Role of miR-99a and miR-125b during Megakaryocyte Development

A thesis Submitted to University of Hyderabad for the Award of

Doctor of Philosophy (Ph.D)

By

Ravinder Kandi
Enrolment No: 11LBPH03

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad, 500 046, India

March, 2016

University of Hyderabad
(A Central University established in 1974 by act of parliament)
Department of Biochemistry
School of Life Sciences
Hyderabad– 500 046, INDIA

"DECLARATION"

I, **Ravinder Kandi**, hereby declare that this thesis entitled **"Role of miR-99a and miR-125b during megakaryocyte development"** submitted by me is based on the results of the work done under the guidance and supervision of **Dr. G. Ravi Kumar** at Department of Biochemistry, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

Ravinder Kandi
Regd. No: 11LBPH03

University of Hyderabad
(A Central University established in 1974 by act of parliament)
Department of Biochemistry
School of Life Sciences
Hyderabad– 500 046, INDIA

"CERTIFICATE"

This is to certify that this thesis entitled **"Role of miR-99a and miR-125b during megakaryocyte development"** is a record of bonafide work done by **Mr. Ravinder Kandi**, a research scholar for Ph.D. programme in the Department of Biochemistry, School of Life Sciences, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Dr. G. Ravi Kumar

(Supervisor)

HEAD

Department of Biochemistry

DEAN

School of Life Sciences

Acknowledgements

First of all, I would like to thank my mentor, ***Dr. Ravi Kumar*** *for inspiring me to pursue doctoral studies and for introducing me to the fascinating field of Stem Cells and Haematological Disorders. His constant support, guidance and the encouragement during the course of the work, and the immense patience that he had invested on me are a constant source of inspiration. I am eternally indebted to him for guiding me to the research problems, and giving me the exceptional freedom and opportunity to work on several different projects. Our association thought me many important aspects of life apart from research. I am extremely lucky to have him as my teacher.*

Furthermore, I am very grateful to members of the doctoral committee, ***Prof. Aparana Dutta Gupta*** *and* ***Dr. Naresh Babu V Sepuri*** *for insightful comments in my work and for many motivating discussions.*

My thanks to the Head, Department of Biochemistry ***Prof. N. Siva Kumar*** *and former Heads* ***Prof. K.V.A. Ramaiah*** *and* ***Prof. O.H. Setty*** *for providing us with good equipment and facilities to carry out the work.*

I thank present Dean ***Prof. P. Reddanna,*** *and former Deans,* ***Prof A. S. Raghavendra, Prof. R. P. Sharma, Prof. Aparna Dutta Gupta*** *and* ***Prof. M. Ramanadham*** *for allowing me to use the school facilities.*

I would like to express my sincere thanks to ***Usha gutti*** *for my data interpretation and manuscript editing.*

I am also pleased to acknowledge the ***Genomics facility*** *(DBT-CREBB) School of Life Sciences and* ***Flow cytometry facility*** *(DST-FIST), Dept. of Biochemistry,* ***Animal house facility,*** *School of Life Sciences, University of Hyderabad.*

I am also thankful to ***Apollo Hospitals*** *and* ***Fernandez Hospitals*** *for their help in providing cord blood and peripheral blood samples.*

I would like to thank all ***Departmental teaching staff*** *for their technical assistance.*

I would like to thank all ***Departmental and school of life sciences non-teaching staff*** *for their help.*

I specially thank my lab mates ***Rambabu, Itishri, Sanjeev Raghuwanshi, Narsaiah, Gopal, Jaswanth, Aneesh*** *and project students for their cooperation and help.*

Acknowledgements

I thank all my friends ***Trinadh, Suman, Sravan, Rameshwar, Suresh, Elsin, venkat, Dayakar, kannan, kiran, rajesh*** *and all my friends, Research Scholars in School of Life Sciences for their support and encourage*

I thank ***DBT, UGC, DST-PURSE, and UPE II*** *for funding the lab; I thank* ***CSIR*** *for supporting me with fellowship.*

I am deeply indebted to my ***family members*** *for their support.*

Ravinder Kandi...

ABBREVIATIONS	
^{o}C	Degree centigrade/Degree Celsius
µg	Micro gram
µl	Micro litre
µM	Micro molar
µm	Micro meter
nM	Nano molar
mg	Milli gram
ml	Milli litre
ng	Nano gram
pg	Pico gram
%	Percentage
min	Minutes
g	Grams
M	Molar
β	Beta
~	Approximately
3'-UTR	3' un-translated region
5'-UTR	5' un-translated region
H2DCFDA	2, 7-dichlorodihydrofluoresceindiacetate
AChE	Acetyl cholinesterase
ALK4	Activin-like kinase 4
ALL	Acute lymphoblastic leukemia
AMKL	Acute megakaryoblastic leukemia
AML	Acute myeloid leukemia
AML1	Acute myeloid leukemia1 protein
P14 ARF	ARF tumor suppressor
B-cells	B -lymphocytes
BCL2	B-cell lymphoma 2
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
Bad	BCL2 Associated Agonist of cell death
BAK1	BCL2-Antagonist Killer 1
BID	BH3 Interacting Domain Death Agonist
BM	Bone marrow
BSA	Bovine serum albumin
CTDSPL	Carboxy-Terminal Domain RNA polymeraseII polypeptide A phosphatase like
CAMs	Cell adhesions molecules
CML	Chronic myeloid leukemia
CD34+	Cluster differentiation 34 positive
CD41	Cluster Differentiation 41
CD61	Cluster Differentiation 61
CFUs	Colony forming units
CSF1	Colony stimulating factor1
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors

CMRPs	Common myeloid repopulating progenitors
CB	Cord blood
CXCR4	C-X-C chemokine receptor type4
Cyc D1	Cyclin D1
CDK4	Cyclin-dependent kinase4
CDK6	Cyclin-dependent kinase6
DTS	Dense tubular system
dNTPs	Deoxynucleotides
DNA	Deoxyribonucleic acid
DGCR8	Di-George syndrome chromosomal [or critical] region 8
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemi-luminescence
FBS	Fetal Bovine Serum
FKBP51	FK 506 binding protein 51
FACS	Fluorescence activated cell sorting
FOXO-3	Fork head box O3
GATA1	Globin Transcription Factor 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly A	Glycophorin A
GMPs	Granulocyte monocyte progenitors
Gfi1	Growth factor independent 1
HSCs	Hematopoietic stem cells
hsa	Homosepian
HRP	horseradish peroxidase
IgG	Immuno globulin G
IGH	Immunoglobulin heavy chain
IGF1R	Insulin- like growth factor 1 receptor
IL6	Interleukin 6
IMDM	Iscove's Modified Dulbecco's Media
Kb	Kilo bases
LMO2	LIM domain only2
LT-HSCs	Long term Hematopoietic stem cells
M-CSF	Macrophage Colony Stimulating factor
MFI	Mean fluorescence intensity
MEPs	Megakaryocyte erythrocyte progenitors
MERPs	Megakaryocyte erythroid repopulating progenitors
MkRPs	Megakaryocyte repopulating progenitors
MKs	Megakaryocytes
mRNA	messenger RNA
miR-125b	Micro RNA-125b
miR-99a	microRNA-99a
miRNAs	microRNAs
MFE	Minimum free energy
MAPK	Mitogen activated protein kinases
MAPKα	Mitogen-activated protein kinases alpha

mmu	Mus musculus
MPPs	Multi potent progenitors
MyRPs	Myeloid repopulating progenitors
NAC	N-Acetyl Cysteine
NOX1	NADPH Oxidase1
NK cells	Natural Killer Cells
NL	New born Liver
NF-E2	Nuclear Factor, Erythroid 2
PB	Peripheral blood
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate buffer saline
PI3K	Phosphoinositide-3 Kinase
PE	Phyco Erythrin
PV	Polycythemia vera
PCR	Polymerase Chain Reaction
Pre-miRNAs	Precursor microRNAs
Pri-miRNAs	Primary microRNAs
PCNA	Proliferating cell nuclear antigen
Pro-MKs	Pro-Megakaryocytes
PSA	Prostate specific antigen
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT-PCR	Real time PCR
RB	Retinoblastoma
RNA	Ribonucleic acid
RISC	RNA Induced Silencing Complex
RNA pol-II	RNA polymerase II
RNA pol III	RNA polymerase III
RT	Room temperature
RPMI	Roswell Park Memorial Institute
RUNX1	Runt-Related Transcription Factor 1
ST-HSCs	Short term Hematopoietic stem cells
SDS-PAGE	Sodium dodecyl Sulfate polyacrylamide gel electrophoresis
SD	Standard Deviation
SDF1	Stromal cell derived factor 1
TAR	Thrombocytopenia-absent radius
TPO	Thrombopoietin
T-cells	Thymocytes cells
TGF	Transforming growth factor
TMD	Transient myeloproliferative disorder
TBS	Tris Buffer Saline
TNF	Tumour necrosis factor
TP53	Tumour protein 53
VCR	Vincristine resistance

INTRODUCTION

1.1 Overview of Hematopoiesis

Hematopoiesis is the process in which hematopoietic stem cell progenitors (HSCs) proliferate and differentiated into all blood cell types. At embryonic stage, it takes place in variety of sites such the liver, spleen, thymus, lymph nodes and bone marrow which gradually shift to bone marrow after birth [**Metcalf, 2007**]. In hematopoiesis, HSCs respond to several extracellular signals and differentiate into cells of different lineages and preserve blood homeostasis. During this process long-term hematopoietic stem cells (LT-HSCs) are able to produce all progenitors and they have unlimited self renewable capacity **(Fig. 1).** Immediate downstream of these cells are short-term hematopoietic stem cells (ST-HSCs) and these cells give rise multi potent progenitor cells (MPPs), the MPP are further produce the common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) these cells progressively lose their self renewable property upon commitment [**Akashi K, 2000**]. These cells mainly follow two pathways, namely, lymphoid and myeloid pathway. In lymphoid pathway, the common lymphoid progenitors (CLPs) differentiate into immune cells such as T-lymphocytes, B-lymphocytes and Nature killer cells. In myeloid pathway, the common myeloid progenitors (CMPs) are able to give rise to granulocyte monocyte progenitors (GMPs) and bi-potent megakaryocyte erythrocyte progenitors (MEPs) that are responsible for production of granulocytes, monocytes and megakaryocytes (MKs), erythrocytes respectively [**Svoboda et al, 2015; Seita et al, 2010**].

Recent studies show that there is an alternative myeloid pathway, in which the LSK $CD150^{+}CD48^{-}CD34^{-}$ subset of HSCs also involve in long term and short term platelet producing cells (Myeloid bypass model) as well as they are able to produce other myeloid lineage cells, but not lymphoid lineage cells [**Sanjuan-Pla et al, 2015**]. Alternative myeloid bypass model suggest the existence of different myeloid repopulating progenitors (MyRPs) as a subset of LT-HSCs, they are megakaryocyte repopulating progenitors (MkRPs),common myeloid repopulating progenitors (CMRPs) and megakaryocyte-erythroid repopulating progenitors (MERPs). These progenitors are involved in long term repopulation and differentiation of cells of particular lineage [**Svoboda et al, 2015; Yamamoto et al, 2013**]. Deregulation of the balance between cell differentiation, proliferation and cell death leads to different hematopoietic disorders. Proliferation and differentiation of hematopoietic stem cell progenitors are coordinated by gene expression programs regulated by exogenous and endogenous factors. Here, we studied megakaryocyte development regulation.

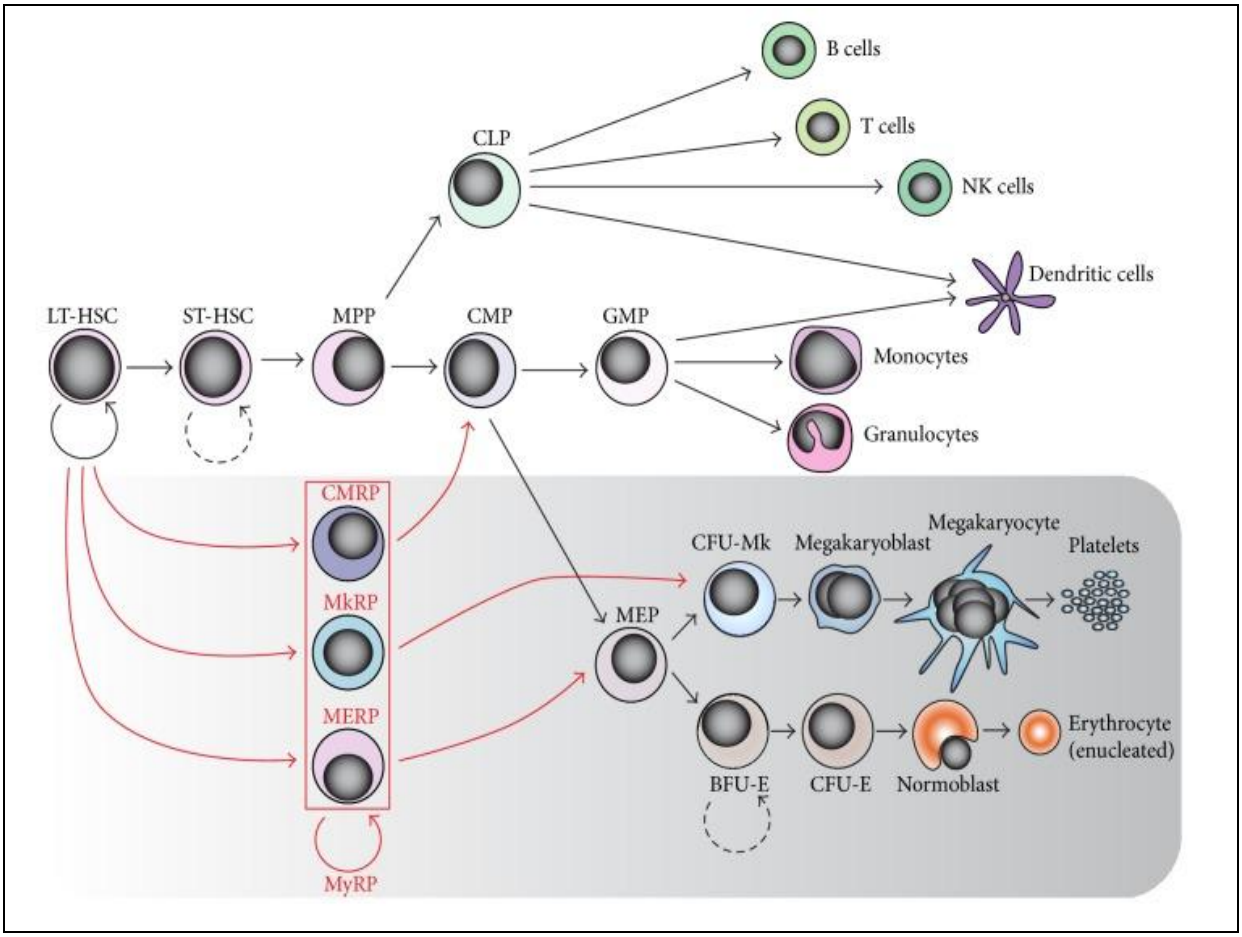


Figure 1. Schematic representation of adult hematopoiesis concerned to megakaryocyte erythrocyte lineage: The conventional hierarchical hematopoiesis are able to produce the common lymphoid progenitors and gives immune cells B- cells T- cells and NK cells where as megakaryocyte-erythroid progenitors (MEPs) are responsible for production of erythrocytes and megakaryocytes. The alternative myeloid bypass model suggests the existence of different myeloid repopulating progenitors (MyRPs) as a subset of LT-HSCs such as CMRPs, MkRPs, and MERPs. These progenitors are able to maintain long-term repopulation and differentiation into the specific lineages **[Adapted from Svoboda et al, 2015]**.

1.2 Megakaryocyte Development

The process of megakaryocyte (MK) development and platelet production is one of the most unique processes in mammalian system. Megakaryocytopoiesis (platelet production process) involves multiple stages that are highly regulated. This process helps the progenitor cells to proliferate, polyploidize and differentiate into megakaryocytes in the presence of thrombopoietin (TPO) [**Liu et al, 2011**]. MKs are large bone marrow cells responsible for production of platelets, which are necessary for blood clotting and to maintain hemostasis.

These are rare myeloid cells (<1% cells) which primarily reside in the bone marrow, but are also found in the peripheral blood and lung. At early stage of development, before bone marrow support for blood cell development, megakaryopoiesis occurs within the yolk sac and fetal liver [**Ogawa, 1993**]. Mks develop from pluripotent HSCs which express CD34 antigen, which is restricted to the formation of megakaryocyte progenitors that develop into mature Mks in the presence of TPO [**Briddell et al, 1989**].

MKs structural features can be discovered by their large size and is better characterized using Wright staining or microscopy studies. Previous reports suggest that based on their size and morphological features they classified as four stages **[Pisciotta et al, 1953; Levine et al 1982; Gewirtz & Schick, 1994; Kaushansky, 2009; Cramer & Vainchenker, 2006].** Which are

Stage I: Megakaryoblast: It has compacted nucleus with rough chromatin organization and a large lobed. These cells may vary in size up to 24 µm in diameter and nuclear to cytoplasm ratio is high **[Cramer et al, 2006].** Electron micrographs shows megakaryoblasts have less α-granules and the demarcation membrane and the endomitotic process gets active. MKs are unable to form pseudopodia and membrane blebbing.

Stage II: Pro-megakaryocyte (Pro-MKs): Pro-MK starts pseudopodia formation and the nucleus to cytoplasm ratio decreases (almost closer to 1:1) compared to megakaryoblast. It has multi-lobulated nuclei arranged in a horseshoe shape. The cell size starts increasing and shows up to 40 µm in size. Electron microscopy studies clearly suggest the formation of demarcation membrane system and granules.

Stage III: Granular megakaryocyte: These cells have lobulated nucleus and shows a low nuclear to cytoplasmic ratio with dense chromatin. Cell size is appximately >50µm. The demarcation membrane system is well developed with abundant granules and organelles.

Stage IV: Mature megakaryocyte: The nucleus become more compact and very lobulated compared to stage II and III MKs. Cell size is found to be >50-60µm. Proplatelet formation can be seen and the demarcation membrane system is homogenously distributed. In this stage, MKs are totally involved in platelet generation.

Platelet Production

In 1906 James Wright discovered MKs as precursor cells for platelets production, described as a process of budding off platelets from extensions of the MKs [**Patel et al, 2005**]. After

polyploidization, MK cytoplasmic maturation takes place with the development of three important structures: the granules (dense and alpha), dense tubular system (DTS) and the demarcation membrane **(Fig. 2).** Fully mature MKs start to shed platelets into blood. Each MK can release more than 5000 platelets with a 7-10 day lifespan in humans [**Tavassoli, 1980, Kleiman et al, 2008, Jennings, 2009]** and around 4-7 days in rats [**Johnson et al, 1977].**

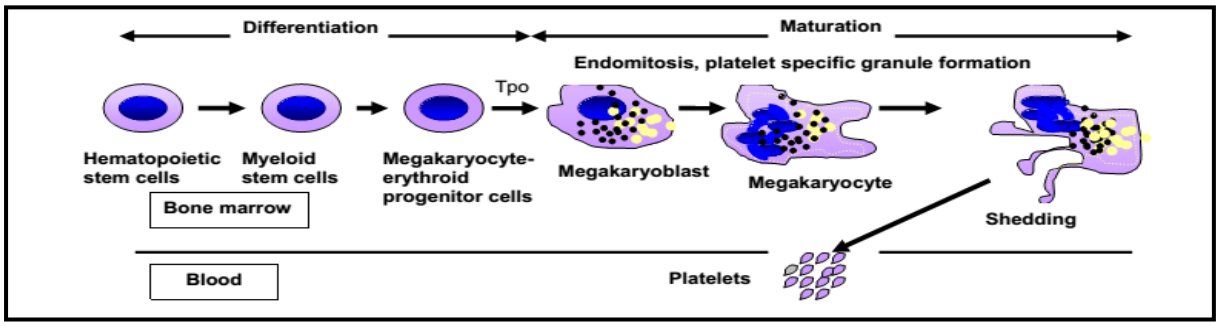


Figure 2. Summary of megakaryocyte maturation and platelet production. [Adapted from Anjali et al, 2008].

1.3 Thrombopoietin and Regulation

Megakaryopoiesis is physiologically regulated by thrombopoietin (TPO) which is an acidic glycoprotein produced primarily in the kidney, liver and bone marrow **(Fig. 3).** TPO is required for MK development and maintenance of constant platelet mass [**Kaushansky, 2005**]. Previous studies suggest that serum TPO levels were inversely proportion to platelet count and MK mass [**Shinjo et al, 1998; Engel et al, 1999**]. TPO is majorly regulated by platelet c-MPL receptor mediated uptake and destruction there by regulate the blood TPO levels. But during thrombocytopenia, TPO levels were increased due to low platelet count but the molecular mechanisms behind this is not clearly understood. All the C-MPL receptors are not responsible for this effect. Interestingly, some endothelial cells showes thrombopoietin receptor which are involved in proliferation and migration of cells in response to TPO [**Shinjo et al, 1998]**.

TPO-MPL signaling cascade well studied and activate several pathways such as MAPK signaling, P38 MAPK, PI3K-AKT signaling pathway and JAK/STAT signaling pathways. It also activates Hif1α which involves in feed back mechanism to decrease the more oxidative stress which suggesting that it regulates glycolytic metabolism. previous studies also showed TPO signal activates αvβ3-integrin which required for adhesion signals **[Kirito et al, 2005; Umemoto et al, 2012; Atsushi, 2014]**.

