Long non-coding RNA: Role in iPSC derived Megakaryocyte Development and Leukemia

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by

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DECLARATION

I, **Swati Dahariya,** hereby declare that this thesis entitled "**Long non-coding RNA: Role in iPSC derived Megakaryocyte Development and Leukemia**" submitted by me is based on the results of the work done under the guidance and supervision of **Dr. Ravi Kumar Gutti**, Professor, Department of Biochemistry, School of Life Sciences, University of Hyderabad. The work presented in this is original and plagiarism free. I also declare that no part or in full of this thesis had been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**Long non-coding RNA: Role in iPSC derived Megakaryocyte Development and Leukemia**" submitted by **Swati Dahariya** bearing enrollment number **16LBPH05** in partial fulfillment of the requirements for the award of Doctor of Philosophy in the Department of Biochemistry, School of Life Sciences is a bonafide work carried out by her under my guidance and supervision.

The thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Parts of this thesis have been:

A. Published in the following publications:

1. **Dahariya, S.**, Paddibhatla, I., Kumar, S., Raghuwanshi, S., Pallepati, A. and Gutti, R.K., 2019. Long non-coding RNA: Classification, biogenesis and functions in blood cells. ***Molecular immunology***, 112, pp.82-92. DOI: 10.1016/j.molimm.2019.04.011.
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September 4, 2019

Dear Sir/Madam,

With great pleasure we can confirm that Ms Swati Dahariya has worked at the Department of Pathology at the University of Cambridge at the laboratory of Dr. Anton Enright. This work was funded under a Newton Bhaba fellowship from the British Council. Swati worked with us from 1st April 2019 until the 31st July 2019.

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Yours Sincerely,

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CONTENTS

ABSTRACT

Megakaryocytes (MKs) are rare polyploid cells found in the bone marrow specially known as mother cell for platelets. Platelets are small colorless enucleated cell fragments that are critical to vascular hemostasis and wound healing as well as in inflammation. Several blood disorders characterized by dysfunctional platelets result in prolonged bleeding time, defective clot formation and bleeding tendency. Thrombocytopenia is one of the most common hematologic disorders, characterized by an abnormally low number of platelets and it affects both children and adults. Thrombocytopenia is sometimes a first sign of hematologic malignancies, infectious diseases, thrombotic micro angiopathies and autoimmune disorders, and is also a common side effect of many medications. There is no specific therapy for the vast majority and only severe cases need to be treated. Currently, *in vitro* generation of MKs from human induced pluripotent stem cell (hiPSC)-derived platelets technology is a good treatment option, could provide an alternative source of platelets for treating thrombocytopenic patients in different disease conditions. Recent advancement in the rapidly evolving field of hematological epigenetics have shown the role of non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long ncRNAs (lncRNAs) in the context of blood cell development. However, there are only few studies which have identified and characterized the lncRNA expression profiles in MKs, the underlying regulatory mechanisms governed by lncRNAs has not been explored for the normal development of MKs lineage as well as in disease condition. We hypothesized that lncRNAs are differentially expressed in MKs, therefore might play a critical role in the normal development and regulation of megakaryocytopoiesis. Our studies aimed to establish a protocol for the generation of MKs in xeno-free and defined conditions, and to determine the lncRNA profile and molecular mechanism in hiPSC-derived MKs, as well as in hyper-proliferative clinical condition i.e. Acute Megakaryocytic Leukemia (AMKL) to gain an understanding on the development of MK and platelet biology. In this approach, we developed a method to produce MKs from hiPSCs-HSCs under a feeder-free and xeno-free condition. Further, we profiled lncRNAs expression in normal MKs and megakaryoblastic leukemic cells. The highest upregulated lncRNA HOTAIRM1 in normal state could promote MK maturation and development in p53 mediated regulation of cyclin D1 via sponging miR-125b. Also in clinical condition, JPX lncRNA could act as sponge for miR-9, miR-17 and miR-106 which leads the translation of TGFβR2 mRNA and to participate in activation of non-canonical TGFβ pathway to promote megakaryoblastic leukemic cells differentiation and development into normal MKs.

List of Symbols and Abbreviations

~	Approximately
/	per
%	Percentage
°C	degree Celsius
µg	Micro Gram
µL	Micro Litre
µm	Micro Meter
µM	Micro Molar
AKT	Serine/Threonine Kinase a.k.a. Protein Kinase B
AML	Acute Myeloid Leukemia
AMKL	Acute Megakaryocytic Leukemia
Bcl-2	B-Cell Lymphoma-2 (Apoptosis Regulator)
BM	Bone Marrow
BMP4	Bone Morphogenetic Protein 4
BSA	Bovine Serum Albumin
BSS	Bernard Soulier Syndrome
Ca^{2+}	Calcium ions
Cb	Cannabinoid
CB	Cord Blood
CBF	Core-Binding Factor
CD	Cluster of Differentiation
cDNA	complementary DNA
CDKs	Cyclin-Dependent Kinases
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
c-Myb	encode nuclear proteins that function as transcriptional trans-activators
CNTFRa	Ciliary Neuro-Trophic Factor Receptor subunit alpha
CO_2	Carbon Dioxide
Cyclin D1	Regulator of cell cycle progression
DAPI	4′,6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA
DNA	Deoxyribonucleic Acid
DMS	Demarcation Membrane System
DS	Down Syndrome
ECM	Extra-Cellular Matrix
endo-siRNAs	endogenous small interfering RNAs
ERK	Extracellular signal-Regulated Kinases
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FC	Fold Change
FGF2	basic Fibroblast Growth Factor

FITC	Fluorescein isothiocyanate
FLI1	Friend Leukemia Integration 1 transcription factor
FLT3	fms-like tyrosine kinase 3
FOG1	Transcription regulator that plays an essential role in erythroid and megakaryocytic cell differentiation
GAPDH	Glyceraldehyd- 3-Phosphate-Dehydrogenase
GATA	A family of transcription factors characterized by their ability to bind to the DNA sequence "GATA"
gp	Glycoprotein
GMP	Granulocyte Monocyte Progenitor
GTPase	Guanosine Tri-Phosphatases
h	Hour
H_2DCF	2′,7′-dichlorofluorescein
H_2DCFDA	2′,7′-Dichlorodihydrofluorescein diacetate
hiPSCs	human-induced Pluripotent Stem Cells
hESCs	human Embryonic Stem Cells
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
iPSCs	induced Pluripotent Stem Cells
IL	Interleukin
JAK	Janus Tyrosine Kinase
kDa	kilo-Dalton
LEF	Lymphoid Enhancer Factor
LIFRb	Leukemia Inhibitory Factor Receptor-b
lncRNAs	long non-coding RNAs
LT	Long Term
M7-AML	Acute Megakaryocytic Leukemia
MAPK	Mitogen-Activated Protein Kinase
MCM	Mini-Chromosome Maintenance
MDS	Myelo-Dysplastic Syndrome
MEP	Megakaryocyte Erythrocyte Progenitor
MEG	maternally expressed, imprinted lncRNA
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
min	Minute(s)
miRNAs	microRNAs
MKs	Megakaryocytes
MKPs	Megakaryocytic Progenitors
mL	Milli Litre
mM	Milli Molar
MPD	Myelo-Proliferative Disorder
MPL	Myelo-Proliferative Leukemia protein
MPPs	Multi-Potent Progenitors
MT1P3	metallothionein 1 pseudogene 3

n	Number of times
N	Ploidy
NAT	Natural Antisense Transcripts
NC	Nitro-Cellulose
ncRNAs	non-coding RNAs
NFE2	Nuclear Factor, Erythroid 2
ng	Nano Gram
NK	Natural Killer
nM	Nano Molar
OSMRb	Oncostatin M Receptor
p21	Cyclin-dependent kinase inhibitor 1
PBS	Phosphate-Buffered Saline
PE	Phycoeryhtrin
PenStrep	Penicillin G and Streptomycin
PI3K	Phosphoinositide-3-Kinase
piRNAs	PIWI associated RNAs
PMA	Phorbol 12-Myristate 13-Acetate
qRT-PCR	quantitative Real Time- Polymerase Chain Reaction
rh	Recombinant Human
RIPA buffer	Radio-Immuno-Precipitation Assay buffer
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RUNX1	Runt-related transcription factor 1
s	Seconds
SCF	Stem Cell Factor
scRNA	small conditional RNA
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate–Poly-Acrylamide Gel Electrophoresis
snoRNA	small nucleolar RNA
ST	Short Term
STAT	Signal Transducer and Activator of Transcription proteins
TBST	Tris Buffered Saline with Tween
TCF	T-Cell Factor
TF	Transcription Factor
TGF-β	Transforming growth factor beta
TLR	Toll-Like Receptors
TPO	Thrombopoietin
tRNA	transfer RNA
VEGF	Vascular Endothelial Growth Factor
WG stain	Wright-Giemsa stain
ZFPM1	Zinc Finger Protein, Multitype 1

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PCR analysis of different cell cycle regulators (Cyc D1, Cyc D2, Cyc E, and p16) expression in PMA-induced MKs as compared to uninduced Dami cells. **C.** DNA index (Cell Ploidy) levels analysis in PMA-induced and -uninduced Dami cells by Flow cytometry (n=3). **D.** The graphical representation of the activation of TGF-βR-induced SMAD-independent ERK1/2 and PI3K/AKT pathways during PMA induced differentiation of Dami cells into MKs, and a crosstalk between JPX lncRNA, miRNAs, and TGF-βR during MK development. Bars represent mean ± SD of three independent experiments. ***72***

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Chapter I: LITERATURE REVIEW

1.1 Hematopoiesis

Hematopoiesis is the process of producing mature and functionally distinct hematopoietic lineage cells (**Figure 1**) (1). This involves differentiation of multipotent, self-renewing stem cells into all lineage cells of the blood. It is a dynamic process essential for the generation of blood cellular components which are required during the development of embryo and throughout adulthood to rejuvenate the blood system. The production of blood cells arises from the sequential commitment of multipotent hematopoietic stem cells (HSCs) into lineage-restricted progenitor cells. HSCs, by definition are cells capable of both self-renewal and differentiation. HSCs lies at the top of the hematopoietic hierarchy because of its ability to apparently limitless capacity to self-renew as well as to differentiate into progenitor cells and mature blood cells progeny of all hematopoietic lineages (2). They give rise to all blood cells by a complex series of differentiation and proliferation events which requires the continuous coordination of many cellular events throughout the life span of the organism (3). Hematopoiesis can be described according to the formation of specific cell types, where erythropoiesis refers to the formation of erythrocytes; myelopoiesis/granulopoiesis is the formation of granular white blood cells, namely neutrophils, eosinophils and basophils; and megakaryopoiesis results in the generation of megakaryocytes (MKs), the precursors of platelets respectively; and lymphopoiesis and monopoiesis are the processes of producing lymphocytes and monocytes (4,5). Cells of all lineages have a limited life-span and are being constantly renewed. In an adult, the production of approximately one trillion different blood cells occurs daily to ensure normal bodily functions (6,7).

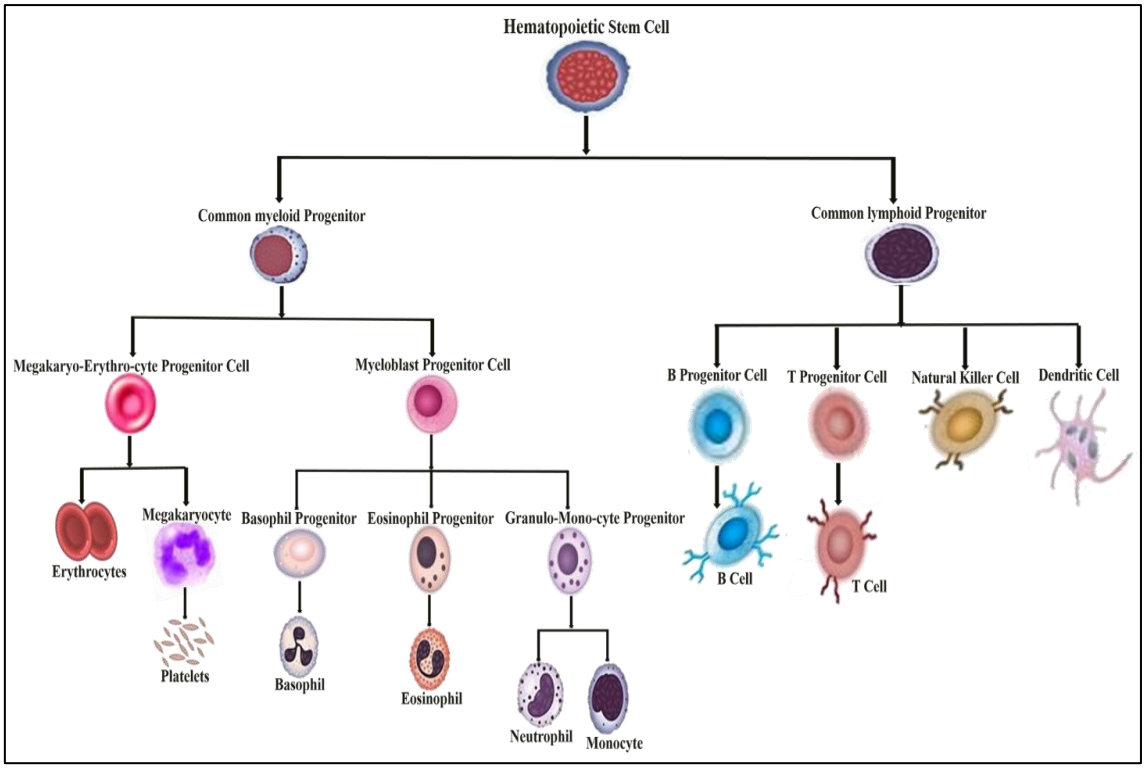


Figure 1: Hierarchical presentation of hematopoietic development. HSCs are self-renewing cells are able to produce progenitors (CMP and CLP) that give rise to multipotent progenitor cells. These progenitor populations are capable to differentiate into all types of blood cells lineages.

1.2 Primitive and Definitive wave of Hematopoiesis

In mammals, based on cell types produced and anatomic location, there are two waves of haematopoiesis: the primitive wave and the definitive wave (8). Primitive haematopoiesis occurs extra-embryonically, within the blood islands of the yolk sac, and it is believed that the primitive hematopoietic progenitors are derived from undifferentiated mesodermal cells called hemingoblasts, which are capable of giving rise to both endothelial cells and blood cells of all types (9–11). Primitive wave occurs transiently, which generate an erythroid progenitor and gives rise to only nucleated erythrocytes and macrophages during early embryonic development (12). Erythrocytes are the predominant cell produced during primitive haematopoiesis, due to the oxygen requirement of newly forming tissues prior to the liver development (9). However, the production of primitive MK progenitors has recently

been described (13). These MK progenitors rapidly give rise to platelets in order to prevent haemorrhage in the developing blood vessels. MKs produced during primitive hematopoiesis also possesses characteristics unique from their definitive counterparts; they are smaller, have lower mean ploidy number (4N versus 16N, where N represents the normal chromosomal compliment) and produce platelets (14).

Contrastingly, definitive hematopoiesis happens later in development, particularly at various time points in various organisms and produces HSCs which are capable of giving rise to all blood lineages. In humans, primitive hematopoiesis is initiated in yolk sac and temporarily shifts into the liver before finally establishing definitive hematopoiesis in thymus and bone marrow. Thereafter, the bone marrow remains the primary hematopoietic organ throughout adult life (6,8,9). Later, HSCs are developed into progenitors which progressively lose potential for specific lineage differentiation. The loss of differentiation potential to one cell lineage results in the commitment of progenitors into alternative cell lineages. HSCs progressively lose their ability to regenerate in response to the earliest differentiation signals. This ability to self-renew further divides the steady state HSC pool into two groups i.e., short-term (ST) and long-term (LT) HSCs. Self-renewal potential of the ST-HSCs is limited and could only provide support for about 6 weeks for the hematopoietic system reconstitution. On the other hand, self-renewal potential of the LT-HSCs is life-long and can provide support for long-term multi-lineage reconstitution of irradiated hosts after transplantation. ST-HSCs can generate multipotent progenitors (MPPs), which can provide support transiently for the production of all mature blood cells types i.e., hematopoiesis. However, they obviously cannot maintain their self-regeneration ability. MPPs can give rise to either common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), based on heterogeneity of the MPP population (4,5). These two lineages are separable at the progenitor level. CMP which, in turn, can differentiate into bipotential progenitors: MK-erythrocyte progenitor (MEP) and

granulocyte-monocyte progenitor (GMP) that ultimately gives rise to erythrocytes and MKs respectively (12,14). Whereas CLPs are believed to be some single-lineage progenitors which are responsible for the production of natural killer (NK), B and T cells (15).

1.3 Megakaryocytopoiesis

MKs are unique bone marrow cells from which platelets are produced. MKs are one of the very largest cells (50-100 μm in diameter) in comparison to other hematopoietic cells. It is also one of the rarest i.e., ~0.05% population (~1 in 10,000 nucleated cells) of the HSCs, found in bone marrow (14). They are platelet-producing cells responsible for the production of platelets in the blood stream. Upon maturation, one MK can produce ~2000 to 8000 platelets. MKs are distinct from other hematopoietic cells in many ways. Firstly, MK expresses specific surface makers such as CD41/CD61, CD42b and CD42a. CD41 is also known as glycoprotein (Gp) IIb, which has been considered as a specific early marker at a stage of a late megakaryocytic progenitor during megakaryocytic differentiation and CD61 can be detected for the mature MK lineage during maturation (16). The cytoplasmic organelles of MKs are also unique, consisting of alpha and dense granules as well as an extensive system of invaginated membranes, which eventually gives rise to platelets (17). The traditional cellular journey of MK development and maturation start from HSCs, through a hierarchical series of progenitor cells (CMP and MEP), which ultimately develop into mature MKs by the process called megakaryopoiesis (**Figure 2**) (14,18). Megakaryopoiesis does not only occur as a stepwise process, but is dynamic and adaptive process catering to biological needs.

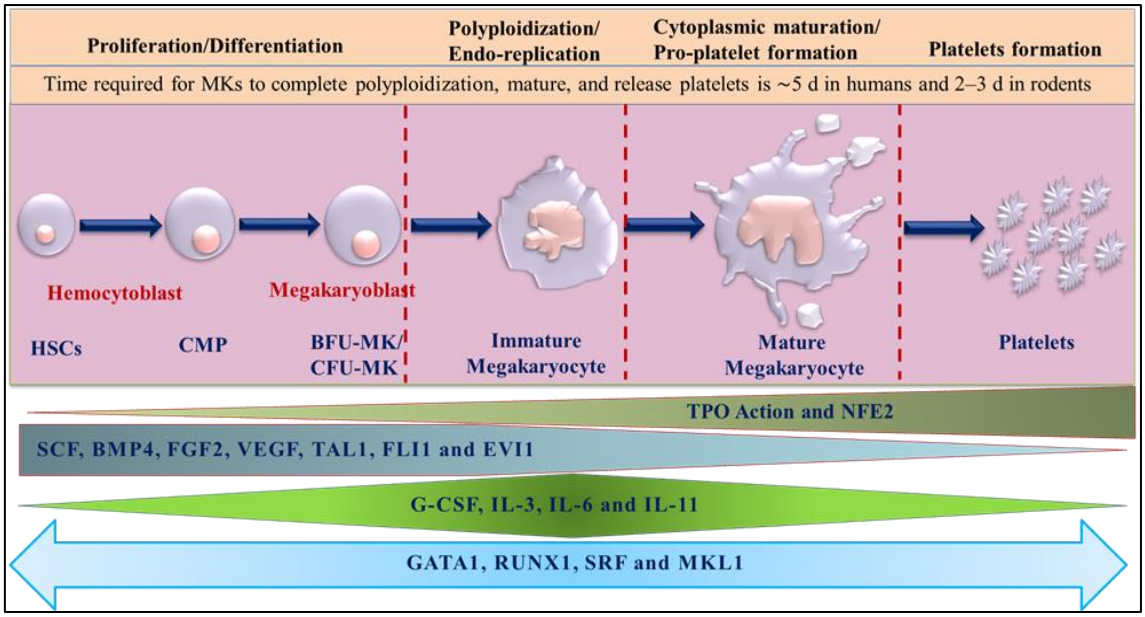


Figure 2: Overview of megakaryopoiesis which originates from the HSCs through successive lineage commitment steps. Thrombopoietin (TPO), number of cytokines and growth factors are most important for megakaryopoiesis and platelet production.

1.3.1 Stages of Megakaryocytopoiesis

Megakaryopoiesis requires the progression through a number of developmental stages. A multipotent HSC becomes committed towards megakaryocytic differentiation, followed by proliferation of MK precursor cells, endomitotic nuclear division, cytoplasmic growth and maturation, the development of structures and organelles unique to platelets, and eventual release of platelets into the blood (18,19). Anyone of these developmental processes can affect the overall platelets generation hence it is a potential focus for regulatory control (20). After MEPs are differentiated into MKs, they undergo a complex maturation via endomitosis mechanism, a process involving nuclear replication without cytoplasmic division and consequently have multiple nuclei, with the average DNA content being 16N, although some MKs have been shown to acquire up to 256 times the normal amount of nuclear material. Unlike regular cell-cycle, in endomitosis, the nucleus fails to divide after S phase which results in diploid pro-megakaryoblasts which turn into tetraploid megakaryoblasts leading to the formation of larger and higher polyploidy pro-MKs and finally mature MKs

(18,21). In addition, MKs creates an invaginated membrane system which is known as demarcation membrane system (DMS) in continuation with plasma membrane and also permeates the cytoplasm. DMS is significant for future platelet formation. DMS functions as a reservoir of cytoplasmic membrane permitting extension of proplatelets into the bone marrow sinuses (22). Cytoplasmic restructuring is the final stage for proplatelets formation that is required for platelet production. Proplatelets (2-4 μm diameter) undergoes repeated dynamic cycles of bending, retraction, extension, and branching, before releasing platelets into the bloodstream (17,20). The end-point of MK maturation is platelet production. Once mature MKs extend long processes membrane called proplatelets into the blood vessel lumen, where shear forces support the shedding of new platelets from the distal ends of the intravascular proplatelets structure within the circulation (23). It has been suggested that platelet release may be a form of apoptosis, as several apoptotic effectors have been shown to facilitate platelet production, such as pro-caspase-3 and -9 and Bcl-2. Following platelet release, the denuded MKs undergoes apoptotic cell death within the bone marrow, and is phagocytosed by macrophages (24,25).

1.3.2 Functions of MKs

Alternative to platelet production, MKs have roles in hematopoiesis; including the ability to behave as immune and inflammatory cells and response towards inflammation and infection (19,26). Mature MK is considered as a mother cell that express several surface markers related to immune function, including members of the TLR family (TLR1, 2, 3, 4, and 6), FcyRIIA (humans only), CD40L (the ligand of CD40 on immune cells) and MHC I (major histocompatibility complex), which is transferred to platelets (16,27–29). Another way in which MKs contributes to inflammation is by packaging cytokines and chemokines into their α-granules and micro particles, which when released contribute to pathogenesis of

pro-inflammatory conditions such as systemic lupus and inflammatory arthritis (30–32). It is not surprising that not only a hematopoietic demand for platelets, also inflammatory and infectious stimuli trigger to quickly generate MKs. Thus, rapid differentiation from an HSC to MK via megakaryopoiesis, results in greater number of platelets (4000-5000) production per MK, which is necessary to replenish the population of platelet pool.

