*PTEN*<br>Bim | Regulation of effector and memory CD8+ T-cell differentiation | Wu et al. [34] |
| miR-150 | *NOTCH3* | Controls of T-cell differentiation | Zhou et al. [188] |
| | | Regulation of differentiation into the memory or effector phenotypes of T cells | Almanza et al. [200] |
| | | Helps in differentiation from DP into CD4+ and CD8+ T-cells | Neilson et al. [189], Ghisi et al. [23], Bellavia et al. [201], Indraccolo et al. [202] |
| | | Regulation of the intrathymic pre-T-cell receptor selection of T-cells | Bellavia et al. [201], Campese et al. [203] |
| miR-155 | *SOCS1* | Regulation of differentiation into the memory or effector phenotypes of T cells | Almanza et al. [200] |
| | | Controls of T-cell differentiation: to favor Th1 responses partially by modulating cytokine production | Rodriguez, et al. [183], Thai et al. [192] |
| | | Control of proliferation and homeostasis of Treg cells by stabilizing the signal of *FOXP3* through the targeting of *SOCS1* | Lu et al. [31] |
| miR-181a | CD69<br>*BCL2*<br>TCR-α<br>(DUSP5, DSP6, SHP2, PTPN22) | Regulation of positive selection by governing the homeostasis of CD4/CD8 lymphocytes and modulation of T-cell sensitivity by increasing TCR signaling to peptide antigens through the downregulation of multiple phosphatases | Chen et al. [18], Fabbri et al. [204], Neilson et al. [189], Liu et al. [205], Li et al. [206], Li et al. [190] |
| | | Regulation of iNKT cell development through the modulation of TCR signaling threshold resulting the increase responsiveness of DP thymocytes to TCR signals | Ziętara et al. [207] |

CD8 T cells are critical mediators of adaptive immune response in an organism against several pathogens. Microbe entry enables these cells to undergo clonal expansion, resulting in production of numerous clones of antigen specific CD8 T cells. Wu et al. made a comparative study of miRNA expression profiles of naïve, effectors, and memory CD8+ T cells and showed that only few miRNAs are dominantly expressed in CD8+ T cells and these miRNAs expression is dynamically regulated during differentiation process. Interestingly, a significant decrease in

miRNA expression level is seen in effector T cells, as antigen stimulation of naïve T cells results in upregulation of several genes [30].

## *T Regulatory Cell Biology*

T regulatory cells are essential components of the adaptive immune response as they are responsible for the )maintenance of immune self-tolerance. )These CD24+ CD4+ cells are characterized with expression of a transcription factor Foxp3. Most of the Foxp3 T regulatory cells are produced as a functionally mature T cell which can effectively bring about immune suppression. Like in all T cell subsets, miRNA has a regulatory role in development of these regulatory T cells also. miRNA deletion can result in certain deleterious effect; one such effect is caused when miR-146a Knockout Tregs are generated.

Foxp3 is the most crucial factor reported in the establishment of regulatory T cell lineage. miR-155 is an extensively studied microRNA in hematopoiesis, mainly in lymphopoiesis. miR-155 regulates SOCS1 (suppressor of cytokine signaling 1), a molecule which is essential for the immune suppressive function regulatory T cells. T reg cells lacking miR-155 exhibited poor expansion in response to IL2. This is due to inhibition in IL-2 signaling by SOCS-1 [31]. These cells are characterized by poorly functional Foxp3+ Tregs in large numbers in the periphery. miR-17-92 cluster is another miRNA class studied to have an impact on T regulatory cell development. It was found that miR-17-92 was required for the accumulation of antigen specific T reg after activation and also for differentiation into effector T cells [32]. These miRNAs also promote T cell survival and Th1 responses, regulate CD8 effectors vs. memory differentiation, and inhibit TGF-β-induced in vitro differentiation into induced Tregs [33]. miR-31 and miR-21 were also addressed to regulate Foxp3 expression in T reg cells, former one inducing a negative effect as it directly binds to the 3′ UTR of Foxp3 mRNA whilst the latter being a positive regulator [34]. miR-155, a multifunctional miRNA, is found to have its profound influence on hematopoiesis, inflammation, and immunity [35]. The role of miR-155 is well studied in immune modulation and in the developmental process of T and B cells.

## *B Cell Development*

The pluripotent hematopoietic progenitor) cell differentiates to form common lymphoid progenitors, which in turn give rise to the B-cell lineage. The stages of B-cell development are early pro-B cell, late pro-B cell, large pre-B cell, small pre-B cell, and mature B cell.

Expression profiling of miRNA in B cell subsets in human and mouse have identified distinct miRNAs and the results has great clinical significance which enabled for an in-depth study in exploring the world of miRNA in B lymphopoiesis and

lymphomagenesis. The studies also indicated that miR-155 has distinct expression profiles and plays a key role in hematopoietic lineage differentiation, inflammation, immunity, cardiovascular diseases, and cancer [36]. Another study has shown that the role of miR-181 expression in lineage negative hematopoietic progenitor cells in mouse doubled the cells belonging to B lymphoid lineage without any significant effect on T lymphoid lineage [37], suggesting it to be a positive regulator of B lymphopoiesis. The same study reported miRNA to be upregulated in B lymphoid and myeloid lineages miR-142 [18].

Dicer ablation conducted by Koralov et al. resulted in inhibition of transition of B cell lineage committed cells from pro-B cell state to pre-B cell state. It was found that miRs related to miR-17-92 cluster were detected in pro-B cells. Bim being the target gene, it is concluded that miR-17-92 cluster is involved in B cell progenitor survival [38]. Overexpression of miR-34a led to prevention of B cell development at the pro-B-cell-to-pre-B-cell transition, leading to a reduction in number of mature B cells. Co-transduction of Foxp1 lacking its 3′ UTR with miR-34a rescued B cell maturation whereas inhibition of Foxp1 causes B cell developmental phenotype through miR-34a activation [39].

## *Natural Killer Cells*

Natural Killer (NK) cells are )large granular lymphocytes which could produce inflammatory cytokines. Bezman et al. have shown the effect of deletion of Dicer and Dgcr8 which resulted in defective NK cell differentiation and functioning suggest that Dicer and a double-stranded RNA binding protein Dgcr8 have a key role in NK cell activation, survival, and function during infection in mouse mounted by cytomegalovirus. The study also reported that an increase in the mRNA level of Bcl-2, an anti-apoptotic factor, was found to be downregulated in cell deficient in Dicer and Dgcr8 and also exhibited an increased rate of apoptosis in peripheral NK cells thereby proving a role for these enzymes in cell survival [40]. Some of the miRNAs studied so far to influence NK cell development include miR-150, miR-583, miR-223, miR-155, miR-181, and miR-15/16.

Lack of miR-150 inhibits the terminal maturation of cells and also decreases the number of total peripheral NK cells. As mentioned above, expression level of this miRNA is low when compared to the differentiated cells and hence suggesting them to have less chance of miR-150 mediated regulation of its development. An ectopic expression of miR-150 showed increase in number of hyperfunctional natural killer cells in miR-150 transgenic mice with no difference in the rate of apoptosis between normal and miR-150 transgenic NK cells [41]. miR-223 is also known to be involved in the developmental regulation of NK cells through granzyme B, a direct activator of proapoptotic molecule Bid thereby initiating caspase activation [42]. Recently, Yun et al. showed that miR-583 had an inhibitory effect on NK cell differentiation [43]. miR-181 mediates NK cell development to a minor extent by regulating NLK (nemo-like kinase), an inhibitor of Notch signaling. Notch signaling is involved in NK cells during the early stages of development [44].

## miRNA in Myelopoiesis

Myelopoiesis is a complex process of hematopoietic CMP development that gives rise to all myeloid lineages; these committed cells further differentiate to give rise to erythrocytes, megakaryocytes, and granulocytes as well as macrophages [45]. Till the past decade, the integrated effects of cytokines regulated signaling pathways and intrinsic TFs were known to control myelopoiesis [46, 47]. However, a number of recent investigations have also evaluated the role of miRNAs in myeloid development and function. Hox9A, a transcription factor, was reported to have a role in normal myeloid differentiation [48] and lymphoid lineage commitment from MPP [49] Loss of function of Hox9A results in contractions in myeloid and lymphoid progenitors and loss of peripheral leukocytes [48], whereas overexpression in mouse bone marrow cells results in AML [214]. miR-126 regulates the expression of Hox9A by binding to the homeobox; miR-126 and hox9A are parallely downregulated during progenitor cell differentiation [50]. Hence, miR-126 appears to control HSCs differentiation and lineage commitment process by regulating Hox9A expression. miR-9 was reported as a critical regulator in cell fate decision in hematopoietic development, its ectopic expression in BM cultures accelerates terminal myelopoiesis, and its inhibition with a miRNA sponge blocks myelopoiesis; miR-9 was also found to enhance myelopoiesis but inhibits lymphopoiesis in mice model [51]. miR-9 inhibits expression of FoxO1 and FoxO3 by targeting 3′ UTR region of the murine *FoxO1* and *FoxO3* genes and FoxO3 inhibits myeloid differentiation induced by miR-9 [51]. miR29a, an important miRNA in hematopoietic development, is highly expressed in HSCs and LSCs and downregulated in more committed progenitor cells; ectopic expression of miR-29a induces CMP and GMP self-renewal, biased myeloid differentiation, and also induces the development of myeloproliferative disorder [52] (Fig. 10.3).

### *miRNAs in Megakaryocytopoiesis*

Traditionally, the mechanistic studies on megakaryocytopoiesis have focused primarily on different cytokines [53] and transcription factors [54, 55]. Over the past many years, numerous laboratories have established the essential role of miRNAs in megakaryocytopoiesis [56–58]. Garzon et al. [58] suggest a regulatory role of miRNAs in megakaryocytopoiesis by targeting different megakaryocytic transcription factors. One study reports the expression profile of 435 miRNAs; among them 13 miRNAs were upregulated and 81 were downregulated during murine MKs differentiation [57]. miR-150 is an important miRNA which plays a role in megakaryocyte-erythrocyte-progenitors (MEPs) fate decision; it favors the commitment of MEP into MKs lineage rather than erythroid lineage [56]. miR-150 is moderately expressed in MEP, and exhibits increased expression in cells undergoing megakaryocytopoiesis whereas progressively downregulated during the erythropoiesis

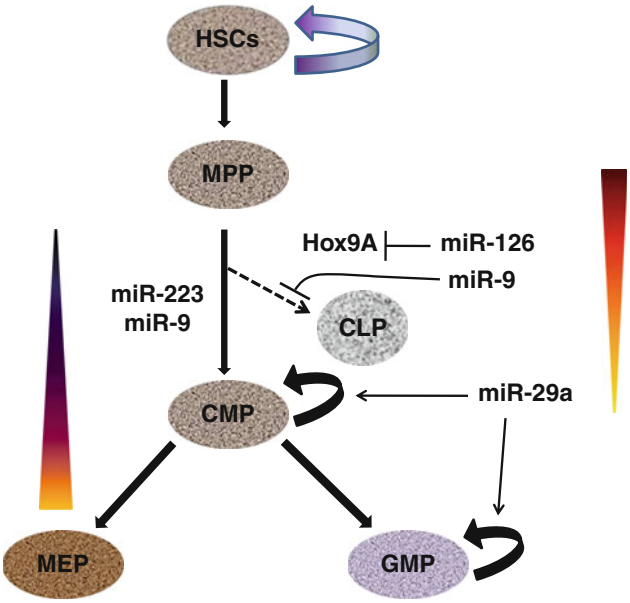


**Fig. 10.3** Schematic representation of the role of miRNAs in CMP differentiation

[56]. Thrombopoietin (TPO) increases the expression of miR-150, which in turn knocked down the expression of c-Myb via its 3′-UTR [59], previous data show that the low levels of c-Myb favors megakaryocytopoiesis [60]. miR-28 is a TPO receptor (MPL) targeting miRNA detected in platelet fraction isolated from myeloproliferative neoplasm patient, the overexpansion of miR-28 downregulates differentiation of CD34+ cells into Mks by targeting TPO signaling [61]. miR-155 is highly expressed in CD34+ cells but the expression is dramatically reduced during the CD34+ cells differentiation into Mks, the decline expression of miR-155 is required for proliferation and differentiation of Mks at progenitor and precursor level, the sustained expression of miR-155 inhibits the Mks differentiation by regulating the targets Meis-1 and Ets-1 [62]. miR-146a was reported as a modulator of megakaryocytopoiesis, the expression of miR-146a dramatically changes when HSCs differentiate into MKs [57, 63, 64]. Opalinska et al. [57] reported that the miR-146a expression level increases when the murine and human derived HSCs induced to differentiate into MKs, but the forced expression of miR-146a had no effects on megakaryocytopoiesis and platelet activation. Labbaye et al. [63] found decreased levels of miR-146 when the human cord stem cells were induced to differentiate into MKs, and decrease in cell ploidy was detected due to forced expression of miR-146a. During megakaryocytopoiesis the level of miR-146a is reduced by PLZF transcription factor; the PLZF binds the promoter region of miR-146a and inhibits its expression, whereas miR146a targets the CXCR4 mRNA and inhibits its translation [63]. Starczynowski et al. [64] found the results of miR-146a expression was similar

to Labbaye et al. [63], but the effect of forced expression on megakaryocytopoiesis was not observed, similar to Opalinska et al. [57]. miR-146a negatively regulates megakaryocytopoiesis by targeting TRAF6, the downregulation of miR-146a increases the activity of TRAF6 which in turn increases the IL-6 level [64]. miR-146b, a member of miR-146 family, is upregulated in progenitor cells undergoing erythroid or megakaryocytic differentiation, and PDGFRA, a negative regulator of erythroid and megakaryocytic differentiation, is a direct target of miR-146b; miR-146b could affect the expression of GATA-1 via downregulating PDGFRA/JNK/JUN/HEY1 pathway, whereas the transcription of miR-146b is positively regulated by GATA-1 [65]. miR-34a was found to be upregulated during megakaryocytic differentiation of K562 cells, but not during erythrocyte differentiation [66] and miR-34a promotes differentiation of bi-potent K562 cells into MKs by targeting c-Myb, CDK4, CDK6 [66], and MEK1 [67]. miR-34a is also upregulated during TPO induced differentiation of CD34+ cells, and its enforced expression increases the number of MKs [66]. Thus, miR34a is illustrated to enhance megakaryocytopoiesis. Recent reports show the downregulation of miR-130a during the CD34+ cells differentiation into MKs targets the MAFB transcription factor, which is upregulated during megakaryocytopoiesis [58]. Thus, miR-130a downregulation may help to MEPs fate decision by upregulating MAFB, which inhibits erythroid differentiation [68]. Several other miRNAs in megakaryocytopoiesis have been identified, but there is no much information related to their activity. miR-27a was found to be upregulated during MKs differentiation by RUNX1, miR27a targets RUNX1 and modulates the steady-state level of RUNX1 during MKs differentiation [69]. In this way, miR-27a and RUNX1 maintain a regulatory feedback loop. miR-10a is downregulated during MKs differentiation and was found to target HOXA1 [58, 70]. Thus, miR-10a appears to inhibit MKs differentiation. miR-181 is upregulated during initial stage of MKs differentiation and after that it is progressively downregulated, miR-181 was found to interrupt the Lin28-Let7 feedback circuit by downregulating the Lin28 in early stage of MKs differentiation [71]. Thus miR-181 appears to promote megakaryocytopoiesis by indirectly promoting the expression of Let-7, but not promoting the erythroid differentiation [71]. Hence, it works as a molecular switch during MEPs progression toward MKs differentiation. miR-223 expression increases during MKs differentiation and it downregulates LMO2 expression by directly targeting LMO2 mRNA, whereas the miR-223 is downregulated during erythrocyte differentiation and forced expression shows suppressive effects on erythroid differentiation [72, 73]. Recent study evidenced the role of miR-125b in regulation of cell proliferation and survival in neonatal megakaryocytes [16] (Fig. 10.4). Represents the summary of study on miRNAs in megakaryocytopoiesis.

## *miRNAs in Erythropoiesis*

Erythropoiesis is the process which produces red blood cells (erythrocytes). Erythropoiesis occurs in bone marrow (in humans) and stimulated by erythropoietin, which is secreted by kidneys in response of decreased oxygen levels in

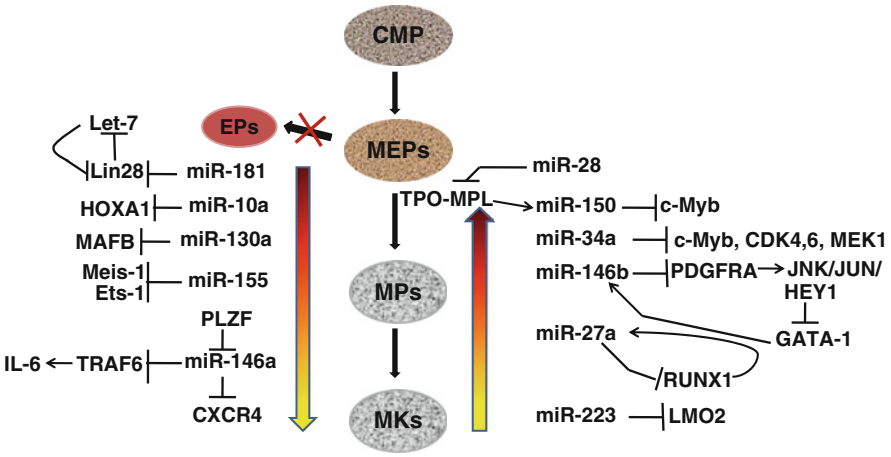


**Fig. 10.4** Schematic representation of the role of miRNAs in megakaryocytopoiesis

circulation. The role of certain miRNAs in erythropoiesis has also been identified in last 6–7 year research work. c-KIT, a receptor of SCF, has important role in early erythropoiesis to regulate survival and proliferation of erythroblasts [74, 75], and its expression is blocked in late erythropoiesis by GATA1 and other mechanisms [75]. Felli et al. [76] first reported that miR-221/222's expression is downregulated during differentiation of CD34+ cells into erythroid precursors, miR-221 and 222 directly targets c-KIT 3′UTR and the expression of both miRs is inversely related to that of c-KIT. Gabbianelli et al. [77] reported that the perinatal HbF switching is controlled by c-KIT. Thus miR-221/222 appears to block HbF switching and downregulates erythroblasts proliferation. One in vitro culture study on peripheral blood CD34+ cells revealed progressive downregulation of miR-221, miR-222, miR-150, and miR-155; upregulation of miR-451 and miR-16 at late stage of erythrocyte differentiation; and biphasic regulation of miR-339 and miR-378 during erythroid differentiation [78]. miR-150 is downregulated during erythropoiesis and directly targets c-Myb which regulates MEP fate decision towards erythrocyte or megakaryocytes [56]. Vegiopoulos et al. [79] reported that c-Myb is promoting MEP commitment toward erythropoiesis and progression of cells from their early to late stage of differentiation. Accumulating data from many erythroid miRNA profiling studies proved that miR-451 is upregulated after erythroid differentiation [78, 80, 81]. The expression of miR-451 is erythroid specific and it transcribed as a bicistronic transcript with miR-144 by GATA-1 transcription factor [82, 83]. miR-451 was found to protect erythroid cells from oxidative stress via inhibiting 14-3-3$\zeta$, which inhibits nuclear accumulation of FoxO3 transcription factor, a positive regulator of erythroid antioxidant genes [84]. Patrick et al. [83] demonstrate that repression of 14-3-3$\zeta$ by miR-451 enhances erythroid differentiation. Recently, Kim et al. [85] identified RAB14 as a novel physiological inhibitor of human erythropoiesis, which is a direct target of both miR144 and miR451; the expression of miR-144 and miR-451 is inversely proportional to that of RAB14. The forced expression

of RAB14 shows the phenocopy of the effects of miR144 and/or miR451 depletion and knockdown expression of RAB14 protects cells from miR-144/451 depletion mediated inhibition of erythropoiesis, the knockdown expression of RAB14 increases the cell number and β-Hb expression during erythropoiesis [85]. miR-233 is downregulated during erythroid differentiation and the expression of its target LMO2 is upregulated, LMO2 is required for erythroid differentiation [72, 73]. Thus miR-223 is an inhibitor of erythroid differentiation. miR-24 regulates the erythrocytic differentiation by controlling the expression of ALK4 receptor by directly targeting 3′UTR region of AKL4 mRNA, miR-24 blocks the expression of activin-mediated accumulation of Hb and decreases the CFU and BFU of CD34+ cells. The expression of miR-24 is downregulated during erythropoiesis [86]. The c-Myb is an important factor for lineage commitment, proliferation, and differentiation. Zhao et al. [87] suggested a negative autoregulatory feedback mechanism between c-Myb and miR-15a. c-Myb binds promoter region of miR-15a and promotes its expression whereas miR-15a downregulates the c-Myb expression by blocking its translation; the forced expression of miR-15a in bone marrow mononuclear cells blocked the erythroid transition from BFU to CFU in vitro [87]. miR-320 was found to inhibit erythroid differentiation by targeting SMAR1 mRNA, which in turn downregulates miR-222 expression by binding promoter region of miR-221/222 [88]. miR-320 is downregulated during erythroid differentiation [80] resulting in upregulation of SMAR1, which downregulates miR-222 expression [88]. miR-222 inhibits erythropoiesis by targeting c-KIT [76]. Thus miR-320 regulates erythropoiesis through SMAR1 and miR-221/222. (Fig. 10.5) represents the summary of the regulatory role of miRNAs in erythropoiesis.