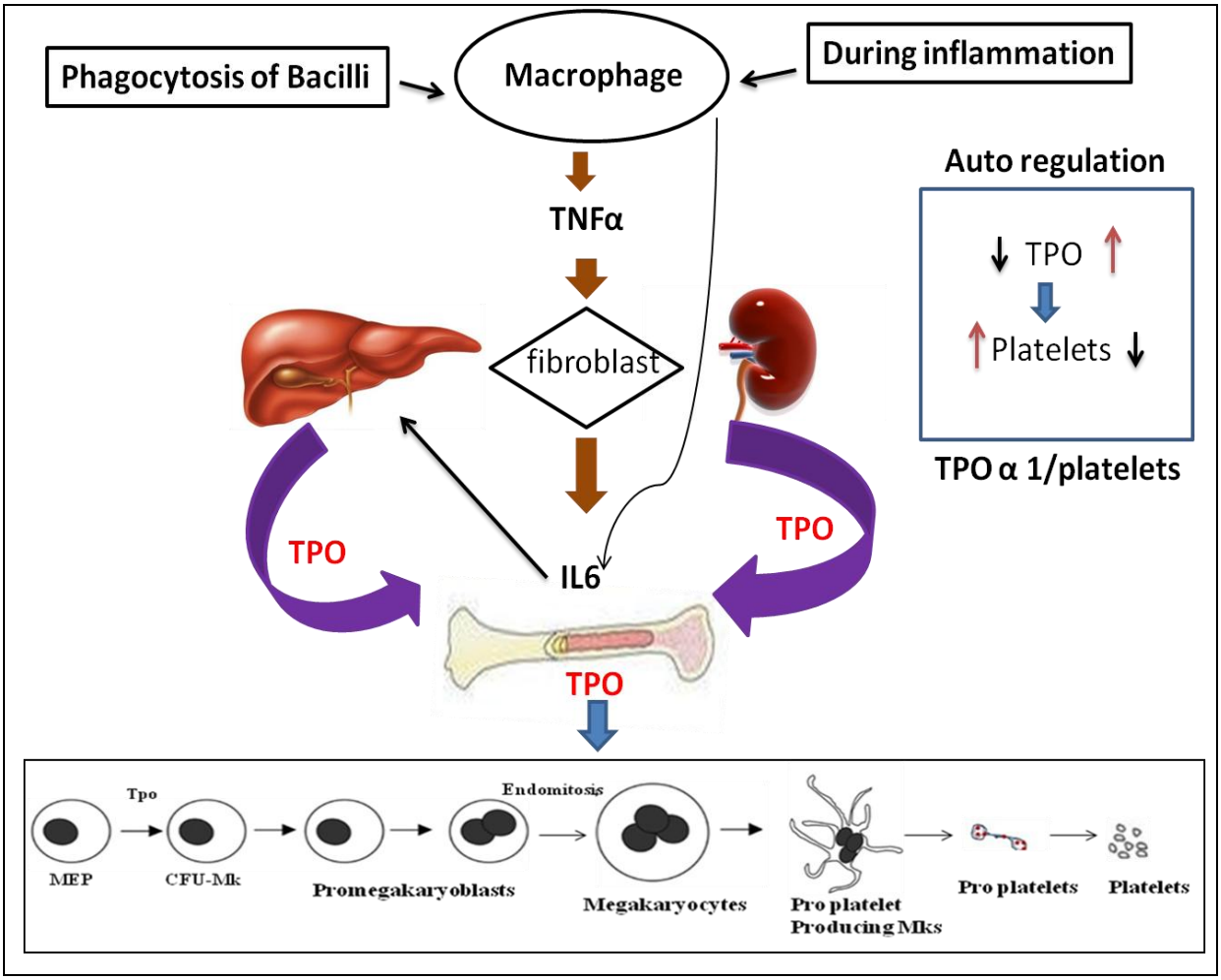


Figure 3. Diagrammatic representation of thrombopoietin levels regulation and megakaryocyte development. The steady state level of hepatic thrombopoietin is controlled by platelet c-mpl receptor mediated uptake destruction of the TPO and reduction in blood circulation. During inflammation, IL-6 is produced from macrophages through TNF-stimulation of fibroblasts and distribute into liver to increase TPO production **[Adapted from Wickrema, 2007].**

1.4 Developmental differences between neonatal and adult megakaryocytopoiesis

Although megakaryocytopoiesis follows the same steps in neonates and adults, there are substantial developmental differences between neonatal and adult megakaryocytes. Specifically, neonatal MKs progenitors are highly proliferative, small size and lower ploidy than adult MKs [**Liu et al, 2011; sola et al, 2007**]. One recent report indicates that the effect of TPO on Mk progenitors differs between neonates and adults. While TPO induced polyploidization in adult megakaryocytes, an inhibitory effect was seen on polyploidization in neonatal Mks (**Pastos et al, 2006**). Furthermore, adults were shown to increase their Mk

number and size in the bone marrow in response to thrombocytopenia, while neonates only increased their Mk number, but not their Mk size **[Sola et al, 2007]**. It has been hypothesized that developmental differences between neonatal and adult MKs contribute to the vulnerability of neonates to develop severe thrombocytopenia. In fact, upto 50% of all neonates admitted to neonatal intensive care units develop thrombocytopenia **[Sallmon et al, 2010]**. Also, Neonates are affected by disorders of megakaryocytopoiesis that are predominantly or exclusively present during this developmental stage, including a syndrome of megakaryocytic hyper proliferation known as transient myeloproliferative disorder (TMD) and the thrombocytopenia associated with the thrombocytopenia-absent radius (TAR) syndrome **[Liu et al, 2011]**. It is therefore highly desired to gain more insights into the molecular mechanisms underlying megakaryocytopoiesis and thrombopoiesis in neonates. Especially, little is known about the regulatory mechanisms underlying the developmental differences between neonatal and adult Mks.

However, the molecular mechanisms that govern these cell-intrinsic differences are unknown. While a developmental expression pattern of some critical transcription factors like GATA-1 and NF-E2 might contribute to the different phenotypes of neonatal and adult MKs **[Mattia et al, 2002]**, a growing body of evidence suggests that a regulation at the post-transcriptional level by small double stranded non-coding microRNAs (miRNAs) is crucially involved in the regulation of stem-cell differentiation in general, and normal as well as malignant hematopoiesis, in particular **[Ambros, 2004; Bartel, 2004; Marcucci et al, 2008]**. miRNAs can silence specific target genes through direct mRNA degradation, translational repression or both. In regard to megakaryocytopoiesis, it was shown that the miRNA expression profile of adult human bone-marrow derived $CD34^+$ cells changes during megakaryocytic differentiation

1.5 micro RNAs

miRNAs are small, endogeneous, higly conserved and non-protein coding RNAs having ~22 nucleotide length which can negatively regulate post transcriptional gene expression through direct mRNA degradation or/and by inhibition of translation by interacting with specific mRNAs [**He et al, 2004**]. In 1993, Ambros discovered first miRNA lin-4 which inhibits lin14 gene expression by binding to 3'untranslated region (3'UTR) region. Lin4 and lin14 regulate early stage of *C.elegans* larval development from first larval stage to second and second to third larval stage respectively [**Lee et al, 1993, Olsen et al, 1999, Wightman et al,**

1993]. Later hundreds of other microRNAs have been identified and sequenced in eukaryotic organisms, suggesting that miRNAs are ubiquitous regulators of gene expression [**Bartel et al, 2004**]. These studies show that miRNAs are regulating over half of all mammalian genes [**Friedman et al, 2009**]. miRNAs are involved in many cellular processes like cell cycle, apoptosis and differentiation [**Ambros, 2004**].

1.6 Biogenesis of miRNAs and Function

Thousands of miRNAs are expressed in a cell and the copy number of miRNAs are more than that of normally found mRNAs [**Lim et al, 2003**]. Most miRNAs are located in intergenic regions or with in the gene. Some of the miRNA exist as clusters, like miR-17-92 and miR-290-295 cluster which are transcribed as singe transcript and express together. [**Chiang et al, 2010; Concepcion et al, 2012; Houbaviy et al, 2005].** miRNAs are transcribed from genomic DNA by RNA polymerase II in the nucleus[**Lee et al, 2004**], whereas, some of the miRNAs are also transcribed by RNA pol III, especially, the one located on upstream regions of Alu sequences [**Borchert et al, 2006**]. During transcription they can produce primary miRNA (Pri-miRNAs) of various sizes upto 1Kb in length, which can be further processed by the microprocessor complex consisting of conserved RNAaseIII endoribonuclease DROSHA and DGCR8 [**Bartel, 2004; Kim et al, 2009**]. Pri-miRNA is cleaved by DROSHA into hair-loop precursor miRNA (pre-miRNA) consist of ~60-70 nucleotides length, which is translocated from nucleus to cytoplasm by means of exportin5 and its cofactor Ran-GTP [**Lee et al, 2003; Yi et al, 2003**]. In cytoplasm, pre-miRNA is further cleaved by Dicer into mature double standard miRNA having ~18-22 nucleotide length [**Ha & Kim, 2015**]. The two strands get detached and the strand which is thermodynamically less stable at 5'end becomes mature miRNA **(Fig. 4).** This mature miRNA combine with RNA Induced Silencing Complex (RISC) to form miRNA-RISC complex. This complex is involved in translational repression by binding to 3'-UTR region of specific mRNA transcript [**Nilsen, 2007**].

In mammals, miRNA target sites are mostly located in the 3'UTR region and rarely in 5'UTR region. Some sites also reside in coding regions, whereas, in plants target sites are mostly in the coding region. The mechanism by which a miRNA can diminish protein expression is unclear[**Undi et al, 2013**]. But the previous reports suggest that miRNA regulates translation by inhibiting iniation or other stages of translation. miRNAs repress the initiation of translation by inhibiting Cap-40s ribosomal subunit association with 60S ribosome subunit

resulting in premature drop off ribosomes and delay in elongation [**Wakiyama et al, 2007; Mathonnet et al, 2007; Kiriakidou et al, 2007**].

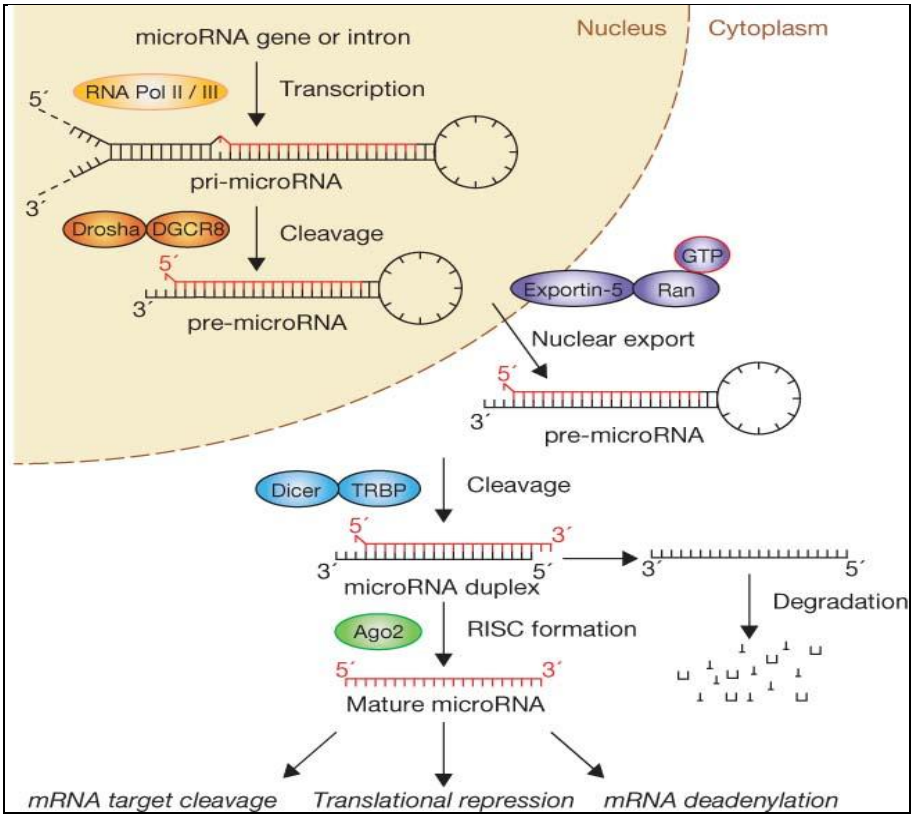


Figure 4. Schematic representation of miRNA biogenesis and functions. miRNA gene transcribed by RNApol II into Pri-miRNAs and are capped and polyadenylated, cleaved by the microprocessor complex DROSHA and its partner DGCR8 into pre-miRNA which in turn is exported to the cytoplasm and further processed by another endoribonuclease, DICER1. Mature miRNA combined with RNA Induced Silencing Complex (RISC) to form miRNA-RISC complex which regulates gene expression by mRNA destabilization or translational repression and mRNA deadenylation [**Adapted from Winter et al, 2009**].

miRNA and target prediction:

Several studies indicate that deregulation of miRNAs results in various disorders including diabetes, cancer, cardiovascular disorders and metabolic disorders. To understanding of miRNA regulation in various disorders enables us to solve the diagnostic and therapeutic challenges. To explore the interaction of miRNA and mRNA many methods have been developed for computional algorithms **(Table 1)** for taget prediction based on their seed sequence complimentarity and thermodynimics **[Undi et al, 2013].**

Database	Target species	Description	website	Ref
Target Scan	Vertebrates	Detection of target genes by perfect complementarity to the seed region	http://www.targetscan.org	**[Lewis et al,2003]**
miRanda	Flies, Vertebrates	Prediction of targets by finding high complementarity regions in 3'UTR	http://www.microrna.org	**[John et al, 2004]**
PicTar	Files, Vertebrates and worms	Filter alignments according to the thermodynamic stability, Seed match, binding energy and conservation	http://pictar.mdc-berlin.de	**[Kerk et al, 2005]**
RNA hybrid	Any Species	Identify miRNA secondary structure prediction and energetically hybridization between miRNA and mRNA	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid	**[Rehmsmeier et al, 2004]**
DIANA-micro T	Any	To predict based on affinity interaction between miRNA and mRNA	http://diana.pebi.upenn.edu/cgi-bin/micro_t.cgi	**[Maragkakis et al, 2009]**
Mi Target	Any	Training data derived from validated miRNA targets from literature.	http://cbit.snu.ac.kr/~miTarget	**[Kim et al, 2006]**
miRTarget2	Humans, mice, Rats, dogs Chickens	Micro array transcriptional profiling data set is used for algorithm training	http://mirdb.org	**[Wang and ElNaga, 2008]**
Target ScanS	Vertebrates	Based on seed sequence complementarity (6-nuclooetids seed match) and conservation of Adenosine	http://genes.mit.edu/targetscan	**[Lewis et al,2005]**
miR walk	Humans, mice and Rats	Identification of targets based on seed sequence complementarity	http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk	**[Dweep et al, 2011]**
miRSystem	Humans and mice	Predict the target genes and pathways	http://mirsystem.cgm.ntu.edu.tw/	**[Lu et al, 2012]**

Table 1. Summary of miRNA target prediction tools based on their seed sequence complimentarity (Undi et al, 2013).

1.7 Micro RNAs in hematopoietic Stem cell development:

The differentiation and commitement of hematopoietic stem cells carefully regulated by complex and interconnected molecular networks. These HSCs generate whole range of mature blood cells and shows different functions which is regulated by both transcriptional and posttranscriptional regulatory mechanisms, and also require for the proper cell diiferntiation by modulation of cell preoliferation and activation. Among these regulatory mechanisms microRNAs are play important roles in hematopoietic system in the past several years **[Montagner et al, 2014]**. Hematopoitic system shows different brach points which is tigtly controlled by environmental influence of nich ,transcriptional factors and others such as miRNAs.

1.8 miRNAs in lympoid cell development

The first breakthrough study about miRNA regulation in immune cell differentiation was miR-181 over expression leads to increase B-lymphocyte whereas, decrease T-lymphocytes. In contrast, forced expression of miR-223 and miR-142 in HSCs leads to 30-40% of cells are T- cell lineage but less effect on B-cell lineage **[Chen et al, 2004]**. miR-155 transfection showed increased $CD8^+$ T cell activation whereas, decreased in differentiation of memory T-cells **[Tsai et al, 2013]**. During initial stage of T-cell differentiation, over expression of miR-17-92 cluster promotes proliferation and expansion,whereas, inhibition of these miRNA cluser leds to reduced proliferation of T-cells **[Wu et al, 2012]**. Recent repots on miRNAs showed high expression of seven miRNAs including miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-7f in T-cell subsets, among these miR-142-3p and miR-142-5p was found to play important role in T-cell development **[Wu et al, 2007]**.

miR-150, is mainly expressed in spleen, thymus and lymph nodes and is up-regulated during the development of mature B and T cells; indeed, prematurely expressed miR-150 impairs B-cell development with moderately enhanced T cell development, miR-150 over expression in HSC do not affect B cell lineage commitment **(Fig 5)**, but over expression blocks the process of pro-B to Pre-B cell differentiation**[Zhou et al, 2007].** Furthermore, reports showed **s**tage specific expression of miR-150 regulates B-cell differentiation by targeting the transcription factor c-myb which is involved in normal lymphocyte development, and ectopic expression of miR-150 impairs the process of lymphocyte development **[Xiao et al, 200**

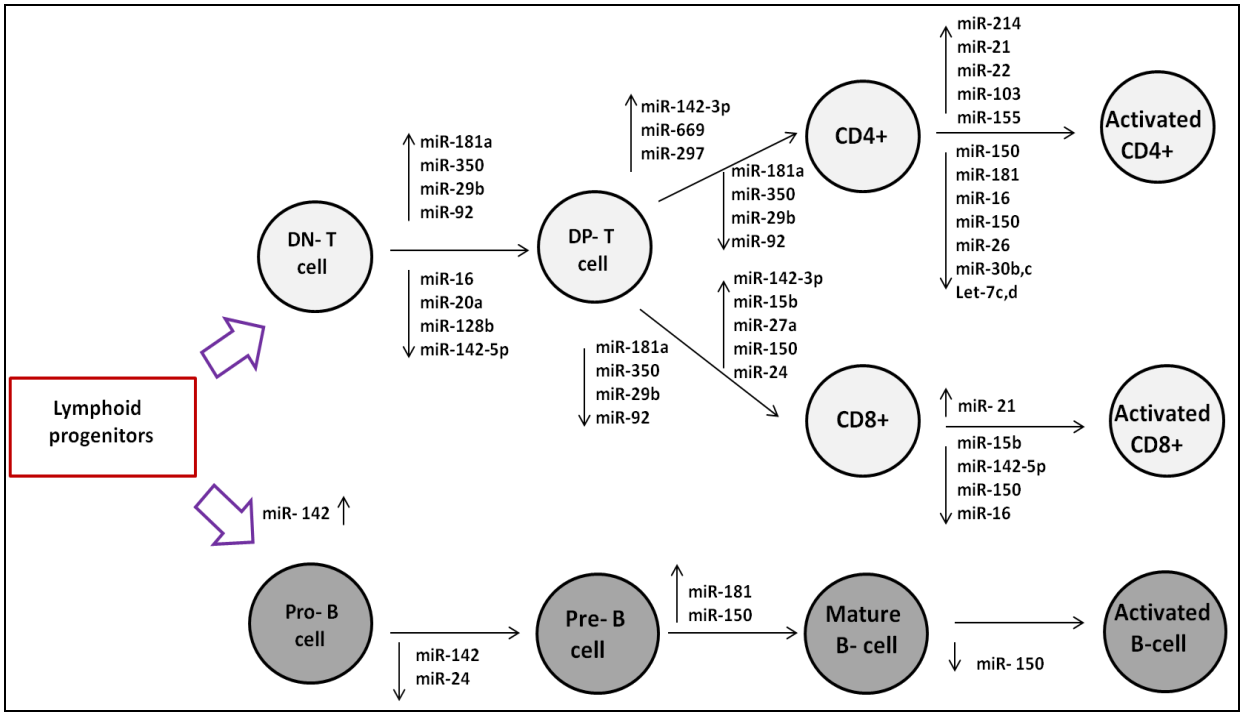


Figure 5. miRNAs in lymphoid progenitor cell differentiation [Sonkoly et al, 2008].

1.9 miRNAs in myeloid cell development

Differential expression studies of murine miRNAs found up-regulation of many miRNAs in stem and progenitor cells compared to differentiated cells, including miR-125b, miR-196a, miR-196b, miR-130a, let-7d, miR-148b, and miR-351, whereas other miRNAs such as miR-140, miR-200c, miR-484, miR-331, and miR-324–5p were down regulated in stem and progenitor cells compared to differentiated cells [**Petriv et al, 2010**]. Comparative study of myeloid and lymphoid cells found miR-223 up-regulation in myeloid cells, whereas, its expression is down-regulated in lymphoid cells. The expression of miR-223 is higher in GMP compared to MEP [**Petriv et al, 2010**] and it show up-regulation in granulocytes compared to monocytes [**Johnnidis, et al, 2008**]. miR-155, miR-222, miR-424 and miR-503 partially promote monocytes differentiation and combination of these four miRNAs show more positive effects on monocytes differentiation compared to their individual activity [**Forrest et al, 2010**].

Wang et al. showed positive correlation between miRNAs-mRNAs like miR17/C13orf25, miR93/MCM7 and miR22/MGC14376 during monocytes differentiation **[Wang et al, 2011]**. miR-155 was found to play role in macrophage differentiation from monocytes [**Hornstein et al, 2010**]. Fontana et al.(2007) reports down regulation of miR-17-5p-20a-106a during monocyte differentiation, and transfection with miR-17-5p-20a-106a results in enhanced blast

proliferation and inhibition of monocytes differentiation and maturation by targeting AML1 protein expression, leading to M-CSF receptor down regulation [**Forntana et al, 2007**]. miR-21 and miR-196b are inhibited by Gfi1 which regulates the normal granulocyte differentiation [**Velu et al, 2009**]. Recent study shows that miR-142-3p is a negative regulator of CSF1 induced macrophage differentiation by targeting Egr2. CSF1 enhance the expression of Egr2 which in turn down regulate mir-142-3p and promotes monocyte differentiation in cooperation with Spi-1/PU1by increasing the expression of cFMS [**Lagrange et al, 2013**].