1.3.3 Platelets

Platelets are enucleate cellular fragments released as the end-point of MK development. The final released platelets are small disc-shaped cellular fragments (1-2 μm), which circulate in the blood. These cells are essential to maintain normal hemostasis. As platelets are often the first line of defense not only during the vascular injury but also plays a vital part in the pathologic and physiologic processes of host defense, wound healing, tumor metastasis and inflammation (29).

1.4 Regulation of Megakaryocytopoiesis

The complexity of the hematopoietic system requires a flexible control mechanism in order to maintain an appropriate level of cellular production in normal circumstances and in response to stress, such as infection or bleeding. The mechanisms responsible for decision making in megakaryocytic differentiation are not well understood. However, it is likely that they involve two distinctive but interrelated pathways. The first pathway involves extrinsic factors such as growth factors, which activate signal transduction pathways through binding to their cell-surface receptors, thereby promoting cellular proliferation. The second pathway involves intrinsic mechanisms such as transcription factors that directly control and co-ordinate gene expression. Further elucidation of the exact molecular mechanisms surrounding lineage determination of MKs will enhance the understanding of both normal haematopoiesis and disease states arising because of deregulation (16,18,26).

1.4.1 Cytokines regulation of Megakaryopoiesis

Megakaryopoiesis is a process in which MKs are developed and ultimately platelets are released. The steady-state megakaryopoiesis is stimulated by the major cytokine Thrombopoietin (TPO), a primary cytokine which is responsible for MKs and platelets development (30,33). TPO is encoded by TPO gene, which is positioned on the long arm of human chromosome 3 (q26.3-27). It is composed of 332 amino acids, highly glycosylated and exhibits a four helical bundle fold. The biological activity of TPO is regulated by binding to its specific cell surface receptor; c-Mpl receptor is expressed on HSC, MK and even on platelets. Binding of TPO to c-Mpl receptor results in receptor dimerization via conformational changes. It also results in activation of various downstream biological signaling pathways, for example: the ERK1/ERK2, MAPK and PI3kinase-Akt pathways. c-Mpl belongs to Type-I family of cytokine receptors, a group of trans-membrane receptors which lack intrinsic kinase activity but associate with the cytoplasmic Janus tyrosine kinases (JAK) protein to its cytoplasmic tail. This in turn results in the phosphorylation of intra-cellular c-Mpl receptor at tyrosine residues itself and active receptor scaffold supports subsequent recruitment and activation of multiple downstream biochemical cascades, including the signal transducers and activators of transcription (STAT) factors, such as STAT5 and STAT3. Upon phosphorylation, these STAT proteins dimerize and translocate to the cell nucleus where they could bind to STAT-responsive transcriptional elements within genes such as cyclin D1, Bcl-xL, p21, etc. (18,26,30,34).

Several studies have shown that along with TPO, other cytokines including stem cell factor (SCF) and interleukins like IL-1, IL-3, IL-6 and IL-11 are also involved to stimulate megakaryopoiesis at different degree. Interleukins such as IL-1, IL-3 and IL-6 which act mainly to initiate the growth of hematopoietic cells has a major effect on the megakaryocytes

production (**Figure 2**) (30). Respective cytokines are produced primarily by bone marrow stromal cells in response to various stimuli. These cytokines signal by forming receptor complexes which consist of common signal-transducing receptor protein, glycoprotein 130 (gp130) in combination with a non-signaling a receptor (IL-6Ra, IL-11Ra, or CNTFRa) or another signal transducing b receptor (LIFRb or OSMRb). For example, the IL-6 family is IL-31 which signals through a specific receptor complex, composed of OSMR and IL-31Ra without gp130 (30,35). This eventually results in initiating the downstream signal transduction like JAK/STAT pathway, involving STAT3, JAK1 and to a lesser extent STAT1, which initiates the small GTPase, Ras and, the MAPK cascade. This ultimately results in the activation of extracellular signal-related kinase (ERK) 1 or 2. Several research studies have reported the significance of TPO-induced MAPK/STAT/JAK signaling which is essential for megakaryopoiesis (36). Previous studies have reported that leukemia inhibitory factor (LIF) and several members of IL-6 family of cytokines, including IL-11 were mainly involved in hematopoiesis and thrombopoiesis as well as in other biological activities such as anti-inflammatory, osteoclastogenic, neurogenic and promotes development of T-cell-stimulated Ig-producing B cells (37) .

1.4.2 Transcriptional Regulation of Megakaryopoiesis

The megakaryopoiesis process is cautiously organized by several transcription factors (TFs), which are responsible for modulating the MK-specific genes expression. Multiple TFs like RUNX1, GATA-1, GATA-2, FOG1/ZFPM1, FLI1, c-Myb and NFE2 are key TFs regulating MK development (**Figure 2**). Few TFs which play a critical role in megakaryopoiesis process and well-studied were briefly described below:

a) ***GATA-binding factor 1 (GATA-1)***

The GATA family of TFs plays significant part in normal hematopoiesis. GATA-1 is the founding member of the GATA family of TFs. This family consists of 6 TFs (GATA-1 through GATA-6). GATA-1, -2 and -3 are highly expressed in cells of the hematopoietic lineage, whereas GATA-4, -5 and -6 are expressed outside the hematopoietic system, mainly in the heart, gut and brain (38). GATA-1, a 47-kDa protein with the corresponding DNA sequence mapped to the X chromosome, is essential for terminal differentiation of both megakaryocytic and erythroid cell lines. GATA-1 DNA binding motifs have been identified in virtually all of the characterized MK-specific promoters, including GPIb-alpha, GPIb-beta, GPIIb, GPV, GPVI, GPIX, c-Mpl, PF4, as well as a number of erythroid promoters (39). Binding sites for GATA-1 can be found in the enhancers of many MK-specific genes. GATA-1 and its co-factor Friend of GATA-1 (Fog1) are essential to promote MK-erythroid differentiation, while at the same time inhibiting the myeloid differentiation and Pu.1 expression (38,40).

Initial evidence suggested that GATA-1 was essential for regulating normal MK development. First, *in vitro* data demonstrated the importance of GATA-1 in MK development, where overexpression of GATA-1 in the murine myeloid cell lines, 416B and M1, resulted in MK production and the expression of MK markers. Similarly, high levels of GATA-1 enabled reprogramming of avian myelomonocytic cells into thromboblasts (avian equivalent to MKs). Despite the *in vitro* evidence supporting an important role of GATA-1 in regulating megakaryopoiesis, mouse chimeras generated from GATA-$1^{-/-}$ ES cells failed to confirm these results. The chimeric mice were still capable of producing terminally differentiated MKs and platelets, although the quality and efficiency of production could not be evaluated accurately. However, the production of mice with a targeted MK-selective loss

of GATA-1 was able to identify the significant role of GATA-1 in MK development (41). Mice with reduced levels of GATA-1 in their MKs exhibit dysregulated megakaryopoiesis, a virtual absence of proplatelets leading to thrombocytopenia (approximately 15% of normal), and platelets with ultrastructural anomalies that are functionally compromised *in vivo* and *in vitro* (41). Further, knock-in mice with compound GATA-1 and GATA-2 mutations, which interrupt the GATA/FOG-1 interaction, showed a total megakaryopoiesis absence, suggesting that GATA-2 might be able to partially compensate for GATA-1 in producing MK (42). Interestingly, the D218 mutation does not appear to compromise binding to either DNA or FOG-1 indicating that this mutation may interrupt the recognition of another important GATA-1 binding partner, such as Fli-1 or Pu.1 (40,43).

Dysregulation of GATA-1 and/or GATA-1 mutations have been linked to human diseases and reinforce the significance of GATA-1 in regular megakaryocytic development. Loss of GATA-1 expression is seen in approximately 90% of patients with MDS. In 20% of these cases the disease progresses to Down syndrome-associated acute megakaryocytic leukemia (DS-AMKL) and the presence of these mutations may represent an early pathogenic event occurring prior to DS-AMKL transformation. More recently, the MKs of patients with idiopathic myelofibrosis have significantly reduced GATA-1 protein levels, compared to control patients (44).

b) ***Friend leukemia integration 1 (FLI-1)***

It is a member of the Ets family of TFs. The human FLI-1 gene contains 9 exons, extending over approximately 120kb, and produces a 452 amino acid protein. The FLI-1 protein contains a conserved 5'activation domain (amino acids 121-196), a 3' Ets domain (amino acids 277-360) and a C-terminal activation domain (amino acids 402-452) (45). FLI-1 is expressed in a variety of hematopoietic cells and tissues, as well as in embryonic

endothelium, skeletal muscle, heart and lung. FLI-1 can affect the developmental programs of various hematopoietic cell lineages, and dysregulation in mice and humans results in tumorigenesis. A number of studies have indicated that FLI-1 is capable of regulating expression of multiple MK proteins including GATA-1, GPIb, GPIIb, GpVI, GPXI, and c-Mpl. Additionally, FLI-1 is capable of trans-activating a number of these MK-specific promoters, including alpha IIb, PF4, c- Mpl, GPIX and GPIIb. There is mounting evidence that FLI-1 plays an indispensable role in regulating MK development.

Induction of an MK-like phenotype in the human erythro-megakaryocytic cell line K562, using PMA, upregulates FLI-1 expression results in increased CD41 and CD61 expression on PMA induced cells. Furthermore, enforced FLI-1 expression in K562 cells leads to an increase in megakaryocytic marker expression and dramatic morphological changes characteristic of MKs. Knockout studies have also shown convincing evidence for a role of FLI-1 in MK development, in which FLI-$1^{-/-}$ mice die by embryonic day 11.5 due to haemorrhage as a result of loss of vascular integrity (45,46). In human, hemizygous loss of FLI-1 results in platelet deficiency and is the cause of Paris-Trousseau/Jacobsen thrombocytopenia (47). FLI-1 boosts the GATA-1 activity at megakaryocytic promoters and suppresses the activity of erythroid factors at erythroid promoters. Therefore, FLI-1 expression might function to limit the MEP to the megakaryocytic lineage (48). More recently, a study of 13 unrelated index cases of thrombocytopenia with dense granule secretion disorder showed that six had unique modifications in FLI-1 and RUNX1, further strengthening the role of FLI-1 in megakaryopoiesis and thrombopoiesis (49). Moreover, it has recently been shown that the overexpression of FLI-1 in $CD34^{+}$ cells derived from PTS patient's rescues MK differentiation and their survival (47,50).

c) ***Runt-related transcription factor 1 (RUNX1)***

During embryogenesis for the HSCs formation, the RUNX1 transcription factor has a significant role in MK development. RUNX1 (previously known as AML1) is the evolutionarily conserved DNA-binding subunit of a transcription complex known as core-binding factor (CBF). The CBF complex is essential for definitive hematopoiesis (51). RUNX-1 belongs to a family of proteins that share a conserved 128-amino-acid runt homology domain, which facilitates DNA interaction and binding with the CBF-β cofactor. RUNX-1 plays a key role in MK differentiation via interacting with additional megakaryocytic factors including GATA-1 and FLI-1 (38). During megakaryocytic induction, the myeloid TF RUNX1 and its cofactor, CBFβ go through up-regulation, depending on ERK signaling and a minimal expression in erythroblasts. Functional studies revealed co-operation among GATA-1, CBFβ, and RUNX1 in the activation of a megakaryocytic promoter. Contrastingly, inducible deletion of GATA-1 by RUNX1-ETO leukemic fusion protein leads to thrombocytopenia and impaired megakaryopoiesis (52,53).

1.4.3 Non-coding RNA mediated Regulation of Megakaryocytopoiesis

Non-coding RNAs such microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) form intricate networks which regulate the differentiation of megakaryocytes both negatively and positively by modulating gene expression of TFs, alone and cooperatively.

The genome of humans and other multicellular eukaryotes is mostly comprised of non-protein coding DNA. 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. In past several years, our knowledge of ncRNA has been expanding and that makes feasible to classify ncRNAs in different categories of regulatory ncRNAs, such as endogenous small interfering RNAs

(endo-siRNAs), PIWI associated RNAs (piRNAs), and lncRNAs. This is transcribed into different categories of ncRNAs that include structural RNAs (rRNAs and tRNAs) and regulatory RNAs (scRNA, miRNA, snoRNA, and lncRNA) **(Figure 3)**. Over the past two decades, many research groups have uncovered the key role of lncRNAs in hematopoiesis **(Table 1)**. They play an important role in the fine-tuning of the translation machinery and in its regulation by modulating the crucial functions in hematopoiesis.

LncRNAs are a class of large size ncRNAs which play the key regulatory role in cellular processes such as proliferation, cell cycle and apoptosis etc. by taking part in modifications of post-transcriptional and post-translational process **(Figure 4)** (54). LncRNAs were arbitrarily defined as long RNA transcripts of more than 200 nucleotides that cannot be translated into proteins. LncRNAs not only originate from their own promoters but also from the promoters shared with divergently transcribed coding or non-coding genes, or from enhancer sequences **(Figure 5)** (55). Majorly lncRNAs show a much more diverse subcellular localization, predominantly they are confined in nucleus rather than cytosol. Based on interactions with DNAs, mRNAs or miRNAs and proteins, lncRNAs can function in numerous ways by acting as signals, decoys, scaffolds and guides **(Figure 6)** (54). Interestingly, like miRNAs, the lncRNAs are differentially expressed in hematopoietic cells including MKs and platelets. Notably, lncRNAs are considered as chief regulators in multiple steps of the megakaryopoiesis process **(Table 2)**. Development of MKs is under control of lncRNAs such as MONC, MT1P3, RBM15-as and MEG family (3,8 and 9) highlighting the importance of MKs transcriptional events for thrombopoiesis (56). Recent study has shown that the lncRNA metallothionein 1 pseudogene 3 (MT1P3) is upregulated in MKs of patients with type 2 diabetes and is positively associated with P2Y12 mRNA levels. Knockdown of MT1P3 with small interfering RNA decreased P2Y12 expression, inhibited platelet aggregation and activation in an animal model of diabetes. Contrastingly, overexpression of

MT1P3 had the opposite effect on P2Y12 levels. Luciferase reporter assays suggested that MT1P3 sponges miR-126, known to be involved in P2Y12 receptor regulation (57,58). In another study lncRNA AS-RBM15 was found to fine-tune the relative proportion of erythroid and megakaryocytic differentiation in the hematopoietic lineage. AS-RBM15 can upregulate translation of the protein RBM15 (involved in MKs differentiation). Transcription of AS-RBM15, lnc-Mega1 and RBM15 is under control of RUNX-1, which is known to suppress erythroid gene expression and stimulate MK differentiation (59). With the advancement of genetics-based technology, more lncRNAs will be identified, explored and studied for their role in the megakaryopoiesis regulation.

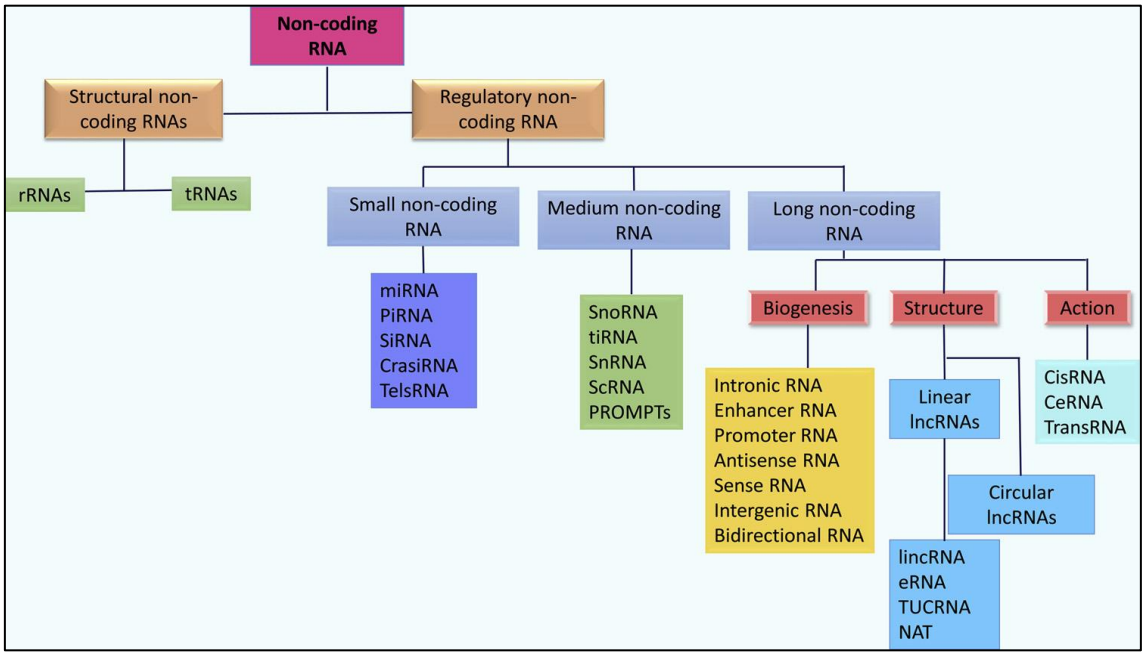


Figure 3: Classification of long non-coding RNAs. Schematic depicts the placement and classification of lncRNAs into classes and sub-classes according to their action, biogenesis and structure.

Table 1: Long non-coding RNAs involved in hematopoiesis. There are lncRNAs are listed with their functions and mechanism involved in normal and disease conditions.

S. No.	LncRNAs	Hematologic disease	Function	Molecular mechanism
1	alncRNA-EC7	Hereditary hemolytic anemias	Potentiates erythroblast proliferation and enucleation during red blood cell development	Promote red cell maturation by regulating neighboring gene encoding BAND 3
2	BGLT3	CML	Regulator of BCR and ABL mediated cellular transformation	Competitive endogenous RNA cross-regulating the expression of the tumor suppressor PTEN
3	CDKN2B-AS1	leukemia	Induced cell cycle disorder, differentiate-on block, and apoptosis arrest in blood cells	Silenced p14, p15, and p16, which are repressors of leukemia
4	DLEU2, DLEU1	CLL	Regulate pivotal oncogenes, tumor suppressors or relevant pathways in malignant hematopoiesis	Epigenetic tumor suppressor mechanism via regulate NF-kB activity.
5	EGOT	Erythro leukemia, myeloid leukemia and Myeloproliferative disorder	Myeloid and erythroid development	Compromises the expression of several proteins major basic protein and eosinophil derived neurotoxin that are important for eosinophil development
6	GAS5	B-cell lymphoma, T-cell leukemia,	Normal cell growth arrest in T-cell lines and non-transformed lymphocytes	Glucocorticoid receptors antagonist. Regulated by mTOR pathway
7	H19	CML, AML, adult T-cell leukemia /lymphoma and leukemia subtypes	During growth and development of HSC reduced quiescence and compromise it's	Genomic imprinting of its neighboring gene IGF2 and Dlk1, also bind

			function Oncogene/ tumor suppressor	PRC2 and Igf2 to promote chromatin remodeling and post-transcriptional processes.
8	IGF1R-AS	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 intra chromosomal enhancer/promoter loop.
9	LincRNA-EPS		Essential for dynamic terminal differentiation of erythroid cells.	Mediated anti-apoptotic activity by repressing many proapoptotic genes e.g. pycard
10	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
11	LncHSC2		involved in HSC self-renewal and T cell differentiation	Close to genes Pml, and Itpkb
12	LUNAR1	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
13	MAFTRR	Different allergies and auto immunity	Key role in T lymphocyte	Increases the expression of MAF
14	MALAT1	Tongue Cancer and Leukemia, Acute Monocytic	Form molecular scaffolds for ribonucleoprotein 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
15	MEG3	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.
16	TUG1	Chronic lymphocytic leukemia and multiple myeloma	Promotes cell proliferation of tumor cells.	PRC2 binding to repress cell-cycle regulation genes. Induced by p53
17	XIST	Fibrosis, leukemia, and histiocytic sarcoma	Required for hematopoietic stem cell survival and function	Not described
18	Neat1	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

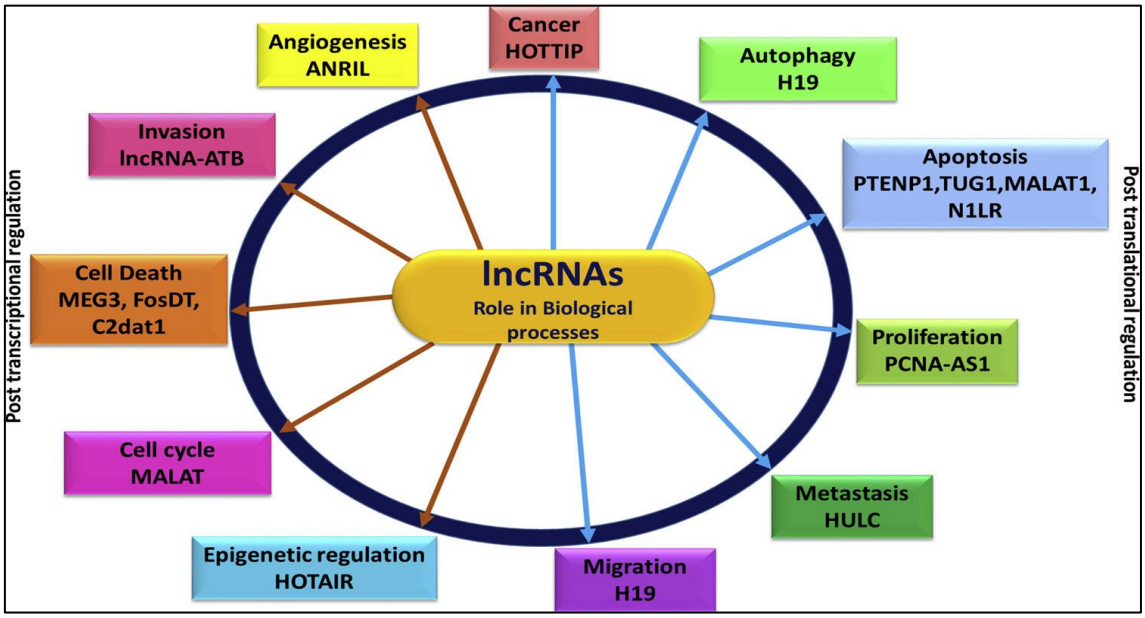


Figure 4: Functions of lncRNA. LncRNAs regulate various cellular processes by post-transcriptional and post-translational modifications as depicted above.