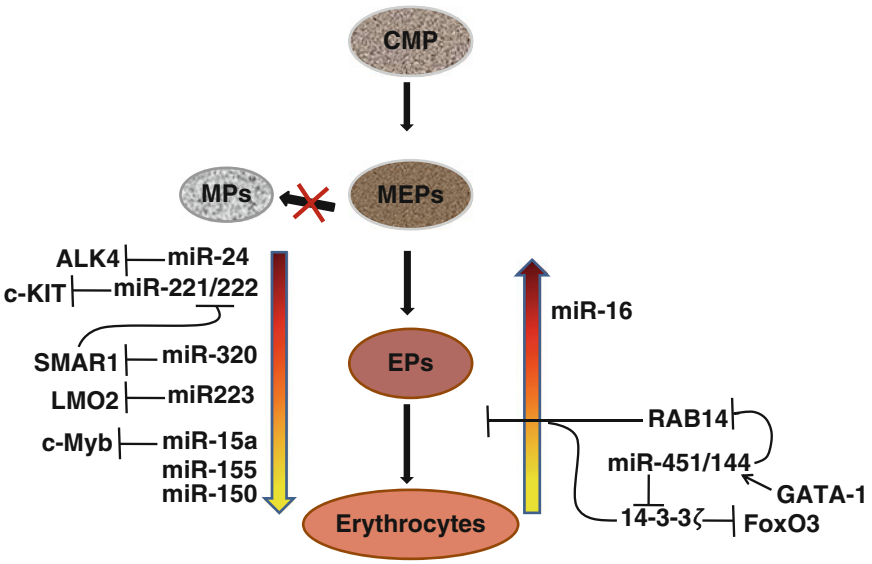


**Fig. 10.5** Schematic representation of the role of miRNAs in erythropoiesis

## *miRNA in Granulopoiesis and Monopoiesis*

Transcription factors are well known to play important role in GMP fate decision; these regulate the commitment of GMP into granulocyte or monocyte. An increasing body of data implicates the importance of various miRNAs in regulatory circuit of these transcription factors. miRNAs regulate these transcription factors activity by downregulating their translation, many TFs also regulates the expression of miRNAs. miR-223 with two other TFs, NFI-A and C/EBPα, was reported to be involved in a regulatory circuit of retinoic acid (RA)-induced differentiation of granulocytes; both NFI-A and C/EBPα compete for binding to the promoter region of *miR-223* gene [89]. NFI-A downregulates promoter activity of *miR-223*, whereas C/EBPα promotes promoter activity of *miR-223* and increases transcription of miR-223 which represses NFI-A translation [89]. Zardo et al. [90] reported in human CD34+ cells that NFI-A expression is also downregulated at transcriptional level by coordinated epigenetic events triggered via recruitment of polycomb (PcG)-RNAi complexes and endogenous miR-223. AML1/ETO, a fusion protein expressed in AML cells, was found to inhibit granulopoiesis by recruiting chromatin remodeling enzymes (HDAC and DNMT) on *pre-miR-223* gene, in turn these enzymes downregulates *pre-miR-223* gene expression via chromatin remodeling [89]. Above reports show that miR-223 upregulates granulocyte differentiation by transcriptional and posttranscriptional targeting of NFI-A gene expression. Johnnidis et al. [91] reported the increased expression of miR-223 during granulocytic differentiation while it decreases during monocytic and erythrocytic differentiation and it was found as the fine-tuner of granulocyte differentiation and function in mice model. GFI-1 is a transcriptional repressor protein which has role in normal granulopoiesis, the GFI-1 deficiency in mice and human has been found to exhibit an arrest in myeloid differentiation [9, 92]. It is reported that GFI-1 binds to the promoter of miR-21 and miR-196b and downregulates their transcription; GFI-1 upregulation in mice GMP cells is associated with dramatic reduction of both miR-21 and miR-196b [93]. miR-21 overexpression in Lin− murine BM cells was found to increase the monocytic colonies and its antagomiR showed apposite effects, whereas ectopic expression of miR-196b was observed with significant decrease in granulocytic colonies [93]. However, coexpression of both miRNAs completely blocked the G-CSF induced granulopoiesis and lead to the accumulation of immature granulocyte and monocyte precursors [93]. Thus, miR-21 appears to act as a promonopoietic factor and miR-196b as an antigranulopoietic factor and both miRNAs with GFI-1 control the fate decision of GMP to granulocytes. Fontana et al. [94] investigated the role of miR-17-5p, 20a, and 106a cluster in monocytic differentiation and maturation of human CD34+ cells, this cluster is downregulated during monocytic differentiation. AML-1, a direct target of this cluster, is unblocked and upregulated during monocytic differentiation, thereby promoting monocytic differentiation and maturation; AML-1 also acts as a suppresser of these three miRNAs by directly targeting their promoter region [94]. Rosa et al. [95] reported that human monocyte/machrophase differentiation is regulated by an interconnected circuit of PU.1, miR-424 and NFI-A; PU.1 activates transcription of miR-424, which in turn induces

monocyte/macrophase differentiation by translational repression of NFI-A, which is an inhibitor of monocytic differentiation. Other reports also found the miR-424 upregulation during monocytic differentiation of leukemia cell lines [96, 97]. Bousquet et al. [98] identified that miR-125 is upregulated in MDS and AML with t(2;11)(p21;q23); miR-125b interferes with primary human CD34+ cell differentiation, and also inhibits granulocytic and monocytic differentiation in HL60 and NB4 leukemic cell lines. Ooi et al. [21] reported that miR-125b expression level is high in mouse HSC, and its expression decreases progressively during lymphoid and myeloid progenitor commitment; the miR-125b expression also decreases during CMP differentiation into GMP. Surdziel et al. [99] reported that the miR-125b affects myelopoiesis in various ways and blocks the G-CSF induced differentiation of granulocytes. Shen et al. [97] reported that miR-424 decreases the miR-125b expression by direct targeting to CDX2 (caudal type homeobox 2) and promotes monocytic differentiation. CDX2 TF was reported as a transcriptional regulator of miR-125b, which binds promoter region of miR-125b and increase its expression [100]. (Fig. 10.6) represents the regulatory role of miRNAs in granulocyte and monocyte differentiation.

## Hematological Disorders

Hematological malignancies account for 9.5 % and lymphomas are more common than leukemia. Hematological malignancies are malignant neoplasms which affect blood, lymph, bone marrow and lymphatic system. Leukemias are classified into

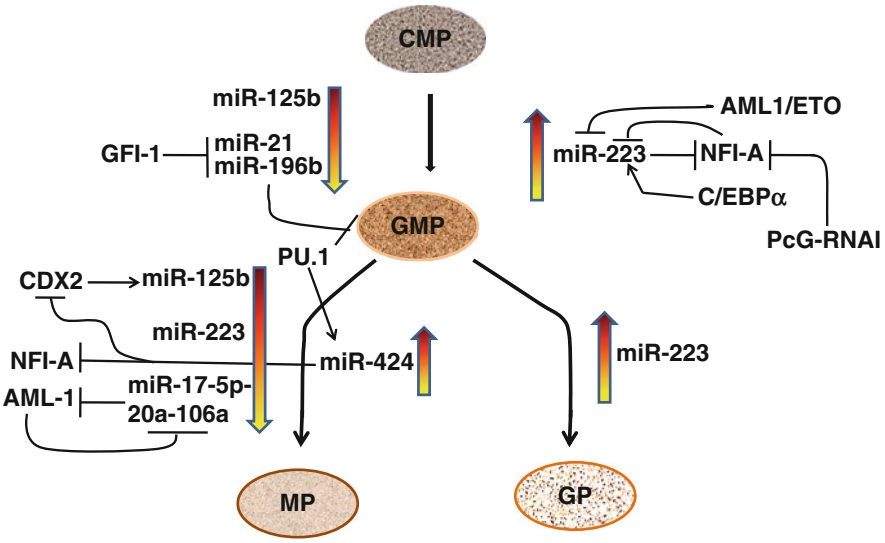


**Fig. 10.6** Schematic representation of the role of miRNAs in granulopoiesis and monopoiesis

acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute monocytic leukemia (AMOL), and so forth. Lymphocytic leukemia and myeloma arise from lymphoid lineage cells, whereas CML, AML, and myelodysplastic syndromes arise from myeloid lineage cells. Lymphomas are classified into Hodgkin's lymphomas and non-Hodgkin's lymphomas. Recent expression profiles of miRNAs studies have evidenced their involvement in the development of hematological malignancies.

## *Acute Myeloid Leukemia*

AML is the most common type of leukemia in adults and is a rapidly growing malignant neoplasm in which immature WBCs are found in blood and bone marrow. Recent study indicated that miR-126/126* is upregulated in this malignancy and induces inhibition of apoptosis and also increases cell viability and proliferation. This study found that tumor suppressor protein PLK2 (polo-like kinase2) which is involved in the cell cycle checkpoint was targeted by miR-126 [101]. miR-125b and miR-99a which are present on chromosome 21 are shown to involve in co-regulation of vincristine resistance in childhood acute megakaryoblastic leukemia [102]. miR-223 regulates granulopoiesis by inhibiting the cell cycle regulator protein E2F1. But in AML E2F1 is a transcriptional inhibitor of miR-223 by binding to the miR-223 promoter [215]. The study has show that mir-223 suppression is not caused by nucleotide sequence change or hypermethylation of DNA, its suppression in AML is caused by impaired miR-223 upstream factors [103]. miRNA-221 inhibits the CDK inhibitor p27 thereby enhancing cell proliferation in AML [104] and overexpression of miRNA 221 has also been associated with several solid tumors. The role of Hypoxia-inducible factor 1 (HIF-1) has been observed in miRNA signaling network by regulating the miR-20a and miR-17 which can inhibit the p21 [105]. Tumor suppressor let-7b miRNA expression was found to be downregulated in AML [104]. Recent studies have shown involvement of several other miRNAs in AML pathogenesis which has been listed in Table 10.3.

## *Chronic Myelogenous Leukemia*

Uncontrolled growth of granulocytes (eosinophils, basophils, and neutrophils) has been observed in chronic myelogenous leukemia or chronic granulocytic leukemia. This leukemia is well characterized by presence of fused BCR-ABL protein present in Philadelphia chromosome. The translocation of BCR gene between chromosome 9 and 22t (9; 22) (q34; q11) [106] leads to fusion protein BCR-ABL. This protein inhibits DNA repair and cause genome instability, which in turn leads to accumulation of genetic abnormalities [106]. Overexpression studies of miR-29b have shown that it decreased the cell growth and colony formation by inhibiting ABL1 and

BCR/ABL1 [107]. The overexpression of miR-138 also inhibits BCR/ABL1 and CCND3 by binding to the coding and 3′UTR regions [108]. The list of miRNAs which are involved in CML pathogenesis is given in Table 10.4.

## *Multiple Myeloma*

Recent studies have evidenced that deregulated miRNAs have associated with myeloma genesis and most of the miRNAs are upregulated in plasma cell of myeloma compared to normal condition [109] and further studies have shown deregulated miRNAs could be used for diagnostic purpose [109]. Ninety-five miRNAs have shown more expression in an analysis of 464 miRNAs in multiple myeloma (MM) condition when compared to healthy individuals in a comparative study of miRNA expression profiles of 52 MM patients and healthy persons [110]. miRNA-15a expression was downregulated in MM cells which could be a tumor regulator [111]. In another study miRNA-15a and miRNA-16 expression

**Table 10.3** miRNAs involved in AML

| miRNA | Targets | Function | References |
|---|---|---|---|
| miR-126* | ADAT2,FOF1, LMO7 | Upregulation leads to chromosomal translocations and inhibits apoptosis | Li et al. [101] |
| miR-126 | TOM1, CKMT2, ZNF131, RGS3 | Targets tumor suppressor PLK2 | Li et al. [101] |
| miR-223 | ANKH, SCN1A, SCN3A, CBFB, CDH11, NEBL, RILPL1, CENPN | C/EBPα regulates its expression in turn it inhibits E2F1 in AML cells | Eyholzer et al. [103] |
| miR-221 | HIPK1, RAB18, DNM3, ZNF547, | Oncogenic miRNA, it inhibits the CDK inhibitor p27 | Cammarata et al. [104] |
| miR-124a | | Target the C/EBPα, silenced and block differentiation gives leukemia phenotype | Hackanson et al. [208] |
| miR-20a and miR-17 | POLQ, KLF12, STK38, CENTD1, NUP35, GNB5, CTSK | Inhibits the p21 | He et al. [105] |
| miR-29b | SCML2, C1orf96, COL3A1, COL7A1, COL11A1 | Tumor suppressor in AML and reduce tumorigenicity | Garzon et al. [216] |
| miR-29b | CD93, HBP1, SNX21, GNS, HMGCR, HNF4G, DNMT3B | Reduced expression of DNA methyltransferases | Garzon et al. [216] |
| miR-196a and miR-196b | FOS, GATA6, HOXB6, HOXC8, ZNF24, CCDC47 | They target ERG expression | Coskun et al. [217] |
| miR-193b | MMP19, ARMC1, ARPC5 | Downregulated in AML and it targets c-Kit | Gao et al. [218] |

**Table 10.4** miRNAs involved in CML

| miRNA | Targets | Function | References |
|---|---|---|---|
| miR-17-92 | IRF9, RAB10, TXNIP, TET2 | Downregulated in imatinib treated CML cells | Venturini et al. [219] |
| miR-21 | TXPAN2, LUM, SUZ12, MSH2, PDZD2 | Antisense inhibition leads to inhibition of migration and cell growth and induces apoptosis | Hu et al. [220] |
| miR-203 | RTKN2, AAK1, MYST4 CD109, IL21, PLD2 | Methylated in AML, CML, ALL, CLL. Inhibit the expression of BCR-ABL | Chim et al. [221] |
| miR-451 | TSC1, ACADSB, GRSF1, MAML1, GDI1, NAMPT | Associated with Bcr-Abl | Lopotová et al. [222] |
| miR-29b | HAS3, SNX24, CD93, SCML2, COL7A1, ZNF396, HMGCR, ICOS | Inhibits ABL1 and BCR/ABL1 there by inhibiting cell growth and colony formation | Li et al. [107] |
| miR-138 | KLF12, H3F3B, MYO5C, NXN, NEBL, PDPN, STK38 | Represses BCR/ABL1 and CCND3, increases by GATA1 | Xu et al. [108] |
| miR-212 | APAF1, EP300, EDNRA, CFL2, NOS1, SOX4, SOX11 | Increases the ABCG2 expression | Turrini et al. [223] |

levels were elevated in the plasma cells (PCs) of diagnosed MM patients [112]. miRNA-29b has been reported to downregulate Mcl-1 and to induce apoptosis of myeloma cells [113]. miRNAs-720, 1308, and 1246 and miRNA-193b-365 cluster were found to be upregulated in PCs of myeloma patients than healthy controls [114, 115].

## *Diffuse Large B-Cell Lymphoma*

Recent dtudies have shown the deregulation of miRNAs in DLBCL. miR-155 has been shown to overexpress in this lymphoma and play key role in progression of pathogenesis [116–118]. The study showed transgenic mice which carry miR-*155* develops high grade B-cell lymphoma after 6 months [119]. Another study showed pre-B cells transformation into malignant when SHIP1 and C/EBPβ were targeted [120–123]. Another study evidenced of showing regression of lymphoma when the inducible promoter of miR-155 was repressed [124]. The clinical importance of miRNA was shown to reduce lymphomas when *miR-155* antagomirs was delivered in the form of nanoparticles into explants of tumors. The growth inhibitory effects of TGF-β1 and BMP2/4 in DLBCL cells were suppressed via SMAD5 when *miR-155* was expressed in in vitro [125]. It was well-studied in in vitro and in vivo xenotransplant models that onco-mir-155 targets SHIP1 to promot TNFα-dependent growth of DLBCL [121], and was also shown to be involved in regulation of

PI3K–AKT pathway by targeting negative regulator PIK3R1 in DLBCL [126]. Differential expression of SHIP1 was observed in two molecular subtypes of DLBCL, activated B-cell like, and germinal center B-cell like DLBCL [127]. CD10 is a prognostic marker for DLBCL, the expression of CD10 is higher in GC subtype whereas the CD10 expression is low or absent in ABC subtype of DLBCL; the ABC type cells show lower prognosis after treatment and higher activity of NF-κB which targets mir-155 and increases expression of this onco-miR which results in down regulation of PU1 and lower expression of CD10 [128]. This study is correlated with previous studies as miR155 expression levels are different between ABC- and GC-type DLBCL. miR-34a is well-studied miRNA which can play a role as tumor suppressor that is linked with p53 network in tumors [129]. Interestingly p53 activates *miR-34a* expression and in turn miR-34a induces p53 via SIRT1 inhibition [130]. The role of tumor suppressor has shown to be involved when treatment of ABC-type DLBCL cell line (U2932) with *miR-34a* lead to regression of tumor growth through suppression of Foxp1 [131].

Lymphoid malignancies and increased tumor aggressiveness was associated with upregulation of *miR-17-92* along with MYC which could be mediated by MYC/*miR-17-92*/E2F circuit [132, 133]. MYC upregulates the miR-17-92 cluster which targets E2F1, while conversely pro-proliferative E2F3 regulates the miR-17-92 cluster [134]. The miR-19 increases the Akt–mTOR pathway through suppression of PTEN and leads to cell proliferation in Eμ-myc model and shown to be a key component of the miR-17-92 cluster [135].

## *Mantle Cell Lymphoma*

Deregulated miRNAs have been demonstrated in pathogenesis of mantle cell lymphoma (MCL) [136–139]. Studies evidence that overexpression of Cyclin D1 in MCL is due to loss of miRNA target sites in the 3′UTR of CCND1 for miR-15/16 and members of the miR-17-92 cluster [140, 141]. MCL is well characterized by higher expression levels of MYC and miR-17-92 cluster, continuous PI3K/AKT pathway, and resistance to apoptosis [66, 139, 142]. In addition to PTEN and BIM, PHLPP2 was shown to be an important regulator of the PI3K/Akt pathway and miR-17-92 cluster [143]. The tumor growth was decreased when silenced the miR-17-92 expression in MCL xenotransplant model lead to suppression of PI3K/Akt pathway. Finally, downregulation of miR-29 was shown to activate CDK4/CDK6, and serves as a potential prognostic marker for this malignancy [136].

## *Hodgkin Lymphoma*

The involvement of miRNAs in Hodgkin lymphoma (HL) progression was demonstrated after miRNA profiling studies in HL cell lines [144–146]. Aberrant miRNAs which are associated with cell proliferation, apoptosis, and the p53 pathway are

identified in HL cell lines using ribonucleoprotein chromatin immunoprecipitation (RIP-ChIP) approach [147]. Upregulated miR-135a in HL cells lead to enhancement of apoptosis and decreases cell proliferation via Bcl-xL inhibition by targeting JAK2 [138]. miRNA-9 is overexpressed in HL and several other cancer. miR-9 targets dicer1 and HuR in HL, the inhibition of mir-9 leads to derepression of HuR and DICER, which results in a decrease in cytokine production by HL cells; the lower levels of cytokines inpair the attraction of normal inflammatory cells by HL cells and also result in decreased tumour growth [148]. Further studies showed that decrease in tumor growth of a xenotransplant model when miR-9 antagomir delivered in tumor. In addition silencing of let-7 and miR-9 in HL cell lines inhibits PC differentiation with decreased expression of PRDM1/BLIMP1 [149]

### *Other B-Cell Lymphomas*

Recent studies demonstrated involvement of miRNA deregulation in mucosa-associated lymphoid tissue (MALT) lymphoma. Methylation of miR-203 leads to transformation of gastritis to MALT lymphoma and provide the clues that ABL1 as a potential target for treatment of this lymphoma. 27-miRNA expression distinguishes gastric DLBCL from MALT lymphoma [150]. Differential expression of miRNAs were shown to differentiate gastritis to MALT lymphoma, i.e., five miRNAs (miR-150, miR-550, miR-124a, miR-518b, and miR-539) were present in gastritis but absent in MALT lymphoma [151]. Another miR-223 expression levels were shown to increase E2A expression in gastric MALT lymphoma [152]. Involvement of miRNAs in B-cell lymphomas now has been studied but yet to be studied in rarer neoplasms such as B-cell primary cutaneous lymphoma, plasmablastic lymphoma, and B-cell prolymphocytic leukemia (B-PLL).

### *T-Cell Lymphomas*

The role of miRNAs in T-cell lymphoma is less studied when compared to B-cell lymphomas. Few studies have been reported on involvement of miRNA in T-cell lymphoma. Recent study showed that more than 100 miRNA are aberrantly expressed in the cutaneous T-cell lymphoma (CTCL), Sézary syndrome (SzS) [153]. QRT-PCR analysis in SzS has shown to miR-223 as a diagnostic marker in clinical study. In addition these studies also showed the role of miR-342 in the pathogenesis of SzS through its targeting of RANKL which was associated with the protection of SzS cells from apoptosis [154].