The role of certain miRNAs has also been identified in erythropoiesis in past 10 years research work. miR-221 and miR-222 were down regulated during the early stage of erythropoiesis, thereby unblocking the expression of c-kit, a receptor of SCF which has important role in early erythropoiesis to regulate proliferation and survival of erythroblast [**Felli et al, 2005, Gabbianelli et al, 2010**]. Another miRNA, miR-223 is also down regulated during erythroid differentiation, which targets LMO2, an essential factor for erythroblast differentiation [**Felli et al, 2009**]. Reports suggest that miR-320 is down regulated during erythroid differentiation and its target SMAR1 3'-UTR region **(Fig 6B)**. SMAR1 is known to regulate erythroid differentiation by targeting miR-221 and miR-222 [**Mittal et al, 2013**]. miR-24 is highly expressed in HSC and down-regulated during erythroid differentiation. It interferes with activin signaling by down-regulating ALK4 expression and reduce the erythroid burst-forming units and colony-forming units [**Wang et al, 2008**]. miR-144 along with miR-451 is found to have role in erythroid development, they show increased expression during erythropoiesis and their expression is regulated by GATA-1 [**Bartel, 2009, Chen et al, 2004, Li et al, 2007, Neilson et al, 2007**]. Human CD34+ HSPCs shows high expression of miR-155 and it is down-regulated during myeloid differentiation [**Georgantas et al, 2006**]. Two individual studies also found inhibitory effects of miR-155 on erythroid differentiation [**Georgantas et al, 2006**] and megakaryocyte differentiation [**Romania et al, 2008**]. miR-150 is moderately expressed in MEP and progressively down regulated during the erythropoiesis, whereas increased expression is observed during megakaryocytopoiesis [**Lu et al, 2008**]. miR-150 regulates erythroblast-megakaryoblast fate decision by targeting c-myb [**Lu et al, 2008**].

Barroga et al. reported that thrombopoietin is a regulator of megakaryocytopoiesis which controls c-myb level by increasing the expression of miR-150 **[Barroga et al, 2008]**. The expression of miR-223, miR-15a and miR-16-1 cluster is down regulated during the

megakaryopoiesis, but after 14 days of culture, the levels were comparable to that of CD34+ cells, whereas, miRNAs like miR-17, miR-20, miR- 106, miR-155,10a 126, 10b and miR-181b were down regulated during megakaryopoiesis [**Garzon et al, 2006**]. CXCR4-SDF-1 signaling play important role in MKs differentiation, where, miR-126 inhibits this signaling by targeting CXCR4 mRNA. PLZF-miR-146a-CXCR4 form a positive feedback loop required for megakaryopoiesis **(Fig 6 A)**. PLZF inhibit the expression of miR-146 and in turn promotes the expression of CXCR4 [**Zhang et al, 2012**].

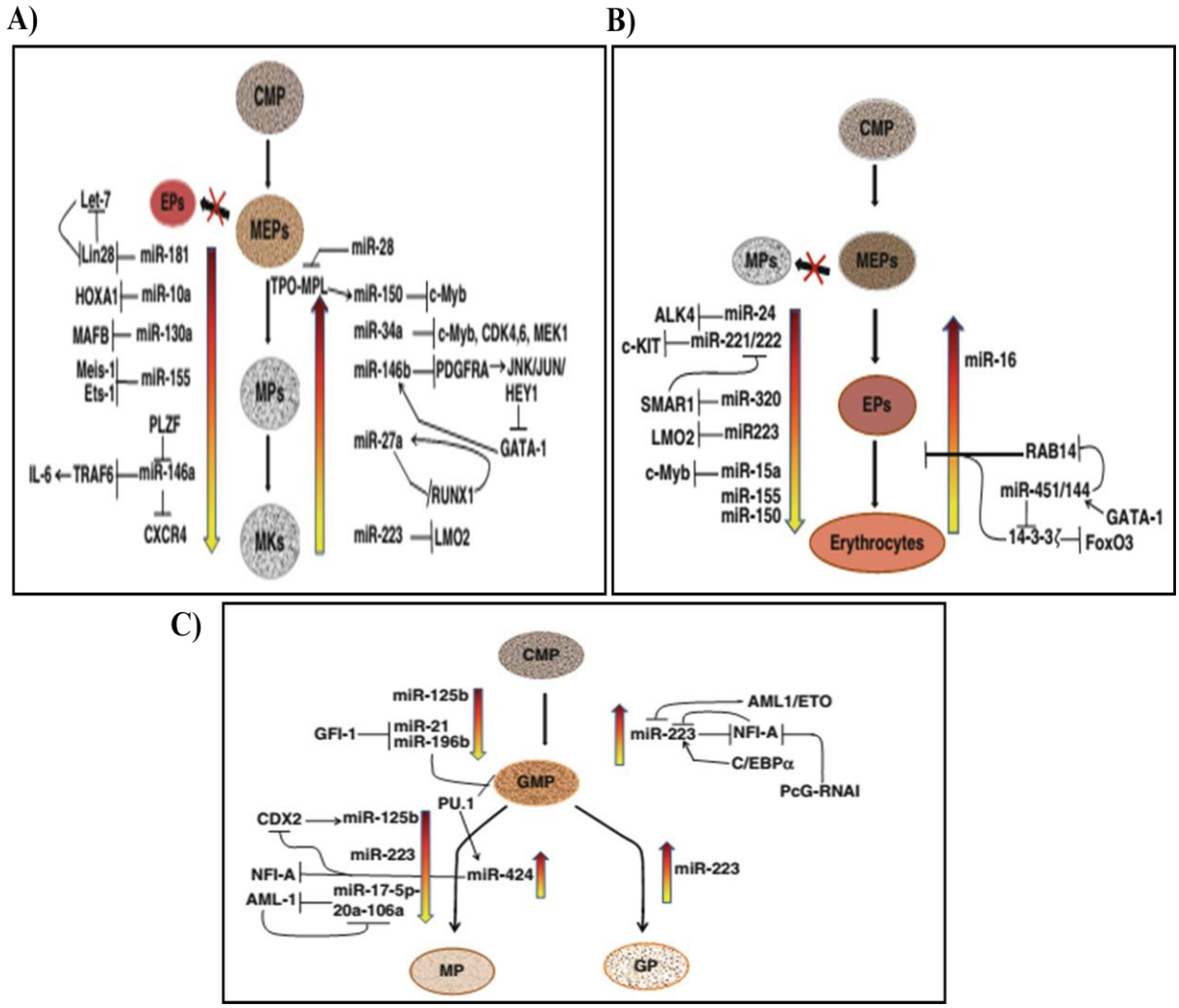


Figure 6. miRNA regulation in myeloid pathways. A) Schematic representation of role of miRNA in megakaryopoiesis B) Schematic representation of role of miRNA in erythropoiesis C) Schematic representation of role of miRNA in grnulopoiesis and monopoiesis. **[Aadapted from Raghuwanshi et al, 2015].**

1.10 miRNAs role in hematological disorders

Common blood disorders include hematopoietic cell malignancies or leukemias and plasma cell dyscrasia, all of which have associated microRNA abnormalities. Hematological malignancies are type of cancers which primarily affects bone marrow, blood and lymph nodes. These are arising from two major blood lineages such as lymphoid (lymphoid leukemia, Lymphomas) and myeloid (acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and myelodysplastic syndromes (MDS)) cell lines. Recent studies suggest that epigenetic complex regulatory programme controls the differentiation of HSCs and generation of different lineages of blood cells from progenitor cells. Aberrant interactions between epigenetic regulators and transcription factors induce alterations in blood cell differentiation system that causes hematological disorders.

Recent studies showed deregulation of small non-coding RNAs particularly miRNAs leading to cause several hematological disorders. Genome wide expression profile analysis in 52 acute myeloid samples with common translocations showed distinct miRNA expression pattern which includes miR-126/126* (t(8;21)/AML1(RUNX1)-ETO(RUNX1T1), and inv(16)/CBFB-MYH11), miR-224, miR-368, and miR-382 (t(15;17)/PML-RARA) and miR-17-92(MLL rearrangements). Among these miR-126/126* was associated with demethylation of promoter of genomic locus **[Li et al, 2008]**. miR-223 is inactivated by hyper methylation of ifs upstream elements in AML.miR-221 is over-expressed in AML and play important role in oncogenesis. Alterations in miR-21, 203, miR-451, miR-138, miR-2121 are related to the chronic myeloid leukemia (CLL) **[Undi et al, 2013]**. Previously, miRNA expression profile analysis in ALL and normal CD19+ samples reported the involvement of miR-204, miR-218,miR-331,miR-181b-1 and miR-128b in hematological malignancies, with miR-128b was more described miRNA in ALL. miR-17-92 cluster was up-regulated in ALL samples **[Francesca et al, 2009; Zanette et al, 2007]** (**Figure 7**). These high throughput studies suggest that differences between the normal and malignant cells, but underlying the molecular mechanisms are not known completely. This information is useful for further studies uncover mechanisms in epigenetic deregulation of miRNA and help in treatment of leukemia.

Computational analysis have predicted of hundreds of single nucleotide polymorphisms (SNPs) located within miRNA-binding sites and experimentally validated. Recent studies have evidenced that there is strong negative selection on SNPs in miRNA binding sites (miRSNPs) when compared to the entire 3′UTR sequence **[Chen et al, 2006; Yu et al, 2007;**

Saunders et al, 2007] and have shown functional significance of those sites. Several reports have evidenced the involvement of miRSNPs with cancer and other diseases.

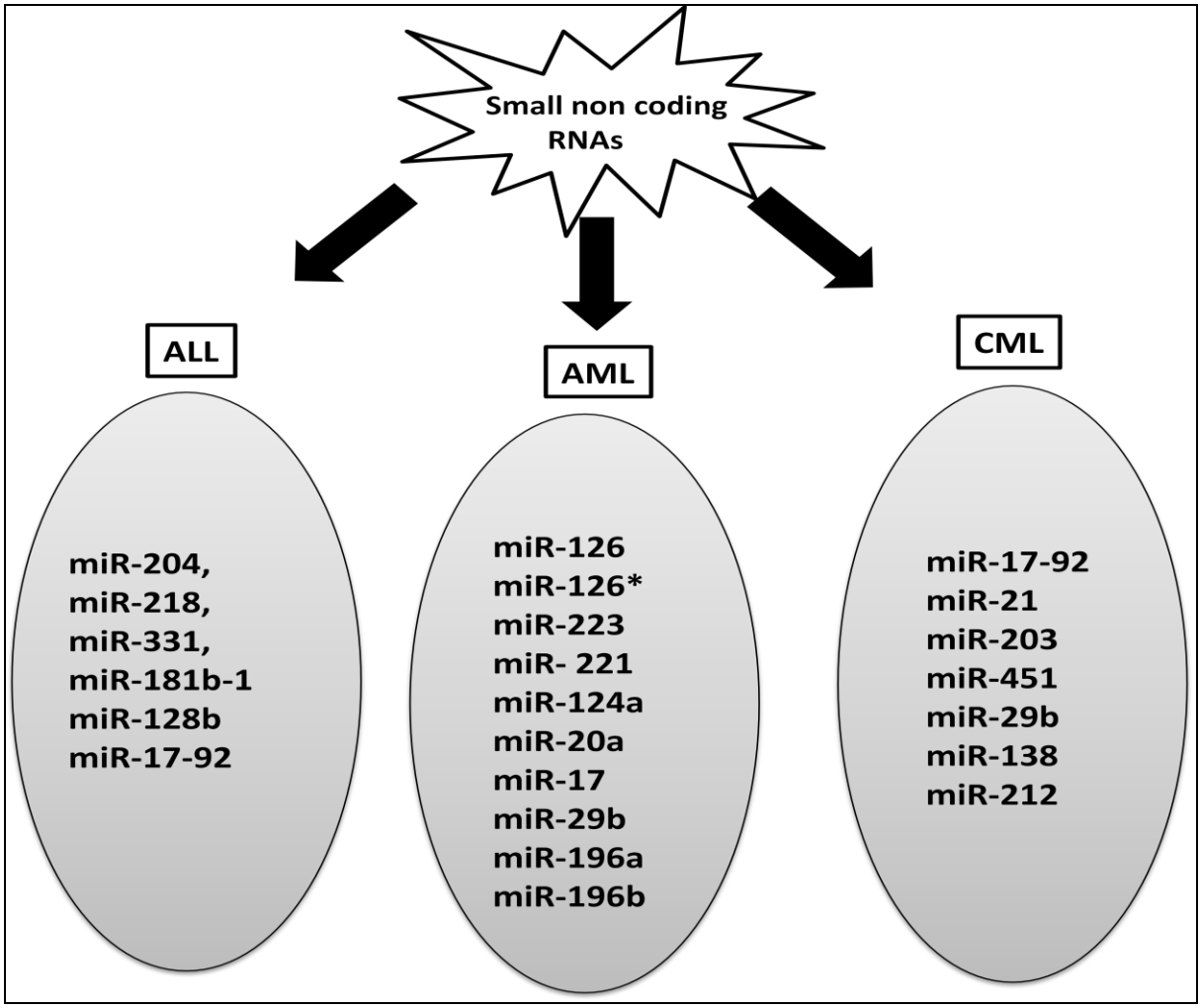


Figure 7.Shematic representation of deregulated miRNAs in ALL, AML and CML.

Recent studies have evidenced involvement of miRNAs as key regulators of signaling cascades that regulate cell fate and the function of normal and pathological lymphoid progenitor/precursor cell. Several studies about miRNA expression profiles of lymphomas could provide the key roles of miRNAs in development of lymphoma pathogenesis. Latest studies have shown miRNA as promising agent in the role of diagnosis, prognosis, and treatment but also become the prospects for novel therapeutic strategies [**Raghuwanshi et al, 2015**]. The role of miRNAs in development and pathogenesis is shown to be evidenced increasingly *in vitro* and *in vivo* that ectopic expression or silencing of specificmiRNAs lead to alter signaling of malignant cell survival. Recent studies have demonstrated that onco-miR silencing by miRNA mimics suppresses the tumour growth.

In current study, we discussed the role of two miRNAs, namely miR-125b and miR-99a which are located on chromosome 21 and are involved in megakaryocyte development.

1.11 hsa-miR-125b

hsa-miR-125b is small non-coding RNA, which is a highly conserved across the species from nematode to humans. Though there is nomenclature difference in nematodes but seed region is conserved in all species. In viral genome, ebv miR-BART21-5p, ebv miR-BART8 and rlcv-miR-rL1-25 have maximum concordance of six nucleotides with miR-125b. It exist as different paralogs and varies in different species, such as in zebra fish, it comprise of three parlogs, whereas, only one copy of miR-125b exists in worms and chimpanzees. Human genomic region has two miR-125 paralogs, such as miR-125b-1 and miR-125b-2. But same mature miRNA sequence is generated from these two precursor structures such as pre-miR-125b-1 (located on chromosome 11q24.1) and pre-miR-125b-2 (located on chromosome 21q21.1). The stem loop structure of miR-125b-1 has 88 bases and is located on inter genic region, whereas, miR-125b-2 has 89 nucleotide length, which is located on intronic region of C21orf34. The mature miR-125b resides between 17th and 38th nucleotide of precursor miR-125b having 22 nucleotide length and sequence of hsa-miR-125b is UCCCUGAGACCCUAACUUGUGA [**Shaham et al, 2012]**.

Along with miR-125b paralogs, it also exist as homologs which differ in their mature sequence, but homologs of miR-125b has same seed region and shows similar functions. In human miR-125a is homolog which is located on chromosome 19, whereas in other species there are three homologs encoded on different chromosomes (miR-125a, miR-125b and miR-125c) having same seed region. Interestingly, mouse and rat encoded miR-351 shows same seed region of miR-125b **(Fig 8)**.

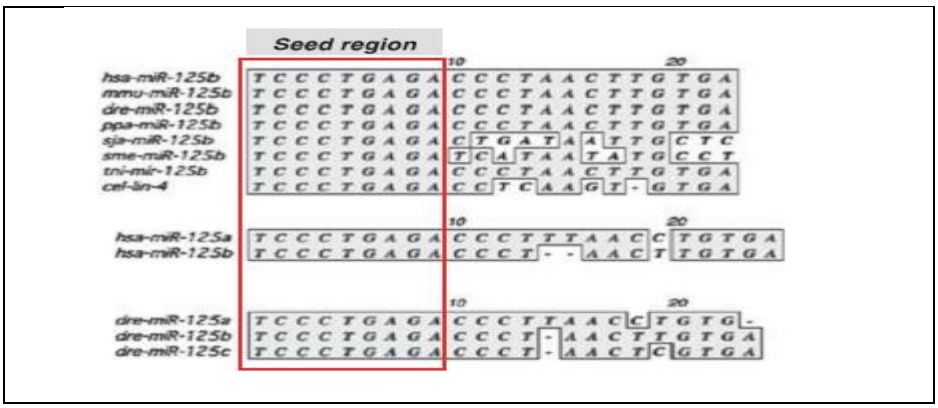


Figure 8. The seed sequence of paralogs and homlogs of miR-125 is conserved in different species [Shaham et al, 2012].

hsa- miR-125b is organized as cluster on different chromosomes along with other miRNAs. MiR-125b-1 paralog located on chromosome 11 cluster with let-7a-2 and miR-100, whereas miR-125b-2 cluster with miR-99a and let-7c located on chromosome 21 **(Fig 9)**. Another cluster on chromosome19 encodes miR-125a, miR-99b and let-7e. All these miRNA clusters are close to each other with distance of less than 1Kb. These clusters suggest that miR-125a has only one promoter and transcribed as one transcript, whereas, miR-125b have alternative promoter to transcribe as alone.

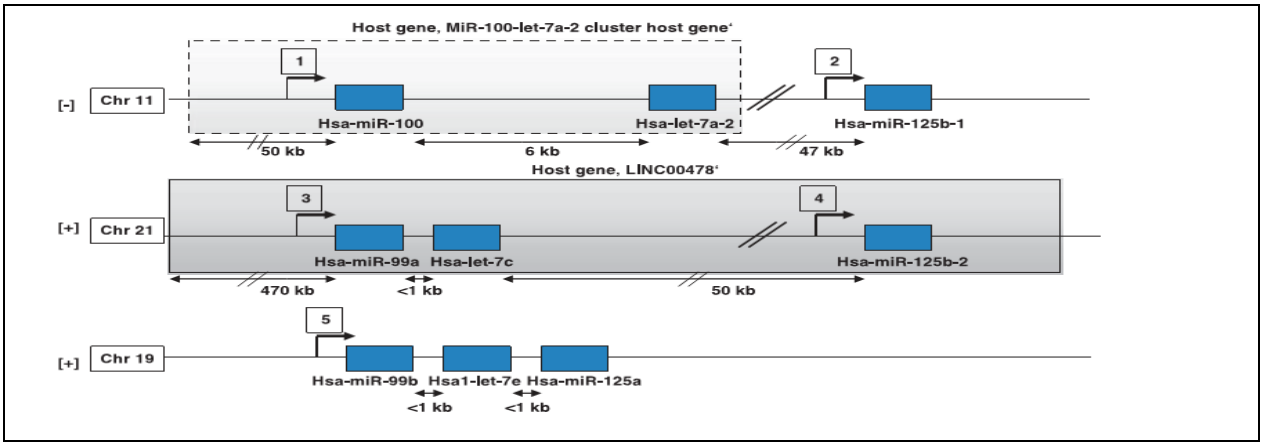


Figure 9. Genomic organization of miR-clusters containing miR-125 shown together along with other miRNA and their potential promoter regions [Shaham et al, 2012].

1.12 miR-125b and functions

miR-125b is the mammalian homolog of lin-4 miRNA which is expressed during embryonic development of *C.elegans* [**Lee et al., 1993**]. Though they performed several studies on lin-4, but the mammalian miR-125b functions are still not characterized, and knockout mouse studies on miR-125b are currently not known. Loss and gain functions of miR-125b family play important role in different cellular processes like cell proliferation, differentiation and apoptosis **(Fig 10)** by regulating different transcription factors [**Bousquet et al, 2012**]. miR-125b is a known oncomir and has been implicated in the pathogenesis of leukemias, B-cell lymphomas, breast cancer, squamous cell carcinoma, urothelial carcinoma, prostate carcinoma and colon cancer **[Guan et al, 2010; Huang et al, 2010; Hui et al, 2010]**. Several microRNAs are located at fragile or cancer-associated genomic regions in the human genome **[Calin et al, 2004]**, It is no surprise that aberrant expression of many different microRNAs has been observed in multiple cancer types. In particular, a significant number of studies have shown that dysregulated expression of miR-125b is correlated with human leukemia. B-cell precursor acute lymphoblastic leukemia (BCP-ALL) showes a genetic aberration with insertion or translocation of miR-125b-1 into the immunoglobulin heavychain (IGH) locus and exhibited elevated miR-

125b-1 expression compared with normal control **[Chapiro et al, 2010; Enomoto et al, 2011; Tassano et al, 2010]**. Also, patients with myelodysplastic syndromes (MDS) who harbor t (2, 11) (p21;q23) or 5q- exhibit increased expression of miR-125b-1 compared with healthy subjects (up to 70-fold) **[Bousquet et al, 2008; Hussein et al, 2010; Votavova et al, 2011].** Similarly, acute myeloid leukemic (AML) patients carrying t (2, 11) (p21;q23) translocation or FLT3 mutation also have higher expression of miR-125b. In pediatric AML patients miR-125b shows ~200 fold up regulation compared to normal CD34+ cells **[Cammarata et al, 2010].**It is also specifically upregulated in acute megakaryoblastic leukemic (AMKL) and transient leukemic (TS) patients with trisomy 21 (Down syndrome) **[Zhang et al, 2009].**The common upregulation of miR-125b in leukemias of different cell origin suggests that it might serve as a cancer inducer or driver of cancer cells.