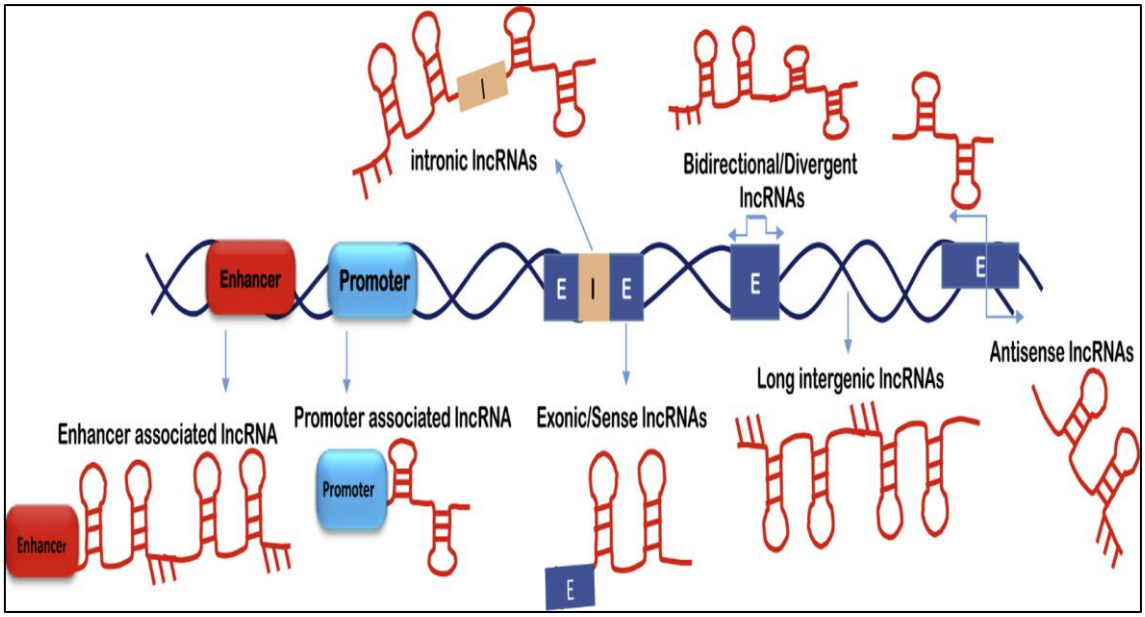
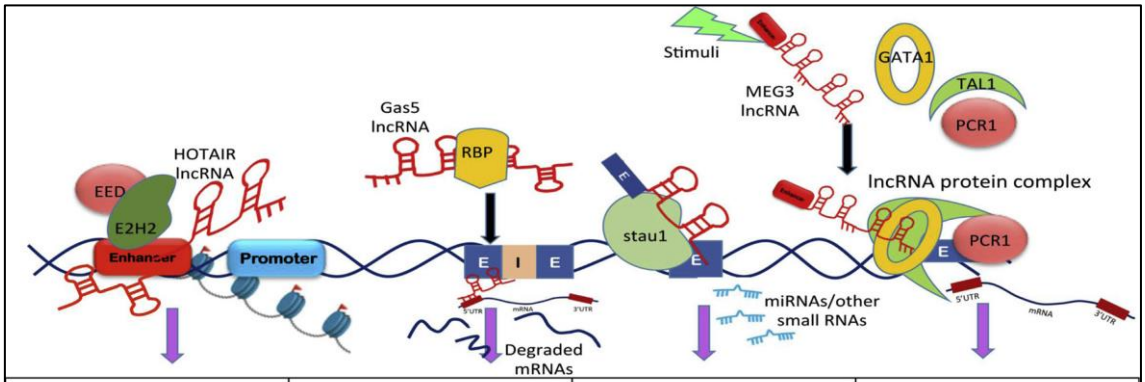


Figure 5: Overview of biogenesis of lncRNA. lncRNAs are pervasively interspersed in the genome with various possible locations. The figure summarizes the diverse range of lncRNAs based on their transcriptional origin: a) whole or partial natural antisense transcripts (NAT), b) coding genes, c) between genes, d) within introns, e) Promoter[blue], and f) Enhancer [red](I=Intron; E=Exon).



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

Figure 6: Mechanisms of lncRNA action. LncRNAs exert or execute their functions by the above depicted molecular mechanisms.

Table 2: Limited number of lncRNAs were explored for their biological functions in MKs.

S. No.	LncRNAs	Role in MK
1	AS-RBM15	Regulator of MK differentiation
2	MONC and MIR100HG	Maintains leukemic growth
3	MT1P3	Regulates platelet activation
4	MEG Family	Regulates leukemogenesis

1.4.4 Cell Cycle Regulation in Megakaryocytopoiesis

MKs are unique among polyploid mammalian cells. Like other types of cells (such as trophoblast cells of human placenta, liver parenchyma and heart muscle cells), MKs can reach higher ploidy through different cell cycle mechanisms. Ploidy describes the state of cells having greater DNA levels such as 4N, 8N, 16N, 32N, 64N and even 128N than the diploid content via escaping complete mitotic division. Higher polyploidization of MKs results in increase of cell size and also facilitate the massive mRNA and protein production as well as membrane synthesis with which the platelet becomes endowed (18,60). MKs polyploidy and differentiation are highly under the control of thrombopoietin based activities and certain cell cycle regulators (34). After MKs differentiation, diploid pro-megakaryoblasts gives rise to tetraploid megakaryoblasts and then successively to bigger promegakaryocytes with higher polyploidy. Finally, they develop into mature MKs through an endomitotic cell cycle which shows variations compared to the normal cell cycle with several mechanisms of genome multiplication (61–63). Endomitosis is characterized by the prophase blockage, without dissolving the nuclear membrane which exclusively leads to colossal chromosomes through repetitive duplication but non-disjunction of the chromosomes. The mechanism of endomitosis is difficult to interpret, it consists of a G1-S phases. Normally, diploid MK progenitor's progress through S phase by interruption, during which the cells enter mitosis (prophase and metaphase) but skip anaphase B and cytokinesis followed by splitting up of

paired sister chromosomes and cleavage furrow formation. Before the cytokinesis completion, the cleavage furrow regresses leading to the formation of tetraploid cell, which re-enters G1 (**Figure 7**). In this way a single cell develops a polyploid and multi-lobulated nucleus (64,65). Later the polyploid MK ceases DNA synthesis completely, further undergoes cytoplasmic maturation process which involves the formation of DMS extensively along with several alpha and dense granules which will ultimately fragment to circulating platelets (18,22).

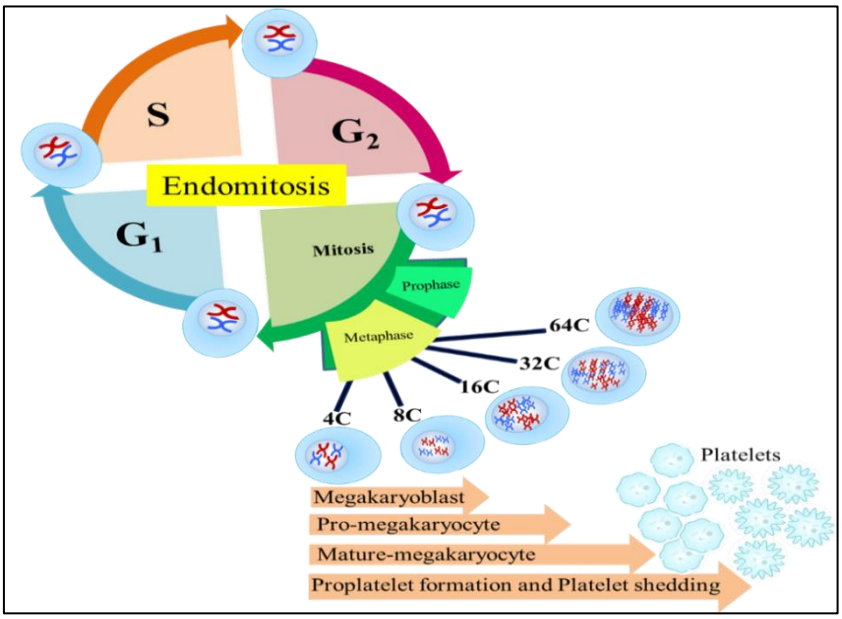


Figure 7: Overview of endomitosis mechanism to regulate megakaryopoiesis. Endomitosis consists of G1, S, G2, and partial M phases, which produces cells with a single giant lobulated nucleus like MKs. MKs on response to developmental signals switch from the mitotic cycle to the endocycle to increase their genomic DNA content without cell division finally promote polyploidy rather than proliferation.

A protein family known as “mini-chromosome maintenance (MCM) proteins” is one of the promising contenders for replication licensing factors. However, they require an additional and incompletely characterized activity known as “loading factor”. MCM together with loading factor are associated with the cell cycle mechanism by regulating through Cyclin-dependent protein kinases (Cdks). Transitions in between the consecutive cell cycle

phases are intermediated via consecutive pulses of Cdks activity. Activation of each Cdks is dependent upon a distinct type of cyclins (63,65).

Few research studies have reported that the switch to polyplodization in MKs is dependent upon the decrease of cyclin B levels and its associated kinase activity of Cdc2. One recent research study has reported the increase in cyclin B1 destruction via the ubiquitin-proteosomal pathway, both in higher ploidy primary murine MKs and in polyploidy megakaryocytic cell lines. It also reported that premature or accelerated cyclin B1 destruction could permit re-entry into the S phase of the cell cycle instead of anaphase entry (62,66). In contrast, some studies have reported the detection of high levels of functional cyclin B1 and Cdc2 but downregulation of the phosphatase Cdc25C, which leads to the common endpoint of downregulation of Cdc2 kinase activity in polyploid human MKs (67,68).

Few studies have observed the upregulated expression of cyclin D family by ploidy-promoting factors (like TPO and phorbol ester) in both primary MKs as well as megakaryocytic cell lines (69). This proves that significant increase in the S phase-linked Cdk complexes and G1-linked components activity will promote several cycles of endomitotic DNA synthesis, leading to the production of higher ploidy cells. An additional significant aspect of the regulation of the cell cycle controlled by cell cycle inhibitors such p53 and p21 which are involved in essential cellular processes (such as cell cycle arrest, DNA repair, transcriptional regulation, differentiation, senescence and apoptosis) is to prevent the proliferation of damaged cells. It is interesting to note that overexpression of tumor suppressor regulators such as p21 and p53 promoting ploidy in the human megakaryocytic leukemia cell lines (61,66,68).

1.5 Signaling Pathways in Megakaryocytopoiesis

Megakaryocytopoiesis is stimulated by multiple growth factors, among them TPO is the most significant. TPO belongs to the four-helix bundle family of cytokines. TPO predominately regulates MK differentiation from the HSC, and thus all progenitor cells primed to become MKs, including HSCs, CMPs, and MEPs, express c-mpl. TPO receptor is called as MPL also known as c-Mpl or CD110.

c-Mpl and TPO are significant for normal MK development and growth along with proper upkeep of other bone marrow progenitor cells (34,70). c-Mpl receptor is 635 amino acid protein encoded by MPL gene. Structurally c-Mpl receptor consists of a 25 amino acid signal peptide (1-25), a 465 amino acid extracellular domain (26-491), a 22 residue transmembrane domain (492-513) and an intracellular domain containing two conserved motifs termed box 1 (528-536) and box 2 (565-574). TPO signaling is initiated when TPO bounds to MPL receptor on MKs. This results in internalization of c-Mpl-TPO receptor ligand complex (71). Activation of the MPL occurs via receptor dimerization which initiates the multiple intracellular signal transduction pathways including 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) (72). Specifically, TPO induces phosphorylation of JAK2 which phosphorylates downstream targets including activation of the transcription factors STAT3/STAT5 (14). Signaling through these pathways causes downstream activation of MK-specific TFs and regulation of expression of MK-specific genes. In knockout mouse and mice models, lack of functional TPO and c-Mpl knockout results in decline of both HSCs and platelets to ~10% of normal, particularly drastic reduction in MKs and platelets. In addition, humans with a total loss of functional c-Mpl have a medium platelet count of $\leq 21 \times 10^9$/L,

which suggests that patients without functional TPO signaling could also retain a form of platelet production and that TPO-independent pathways of megakaryopoiesis in mammals exist (73,74).

Although residual platelet production persists in the absence of TPO signaling, TPO-independent regulators of megakaryopoiesis remain elusive. However, some alternative pathways have been shown to regulate megakaryopoiesis such as canonical and non-canonical Wnt signaling pathways, Notch signaling, Cannabinoid and TGF-β signaling pathways. In MKs, canonical and non-canonical Wnt signaling pathways are present and functional. Wnt signaling is necessary for MK proliferation and maturation. It also significantly supports formation of proplatelets from the mature MKs, which is facilitated by dose- and time-dependent increase in expression of β-catenin. This acts as a transcriptional co-activator in complex with TFs such as lymphoid enhancer factor/T-cell factor (LEF/TCF) family members for regulating gene expression (75,76). Even though these pathways function in a β-catenin dependent/independent manner, some interaction can be observed in some contexts in between non-canonical and canonical signaling. In general, T-cell lineage fate is determined by Notch signaling, in part via negatively regulating myeloid lineage and B cell development. However, the available evidence also suggests that Notch signaling can positively regulate megakaryopoiesis and plays a more multifaceted part in several aspects such as decisions of cell-fate w.r.t. myeloid progenitors.

Induction of pharmacologic agents which specifically inhibit the Notch pathway can revoke megakaryocytic differentiation confirmed the significance of Notch-mediated transcriptional activation role for megakaryopoiesis (77). Recently, TPO has been reported to play a significant role in the regulation of TGF-β synthesis and in MKs release, both *in vivo* and *in vitro*. TGF-β is a multifunctional growth factor which plays a vital part in regulating a

broad variety of biological mechanisms such as differentiation and development, cell adhesion and migration in various cellular systems, synthesis and release of extracellular matrix (ECM) and as a regulator in cytoplasmic Ca^{2+} influx. TGF-β is expressed ubiquitously. Previous reports show that components involved in TGF-β core pathway as well as its effectors are upregulated significantly in differentiating megakaryocytic cell line model. Additionally, the presence of canonical TGF-β pathway during the maturation of MKs was confirmed by the increased levels of phospho-SMAD2/3 (pSMAD2/3) (78). Interestingly, recent study has identified the Cannabinoid (Cb) signaling as a mediator of megakaryopoiesis. Endocannabinoids, well-known neurotransmission regulators are reported for their involvement in differentiation and maturation of MKs by stimulating the Cb receptors. Cb receptors are associated with a significant role in megakaryocytopoiesis by altering mitochondrial functions, ROS production and triggering MAPK activation (79). Over all, this information's suggest not only the importance of TPO signaling in megakaryopoiesis, but also the role of other signaling pathways as positive regulators of MK lineage development.

1.6 Clinical correlates

Acute myeloid leukemia (AML) is a group of blood cancers in which bone marrow generate a large number of immature and abnormal blood cells. The number of these immature blood cells (called blasts) builds up in the body. Acute megakaryocytic leukemia (M7-AML) is a very rare type of leukemia which are identified with poor prognosis. M7-AML is a pediatric leukemia characterized by a high number (>20%) of proliferating megakaryoblasts which are associated with extensive myelofibrosis of the bone marrow. In contrast to AML in children with Down syndrome (DS-AML), non-DS-AML subgroup associated with poor prognosis. Different types of leukemia have different treatment options

and outlooks. However, M7-AML remains frustratingly difficult to treat because there is no exact known way to prevent M7-AML. Modern treatment can significantly improve quality of life and may extend survival.

Dysregulated development of MKs could result in irregular platelet number and the generation of functionally defective platelets as a result deviation in the normal range of platelets (80). As such, variation in normal range of platelet counts can lead to several pathological situations, for example, too few platelets (thrombocytopenia) can result in bleeding, whilst too many platelets (thrombocytosis) can increase the risk for thrombotic events, including myocardial infarction , peripheral ischemia, and stroke (81). Deviant MK development can result in deregulated platelet number and functionally defective platelets, as seen in myelodysplastic syndromes (MDS), myeloproliferative disorders (MPD) and Bernard Soulier Syndrome (BSS), to name just a few (81). Due to secondary effect of a progressive myeloproliferative cancer MDS and MPD, an increased risk of some rare kinds of subtype of AML such as Acute Megakaryocytic Leukemia (AMKL). It is predominant AML among children compare to adults. However, the exact molecular mechanisms responsible for AMKL defects remain elusive.

Intensive multidrug chemotherapy and allogeneic bone marrow transplantation are common known treatment approaches for AMKL. Nevertheless, patients with AMKL have a dismal prognosis. Therefore, the process in understanding molecular mechanisms of the disease has led to significant insights into the molecular genetics and cellular pathophysiology of these disorders. As AMKL is not a common disease and heterogeneity of the disease create challenges in finding appropriate procedures of targeted therapy. Therefore, Differentiation therapy seems to be a particularly attractive solution for AML patients. A variety of agents stimulate differentiation of the leukemic cells into normal cells. Because of

forced differentiation of leukemic cells towards healthy cells seemed an attractive solution to clinicians and researchers. As lncRNAs, offer an added level of control between differentiation and proliferation by regulating gene expression post-transcriptionally by targeting mRNAs/miRNAs via protein translation inhibition. Many lncRNAs have been related to the specification of hematopoietic cell lineages, and have been found altered by chromosomal translocations associated with leukemia. Therefore lncRNAs could be potential therapeutic targets and can be beneficial to AMKL patients.

1.7 Alternative source of MKs

Platelets are generated from MKs whose main role is to maintain hemostasis and also to halt haemorrhage through localizing clot formation at the vessel injury site (20). The primary mechanisms of hemostasis, clot formation and blood coagulation are dependent upon the adequate supply of platelets within the bloodstream of the individual. A reduction in number of platelets (thrombocytopenia) can be caused due to the failure of bone marrow (acquired, in case of post-cancer treatment or inherited) or serious bleeding in peripheral regions after surgery or trauma which ultimately results in life-threatening haemorrhage. The most efficient approach to increase a patient's platelet count is through transfusion, however limitations in the supply of platelets is a recurring concern. An inadequate shelf-life (i.e. 5 days) and the necessity of storage at room temperature which increases the risk of bacterial contamination is a difficult issue in order to maintain adequate supplies. Additionally, individuals who receive several platelet transfusions, for example individuals with multiple cancers often develop platelet refractoriness because of HLA alloreactivity. Hence, requires additional transfusions with HLA-matched donor platelets (82). Developing alternate sources for non-immunogenic, high-quality platelets could help to decrease chronic shortages in the platelet supplies and also reducing the risks of refractoriness. The large scale production of

platelets and MKs *ex vivo* will be greatly appreciated in the treatment of thrombocytopenia which has been the focus of many studies. Therefore, one strategy to accelerate platelet recovery is to implant the individual with sufficient number of MKs which are committed for repopulating cells until hematopoietic reconstitution occurs. It has been reported that a higher proportion of megakaryocytic progenitors (MKPs) in transplanted grafts positively affects the platelet recovery. Human $CD34^{+}$ cells from umbilical cord blood (CB) and bone marrow (BM) are proficient to produce platelets and MKs, however proliferation ability of these cells is limited and generation of platelets and MKs is dependent on donor (83,84). Because of MK rarity and clinical limitations, recent developments in cell culture techniques and the advent of stem cell technology has enabled generation of MKs from human pluripotent stem cells including induced pluripotent stem cells (hiPSCs) and embryonic stem cells (hESCs), which have self-renewal ability and get differentiated into all somatic cell types, making it a viable option for cell source (**Figure 8**). hiPSCs and hESCs have been widely used to generate both platelets and MKs through xeno and feeder-free conditions *ex vivo* (85,86). Potential for unlimited capacity for renewal, iPSCs are an attractive source for *in vitro* generation of megakaryocytes as well as other cells which can be further applicable for drug screening, cell therapy and disease modelling etc. (**Figure 8**). This has led to the belief that *ex vivo or in vitro* generation of MKs from human iPSCs provides a viable and renewable cell source for platelets generation in the treatment of individuals with thrombocytopenia and allows a proper understanding of MK and platelet biology (87,88).

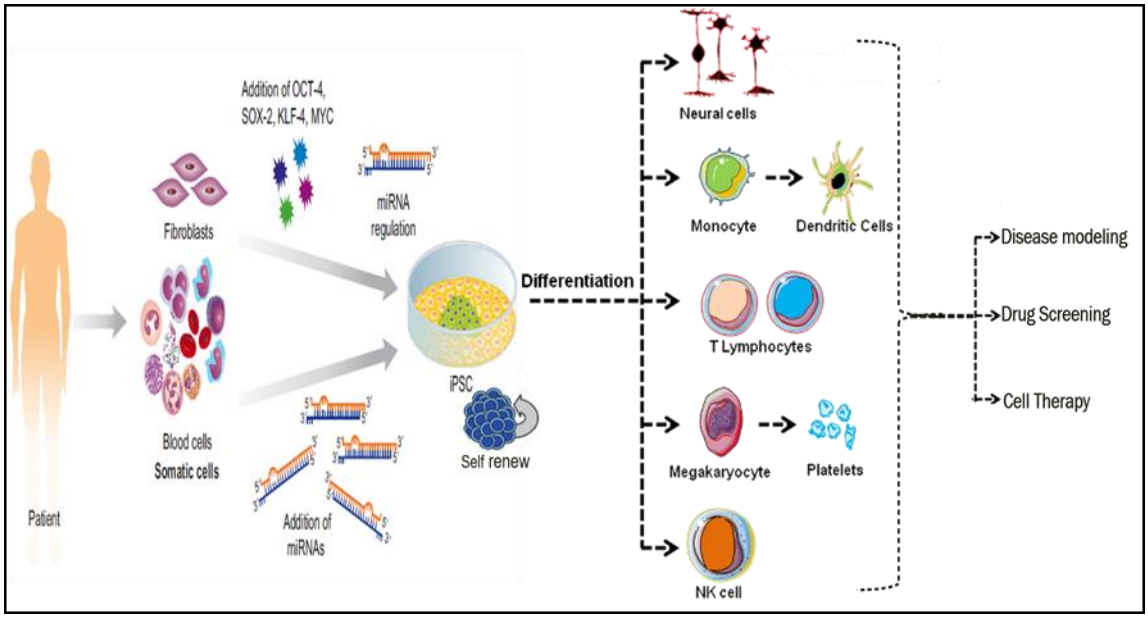


Figure 8: iPSCs as a potential tool for generation of MKs from any mature somatic cells. iPSCs can be reprogrammed by addition of certain Yamanaka's key TFs with certain miRNAs to improve their efficiency to generate different types of cells including MKs.

Chapter II:
OBJECTIVES OF THE WORK

Megakaryocytes (MKs) are rare polyploid cells found in the bone marrow and finally produce platelets. A rupture of single MK can release ~4000-5000 platelets into the circulation. Platelets are small colorless, enucleated cell fragments that are critical to vascular hemostasis and wound healing as well as in inflammation. Therefore, to maintain normal platelet count (5-45 x 10^4 platelets/μL of blood) is very important. Several blood disorders characterized by dysfunctional platelets can lead to bleeding tendency, defective clot formation and extended bleeding time. Thrombocytopenia is one of the most common hematologic disorders, characterized by an abnormally low number of platelets due to several reasons. It affects both children and adults. Thrombocytopenia is sometimes a first sign of autoimmune disorders, thrombotic micro-angiopathies, infectious diseases, and hematologic malignancies. It is also a common side effect of many medications. As a rule, there is no specific therapy for the vast majority and only severe cases need to be treated. Currently *ex vivo* generation of MKs from human induced pluripotent stem cell (hiPSC)-derived platelets technology is a good treatment option, which could provide an alternative source of platelets for treating thrombocytopenic patients in different disease conditions and allows a better understanding of MK and platelet biology. Recent advancements in the rapidly evolving field of epigenetics has shown the role of ncRNAs in hematological epigenetics, mostly focusing on the mechanisms by which miRNAs and lncRNAs impact the epigenome in the context of blood cell development.

However, there are only few studies which have identified and characterized the lncRNA expression profiles in MKs, the underlying regulatory mechanisms governed by lncRNAs has not been explored for the normal development of MK lineage. We hypothesized that lncRNAs are differentially expressed in hiPSC generated MKs, therefore might play an essential part in the normal regulation of megakaryocytopoiesis. Our studies aimed to establish a protocol for the generation of MKs in xeno-free and defined conditions, and to

determine the lncRNA profile and molecular mechanism in hiPSCs-derived MKs, to gain an understanding on the normal development of MK. Furthermore, we focus our study to understand the potential function of respective lncRNA in hyper-proliferative clinical condition i.e. Acute Megakaryocytic leukemia (AMKL).

Based on these preliminary findings we framed our study into following **objectives:**

1. To generate and characterize the MKs derived from iPSCs using blood cells.
2. Elucidate the role of lncRNA in normal development of MK lineage derived from iPSCs.
3. Study the role of lncRNA in hyper proliferation (AMKL) during treatment by using Phorbol 12-myristate 13-acetate (PMA).