Downregulation of miR-150 is shown to involve in NK/T-cell lymphomagenesis [155]. This miR-150 regulates NK cells via Myb inhibition and other T-cell subsets through NOTCH3 targeting [23, 155]. miR-150 enhances apoptosis and decreases cell proliferation through DKC1 and AKT2 and causes downregulation of BIM, p53, and phosphorylated AKT expression levels when this miR was introduced into

NK/T cell lymphoma. Overexpression studies of miR-122 in CTCL induced AKT phosphorylation coupled with a decreased sensitivity to chemotherapy-induced apoptosis as well as inhibition of p53. Another study has shown that overexpression of miR-21 and miR-155 lead to enhancement of PI3K–Akt pathway activity [122] whereas CTCL enhances AKT phosphorylation which couple with a decreased sensitivity to chemotherapy-induced apoptosis as well as inhibition of p53 [156]. Further studies have shown the increased apoptosis after miR-21 silencing in SzS cells, in this way miR-21 might represent a therapeutic target for the treatment of SzS [157].

## Polymorphisms in miRNA-Binding Sites in Hematological Malignancies

Computational analysis have predicted of hundreds of single nucleotide polymorphisms (SNPs) located within miRNA-binding sites and experimentally validated. Recent studies have evidenced that there is strong negative selection on SNPs in miRNA binding sites (miRSNPs) when compared to the entire 3′UTR sequence [158–160] and have shown functional significance of those sites. Several reports have evidenced the involvement of miRSNPs with cancer and other diseases [161, 162]. First report of association of miRSNPs in hematological malignancies was reported in 2012 [163]. Bioinformatics analysis has shown that 111 putative miRSNPs are associated with 137 leukemia-associated genes.

Bioinformatics analysis of SNPs in the 3′UTRs of 137 leukemia-associated genes revealed 111 putative miRSNPs. 10 miRSNPs have been observed in patients of childhood acute lymphoblastic leukemia (ALL), adult CML and AML, and healthy controls. This study concluded that polymorphic genotypes of ETV6_rs1573613 and TLX1_rs2742038 have been observed and this association would enhance the risk of disease. Carriers of the variant allele of PML_rs9479 were at lower risk of ALL and AML [164]. The involvement of SNPs in polymorphisms in miRNA binding sites of hematological disorders should be validated in in vivo and the mechanisms by which the miRSNPs regulates hematological disorders.

## Key Modulators in Therapeutics

Recent studies have evidenced involvement of miRNAs as key regulators of signaling cascades that regulate cell fate and the function of normal and pathological lymphoid progenitor/precursor cells (Table 10.5). Several studies about miRNA expression profiles of lymphomas could provide the key roles of miRNAs in development of lymphoma pathogenesis. Latest studies have shown miRNA as promising agent in the role of diagnosis, prognosis, and treatment but also become the prospects for novel therapeutic strategies.

**Table 10.5** miRNAs, lymphoma-associated or treatment response correlated

| Disease | miRNA | UP or DR | Response | References |
|---|---|---|---|---|
| DLBCL vs. FL | miR-150, miR-17-5p, miR-145, miR-328 vs. miR-9/9*, miR-301, miR-338 and miR-213 | | Differentially expressed miRNAs in DLBCL and FL identify signatures respectively in DLBCL and FL | Roehle et al. [209] |
| DLBCL/FL/RNL | mir-330, mir-17-5p, mir-106a and mir-210 | | Correctly identifies 98 % of DLBCL, FL and RLN | Roehle et al. [209] |
| FL-t vs. FL-nt | miR-223, miR-217, miR-222, miR-221, let-7i, miR-7b | UP | Differentially expressed ($p<0.05$) between FL-t and FL-nt, accurately (89 %) predict transformation of FL cases | Lawrie et al. [145] |
| ABC-DLBCL vs. GC-DLBCL | miR-155, miR-21 and miR-221 | UP | More highly expressed in ABC- than GC-subtypes, distinguish between ABC- and GC-DLBCL cases ($p<0.05$). miR-21 expression is an independent prognostic indicator in de novo DLBCL ($p<0.05$) | Lawrie et al. [117] |
| FL | miR-9, miR-301, miR-213, miR-9*, miR-330, miR-106a, miR-338, miR-155, miR-210 | UP | Significantly higher expression in FL than RLN | Roehle et al. [209] |

(continued)

**Table 10.5** (continued)

| Disease | miRNA | UP or DR | Response | References |
|---|---|---|---|---|
| DLBCL-dn vs. DLBCL-t | miR-27a, miR-19b, miR-25, miR-18a, miR-636, miR-92, miR-621, miR-526c, miR-766, miR-299-5p, miR-380-3p, miR-129, miR-588 | UP | More highly expressed ($p < 0.05$) in DLBCL *de novo* than DLBCL-t cases, correctly predict transformation >85 % | Lawrie et al. [145] |
| DLBCL | miR-210, miR-155, miR-106a, miR-17-5p | UP | Significantly higher expression in DLBCL than RLN | Roehle et al. [209] |
| | miR-150, miR-145, miR-328, miR-139, miR-95, miR-99a, miR-10a, miR-149, miR-320, miR-151, let-7e (miR-17-3, miR-595, miR-663) | DR/lost | Significantly lower expression in DLBCL than RLN miR-17-3, miR-595, miR-663 most significantly lost in DLBCL | Di Lisio et al. [137] |
| HL | miR-17-92 cluster members, miR-16, miR-21, miR-24, and miR-155. miR-150 | UP | The HL-specific miRNAs upregulated | Gibcus et al. [146] |
| | | DR | Only miR-150 is significantly downregulated in HL compared with NHL | |
| cHL vs. HL EBV+ | miR-96, miR-128a, miR-128b | DR | Selectively downregulated in HL lymph nodes of EBV+ HL patients | Navarro, et al. [210] |

(continued)

**Table 10.5** (continued)

| Disease | miRNA | UP or DR | Response | References |
|---|---|---|---|---|
| cHL prognosis | miR-135a | | Expression of miR-135a in HL lymph nodes correlates with clinical outcome. Patients with low miR-135a expression had a higher probability of relapse and a shorter disease-free survival | Navarro et al. [138] |
| | miR-21, miR-30e, miR-30d and miR-92b | | To identify two different risk groups for 5-year FFS | Sánchez-Espiridión et al. [211] |
| B-CLL poor prognosis | miR-29 | | | |
| | miR-15a, miR-195, miR-221, miR-23b, miR-155, miR-24-1, miR-146, miR-16-1, miR-16-2 | UP | Significant relationship between the expression of 9 miRNAs and the time from diagnosis to beginning of chemotherapy | Calin et al. [212] |
| CLL vs. B-NHL | miR-182, miR-199a*(5p), let-7 family, miR-424, miR-10a, miR-7, mir-126, miR-218, MiR-197, miR-595, miR-483 | DR/UP | miR-197 the most highly expressed miRNA, miR-595 and miR-483 also upregulated | Di Lisio et al. [137] |
| BL vs. B-NHL | miR-17-3p, miR-18a, miR-19a, miR-19b, miR-92 | UP | Up/Downregulated in BL vs. NHL | Di Lisio et al. [137] |
| | let-7 family, miR-29 (a, b, c), miR-155, miR-146a | DR | | |

(continued)

**Table 10.5** (continued)

| Disease | miRNA | UP or DR | Response | References |
|---|---|---|---|---|
| DLBCL prognosis | miR-637, miR-608 and miR-302 | | Poor prognosis | Lawrie et al. [145] |
| | miR-330, miR-30e, miR-425, miR-27a, miR-24, miR-23a, miR-199b, miR-199a* and miR-100 | | Better outcome | Lawrie et al. [145] |
| | miR-21, miR-127, miR-34a, miR-195, let-7 g, miR-19a, miR-27a | | Correlate with EFS and OS | Roehle et al. [209] |
| DLBCL drug sensitivity | miR-181a, miR-222 and miR-18a | | Independent prognostic indicators of survival in R-CHOP treated DLBCL | Alencar et al. [213] |

The role of miRNAs in development and pathogenesis is shown to be evidenced increasingly in vitro and in vivo that ectopic expression or silencing of specific miRNAs lead to alter signaling of malignant cell survival. Recent studies have demonstrated that onco-miR silencing by miRNA mimics suppresses the tumour growth. First cancer-targeted miRNA drug MRX34, a liposomal formulation loaded with tumor suppressor miR-34a mimic, entered a Phase I clinical trial in patients with primary liver cancer or those with liver metastasis from other cancers in April 2013 [165].

miRNAs are generally classified as oncomirs or tumor suppressor genes based on their function, i.e., oncomirs or tumor suppressor genes provide approach for therapeutics development. Silencing or suppression of tumor-inducing miRNAs and replacement of downregulated miRNA by re-introducing miRNAs with tumor suppressor function can be used as therapeutic agents.

Anti-miRNAs have been synthesized to silence or block a target miRNA that acts as an oncogene. Cholesterol-conjugated "antagomirs," polylysine-conjugated peptide nucleic acids (PNAs), and locked nucleic acid oligonucleotides are chemically modified anti-miRNAs that have demonstrated effectiveness in in vivo treatment [166–168]. Anti-miRNAs have been used against miR-155, miR-17-92 cluster, miR-9, and let7 which are overexpressed in many lymphomas.

miR-17-92 cluster overexpression demonstrates to significantly enhance resistance to radiotherapy in human MCL, and become potential molecular target for

improving the effectiveness of conventional treatments. Another evidence that antagomir 17-5p abolish the growth of therapy-resistant neuroblastoma cells which express the miR-17-92 cluster at an elevated level [169, 170].

Even though antisense oligonucleotides have been shown to silence many oncomiRs such as miR-155, miR-17-92, and miR-21, yet the use of anti-miRNAs as drug in clinical practice is still a long way and a number of obstacles have to be overcome before implementation of these therapeutic strategies in human diseases. LNA-modified anti-miRNAs could enhance the specificity and reduce effective doses of anti-miRNA molecules [46]. The incorporation of endonucleolytic DNAzyme motif in LNA-based anti-miRNAs and siRNAs can enhance the catalytic efficiency of these anti-miRNAs and siRNAs [171]. Several parameters such as stability, safety, and successful delivery of therapeutic anti-miRNAs to the appropriate tissue and into the cells need to be evaluated [172].

Babar et al. [173] have developed technology based on nanoparticles coated with a cell-penetrating peptide encapsulating antisense peptide nucleic acids to systemically deliver anti-miR-155 (antagomir) in a B-lymphoma mouse model. In this study, overexpression of miR-155 was induced in the lymphoid tissues of a deficient miR-155 mouse model, causing disseminated lymphoma characterized by clonal transplantable pre-B-cells. In this inducible system, nanoparticle-based therapy targeting miR-155 resulted in the ability to reduce lymphoma growth. In particular, nanoparticles injected in situ or systemically produced a rapid regression of lymphadenopathy, partially due to apoptosis of the malignant B-lymphocytes.

Another approach is exogenous administration of short double-stranded miRNA mimics or endogenous miRNAs into diseased tissues to regulate miRNAs in order to regain physiological levels of downregulated miRNAs in systematic approach or tissue specific approach. In several malignancies, including lymphomas and leukemia, frequent aberration of miR-34 gene expression is demonstrated which has tumor suppressor function. So developments of miR-34a mimic formulations are become promising drug in treatment of solid tumors.

Craig et al. have shown the relevance of miR-34 deregulation in lymphomagenesis by inoculating ABC-type cell line (U2932) with low expression of miR-34a into humanized immunodeficient NOD/SCID/IL2RG−/− mice [131]. The tumor growth was decreased by 76 % in this mouse with U2932 lymphoma and also showed apoptosis of malignant B-lymphocytes when intravenous injections of a neutral lipid emulsion (NLE)/miR-34a formulation in this mouse [131]. In addition, miR-34a along with cytotoxic chemotherapeutic agents enables chemosensitivity mainly through modulation of p53 in many cancers and hematological malignancies [173, 174]. Recent studies evidenced the involvement of restoration of tumor suppressor effects of miRNA using epigenetic drugs 5-aza-2′-deoxycytidine and zeburaline that change the DNA methylation status. Epigenetic modifications such as hypermethylation could downregulate the miR-34a in hematological malignancies NK/T-cell lymphoma. The down-regulated expression of miR-34 showed re-expression when pharmacological reversal of epigenetic silencing by epigenetic inhibitors was employed in lymphoma [175]. Epigenetic downregulation of miR-124 in acute lymphoblastic leukemia (ALL) showed poor prognosis in ALL patients

due to a high relapse and mortality rate [176]. Recent study showed that 5-aza-2-deoxycytidine and 4-phenylbutyric acid (PBA) drugs activate the expression of miR-127 and decrease the proto-oncogene BCL6 in Ramos lymphoma cell line and other cancer cell lines [177]. The downregulation of proto-oncogene BCL6 suppresses PRDM1 which is implicated in DLBCL [178]. The histone modification restores the function of miR-127 and shown clinical importance to treat cancer.

## Conclusion

Studies have evidenced the role of miRNA in regulation of all stages of hematopoiesis and differentiation. The mode of action of several miRNAs is not yet understood due to its pleiotrophic nature of function, even though several miRNAs have been known. Increasing evidences showed the role of miRNA based interventions in treatment of hematological disorders could provide a powerful therapeutic approach. Several substantial obstacles such as potential off-target effects, efficacy, and safety are needed to overcome before entering into clinical trials. Hematological malignancies from different origin have distinct miRNA profile which help to distinguish different hematological entities and could be useful for diagnosis, and evaluation for therapeutic potential. Recent decade has shown tremendous progress in research to understand the role of miRNAs in developmental hematopoiesis and molecular mechanism of hematopoietic malignancies. This chapter updates the miRNAs become crucial regulators in diversity of regulatory pathways and also involve in the regulation of positive and negative regulatory feedback loops. Moreover this chapter also illustrates that involvement of aberrant expression of miRNA in several hematological disorders in association with transcriptional repressors or activators.

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# Endoplasmic reticulum stress induced apoptosis and caspase activation is mediated through mitochondria during megakaryocyte differentiation

Narasaiah Kovuru, Sanjeev Raghuwanshi, Durga Shankar Sharma, Swati Dahariya, Adithya Pallepati, Ravi Kumar Gutti*

*Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad 500046, TS, India*

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ABSTRACT

Megakaryocytopoiesis involves the process of the development of hematopoietic stem cells into megakaryocytes (MKs), which are the specialized cells responsible for the production of blood platelets. Platelets are one of the crucial factors for hemostasis and thrombosis. In terminally differentiated MKs, many molecular process such as caspase activation and a massive cytoskeletal rearrangement drive the formation of cytoplasmic extensions called proplatelets. These cytoplasmic extensions packed with granules and organelles are then released from the bone marrow into the blood circulation as platelets. Classically, caspase activation is associated with apoptosis and recent reports suggest their involvement in cell differentiation and maturation. There is no clear evidence about the stimulus for caspase activation during megakaryocyte development. In the current study, we attempted to understand the importance of endoplasmic reticulum stress in the caspase activation during megakaryocyte maturation. We used human megakaryoblstic cell line (Dami cells) as an experimental model. We used PMA (Phorbol 12-myristate 13 acetate) to induce megakaryocytic differentiation to understand the involvement of ER stress and caspase activation during MK maturation. Further, we used Thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) as a positive control to induce ER stress. We observed larger and adherent cells with the increased expression of megakaryocytic markers (CD41 and CD61) and UPR markers in PMA or Thapsigargin treated cells as compared to control. Also, Thapsigargin treatment induced increased caspase activity and PARP cleavage. The increased expression of megakaryocyte maturation markers alongside with ER stress and caspase activation suggests the importance of ER stress in caspase activation during MK maturation.

## 1. Introduction

Megakaryocytopoiesis is a tightly regulated developmental process of megakaryocyte (MK) differentiation and maturation from multipotent hematopoietic stem cells (HSCs), this process is govern by various transcription factors and cytokines (Woolthuis and Park, 2016; Raghuwanshi et al., 2018). During maturation process, MK-progenitors undergo successive round of endomitosis and acquires polyploidy, the ploidy varies with the age and the physiological demand of the platelet (Mazzi et al., 2018). Finally, platelets are released into the blood stream from the terminally differentiated MKs (Abd-Elrahman et al., 2013). Recent reports suggest non-apoptotic functions of caspases in myeloid cell differentiation (Solier et al., 2017). Clarke et al. (2003) reported that constitutive formation of the proplatelet-bearing MK is caspase-dependent involving mitochondrial release of cytochrome *c*. Other studies elaborated that both MK frequency and production of functional platelets were significantly reduced by the broad-spectrum caspase inhibitor, zVAD-fmk, these studies implying a role for caspase-directed apoptosis in the formation of proplatelet extensions by MKs and the subsequent production of functional platelets (De Botton et al., 2002).

It is widely accepted that to produce platelets, MKs activate apoptotic pathways (Kaluzhny and Ravid, 2014; Fadeel and Orrenius, 2005; Patel et al., 2005; Siegel, 2006; Galluzzi et al., 2008; Solary et al., 2008). The role of apoptotic pathways in the development and function of the MK and platelets has generated renewed interest in recent years (Kile, 2014). Recently, it was observed that MKs and platelets both depends on mitochondrial apoptotic pathway for their development and viability (McArthur et al., 2018). But, the important question is that how apoptosis is initiated in these cells.

Classically caspase activation and apoptosis occurs in response to any assaults to cells such as potential harm full mutations, viral infection. Primarily apoptosis occurs in two different ways namely, intrinsic

* Corresponding author.
*E-mail address:* guttiravi@gmail.com (R.K. Gutti).

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and extrinsic apoptosis. Intrinsic apoptosis involves signal that comes from the mitochondria, whereas extrinsic apoptosis involves signal that comes from the plasma membrane (Elmore, 2007). In addition to these, alternative mechanisms are also there for the caspase activation and apoptosis, this could be achieved by the endoplasmic reticulum stress (Sano and Reed, 2013).

In current study, we used PMA (Phorbol 12-myristate 13-acetate) induced Dami cells (Megakaryoblastic cells) to investigate the mechanism of MK maturation (Greenberg et al. 1988). We found that PMA induces several cellular process simultaneously such as ER stress, caspase activation and megakaryocytic maturation in Dami cells. We have also used Thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) as a positive control to induce ER stress (Foufelle and Fromenty, 2016). Upon its treatment, we observed increased expression of ER chaperons and megakaryocytic markers. We observed that MK maturation maybe induced by ER stress induced caspase-dependent pathways.

## 2. Materials and methods

### 2.1. Cell culture

Human Megakaryoblastic cells (Dami cell line) were cultured in RPMI-1640 medium (Gibco Invitrogen, CA) containing 10% fetal bovine serum (FBS) and 1% antibiotic and anti-mycotic (life technologies, Inc.). Cells were grown in humidified environment under 5% carbon dioxide. To induce megakaryocyte maturation, Dami cells were treated with different concentrations of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich).

### 2.2. Morphological analysis using the microscopy

Cells were cultured with PMA, Thapsigargin and without PMA & Thapsigargin (control) for 48 h. Morphological features such as cells size and adherence were observed under light microscope (Olympus Pvt. Ltd.). Cell size was measured using ImageJ software, where up to 15 frames were counted.

### 2.3. Quantitative Real-time PCR (qRT-PCR) analysis

Total RNA was isolated from cells using RNeasy mini kit method (Qiagen) according to manufacturer's instructions and reverse transcribed using Primescript 1st Strand cDNA Synthesis Kit (Takara) following the manufacturer's instruction. The first strand cDNA was used as a template for real time PCR (Applied Biosystems StepOne Plus System) using the SYBR Green qPCR master mix (Kapa Biosystems). The cycling program was set as follows: denaturation at 95 °C for 10 min, 35 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s for extension. Specific primers were used for quantification of gene expression of CD41, CD61, CD42b, Bip and Chop. Actin was used as internal control. Each sample was normalized with actin and expressed as relative compared to controls (n = 3).

### 2.4. Western blotting

Cells were cultured in RPMI1640 media and protein was extracted with RIPA buffer (Sigma) with protease inhibitor cocktail (invitrogen). 75 μg protein were subjected to separation on 10% or 12% polyacrylamide gel, transferred onto nitrocellulose membrane (Millipore) and probed with specific primary antibodies such as PARP, Bax, Survivin, Bcl2, Bim (Cell Signaling Technology) and β-Actin (Santa Cruz Biotechnology). Detection of the proteins using specific primary antibodies were carried out with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz) and developed with enhanced Super Signal Chemiluminescent Substrate (Thermo Scientific).

### 2.5. Mitopotential

Mitochondrial potential analysis of both the PMA treated and untreated Dami cells was performed after 48 h of culture by using Mitopotential assay kit-MCH100110 (Millipore) with the help of Muse™ Cell Analyzer (Millipore) following the manufacturer's instructions.

### 2.6. ROS measurement

$1 \times 10^5$ cells were treated for 48 h with PMA and were collected, washed with PBS twice. These cells were resuspended in 100 μl of medium and 20 μM DCFDA (2′,7′-dichlorofluorescein diacetate) was added and incubated for 30 min at 37 °C. This was further washed with PBS and resuspended in 100 μl of 1× PBS and fluorescence was measured at 485 nm excitation and 535 nm emission respectively using the multi-mode plate reader (Molecular Devices).

### 2.7. MAPK analysis

MAPK analysis was performed using Muse™ Cell Analyzer (Millipore). Cells were collected after 30 min of treatment with PMA and washed with PBS. Cells were analyzed using Muse® MAPK Activation Dual Detection Kit (EMD Millipore) on Muse™ Cell Analyzer (Millipore) following the manufacturer's instructions.