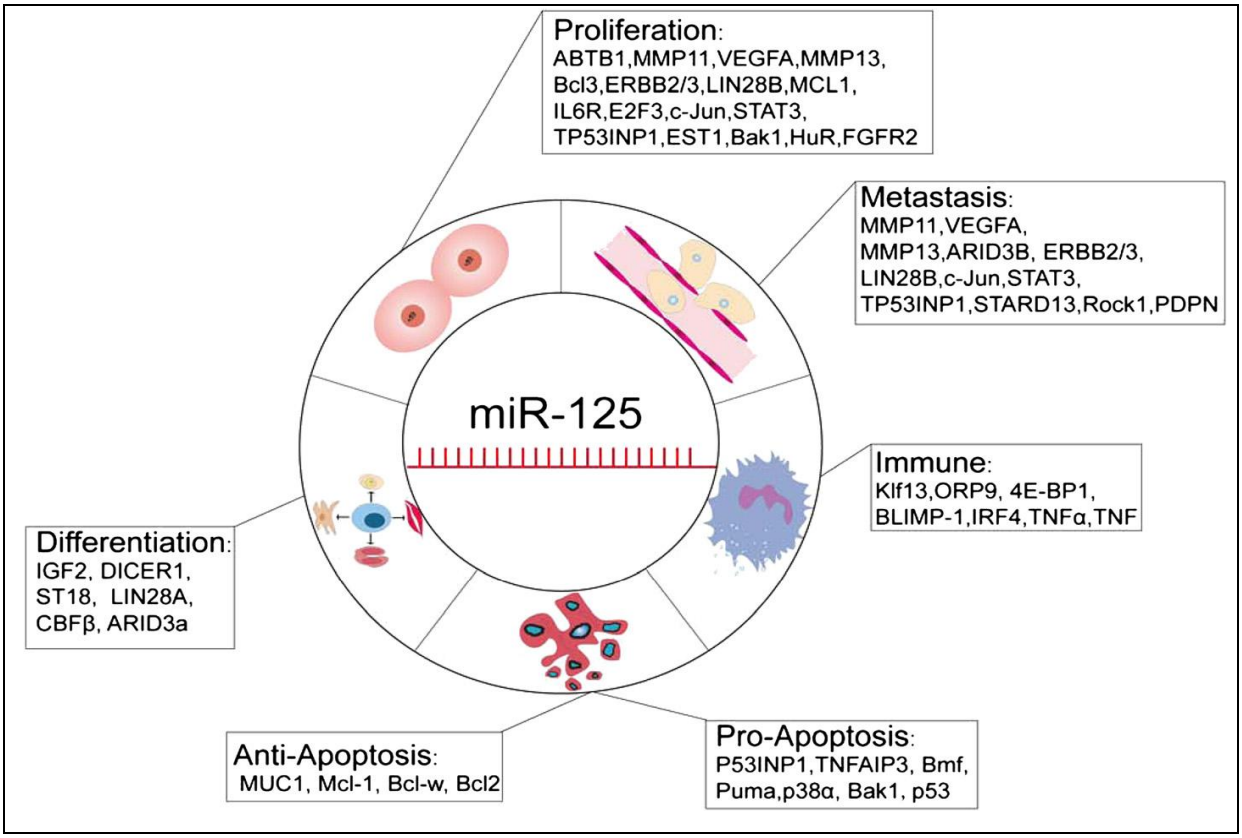


Figure 10. miR-125b roles in different physiological processes by regulating several target genes [Adapted from Yu-meng sun et al, 2013].

It is also involved in hematopoiesis by regulating cell proliferation and differentiation [**Connell et al, 2010**]. Previous studies showed that miR-125, miR-99a and let-7 levels are lower during HSC differentiation. The expression pattern of these miRNAs are as follows in mouse model: HSCs > multiplepotent progenitors (MPPs) > common myeloid progenitors (CMPs) >granulocyte macrophage progenitors (GMPs) >megakaryocytic erythroid progenitors (MEPs) [**Ooi et al, 2010**].

1.13 hsa-miR-99a

Hsa-miR-99a has two homologs, hsa-miR-99a and hsa-miR-99b, which contains same seed region **(Fig. 11).** hsa-miR-99a is located on chromosome 21, whereas, hsa-miR-99b is located on chromosome 19 and is associated with other miRNAs, hsa-miR-125b and hsa-miR-125a respectively.

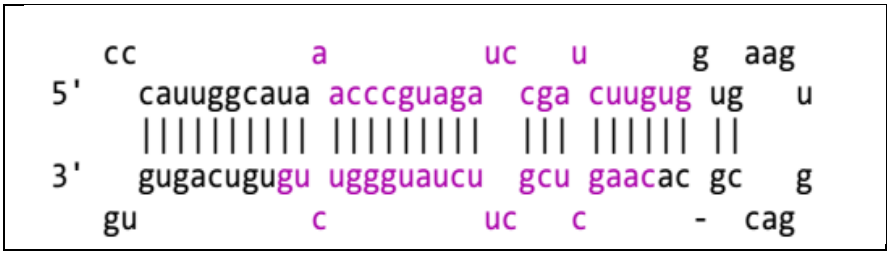


Figure 11: Stem loop structure of hsa-miR-99a

1.13 miR-99a and its roles:

Previous reports suggest that miR-99a is upregulated in Acute myeloid leukemia (AML) and Chronic myeloid leukemia (CML) patients. It acts as oncogene and promotes cell proliferation by inhibiting the apotosis in K562 and HL60 cells. But in Prostate cancer cell line, miR-99a family members, miR-99a, miR-99b and miR-100 were down regulated. Transfection of these miRNAs decrease the growth of cells by inhibiting prostate-specific antigen (PSA). Several reports in the past show that miRNA might function as an oncogene or tumor suppressor in different cancers. MiR-100 and miR-99a are aberrantly expressed in acute leukaemia and vincristine resistant acute lymphoblastic leukemia**[Li et al, 2013; Akbari Moqadam et al, 2013].** Increased expression of mTOR kinase in endometrioid endometrial cancer coexists with down-regulation of its targeting miRNAs, miR-99a, miR-100 and miR-199b **[Torres et al, 2012]**. miR-99a/100, let-7, and miR-125b functionally converge at the combinatorial block of the transforming growth factor β (TGFβ) pathway by targeting four receptor subunits and two SMAD signaling transducers. In addition, down-regulation of tumor suppressor genes adenomatous polyposis coli *(*APC*)*/APC2 stabilizes active β-catenin and enhances Wnt signaling. By switching the balance between Wnt and TGFβ signaling, the concerted action of these tricistronic miRNAs promoted sustained expansion of murine and human HSCs in vitro or in vivo while favoring megakaryocytic differentiation **[Emmrich et al, 2014]**.

OBJECTIVES

Thrombocytopenia, defined as platelet count <150 x 10^9/L is common among sick infants (upto 50% of infants admitted to the Neonatal Intensive Care Unit). Neonates are affected by disorders of megakaryocytopoiesis that predominantly or exclusively present during this developmental stage, including a syndrome of megakaryocytic hyper proliferation known as transient myeloproliferative disorder (TMD) and the thrombocytopenia associated with the thrombocytopenia-absent radius (TAR) syndrome. It has been hypothesized that developmental differences between neonatal and adult megakaryocytes (MKs) contribute to the vulnerability of neonates to develop severe thrombocytopenia. The regulatory mechanisms underlying these developmental differences are unclear, but we hypothesize that epigenetics may play a critical role in the regulation of developmental megakaryocytopoiesis. Recent advancements in the rapidly evolving field of epigenetics have shown extensive reprogramming of every component of the epigenetic machinery including DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs, specifically microRNA expression. microRNAs (miRNAs) are short, endogenous non-coding RNAs. The abnormal expression of miRNAs may be valuable for the diagnosis and treatment of various diseases. We were interested in miRNA-regulated gene targets, which enabled us to understand miRNA functions. We hypothesize that miRNAs are differentially expressed in neonatal and adult megakaryocytes and, therefore, might contribute to their phenotypic differences. Our preliminary studies shows ten miRNAs were expressed at significantly higher levels in CB compared to PB-MKs (2 to 21 fold, $p<0.05$). Interestingly, we found that two miRNA, namely miR-99a and miR-125b are located on same chromosome 21. We further investigated how miR-99a and miR-125b regulates in neonatal megakaryocyte proliferation. Based on these preliminary findings we framed the following **objectives**:

1) **Assessment of role of miR-99a and its regulated signaling pathway during megakaryocyte development.**

2) **To investigate the role of miR-125b and its regulated signaling pathway during megakaryocyte development**

3) **To establish murine model of megakaryocytopoiesis**

   a) **To understand the differential expression of miR-99a and miR-125b at neonatal and adult stages.**

   b) ***In silico*** **analysis of miR-99a and miR-125b regulated signaling pathways.**

MATERIALS AND METHODS

Materials and methods

Human Cell Cultures

Human cord blood (CB) and peripheral blood (PB) $CD34^+$ cells were obtained from TranScell Technologies, India (n=3 for each group). The cells were cultured in serum free Iscove's Modified Dulbecco's Media (IMDM) with thrombopoietin 50ng/mL (PeproTech Inc.) as previously described [**Pastos et al, 2006**]. After 14 days of culture, we obtained >90% of MKs expressing $CD41^+$ by flow cytometric analysis.

For the study, we majorly used two cell lines, DAMI and K562. The megakaryoblastic cell line **DAMI** is derived from the peripheral blood of a patient with megakaryoblastic leukaemia. These cells have characteristics of megakaryoblasts or immature megakaryocytes, displaying many of the morphologic and biochemical features of the megakaryocytic lineage, whereas, **K562 cells** were the first human immortalised myelogenous leukemia line and are of the erythroleukemia type. The Dami and K562 are suspensions cells that were obtained from CMC Vellore (India) and National Center for Cell Science (Pune, India) respectively. These cell lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (GIBCO), 10% fetal bovine serum (FBS), 1% antibiotics and maintained in 5% CO2 incubator at 37^oC. These cells can be induced to differentiate further along the megakaryocytic lineage by the tumor-promoting phorbol ester, phorbol myristate acetate (PMA). PMA-stimulated DAMI cells increase their ploidy and the expression of GP IIb/IIIa and Ib, both characteristic markers of the megakaryocytic membrane.

All investigations were performed in accordance to the local laws and regulations. This work was carried out with the Institutional Ethics Committee (IEC) approval.

Megakaryocyte cultures from mice Newborn liver and Adult bone marrow.

Wild types (C57BL/6-SV129J) were housed in pathogen-free, temperature- and light-controlled conditions (20 °C), with an alternating light-dark cycle with 14 h of light and 10 h of darkness). All of the animal studies were approved by the University of Hyderabad IAEC. The animals were killed by asphyxiation with CO_2 and decapitated. We obtained newborn liver (NL) cells from one day old pups, and bone marrow (BM) cells from 6 week old adult mice. Single cell suspensions generated from these samples were cultured for five days in DMEM medium supplemented with 10% fetal calf serum and 50 ng/mL of Tpo. After this culture period, MKs were enriched by passing through an albumin gradient, and the purity of

the collected cells was determined by acetylcholinesterase (AChE) staining. More than 80% of the cells collected with this process were MKs (AChE+).

Trypan blue exclusion test of cell viability

The trypan blue exclusion test is useful for the number of viable cells present in a cell suspension. Dami cells were nucleofected with miR-99a mimetic and miR-99a inhibitors along with scrambled control for 48h. After nucleofection, harvest cells and re suspended in the 1ml of PBS. One part of the cell suspension was mixed with one part of 0.4% trypan blue and was incubated for 3 min. Living cells were unstained and appeared clear while dead cells were stained blue.

Megakaryocyte maturation markers by FACS analysis

K562 cells (1×10^6 cells) were treated with 40nM PMA for 72 hours and then the cells were harvested. These cells were washed two times with PBS and re-suspended in 100µl PBS. Then 10 µl of mouse anti-human PE-conjugated CD61 or CD41 monoclonal antibody was added to 100 µl of the cell suspension, and incubated for 30 min in dark at 4^oC. After washing, we analyzed the cells by flow cytometry for three independent samples.

Total RNA and miRNA isolation

Total RNA including miRNA were extracted from mature CB and PB MK cells (n=3 each) or transfected cells using the miRNeasy mini kit (Qiagen), according to the manufacturer's instructions. The RNA extraction was performed by using QIAzol (Qiagen) to lyse the cells. Chloroform (Sigma) was added and the samples were thoroughly mixed followed by centrifugation at 12000g for 15min. This resulted in a phase separation and the RNA was obtained in the upper water phase. The upper phase was transferred to a Qiagen Spin Column (Qiagen) and the RNA was washed with RPE and RWT buffers which was bound to the filter in the Spin Column. Finally the RNA was eluted using RNase free water. RNA was quantified using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific).

C-DNA synthesis

1) Preparation of cDNA from mRNA

Gene expression can be quantified by measuring the expression of its transcribed mRNA. Before mRNA can be quantified by qRT-PCR (qPCR), the mRNA needs to be transcribed into cDNA. The enzyme reverse transcriptase is used for this. The template for the cDNA

synthesis is RNA and the primers bind either to the poly (A) tail of the mRNA or randomly to the RNA and transcribe it to cDNA.

Reaction I

Component	Volume
OligodT(50uM)/ random hermers	1 µl
10uM dNTPs	1 µl
RNA 10pg-50ug	Variable

Table 1: Reverse transcription reaction 1 components for mRNA into cDNA

After cDNA synthesis, we made up the volume to 13 µl reaction with distilled water and incubated at 65^0 C at 5min.

Reaction II

Component	Volume
5x first standard Buffer	4 µl
0.1M DTT	1 µl
RNase Out	1 µl
Super Script III RT	1 µl

Table 2: Reverse transcription reaction 2 components for mRNA into cDNA.

Collected Reaction-I by brief centrifugation and added 7 µl of Reaction II followed by brief mixing. We Incubated the samples at 50^0 C for 30 min and inactivated the reaction by heating at 70^0 C for 15 min.

2) Preparation of cDNA from miRNA

For miRNA quantification, total RNA (1 µg) was reverse transcribed using miScript II RT c-DNA synthesis kit (Qiagen).

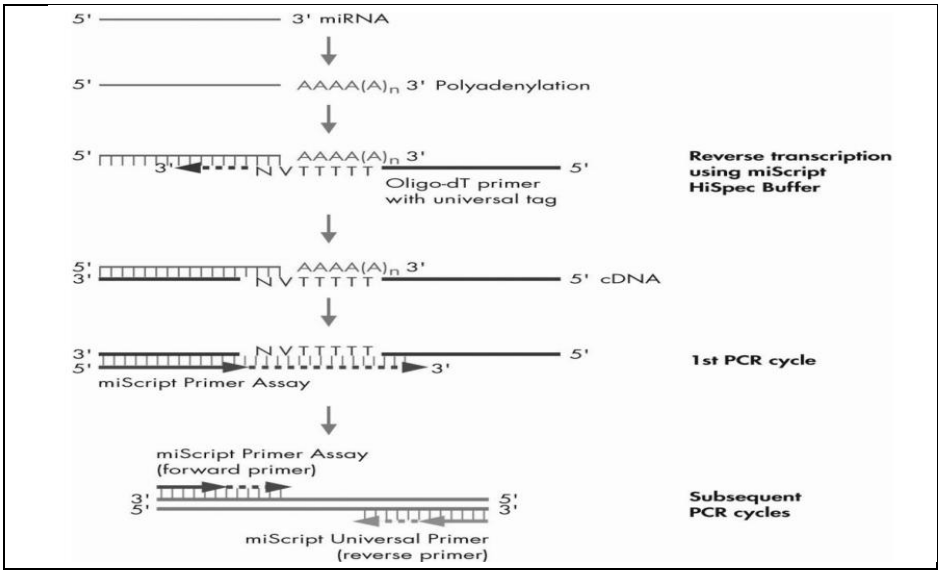


Figure 1. Synthesis of cDNA from mature miRNAs and subsequent detection. In a reverse-transcription reaction using miScript, HiSpec Buffer and mature miRNAs are polyadenylated by poly (A) polymerase and subsequently converted into cDNA by reverse transcriptase with oligo-dT priming. The cDNA is then used for real-time PCR profiling of mature miRNA expression [EN-miScript-miRNA-PCR-Array-Handbook.pdf].

Total RNA containing miRNA was used as the starting material. The miScript II RT has two buffers 5x miScript HiSpec Buffer (cDNA for mature miRNA) and 5x miScript HiFlex Buffer (cDNA for miRNAs in parallel with precursor miRNAs, mRNAs). Prepare the reverse-transcription master mix on ice having following components:

Component	Volume
5x miScript HiSpec Buffer or 5x miScript HiFlex Buffer	4 µl
10x Nucleics Mix	2 µl
RNase-free water	Variable
miScript Reverse Transcriptase Mix	2 µl
Template RNA	Variable
Total volume	**20 µl**

Table 3: Reverse transcription reaction components for miRNA to cDNA

Added the template RNA to each tube containing reverse-transcription master mix. We mixed the components gently, briefly centrifuged, and then placed the samples on ice. Incubated for 60 min at 37ºC and further incubated for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and placed on ice.

Real time PCR analysis

The first strand cDNA was used as a template in real time PCR with SYBR Green Master Mix in ABI StepOne Plus system (Applied Biosystems) to detect the CTDSPL, CD41 and CD61 expression with respective specific primers. The cycling program was set as follows: denature at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the PCR products was verified by melting curve and agarose gel analyses. Each sample was assayed in triplicate, normalized to the level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and expressed as relative to PB and control miRNA (n=3) expression. Analysis of miR-125b and miR-99a miRNA was also determined using a quantitative PCR following the manufacturer's instructions with an ABI StepOne Plus system (Applied Biosystems). Values (n=3) were normalized against U6, and CB miRNA values were expressed as relative to miR-125b and miR-99a levels in PB cells at the same stage of differentiation.

Bioinformatics

Identification of putative targets was performed by using the target prediction algorithms *miRanda, TargetScan, RnaHybrid* and *PicTar* as well as the *David* database for functional pathway analysis.

Cell Transfection

MiR-99a mimetic and miR-99a inhibitor (miScript, Qiagen) was nucleofected into DAMI cells using Amaxa Nucleofector (Lonza), according to the manufacturer's instruction. Cells were recovered after 48h post nucleofection and miRNA and protein lysates were prepared. Cy3-labeled Anti-miR Control is a non targeting negative control for monitoring nucleofection efficiency in nucleofection experiments (Approx. 70%).

Western Blot

Proteins were extracted from at least three samples after 14 days of culture using using radioimmunoprecipitation assay (RIPA) buffer (sigma) in the presence of a mixture of protease inhibitors (Roche Applied Sciences). The extracts (50 µg) were subjected to separation on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Millipore) by electro-blotting. The membranes were blocked for 1h at room temperature in TBS-T buffer with 5% non-fat dried milk. These blots were incubated with specific primary antibodies (1:1000) CTDSPL (Abcam), p53, p21, BAK1, CDK6, Cyclin D1, Cyclin D2, Cyclin D3, E2F1, pRB, Bcl2, Bad (Santa Cruz) and β-Actin (Sigma) overnight at 4°C. The blots were washed three times for 10 min with TBS-T buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000), Goat anti-rabbit IgG and anti-mouse IgG (Cell Signaling) for 1h at room temperature. After washing these blots were developed using enhanced chemi-luminescence reagents (ECL, Pierce Biotechnology, Inc., Rockford, IL) and visualized using versa doc instrument (Bio-Rad). To quantify protein expression levels, we used Image-J software and measured the integrated density of the protein signal normalized to actin levels.

Determination of intracellular ROS

2, 7-dichlorodihydrofluoresceindiacetate (H2DCFDA) is a cell permeable dye was used to monitor the intracellular ROS levels from the PMA-activated K562 (1×10^6 cells/ml). Here, we measured the ROS from different experimental time points such as control, 24h, 48h, 72h treated with PMA (100ng/ml). Briefly, cells were washed with PBS and incubated with H2DCFDA dye (10µM) in PBS for 15 min in dark at room temperature. Green fluorescence of 2, 7-dichlorofluorescein (H2DCF) was measured using a flow cytometry (BD LSR FORTESSA) and mean fluorescence intensity (MFI) represented as bar graphs.

Statistical Analysis

Three independent experiments were carried out. Two-tailed Student T-tests were used to determine the statistical significance of differences between neonates and adults. Data was expressed as mean ± SD. P-value <0.05 was considered to be statistically significant.

Objective 1

Objective1. Assessment of role of miR-99a and its regulated signaling pathway during megakaryocyte development.

Growing body of evidence indicates that miRNAs are crucially involved in the regulation of differentiation in normal as well as malignant hematopoiesis. MiRNAs can silence specific target genes through direct mRNA degradation, translational repression or both. In regard to megakaryocytopoiesis, it was shown that the miRNA expression profile of neonatal and adult human bone-marrow derived $CD34^+$ cells changes during megakaryocytic differentiation. Previous studies on acute megakaryoblastic cancer cell lines have shown a significant up-regulation of miR-99a compared to in vitro-differentiated MKs indicating its possible role in cell proliferation **[Garzon et al, 2006]**. Reports suggested the involvement of miR-142 as actin cytoskeleton regulator of MK and chromosome 21 locus miRNAs such as miR-125b and miR-99a in megakaryoblastic leukemia **[Kandi et al, 2013; Pastos et al, 2006; Hu et al, 2012]**. Based on these findings highlighting the regulatory importance of miRNAs during megakaryocytopoiesis, we hypothesized that the miR-99a and miR-125b expression profiles between neonatal and adult MKs will differ and could contribute to their substantial biological differences. In order to better understand role of miRNA's at distinct developmental stages of megakaryopoiesis, we initiated a comparative study using cultured human neonatal and adult MKs.

miR-99a as a molecular regulator of developmental differences and proliferation

We compared miR-99a levels in primary MKs derived from cord blood (CB) and peripheral blood (PB) by quantitative PCR and found ~12-fold up-regulation (on day 14) in neonatal megakaryocytes compared to adults ($n=3$, $p<0.05$). Taken together, this data provide compelling evidence that miR-99a is differentially expressed in developmentally different CB- and PB-derived MKs.