Chapter III: MATERIALS and METHODS

3.1 hiPSCs Culture

hiPSCs-HSCs were obtained from inStem CSCR, Vellore, India. Purified HPCs (1 x 10^5/mL) were cultured (5% CO_2 incubator at 37 °C) in 1 mL serum free: StemPro-34TM basal media (1X) supplemented with PenStrep (1X), L-Glutamine (2 mM), rhSCF (100 ng/mL), rhFLT3 (100 ng/mL), rhIL-3 (20 ng/mL), and rhIL-6 (20 ng/mL). Expansion media was changed at 2-day intervals.

For MKs differentiation, hiPSCs-HSCs on 5th day of culture were collected and transferred to MK differentiation media: StemPro-34^{TM} basal media supplemented with PenStrep (1X), L-Glutamine (2 mM), FGF2 (10 ng/mL), BMP4 (10 ng/mL), SCF (20 ng/mL), VEGF-2 (10 ng/mL), TPO (50 ng/mL), IL-11 (10 ng/mL) and IL-3 (10 ng/mL). This standardized differentiation system provides a simple platform to produce MKs in feeder free condition within 5–10 days. Half media was replaced at every 2-day intervals.

3.2 Cell line Cultures

Dami cells (Megakaryoblastic cells) were used to investigate the effects of PMA on megakaryocyte differentiation. Cells were cultured in RPMI-1640 media (Gibco, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic (Sigma Aldrich) at 37 °C in 5% CO_2. To investigate the effects of PMA on cell growth and differentiation, cells were treated with 100 nM concentration of PMA (Sigma–Aldrich) followed by quantification of cells by hemocytometer using trypan blue stain (Mediatech).

K562 cells were cultured in RPMI-1640 media with 1% antibiotic antimycotic solution, 10% FBS and maintained in 5% CO_2 incubator at 37 °C. To induce megakaryocytic

differentiation, K562 cells were treated with 50 nM PMA for 72 h. After 72 h cells were collected and used for RNA and protein isolation.

3.3 Microscopy

3.3.1 Phase contrast micrographs: Images (Zeiss LSM 510 Confocal Microscope, Olympus with 405 LASER) were taken at 10th day to evaluate morphological changes on hiPSCs-HSCs derived MKs.

3.3.2 Bright-field imaging: To evaluate morphological changes in PMA-induced megakaryoblastic Dami cells. Dami cells upon 72 h of PMA treatment were visualized and imaged under inverted light microscope.

3.3.3 Immunofluorescence microscopy: MK-specific marker, CD41 surface antigen expression was evaluated by immunostaining of cells with anti-CD41 FITC (BD Pharmingen). Cells (1×10^6) were fixed with paraformaldehyde (2%) and permeabilized with 1% Triton X-100, and blocked with 2% BSA for 60 min. After blocking, cells were incubated for one hour with anti-CD41 FITC followed by washing with PBS to remove all unbound antibodies. Finally, cells were mounted with DAPI and detected under confocal microscope (Carl Zeiss Micro imaging).

3.4 Ploidy (DNA index) analysis

To observe the multi-lobed polyploid nucleus of differentiated MKs, harvested cells were rinsed with 1X PBS and fixated on glass slides. Wright-Giemsa (WG; Baxter) staining was performed as per the manufacturer's instructions. The images were captured using Zeiss LSM 510 confocal microscope, in conjunction with the Zeiss ZEM imaging software.

To properly calculate the DNA Index, the cells were collected and stained (1 x 10^6 cells/mL) with reagents of Muse Cell Cycle Assay Kit (Merck Millipore, USA) as per the manufacturer's instructions. The assay kit comes with propidium iodide (PI) which is detected in the Yellow detector in linear mode. Although PI is undoubtedly the most regularly used dye to quantitatively assess DNA content which was analyzed by using a flow cytometer (Muse Cell Analyzer; Merck Millipore, Germany). A histogram plot of DNA content against cell numbers gives the classical DNA profile for a cell culture. The experiments were performed in triplicates and contained a minimum of 5000 events per run.

3.5 Flow cytometry analysis

iPSCs-HSCs derived MK Cells (1 x 10^6/mL) were prepared in ice-cold 1X PBS with 0.1% FBS. Cells were harvested and labelled with PE (Phycoeryhtrin)-tagged anti-CD34 and FITC (Fluorescein isothiocyanate) -tagged anti-CD41 (BD Biosciences), and PE-tagged anti-CD61 (BD Biosciences. Ig isotype controls (FITC and PE) were used as control for flow cytometry. All samples were analyzed for $CD34^+$, $CD41^+$, and $CD61^+$ expression using the FACS Aria^{TM} III flow cytometer (BD Biosciences). iPSCs-HSCs were used as control.

Dami Cells (1 x 10^6) were harvested and rinsed with ice cold 1X PBS with 0.1% FBS. Further, cells were fixed with paraformaldehyde. After blocking with 5% mouse serum and 1% BSA, cells were stained with FITC-conjugated anti-human CD41 (BD Biosciences) and isotype-matched IgGs (negative controls). Finally, cells were washed with 0.1% FBS in 1X PBS and analyzed using FACS Aria™ III flow cytometer (BD Biosciences).

3.6 RNA isolation and qRT-PCR analysis

Total RNA was prepared from harvested cells by using miRNeasy mini kit (Qiagen) as per the manufacturer's protocol. cDNA was prepared from 1 μg of total RNA using

random hexamer assays of EasyScript^{TM} cDNA Synthesis Kit (ABM) as per the instructions provided by the manufacturer. Further, the cDNA was used for the qRT-PCR quantification; qRT-PCR was performed with specific primers (**Table 3**) and SYBR Green FAST qPCR Master Mix (Kappa Biosystems) using Step One Plus^{TM} Applied Biosystems quantitative real-time PCR system. PCR cycle conditions were: initial denaturation at 52 °C (2 min) and 95 °C (8 min), followed by 42 cycles of 56 °C (30 s) and 72 °C (30 s). Ct-values were normalized against internal control GAPDH (Glyceraldehyd- 3-Phosphate-Dehydrogenase). miScript Primer Assays (Qiagen) were used to analyze the expressions of miRNAs by qRT-PCR, U6 was used as internal control for data normalization. Relative quantification of genes was calculated by the comparative ΔΔCt method ($2^{\wedge(-\Delta\Delta Ct)}$) and represented as mean ± standard deviation (SD) of three independent experiments.

Table 3: List of primers used in this work.

Names of Genes and lncRNAs	Primer Type	Primer Sequence
CD41	Forward	TCAACCCTCTCAAGGTGGAC
	Reverse	GCAGCACAAACTGATCCAGA
CD61	Forward	CCTGTTGGGAGTGAGGATGT
	Reverse	AGAGCTGCCAATAAGGCAAA
GAPDH	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA
18S RNA	Forward	CTCGGCAACGGATATCTCG
	Reverse	GCCCTCAACCTAATGGCTTC
RUNX1	Forward	AACCTCGAAGACATCGGCAG
	Reverse	GGCTGAGGGTTAAAGGCAGT

p21	Forward	ATTCAGCATTGTGGGAGGAG
	Reverse	TGGACTGTTTTCTCTCGGCT
p53	Forward	TGCGTGTGGAGTATTTGGATG
	Reverse	TGGTACAGTCAGAGCCAACCTC
TGF-β1	Forward	GCAACAATTCCTGGCGATAC
	Reverse	TAGTGAACCCGTTGATGTCC
TGFβR1	Forward	GTGACAGATGGGCTCTGCTT
	Reverse	GAGGGTGCACATACAAACGG
TGFβR2	Forward	AGACGTTGACTGAGTGCTGG
	Reverse	TTAGGGAGCCGTCTTCAG
p16	Forward	CCCAACGCACCGAATAGT
	Reverse	ACCAGCGTGTCCAGGAAG
Cyc E	Forward	TTCTTGAGCAACACCCTCTTCTGC
	Reverse	TCGCCATATACCGGTCAAAGAAAT
Cyc D1	Forward	TGAACTACCTGGACCGCT
	Reverse	GCCTCTGGCATTTTGGAG
Cyc D2	Forward	CAGAAGGACATCCAGCCGT
	Reverse	TCGGGACTCCAGCCAAGAA
JPX	Forward	GCACCACCAGGCTTCTGTAAC
	Reverse	GGGCATGTTCATTAATTGGCC
MEG3	Forward	GCTGATGAACCAGGCGGAGG
	Reverse	TTTGTCTCCCCTGAGTCCAC
TncRNA	Forward	GCTGGAGTCTTGGGCACGGC

	Reverse	TCAACCGAGGCCGCTGTCTC
UCA-1	Forward	GCCCAAGGAACATCTCACCAATTT
	Reverse	TTGAGGGGTCAGACTTTTGACAAGG
UM9-5	Forward	GTCTCCATTTCACAGGAAGAAACA
	Reverse	GCTAACTCAGTCTCTTACTGAGA
HOTAIRM1	Forward	AGGGGGTTGAAATGTGGGTG
	Reverse	CTTGAAAGTGGAGAAATAAAGTGCC
MiR-125b	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
MiR-106b	Forward	CTCAACTGGTGTCGTGGAGTCGGC
	Reverse	ACTGCTAAAGTGCTGACAGTGCA
MiR-17	Forward	CATCTACTGCCCTAAGTGCTCCTT
	Reverse	GCTTGGCTTGAATTATTGGATGA
MiR-9	Forward	CAGAGAAGGGCAGTGGAGAC
	Reverse	ACGACAGAGACCGAAAAAGG
LUST	Forward	GCTTCAACACTGCGTGACAA
	Reverse	CGTGGAATCAAATGGAGTGG

3.7 RNA isolation and lncRNA expression profiling array

For lncRNA profiling, total RNA was isolated from cultured cells using the miRNeasy mini kit (Qiagen) following the manufacturer's instructions, and total RNA input (1 μg) was reverse transcribed by using Human LncProfilers™ cDNA synthesis Kit (System Biosciences), as per instructions specified by the manufacturer. For lncRNAs profiling, the Human LncProfilers™ qPCR Array was used. Ct values were normalized to 18S RNA,

differentially expressed lncRNAs between control group and differentiated MKs were recognized via ΔΔCt analysis software (fold change (FC) ± 2 and $p<0.05$) in SBI website (www. systembio.com/ LncRNA).

3.8 Protein isolation and Western blot assay

Total proteins were extracted from cells by utilizing pre-chilled RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitors (G-Biosciences; Geno Technology). Proteins (50 µg) were separated on 6-12% SDS-PAGE and transferred to nitrocellulose (NC) membranes (Millipore). Membranes were blocked with 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with specific primary antibodies (PI3K, AKT, Cyc D1, p16, p21, pERK1/2, p53, Bcl2, Bad, Cyt C, Bax, Caspase 3, 9, and PARP; β-actin (Biosciences, Sigma and Santa Cruz). After washing with TBST, NC membranes were incubated (1h at room temperature) with suitable peroxidase-conjugated secondary antibody. A chemiluminescent solution, Pierce™ ECL Substrate (Thermo Fisher Scientific) was used to develop protein bands on NC membranes and visualized by Chemidoc Imaging System (BioRad) as per the instructions of manufacturer. ImageJ software was used to quantify the relative protein levels using western blot band density and β-actin was used for normalization.

3.9 Determination of intracellular Reactive oxygen species (ROS)

2, 7-dichlorodihydrofluoresceindiacetate (H_2DCFDA) is a cell permeable dye was used to monitor the intracellular ROS levels of the PMA-activated K562 (1×10^6 cells/mL). Here, we measured the ROS from different experimental time points such as control, 24 h, 48 h, and 72 h treated with PMA (50 ng/mL). Briefly, cells were washed with PBS and incubated with H_2DCFDA dye (10 µM) in 1X PBS for 15 min in dark at room temperature. Green fluorescence of 2, 7-dichlorofluorescein (H_2DCF) was measured using a flow

cytometry (BD LSR FORTESSA) and mean fluorescence intensity (MFI) represented as bar graphs.

3.10 Small interfering RNA (SiRNA) transfection

3.10.1 HOTAIRM1-siRNA transfection: K562 cells were maintained in RPMI-1640-GlutaMAX^{TM} Supplement media (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS and 1% antibiotic-antimycotic solution in a humidified incubator at 37 °C with 5% CO_2. The cells were cultured in 24-well plate (1×10^5 cells/well) overnight. To induce megakaryocytic differentiation of K562, 50 nM PMA was used. LncRNA HOTAIRM1-siRNA were designed and synthesized from sigma-aldrich.com, and transfected to PMA-induced K562 cells by X-tremeGENE 360 Transfection Reagent (Sigma-Aldrich) and negative controls (non-targeting or nonsense SiRNA control sequence). The final concentration of each si-HOTAIRM1 in the transfection sample was 25 nM, as per the manufacturer's instructions. To verify if there were any off target effects, expression of lncRNA-HOTAIRM1 was examined using qRT-PCR after three HOTAIRM1-siRNA mixture transfections, which can efficiently evade off target effects. After 48 h transfection of PMA-induced K562 cells with human SiRNA-HOTAIRM1, cells were processed for subsequent experiments.

3.10.2 JPX-siRNA transfection: A total of 1×10^5 dami cells/well were cultured in a 24-well plate for 24 h in an incubator at 37°C. For rescue experiments, cells were pretreated with 100 nM PMA for another 48 h prior to transfection at 37 °C. Then transfected with JPX-siRNA and negative control-siRNA according to the manufacturer's protocols, as above-mentioned. Subsequently, cells were harvested after 48 h for further experiments.

Statistical analyses

All the experiments were executed in triplicates (n = 3). Results were represented as mean ± SD of three individual experiments. Student's t-test was performed to examine significance variance between the analyzed groups. Statistical significance of data presented by p-value (**$p<0.002$, *$p<0.05$).

Chapter IV: RESULTS and DISCUSSION

OBJECTIVE 1:
To generate and characterize the megakaryocytes derived from iPSCs using blood cells

Objective 1: To generate and characterize the megakaryocytes derived from iPSCs using blood cells.

Development of all types of blood cells has long been viewed as a hierarchical paradigm which originates from HSCs in bone marrow (3,7). It is believed that HSCs supports life-long hematopoiesis because of its higher capacity of self-renewal and multipotent features. HSCs are responsible for the generation of common myeloid progenitor (CMPs), which further differentiate into MKs through successive lineage commitment steps and develop as mature MKs via megakaryopoiesis (12–14,89). During MKs maturation process, with increase in cell size they replicate their DNA content without cell division via a unique process known as endomitosis, and develop into a big cell with ploidy nucleus, which further develop an extensive internal DMS, and finally form pro-platelet processes which sheds into platelets (18,61,62,66,68,69).

MKs are the only source to produce platelets in our body by the process of thrombopoiesis. Platelets are the end product of MKs, they are colorless, small enucleated blood cells with the limited shelf life (7–9 days). Platelets are critical players to maintain physiological homeostasis, immunity, blood clotting and wound healing therefore to maintain the normal range of platelets i.e. 150–450 × 10^9/blood in our body is very important (18,29,34,76). Bone marrow failure (inherited or acquired, such as post-cancer treatment), defects in thrombopoiesis, severe peripheral bleeding after trauma or surgery, chemotherapy, and potentially leads to life-threatening hemorrhages can cause decrease in platelet count results life-threatening thrombocytopenic condition (47,80,81).

Cord blood transplantation and MKs engraftment are often poor and delayed process. Platelet transfusion is an important therapeutic approach for patients with severe thrombocytopenia (82,90). However, most medical facilities, clinics and blood centers often

suffer from imbalance of supply-demand for platelet transfusion due to the shortage and dependence on volunteer donors. In addition, there is inevitable risk of pathogenic contamination in the products from donors with the limited shelf life of platelets. Altogether, for worldwide health organizations, it reflects pandemic challenge with financial, logistical and biosafety term. To overcome such challenges, very limited number of studies have shown *in vitro* generation of platelets from hiPSCs as an alternative source and most feasible therapeutic option for the treatment of thrombocytopenic patients (82,87,88,90,91). Based on hiPSC capacity to differentiate towards MKs and platelets upon adequate stimulation opens the way for remarkable opportunities for basic research and clinical applications with safety advantages. We hypothesize to generate MKs from hiPSCs to match the needs of large amounts of MKs/platelets via optimizing the existing protocol reported by Liu et al. in 2015 (84,88). In order to generate large quantities of particular interest of functional MKs, we initiated differentiation protocols by culturing and characterizing hiPSCs derived from human blood cells.

4.1 MKs differentiation from iPSCs-HSCs under xeno-free and defined conditions

In present study, we have followed an approach for efficient differentiation of MKs from hiPSCs-HSCs under xeno-free and defined conditions by modifying the existing culture conditions (**Figure 9**), which were reported in recent studies [5,6,17]. In this culture system, before inducing MK differentiation of hiPSCs-HSCs, selective expansion media for 5 days was used. Further hiPSCs-HSCs were able to undergo megakaryopoiesis to differentiate and develop into MKs in megakaryopoiesis defined media supplemented with supporting growth factors and cytokines (FGF2, BMP4, SCF, VEGF-2, TPO, IL-11, IL-3) which were essential for MK differentiation and growth. Here we showed that hiPSCs-HSCs were successfully

differentiated into MK population on day 10, further hiPSCs-HSCs derived MKs were examined for their morphological and biochemical specific properties on the molecular level.

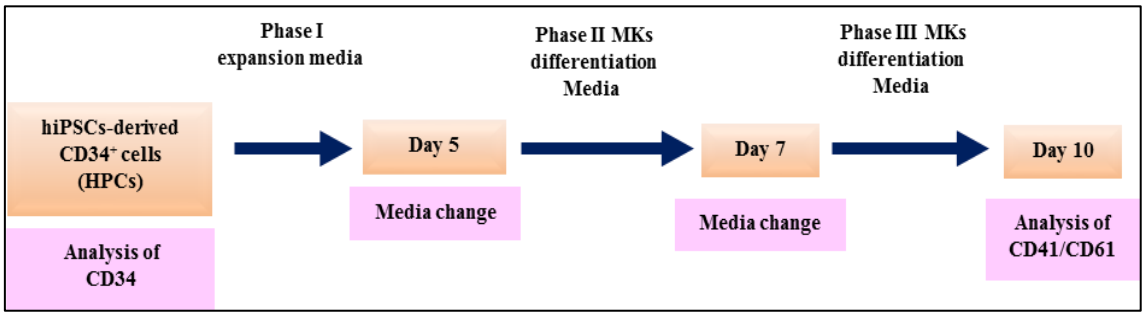


Figure 9: Schematic representation of the successive iPSCs-HSCs differentiation protocol for MKs differentiation. In phase I, hiPSCs-HSCs were cultured in expansion media for 5 days. Phase II, to induce MKs differentiation hiPSCs-HSCs were cultured in megakaryopoiesis defined media supplemented with supporting growth factors and cytokines (FGF2, BMP4, SCF, VEGF-2, TPO, IL-11, IL-3) up to 10 days.

4.2 Characterization of MKs specific features

We have successfully generated MKs population from hiPSCs-HSCs at day 10, with higher efficiencies to those previously reported (83,87,88). After 10^{th} day cells have shown their MK-like specific features by demonstrating typical MK morphology and MK specific gene expression which was analyzed by FACS, qRT-PCR, and Giemsa staining and cell cycle analysis.

For morphological characterization, cells were observed with increased cell size as compared to day 0 (**Figure 10A**) by using bright field microcopy. The surface expression of MKs specific cell markers $CD41^+/CD61^+$ were further assessed by Flow cytometry. These results showed about 80.3% of non-stimulated cells (day 0) expressed CD34 surface antigen specific to HSCs. Interestingly, >90% of the cells were enumerated highly pure population of MKs ($CD41^+/CD61^+$; **Figure 10B**) at day 10 by FACS analysis. The platelet surface antigens, CD41 and CD61 are commonly used to define megakaryocyte differentiation and maturational stages [18,19]. Polyploidization is a crucial event of megakaryocytopoiesis, and

the large, polyploid-multilobed nucleus is considered as the hallmark of matured MKs (60,61,66). Therefore, a population of FACS-sorted MKs ($CD41^{+}/CD61^{+}$) were examined for cellular morphology and multilobed ploidy states of nucleus was followed by WG staining by using bright field microcopy. The images of WG-stained smears of cells confirmed at day 10 differentiated MKs were large in size with polyploid multilobed nucleus compared to day 0 (**Figure 10C**).

In addition, increased ploidy levels by cell cycle analysis was detected in hiPSCs-HSCs derived MKs as compared with hiPSCs-HSCs on 10^{th} day (**Figure 10D**). To further characterize hiPSCs-HSCs derived MKs at a molecular level, we assessed the expression of MK specific cell surface markers (CD41/CD61) by quantifying their mRNA levels using qRT-PCR. We observed after 10^{th} day of treatment with cytokines as compared to day 0 cells consistently increased expression of both the CD41 (~6 fold) and CD61 (~15 fold) MK-marker mRNAs (**Figure 10E**). Fortunately, we were able to demonstrate for the first time MK differentiation from hiPSCs-HSCs derived from Indian origin blood cells by using simple protocol.

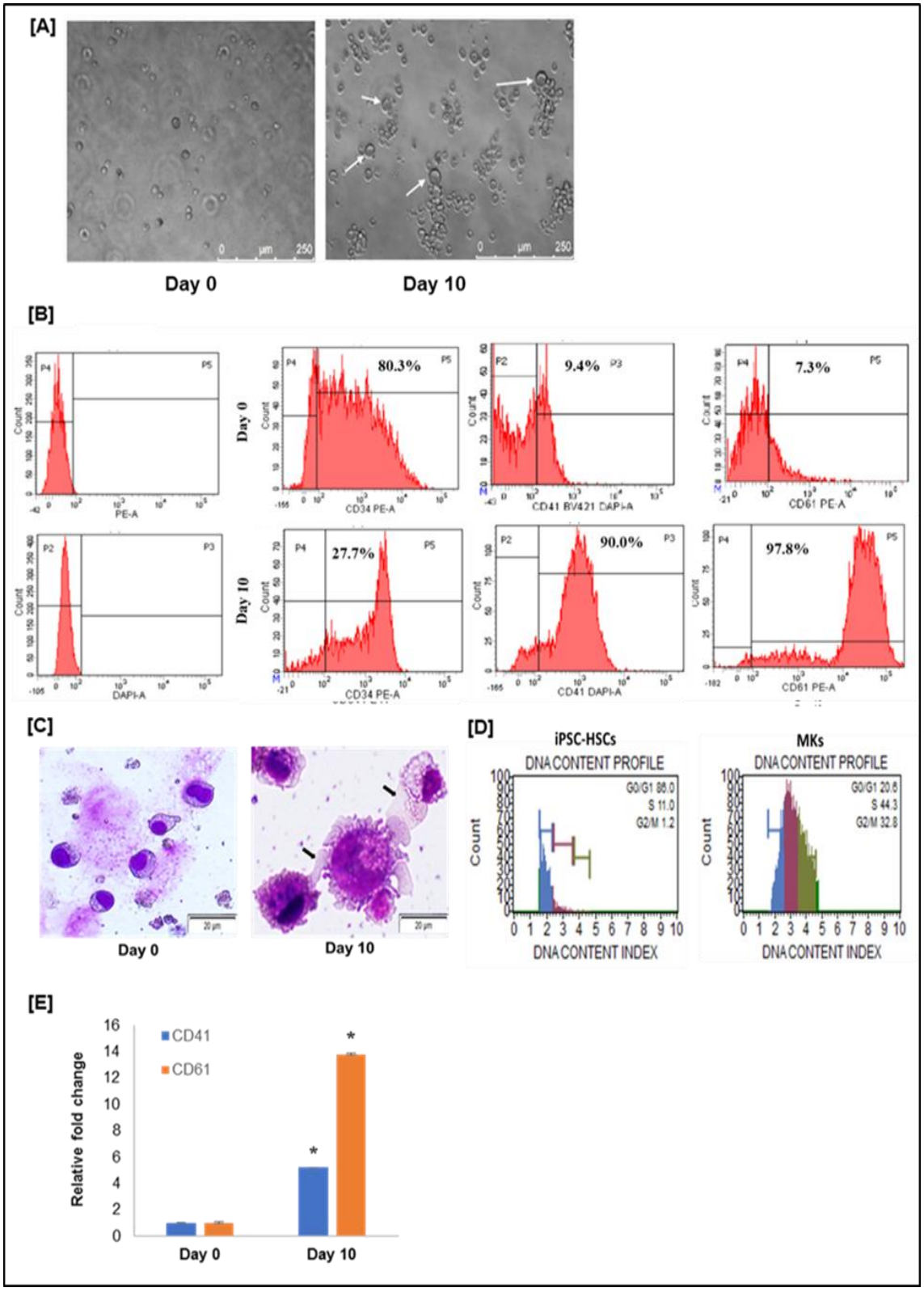


Figure 10: Characterization of hiPSCs-HSCs derived MKs. A. Phase contrast images of cells on days 0 and 10. **B.** Representative flow cytometry histograms of the cultured suspension cells on day 0 and day 10. The suspension cells on day 0 expressed CD34 marker and on day 10 maximum cells expressed MK markers CD41 and CD61. **C.** Photomicrograph of cells stained with WG showing the cell and nuclear size were increased for MKs on day

10. **D.** DNA content (polyploidy status) was increased in MKs compared to iPSCs-HSCs which was analyzed by MUSE analyzer. **E.** qRT-PCR results showing fold induction in the expression of CD41 and CD61 in cells of day 10 as compared to the cells of day 0 (n=3; *p<0.05). Bars represent mean ± SD of three independent experiments.