### 2.8. Annexin V assay

Apoptosis was detected through Annexin V staining using Muse Cell Analyzer (Millipore) following manufacturers protocols (Annexin V/Dead Cell kit MCH100105). The assay works on the principle of translocation of phosphatidyl serine to the outer surface of the cell membrane.

### 2.9. Caspase activity assay

For the caspase activation assay 100 μg of protein was taken and volume was made up for 100 μl with caspase buffer (20 mM HEPES pH 7.4, 10% sucrose, 100 mM NaCl, 10 mM DTT, 0.1% CHAPS, 1 mM EDTA) and 5 mM caspase-3 substrate (N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-tri fluoromethylcoumarin) (Sigma) was added. Incubation was performed at 37 °C for 1 h. Samples were then read on multi-well fluorescence plate reader (Molecular Devices) at an excitation of 400 nm, emission wavelength of 450–490 nm and the results were plotted as fluorescence units.

### 2.10. Statistical analysis

T-test was used for pair-wise comparison for determining the significance. Bars represent mean ± SE of three independent experiments, qRT-PCR data was analyzed by standard ΔΔ Ct method.

## 3. Results and discussion

### 3.1. The effect of PMA on the differentiation of Dami cells to megakaryocytes

In vitro megakaryocytic differentiation of the Dami cell line was induced by PMA. Dami cells were seeded in 60 mm plates at a density of $10^5$ cells in RPMI 1640 medium containing 10% FBS. Differentiation was induced on next day of cell seeding by the addition of 40 nM PMA in DMSO. Control cells received the same concentration of DMSO alone. Culture conditions were followed as previously described (Undi et al., 2017). Treatment of Dami cells with PMA resulted in the emergence of typical megakaryocytic features such as increased cell size and adherence (Fig. 1A). Further, the induction of megakaryocytic markers, CD41, CD42b and CD61, was significantly higher (~5 fold) in PMA

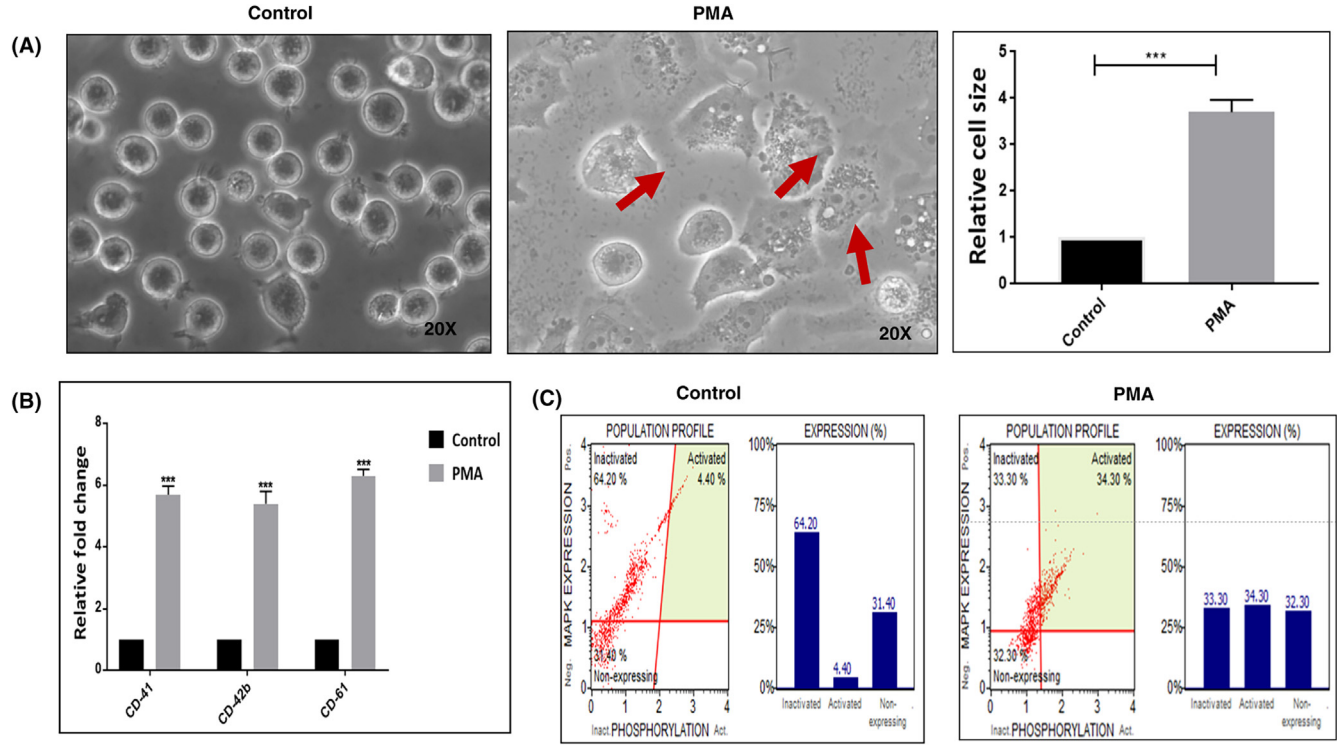


**Fig. 1.** Maturation of megakaryocytes and induction of MAPK signaling was observed with PMA treatment in Dami cells. (A) Megakaryocytic morphological features such as increased cell size and adherence were observed with PMA as compared with untreated control. (B) Increase in megakaryocyte differentiation markers, CD41, CD42b and CD61, expression was observed in quantification by qRT-PCR. (C) MAPK signaling pathway is significantly more active in PMA treatment as compared to untreated control. Bars represent mean ± SE of three independent experiments (n = 3; ***$P$ < .002).

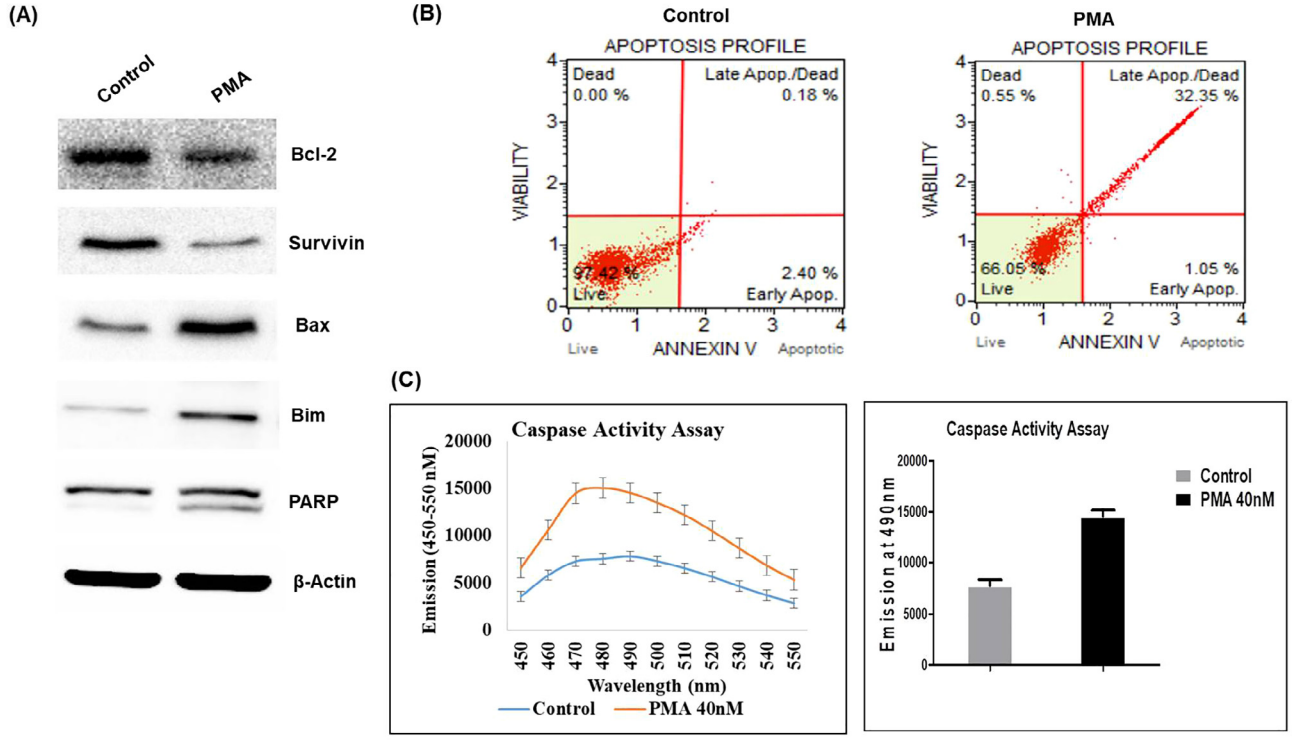


**Fig. 2.** Apoptosis like phase observed in Dami cells during PMA induced maturation. (A) Pro-apoptotic proteins Bax and Bim were up regulated and anti-apoptotic components Bcl-2 and survivin were down regulated in PMA treated cells compared to control cells. Increase in the cleaved product of caspase-3 target PARP was observed in the PMA treated cells. (B) Annexin V staining assay by Muse cell analyzer observed more Annexin V positive cells in PMA treated compared to control cells in right side upper quadrant cells (C) Caspase-3 activity was significantly high in PMA treated compared to control cells. Bars represent mean ± SE of three independent experiments (n = 3).

treated Dami cells as compared to control (Fig. 1B, n = 3, ***p < .002). These results indicated that 40 nM PMA-treated Dami cells were differentiated into a megakaryocytic lineage successfully. Similar to the results of current study, Greenberg et al. (Greenberg et al., 1988) also reported that in response to PMA, the proportion of Dami cells with high ploidy levels increased along with increased expression of megakaryocyte specific markers. Taken together, this data suggests that PMA may promote megakaryocyte maturation and could serve as a superior model to study human megakaryocyte biochemistry and differentiation.

### 3.2. PMA increased the activity of MAPK in Dami cells

Increased MAPK pathway activity has been noted during megakaryocyte differentiation from cord blood and adult peripheral blood derived CD34+ cells (Liu et al., 2011). To test the MAPK activity in PMA induced MKs, we treated Dami cells with PMA (40 nM), after 30 min of treatment cells were analyzed by Muse™ Cell Analyzer (Millipore). We observed a significant increase in the MAPK activity in PMA induced Dami cells as compared to control (Fig. 1C). In same line, previous findings also suggest that PMA-induced megakaryocytic differentiation of K562 cell requires the activity of the MAPK pathway (Shelly et al., 1998).

### 3.3. PMA induced megakaryocytic differentiation activates apoptotic pathways

Megakaryocyte maturation and platelet production is a complex process involving several transcription factors and cytokines. However, activation of caspases has been shown to be prerequisite for proplatelets formation and platelet shedding (Elmore, 2007; De Botton et al., 2002). In current study, treatment of the Dami cells with PMA results in induction and activation of the pro-apoptotic components such as Bax and Bim, whereas anti-apoptotic components Bcl-2 and survivin were down regulated (Fig. 2A). Moreover, in our study, we observed a significant increase in the Annexin V positive cells in PMA induced- as compared to control-Dami cells (Fig. 2B). Annexin V assay works on the principle of translocation of phosphatidyl serine to the outer surface of the cell membrane, which is often associated with apoptosis (Huang et al., 2014). Further, caspase activity assay using the substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-tri fluoromethylcoumarin showed ~2 fold increase in the florescence in PMA treated cells as compared to control (Fig. 2C). We have also observed the cleaved product of PARP (Poly-ADP-ribose polymerase) in PMA treated Dami cells (Fig. 2A), Caspase activation and PARP cleavage are known markers of apoptosis (Lyakhovich and Surrallés, 2010). Kozuma et al. (2009) have noticed canonical apoptotic cascade mediated through caspases in megakaryocytes; other reports have also suggested that megakaryocytes possess a functional apoptotic caspase cascade (De Botton et al., 2002; White and Kile, 2010; Kozuma et al., 2009, 2007; Sarvothaman et al., 2015). Results of our study further confirmed that the apoptosis cascade components likely involved in the MK maturation.

### 3.4. PMA induced megakaryocytic differentiation involve ROS production through altered mitopotential

Mitochondria are the major intracellular source of ROS production, ROS is generated by mitochondria as a by-product of superoxide anion, and in previous studies it has been demonstrated that ROS is necessary for megakaryocytic development under PMA stimulation (Huang et al., 2014; Tan et al., 2010; Sardina et al., 2010; Hirose et al., 2013). In order to understand whether PMA induces ROS production, Dami cells were treated with 40 nM PMA for 48 h and the ROS levels were measured. Treatment of Dami cells with PMA increased the ROS production by ~3 fold as compared with control (Fig. 3A). Further, reduced mitochondrial membrane potential (depolarized mitochondrial membrane) was observed with PMA treated cells as compared with control (Fig. 3B). Our observations suggest that PMA could induce mitochondrial ROS generation and increases the permeability of the mitochondrial membrane, thereby inducing megakaryocytic maturation. Reactive oxygen species has been shown to play an important role in megakaryocyte maturation and platelet production and ROS may be generated by mitochondria as a by-product of superoxide anion (Motohashi et al., 2013; Chen et al., 2013).

### 3.5. ER stress induces apoptosis during the megakaryocyte maturation

Reports suggest that Endoplasmic Reticulum (ER) stress triggers unfolded protein response (UPR) and prolonged UPR results in activation of caspases and apoptosis pathway (Tsai and Weissman, 2010; Bravo et al., 2013; Hetz et al., 2015). In current study, we treated Dami cells with PMA (40 nM) and Thapsigargin (1 μM) separately and after 48 h of incubation cell morphology, megakaryocyte specific markers (CD41, CD61 and CD42b) and UPR markers (Bip and CHOP) were analyzed. We observed larger and adherent cells (Fig. 4A) with the increased expression of both the megakaryocytic markers (Fig. 4B) and UPR markers mRNAs (Fig. 4B) in PMA and Thapsigargin treated cells as compared to control. Further, thapsigargin treatment induces increased caspase activity and PARP cleavage (Fig. 4C). Thapsigargin is an inhibitor of the endoplasmic reticulum Ca2+-ATPase and upon its treatment results in a decrease in ER calcium levels leading to reduced activity of calcium-dependent ER chaperones and accumulation of unfolded protein (Sehgal et al., 2017). ER stress has been known to be involved in plasma cell differentiation (Ma et al., 2010) and megakaryocyte maturation (Lopez et al., 2013). Moreover, the UPR marker, CHOP transcriptionally attenuates BCL2 anti-apoptotic family of protein. Pro-apoptotic Bcl-2 family members such as BAX are associated with the ER membrane and stimulate the activation of apoptotic pathways. In this study, we further confirm the ER stress and UPR during megakaryocytic maturation of PMA and Thapsigargin induced Dami cells. This UPR could be a signal for the Caspase cascade and maturation of cells.

## 4. Conclusion

The mechanism underlying the activation of caspases during megakaryocyte maturation is not explored largely. We studied caspase activation by PMA induction in Dami cells which serve as a superior model to study human megakaryocyte biology and differentiation. We report that ER stress triggers unfolded protein response (UPR) and prolonged UPR results in apoptosis leading to the activation of caspase. PMA induction resulted in activation of the pro-apoptotic proteins and down regulation of anti-apoptotic components. Increase in the caspase activity and cleaved product of PARP was observed in PMA treated cells along with increased ER stress and maturation markers. These findings suggest that ER stress is a possible mechanism underlying the activation of caspases and maturation of megakaryocytes.

## Conflict of interest

Authors have no conflict of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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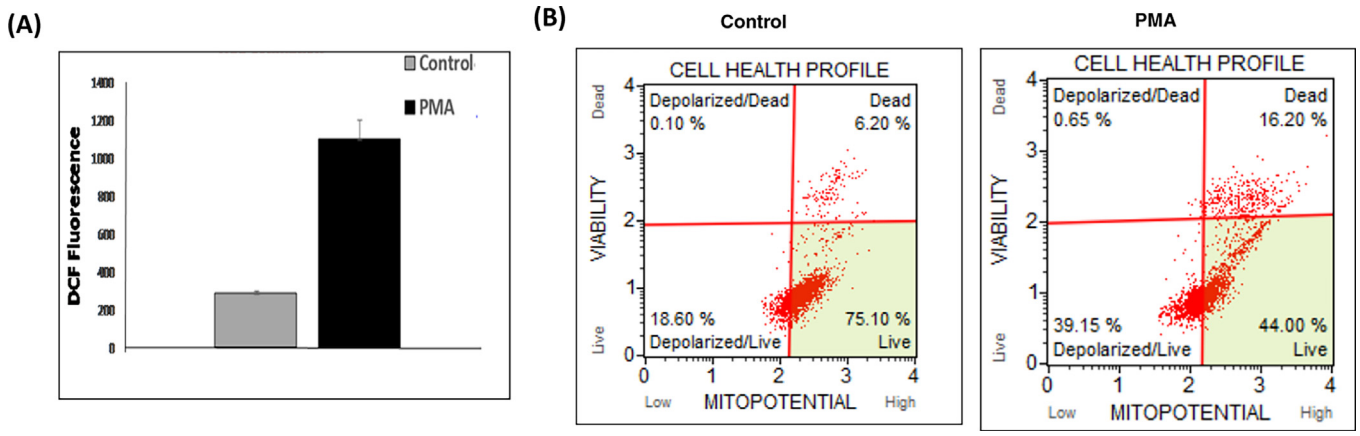


**Fig. 3.** Dami cells undergo depolarization and increased ROS production in PMA treated cells. (A) ROS measurement using DCFDA showed increased levels in PMA treated compared to control cells. (B) Depolarized cells in lower left quadrant were more in PMA treated compared to control cells (n = 3).

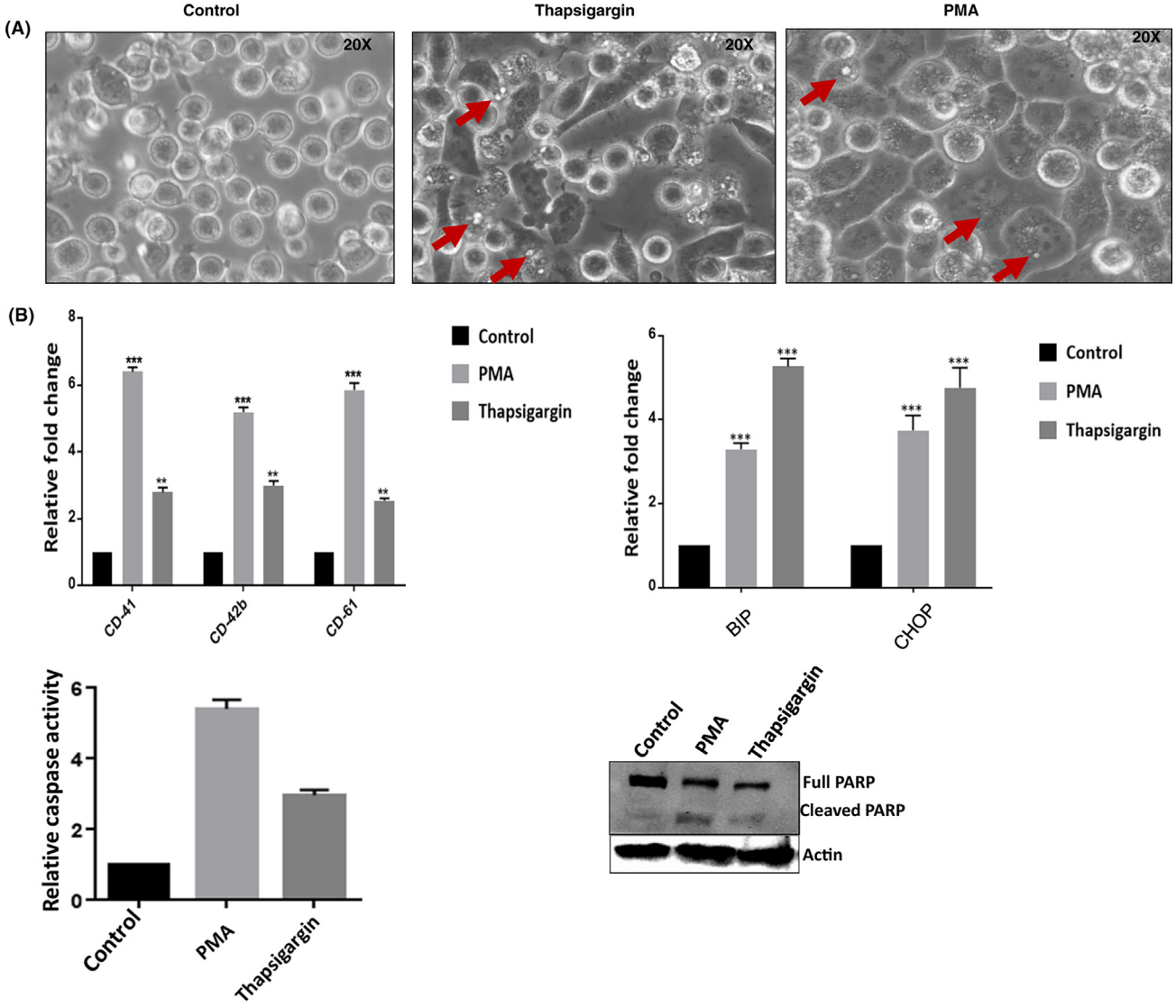


**Fig. 4.** ER stress induces apoptosis during the megakaryocyte maturation. (A) Morphological analysis suggests megakaryocytic features such as increased cell size and adherence were observed in both PMA and Thapsigargin treatments as compared to control cells. (B) Megakaryocyte specific marker (CD41, CD42b and CD61) were found to be increased in PMA and Thapsigargin treated Dami cells by qRT-PCR analysis. Elevation in the expression of UPR markers (Bip and CHOP) was observed. (C) Increase in caspase enzymatic activity and PARP cleavage was observed by western blot analysis in PMA and Thapsigargin treated Dami cells. Bars represent mean ± SE of three independent experiments (n = 3; ***$P < .002$).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2019.10.009.