We were interested in determining whether the differences in miR-99a expression between CB and PB cells are due to endogenous developmental differences in the $CD34^+$ progenitors or rather appear at some point during megakaryocytic differentiation as a consequence of growth factor (Tpo) treatment. To determine whether these differences were present at all stages of MK development, we harvested CB and PB cells on days 0, 7, 11, and 14 of MK culture, isolated miRNA, and determined miR-99a expression levels by quantitative PCR. Values in each sample were normalized against U6 (miRNA internal control), and CB miR-99a levels were expressed as relative values to the PB levels at the same stages of culture.

This analysis revealed that neonatal MKs expressed higher miR-99a at all levels, with highest (~12 fold, $p<0.05$) at day 14 in mature neonatal MKs (**Fig.1A**). We observed significant ($p<0.01$) down-regulation of miR-99a during different stages of differentiation in CB-MKs (**Fig. 1B**).

Our discovery that miR-99a expression was significantly down regulated in PB-MKs compared to CB-MKs led us to hypothesise that higher levels of this miRNA could be one mechanism utilized by CB-MKs to proliferate at much higher rate than PB-MKs. The expression levels of miR-99a in CB-derived cells were consistently higher indicating true developmental differences between the two hematopoietic progenitors. Since our culture system only employs Tpo as a growth factor, these results suggest miR-99a regulation downstream of Tpo by yet unknown molecular mechanisms.

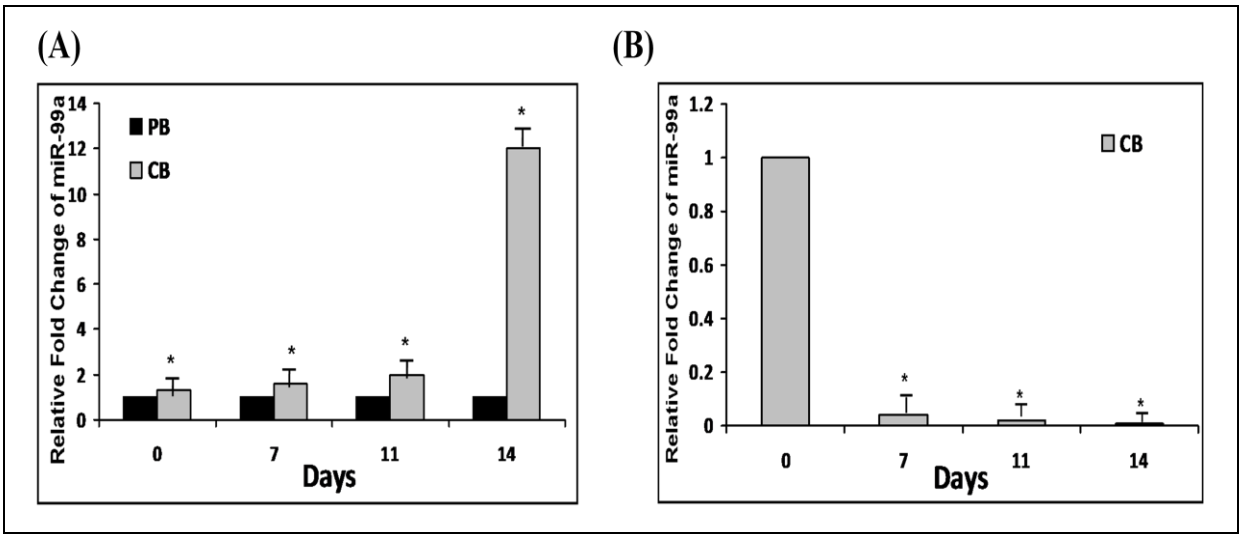


Figure 1: Expression of miR-99a and its target in CB- vs PB-MKs during differentiation of MK. (A) miR-99a levels in CB cells at different stages (day 0, 7, 11, 14) of MK differentiation in culture, expressed as relative to miR-99a levels in PB cells at the same stage of differentiation. Bars represent means ± SD of three independent experiments (*$p<0.05$). (B) miR-99a levels in CB cells at different stages (day 0, 7, 11, 14) of MK differentiation in culture, expressed as relative to miR-99a levels on day 0 of CB cultures. The differences between the groups were statistically significant at all time points (n=3, *$p<0.01$).

CTDSPL 3'-UTR is a target of miR-99a and regulated pathways by in silico approach.

To understand the relation between miRNA and mRNA association several computational based databases have been developed which predict possible targets of miRNAs by using

different algorithms. All these tools recognize miRNA targets based on seed region of miRNA and 3'UTR of target genes. Further we explored putative target of miR-99a using bioinformatics tools Target Scan, PicTar and miRBase. We found that CTDSPL as potential target of miR-99a from all the data bases. RNA hybrid tool also suggested miR-99a and CTDSPL duplexes showed minimum free energy (**Fig 2B**). Where CTDSPL 3'UTR binding site showed sequence conservation in several species and seed sequence complementarity to miR-99a **(Table1).** CTDSPL is an enzyme that controls the RNA polymerase II transcription machinery and could involve in cell cycle arrest by dephosphorylating RB.

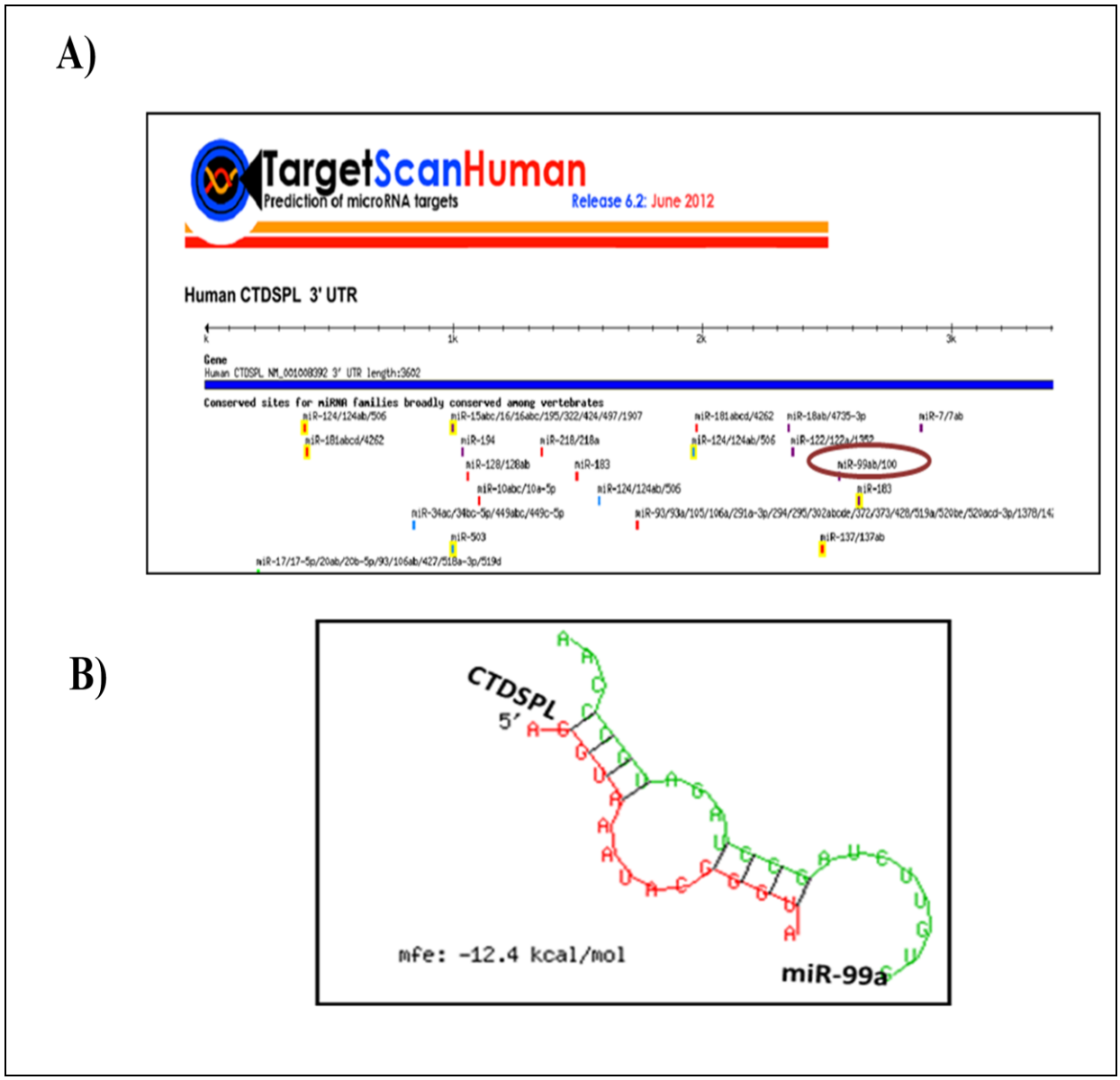


Figure 2: Bioinformatic analysis of miR-99a. (A) CTDSPL as target of miR-99a by target Scan. (B) Minimum free energy (MFE) duplexes of the miR-99a and CTDSPL 3'UTR analyzed by RNA hybrid.

Species	CTDSPL 3'-UTR seed (nt 2345-2352)
Human	AAGGUAAA*UACGGGU*
Chimpanzee	AAGGUAAA*UACGGGU*
Rhesus	AAGGUAAA*UACGGGU*
Mouse	AAGGUAAA*UACGGGU*
Rat	AAGGUAAA*UACGGGU*
Bush baby	AAGGUAAA*UACGGGU*
Guinea pig	AAGGUAAA*UACGGGU*
Shrew	AAGGUGAA*UACGGGU*
Hedgehog hog	AAGGUCAA*UACGGGU*
Dog	AAGGUAAA*UACGGGU*
Horse	AAGGUAAA*UACGGGU*
Cow	AAGGUAAA*UACGGGU*
Armadillo	AAGGUAAA*UACGGGU*
Elephant	AAGGUAAA*UACGGGU*
Tenrec	AAGGUAAA*UACGGGU*
hsa-miR-99a	CUAGCCUAG*AUGCCCA*

Table1: Conservation of miR-99a binding to CTDSPL across species. miR-99a binding site is fully conserved in different species which is complementary to seed region of CTDSPL gene.

In silico analysis of miR-99a regulated signaling pathways

In silico analysis showed that hsa-miR-99a was regulating 16 signalling pathways, particularly, cancer associated pathways, wnt signalling, calcium and actin cytoskeleton pathways **(Chart 1).** These pathways have previously been known to be regulators of MK development. Association with cancer related pathways suggests its involvement in cell proliferation.

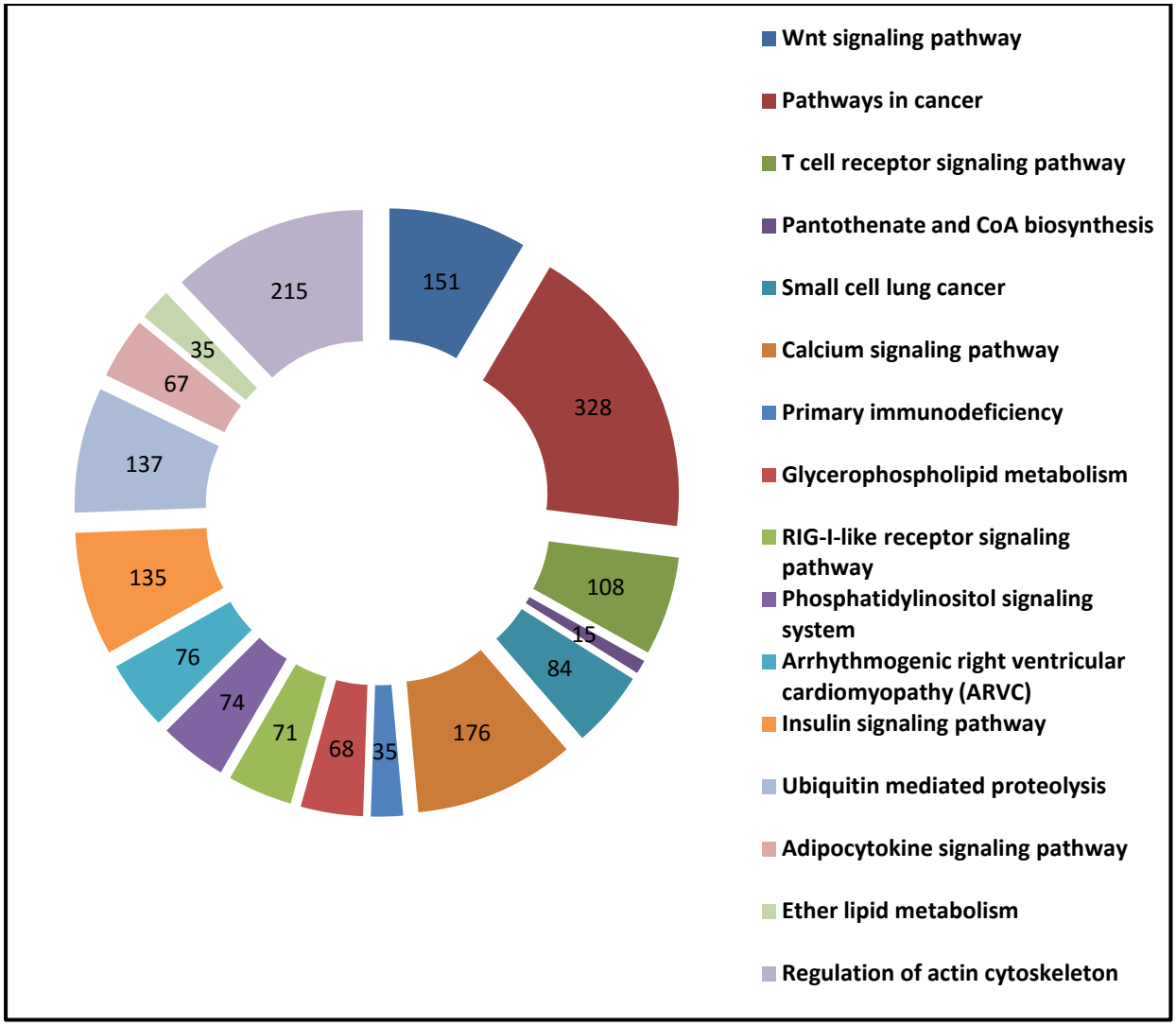


Chart 1. hsa-miR-99a regulated signalling pathways by *in silico* analysis.

Expression of CTDSPL mRNA and protein in CB- vs PB-MKs

We furthermore focused our analysis to explore the relationship between miR-99a and CTDSPL. Our microarray studies (Unpublished) carried out using *Affymetrix*® chips (human U133 plus 2.0), and data analyzed with the *Rosetta Resolver*® program using hierarchical clustering based on z-scores also showed CTDSPL as differentially expressed gene (3.4-fold difference, $p<0.01$). We reconfirmed the results using quantitative PCR using CB and PB MKs and found ~5-fold higher levels ($n=3$, $p<0.05$) of CTDSPL in PB compared to CB MKs (**Fig. 3A**). In addition, CTDSPL protein levels were approximately 4-fold higher ($p<0.05$) in PB compared to CB MKs (**n=3, Fig. 3B**).

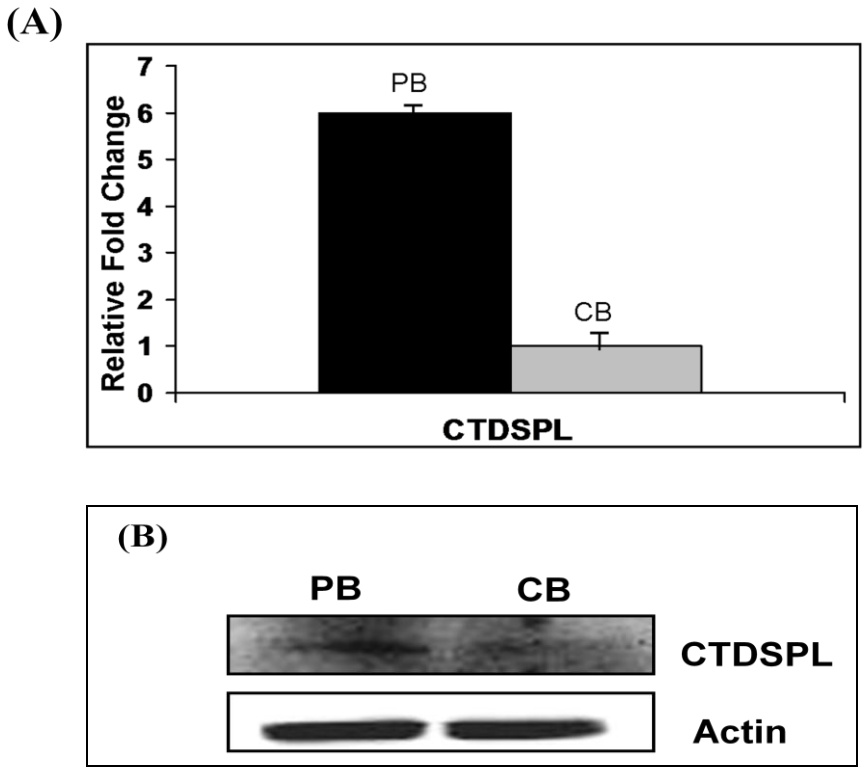


Figure 3: Expression of CTDSPL mRNA and protein in CB- vs PB-MKs. (A) Real time PCR quantification of the CTDSPL in CB and PB-MKs (day 14), (*p<0.05). (B) CTDSPL protein levels were lower in CB-MKs compared to PB MKs.

Transfection of miR-99a mimetic and inhibitor

To validate whether CTDSPL is a target of miR-99a, we performed nucleofection studies of miR-99a. DAMI cells (1 x 10^6 cells) were seeded in 6-well plate and miR-99a mimetic (20nM) and cy3-labeled negative control were nucleofected using standard methods. After nucleofection of miR-99a mimetic, RNA and protein lysates were prepared to validate CTDSPL as target. miR-99a was up regulated (~8 fold) in nucleofected cells as compared to control by real-time analysis (**Fig 4A**). CTDSPL protein levels were decreased in nucleofected DAMI cells upon introduction of miR-99a mimetic (**Fig 4B**). Furthermore, we performed knock out studies in DAMI cells using miR-99a inhibitor to validate the CTDSPL target protein levels. miR-99a was down regulated (~6 fold) in nucleofected cells compared to control cells (**Fig 4C**). CTDSPL protein levels were increased in miR-99a inhibitor cells as compared to control cells (**Fig 4D**). These results indicate that CTDSPL is a target of miR-99a.

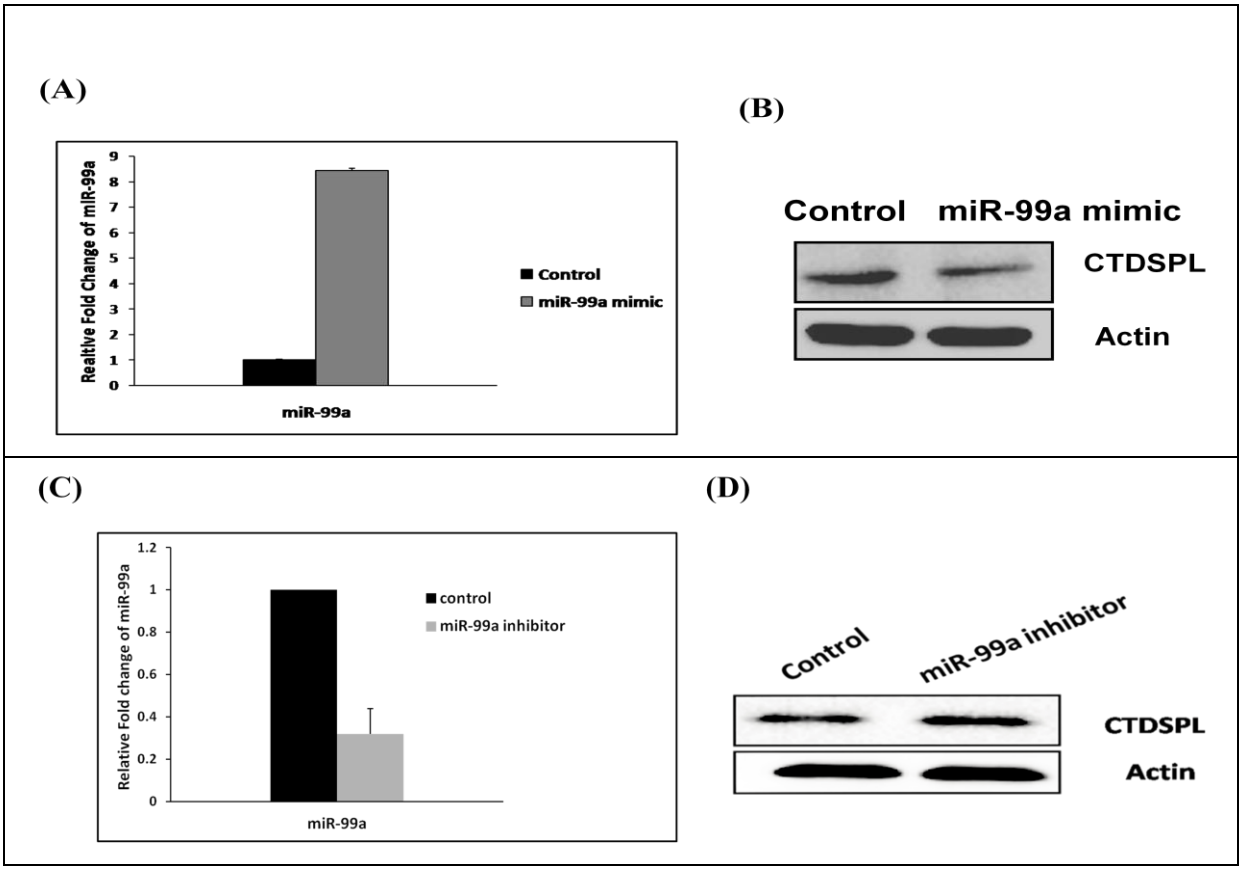


Figure 4: Human miR-99a regulates the expression of CTDSPL. (A) DAMI cells were nucleofected with miR-99a mimic (20nM) and Cy3-labeled Anti-miR control for 48 h. Relative levels of miR-99a was measured by real time PCR and normalized to the endogenous control U6. The expression level of miR-99a was 8-fold higher in nucleofected cells relative to control cells. (B) CTDSPL protein expression was down-regulated in cells over expressing miR-99a, as shown in western blot. C) Real time PCR quantification showed miR-99a levels in control and miR-99a inhibitor nucleofected cells. D) CTDSPL protein expression was up-regulated in cells expressing miR-99a inhibitor by western blot analysis.

miR-99a regulation in proliferation

To understand functional role of miR-99a, we checked the cell number in nucleofected cells by using trypan blue exclusion assay. Proliferation marker PCNA (proliferating cell nuclear antigen) expression was checked by using western blot analysis. Significant increase in the cell number was observed in miR-99a mimetic nucleofected cells, whereas, decrease in cell number was observed in miR-99a inhibitor nucleofected cells as compared to control (**Fig 5A**).