4.3 Conclusion

In our study, we have developed a differentiation system for efficient MK production from hiPSCs-HSCs under feeder-free and serum-free conditions. Our protocol comprises one step including 2 phases: (i) expansion of hiPSC-HSCs in the presence of expansion media, and (ii) differentiation/maturation phase of cultured MKs in the presence of suitable growth factors and cytokines. In the first phase of hiPSCs-HSCs expansion, we used commercially available expansion media to get sufficient hiPSCs-HSCs which we used for MK differentiation, based on pioneer studies (92,93). We have since improved this method.

Although our study was focused on the generation of MKs from hiPSCs-HSCs, in phase two, we used cocktail of certain cytokines with relevant growth factors which are essential for MKs differentiation as well as complete development (88,94). Our study likely resulted in high yield of hiPSC-HSCs derived MKs, which were capable to form platelets in feeder free and xeno free culture system compared to previous reports. We found on day 10, cells were resembling the features of typical MKs like large cell having polyploid nucleus with proplatelet forming structure. At day 10, culture had higher ploidy DNA content, suggesting a close similarity of hiPSC-HSCs derived MKs with normal MKs. The major reason to optimize the previous reported protocols (87,88,91,94,95) is to reduce contamination while changing different types of media for specific stages of differentiation as well as to significantly increase the yield of pure population of MKs. As we expected, the percentages of $CD41^{+}CD61^{+}$ cells on day 10 was higher with stimulation than without stimulation of MK differentiating cocktail. Also, generation of HSCs from hiPSCs and then

HSCs used for MK generation was cumbersome, time taking, complex and costly process for clinical applications.

Our approach indicates the feasibility to generate efficient MKs directly from hiPSC-HSCs in simple as well as cost effective manner. Our study is primarily focused on the generation of MKs from hiPSC-HSCs by using basic and defined MK differentiation system which can be used as convenient way for future development of allogeneic transfusion of MKs/platelets. Also, our experimental system should provide a convenient platform to differentiate MKs for studying the genetic influences and molecular regulation of megakaryopoiesis in the future.

Chapter V: RESULTS and DISCUSSION

OBJECTIVE 2:
Elucidate the role of lncRNA in normal development of megakaryocyte lineage derived from iPSCs

Objective 2: Elucidate the role of lncRNA in normal development of megakaryocyte lineage derived from iPSCs.

LncRNAs are ncRNAs of more than 200 nts in length which have been implicated in numerous biological processes, including regulation of gene expression, cell-cycle, transcriptional/translational and epigenetic regulation (54). Interestingly, differential expression pattern of lncRNAs have been associated with the regulatory processes of development, differentiation and maturation of blood cells. Growing evidences indicate several lncRNAs act as key regulators in normal and malignant hematopoiesis regulation such as XIST, HOTTIP, NeST and EGO etc. (96). Recent report suggest the crucial role of miRNAs in the regulation of MK development and platelet biogenesis (97,98). Growing evidences show lncRNAs have gained significant research interest by their newly defined roles via tethering with DNA, RNA or protein in the development of several blood lineages (54). Few studies have demonstrated the existence of lncRNAs in aberrant gene expression associated with different blood cells and characterized them. In contrast to miRNAs, the expression profiling and role of lncRNAs has not been fully investigated in MK. Limited information is known about the participation of lncRNAs in megakaryocytopoiesis. LncRNAs involvement in regulatory mechanism of MK development is still unexposed. Therefore, lncRNAs expression profile may help to identify key lncRNAs which potentially involve in megakaryocytopoiesis. Also, it will pave the way to better understand the regulatory role lncRNAs in MK development, which is currently not fully understood.

In order to know the list of significantly expressed lncRNAs in MK, experiment was designed to profile lncRNAs expression pattern between hiPSC-HSCs vs. matured MKs derived from hiPSC-HSCs. We hypothesized that significant lncRNAs that were

differentially expressed in MKs could have possible function and regulatory mechanism that is implicated in MK development.

5.1 Differential expression analysis of lncRNAs in iPSCs-HSCs vs MKs

Following the confirmation of the MK differentiation from hiPSC-HSCs, we have performed the expression profile of lncRNAs in control (hiPSC-HSCs) and experimental (MKs) groups using Human LncProfilers™ qPCR Array (SBI). The differential expression of lncRNAs between hiPSC-HSCs and MKs was analyzed via ΔΔCt analysis software (www.systembio.com/LncRNA). The biological significance threshold was set to FC of ±2-fold, and the statistical significance was set to p-value of <0.05. Heat map showing the Ct values of 90 lncRNAs which were profiled in hiPSCs-HSCs and MKs groups (**Figure 11A**). Of the 90 lncRNAs analyzed in MKs, only 26 (28.88%) lncRNAs showed statistically significant difference with respect to hiPSC-HSCs (n=3; p<0.05; FC ±2.0; **Figure 11B**). Of these 26 differentially regulated lncRNAs, 24 (26.66%) genes were up-regulated, whereas only 2 (2.22%) genes were down-regulated (**Figure 11B**). HOTAIRM1 was identified as the highest up-regulated lncRNA in MKs compare to hiPSC-HSCs.

Further we validated Array-Based LncRNA Expression Profile using lncRNA specific primers through qRT-PCR. To confirm these significant lncRNAs signature performance in MKs, we re-validated the expression of randomly selected top 3 lncRNAs (HOTAIRM1, LUST and TncRNA) out of 24 upregulated lncRNAs by qRT-PCR. We have quantified expression of the highest upregulated lncRNAs HOTAIRM1, LUST and TncRNA, and similar distinctions between hiPSC-HSCs and MKs were observed in qRT-PCR result (n=3; p<0.02; **Figure 11C**). In both experiments more than 2-fold difference in the expression levels of lncRNAs was observed. These results support the use of qRT-PCR array as

screening tool for lncRNAs expression profile and emphasize the need of validation of the array results.

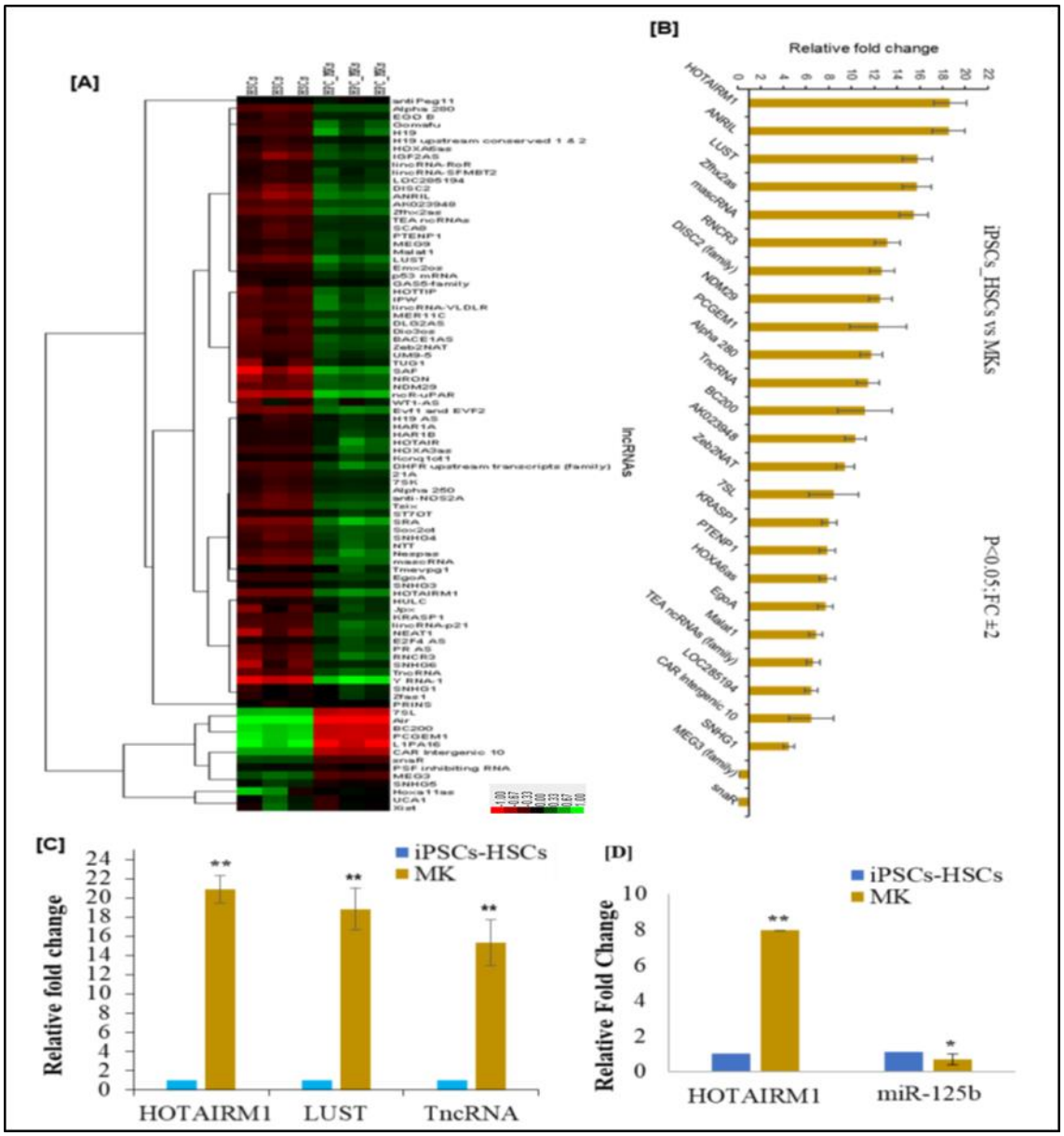


Figure 11: LncRNA profiling and analysis of differentially expressed lncRNAs in MKs. **A.** Visualization of ΔΔCt values of lncRNAs in iPSCs-HSCs and MKs using heat map (n=3). **B.** qRT-PCR showing top 26 (24 up- and 2 down-regulated) most differentially expressed lncRNAs in iPSCs-HSCs and MKs (n=3; *p<0.05). **C.** Validation of lncRNA array data using qRT-PCR. The qRT-PCR reactions for HOTAIRM1, LUST, and TncRNA were repeated three times in iPSCs-HSCs and MKs (n=3; **p<0.02). **D.** qRT-PCR results showing fold induction in the expression of HOTAIRM1 and miR-125b in iPSCs-HSCs and MKs (n=3; **p<0.002, *p<0.05). Bars represent mean ± SD of three independent experiments.

5.2 HOTAIRM1 as a natural sponge/decoy, likely involved in the regulation of p53 mRNA and protein expression by competing with miR-125b during megakaryocytic differentiation

Most recent studies have shown the functional significance of different lncRNAs in the regulation of major biological processes, such as cell pluripotency, chromatin remodeling and cancer progression and development (99–103). Although a number of lncRNAs have been identified in different lncRNA profiling studies, only few are experimentally validated in human hematopoiesis (104). Recent studies have shown that HOTAIRM1 is involved in myeloid differentiation; however, the underlying mechanism of HOTAIRM1 mediated regulation in megakaryocytic maturation has not been determined.

In this study, we used a well-defined PMA induced megakaryocytic differentiating K562 cell line model to explore the lncRNA-HOTAIRM1 regulated mechanism in MKs. PMA-induced megakaryocytic differentiation of K562 cells is a classic model to study the differentiation of MKs. This process is accompanied by the cell growth arrest, increased expression of MK specific markers, changes in cell morphology and other changes (78,105). Interestingly, validation results confirmed HOTAIRM1 expression was significantly increased (~20-fold; $p<0.002$; **Figure 11C**) in hiPSC-HSCs derived MKs as compared to control iPSC-HSCs. In addition, we observed the expression pattern of HOTAIRM1 in PMA induced megakaryocytic K562 cells is closely related to its expression in hiPSC-HSCs derived MKs (~4.7-fold; $n=3$; $p<0.002$; **Figure 12A**). Interestingly HOTAIRM1 was significantly upregulated in both model systems. Previously, it has been demonstrated that under ordinary circumstances the expression of HOTAIRM1 is restricted to the myeloid lineage and it is involved in the differentiation of myeloid cells (106). Moreover, recent studies have shown the regulatory interactions between p53 and HOTAIRM1 (107,108). The

lncRNA HOTAIRM1 is regulated by p53-dependent alterations in chromatin state during differentiation of human embryonic stem cells (hESCs).

Remarkably, it has been noticed that lncRNA HOTAIRM1 is induced by p53 only when hESCs were differentiated toward definitive ectoderm and mesoderm, but not endoderm lineages (109). In present study, we found that during PMA induced megakaryocytic differentiation in K562 cells, p53 protein levels were significantly increased (~7-fold; n=3; **p<0.02, *p<0.01 **Figure 12D and 12E**). It has been demonstrated that the lncRNA-HOTAIRM1 acts as a sponge/decoy for miR-125b and other miRNAs, keeping them away from p53 mRNAs (110). We have also found significant interactions between lncRNA HOTAIRM1 and miR-125b (**Figure 12B**) using IntaRNA tool (http://rna.informatik.uni-freiburg.de/IntaRNA). miR-125b is a well-known oncomiR which negatively regulates p53 (111,112). We have also found the potential target gene of miR-125b is p53 (**Figure 12C**) by using TargetScan Human (http://www.targetscan.org/vert_72/). miR-125b was also reported with increased expression in acute megakaryocytic leukemia associated with Down's syndrome (DS) (112). In present study, miR-125b expression level was significantly low in both iPSC-HSCs derived MKs and PMA induced MKs in comparison to control groups (iPSC-HSCs and untreated K562 cells) (n=3; p<0.002; **Figure 11D and 12A**). Considering these observations, a possible explanation may be that the potential role of p53 dependent expression of differentiation-specific lncRNA HOTAIRM1 can be to protect p53 mRNA from miR-125b mediated translational arrest during MK maturation (**Figure 13E**).

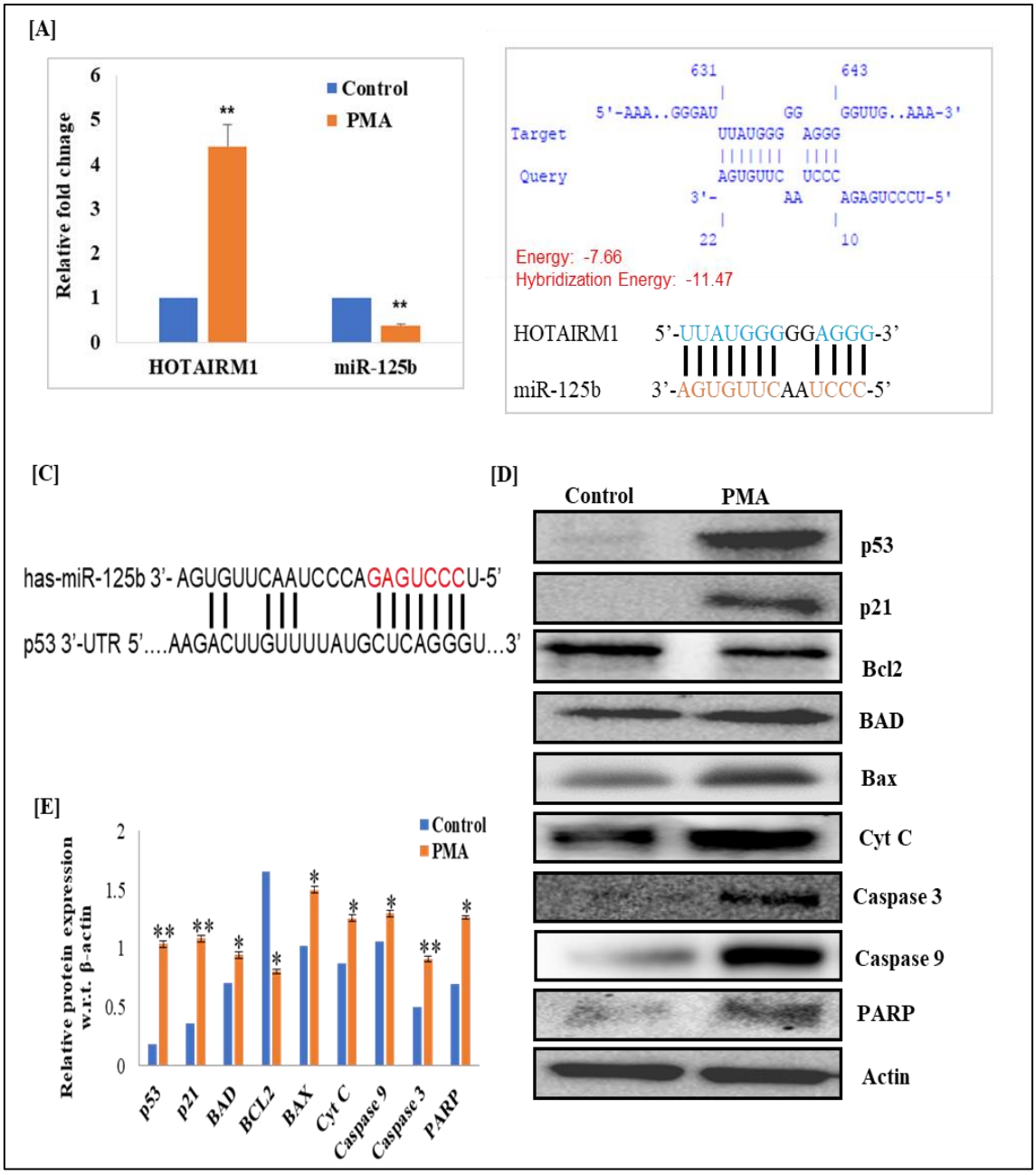


Figure 12: p53, HOTAIRM1 and miR-125b interaction study in PMA induced megakaryocytic differentiating cell line model. A. qRT-PCR results showing the differential expression of HOTAIRM1 and miR-125b in PMA-induced MKs as compared to uninduced (control) K562 cells (n=3; **p<0.002). **B.** HOTAIRM1 and miR-125b sequence interaction. Minimum free energy (mfe) duplex of miR-125b and HOTAIRM1 was analyzed by using IntaRNA tool. **C.** RNAhybrid prediction analysis of miR-125b with its target gene p53 by using TargetScan tool. **D.** The protein levels of p53, p21 and including components of intrinsic apoptosis pathway were increased in PMA induced MKs as compared control cells (n=3; **p<0.02). **E.** Quantification of protein expression w.r.t. β-actin (n=3; **p<0.02, *p<0.01). Bars represent mean ± SD of three independent experiments.

5.3 HOTAIRM1 up-regulates the p53 expression and involved in MK maturation via regulating downstream cell cycle target genes p21, cyclin D1 along with apoptosis regulators and ROS

To understand the functional role of p53 in MKs, we have studied the expression levels of three known transcriptional targets of p53 (p21, BAD, and Bcl-2). In present study, p21 and BAD were upregulated and Bcl-2 was downregulated in both gene and protein expression analysis by qRT-PCR and western blotting in PMA induced MKs in comparison to control (n=3; **p<0.02, *p<0.01; **Figure 13D and 13E**). Further we have also observed the increased production of ROS by flow cytometry and MK specific late marker CD61 expression by qRT-PCR at different time points during megakaryocytic maturation (n=3; **p<0.02; **Figure 13A and 13B**). In the present study, p53, p21, BAX and BAD were upregulated in PMA induced megakaryocytic cells (n=3; **p<0.02, *p<0.01; **Figure 12D and 12E**). p21 has been extensively studied in MKs, and it is generally reported that the expression of p53 and p21 is increased during MK maturation (66,113). BAD and BAX belongs to the Bcl-2 family members as pro-apoptotic proteins, which inhibit the anti-apoptotic function of Bcl-2 (114). Whereas, Bcl-2 expression was down-regulated, and it is known to be negatively affected by p53 (n=3; **p<0.02, *p<0.01; **Figure 12D and 12E**) (66). Members of the Bcl-2 family have been shown by different research groups to be directly involved in megakaryocyte maturation and apoptosis (25,114). In same line, our results are also demonstrating the activation of caspase cascade and PARP cleavage known as markers for the components of classical intrinsic apoptosis pathway during terminal maturation of MKs (n=3; **p<0.02, *p<0.01; **Figure 12D and 12E**) (25,115–117).

In the context of megakaryocytopoiesis, previous studies have also reported the elevated ROS levels during the megakaryocytic differentiation and maturation (78,118). In

recent studies, ROS production was also associated with the p53 expression which depended on miR-125b status of expression (119). Further, to understand whether ROS generation is important for megakaryocyte maturation, we have carried out PMA-induced cell differentiation experiments using ROS quencher N-Acetyl-L-cysteine (NAC) which associates directly with ROS to functions as a scavenger of oxygen free radicals there by reducing the intra cellular ROS levels. PMA induced cells showed that ROS down regulates miR-125b expression in matured MKs and show higher megakaryocytic marker CD61 expression in comparison to control (n=3; $p<0.05$; **Figure 13C and 13D**). Cells treated with NAC showed no significant change in the expression of CD61 marker in comparison to control (n=3; $p<0.05$; **Figure 13C**). However, upon addition of PMA and NAC together cells expressed higher level of CD61 as compared to NAC treated cells (n=3; $p<0.05$; **Figure 13C**), however, the expression was lower as compared to PMA induced cells. Taken together, this data supports the notion that ROS production is required for megakaryocytic maturation which associated with the p53 expression depended on miR-125b status (n=3; $p<0.05$; **Figure 13C and 13D**).