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# Long non-coding RNA: Classification, biogenesis and functions in blood cells

Swati Dahariya, Indira Paddibhatla, Santosh Kumar, Sanjeev Raghuwanshi, Adithya Pallepati, Ravi Kumar Gutti*

*Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad, 500046, TS, India*

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ABSTRACT

While there exist some long non-coding RNAs (lncRNAs) that are structurally similar to mRNAs (capped, spliced, poly a tail), not all of the lncRNAs exhibit these features. Structurally, lncRNAs are classified under the regulatory non-coding RNAs category these lncRNA molecules operate as signals, decoys, guides, and scaffolds. In eukaryotes, lncRNAs are transcribed by RNA Polymerase II and RNA Polymerase III at several loci of the genome. Unlike other protein-coding mRNAs, lncRNAs exhibit functional uniqueness by participating in and modulating the various cellular processes such as, histone modification, DNA methylation, and cellular transcription (Wei et al., 2017). LncRNA alters chromatin structure and DNA accessibility, thereby regulating patterns of gene expression (Wang et al., 2011b). Disordered lncRNA with quantitative or qualitative alterations lead to the progression of numerous diseases including blood associated diseases. LncRNAs not only regulate lineage commitment such as cardiovascular lineage but also contribute for the hematopoietic stem cell development with a significant role in myeloid and lymphoid lineage commitment. However, the key molecular functions of lncRNAs in hematopoiesis are still unclear, particularly, their functional role during megakaryocyte development from hematopoietic stem cells (HSCs) is largely unexplored. This review summarizes the current status of knowledge on lncRNAs classification, biogenesis and its role in blood cells.

## 1. Introduction

Many years of research on the transcriptome, accompanied by an advancement in sequencing technologies such as microarrays, deep RNA sequencing, and next-generation sequencing, have provided a mechanistic paradigm of eukaryotic gene expression (Wang et al., 2009;

*Abbreviations:* rRNA, ribosomal RNA; tRNA, transfer RNA; ScRNA, small cytoplasmic/ conditional RNA; miRNA, MicroRNA; SnoRNA, small nucleolar RNA; lncRNA, long non-coding RNA; ncRNA, non-coding RNA; EndoSiRNA, endogenous small interfering RNA; PiwiRNA, Piwi-interacting RNA; ENCODE, encyclopedia of DNA elements; cisRNA, Cis-regulatory RNA; Tels RNA, telomere specific small RNA; prompts, promoter-upstream transcripts; tiRNA, transcription initiation RNA; SnRNA, small nuclear RNA; lincRNA, long intergenic non-coding RNA; CeRNA, competing endogenous RNA; HOTAIR, HOX transcript antisense RNA; PTV1, poly tropic virus 1; HOXa11-as, Homeo BoxA11-antisense RNA; MEG3, maternally expressed 3; MIAT, myocardial infraction associated transcript; PFL2, profilin 2; H19, imprinted maternally expressed transcript; ARSR, activated in RCC with sunitinib resistance; RNAi, RNA interfernce; NEAT1 RNA, nuclear enriched abundant transcript 1 RNA; DLEU2 RNA, deleted in lymphocytic leukemia 2; AlncRNA-EC7, bloodlinc; LincRNA EPS, LincRNAerythro id prosurvival; GATA1, globin transcription factor; TAL1, T-cell acute lymphocytic leukemia 1; KLF1, krupple like factor 1; PACER, P50 associated COX2 extragenic RNA; NRON, non-protein coding repressor of NFAT; Gas5, growth arrest specific 5; TINCR, terminal differentiation induced non-coding RNA; TP53COR1 (Linc-p21), tumor protein p53 pathway corepressor 1; XIST, X inactive specific transcript; PRC2, polycomb repressive complex 2; FENDRR, fetal-lethal (FOX1F1 adjacent) non-coding developmental regulatory RNA; TrxG/MLL, trithrox group/ myeloid lymphoid or mixed lineage leukemia group protein; LncHSC1, long non-coding RNA hematopoietic stem cell 1; ANRIL (CDKN2B-AS1), antisense non-coding RNA in the INK4 locus; CBX7, chromo box 7; Ink4, inhibitor of CDK4; LSD1/COREST/REST, RE1 silencing transcription factor; Dacor1, DNMT1 associated colon cancer repressed lncRNA 1; HOTAIRM1, HOX transcript antisense RNA myeloid specific 1; LUNAR1, leukemia associated non-coding IGF1R activated RNA; PARTICLE, promoter of MAT2A antisense radiation induced circulating lncRNA; DUM, developmental pluripotency associated 2 upstream binding muscle lncRNA; TARID, TCF21 antisense RNA inducing demethylation; HDAC1, histone deacetylase 1; EZH2, enhancer of ZEST polycomb repressive complex 2; DNMT3A, DNA methyl transferase 3 alpha; CRNDE, colorectal neoplasia differentially expressed; SPRINGHTLY, formerly sprouty 4 intron; HOST2, human ovarian cancer specific transcript 2; BGLT3, beta globin locus transcript 3; TUG1, taurine up-regulated 1; LINK A, long intergenic non-coding RNA; CHAST, cardiac hypertrophy associated transcript; TNBC, triple negative breast cancer; HBEGF, heparin binding EGF like growth factor; EGFR-GPNMB, epidermal growth factor receptor glycol protein non metastatic B; EGO, eosinophil granule ontogeny; LINC00173, long intergenic non-protein coding RNA 00173; AS-RBM15, antisense RNA binding protein; RUNX1, runt related transcription factor 1; LncMEGA1, transcription activation of an antisense long non-coding RNA

* Corresponding author.
*E-mail address:* guttiravi@uohyd.ac.in (R.K. Gutti).

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Lowe et al., 2017; McGettigan, 2013; Martin and Wang, 2011; Qian et al., 2014). This led to tremendous progress in understanding the intricate structure of the genome and transcriptome. The genome of humans and other multicellular eukaryotes is mostly comprised of non-protein coding DNA (Kaikkonen et al., 2011; Palazzo and Lee, 2015a). This is transcribed into different categories of non-coding RNAs that include structural RNAs (rRNAs and tRNAs) and regulatory RNAs (scRNA, miRNA, snoRNA, lncRNA) (Hemberg et al., 2012). Only a fraction (~2%) of the genome is translated into coding transcripts that include the coding RNAs (Palazzo and Lee, 2015). Non-coding RNAs (ncRNAs) are found to deliver housekeeping functions in several biological processes by taking part in the regulatory mechanism of gene expression at the transcriptional and post-transcriptional level (Palazzo and Lee, 2015; Mattick and Makunin, 2006; Esteller, 2011). The lncRNA sequence may be present as introns and intergenic region in the same set of genes that encode proteins (Brown et al., 2008; Deveson et al., 2017). Two decades back the discovery of small ncRNA opened the avenues for the existence of other functional ncRNAs. This led to some uncertainty about the uncharacterized world of functional ncRNAs and junk RNA (Palazzo and Lee, 2015; Wang et al., 2011a). In past several years, our knowledge of ncRNA has been expanding and that makes feasible to classify ncRNAs in different categories of regulatory non-coding RNAs, such as endogenous small interfering RNAs (endo-siRNAs) (Castel and R a, 2013; Okamura and Lai, 2008), PIWI-associated RNAs (piRNAs) (Okamura and Lai, 2008; Costa, 2008), and long non-coding RNAs (lncRNAs) (Long et al., 2017). But, the functional significance of these ncRNA is yet to be deciphered for further characterization. Recent scientific literature provides barely sufficient data to postulate the consequential role of lncRNAs in blood cell development and blood-related diseases (Alvarez-Dominguez and Lodish, 2017). Over the past two decades, many research groups have uncovered the key role of lncRNAs in hematopoiesis. LncRNAs are a class of large size non-coding RNAs which play the key regulatory role in cellular processes such as proliferation, differentiation, and development. They are also known to be involved in the development of several diseases with their changed expression levels such as *GAS5LincRNA-p21, HOTAIR, MALAT1, NEAT1,* and *H19* in colorectal cancer, *HOTAIR, MALAT1, NEAT1, BANCR,* and SOX2-OT in lung cancer and *HULC, HOTAIR, HOTTIP* and *ZFAS1* in liver cancer and so many lncRNAs in breast, pancreatic, ovarian cancers and leukemia (Bhan et al., 2017; Ohtsuka et al., 2018). This review is a compendium of literature on lncRNAs, their expression and function in blood cells with a specific focus on megakaryocytes. Also, the review highlights the mechanism through which lncRNAs participate in different regulatory functions.

## 2. Classification of non-coding RNAs

Human genome analysis contributed to the first discovery of long sequences of ncRNA such as tRNA and rRNA (Wright and Ea, 2011). In addition to this, other lncRNA sequences were also identified whose function is not found in proteins translation machinery. When these long sequences of non-coding RNA were compared to ENCODE consortium data, it was found that several classes of ncRNA molecules are generated through pathways similar to that of protein-coding genes (Derrien et al., 2012; Harrow et al., 2012; Consortium et al., 2012). These findings in relation to previous studies sparked on the diversity of non-coding RNA, ncRNAs are classified into two major categories: structural non-coding RNAs and regulatory non-coding RNAs (Fig. 1). Structural non-coding RNAs comprise of rRNAs and tRNAs. Regulatory non-coding RNAs are further divided into three classes, small, medium and long non-coding RNAs (Ponting et al., 2009; Alvarez-Dominguez and Lodish, 2017). further, miRNA, siRNA, piRNA, cisRNA, telsRNA were considered as short non-coding RNA with a size between 20–50 nucleotides, and snoRNA, prompts, tiRNA, snRNA and many more (Fig. 1) are classified as medium non-coding RNA with a size between 50–200 nucleotides (Nagano and Fraser, 2011; O'Day and Lal, 2010). And a large class of RNA with maximum regulatory potency containing greater than 200 nucleotides are classified as long non-coding RNA (Derrien et al., 2012; Wang et al., 2014). With increasing studies on highly abundant and functionally important categories of lncRNAs (such as intronic, antisense, lincRNA, cisRNA, ceRNA etc), we provide, with sufficient clarity, the classification of ncRNAs and list out all the existing lncRNAs (Fig. 1) (Ma et al., 2013; Gullerova, 2015).

## 3. LncRNAs (long non-coding RNAs)

Generally lncRNAs are defined as long RNA transcripts of more than 200 nucleotides that cannot be translated into proteins (Guo et al., 2016). These are also referred as competing endogenous RNAs (ceRNAs) (Wang et al., 2018a) e.g. *HOTAIR* (Liu et al., 2014; Cai et al., 2017), *PTV1* (Huang et al., 2017), *HOXa11-AS* (Cui et al., 2017), *MEG3* (Peng et al., 2015; Yan et al., 2017), *MIAT* (Qu et al., 2017; Yan et al., 2015), *PFL* (Liang et al., 2018), *MALAT1* (Wang et al., 2016; Qu et al., 2017; Liang et al., 2018), *H19* (Liang et al., 2016) *and* ARSR (Qu et al., 2016). They play an important role in the fine-tuning of the translation machinery and in its regulation by modulating the crucial functions of other ncRNAs such as miRNAs, snoRNAs etc. (Long et al., 2017; Paraskevopoulou, 2016; Nitzan et al., 2014). lncRNAs are not only originate from their own promoters but also originate from the promoters shared with divergently transcribed coding or non-coding genes, or from enhancer sequences (Al-Tobasei et al., 2016; Hu et al., 2012). Presence of lncRNAs is reported in many organisms like zebrafish (Pauli et al., 2012), tetrapod (Jenkins et al., 2015), roundworm (Nam and Bartel, 2012), mammal (Chen et al., 2016) and other organisms (Table 1). Previous studies revealed large-scale catalog of lncRNAs as putative transcripts within the human genome that do not encode proteins but many of them show poly-A + tail and 5′cap (Derrien et al., 2012). However, recent studies using Ribosome profiling, or Ribo-Seq, and mass spectrometry showed the presence of lncRNA with an open reading frame, that unveils translational ability to encode proteins (Wang et al., 2017a; Bazin et al., 2017). Few studies also shed light on the existence of lncRNAs with a high probability of coding peptides based on their location to nearby protein-coding genes with shorter and fewer exons (Fadista et al., 2014; Jia et al., 2010; Liu et al., 2018; Vance and Ponting, 2014). lncRNAs possess distinctive evolutionarily conserved patterns, with a higher degree of tissue specificity (Vance and Ponting, 2014). Many years of research identified a multitude of regulatory paradigms controlled by several identified lncRNAs that affect the wide range of cellular activities and functionally associated with normal development and pathophysiology of quite a few diseases (Cipolla et al., 2018; Melissari and Grote, 2016; Giroud and Scheideler, 2017). These include several types of cancer, neurological and cardiovascular conditions, immunological and metabolic disorders (Cipolla et al., 2018). Due to RNA sequencing and other new techniques, a greater number of lncRNA classes have been observed (Kung et al., 2013). Several classes of lncRNAs have been discovered on the basis of diverse parameters such as transcript length, association with annotated protein-coding genes, and mRNA resemblance among others (Zhang et al., 2014a; Costa, 2005; Kumari and Sampath, 2015). However, several challenges have been encountered during the annotation and interpretation of non-coding transcriptome data due to the absence of an unambiguous classification framework. These classes of RNA molecules are distinguished from other RNAs based on certain unique features (Hu et al., 2015). These include alternative forms of biogenesis (Spurlock et al., 2016), unique regulatory mechanisms (Marchese et al., 2017; Wang and Chang, 2012), cis-regulatory activities (Kornienko et al., 2013; Pelechano and Steinmetz, 2013), and functionally structured RNA-binding domains (Zampetaki and Albrecht, 2018). In spite of various research groups focusing on lncRNAs, there still exists unidentified and unaccounted information regarding the function and structure of lncRNA.

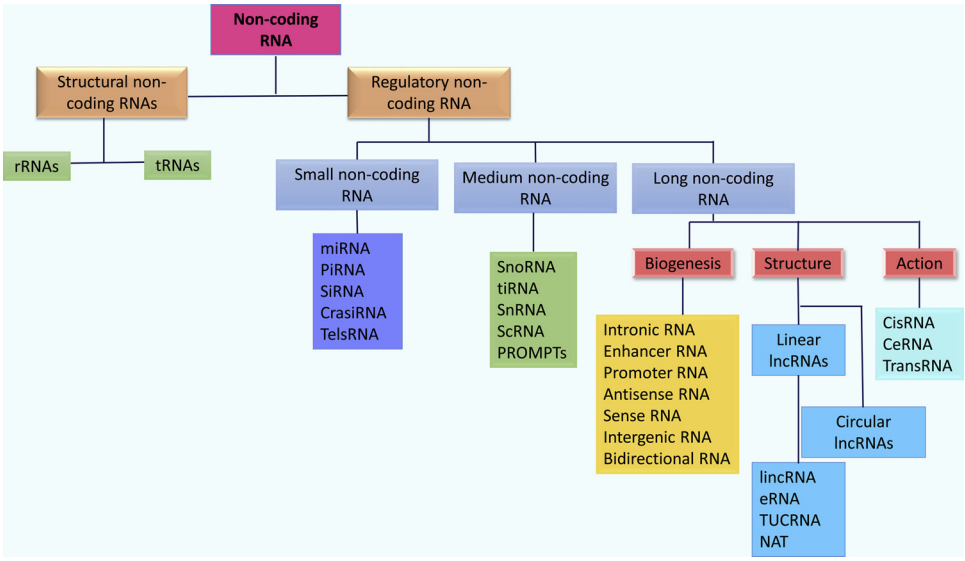



**Fig. 1.** Classification of long non-coding RNAs. Schematic depicts the placement and classification of long non-coding RNAs into classes and sub-classes according to their action, biogenesis and structure.
***Abbreviations:*** ncRNA: non-coding RNA, tRNA: transfer RNA, miRNA: micro RNA, piRNA: piwi RNA, siRNA: small interfering RNA, tiRNA : transcription initiation RNA, crasiRNA : Centromere repeat associated small interacting RNA, telsRNAs : telomere-specific small RNA, snoRNA : small nucleolar RNA, snRNA : small nuclear RNA, scRNA: small cytoplasmic RNA, cis-lncRNA: cis-acting long non coding RNA, trans-lncRNA : trans-acting long non coding RNA, ceRNA : competing endogenous RNA, eRNAs: enhancer-derived RNAs, NATs: natural antisense transcript, TUCRNAs: transcribed ultraconserved RNAs, lincRNAs : Long intergenic noncoding RNAs.

### 3.1. Biogenesis

Long non-coding RNAs play an important role in different aspects of human development and disease (Schmitz et al., 2016; Delás and Hannon, 2017). Understanding the biogenesis of lncRNAs is beneficial in not only differentiating it from other types of RNAs but also to decipher its functional significance. The biogenesis of lncRNAs is cell type- and stage-specific which is under the control of cell type- and stage-specific stimuli (Akerman et al., 2017; Morán et al., 2012). Different classes of lncRNAs transcribed from several DNA elements such as enhancers, promoters, and intergenic regions in eukaryotic genomes (Wu et al., 2017) (Fig. 2). Different mechanisms involved in lncRNA biogenesis such as cleavage by ribonuclease P (RNaseP) to generate mature ends, formation of small nucleolar RNA (snoRNA) and protein (snoRNP) complex caps at their ends, and the formation of circular structures (Vicens and Westhof, 2014; Chen, 2016). Recently unique sub-nuclear structures known as “paraspeckles” have been identified around specific lncRNAs during their biogenesis (Naganuma and Hirose, 2013). RNAi analyses of 40 paraspeckle proteins (PSPs) brought out the discovery of four PSPs that are required for paraspeckle formation (Yamazaki and Hirose, 2015; Krol, 2017). Overall, mechanisms of synthesis and regulation of different lncRNAs are not completely uncovered, however the next few years will deepen our insight into the biogenesis and functions of lncRNAs with the help of various techniques, such as ChIRP-Seq (Chromatin Isolation by RNA purification), crosslinking immunoprecipitation (CLIP), ribosome profiling, RNA structure mapping, and phylogenetic lineage tracing, targeted genome engineering by CRISPR and advanced genetic screens (Yoon and Gorospe, 2016; Salehi et al., 2017; Li et al., 2014a).