Similarly, in trypan blue exclusion assay, we observed increased cell number in miR-99a mimetic nucleofected cells **(Fig. 5B),** whereas, decreased cell number in miR-99a inhibitor

nucleofected cells compared to control cells **(Fig. 5C).** The proliferation marker PCNA was expressed higher in miR-99a nucleofected cells compared to Cy3 control (**Fig 5D**) where as lower expression of PCNA in miR-99a inhibitor cells compared to control(**Fig 5E**). Further we checked expression of PCNA in primary cells and we observed up regulation of PCNA in CB compared to PB MKs (**Fig 5F**). These results suggest miR-99a might be involved in regulation of cell proliferation.

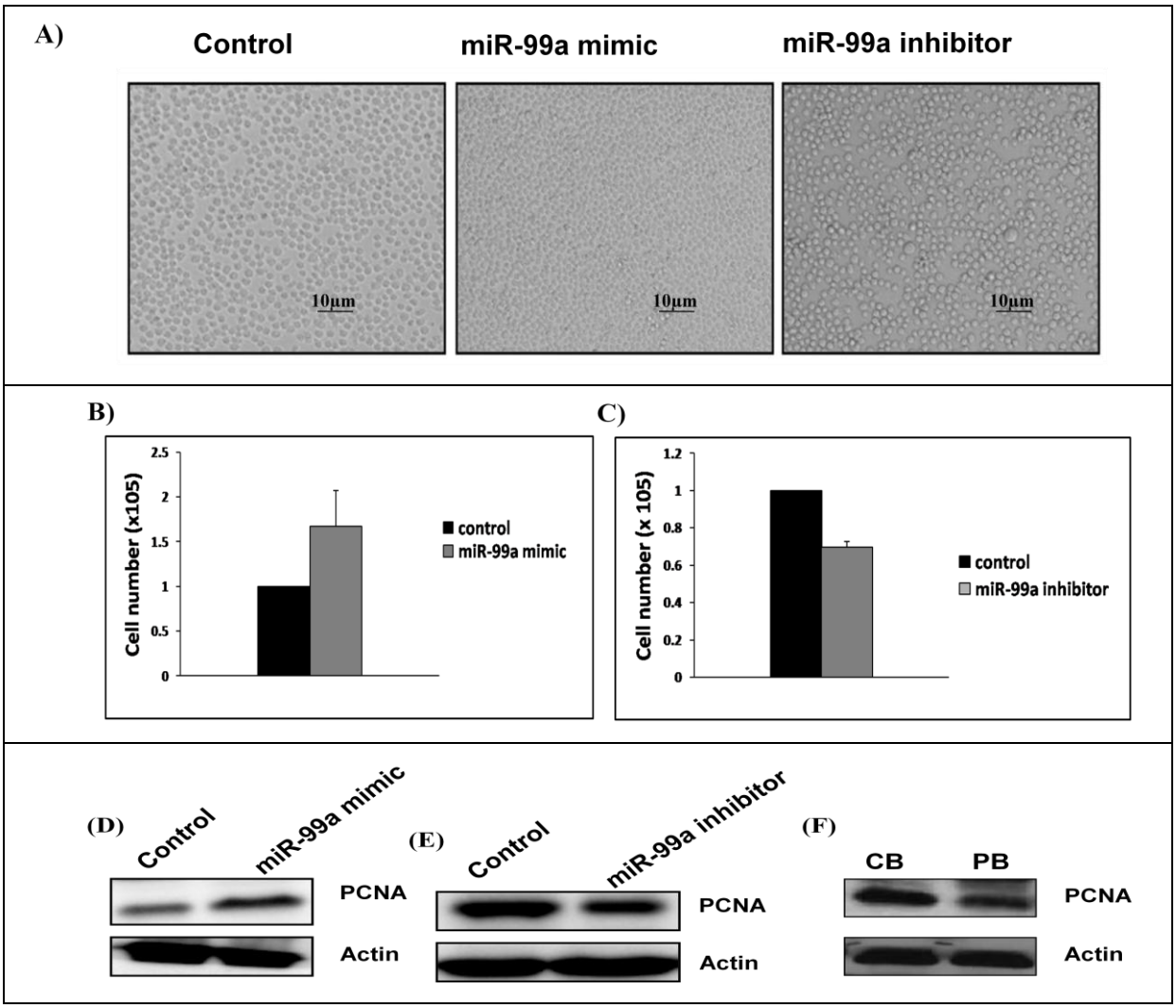


Figure 5: Human miR-99a regulates the Mk proliferation (A) Microscopy revealed increase in cell number in miR-99a mimic cells and decreased cell number in miR-99a inhibitor nucleofected cells compared to control. **(B&C)** Quantification of cell number in miR-99a mimetic and inhibitor nucleofected cells by trypan blue **(D&E)** PCNA protein expression was up- regulated in miR-99a mimetic nucleofected cells and decreased in miR-99a inhibitor compared to Cy3-control. **(F)** PCNA protein levels were higher in CB-MKs compared to PB MKs.

The CTDSPL gene is involved in cord blood megakaryocyte proliferation via the dephosphorylation of RB and the release of E2F1

Our discovery that miR-99a expression was significantly up regulated in CB-MKs compared to PB-MKs led us to hypothesise that higher levels of this miRNA could be one mechanism utilized by CB-MKs to proliferate at much higher rate than PB-MKs. However, the function of CTDSPL in megakaryocyte has not yet been elucidated. Based on the known role of CTDSPL, a target of miR-99a, we believe that the lower levels of CTDSPL in CB could be partially responsible for the high proliferative rate of neonatal MK progenitors. CTDSPL is identified as phosphatase-like tumor suppressor gene that dephosphorylates the RB1 serine on Ser-807 and Ser-811 [**Kashuba et al, 2004**]. We therefore evaluated protein expression levels of CTDSPL downstream regulators such as pRB, Cyclin D1, D2, D3 and E2F1 in mature CB- and PB-derived MKs. We found higher protein expression of pRB, Cyclin D1, D2, D3 and E2F1 in CB-MKs suggesting the possible role of CTDSPL in megakaryocyte regulation (**Fig 6A**). We believe CTDSPL, regulated by miR-99a, in turn lead to enhanced pRB expression and release the E2F1, which induce the G1/S transition by increasing Cyclin expression such as CyclinD1, Cyclin D2 and Cyclin D3 (**Fig. 6B**).

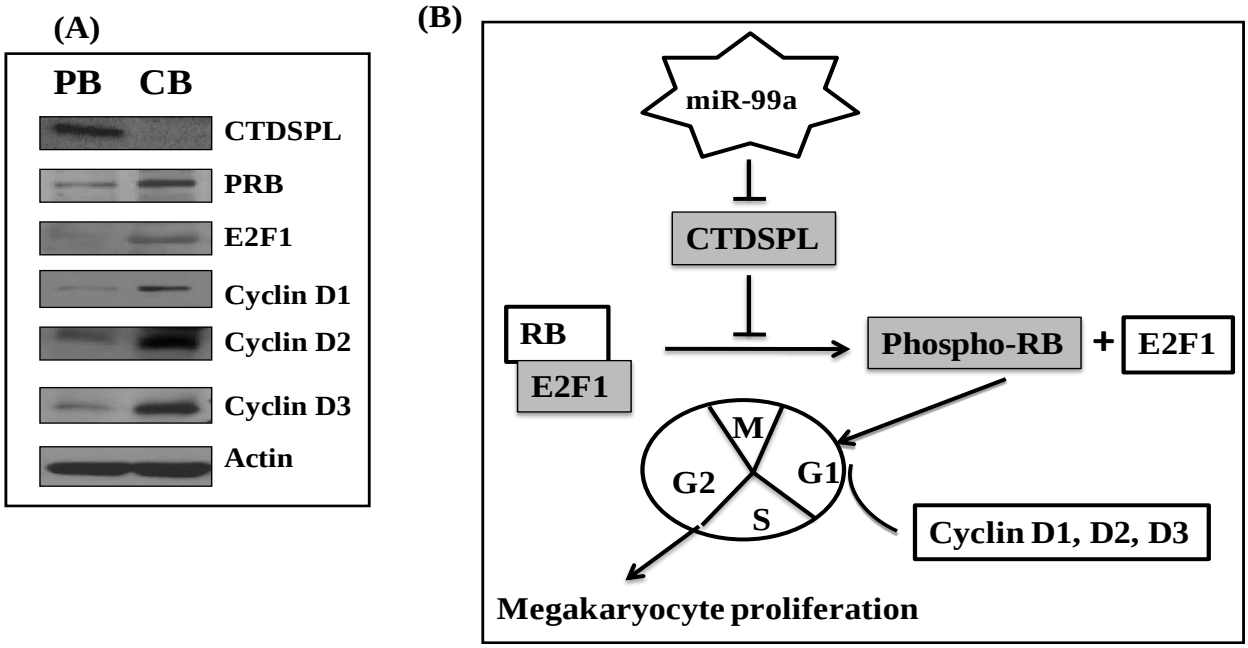


Figure 6: Western analysis of cell cycle regulators expression in CB and PB. (A) Protein expression analysis of CTDSPL and its down-stream regulators pRB, E2F1, Cyclin D1, D2 and D3 in CB and PB-MKs. (B) Schematic representation of a pathway modulated by miR99a targeted gene CTDSPL in megakaryocyte development.

Discussion

Several reports have suggested substantial biological differences between neonatal and adult MKs in regard to their proliferative potential, ploidy, maturational status, and response to Tpo **[Pastos et al, 2006]**. It has been demonstrated that smaller neonatal megakaryocytes produce less platelets than larger megakaryocytes. Also, neonates are affected by megakaryocyte disorders such as transient myeloproliferative disorder (TMD) and thrombocytopenia associated with the TAR syndrome [**Sallmon et al, 2010**]. However, the mechanisms underlying the higher proliferative rate of neonatal megakaryocytes are unclear. miRNAs have been identified as crucial players in various developmental systems and their differential expressions are reported in megakaryocytes. In current study, we have identified miRNA regulating the megakaryocyte development and elucidated its mechanism of action by targeting the regulatory proteins of cell cycle. Previous studies on acute megakaryoblastic cancer cell lines have shown a significant up-regulation of miR-99a compared to *in vitro*-differentiated megakaryocytes indicating its possible role in cell proliferation [**Garzon et al, 2006**]. In our recent studies, we have found that miR-99a down regulates the targets FKBP51 and IGF1R in CMK cell lines which in turn increase the p-Akt (Ser473) levels **[Kandi R et al, 2014]**. Differential up-regulation of miR-99 was also reported in Osteosarcoma (malignant bone tumor) compared to osteoblast cell lines **[Hu et al, 2012]**. Several reports in the past show that miRNA might function as an oncogene or tumor suppressor in different cancers. MiR-100 and miR-99a are aberrantly expressed in acute leukaemia and vincristine-resistant acute lymphoblastic leukemia [**Li et al, 2013; Akbari et al, 2013**]. Our studies are in agreement with the studies of Zhang et al [**Zhang et al, 2013**] who have shown that miR-99a plays a potential oncogenic role in AML and CML cell lines. They demonstrated that proliferation of these cell lines is promoted, whereas, their apoptosis is inhibited by miR-99a via regulating CTDSPL.

Conclusions

Our findings suggest that miR-99a play a significant role in proliferation of neonatal MKs and its development, mediated by repressing the tumor suppressor protein CTDSPL. Characterizing miR-99a/CTDSPL will help in defining pathways involved in MK development and give a better understanding of neonatal MK disorders and enhance our understanding of adult hematopoietic disorders potentially associated with re-activation of fetal pathways (i.e. myeloproliferative disorders).

Objective 2

Objective 2. Assessment of role of miR-125b and its regulated signaling pathway during megakaryocyte development.

MicroRNAs (miRNAs) are small, non-coding RNA of 20–22 nucleotides in length which can silence specific target genes through direct mRNA degradation, translational repression, or both. miRNAs are involved in several cellular processes like cell cycle, apoptosis, drug resistance and differentiation. Five miRNAs (miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) are encoded on chromosome 21, and are potential players in the pathogenesis of transient myeloproliferative disorder (TMD). TMD of Down syndrome is a hematologic abnormality which is characterized by an uncontrolled proliferation of myeloblasts in bone marrow and peripheral blood that affects babies with trisomy of chromosome 21**[Roy et al, 2009]**. Interestingly, four of the five miRNAs encoded in chromosome 21 (miR-99a, let-7c, miR-125b-2, and miR-155) play a crucial role in megakaryocyte development. As we could observe significant difference in miR-99a levels, we hypothesized that the co-expressing miR-125b profiles between neonatal and adult MKs will differ and could contribute to their substantial biological differences.

miR-125b is developmentally regulated in CB and PB megakaryocyte development.

We hypothesized that miRNAs are important participants in developmental processes and hereby used this to study the regulatory mechanisms in neonates vs. adults. miR-125b was reported to be over-expressed in TMD and AMKL samples compared to normal MKs and act as potential oncomiR [**Klusmann et al, 2010**]. Based on these reports, we compared miR-125b levels in primary MKs derived from cord blood (CB) and peripheral blood (PB) by quantitative PCR and found ~4-fold up-regulation (on day 14) in neonatal megakaryocytes compared to adults ($n=3$, $p<0.05$). Taken together, this data provide compelling evidence that miR-125b is differentially expressed in developmentally different CB- and PB-derived MKs.

We harvested CB and PB cells on days 0, 7, 11, and 14 of MK culture, isolated miRNA, and determined miR-125b expression levels by quantitative PCR. Values in each sample were normalized against U6 (miRNA internal control), and CB miR-125b levels were expressed as relative values to the PB levels at the same stages of culture. This analysis revealed that neonatal MKs expressed higher miR-125b at all levels (**Fig 1**).

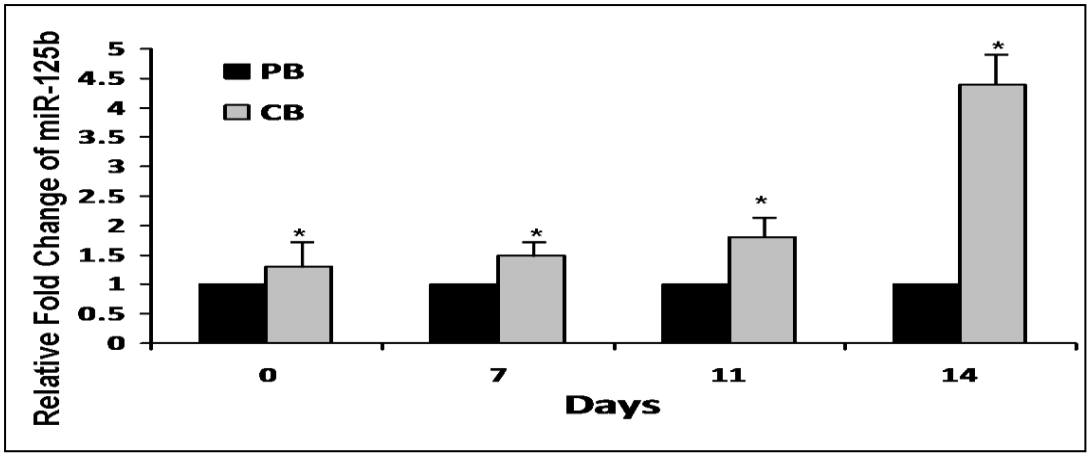
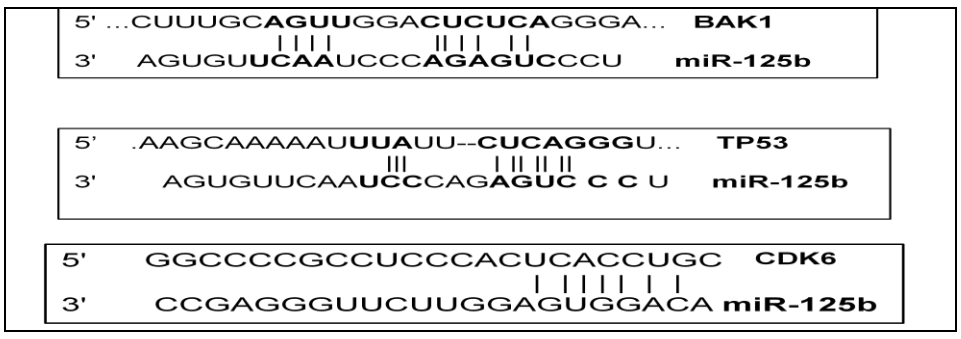


Figure 1. Expression and target studies of miR-125b during MK differentiation. miR-125b levels (n=3, *p<0.05) in CB cells at different stages (day 0, 7, 11, 14) of MK differentiation in culture, Expressed as relative to miR-125b levels in PB cells at the same stage of differentiation.

Identification of putative targets of mi-125b by bioinformatic tools.

The clarification of miRNA targets remains a major issue in functional examination of miRNAs. A putative target of miR-125b was explored using TargetScan, PicTar and miRBase (**Fig 2**). Three classes (one from each class) of targets viz. Tumor Suppressor, Apoptosis and Cell cycle regulator were selected. P53, BAK1 and CDK6 were most potential target among several putative targets. P53 is tumor suppressor gene which is involved in cell cycle regulation. BAK1 act as pro-apoptotic regulator that is involved in a wide variety of cellular activities.



Figere 2. miR-125b complementary to the 3' UTR region of BAK1 and TP53 and CDK6 by Target Scan.

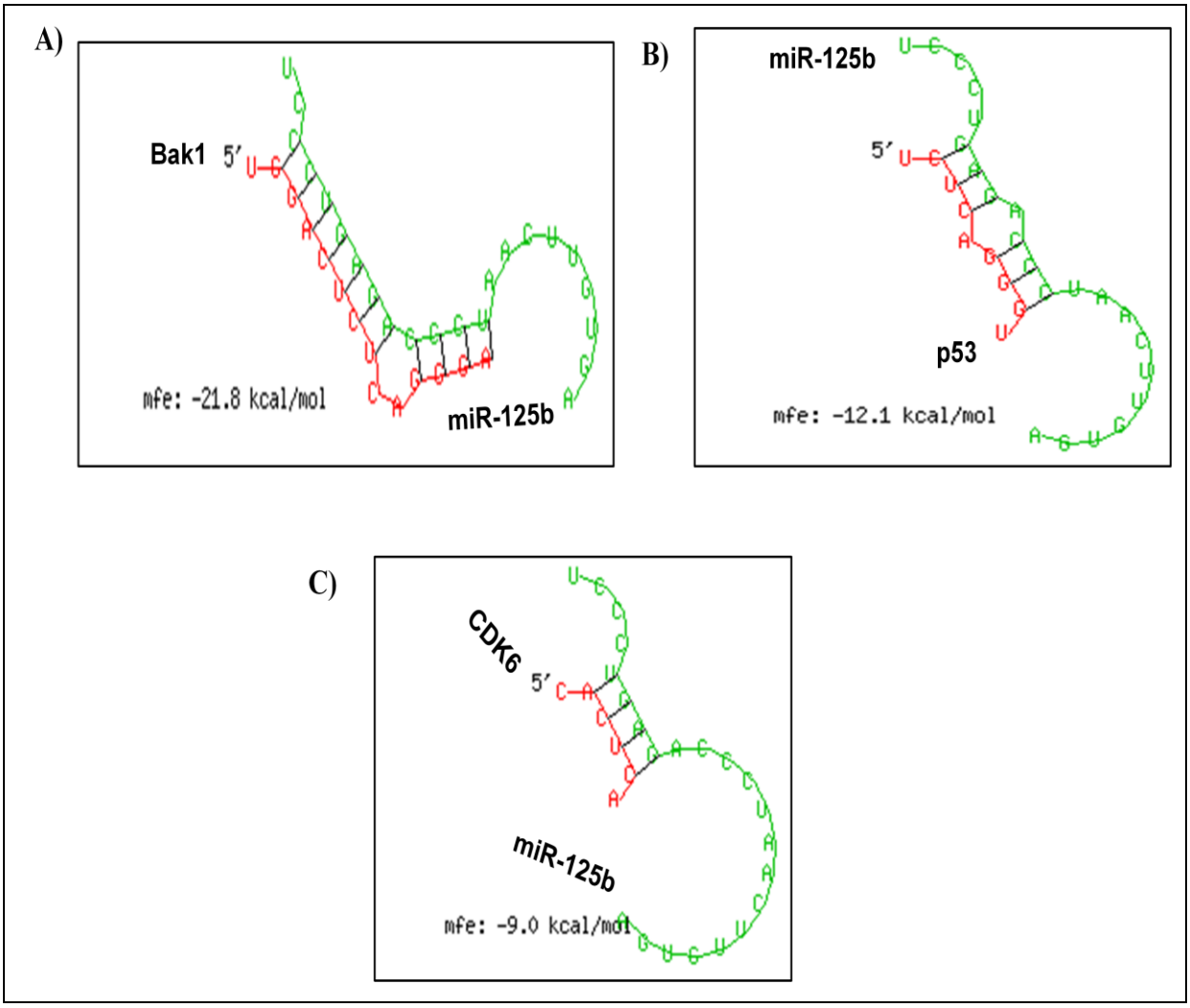


Figure 3. miR-125b complementary to the 3'-UTR region of BAK1 and TP53 and CDK6 by RNA hybrid. A) Minimum free energy (MFE) duplexes of the miR-125b and BAK1 3'UTR. B) Minimum free energy (MFE) duplexes of the miR-125b and p53 3'UTR. C) Minimum free energy (MFE) duplexes of the miR-125b and CDK6 3'UTR.