Our results were consistent with previous reports, that identified the functional role of p53 in the regulation of endomitosis and polyploidization by decelerating the cell cycling and promote apoptosis to initiate terminal maturation of MKs (113). Thus, we report that the interactions between HOTAIRM1, p53 and miR-125b could be involved in the regulation of cell cycling, ROS production, and activation of intrinsic apoptosis during megakaryocytopoiesis (**Figure 13E**).

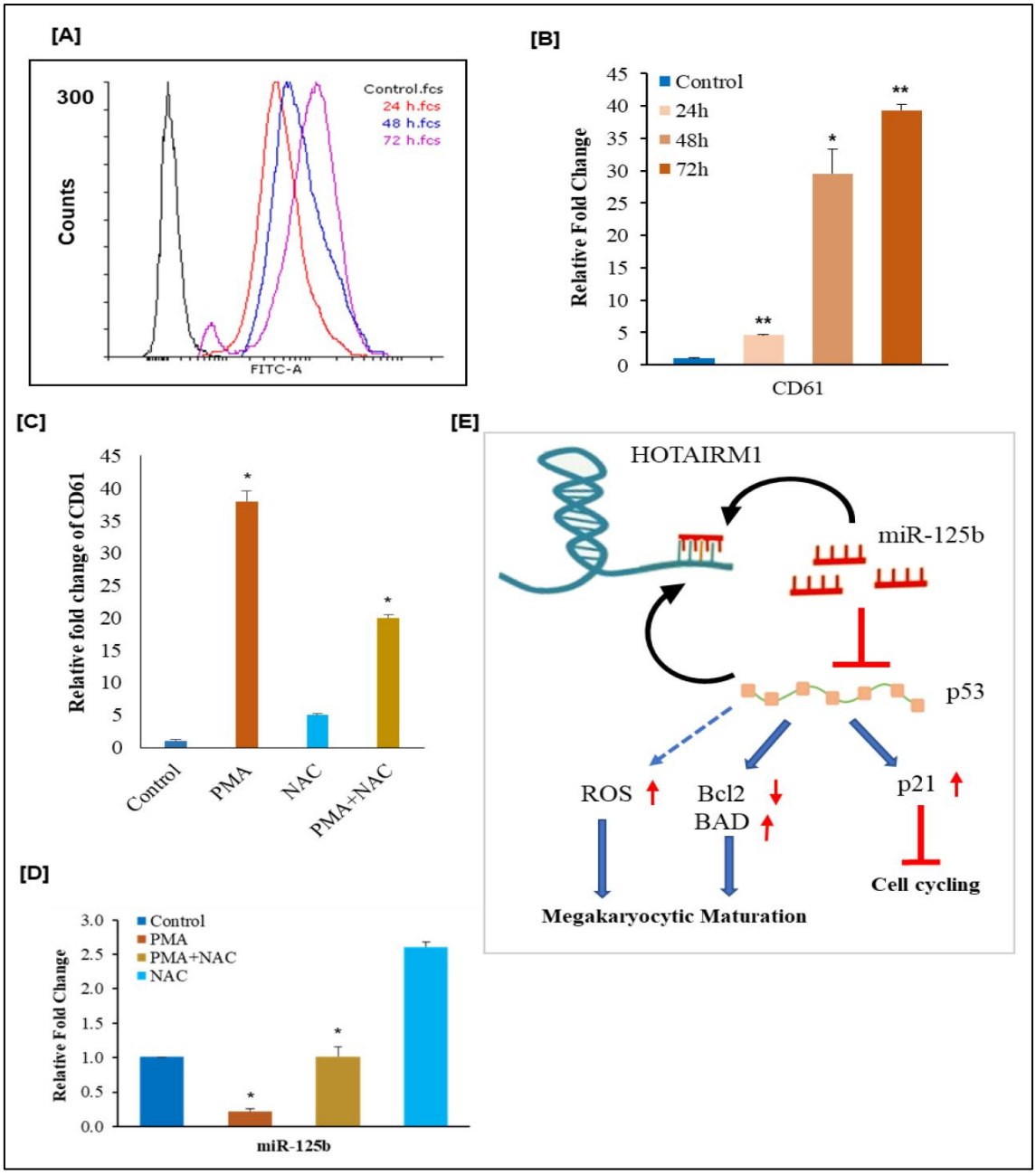


Figure 13: PMA-induced MK differentiation involves ROS production during maturation. A. For measurement of intracellular ROS production DCFH-DA assay was performed, cells were analyzed by Flow cytometry (n=3). **B.** qRT-PCR results showing the increased expression of MK marker CD61 during PMA-induced megakaryocytic maturation of K562 (n=3; **p<0.02). **C.** In qRT-PCR analysis MK marker CD61 expression was increased in PMA treated cells as compared to control, whereas, decreased in NAC treated cells (n=3; **p<0.05). **D.** miR-125b expression was increased in ROS scavenger NAC treated cells as compared to untreated control, whereas, decreased in PMA treated cells (n=3; **p<0.05). **E.** The graphical representation of the crosstalk between lncRNA (HOTAIRM1), miRNA (miR-125b) and p53 during MK development. Bars represent mean ± SD of three independent experiments.

5.4 Suppression of lncRNA HOTAIRM1 expression by siRNA induce impairment in the differentiation and maturation of PMA-induced K562 cells

To determine the function of lncRNA HOTAIRM1 in MKs differentiation and maturation, we used siRNA-HOTAIRM1 to down regulate the lncRNA HOTAIRM1 expression and detected its effects on cell cycle regulators (p53, p21 and cyclin D1) and MK specific cell surface markers including miR-125b expression. After 48 h of transfection, cells were collected and observed under Bright field microscope, both the control group and the group transfected with siRNA-HOTAIRM1, there is no large sized cells like MKs were observed (**Figure 14A**). Next, we investigated gene expression for cyclin D1, p53 and p21 by qRT-PCR analysis that revealed inhibition of lncRNA HOTAIRM1 expression in si-RNA transfected K562 cells could not generate cyclin D1, p53 and p21 mRNAs respectively, as a result their protein levels were significantly reduced ((n=3; **p<0.02, *p<0.01; **Figure 14B, 14C and 14D**). Also, miR-125b level was significantly upregulated in the si-HOTAIRM1 treated PMA-induced megakaryocytic K562 cells as compared to control (n=3; **p<0.02, *p<0.01; **Figure 14B**). To assess the role of lncRNA HOTAIRM1 on MK maturation, we investigated the effects of downregulated lncRNA HOTAIRM1 expression on MK specific cell surface markers (CD41 and CD61). qRT-PCR analysis showed that CD41 and CD61 expression were decreased more significantly in the si-HOTAIRM1 treatment group (n=3; **p<0.02, *p<0.01; **Figure 14B**). These results may suggest that downregulation of lncRNA HOTAIRM1 expression might affect MK differentiation and maturation by influencing the expression of miR-125b, which might impact on p53 and p21 expression to stimulate MK maturation.

These data suggest that transfection of K562 cells with HOTAIRM1-siRNA results in increased expression of miR-125, because of that the expression of its putative targets of p53

were down-regulated. Alternatively, in both the control group and the group transfected with si-HOTAIRM1, no visual changes were observed. The expression of MK-specific markers (CD41 and CD61) were also altered after the si-HOTAIRM1 transfection in K562 cells, which suggest MK differentiation did not take place. These data suggest lncRNA HOTAIRM1 might promote p53 gene expression by decoying the expression of miR-125b, as a result influencing differentiation and maturation of MKs. Thus, our findings revealed the functional role of HOTAIRM1 lncRNA in p53 mediated regulation of cyclin D1 as well as ROS production, and to trigger intrinsic apoptosis pathway during megakaryocytopoiesis by decoy function on miR-125b (**Figure 13E**).

5.5 Conclusion

In this study, we present a differential lncRNAs expression profile from MKs derived from hiPSC-HSCs, which will provide the foundation for the future studies of the biological functions of lncRNAs in the development of MKs. We found that most of the differentially expressed lncRNAs were upregulated in MKs compared to hiPSC-HSCs. Further, to confirm these significant lncRNAs signature performance, we validated the expression of 3 lncRNAs (HOTAIRM1, LUST and TncRNA) out of 24 upregulated lncRNAs by qRT-PCR and similar distinctions between hiPSC-HSCs and MKs were observed. LncRNAs play crucial role in gene expression regulation during development and differentiation of cells. The upregulated lncRNAs identified in the present study may have functional role in the regulation of gene expression during differentiation/maturation process of MKs.

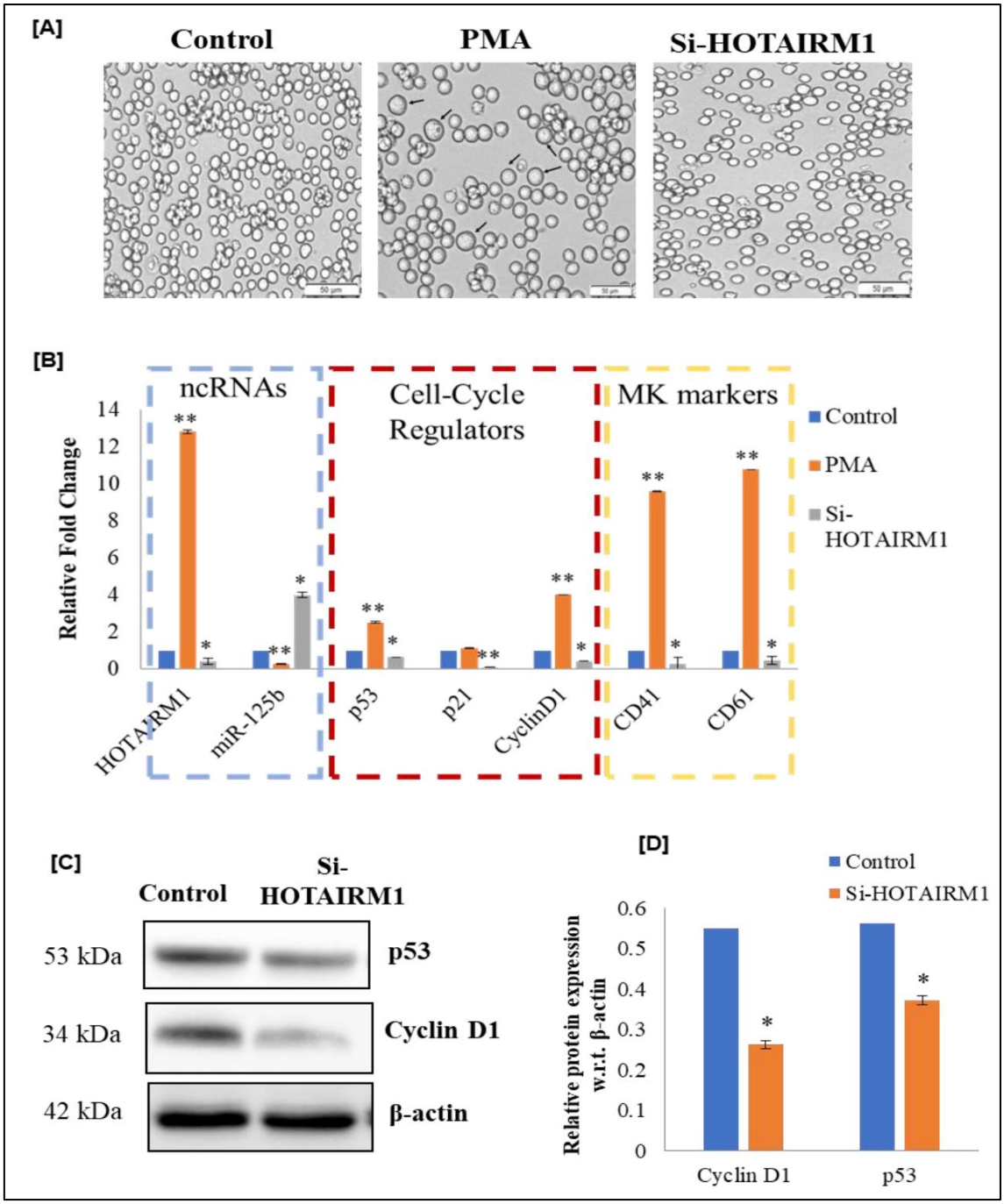


Figure 14: Suppression of lncRNA HOTAIRM1 expression induces impairment the differentiation and maturation of PMA-induced K562 cells. A. Morphology of Si-HOTAIRM1 transfected cells showing similar morphology to control as compared to PMA-induced megakaryocytic cells. (20X; 50 µm). **B.** qRT-PCR results shows the Si-HOTAIRM1 transfection elevated the level of miR-125b which further reduced the expression of its respective target genes (n=3; **p<0.02, *p<0.01). **C.** Si-HOTAIRM1 transfection significantly inhibits the protein expression of p53 and Cyclin D1 was analyzed by western blot. **D.** Bar shows the actual mean band intensity of the blots (n=3; *p<0.01).

In the current study, HOTAIRM1 was identified and confirmed as the highest upregulated lncRNA in hiPSC-HSCs derived MKs. In addition to interacting with proteins as a scaffold, lncRNA-HOTAIRM1 could exert its functional effects on several gene expressions by acting as a miRNA sponge/decoy for number of miRNAs. We also found that the treatment of K562 cells with PMA (a model to study MK development) also induces HOTAIRM1 expression similar to hiPSC-HSCs derived MKs. Further, we noticed lncRNA HOTAIRM1 dependent p53 expression. miR-125b leads to significant upregulation of p53 in PMA induced MKs. Importantly, the present study demonstrates that during MK maturation lncRNA HOTAIRM1 may be involved in the transcriptional regulation of p53, which in turn could acts as a decoy for miR-125b, keeping them away from p53 mRNA. p53 also regulates the expression of apoptotic genes and promote the ROS production which is essential during megakaryopoiesis. Thus, p53 along with HOTAIRM1 likely via regulating p21, components of Bak/Bax pathway expression and ROS production augments the maturation and apoptosis in MKs development. However, further experiments are necessary to validate the associations between HOTAIRM1, p53 and miR-125b, including the functional effects of these associations in MKs development.

Chapter VI: RESULTS and DISCUSSION

OBJECTIVE 3:
Study the role of lncRNA in hyper proliferation (megakaryoblastic leukemia) during treatment by using Phorbol 12-myristate 13-acetate (PMA)

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Growing body of evidences indicate lncRNAs emerge as vital component to regulate the normal as well as malignant hematopoiesis. According to National Institute for Health and Care Excellence, thrombocytopenia (low platelets) is commonly encountered in various undiagnosed hematopoietic malignancy like acute myeloid leukemia (AML). Acute megakaryocytic leukemia (AMKL) is one of the rarest life-threatening sub-types of AML, which is characterized by abnormal megakaryoblasts that frequently demonstrates extensive myelofibrosis with irregular proliferation and low percentage (~ 20%) of megakaryoblasts in the bone marrow. Compared to adults, it is very common in children specially children with Down Syndrome (120). It is very challenging to diagnose therefore it creates difficulty in prognosis and clinical management for treatment.

One approach to treatment of different leukemic cases is chemically-induced differentiation of blasts (leukemia cells), this approach is also named as differentiation therapy. In recent studies, different transcriptional factors, cell cycle regulators, and ncRNAs have been recognized with aberrant expression in leukemia blasts, the aberrant expression of these components is most likely involved in leukemogenesis via blocking blasts differentiation. Previous analyses have shown that the human megakaryoblastic cells can be differentiated into cells with MK-like characteristics by PMA (121). Considering all mentioned information, we hypothesized treatment of AMKL via inducing differentiation with PMA in megakaryoblastic leukemic cells, that can overcome blocks in differentiation and lead to terminal maturation of MKs, which finally leads the production of platelets. Also to understand the regulatory mechanism of lncRNAs responsible for blasts differentiation, we

initiated with lncRNA profiling in Dami cells (Megakaryoblastic cells) treated with and without PMA.

6.1 PMA induces megakaryocytic differentiation in Dami cells

Recent studies have reported that PMA resulted in a dose-related inhibition of growth and a stimulation of differentiation in different myeloid leukemia cells (78,122). In present study, in order to access the PMA-induced MK differentiation, we have treated Dami cells with PMA (100 nM), PMA treated cells were grown for 72 h and examined for MK related morphological features and cell surface antigens expression. Post 72 h of PMA stimulation, cell proliferation stopped, a marked increase in cell size, adherence and granularity was observed for PMA-treated Dami cells, which are typical features of megakaryocytic differentiation (n=3; **Figure 15A**). Megakaryocytic differentiation was further confirmed by analyzing the MK specific surface markers.

In FACS study, we analyzed megakaryocytic surface antigen CD41, post 72 h of PMA stimulation more than 85% of cells were CD41 positive (n=3; **Figure 15B**). MK surface antigen CD41 (DAPI/CD41-FITC) expression was also visualized under fluorescence microscope; we observed more CD41-FITC positive cells with increased fluorescence and cell size upon PMA stimulation as compared to control (n=3; **Figure 15D**). In qRT-PCR study, we further confirmed that MK markers CD41 and CD61 mRNA levels were significantly increased in PMA stimulated Dami cells as compared to unstimulated control Dami cells (n=3; $p<0.02$; **Figure 15C**). Moreover, we have also analyzed the megakaryocytic transcription factors RUNX1 and GATA1 mRNA expressions; interestingly, the expression levels of both the transcription factors encoding mRNAs were significantly high in PMA-induced megakaryocytic Dami cells as compared to uninduced Dami cells (n=3; $p<0.02$; **Figure 15E**). The results of the present study confirmed that

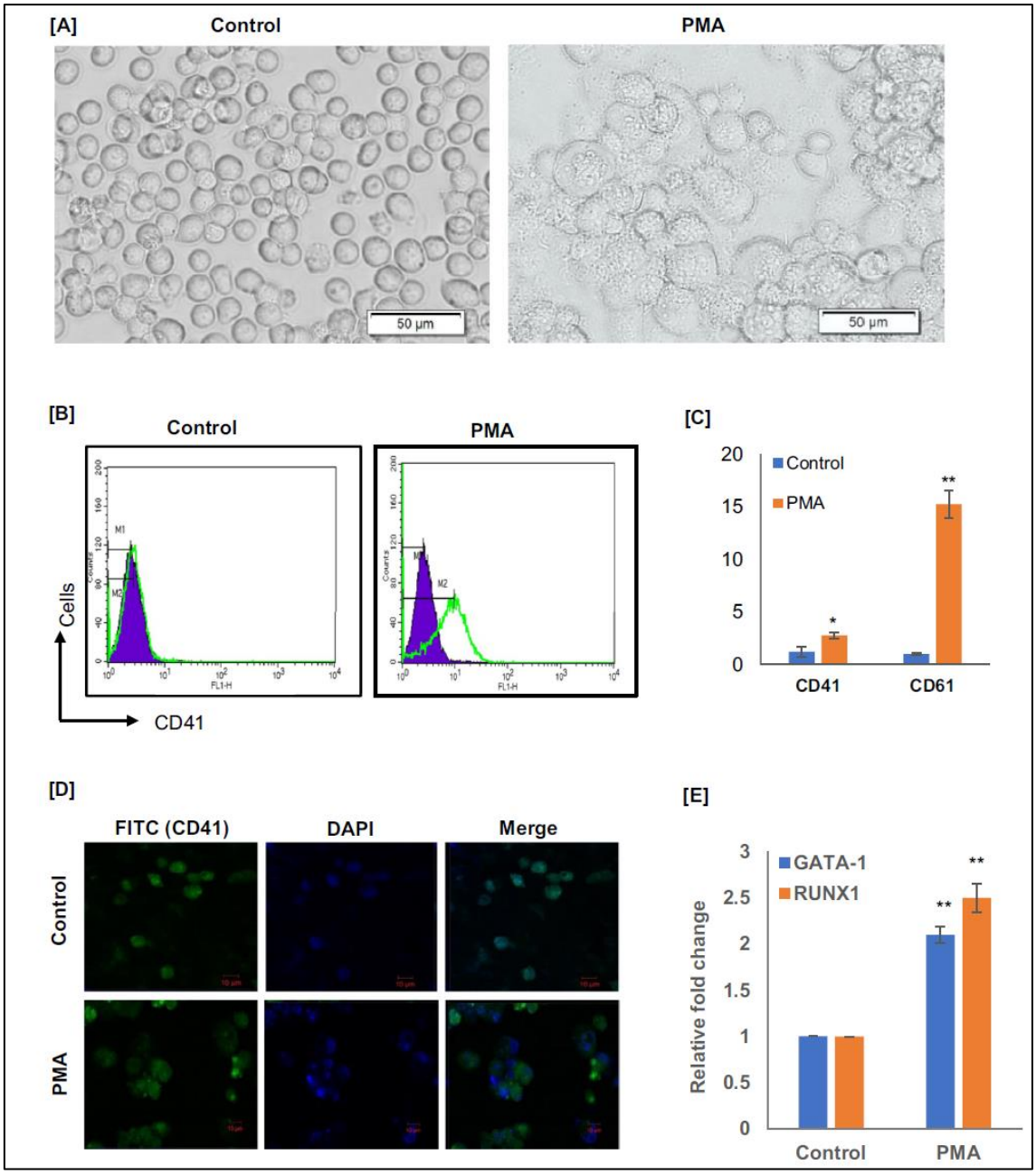

Figure 15: PMA-induced differentiation of megakaryoblastic Dami cells into megakaryocytes (MKs). A. Photomicrograph of cells showing morphological differences (cell size and adherence) in Dami cells upon 72 h of PMA treatment as compared to unstimulated Dami cells ((20X objective; scale = 50µm)). **B.** Representative flow cytometry histograms of the cultured suspension cells on day 3, maximum Dami cells expressed MK markers CD41 upon PMA stimulation as compared to unstimulated Dami cells. **C.** qRT-PCR results showing fold induction in the expression of MK markers CD41 and CD61 in Dami cells upon PMA stimulation as compared to unstimulated control cells (n=3; *p<0.05; **p<0.02). D. Photomicrograph of cells stained with CD41-FITC and DAPI showing the expression of MK surface antigen CD41. **E.** Differential expression analysis of megakaryocytic transcription factors RUNX1 and GATA1 mRNA. Bars represent mean ± SD of three independent experiments.

PMA stimulation of Dami cells significantly established the megakaryocytic features – increased cell size, adherence, the expression of surface MK specific surface antigens and megakaryocytic transcription factors. Thus, the present study results have confirmed PMA as a potential drug that can inhibit the proliferation of megakaryoblastic leukemic cells and induce MK differentiation.

6.2 Differential expression of lncRNAs is associated with PMA-induced megakaryocytic differentiation

Recent technical advancement of high-throughput sequencing and the rapid development of biological techniques have shown that lncRNAs can regulate gene expression at multiple levels, including transcriptional and post-transcriptional levels. Notably, several lncRNAs have been identified with clinical potential as tumor-suppressors and key targets in the diagnosis, prognosis, and treatment of leukemia (123). Currently, there are only limited studies which have identified and characterized the lncRNA expression profiles in myeloid leukemia cells (124–126), also the expression of lncRNAs has not been explored in megakaryoblastic leukemia cells. Understanding the dynamics of lncRNAs expression during MK differentiation will allow us to identify novel therapeutic targets for megakaryoblastic leukemia.