### 3.2. Functional role and mechanism of action

In the previous studies many members of the lncRNA group have been functionally characterized in different types of cells and disease conditions (Li et al., 2014a; Neguembor et al., 2014) There is a considerable number of lncRNAs with the potential to participate in both normal and aggressive disease state by regulating the biological processes such as cell differentiation, cell lineage determination, organogenesis, and tissue homeostasis along with cell pluripotency induction, X-inactivation and gene imprinting (Kazemzadeh et al., 2015). Notably, a large number of lncRNAs have been associated with numerous diseases, such as diabetes, rheumatoid arthritis, neurodegenerative disorders, atherosclerotic coronary artery disease, and various types of cancer (Wu et al., 2014; Li et al., 2013). Knowledge of lncRNAs’ functions is adding another layer of complexity to our understanding of the mechanism of lncRNA. However, according to the current research, lncRNAs are involved in both the activation and inhibition of the gene expression. This is attained through a range of diverse molecular

**Table 1**
LncRNAs examples in vertebrates and invertebrates. lncRNAs and their length in different organisms (vertebrates and invertebrates).

| Animal kingdom | Length of lncRNAs | Organisms | Examples of lncRNAs | References |
|---|---|---|---|---|
| **Vertebrate** | 2.3-17.2 *1000 basepairs. | Mammal eg. Mus musculus, | *Xist, Tsix, H19, Air, HOTAIR, Nron, AK043754, AK082072, AK082467, TCONS_00062870* | (Kutter et al., 2012; Raghuwanshi et al., 2018; Tran et al., 2016) |
| | | Sus | *TCONS_00244288 TCONS_00791385 TCONS_00323177 TCONS_791379 TCONS_00815876 TCONS_00512109 TCONS_853343* | |
| | 556 basepairs | Actinopterygii e.g. Danio rerio | *HoxAa, myo18a* | (Ma et al., 2007; Nitzan et al., 2014) |
| | 500 basepairs | Aves e.g. Gallus gallus domesticus, Didelphimorphia | *AK043754, AK082072, AK082467 and Rmst* | (Raghuwanshi et al., 2018) |
| **Invertebrates** | 415-378 basepairs | Chromadorea e.g. C. elegans | *linc-41, NATs* | (Garzon et al., 2014; Hu et al., 2012) |
| | | Insecta eg. Drosophila melanogaster | *roX1 and roX2* | (Hu et al., 2016) |

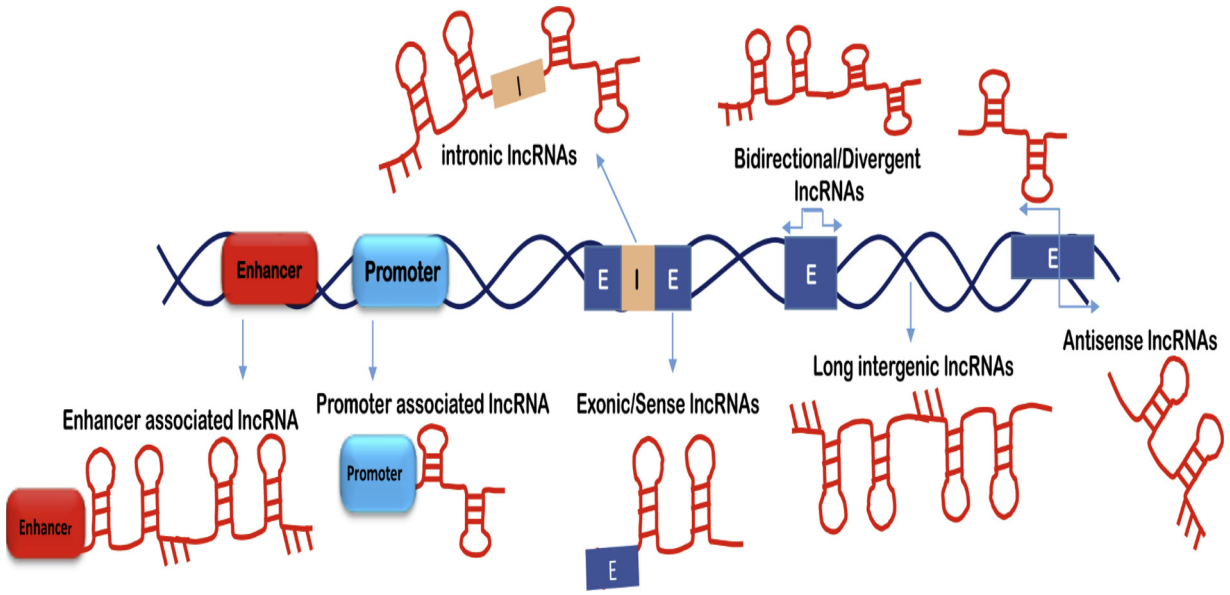
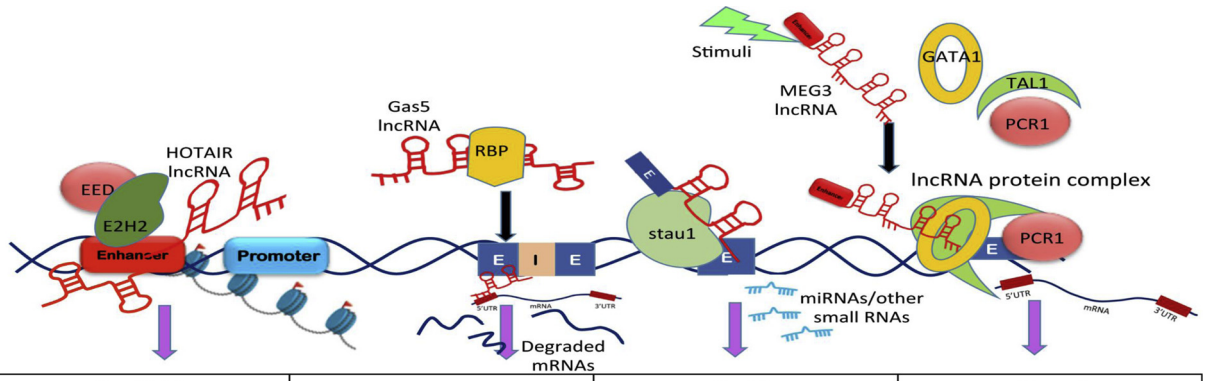



**Fig. 2.** Overview of biogenesis of lncRNA. lncRNAs are pervasively interspersed in the genome with various possible locations. The figure summarizes the diverse range of lncRNAs based on their transcriptional origin: a) whole or partial natural antisense transcripts (NAT), b) coding genes, c) between genes, d) within introns, e) Promoter[blue], and f) Enhancer [red](I = Intron; E = Exon) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).




| Scaffold | Decoy | Guide | Signal |
|---|---|---|---|
| Chromatin modification and epigenetic modification also recruitment of chromatin remodeler. e.g. HOTAIR, ANRIL, and PARTICLE etc. | Transcriptional inhibition / mRNA degradation and RNA-RNA interaction, transcriptional and translation inhibition. e.g. HOTTIP, GAS5, and KCNQ1OT1 etc. | Precursors of miRNAs and other small and medium RNAs or RNA Processing such as splicing, editing, turnover and miRNA sequestration. e.g. MALAT1, GAS5, XIST, FENDRR, and lncHSC1-2 etc. | Transcription/Translation activation for gene activation, modulation of miRNA regulation e.g. NRON, BAND, DLEU2, PACER, and lincRNA COX2 |

**Fig. 3.** Mechanisms of lncRNA action. LncRNAs exert or execute their functions by the above depicted molecular mechanisms.

mechanisms that are summarized as the four manners, (signal, decoy, guide, and scaffold) (Rinn and Chang 2012; Wang and Chang, 2012; Quinn and Chang, 2015) (Fig. 3).

In previous studies it was observed that lncRNAs crosstalk with many epigenetic factors or interface with them to regulate gene expression and modulate nuclear architecture (Hanly et al., 2018; Heo et al., 2013; Mercer and Mattick, 2013). LncRNAs also helps in developmental processes by co-regulating their own and neighboring protein-coding gene expression (Quinn and Chang, 2015; Luo et al., 2015). Some lncRNAs have been found to participate in signal transduction via playing role as a regulator to initiate, elongate or to terminate combinatorial actions of transcription factors (Long et al., 2017). There exist few lncRNAs that are involved in significant biological events such as multi-dimensional spectra of post-transcriptional RNA modification. These modifications include splicing, editing, localization of mRNA

*alncRNA-EC7, BAND3, DLEU2,* and *lincRNA-EPS* lncRNAs are transcribed from the enhancer region that has a crucial role in the activation of the neighboring gene (Zhang et al., 2009). These lncRNAs also have a function in the development of red blood cells via stimulating erythroid gene expression and prompting erythroid progenitors proliferate (Marchese et al., 2017; Wang and Chang, 2012; Schmitz et al., 2016; Koerner et al., 2009; Delaval and Feil, 2004; Alvarez-Dominguez et al., 2014, 2017). In addition, studies have also demonstrated that these lncRNAs interface epigenetic regulation of erythropoiesis by targeting the key transcription factor (GATA1, TAL1, and KLF1) and catalytic proteins or subunits of large chromatin remodeling machinery as well as miRNAs (Tian et al., 2016). Recently it was observed that lncRNA maternally expressed gene 3 (MEG3) which functions as a tumor suppressor is significantly downregulated in AML. MEG3 is transcriptionally activated by Wilms' tumor 1 (WT1) and its cofactor

**Table 2**
Long non-coding RNAs in hematopoiesis. list of all the LncRNAs (with functions) involved in human hematopoiesis and associated diseases.

| lncRNA | Aliases for lncRNA gene | Abbreviation | Hematologic disease | Function | Molecular mechanism | References |
|---|---|---|---|---|---|---|
| alncRNA-EC7 | Bloodlinc, and Gm11616 | Erythroid developmen- al long intergenic non-protein coding transcript | Hereditary hemolytic anemias | Potentiates erythroblast proliferation and enucleation during red blood cell development | Promote red cell maturation by regulating neighboring gene encoding BAND 3 | (Luo et al., 2015) |
| BGLT3 | BGL3, CTD-2643I7.1 LncRNA-BGL3, and LINC01083 | Beta globin locus transcript 3 | CML (chronic myeloid leukemia) | Regulator of BCR and ABL mediated cellular transformation | Competitive endogenous RNA cross-regulating the expression of the tumor suppressor PTEN | (Kuvardina et al., 2015) |
| CDKN2B-AS1 | ANRIL, P15AS, NCRNA00089, CDKN2B-AS, and PCAT12 | CDKN2B antisense RNA 1 | leukemia | Induced cell cycle disorder, differentiati-on block, and apoptosis arrest in blood cells | Silenced p14, p15, and p16, which are repressors of leukemia | (Zhao et al., 2014; Zhan et al., 2018) |
| DLEU2, DLEU1 | MIR15AH-G, TRIM13O-S, BCMSUN, RFP2OS, ALT1, LEU2, RFP2, and DLB2 | Deleted in lymphocytic leukemia 2 | CLL (chronic lymphocytic leukemia) | Regulate pivotal oncogenes, tumor suppressors or relevant pathways in malignant hematopoiesis | Epigenetic tumor suppressor mechanism via regulate NF-kB activity. | [180] |
| EGOT | EGO, and NCRNA00190 | Eosinophil granule ontogeny transcript | Erythro leukemia, myeloid leukemia and Myeloproliferative disorder | Myeloid and erythroid development | Compromises the expression of several proteins major basic protein and eosinophil derived neurotoxin (EDN) that are important for eosinophil development | (Lin et al., 2016) |
| GAS5 | NCRNA00030, and SNHG2 | Growth arrest specific 5 | B-cell lymphoma, T-cell leukemia, Acute myeloid leukemia | Normal cell growth arrest in T-cell lines and non-transformed lymphocytes | Glucocorticoid receptors antagonist. Regulated by mTOR pathway | [181, 182] |
| H19 | D11S813E, ASM, ASM1, NCRNA00008, and LINC00008 | H19, imprinted maternally expressed transcript | CML, AML, adult T-cell leukemia /lymphoma and leukemia subtypes | During growth and development of HSC reduced quiescence and compromise it's function Oncogene/ tumor suppressor | Genomic imprinting of its neighboring gene IGF2 and Dlk1, also bind PRC2 and Igf2 to promote chromatin remodeling and post-transcriptional processes. | [183] |
| IGF1R-AS | IRAIN | IGF1R antisense imprinted non-protein coding RNA | Leukemia, Acute Myeloid and Pancreatic Cancer | Promoting cell growth through the PI3K/Akt signaling pathway | Interacts with chromatin DNA at promoter and enhancer regions of the IGF1R gene to form an intrachromosomal enhancer/promoter loop. | [184] |
| LincRNA-EPS | | LincRNA erythroid prosurvival | | Essential for dynamic terminal differentiation of erythroid cells. | Mediated anti-apoptotic activity by repressing many proapoptotic genes e.g. pycard | [185] |
| LncHSC1 | | | | Essential in myeloid differentiation and HSC self-renewal and lineage commitment | Regulated by hematopoietic TFs including Erg, Fli1, Lmo2, Meis1, Gata2, Runx1, PU.1, Scl, Lyl1, and Gata2 | (Quinn and Chang, 2015) |
| LncHSC2 | | | | involved in HSC self-renewal and T cell differentiation | Close to genes *Pml,* and *Itpkb* | (Quinn and Chang, 2015) |
| LUNAR1 | Leukemia-Induced Noncoding-Activator RNA, and AC036108.4 | Leukemia-associated non-coding IGF1R activator RNA 1 | Acute T Cell Leukemia and Diffuse Large B-Cell Lymphoma. | Controlling leukemogenesis | NOTCH1-regulated. Activation of IGF1R expression in cis by recruitment of the Mediator complex and RNA polymerase II to the IGF1R enhancer | [186] |
| MAFTRR | Linc-MAF-4 | MAF transcriptional regulator RNA | Different allergies and auto immunity | Key role in T lymphocyte | Increases the expression of MAF and skews T-cell differentiation toward the Th2 phenotype | [187] |
| MALAT1 | NCRNA00047, LINC00047 PRO2853, NEAT2, and HCN | Metastasis associated lung adenocarcinoma transcript 1 | Tongue Cancer and Leukemia, Acute Monocytic | Form molecular scaffolds for ribonucleopro-tein complexes | Promote cell cycle progression and proliferation and maintaining undifferentiated status of early-stage hematopoietic cells by regulating the expression of oncogenic transcription factor B-MYB | [188] |
| MEG3 | GTL2, NCRNA00023, LINC00023, and onco-lncRNA-83 | Maternally expressed 3 | Kagami-Ogata Syndrome and Phaeochromocytoma, acute myeloid leukemia | Involve in myeloid differentiation and inhibits tumor cell proliferation by recruiting the PRC2 complex. | Regulation of the Rb-p16INK4a pathway, regulate tumor suppressor p53 pathway. | [189] |
| TUG1 | FLJ20618, NCRNA00080, LINC00080, and TI-227H | Taurine up-regulated 1 | Chronic lymphocytic leukemia and multiple myeloma | Promotes cell proliferation of tumor cells. | PRC2 binding to repress cell-cycle regulation genes. Induced by p53 | [190] |
| XIST | NCRNA00001, DXS1089, swd66, SXI1, LINC00001, LINC00001, DXS1089, and DXS399E | X inactive specific transcript | Fibrosis, leukemia, and histiocytic sarcoma | Required for hematopoietic stem cell survival and function | Not described | [191] |

(*continued on next page*)

**Table 2** (*continued*)

| lncRNA | Aliases for lncRNA gene | Abbreviation | Hematologic disease | Function | Molecular mechanism | References |
|---|---|---|---|---|---|---|
| Neat1 | Non-Protein Coding RNA 84, MENepsilon/Beta NCRNA00084, LINC00084, TncRNA, and VINC | Nuclear Paraspeckle Assembly Transcript 1 | Relapsing-remitting multiple sclerosis and Dengue | Act as a transcriptional regulator for numerous genes involved in cancer progression and viral infection | Forms the core structural component of the paraspeckle sub-organelles | [192] |

Ten-eleven translocation-2 (TET2). TET2 mutations dysregulate the WT1-MEG3 exis and promote leukemogenesis (Lyu et al., 2017; Williams et al., 2011). Taken together, it is observed that several lncRNAs' expression prolongs as a molecular signal in cellular function.

LncRNAs exhibit decoy type of molecular activity by binding to transcription factors or proteins and thereby titrate away protein factors from their action on target DNA. Some lncRNAs execute themselves as a sponge or molecular sink for several RBPs (RNA binding Proteins) (He et al., 2016). These RBPs function as chromatin modifiers, transcription factors and also regulators of their neighboring protein-coding genes (He et al., 2016). LncRNAs like *snoRNAs, MALAT1* and *NEAT1*. functioning as a sponge or molecular sink to miRNAs (Wang et al., 2016; Liu et al., 2017a; Xiao et al., 2015), also can mediate changes in the transcriptome of cells by negatively acting on effector molecules such as transcription factors, cell receptors, growth factors and splicing regulators. (Fu, 2014; Tripathi et al., 2010; Cooper et al., 2014). In B-cell and T-cell leukemia, lncRNA Gas5 (Growth arrest-specific 5) functions as a tumor suppressor by targeting cancer-promoting miRNAs like miR21 (Wen et al., 2017). Gas5 was also shown to act as a growth arrest- and starvation- associated repressor of the glucocorticoid receptor (GR) (Kino et al., 2010). LncRNA Gas5 was also recognized for growth arrest in both leukaemic and untransformed human T-lymphocytes.(Williams et al., 2011). *LncRNAs such as HOTAIR and linc-p21,* previously described as crucial regulators of the epigenime, they function as the RNAs which recruit chromatin modifiers to regulate gene expression via affecting the process of transcription (Hung and Chang, 2011; De Lucia and Dean, 2011). Another example of guide lncRNA is *FENDRR* (Fetal-lethal noncoding developmental regulatory RNA). Its function is clearly delineated in mouse mesoderm lineage development (Grote et al., 2013). It is associated with PRC2 and TrxG/MLL complexes via dsDNA/RNA triplex formation. It thereby brings PRC2 and TrxG/MLL along with itself close to an associated target gene (*Foxf1* and *Pitx2*) involved in differentiation of lateral mesodermal lineage derivatives including blood cells (Grote et al., 2013; Grote and Herrmann, 2013). Recently reported lncRNAs, *lncHSC1* and *lncHSC2*, in an in vitro study using human hematopoietic stem cells, are involved in self-renewal and in early lineage decisions specifically for myeloid differentiation, while *lncHSC6* has a crucial role in HSC differentiation (Luo et al., 2015). *lncHSC4* is also important for blood cell differentiation and the development of erythroid lineage by regulating Kif1, GATA1, and Tal1, but the molecular mechanism is not clear (Luo et al., 2015). LncRNAs have an impact primarily on transcriptional regulation, while *HOTAIR, ANRIL* represents a prototype of a lncRNA which directly modulates the activity of transcription factors and multiple effector molecules by serving as a scaffold for their assembly (Rinn and Chang 2012; Quinn and Chang, 2015; Spitale et al., 2011). It would be interesting to investigate further how lncRNAs achieve transcriptional regulation through the assembly of scaffolding complexes, which may have the action on transcriptional activation or repression for both the development of an organism and on-set of disease (Chen et al., 2015; Wierzbicki, 2012). Some interesting studies on lncRNAs such as *ANRIL* or *CDKNB2-AS1* which tightly interact with multiple components in polycomb repressive complex-1 (PCR1) and polycomb repressive complex-2 (PCR2) and form heterochromatin surrounding the INK4 locus and that results in INK4 gene silencing through chromatin methylation (Aguilo et al., 2011). *HOTAIR* lncRNA is also closely associated with chromatin remodeling complexes by binding to PRC2 and LSD1/CoREST/REST complex at both the 5′ domain and 3′ domain, respectively (Arrial et al., 2009; Bhan and Mandal, 2015). Through histone methylation lncRNAs, such as *XIST* (Corbel et al., 2013), *Dacor1, HOTAIRM1, Fendrr, LUNAR1 (Leukemia-Associated Non-Coding IGF1R Activator RNA 1)* and *PARTICLE* mediate chromatin remodeling gene expression (Schmitz et al., 2016). LncRNAs, *PARTICLE* (O'Leary et al., 2015), *DUM* and *lincP21* (Bao et al., 2015) and many more are involved in epigenetic mechanism in coordination with multiple DNA methyltransferases (DNMTs) and histone methyltransferases (SETDB1) (Zhao

**Table 3**
**Role lncRNAs in erythro-megakaryopoiesis lineages and in hematopoietic tumors.** Role of few lncRNAs involved in fundamental processes of gene regulation, differentiation, apoptosis of stem cells and progenitors of Erythro-megakaryopoiesis and their applications in hematopoietic tumors.

| Sl.No. | LncRNAs | Function/Role | References |
|---|---|---|---|
| 1 | AS-RBM15 | Proliferation and terminal differentiation of erythrocytes | (de Freitas and da Costa Maranduba, 2015; Staerk and Constantinescu, 2012) |
| 2 | LincRNA-EPS | Cell survival, proliferation and differentiation of erythrocytes, anti-apoptotic activity during erythropoiesis | (Cipolla et al., 2018; Katsushima et al., 2016) |
| 3 | Shlnc-EC6 | Erythropoiesis | (Anon., 2019) |
| 4 | HOTAIRM1, EGO | Myeloid differentiation | 192 |
| 5 | LINC00173 | Regulate blood homeostasis | (Trimarchi et al., 2014) |
| 6 | LRRC75A-AS1, CRNDE, LINC01268, HOTAIRM1, H19, LINC00926, FAM30A | Prognostic indicator in Acute Myeloid Leukemia (AML) | 193 |
| 7 | AL356475.1, AC011498.1, AC092811.1, LINC00158, LINC00504 and UCA1 | Biomarker for cytogenetically normal acute myeloid leukemia | 194,195,196 |
| 8 | Fas-AS1 or Saf | Modulates a critical cell death program in erythropoiesis | 197 |
| 9 | XIST | Absence cause hematologic cancers | 191 |
| 10 | H19 | Regulates hematopoietic stem cell quiescence | 183 |
| 11 | Lnc-DC | Positively regulates differentiation of myeloid DC progenitors | 198 |

et al., 2016a; Merry et al., 2015). Even with all the prevailing literature on lncRNAs, we suggest future work to provide new insights to our understanding of the possible role of lncRNAs in the development of blood lineages.