Furthermore, we also check the minimum free energy levels during association of miRNA and 3'-UTR region of mRNA. We found that miR-125b interaction with P53, BAK1 and CDK6 showed -21.8Kcal/mol, -12.1 Kcal/mol and -9.0 Kcal/mol respectively.

In silico analysis of miR-125b regulated signaling pathways

In silico analysis showed that hsa-miR-125b was regulating 19 signalling pathways, particularly, cancer associated pathways, wnt signalling, mTOR signalling pathways **(Fig 4).** These pathways have previously been known to be regulators of MK development. Association with cancer related pathways suggests its involvement in cell proliferation.

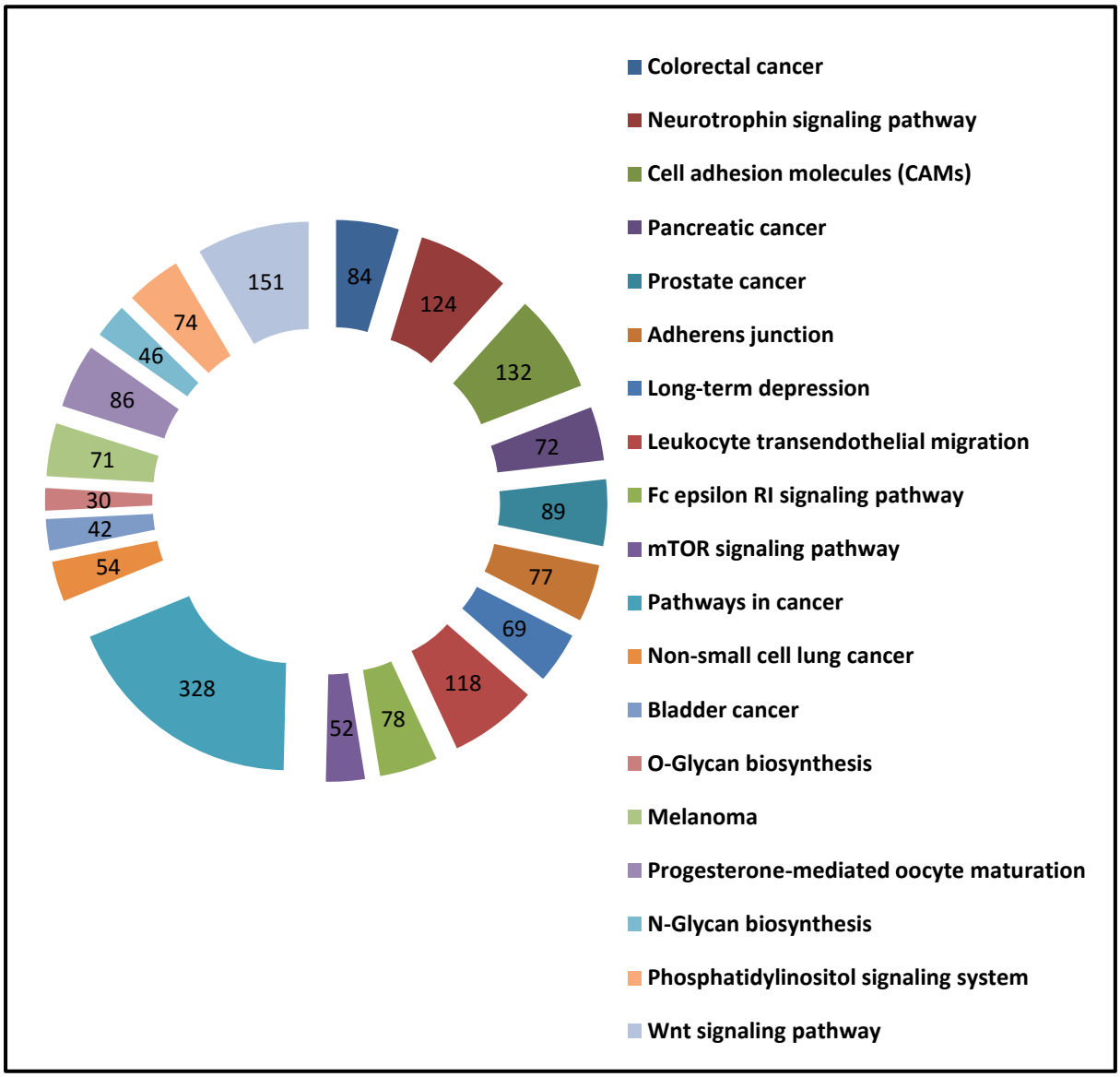


Figure 4. hsa-miR-99a regulated signalling pathways by *in silico* analysis.

Expression studies of mRNA and protein in CB- vs PB-MKs.

miR-125b has been shown to target key proteins regulating apoptosis, innate immunity, inflammation, and hematopoietic differentiation. We predicted that miR-125b targets p53, BAK1 and CDK6 expression in CB-MKs. We confirmed the results using western blot analysis and found P53, BAK1 and CDK6 protein levels were higher in PB compared to CB MKs (**n=3, Fig. 5 A&B**).

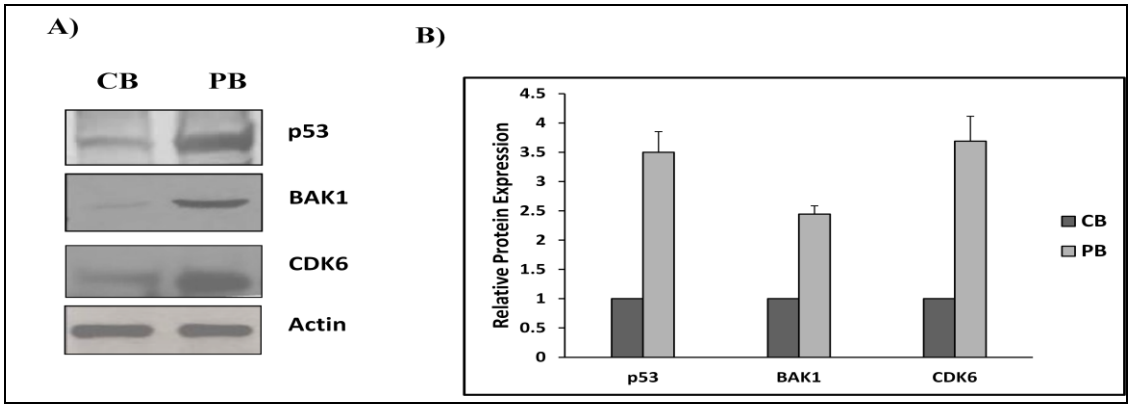


Figure 5. Expression studies of mRNA and protein in CB- vs PB-MKs. A) Increased miR-125b activity in primary CB-MKs results in decreased expression of p53, BAK1 and CDK6 in CB-MK cells. B) Relative protein expression levels of p53, BAK1 and CDK6 by image j software (n=3).

Based on the known role of p53, a target of miR-125b, we hypothesized that the lower levels in CB would be at least partially responsible for the high proliferative rate of neonatal MK progenitors. Several reports revealed that p53 is involved in the regulation of cell growth and differentiation **(Lin et al, 2004)**. However, the function of p53 in megakaryocyte development has not yet clearly known. We therefore evaluated protein expression levels of downstream targets of p53, such as, p21, Cyclin D1, Bcl2 and Bad in mature CB- and PB-derived MKs. We found higher protein expression of Cyclin D1 and Bcl2 in CB-MKs, whereas, lower expression of p53, p21 and Bad in PB-MKs suggesting the possible role of p53 in megakaryocyte regulation (**Fig. 6**). We believe p53, a target regulated by miR-125b, in turn lead to megakaryocyte proliferation.

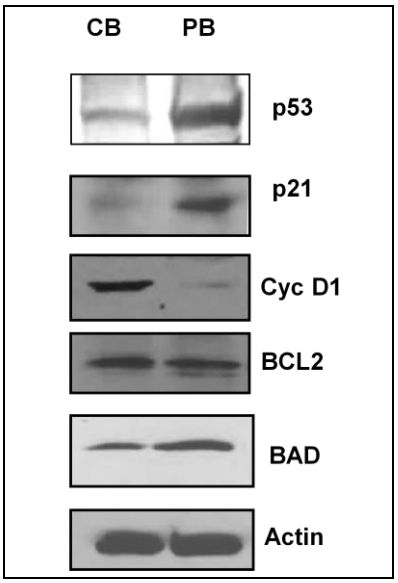


Figure 6. Western analysis of cell cycle regulators expression in CB and PB. (A) Protein expression analysis of p53 and its down-stream regulators p21, Cyclin D1, BCL2 and BAD in CB and PB-MKs (n=3).

Discussion

Previous reports support the existence of substantial biological differences between neonatal and adult MKs in regards to their proliferation and maturation; previous investigators have also suggested that the neonatal and adult MKs show different biological response to TPO. It is known that neonatal MKs are smaller than adult MKs and smaller MKs produce less platelets compared to larger MKs [**Pastos et al, 2006**]. Based on these observations, we have reported that the neonatal MKs progenitors are more proliferative and experience rapid terminal maturation compared to adult MKs progenitors [**Mattia et al, 2002**]. Hence, it is widely accepted that these different mechanisms allow neonates to maintain low platelet counts and contribute to the disorders of neonatal megakaryocytopoiesis that are predominantly present during this developmental stage, including a syndrome of megakaryocytic hyper-proliferation known as transient myeloproliferative disorder (TMD) and the thrombocytopenia associated with the thrombocytopenia-absent radius (TAR) syndrome [**Liu et al, 2011; Sallmon et al, 2010, Ferrer-Marin et al, 2010**]. However, the mechanisms underlying the higher proliferative rate of neonatal megakaryocytes are unclear. We hypothesize that small non-coding miRNAs may play a critical role in the regulation of developmental megakaryocytopoiesis. Recent studies revealed that miRNAs have an important role in megakaryocyte development. In the current study, we found that the miR-125b expressed in neonatal MKs at levels ~4.5-fold higher than in adult MKs. Reports show that miR-125b play a significant role in cell growth and correlates with the prognosis of patients with tumors. In 2011, Schotte et al. reported different miRNA signatures in pediatric acute lymphoblastic leukemia (ALL) with various genetic subtypes and drug-resistance. MiR-125b, miR-99a, and miR-100 were up-regulated ~20-fold in vincristine and daunorubicin-resistant ALL cells **[Schotte et al, 2011]**. Recent reports suggested that co-expression of miR-125b in combination with miR-100, or miR-99a, or miR-100 and miR-99a induces significant vincristine (VCR) resistance in ETV6-RUNX1-positive Reh leukemic cells. miR-125b have also been previously shown to be up-regulated in acute megakaryoblastic cell lines **[Akbari et al, 2013]**, but there is no evidence of its involvement in regulation of normal neonatal megakaryocytopoiesis. Previous studies demonstrated that

miR- 125b plays an important role in megakaryocyte development by increasing cell proliferation and self renewal of megakaryocyte and erythrocyte progenitors [**Klusmann et al, 2010**]. Several reports suggest that miR-125b acts oncomir regulating different cancers **[Bousquet et al, 2010]**.

Based on these reports, we compared miR-125b levels in primary MKs derived from CB and PB by Real-time PCR and found ~4.5-fold up-regulation in CB compared to PB MKs. We confirmed our hypothesis of miR-125b involvement, first by searching for putative targets using several computational databases and consistently identified p53, BAK1 and CDK6 as a direct miR-125b target. This is further re-confirmed by checking the expression of p53, BAK1 and CDK6 protein levels in both CB and PB derived MKs. Previous studies showed miR-125b acts as oncomir by inhibiting $P14^{Arf}$ and stimulating prostate cancer cell proliferation through p53 dependent or independent pathways [**Amir et al, 2013**]. Recent report suggests that miR-125b down-regulates apoptosis-associated proteins in p53 pathway including BAK1, TP53INP1 and it blocks the myeloid differentiation in part by targeting CBFβ [**Bousquet et al, 2012**]. miR-125b has been shown to target key proteins regulating apoptosis, innate immunity, inflammation and hematopoietic differentiation **[So et al, 2013]**.

Our results showed that miR-125b could promote CB-MK proliferation by involving in the cell cycle control to promote cell proliferation and may block terminal differentiation and consequently facilitate the development of megakaryocyte. These effects characterize a new role for miR-125b. There are various reports that suggest CB-MK progenitors are highly proliferative than PB-MKs (**Liu et al, 2011; sola et al, 2007**). Further, we looked into the molecular pathway that might be controlled by miR- 125b. As expected, expression of p53, BAK1, CDK6 protein is in inverse correlation with miR- 125b in cord blood MKs. P53 is an important candidate tumour suppressor gene [**Solozobova et al, 2011**]. However, the involvement of p53 in the regulation of neonatal megakaryocyte cell proliferation and growth has not yet been studied. We believe miR-125b in megakaryocytes might control cell cycle progression by regulating p53 signalling pathway thereby regulating proliferation. As expected, we found lower protein expression of p53, p21, CDK6 and BAK1 in CB-MKs suggesting the possible role of p53 in megakaryocyte regulation **(Fig 11)**. Previous studies have also reported that p53 regulates p21 expression (**Li et al, 1994**). In the present study, in CB MKs, a high expression level of miR-125b represses the expression of p53 which in turn leads to regulating downstream targets of p53 thus increasing proliferation and leading to megakaryocyte progression.

These findings mainly suggest that miR-125b might play significant role in proliferation of cord blood megakaryocytes and its development, mediated by repressing the p53. This study extends our knowledge about the regulation of p53, a tumour suppressor protein in MKs. Characterizing miR-125b/p53 axis further will help in defining other related pathways involved in developmental megakaryocytopoiesis and give a better understanding of neonatal MK disorders and enhance our understanding of MK biology as a whole. In addition, this may provide new mechanistic insights and therapeutic targets for adult hematopoietic disorders potentially associated with re-activation of fetal pathways (i.e. myeloproliferative disorders).

Establishment of K562 cell differentiation model by using phorbol-12-myristate-13-acetate (PMA).

To understand megakaryocyte differentiation and its regulation, we have standardized K562 cell line model for furthermore studies. K562 cells were induced with hemin which differentiate them into erythrocytes and thereby show increased expression of erythrocyte specific marker Gycophorin-A (Gly-A), whereas, PMA (phorbol-12-myristate-13-acetate) induction of K562 cells leads to expression of megakaryocyte specific markers such as CD41 and CD61. PMA induced megakaryocytic differentiation of K562 cells is a classic model to understand the differentiation of blood cells such as megakaryocytes. During this process, the changes in cell morphology, including, gaining of adhesion properties, cell growth arrest, lobulated nucleus, specific markers (CD41, CD61) expression on the cell surface of megakaryocytes and other changes (**Fig 7**).

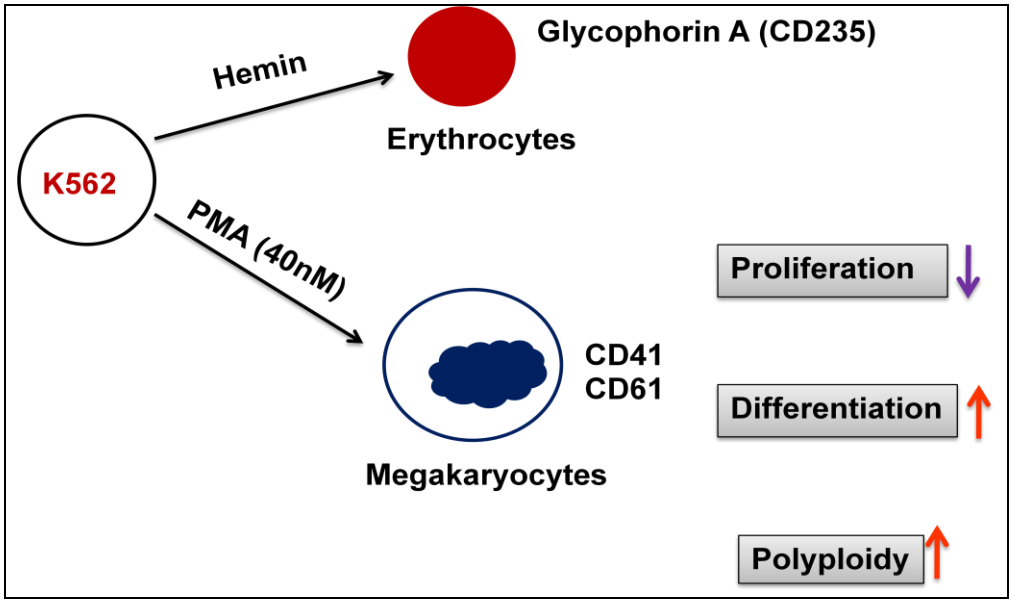


Figure 7. Schematic representation of chemically induced K562 cells mimics like megakaryocyte-erythrocyte progenitors. K562 cells differentiate into megakaryocytes after treatment with PMA, whereas, induction with hemin differentiate them into erythrocyte cells.

In vitro megakaryocyte differentiation of K562 cells upon PMA induction

In vitro differentiation of the K562 human leukemia cell line was induced by using chemical PMA (40nM). These cells were seeded in 60mm plates at density of 1 million cells in RPMI

medium supplemented with 10% fetal bovine serum. Following 24h, 48h and 72h of culture, cells were also collected to study the megkaryocyte specific markers. After PMA induction of K562 cells, a consistent increase in the expression of MK specific markers (CD41 and CD61) was observed with increasing time. Distinct morphological changes were observed by microscopy with changing time, with the largest cell size and lobulated nucleus at 72h treatment(**Fig.8**).

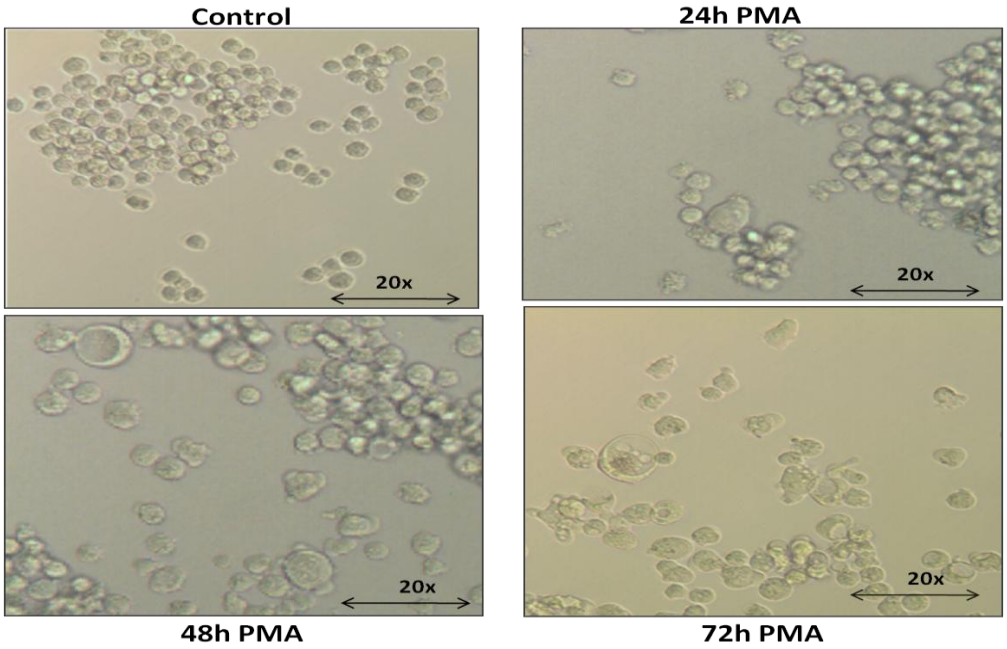


Figure 8. Cell morphology studies by microscopy. K562 cell size increased upon PMA treatment (different time course 0h, 24h, 48h and 72h).

We further confirmed CD 41 and CD 61 expression by using the Real time PCR analysis and found significant increase in MK specific markers with increasing time after PMA treatment (**Fig 9**).

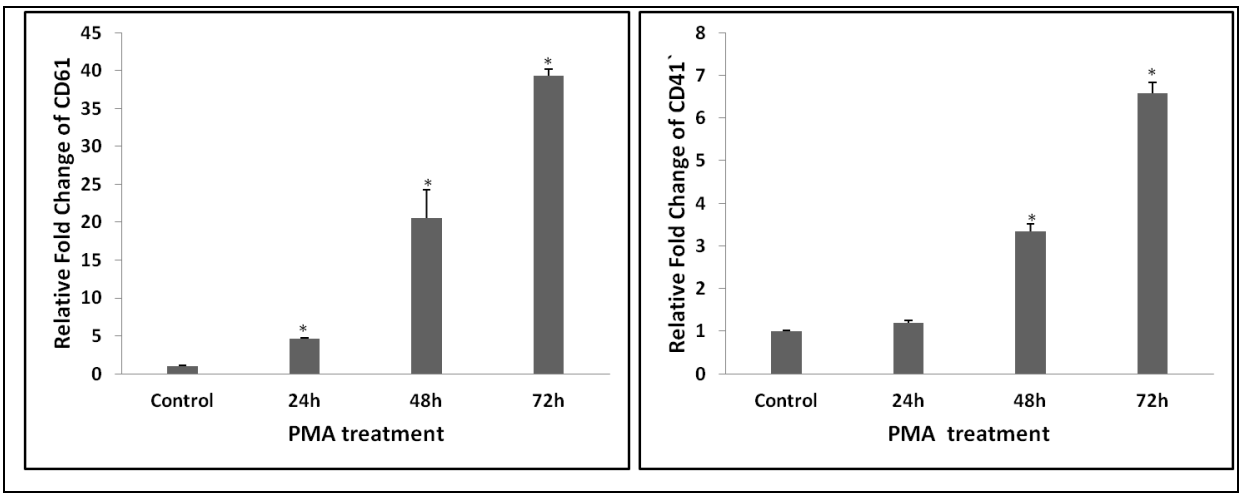


Figure 9. Megakaryocyte specific markers expression by Real time PCR analysis. Increased megakaryocytic marker (CD61 and CD41) expression in PMA induced cells at different points 0h, 24h, 48h and 72h (n=3).