In an effort to explore the expression profile of differentially expressed lncRNAs, a qRT-PCR-based lncRNA Profiler (System Biosciences) was used, which comprise of 90 lncRNAs. The differential expression analysis was performed in unstimulated Dami cells (control) versus PMA stimulated differentiated megakaryocytic Dami cells (MKs). Heat map shows the differentially expressed lncRNAs profile of control versus differentiated megakaryocytic cells (n=3; **Figure 16A**). Further, the data was presented as bar graph which shows statistically significant ($p<0.05$) differentially expressed (FC of ± 2-fold) lncRNAs

expression (n=3; **Figure 16B**). For the first time, we have investigated the differential expression profile of 90 lncRNAs in megakaryoblastic cells versus PMA-induced MKs. A total of 30 lncRNAs were identified with statistically significant differential expression after PMA treatment (n=3; $p<0.05$; **Figure 16B**); JPX being the highest up-regulated lncRNA in PMA-induced MKs as compared to control megakaryoblastic Dami cells.

Further, we did validation of array-based lncRNA expression profile by qRT-PCR using lncRNAs (JPX, MEG9, TncRNA, UM9-5, and UCA1)-specific primers, all tested lncRNAs were showing significant up-regulation in PMA-induced MKs as compared to control (n=3; *$p<0.05$; **Figure 16C**). Moreover, the expression levels of these lncRNAs were similar to that observed in array profile. Furthermore, the expression levels of the highest upregulated JPX lncRNA were investigated in cytokine-induced iPSCs-MKs (n=3; *$p<0.02$; **Figure 16D**) and the results were consistent with our array findings. The increased expression of respective lncRNAs after PMA induction of megakaryoblastic Dami cells and cytokine induction of HPCs emphasize a comprehensive understanding of their functional role in MK development. Moreover, in new megakaryoblastic leukemia treatment strategies these lncRNAs could be the potential therapeutic options, and prognostic markers for monitoring the pathogenesis of megakaryoblastic leukemia.

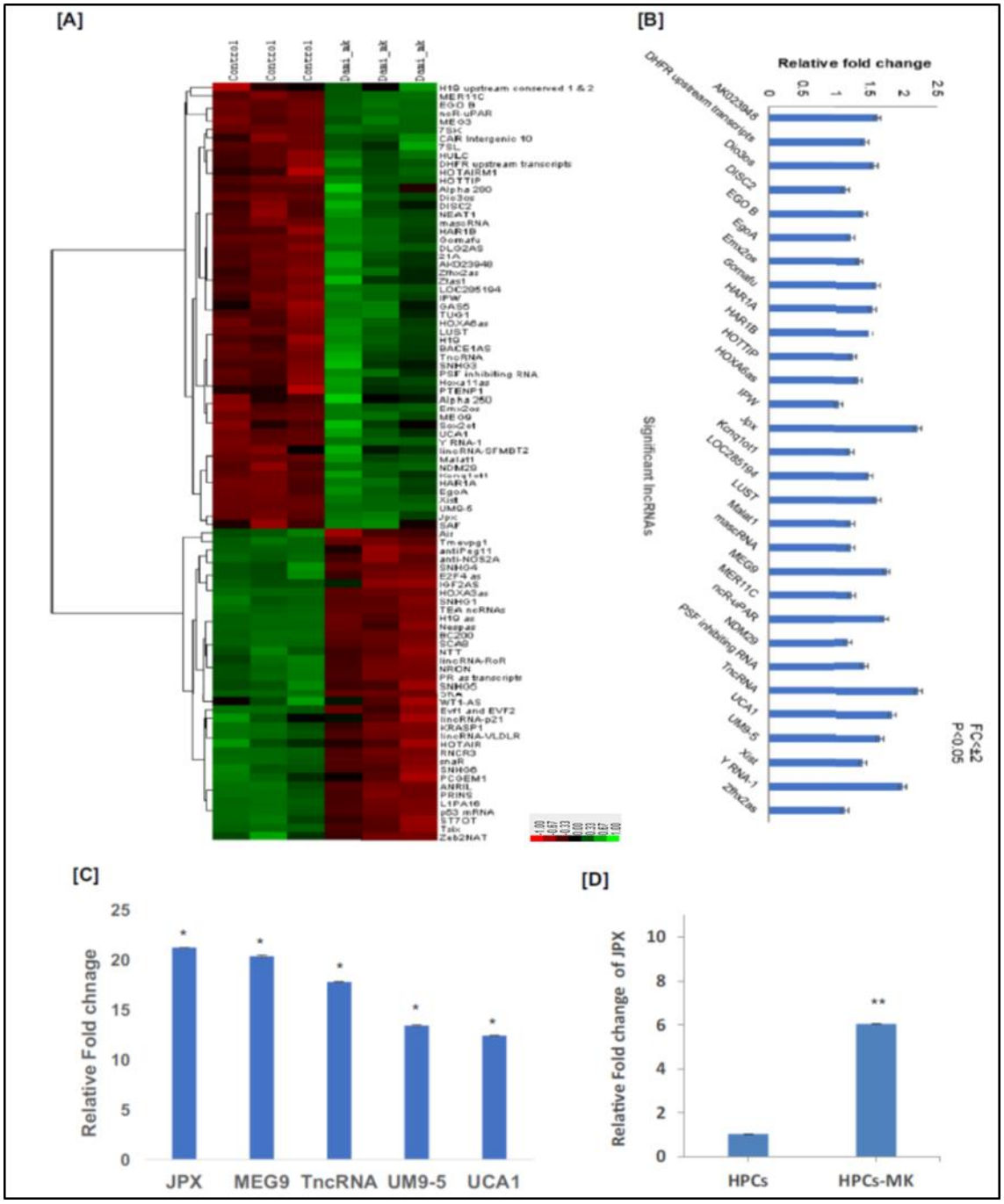

Figure 16: LncRNA profiling and analysis of differentially expressed lncRNAs in PMA-induced MKs. A. Visualization of ΔCt values of lncRNA genes in control (Dami) and PMA-induced MKs (Dami-MKs) using heat map (n=3). **B.** Array Data showing top 30 most differentially expressed genes in Dami and Dami-MKs (n=3; *p<0.05). **C.** Validation of lncRNA array data using qRT-PCR. qRT-PCR reactions with differential expressions of JPX, MEG9, TncRNA, UM9-5, and UCA1 in Dami and Dami-MKs (n=3; *p<0.05). **D.** The expression levels of the highest upregulated JPX lncRNA were validated in HPCs derived MKs by using qRT-PCR (n=3; *p<0.02). Bars represent mean ± SD of three independent experiments.

6.3 LncRNA JPX/miRNAs/TGF-βR axis correlates with the growth and differentiation of Dami cells

Previous studies from our lab confirmed PMA-induced megakaryocytic cells could be a potential tool in understanding the molecular mechanism associated with MK differentiation and growth. Interestingly, we have also identified that the expression of lncRNAs is associated with megakaryocytic differentiation, JPX was the highest upregulated lncRNA in PMA-induced MKs as compared to uninduced megakaryoblastic Dami cells. In the previous study, JPX lncRNA was identified as differentially expressed between leukemia patients in favorable and intermediate/normal risk categories, the expression was statistically significant upregulation in the cytogenetically favorable risk category as compared to intermediate/normal risk category (96).

The functional role of non-coding RNAs (specifically miRNAs) has been widely studied in last decade. The lncRNAs are a class of non-coding RNAs, that are involved in various biological processes such as chromatin interactions, transcription and translation regulation, and the regulation of protein interactions (54,96). In different studies, lncRNAs have been identified in the regulation of gene expression by acting as a guide by competing RNA for miRNAs. These lncRNAs act as a molecular sponge, they allow the protein expression by competing with miRNAs for mRNA binding. Further, in order to understand the functional role of JPX in molecular regulation of miRNAs during MK maturation, we have performed lncRNA-miRNA interaction study. In this interaction study, we have used IntaRNA online tool (http:// rna. Infor matik.unifreiburg.de/IntaRNA). The interactions were performed between JPX lncRNA and three commonly known oncogenic miRNAs (miR-9-5p, miR-17-5p, miR-106-5p) those were previously reported to be involved in leukemia development (127–129).

Interaction study identified significant intersections between JPX sequence and all three selected mature miRNAs sequences (**Figure 17A and 17B**). During MK differentiation or maturation, the JPX lncRNA may function as a miRNA-sponge, thereby regulating the expression of miRNA target genes after transcription. Interestingly, in target scan analysis (http:// www.targetscan.org/vert_72/) TGF-β receptor mRNA was identified as the confirmed common target of miR-9-5p, miR-17-5p, and miR-106-5p (**Figure 17C**). Further, we analyzed the expression of TGF-β and TGF-β receptor (TGF-βR), the expression levels of both the TGF-β and TGF-βR mRNAs were consistently high in PMA-induced MKs as compared to uninduced megakaryoblastic Dami cells ($n=3$; $p<0.02$; **Figure 17D**). TGF-β receptor signaling is involved in multiple biological functions, TGF-β is a potent inhibitor of human myeloid leukemia cells (130–132), TGF-β signaling has also been recently reported to be involved in MK maturation (78). Considering the JPX-miRNA-TGF-βR mRNA interaction data, a possible explanation may be that JPX involved in the regulation of TGF-β receptor expression by competing with TGF-βR targeting miRNAs during megakaryocytic differentiation (**Figure 17E**). Thus, JPX could be a molecular signature and a potential therapeutic target in megakaryoblastic leukemia (AMKL).

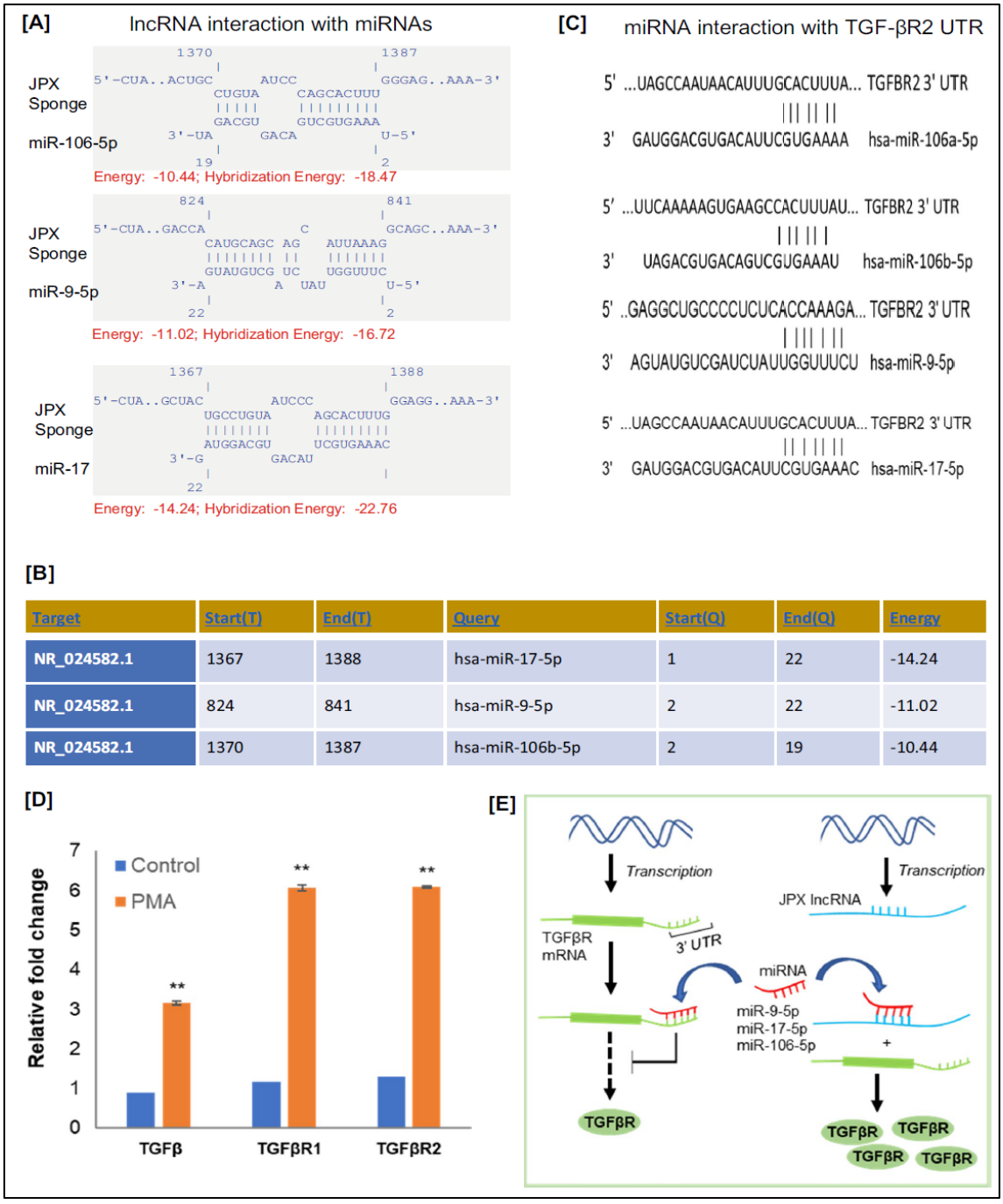

Figure 17: JPX-lncRNA-miRNAs and miRNAs-TGF-βR mRNA interaction study in PMA-induced megakaryocytic differentiating cell line model. A. JPX-lncRNA-miRNAs sequence interactions. **B.** Minimum free energy (mfe) duplex of JPX-lncRNA miRNAs analyzed by IntaRNA tool. **C.** Target scan analysis showing miR-9-5p, miR-17-5p, and miR-106-5p interaction with TGF-βR. **D.** qRT-PCR results showing the differential expression of TGF-β1, TGF-βR1, and TGF-βR2 in PMA-induced MKs as compared to uninduced (control) Dami cells (n=3, **p<0.02). **E.** The graphical representation showing the effect of JPX-lncRNA on the expression of TGF-βR by titrating miRNAs away from TGF-βR mRNAs. Bars represent mean ± SD of three independent experiments.

6.4 TGF-βR signaling activates SMAD-independent pathways during PMA-induced megakaryocytic differentiation

In several studies, TGF-β has been reported as one of the potential regulators of apoptosis, proliferation, differentiation, and matrix formation (133). Specifically, in recent studies, the activation of TGF-βR/SMAD pathway has been reported with the functional significance in MK maturation (78). The TGF-βR signaling involves various ligands, receptors, SMADs, and interacting partners. TGF-β is also known to activate various non-SMAD or SMAD-independent pathways such as ERK1/2, Jun-N terminal kinase (JNK), and p38 and PI3K kinase (134,135).

In the present study, we examined the components of SMAD-independent ERK1/2 and PI3K/AKT pathways. In protein expression analysis by western blot, the expression levels of pERK1/2 and PI3K/AKT were increased in PMA-induced MKs as compared to control (n=3; $p<0.02$; **Figure 18A**). In previous studies, PI3K/AKT and ERK1/2 pathways have been associated with MK maturation, these are the main pathways activated by TPO receptor signaling during MK development (136). Moreover, PI3K/AKT is well known to be involved in ploidy, size, and cytoplasmic maturation of MK (136–138). Further, we have analyzed the expression levels of different cell cycle regulators which are well known to be regulated by PI3K/AKT pathway. Interestingly, the protein expression levels of cyclin D1 and p21 were increased; however, the expression levels of p16 were reduced (n=3; $p<0.02$; **Figure 18A**). Similar expression profile of cyclins and Cdk inhibitor p16 was observed in our qRT-PCR study (n=3; $p<0.05$; **Figure 18A**). Furthermore, to understand the PMA-induced MK maturation, we have analyzed the ploidy by flow cytometry of PI-stained cells. We observed that post-PMA stimulation most of the cells reaching a ploidy class of 4 N and 8 N compared to unstimulated control cells (n=3; **Figure 18C**).

The megakaryocytic transcription factor RUNX1, which plays crucial role in MK ploidy development and maturation, was also observed with increased expression in PMA-induced cells (n=3; $p<0.05$; **Figure 18E**). The PI3K/AKT pathway is a strong activator of cyclin D1, an important regulator of cell cycle (G1/S phase) progression (139). In previous studies cyclin D1 has been well defined for its role in growth and polyploidization of MKs (140), whereas, p16 is a well-known inhibitor of cell cycle progression, the stability and function of p16 can be modulated by PI3K/ AKT signaling. On the other hand, studies suggest that the function of p21 is more complex in the regulation of cyclin/Cdks, during cell cycle progression p21 function appears to ensure appropriate Cdk activation and has both positive and negative effects on cell cycle progression. In different studies the activity of p21 is also associated with PI3K/AKT pathway activation. The present data suggest the possible role of TGF-βR-activated SMAD-independent ERK1/2 and PI3K/AKT pathways in the regulation of the expression and activity of different transcription factors and cell cycle regulators in PMA-induced megakaryocytic maturation and ploidy development.

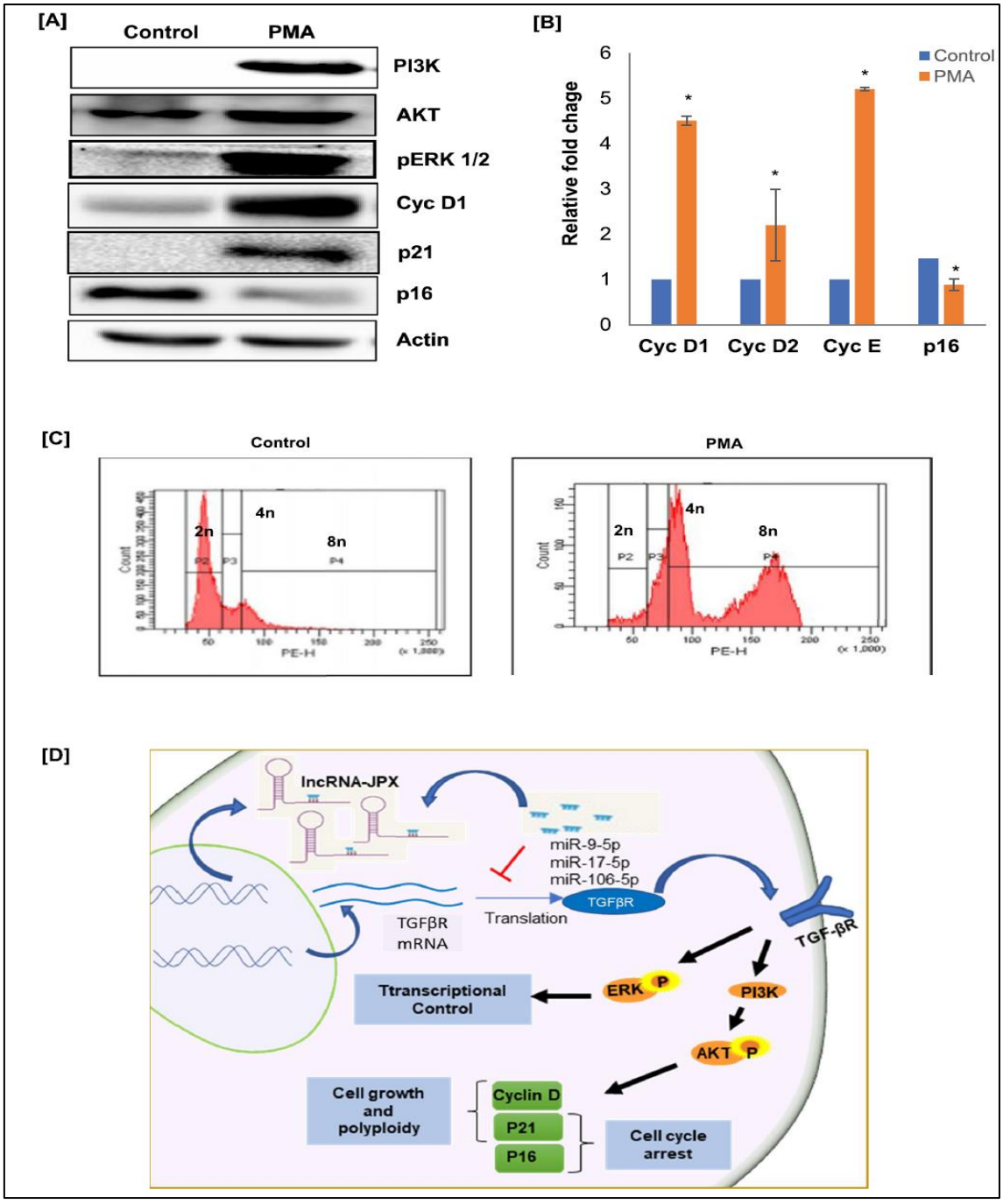

Figure 18: TGF-βR signaling activates SMAD-independent pathways during PMA-induced megakaryocytic differentiation. A. pERK1/2, PI3K/AKT, Cyc D1, p21, and p16, protein levels were quantified in control cells and PMA-induced MKs (n=3; $p<0.02$). **B.** The qRT-PCR analysis of different cell cycle regulators (Cyc D1, Cyc D2, Cyc E, and p16) expression in PMA-induced MKs as compared to uninduced Dami cells. **C.** DNA index (Cell Ploidy) levels analysis in PMA-induced and -uninduced Dami cells by Flow cytometry (n=3). **D.** The graphical representation of the activation of TGF-βR-induced SMAD-independent ERK1/2 and PI3K/AKT pathways during PMA induced differentiation of Dami cells into MKs, and a crosstalk between JPX lncRNA, miRNAs, and TGF-βR during MK development. Bars represent mean ± SD of three independent experiments.

6.5 Conclusion

In this study, we assessed the molecular mechanisms associated with the PMA-induced differentiation of megakaryoblasts into MKs. For this purpose, MKs generated upon PMA stimulation of Dami cells (megakaryoblastic leukemic cells) were used (**Figure 15**). Notably, several lncRNAs have been identified with clinical potential in the diagnosis, prognosis, and treatment of leukemia. Currently, there are only few studies which have identified and characterized the lncRNAs expression profiles in myeloid leukemia cells; however, the expression of lncRNAs has not been explored in megakaryoblastic leukemia condition (AMKL).

To elucidate the functional role of lncRNA in MK differentiation, we have performed the differential lncRNAs expression profiling in megakaryoblasts leukemic cells and PMA-induced MKs. A total of 30 lncRNAs were identified with statistically significant differential expression after PMA treatment, JPX was the highest upregulated lncRNA in PMA-induced MKs (**Figure 16**). JPX lncRNA is a molecular switch for X chromosome inactivation, JPX is also known for its activities as a suppressor or promoter of cancer cells proliferation by sponging different miRNA. Further to understand the functional role of JPX in molecular regulation during MK differentiation, we have performed interaction studies with three commonly known oncogenic miRNAs (miR-9-5p, miR-17-5p, miR-106-5p); previously, these miRNAs have been reported with their oncogenic properties in leukemia cells.

The intersection study identified strong interactions between JPX and putative miRNAs (**Figure 17**). Interestingly, TGF-βR, a confirmed common target of miR-9-5p, miR-17-5p, and miR-106-5p, was identified with increased expression during megakaryoblastic cells differentiation into MKs. A possible explanation of co-expression and interaction data may be that JPX involved in the regulation of TGF-βR expression by sponging TGF-βR

targeting miRNA in MKs. ERK1/2 and PI3K/AKT are the main pathways activated by TPO receptor during MK development; TGF-βR signaling is also well known to activate non-SMAD pathways such as ERK1/2 and PI3K/AKT. In the present study, we report the activation of SMAD-independent ERK1/2 and PI3K/AKT pathways and differential regulation of their targets (cyclin D1, p16, and p21) during PMA-induced megakaryoblastic cells differentiation into MKs (**Figure 18A**). Moreover, we have identified cells with high ploidy (**Figure 18C**). Also increased expression of megakaryocytic transcription factors RUNX1 and GATA1 (**Figure 18E**) upon PMA stimulation.