## 4. LncRNA and diseases

LncRNAs is a crucial class of ncRNAs, involved in modulating gene expression in diverse diseases conditions (Tables 2 and 3). LncRNAs play a role in affecting the fundamental features of the epigenome that is reflected during the expression of protein-coding as well as non-coding transcripts. A series of independent studies demonstrated that lncRNAs serve as the source of various short regulatory RNAs (Röther and Meister, 2011). However, mounting evidence show the requirement of various lncRNAs in discrete cellular functions such as cell differentiation and development through different regulatory pathways (Fatica and Bozzoni, 2014). In human cells there are some examples of lncRNAs, such as *SNHG7* (Shan et al., 2018), *CRNDE* (Wang et al., 2017b), *PVT1* (Zeng et al., 2015), *AFII3014* (Zeng et al., 2017), *SHG1 and NCKI-AS1* (Li et al., 2018), *SPRIGHTLY* (Zhao et al., 2016b), *HOST2* (Liu et al., 2017b; Wang et al., 2018b), and *BX35664* (Liu et al., 2016), whose expression has remarkable effects on numerous vital cellular processes. LncRNAs impact cellular processes by affecting the regulatory mechanism of mRNA transcription, alternative splicing and translation that tear down the physiological processes which are associated with cell proliferation, pluripotency, differentiation (Boland et al., 2014; Guttman et al., 2011), and apoptosis (Rossi and Antonangeli, 2014) (Fig. 4). Many of lncRNAs and their products have been linked to clinical disease phenotypes via the regulation of alternative splicing, silencing and post-transcriptional modification of mRNA (Li et al., 2014b). In past couples of years, siRNA mediated exploitation of lncRNAs has been shown with significant potential for effective diseases treatment (Prabhakar et al., 2017). This opens a new avenue for future research to dwell into using lncRNA as a potential therapeutic target. Because of its inhibitory functions with tissue specificity in the regulation of specific target gene, lncRNAs serve as potential therapeutic value (Gomes et al., 2017). Various types of lncRNAs *HOTAIR, ceRNAs, BGLT3,* and *CDKN2B-AS1* have altered their own expression in various disease conditions such as cancers, leukemia, diabetes, and autoimmune diseases (Yuan et al., 2017). Currently, some studies revealed that inhibition of upregulated lncRNAs like *TUG1 (taurine up-regulated 1)* (Viereck et al., 2016), *LINK-A* (long intergenic non-coding RNA for kinase activation), and *CHAST* (cardiac hypertrophy-associated transcript) (Viereck et al., 2016) by several methods like RNA interference (RNAi), degradation by RNase H, activating antisense oligonucleotides (ASOs), or gross deletion/alteration at the DNA level using CRISPR/Cas9 genome editing methods may be effective for the treatment of various diseases (Anon., 2019). For example, significantly reduced expression of *TUG1* an intergenic lncRNA, found in placental abnormalities triggers the poor transformation of the spiral arteries in the trophoblast. The silencing/knocked down *TUG1* expression levels through the transfection of *TUG1* siRNAs, lead to a suppressive effect on trophoblasts by inhibiting cell proliferation, invasion, migration and promoting cell apoptosis (Xu et al., 2017). Furthermore, *TUG1* is highly expressed in GSCs (glioma stem cells) and maintained the stemness features of glioma cells, triggered through the Notch signaling pathway (Katsushima et al., 2016). Targeting *TUG1* by antisense oligonucleotides (ASO) with a potent drug delivery system (DDS), provided an effective and selective antitumor effects to control the population of glioma cells (Katsushima et al., 2016). A cytoplasmic lncRNA, *LINK-A* is necessary for the progression of tumor formation and metastasis of many cancer types including Triple-Negative Breast Cancer (TNBC). *LINK-A* is required for the proper activation and stabilization of Hypoxia-Inducible Factors (HIF1αs) targeted transcriptional program. This program is involved in TNBC progression, recurrence, and metabolic reprogramming (Lin et al., 2016). Furthermore, in cardiomyocytes, upregulated *CHAST* as a budding lncRNA candidate influences cardiomyocyte hypertrophy, GapmeR-mediated silencing of *CHAST* was sufficient to prevent and attenuate TAC (Transverse aortic constriction) induced pathological cardiac remodeling with no early signs on toxicological side effects, which highlight a therapeutic role of lncRNAs in heart diseases (Viereck et al., 2016). Although several groups have investigated possible roles for lncRNAs as key regulators of the pathogenic and oncogenic states of acute leukemia. *LUNAR1* lncRNA over-expression is associated with T cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma disease conditions. Knockdown of *LUNAR1* significantly supports a mechanism that control cell division of cancerous cells (Trimarchi et al., 2014).

## 5. LncRNAs in hematopoiesis and perspective role in megakaryocytopoiesis

LncRNAs play an essential role in maintaining the hematopoietic stem cell pool in humans. They are known to be paramount for lineage-specific differentiation. For example: *shlnc-EC6* is a lncRNA associated with erythroid differentiation (Wang et al., 2015), *HOTAIRM1* is cell-cycle regulator during myeloid maturation in addition to granulocytic differentiation (Wei et al., 2016; Zhang et al., 2014b). EGO positively regulates several genes important for eosinopoiesis (Wagner et al., 2007). *LINC00173* is subjected to in vitro granulocytic differentiation, myeloid colony-formation and function (Schwarzer et al., 2017) (Table 2). Mouse and human hematopoiesis share similar development

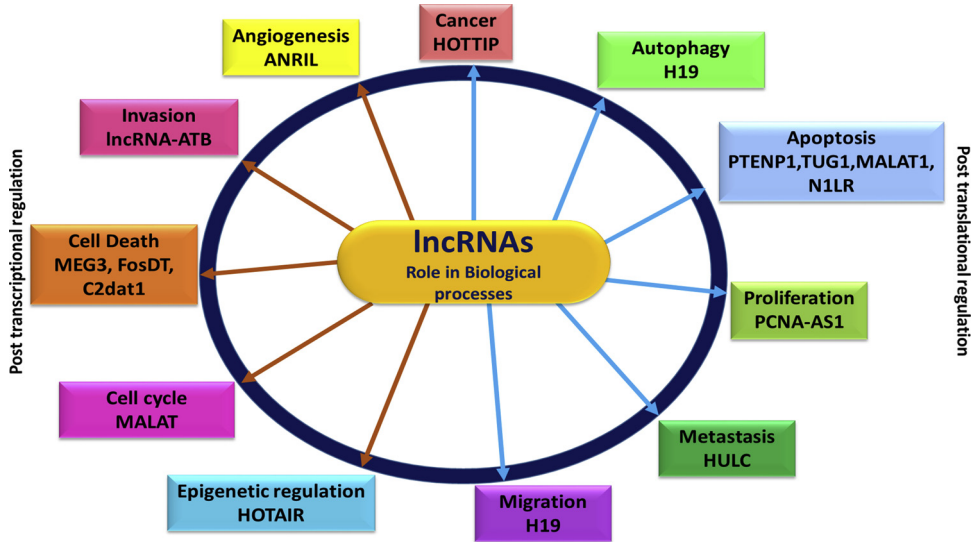



**Fig. 4.** Functions of lncRNA. LncRNAs regulate various cellular processes by post-transcriptional and post-translational modifications as depicted above.

process (Staerk and Constantinescu, 2012; de Freitas and da Costa Maranduba, 2015). However, the lncRNAs involved in mouse and human hematopoiesis are poorly conserved, indicating that lncRNAs evolved differently (Kutter et al., 2012). Finally, lncRNAs represent a vastly unexplored area in the development of megakaryocytes. Megakaryocytes (MKs), among the largest cells in the bone marrow, are generated from hematopoietic stem cells (HSCs) in a sequential process called megakaryocytopoiesis in which HSCs undergo MK-progenitor (MP) commitment and maturation to terminally differentiated MK. Megakaryocytopoiesis is controlled by a complex network of bone marrow niche factors (Raghuwanshi et al., 2018). Among the multiple classes of regulatory factors, lncRNAs are an important regulatory factor in MK development and platelet production. Very little is noted about the role of lncRNAs in megakaryocytopoiesis. Past studies showed that some of the lncRNAs are aberrantly expressed in myeloid progenitors and MK (Table 3). The first lncRNA reported in MK is *AS-RBM15* located on chromosome 1 and has total nucleotide length of 52,797 bp. The transcript of *AS-RBM15* is transcribed in an antisense manner and overlapped with 5′ UTR of a protein-coding gene RBM15 *(RNA Binding Protein 15)* (Tran et al., 2016). RBM15 plays a major role in the growth and apoptosis of cells especially blood cells, by regulating several signaling pathways such as Notch and Wnt signaling (Kazemzadeh et al., 2015). The expression of RBM15 is highly marked during hematopoiesis specially in myeloid differentiation and other hematopoietic cell lineages via a mechanism that is mediated by stimulation of Notch signaling via RBPJkappa (Ma et al., 2007) and occurs at more moderate levels during megakaryopoiesis. The differential expression of RBM15 plays a key role in the development of myeloid, megakaryocytic, and progenitor compartments and disorders related to their abnormal functioning (Ma et al., 2007). Recent emerging evidence indicates that lncRNA, *AS-RBM15* is an anti-sense RNA transcript involved in the regulation of the transcription and translation of the RBM15 protein in megakaryocytes (Garzon et al., 2014). RBM15 is a very important controlling component of MK terminal differentiation (Hu et al., 2016). Furthermore, RUNX1 is a transcription factor that represses the erythroid gene expression program during megakaryocytic differentiation (Kuvardina et al., 2015). It counter balances the activity of RBM15 and *AS-RBM15* as a result of the expression pattern of erythroid genes. These genes are restricted by epigenetic repression of the erythroid master regulator krueppel-like factor 1 (KLF1) during megakaryocytic differentiation (Lyu et al., 2017). Not only the activation of lncRNA *AS-RBM15* but also *lnc-MEGA1* has a direct regulatory role in the induction of RBM15 protein synthesis by putative RNA interaction with polysome containing mRNA (Zhao et al., 2014). Moreover, current studies demonstrated that high level of *AS-RBM15* expression indicates that the function of RUNX1 influences the balance between erythroid and megakaryocytic differentiation (Tran et al., 2016). Taken together, we could reveal that *AS-RBM15* is a key player in the network of transcription factor RUNX1 that promotes proliferation and differentiation of megakaryocytes and inhibits the erythroid gene expression program (Tran et al., 2016; Zhao et al., 2014).

Scientific evidences showing that lncRNAs biological functions should not be ignored. These lncRNAs are crucial and required as a combination of complementary approaches. These approaches vary depending on their mode of action in megakaryocyte development and disease processes. Furthermore, these lncRNAs may be involved in multiple pathways related to MK developmental processes.

## 6. Conclusions and perspective

Although thousands of lncRNAs are expressed during hematopoiesis, the studies of specific lncRNAs with experimentally verified functions are limited. This increasing requirement for deciphering the exact functions of lncRNAs in megakaryocyte development rises further investigative questions. Fewer studies to date have outlined their significance in erythro-megakaryopoiesis with regards to lineage-specific development.

In the presence of varied physiological conditions, it is crucial to have primarily an in-depth understanding of the changes in the lncRNA transcriptome in MKs with specific focus on how these alterations indicate their importance in megakaryocyte development and progression. Such studies might help in the identification of novel cellular pathways involved in the production of mature MKs from hematopoietic stem-progenitor cells (HSPCs). It is important to comprehend lncRNA profile since it can provide information on the direct inference of MK lineage relationship and functional similarity with the hematopoietic hierarchy, including erythroid progenitor and stem cell populations. Further, lncRNA profiling in MK populations may prove to be not only novel but also, powerful approach for identifying and predicting their new developmental roles in terminal megakaryopoiesis followed by platelet formation. In conclusion, at present limited studies exist which explore the significance of lncRNAs in modulating transcriptional and post-transcriptional regulations during megakaryopoiesis. Thus, future studies are necessitated to fully explore the importance of these unique classes of RNA molecules in different aspects

of platelet biogenesis.

## Conflict of interest

Authors have no conflict of interest.

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LETTER TO THE EDITOR

# Exosome mediated differentiation of megakaryocytes: a study on TLR mediated effects

**Narasaiah Kovuru**[1] · **Sanjeev Raghuwanshi**[1] · **Ravi Kumar Gutti**[1]

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## Abstract

Megakaryocytes are large polyploid bone marrow cells whose function is to produce circulatory platelets. Megakaryocytes are also known to release extracellular vesicles (EVs) of varying sizes. Toll like receptors (TLRs), present on the sentinel cells are essential components of the innate immune response, these receptors are also expressed by platelets and megakaryocytes. Our data provide the evidence that TLR-2 induced MKEVs are able to recapitulate TLR-2 signalling in megakaryocytic cell line (Dami cells) and that likely induces megakaryocytic maturation by increasing the production of cytokines involved in MK maturation. TLR-2 induced MKEVs may be involved in replenishment of the immune effector platelets in circulation and its progenitor megakaryocyte in bone marrow for the physiological need of the platelets by inducing the maturation of megakaryocyte.

**Keywords** Megakaryocyte · Extracellular vesicles · Exosomes · TLR-2

Extracellular vesicles (EVs), membrane vesicles of varying size, including exosomes, microparticles and apoptotic bodies, are a newly-discovered way by which cells participate in paracrine and endocrine signaling as well as transporting cargos [1]. These EVs are capable of transferring various bioactive macromolecules including membrane proteins, cytosolic proteins and RNA to the recipient cells via fusion or endocytosis following specific receptor recognition [2]. In addition to the cell-intrinsic programs, hematopoietic stem and progenitor cells (HSPCs) fate decisions are regulated by many paracrine and endocrine mediators, including exosomes and microparticles. Several recent reports have supported a role for EVs in the regulation of hematopoiesis [3]. A recent report by Jiang et al. [4] demonstrated that megakaryocyte (MK)–microparticles (MKMPs) can induce the differentiation of HSPCs into functional MKs. Moreover, Srinivasan et al. [5] reported that exosomes from TLR stimulated cells can recapitulate TLR activation in distal cells in vitro.

Toll like receptors (TLRs) are a family of pattern recognition proteins that play a central role in pathogen recognition and shaping the innate immune response. The importance of TLRs for megakaryopoiesis and thrombopoiesis has been demonstrated using a TLR4 gene locus knockout mice model [6]. Recently, we have also observed that TLR-2 induction leads to MK maturation in vitro [7]. Interestingly, MKMPs are the most abundant MPs in circulation [8]. Further, this could be exploited to understand the stimulus specific EVs signatures and their effects on MK differentiation. Considering the role of TLR-2 and MPs in MK development, we hypothesise that exosomes from TLR-2 stimulated Dami cells can induce TLR-2 activation and megakaryocytic differentiation in Dami cells.

To test the role of TLR-2 agonist in MKs differentiation, we cultured Dami cells in RPMI media with 10% FBS (Gibco) and 1% antibiotics, cells were treated with 10 µg/ml of TLR-2 agonist, Zymosan (Sigma) and maintained for 48 h in humidified incubator with 5% carbon dioxide. Further, these cells were centrifuged at 1000×*g* for 3 min to separate cells, cell debris and platelet-like particles (PLPs). Further, the supernatant was used for the isolation of MKMPs using protocol of Jiang et al. [4].

TLR-2 agonist, Zymosan increased the adherence of cells and shedding of MPs (Fig. 1a) as compared to untreated (control) culture. More specifically, we observed

✉ Ravi Kumar Gutti
guttiravi@gmail.com

[1] Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad, TS 500046, India

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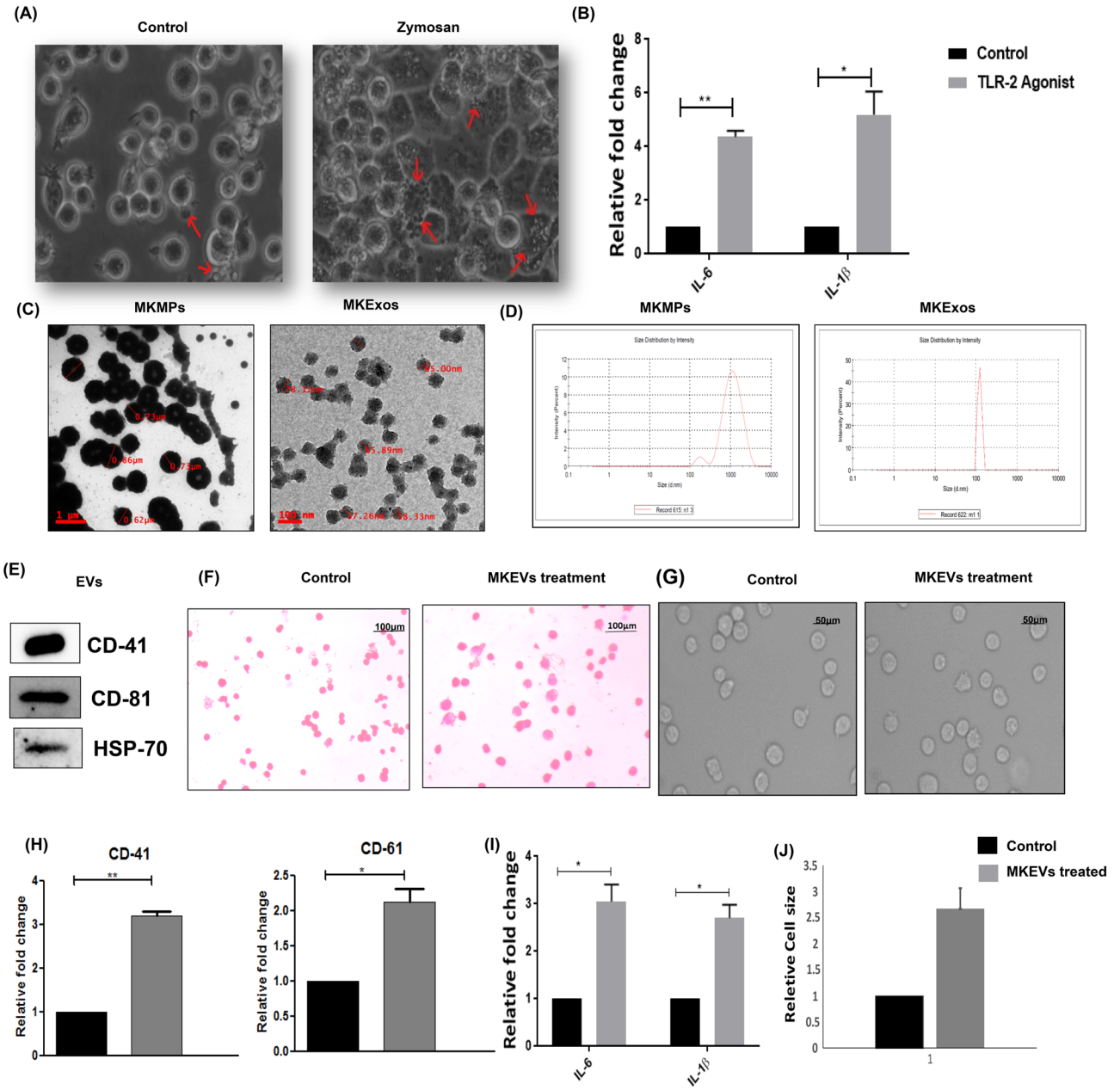


**Fig. 1** TLR-2 induced MKEVs induces the megakaryocytic maturation. **a** Megakaryocyte maturation (increased cell size and adherence) was observed with Zymosan treatment in Dami cells. **b** Inflammatory cytokines, IL-6 and IL-1β levels were elevated in Zymosan treated Dami cells as compared to control. **c** Micro particles were observed under TEM and they were in the size range of 700–1000 nm. **d** Exosomes were analysed using DLS and were found to be of size range 70–100 nm. **e** Micro particles showed expression of integrin CD-41 and HSP70. **f** Morphological analysis showed megakaryocytic features such as increased cell size and adherence upon MKEV treatment as compared to untreated control. **g** MKEVs did not show any effect on cell morphology and adherence of THP-1 cells. **h** TLR-2 induced MKEVs induce the megakaryocyte maturation markers (CD-41 and CD-61) expression. **i** Increased cytokine (IL-6 and IL-1β) levels were observed in TLR-2 induced MKEVs treated Dami cells as compared to control. Bare represents mean SD of three independent experiments. ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.002$). **j** Increased cell size was observed in TLR-2 induced MKEVs treated Dami cells as compared to control which was quantified using ImageJ

an elevated levels of IL-6 and IL-1β (inflammatory cytokines) mRNA in Zymosan treated Dami cells as compared to control (Fig. 1b; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.002$).

To study the size of MKMPs or exosomes (MKExos), transmission electron microscopy (TEM) was performed on Zeiss Libra 120 and images were acquired using a Gatan Ultrascan 1000 CCD. A uniform population of MKMPs and

MKExos were obtained showing that MKMPs are larger as compared to MKExos (Fig. 1c). Further, characterization of MKMPs and MKExos size was performed using dynamic light scattering (DLS). MKMPs or MKExos were suspended in PBS (filtered using 0.22 µm filter) that contains no particles. DLS measurement of suspensions of MKMPs or MkExos were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments) with 633 nm He–Ne laser with a fixed angle of 173° and automatic attenuator. DLS size distribution shows that MKMPs are in the range of 700–1000 nm, whereas MKExos are in the range of 70–100 nm (Fig. 1d). Moreover, MPs specific markers (HSP-70) and MK specific marker (CD41) were detected in TLR-2-induced MKEVs (Fig. 1e) along with exosome specific marker CD81.

In order to study the effect of TLR-2 induced EVs on the recipient cell differentiation, megakaryocytic Dami cells and monocytic THP-1 cells were cultured separately in the presence of TLR-2 induced MKEVs (100 µg/ml) and for control cells same volume of PBS was added. After 48 h of incubation, microscopic analysis of morphological features showed increased cell size in TLR-2 induced MKEVs treated Dami cells as compared to control (Fig. 1f, j). Whereas, in case of other myeloid cell type (THP1, monocyte) the TLR-2 induced MKEVs could not induce adherence and morphological changes as compared to control (Fig. 1g). More specifically, we checked the effects of TLR-2 induced MKEVs on MK specific markers and inflammatory cytokines expression by qRT-PCR, we observed that MK maturation markers, CD41, and CD61, mRNA expression was significantly higher in TLR-2 induced MKEVs treated Dami cells as compared to control (Fig. 1h; $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.002$). Also, IL-6 and IL-1β levels were elevated in TLR-2 induced MKEVs treated Dami cells as compared to control (Fig. 1i; $n = 3$, $^{*}p < 0.05$).

In conclusion, our data provide the evidence that TLR-2 agonist, Zymosan induced MKEVs can promote megakaryocytic maturation process in Dami cells. TLR-2 agonist was previously reported for its role in megakaryocytic maturation of Dami cells [7], we have also observed similar results. Further, our data provide the evidence that TLR-2 induced MKEVs are able to recapitulate TLR-2 signalling in megakaryocytic Dami cells and that likely induce megakaryocytic maturation of Dami cells by increasing the production of cytokines involved in MK maturation. TLR-2 induced MKEVs could be involved in replenishment of the immune effector platelets in circulation by inducing the maturation of megakaryocyte.

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**Author contributions** N.K. designed and performed experiments, and prepared the manuscript. S.R. prepared the manuscript and R.G. designed and supervised the study.

## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

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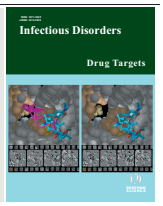
**REVIEW ARTICLE**

# Current Updates on Role of Lipids in Hematopoiesis




Priyanka Bansal[1,#], Priyanka Dahate[1,#], Sanjeev Raghuwanshi[1], Durga Shankar Sharma[1], Narasaiah Kovuru[1], Usha Gutti[2], Nagendra Sastry Yarla[2] and Ravi Kumar Gutti[1,*]

*[1]Stem Cells and Haematological Disorders Laboratory, Department of Biochemistry, School of Life Sciences, University of Hyderabad, (PO) Gachibowli, Hyderabad-500046 (Telangana), India and [2]GITAM Institute of Science, GITAM University, Visakhapatnam- 530 045 (Andhra Pradesh), India*

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**Abstract:** Hematopoiesis is the process which generates all the mature blood cells from the rare pool of Hematopoietic stem cells (HSCs). Asymmetric cell division of HSCs provide it dual capacity for self-renewal and multi-potent differentiation. Hematopoiesis is a steady state process in which mature blood cells are produced at the same rate at which they are lost, establishing a homeostasis. HSCs are regulated through their environmental niche, cytokine signalling, and the orchestrated activities of various transcription factors. However, there is very little information available about the signal transduction events that regulate HSC function; in particular, the effects of bioactive lipids and lipid mediators are not well understood. Recent studies have added an important aspect of this process, introducing the role of lipids in cell fate decisions during hematopoiesis. The mechanisms of bioactive lipids and their derivatives have been studied extensively in signal transduction and various other cellular processes.

This review focuses on various categories of lipids and their regulatory mechanisms in HSCs and their comment into different blood cells. Moreover, we also discuss the role of lipid signalling specifically in megakaryocyte and platelets.

**Keywords:** HSC, Megakaryocyte, Eicosanoids, Phosphatidylinositides, Ceramide, Hematopoiesis.

## 1. INTRODUCTION

Hematopoiesis is a remarkable process that generates mature blood cells. Hematopoietic stem cells are the common progenitor cells from which different lineages of blood cells arise [1]. This occurs due to finely orchestrated cell signalling networks which involve many factors, including lipids, cytokines and transcription factors. The role of lipid bridges and lipid metabolism in signal transduction of HSCs was not elucidated in detail in past. Until recently, lipids were considered to be a source of energy and components of membranes. Lipids can be converted into derivatives that either complement or antagonize each other's cell signalling function. Research in the past10-15 years have established their role as mediators of inflammatory response and regulators of cell membrane polarity. They are critical for cell fate decisions during stem cell differentiation by inducing apoptosis or sustaining cell survival and polarity [2]. Lipid has always been an important part of the cell since they regulate many vital processes in hematopoiesis. Bioactive lipids like Ceramide-1-Phosphate (C1P) and Sphingosine-1- Phosphate (S1P) are involved in migration and homing of HSPCs in bone marrow niche. Phospholipids like

*Address correspondence to this author at the Department of Biochemistry, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad- 500046 (Telangana), India;
Tel: +91-9052665597; E-mail: guttiravi@gmail.com
#Both authors contributed equally.

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phosphatidylinositols actively participate in the stem cell differentiation and also help in maintaining their pluripotency. In this review, we discuss the core of lipid signal transduction pathways that support the stem cell survival and differentiation, highlighting the importance of lipids in megakaryocytopoiesis.

## 2. BIOLOGICAL ROLE OF LIPIDS IN HEMATOPOIESIS

### 2.1. Eicosanoids

Eicosanoids is a family of bioactive lipid signaling molecules derived from ω-3 and ω-6 polyunsaturated fatty acids [1]. Eicosanoids are generated in a process of membrane phospholipids hydrolysis by the phospholipase A2 to ω-3 and ω-6 fatty acids that next are converted into eicosanoids: prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), prostacyclins (PCs), and lipoxins (LXs). The PGs are synthesized from arachidonic acid (Polyunsaturated ω-6 fatty acid) by the action of cyclooxygenase (Cox)-1 or -2, which generates a precursor PG (PGH2). This precursor PG is further processed by isoform-specific cytosolic or microsomal prostaglandin synthase (c/m PGES) to form PGE2 or one of many other prostaglandins [2, 3]. Among all the isoforms of prostaglandins, PGE2 is the best characterized isoform.

PGE2 is a lipid signaling molecule made by the oxidation process of 20-carbon fatty acid, PGE2 is involved in paracrine signaling through any of its four specific and highly conserved G-protein coupled E-prostanoid (EP) receptors (EP1-EP4) [4]. Signalling through these receptors accounts for multiple, sometimes opposing responses. The Gs-coupled EP2 and EP4 receptors were initially known to increase intracellular cAMP levels, however recent reports show that EP2 and EP4 receptors also activate PKA signaling [5]. The Eq-coupled EP1 receptor leads to increased intracellular calcium levels. The EP3 receptor isoforms– EP3 α, β, and Y – are coupled with different G-proteins, but the major effector is the EP3-Gi complex, which has negative effects on intracellular cAMP levels (Fig. **1**) [6-8].

Recently, PGE2 has been shown to support HSC expansion, North TE *et al*. [9] revealed the role of PGE2 in the regulation of vertebrate HSC homeostasis. Role of PGE2 in hematopoietic has long been reported. However, reports in the last two decades resolved the conflicting data and come with few new insights. Pelus and Hoggatt [10] reported that the PGE inhibits *in vitro* growth of HPC (hematopoietic progenitor cell), defined as colony forming unit-granulocyte/macrophage (CFU-GM). In a selective feedback inhibition loop to maintaining appropriate HPC proliferation, PGE2 acts as a negative regulator of myeloid expansion to counterbalance positive signalling from the colony-stimulating factors [11-13]. PGE2 improves proliferation of multipotential progenitor cells which differentiate into granulocytes, erythrocytes, monocytes and megakaryocytes. In the light of effects on myelopoiesis, PGE2 is also observed in stimulating erythropoiesis by increasing erythropoietin production from the kidney and by accelerated proliferation of the erythroid progenitor cells [14-19].

The PGE2 is well known to improve HSCs homing efficiency, cell survival and self-renewal. The effects of PGE2 on HSCs homing and cell survival are associated with anti-apoptotic mechanism. This anti-apoptotic mechanism involves the lower levels of annexin-V and active caspase-3. Survivin is known to regulate HSC entry and progression through cell cycle. The PGE2 enhances the survival of cell by increasing the level of survivin, an inhibitor of active caspase-3 [20]. The PGE2 also up-regulates the expression of CXC chemokine receptor 4 (CXCR4) in HSCs and increases chemotaxis in response to ligand stromal cell-derived factor-1α (SDF-1α) gradient. The CXCR4 and SDF-1α interection is an important axis regulating the HSC and HPC tethering and trafficking to and from the bone marrow niche. Thus, coordinated effects of PGE2 on homing and survival pathway enhance the HSC engraftment. More interestingly, the transcription factor hypoxia-inducible factor 1α (HIF-1α), which increase the expression of survivin and CXCR4, is stabilized by PGE2 [10, 20, 21].

Other eicosanoids, such as Leukotrienes are also produced in the marrow microenvironment by oxygenation of arachidonic acid by 5'-Lipoxygenase (LOX) and involved in localized signaling. Leukotrienes (LTB4, LTC4 and LTD4) were reported for their role in enhancing HPC proliferation. Moreover, LTB4 characterized for their role in the regulation of hematopoiesis, LTB4 signaling decreases HSCs self-renewal and increases

their differentiation [20]. Estrov *et al*. [22] reported the inhibitory effects of LTB4 and LTC4 on early and late erythroid progenitor cells. These reports suggest that PGs and LTs have diverse effects on hematopoietic cells.

### 2.2. Phosphatidylinositides

Phosphatidylinositides (PIs) is a family of phospholipids in membrane derived from membrane lipid phosphatidylinositol and play a principal role in a number of signal transduction pathways [23]. The PIs derivatives phosphatidylinositol 4-monophosphate (PI(4)P) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)$P_2$) are produced through canonical PI pathway and function as precursors of the intracellular second messengers inositol 1,4,5-trisphosphate (IP(3)) and diacylglycerol (DAG). The PI(4,5)$P_2$ is cleaved by various phospholipases C (PLC) to generate DAG, which is an activator of PKC and IP(3), which triggers $Ca^{2+}$ release from endoplasmic reticulum [24]. The class I phosphatidylinositol-3-kinase (PI3K) acts *via* either G-coupled protein receptor family or tyrosine receptor kinase, generating PI(3,4)$P_2$ (Phosphatidylinositols 3,4 bisphosphate) and PI(3,4,5)$P_3$ (Phosphatidylinositol 3,4,5 triphosphate), which are known to be activators of Akt/PKB cell signalling pathway for cell survival and differentiation [25-30]. The class I PI3Kα and PI3Kβ show ubiquitous expression, whereas class I PI3Kδ and class PI3Kγ are predominantly found in different hematopoietic lineages [31]. The restricted expression of PI3Kδ and class PI3Kγ in hematopoietic lineages suggest their function as critical regulators in cells of different myeloid and lymphoid lineages [32]. The phosphatase and tensin homolog (PTEN) is a tumour suppressor lipid phosphatase, reducing pluripotency of stem cells [33-35]. PTEN also controls the growth of embryonic stem (ES) cells and embryonic tissues by converting PIP3 to PIP2. The deletion of PTEN results in the up-regulation of the Akt/PKB-to-β-catenin pathway and increased pluripotency or malignancy [33, 34] (Fig. **1**).

### 2.3. Ceramide

Ceramides are a family of waxi lipid molecules found in the cell membrane, they are composed of sphingosine and fatty acid. Recently, the role of ceramides has been revealed in cell proliferation, differentiation, and apoptosis by regulating various signaling mechanisms [36, 37]. Ceramide and glycolipid group interacts with other membrane lipids, such as cholesterol and sphingomyelin, to form specialized microdomains e.g. rafts, caveolae, or lipid-enriched microdomains. Cell surface microdomains in stem cells are emerging as an important area of research as it provides a platform for signal transduction and cell adhesion [25-28]. In the presence of increased level of apoptotic proteins Par-4, ceramide-associated atypical protein kinase C (aPKC) binds to Par-4 and forms a ceramide-aPKC apoptosis complex. This complex reduces the activity of aPKC and its downstream targets, NF-κB and Akt which results in the activation of Bax/Bad and reduced expression levels of Bcl-2 [30]. The deficiency of ceramidase (ACDase) activity results in ceramide accumulation, inherited disorder of lipid metabolism called farber disease (FD). Alayoubi AM *et al*. [38] generated the model of systemic ACDase deficiency and accumulated ceramide. The ACDase defects showed altered hematopoiesis with the increased levels of white blood cells. The exact function of ceramide in HSCs is still ambiguous however, the ceramide derivatives are well known for their regulatory role in hematopoiesis (Fig. **2**).

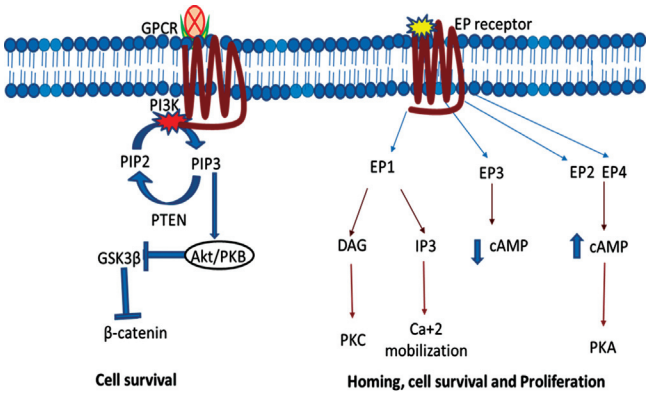


**Fig. (1).** (A) PI3K generates PIP3 from PIP2, which results in the up-regulation of the Akt/PKB-to-β-catenin pathway have role in cell survival. (B) EP receptors and PGE2 mediated signaling pathways. EP1 activates PLC, resulting in the production of DAG and IP3. DAG activates PKC while IP3 play important role in Ca2+ mobilization. EP2 and EP4 activate adenylate cyclase (AC) which results in elevated cAMP synthesis and PKA activation. EP3 negatively regulates AC and thus cAMP levels are reduced. The activation of these signaling pathways by the EP receptors in blood cells results in homing, cell survival and proliferation.

The bioactive lipids such as Ceramide-1-Phosphate (C1P) and its derivative Sphingosine-1-Phoshphate (S1P) are shown for their activity in HSPCs (hematopoietic stem progenitor cells) trafficking into BM [39]. The concept of homing and mobilization of HSPCs into BM is based on the chemotactic 'tug of war' concept where varying concentration of C1P & S1P mediates migration of cells [33]. However, SDF-1α and its receptor CXCR4 were emerged as a chief regulator in HSPCs trafficking, bioactive lipids such as SIP and CIP also help in the migration of HSPCs into BM. The complement cascade is an important modulator of stem cell trafficking, the activation of complement cascade elevates the levels of S1P & C1P in bone marrow niche [40]. Apart from being recognized as chemoattractant for HSPCs [48-50], S1P is also involved in trafficking of early B-lymphoid cell progenitors from BM [41, 42], in mobilization of T lymphocytes between peripheral blood [41, 43] and lymphoid organs and a regulator in trafficking of myeloid progenitors between BM and peripheral organs [44].

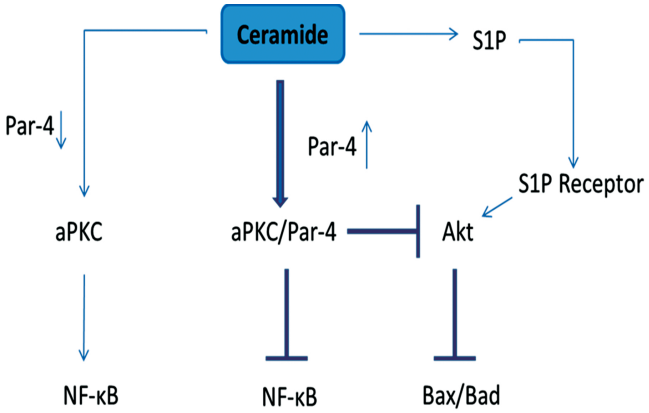


**Fig. (2).** Ceramide mediated cell-signaling pathways. (A) In the absence of PAR-4 ceramide activates aPKC (B) with the higher levels of PAR-4 ceramide induces inhibition of aPKC by PAR-4, which may inactivate cell survival pathways (Akt and NF-κB signaling), and induces apoptosis. This may be antagonized by S1P-mediated activation of Akt.

## 3. LIPIDS IN MEGAKARYOCYTOPOIESIS

Megakaryocytopoiesis is a tightly regulated process of megakaryocyte (MK) progenitor commitment and maturation that results in the formation of the rarest and intriguing population among the blood cells, the megakaryocytes. Megakaryocytes release platelets that have a crucial role in hemostasis. Megakaryocytes develop from hematopoietic stem cells under the influence of the cytokine, thrombopoietin (TPO), which specifically regulates maturation and platelet production [45]. The HSC forms the common myeloid progenitors and then forms megakaryocyte/erythrocyte progenitor (MEP), which gives rise to burst forming unit-erythroid (BFU-E) and high proliferative potential-colony forming unit-megakaryocyte (HPP-CFU-MK). HPP-CFU-MK then gives rise to BFU-MK which forms CFU-MK. CFU-MK gives rise to immature megakaryocytes, the megakaryoblasts, which further differentiate into mature megakaryocytes, develop proplatelets, and finally shed platelets [46]. Megakaryocyte differentiation is characterized by expression of CD61 and increased expression of CD41 and CD42 [47].

A study has reported that an unregulated PAF-like lipid production in the bone marrow could influence trafficking, activation, and gene expression patterns in neighbouring cells [48]. Inflammatory phospholipids such as platelet activating factor (PAF) regulates platelet activation. PAF binds to its receptor PAF receptor-1 (PAFR-1) *via* a short *sn*-2 acetyl residue [49-50]. However, this interaction is not specific, allowing other oxidatively modified phospholipids with short *sn*-2 residues to stimulate cells *via* PAFR-1. These lipids are referred as PAF like lipids since they act *via* PAFR-1 receptor [51]. Both PAF and PAF-like lipids are substrates of a small subset of phospholipases A2, the PAF-acetylhydrolases (PAF-AHs), which quench signaling by both types of PAFR ligands [52].

PAF is present in human bone marrow where it regulates the function of stem cell progenitors [53]. It is reported that PAF directly affects the growth of human myeloid and erythroid progenitors [54]. $CD34^+$ cells that differentiate into megakaryocytes also respond to PAF [55]. Human $CD34^+$ cells generate PAF-AH activity as they differentiate into megakaryocytes. Accumulation of PAF-AH in megakaryocytes prevents accumulation of bioactive PAF-like lipids. Mature circulating platelets possess PAF-AH activity; it may also be possible that megakaryocytes produce PAF-AH to incorporate the enzyme into platelets during thrombopoiesis [48].

Accumulation of PAF-AH activity may provide a mechanism to modulate and control signals de-

livered through the PAFR in human bone marrow. When endogenous PAF-AH activity is quenched or stimulated with cPAF, (a stable analog of PAF) megakaryocytes adhere and spread more readily on immobilized fibrinogen. Both of these conditions are associated with increased activation of $\alpha_{IIb}\beta_3$ integrins and binding of soluble fibrinogen [48].

Similar to its effect on cell adhesion, spreading, and $\alpha_{IIb}\beta_3$ activation, cPAF enhances migration of megakaryocytes through a porous membrane in a PAFR-dependent manner. This indicates that stimulation of the PAFR may modulate the movement of megakaryocytes as they migrate from osteoblastic niches to vascular beds within the bone marrow to produce proplatelets. This may describe the chemoattractant properties of the PAFR signalling system. Aberrant signalling through the PAFR may also influence packaging of critical proteins and/or mRNA transcripts into platelets during thrombopoiesis because platelet formation is a regulated process where both proteins and mRNAs are transported by microtubule-associated processes along cellular extensions to buds that will become proplatelets [48].

Den Dekker *et al.* [56] have showed the role of PIP5KIγ during megakaryocyte differentiation. PIP5KIγ (Phosphotidylinositol-4-phosphate 5-kinase I) belongs to the family of phosphatidylinositol phosphate kinase (PIPK) which catalyzes addition of one phosphate residue on PI(4)P (Phosphatidylinostol-4-monophosphate) to synthesize $PI(4,5)P_2$ (Phosphatidylinositol-4,5-bisphoshphate). $PIP_2$ is present in ample amounts in plasma membrane where it produces secondary messenger: $IP_3$ (Inositol triphosphate) & DAG (Diacylglycerol). $PIP_2$ also governs the dynamics of cytoskeletal in megakaryocytes in all eukaryotic cells. PIP5KI have three isoforms: α, β and γ where each isoform directs its own distinct function in cell. PIP5KIγ has been found to be associated with the megakaryocytes actin and platelet dynamics. Studies suggest that deletion of PIP5KIy results in blebbing of plasma membrane in megakaryocytes since it is associated with plasma membrane and cytoskeleton [56]. Studies have also demonstrated that same phenotype is observed in megakaryocytes lacking talin1, a protein involved in cell membrane attachment to the environment because PIP5KIγ binds to talin1. Talin1 is able to bind β-integrins, leading to their activation and aggregation [57].

Current reports suggest that lipids play an important role in megakaryocytopoiesis and platelets but how the above two pathways crosstalk with each other or with other lipids in increasing the megakaryocyte differentiation marker is still a mystery that remains to be solved.

## CONCLUSION

Lipid signalling plays an important role in the development of blood cells from HSCs. Many lipid derivatives revealed as important regulators in promoting stem cell survival and lineage commitment, also regulating polarity and trafficking of HSCs and their progenitor cells between bone marrow and peripheral blood. Lipids are acquired both extracellularly from bone marrow niche or intracellularly synthesized by the cell during differentiation process in a regulated manner. Different lipids activate stem cell in different way but how all lipids are interconnected is not known. How one lipid or its derivatives will complement or antagonize other lipid function affecting the same pathway decide their fate remains a question. More in depth studies are required to be done to understand the web of lipids in hematopoietic stem cell development.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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# MicroRNA-9 Regulation of RUNX1/TGFβ Axis during Megakaryocyte Maturation: A study on Development Perspective

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