In previous studies, PI3K/AKT and ERK1/2 pathways have been associated with MK maturation. Moreover, PI3K/AKT is well known to be involved in ploidy, size, and cytoplasmic maturation of MK. In conclusion, JPX lncRNA is likely involved in the regulation of TGF-βR by sponging miRNAs. Thus, by unblocking the TGF-βR expression, JPX may augment the activity of ERK1/2 and PI3K/AKT pathways and that in turn enhance the polyploidization and terminal maturation of MKs. These data present that JPX-lncRNA could be a molecular signature and a potential therapeutic target in megakaryoblastic leukemia (AMKL). However, further experimental validations are required to confirm the interactions between TGF-βR, JPX and miRNAs and the functional effects of these interactions in MK development.

Chapter VII:
SUMMARY

Megakaryocytes (MKs) are rare polyploid cells found in the bone marrow which produce platelets. A single MK rupture and release approximately 4000-5000 platelets into the circulation. Platelets are small colorless enucleated cell fragments that are critical to vascular hemostasis and wound healing as well as in inflammation. To maintain normal human platelet count range from 150,000 to 450,000 platelets per microliter of blood is very important. Several blood disorders characterized by dysfunctional platelets result in prolonged bleeding time, defective clot formation and bleeding tendency.

Thrombocytopenia is one of the most common hematologic disorders, characterized by an abnormally low number of platelets and it affects both children and adults. Thrombocytopenia is sometimes a first sign of hematologic malignancies, infectious diseases, thrombotic micro angiopathies and autoimmune disorders, and is also a common side effect of many medications. There is no specific therapy for the vast majority and only severe cases need to be treated.

Currently, *in vitro* generation of MKs from human induced pluripotent stem cell (hiPSC)-derived platelets technology is a good treatment option, could provide an alternative source of platelets for treating thrombocytopenic patients in different disease conditions and allows a better understanding of MK and platelet biology.

Recent advancement in the rapidly evolving field of epigenetic have shown the role of non-coding RNAs (ncRNAs) in hematological epigenetics, mostly focusing on the mechanisms by which microRNAs (miRNAs) and long ncRNAs (lncRNAs) impact the epigenomes in the context of blood cell development. However, there are only few studies which have identified and characterized the lncRNA expression profiles in MKs, the underlying regulatory mechanisms governed by lncRNAs has not been explored for the normal development of MKs lineage.

We hypothesized that lncRNAs are differentially expressed in MKs, therefore might play a critical role in the normal development and regulation of megakaryocytopoiesis. Our studies aimed to establish a protocol for the generation of MKs in xeno-free and defined conditions, and to determine the lncRNA profile and molecular mechanism in hiPSC-derived MKs, to gain an understanding on the normal development of megakaryocyte. Furthermore, we focus our study to explore the potential role of respective lncRNA in hyper-proliferative clinical condition i.e. Acute Megakaryocytic leukemia (AMKL).

Based on these preliminary findings we framed our study into following objectives:

1. To generate and characterize the megakaryocytes derived from iPSCs using blood cells.
2. Elucidate the role of lncRNA in normal development of megakaryocyte lineage derived from iPSCs.
3. Study the role of lncRNA in hyper proliferation (Acute Megakaryocytic leukemia) during treatment by using Phorbol 12-myristate 13-acetate (PMA).

Objective 1: To generate and characterize the MKs derived from iPSCs using blood cells.

In our study, we have developed a differentiation system for MK production from hiPSC-HSCs under a feeder-free and serum-free condition. Our protocol comprises following phases: (i) expansion of hiPSC-HSCs in the presence of expansion media, and (ii) differentiation/maturation phase of cultured MKs in the presence of suitable growth factors and cytokines **(Figure 1a)**. In the first phase of hiPSC-HSCs expansion, we used commercially available expansion media to get sufficient hiPSC-HSCs which we used for MK differentiation, based on pioneer studies. We have since improved this method. Although our study was focused on the generation of MKs from hiPSC-HSCs, we used cocktail of certain cytokines along with relevant growth factors which are essential for MKs

differentiation. Our study resulted in highly pure hiPSCs-derived MKs, which were capable to form platelets in feeder free and xeno free culture system compared to previous reports. We found on day 10, cells were resembling the features of typical MKs like large cell having polyploid nucleus with pro-platelet forming structures **(Figure 1b)**. Although, at day 10 of culture had higher polyploid DNA contents, suggesting a close similarity of hiPSCs- derived MKs with normal MKs. The major reason to optimize the previous reported protocols was to reduce contamination while changing different types of media for specific stages of differentiation as well as to increase the yield of pure population of MKs. As we expected, the percentages of $CD41^{+}$ and $CD61^{+}$ cells on day 10 was higher with stimulation than without stimulation of MK differentiating cocktail. Also, generation of HSCs from hiPSCs and then HSCs used for MK generation is time taking, complex and cost effective process for clinical applications. Our approach indicates the feasibility to generate MKs directly from hiPSC-HSCs in safe as well as cost effective manner. Our study primarily focused on the generation of MKs from hiPSC-HSCs by using basic and defined MK differentiation system which can be used for development of transfusion of MKs/platelets. Also, our experimental system should provide a convenient platform to differentiate MKs for studying the genetic influences and molecular regulation of megakaryopoiesis in the future.

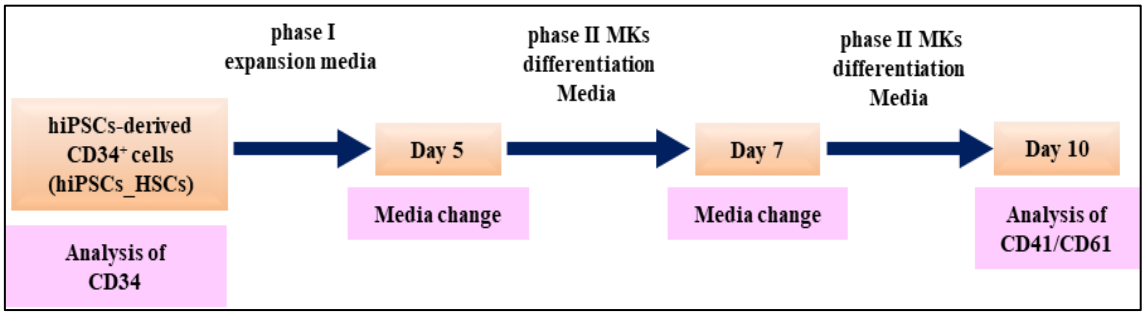

Figure 1a: Schematic representation of the successive iPSCs-HSCs differentiation protocol for MKs differentiation. In phase I, hiPSCs_HSCs were cultured in expansion media for 5 days. Phase II, to induce MKs differentiation hiPSCs_HSCs were cultured in megakaryopoiesis defined media supplemented with supporting growth factors and cytokines (FGF2, BMP4, SCF, VEGF-2, TPO, IL-11, IL-3) up to 10 days.

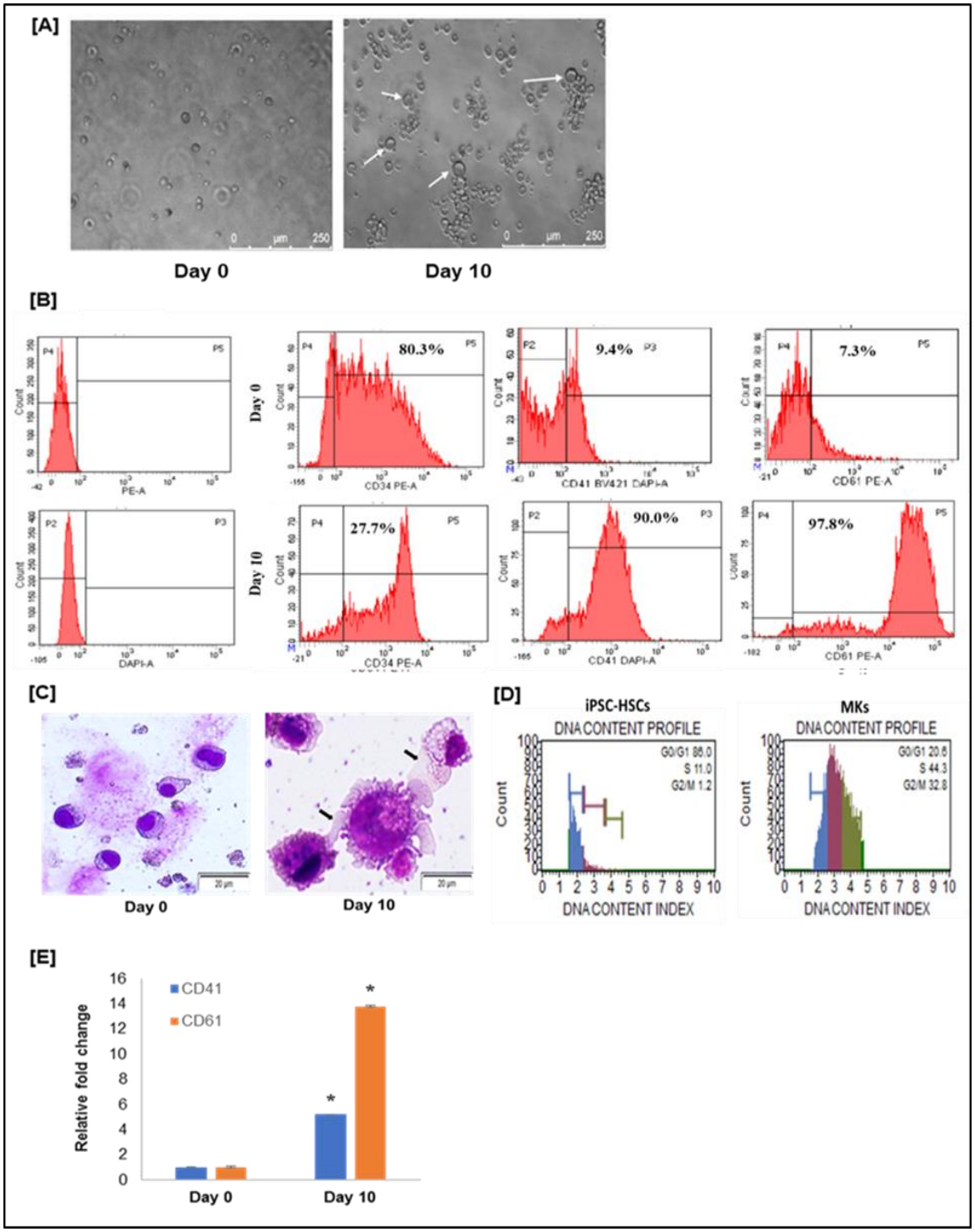

Figure 1b: Characterization of hiPSCs-derived MKs. A. Phase contrast images of cells on days 0 and 10. B. Representative flow cytometry histograms of the cultured suspension cells on day 0 and day 10. The suspension cells on day 0 expressed CD34 marker and on day 10 maximum cells expressed MK markers CD41 and CD61. C. Photomicrograph of cells stained with WG showing the cell and nuclear size were increased for MKs on day 10. D. DNA content (polyploidy status) was increased in MKs compared to iPSCs-HSCs which was analyzed by MUSE analyzer. E. qRT-PCR results showing fold induction in the expression of CD41 and CD61 in cells of day 10 as compared to the cells of day 0 (n=3; *p<0.05). Bars represent mean ± SD of three independent experiments.

Objective 2: Elucidate the role of lncRNA in normal development of megakaryocyte lineage derived from iPSCs.

LncRNAs are long ncRNAs of more than 200 nts in length which have been implicated in numerous biological processes, including regulation of gene expression, cell-cycle and transcriptional/translational process, and epigenetic regulation. Interestingly, differential expression pattern of lncRNAs have been associated in the regulatory process of development, differentiation and maturation of blood cells. Growing evidences indicate that several lncRNAs act as key regulators in normal and malignant hematopoiesis regulation such as XIST, HOTTIP, NeST EGO etc. In the past 2 decades, the crucial roles of miRNAs have been demonstrated in the regulation of MK development and platelet biogenesis. Currently growing evidences revealed lncRNAs have gained significant research interest by their newly defined roles via tethering with DNA, RNA or protein in the development of several blood lineages.

Few of studies have also demonstrated the existence of lncRNAs in aberrant gene expression associated with different blood cells and characterized them. In contrast to miRNAs, the expression profiling and role of lncRNAs has not been fully investigated in MK. Limited information is known about the participation of lncRNAs in megakaryocytopoiesis. Although, several details about lncRNAs involvement in regulatory mechanism of MK development process are still unexplored. Therefore, the profiling of lncRNAs expression in MK is of our particular interest and lncRNAs expression profile may help to identify key lncRNAs which potentially involve in megakaryocytopoiesis. We have investigated the expression and functional significance of lncRNAs in hiPSCs-derived MKs which remains unclear in MK biology. The aim of this study was to identify lncRNAs that may play an important role in megakaryopoiesis. We have performed lncRNAs expression profiling by using the Human LncProfilers™ qPCR Array Kit and spotted 26 differentially regulated lncRNAs (FC ±2.0; $p<0.05$) in hiPSC-derived MKs as compared to hiPSCs-HSCs.

In addition, an independent K562 cells as a classic *in vitro* model of megakaryocytopoiesis for the molecular studies was used to validate the number of lncRNAs by qRT-PCR analysis. HOTAIRM1 (HOX antisense intergenic RNA myeloid 1) was the highest upregulated (~20 fold) lncRNA in hiPSC-derived MKs. Furthermore, we have studied the potential mechanism of HOTAIRM1 based on the interactions between HOTAIRM1, p53 and miR-125b in K562 cells. Our results demonstrated that during MKs maturation HOTAIRM1 may be involved in the transcriptional regulation of p53, via acting as a decoy/sponge for miR-125b (keeping them away from p53 mRNA). Thus, the interaction between HOTAIRM1, p53 and miR-125b is likely involved in controlling cell cycling, ROS production, and apoptosis to support terminal maturation of MKs **(Figure 2)**. Our findings suggest the regulatory role of HOTAIRM1 in p53 mediated regulation of cyclin D1 during megakaryocytopoiesis to promote MK maturation by decoy/sponging miR-125b.

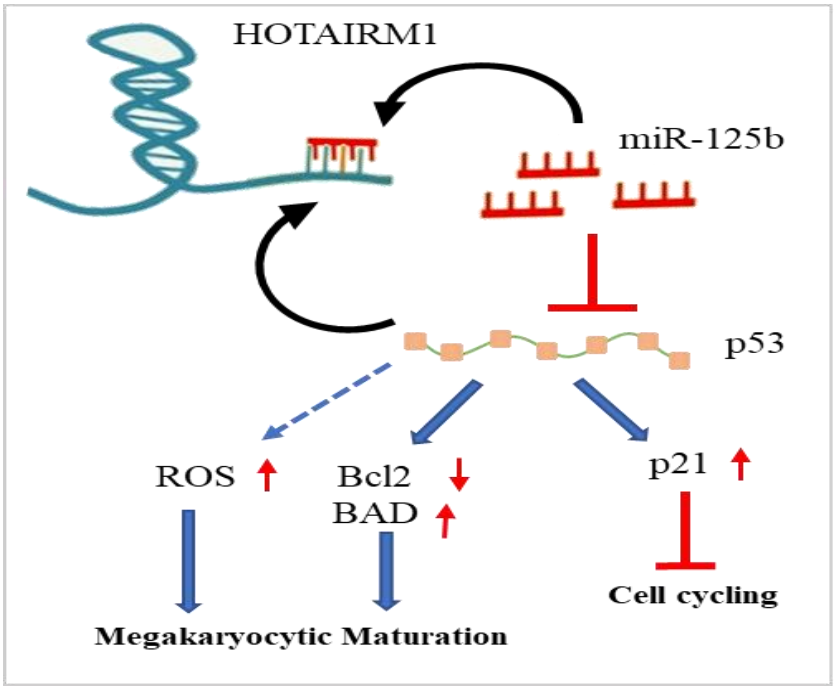

Figure 2: The functional role of HOTAIRM1 during megakaryopoiesis via interacting with p53 and miR-125b. The graphical representation of the crosstalk between lncRNA (HOTAIRM1), miRNA (miR-125b) and p53 during MK development.

Objective 3: Study the role of lncRNA in hyper proliferation (Acute Megakaryocytic leukemia) during treatment by using Phorbol 12-myristate 13-acetate (PMA).

Acute megakaryocytic leukemia (AMKL) is one of the rarest sub-types of acute myeloid leukemia (AML). AMKL is characterized by high proliferation of megakaryoblasts and myelofibrosis of bone marrow, this disease is also associated with poor prognosis. Previous analyses have reported that the human megakaryoblast cells can be differentiated into cells with MK-like characteristics by PMA. However, little is known about the mechanism responsible for regulating this differentiation process. We performed lncRNAs profiling to investigate the differently expressed lncRNAs in MK blast cells treated with and without PMA and examined those that may be responsible for the PMA-induced differentiation of megakaryoblastic cells (Dami cells) into MKs. We found 30 out of 90 lncRNA signatures to be differentially expressed after PMA treatment of megakaryoblast cells, including the highly expressed JPX-lncRNA.

Further, *in Silico* lncRNA-miRNA and miRNA-mRNA interaction analysis revealed that the JPX is likely involved in unblocking the expression of TGF-β receptor (TGF-βR) by sponging oncogenic miRNAs (miR-9-5p, miR-17-5p, and miR-106-5p) during MK differentiation. Further, we report the activation of TGF-βR-induced non-canonical ERK1/2 and PI3K/AKT pathways during PMA-induced MK differentiation and ploidy development. The present study demonstrates that TGF-βR-induced non-canonical ERK1/2 and PI3K/AKT pathways are associated with PMA-induced MK differentiation and ploidy development; in this molecular mechanism, JPX lncRNA could act as a sponge/decoy for following miR-9-5p, miR-17-5p and miR-106-5p, keeping them away from TGF-βR mRNAs. This study reveals the activation of ERK1/2 and PI3K/AKT pathway in PMA-induced megakaryoblast differentiation into matured MK **(Figure 3)**. The identified differentially expressed lncRNA signatures may facilitate further study of the detailed molecular mechanisms associated with

MK development. Thus, our data provide several targets with therapeutic potential for the modulation of the differentiation of megakaryoblasts in AMKL.

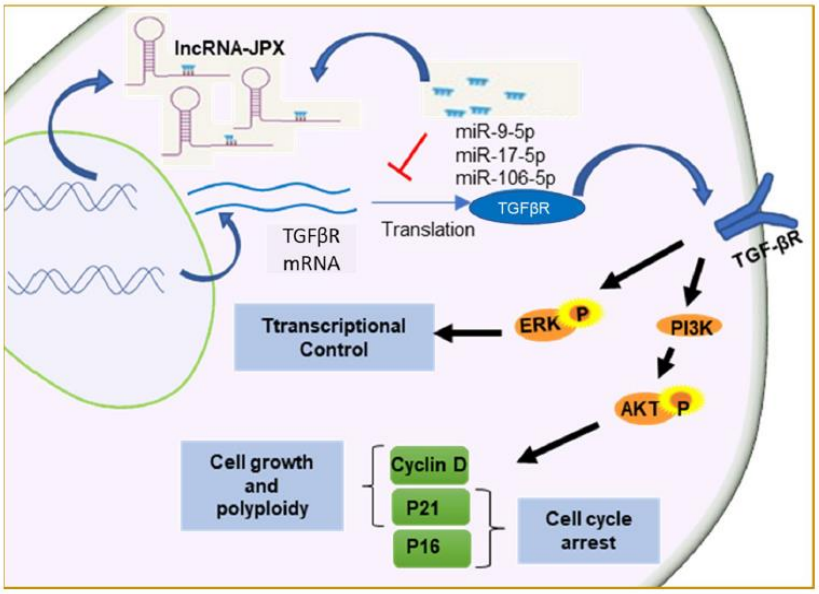

Figure 3: TGF-βR signaling activates SMAD-independent pathways during PMA-induced megakaryocytic differentiation. The graphical representation of the activation of TGF-βR-induced SMAD-independent ERK1/2 and PI3K/AKT pathways during PMA-induced differentiation of Dami cells into MKs, and a crosstalk between JPX lncRNA, miRNAs, and TGF-βR during MK development.

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

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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

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ORIGINAL ARTICLE

Megakaryoblastic leukemia: a study on novel role of clinically significant long non-coding RNA signatures in megakaryocyte development during treatment with phorbol ester

Swati Dahariya[1] · Sanjeev Raghuwanshi[1] · Anjali Sangeeth[1] · Mahesh Malleswarapu[1] · Ravinder Kandi[1] · Ravi Kumar Gutti[1]

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Abstract

Acute megakaryocytic leukemia (AMKL) is one of the rarest sub-types of acute myeloid leukemia (AML). AMKL is characterized by high proliferation of megakaryoblasts and myelofibrosis of bone marrow, this disease is also associated with poor prognosis. Previous analyses have reported that the human megakaryoblastic cells can be differentiated into cells with megakaryocyte (MK)-like characteristics by phorbol 12-myristate 13-acetate (PMA). However, little is known about the mechanism responsible for regulating this differentiation process. We performed long non-coding RNA (lncRNA) profiling to investigate the differently expressed lncRNAs in megakaryocyte blast cells treated with and without PMA and examined those that may be responsible for the PMA-induced differentiation of megakaryoblasts into MKs. We found 30 out of 90 lncRNA signatures to be differentially expressed after PMA treatment of megakaryoblast cells, including the highly expressed JPX lncRNA. Further, in silico lncRNA-miRNA and miRNA-mRNA interaction analysis revealed that the JPX is likely involved in unblocking the expression of TGF-β receptor (TGF-βR) by sponging oncogenic miRNAs (miR-9-5p, miR-17-5p, and miR-106-5p) during MK differentiation. Further, we report the activation of TGF-βR-induced non-canonical ERK1/2 and PI3K/AKT pathways during PMA-induced MK differentiation and ploidy development. The present study demonstrates that TGF-βR-induced non-canonical ERK1/2 and PI3K/AKT pathways are associated with PMA-induced MK differentiation and ploidy development; in this molecular mechanism, JPX lncRNA could act as a decoy for miR-9-5p, miR-17-5p, and miR-106-5p, titrating them away from TGF-βR mRNAs. Importantly, this study reveals the activation of ERK1/2 and PI3K/AKT pathway in PMA-induced Dami cell differentiation into MK. The identified differentially expressed lncRNA signatures may facilitate further study of the detailed molecular mechanisms associated with MK development. Thus, our data provide numerous targets with therapeutic potential for the modulation of the differentiation of megakaryoblastic cells in AMKL.

Keywords Megakaryocyte · Leukemia · PMA · LncRNA · miRNA · TGF-βR · ERK1/2 · PI3K · AKT

Introduction

Leukemia is a metastasizing blood cell disease characterized by abnormal proliferation, apoptosis repression, and differentiation blockage in hematopoietic stem/progenitor cells [1–3]. Acute megakaryocytic leukemia (AMKL) is one of the rarest sub-type of acute myeloid leukemia (AML). AMKL is characterized by abnormal megakaryoblasts with high cell proliferation and extensive myelofibrosis [3]. This disease is rare in adult population, only 1% of all AML patients, but this disease is more common in children, comprises between 4-15% of all AML cases [4]. AMKL is associated with poor prognosis, diagnosis and clinical management are also the challenges which are associated with AMKL.

One approach for the treatment of different leukemia cases is chemical-induced differentiation of leukemia blasts, this approach is also referred to as differentiation therapy [5]. Retinoic acid (RA) is one of the examples of the successful differentiation therapy for acute promyelocytic leukemia. In recent studies, different transcriptions factors, cell cycle

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Dear Dahariya,

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Thanking you,

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