Furthermore, we analysed cell surface markers expression using flowcytometric analysis using CD61 and C41-PE conjugated antibodies. We observed clear shift in both the markers in PMA treated cells and found significant increase in mean fluorescence intensity values in PMA treated compared to control cells (**Fig 10 A&B**). These results clearly suggest that 40nM PMA induced K562 cells were differentiated into megakaryocytic cells successfully.

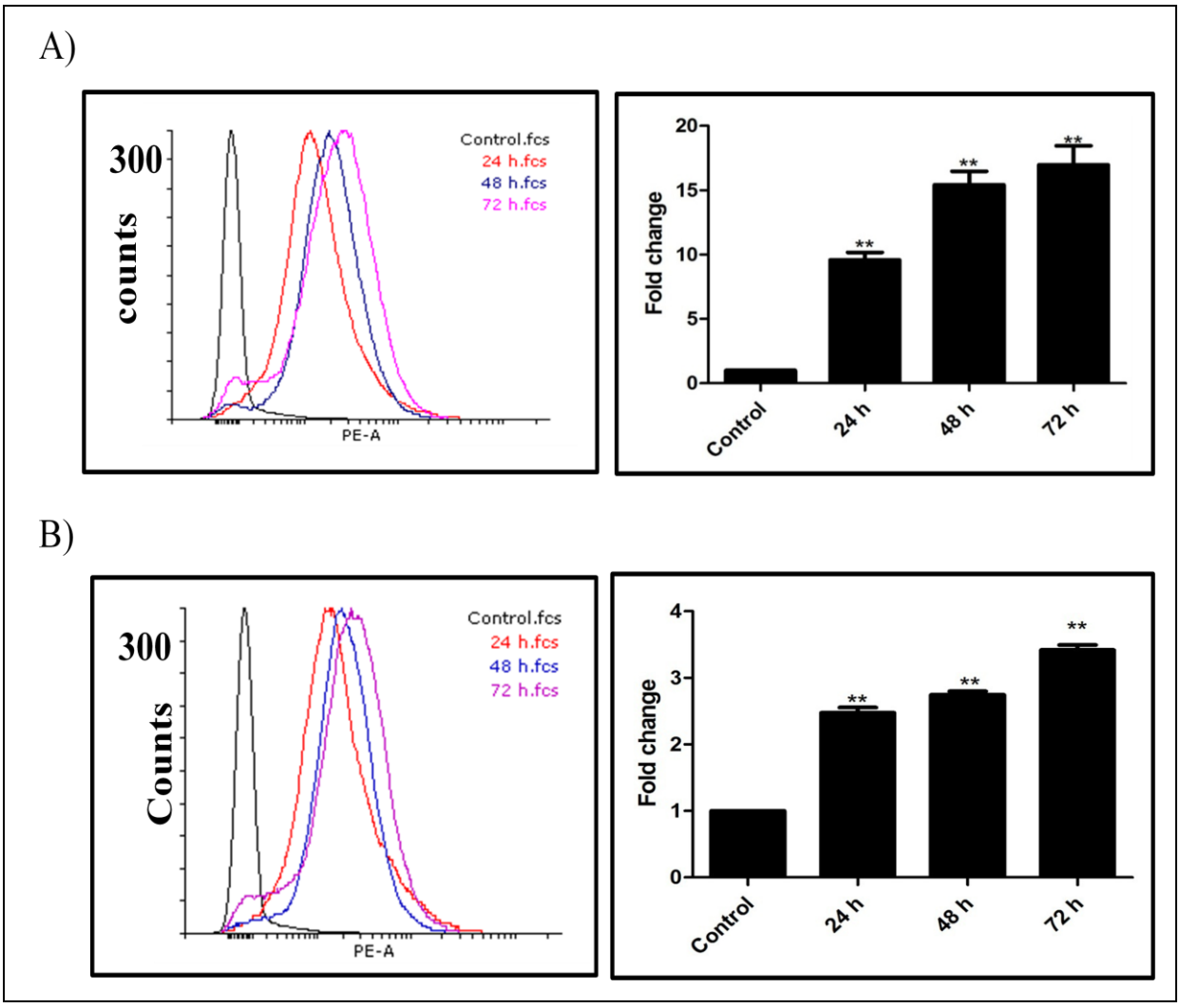


Figure 10. Megakaryocyte markers level by flow cytometry analysis. A&B) Increased CD61 and CD41 expression with increasing time course treatment of PMA (n=3) by flow cytometry analysis (CD61 and CD41 both are PE conjugated antibodies).

ROS formation regulates megakaryocyte differentiation

During megakaryocyte differentiation, the cells migrate to the vascular niche from the osteoblastic niche in the bone marrow, leading to change in reactive oxygen species (ROS)-dependent oxidation state in the microenvironment. This suggests that ROS can distinctly influence platelet generation and function in a microenvironment-dependent manner. Also, previous reports suggest that ROS is generated in response to PMA treatment. In order to understand ROS relationship with miRNA in MK development, we treated K562 cells with PMA (40nM) and analysed the intracellular ROS production by flow cytometry at different time points ((24h, 48h, 72h). We observed significant increase in ROS levels with increasing time in PMA treated K562 cells (**Fig 11**).

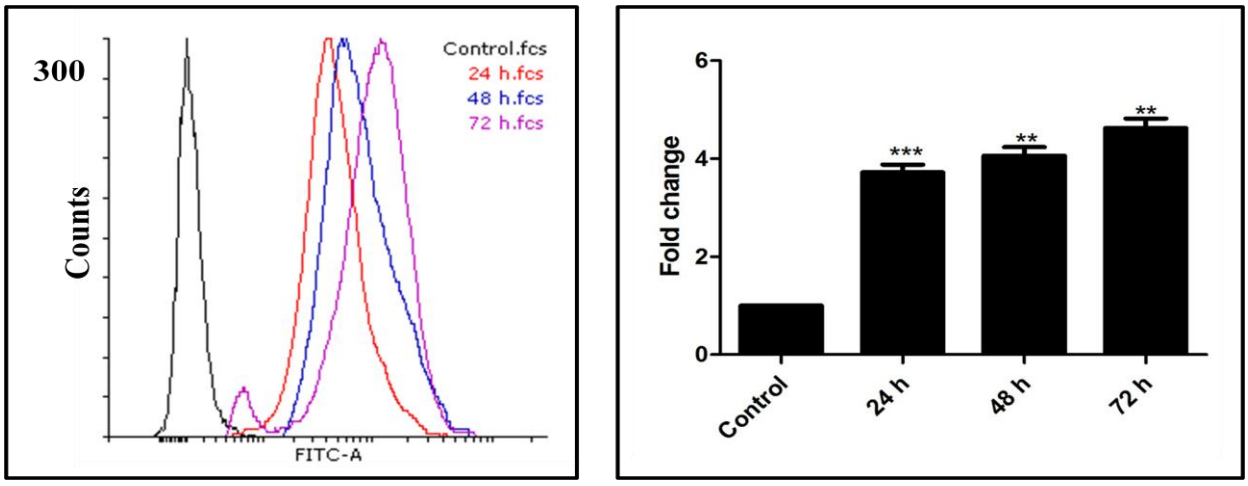


Figure 11. Measurement of ROS levels in PMA induced-K562 cells. For measurement of intracellular ROS production, cells were washed and incubated with 10uM 2', 7'-dichlorofluorescein diacetate (DCFH-DA) analysed by Flow cytometry. These cells were analysed before and after MK differentiation and evaluated for the ROS production by measuring the fold change in the DCFH-DA fluorescence relative to control cells (n=3).

To understand whether ROS generation is important for megakaryocyte differentiation, we have carried out cell differentiation experiments using ROS quencher N-acetyl-L-cysteine (NAC) which interacts directly with ROS to functions as a scavenger of oxygen free radicals thereby reducing the intra cellular ROS levels. In differentiation experiments, PMA induced cells showed higher megakaryocytic marker (CD41 and CD61) expression and decreased erythrocyte marker (Gly A) expression. NAC treated cells showed reduced expression of the the megakaryocyte specific markers such as CD 41 and CD 61. Increased expression of erythroid specific marker Gly A was observed in the presence of antioxidant. Upon addition

of PMA and NAC together cells expressed higher MK specific markers compared to only NAC treated cells, whereas, expression was lower in only PMA induced cells and vice versa was observed in erythrocyte specific marker expression. Taken together, this data support the notion that ROS production is required for megakaryocytic differentiation (**Fig 12**).

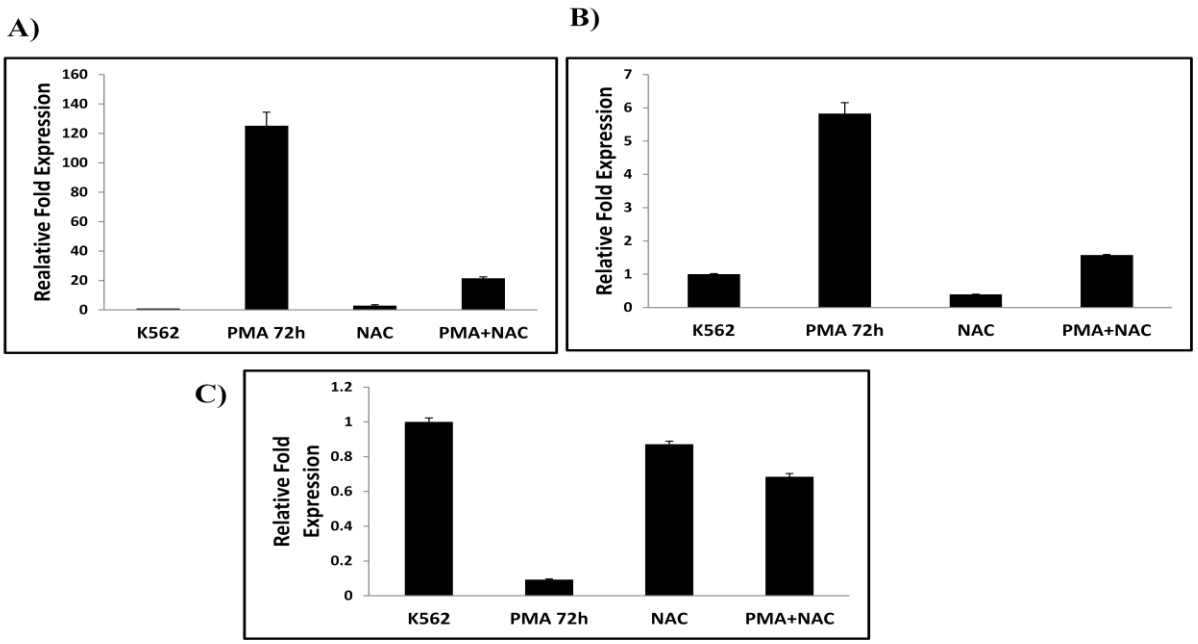


Figure 12. Expression levels of megakaryocyte and erythrocyte markers in the presence of antioxidant NAC. A) CD 61 expression was increased in PMA treated cells, whereas, decreased in NAC treated cells. B) CD41 levels were higher in PMA induced cells and lower in antioxidant induced cells. C) Gly A showed decreased expression in PMA, whereas increased in NAC treated cells (n=3).

Role of miR-125b and its target p53 during megakaryocyte differentiation

We harvested control and differentiated cells upon PMA induction (72h) and extracted miRNA to determine miR-125b expression levels by quantitative PCR. Values in each sample were normalized against U6 (miRNA internal control), and control miR-125b levels were expressed as relative values to the differentiated cell levels at 72h PMA treatment of culture. This analysis revealed that miR-125b expression decreased in PMA treated cells compared to control cells **(Fig 13 A)**. The clarification of miRNA targets remains a major issue in functional examination of miRNAs. As previously discussed, p53 was most potential target among several putative targets. P53 is tumour suppressor which is involved in cell cycle regulation. We confirmed the p53 protein levels by using western blot analysis and

found that p53 protein expression was up regulated in PMA differentiated cells compared to control **(Fig 13 B).**

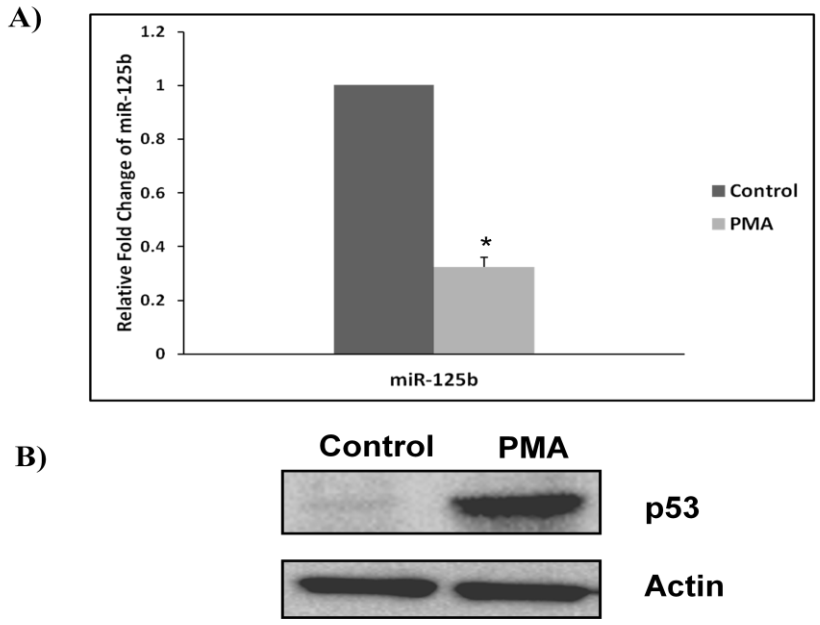


Figure 13. Expression of miR-125b and its target gene p53 during K562 differentiation. A) hsa-miR-125b levels were low in PMA treated cells compared to control B) Decreased miR-125b activity in PMA treated K562 cells results in increased expression of p53 in differentiated K562 cells (n=3).

miR-125b/p53 axis mediates cell cycle regulation during K562 cell differentiation

Our discovery that miR-125b expression was significantly down regulated in differentiated cells compared to undifferentiated cells has led us to hypothesize that lower levels of this miRNA could be one mechanism utilized by differentiated K562 cells. Based on the known role of p53, a target of miR-125b, we further studied the p53 signaling pathway. Previous reports suggest that over-expression of p21 in human K562 myeloid leukemia cells, induces megakaryocytic differentiation with increased ploidy. P21 is known to be downstream regulator of p53 signalling **[Li et al, 1994]**.

P53 plays a major role in several physiological processes such as differentiation, cell cycle arrest, DNA repair and apoptosis. We therefore evaluated protein expression levels of P53 and its downstream regulators P21, BAD. We also analyzed Mdm2, which inhibits the P53 expression in differentiated and undifferentiated K562 cells. We found higher protein expression of P53, P21, BAD in differentiated K562 cells suggesting the possible role of P53

in megakaryocyte regulation. We hypothesized that P53, regulated by miR-125b, in turn lead to enhanced p21 expression, which inhibits the G1/S transition **(Fig 14).**

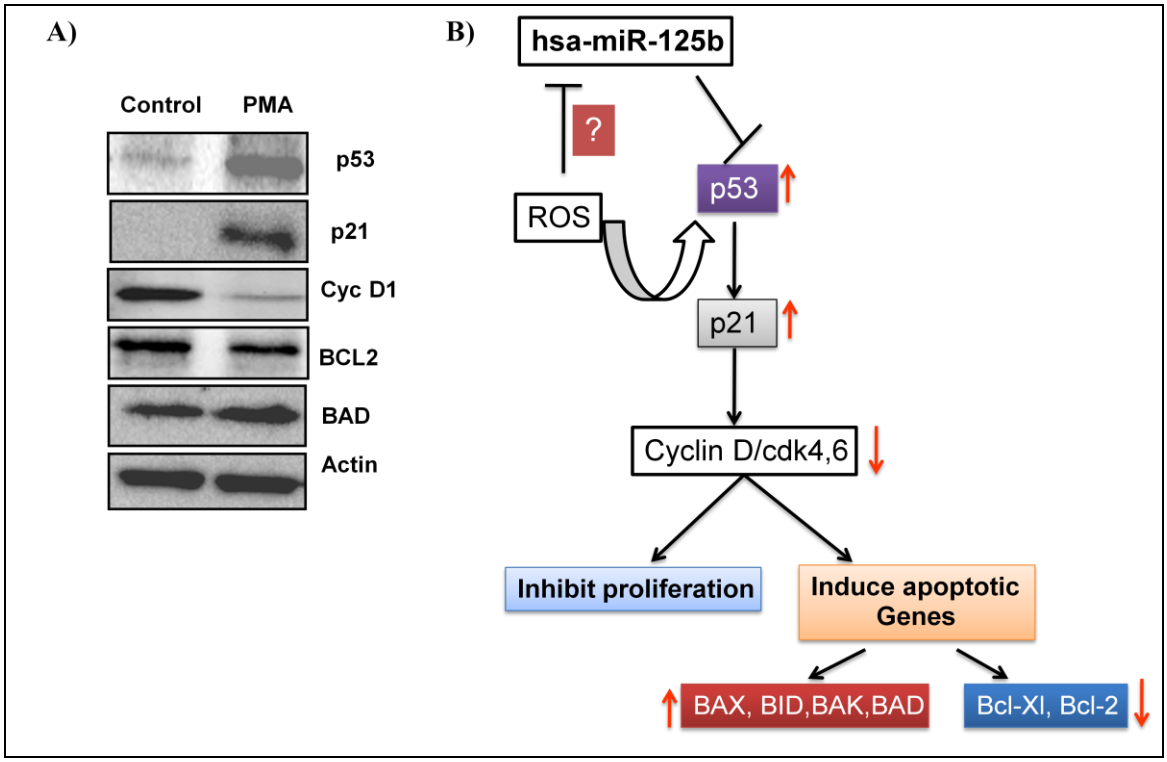


Figure 14. Western blot analysis of apoptotic and cell cycle regulators. A) miR-125b targeted gene P53 regulates cell regulatory proteins in K562 cell differentiation (n=3). B) Schematic representation of p53 pathway modulated by miR-125b during K562 cell differentiation.

ROS inhibits miR-125 expression

Previous reports suggest increased ROS levels are responsible for inhibition of the miR-199 and miR-125b expression in ovarian cancer cells **[He et al, 2012]**. Known role of miR-125b functions, we first observed miR-125b expression in K562 cell differentiation. Further we want understand whether ROS regulates miR-125b expression in PMA induced K562 cell differentiation. Initially, we cultured cells K562 cells with same cell numbers and treated these cells with 40nM PMA alone, antioxidant NAC 200µM alone and both PMA+NAC together. As previously discussed, PMA induced cells increased ROS levels. We further measured miR-125b expression levels by using Real-time PCR analysis. In PMA treated cells, we found down regulation of miR-125b levels compared to control cells. Furthermore, we also noted that reduced miR-125b levels in PMA+ NAC treated cells compared to NAC

induced K562 cells. We demonstrated that miR-125b might regulated by intracellular ROS which is generated by PMA **(Fig 15).**

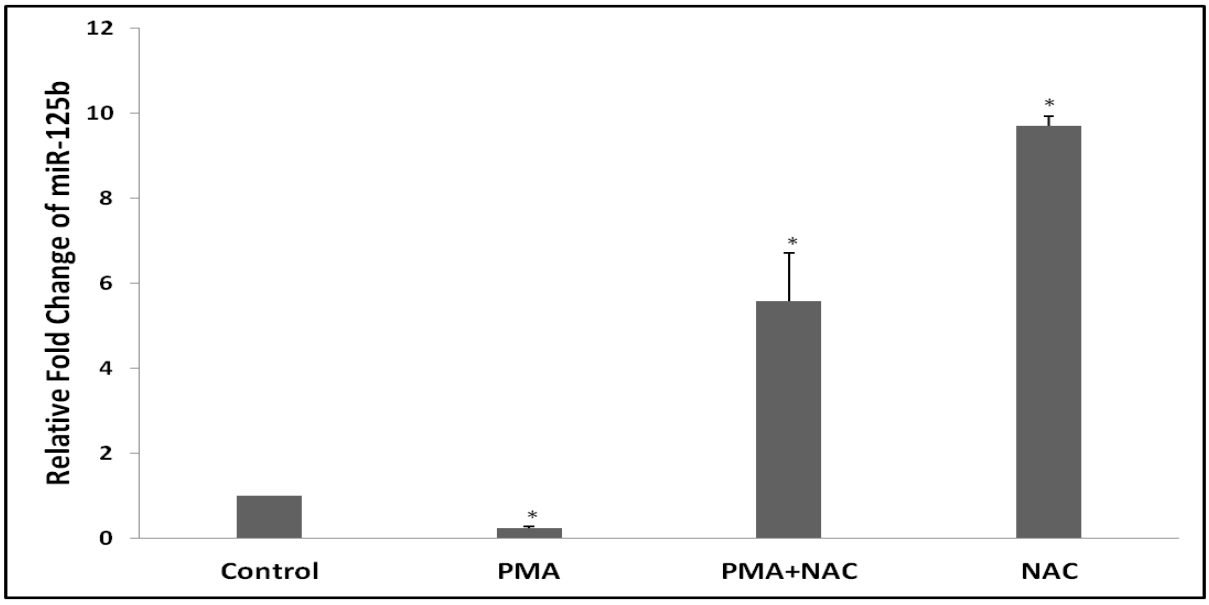


Figure 15. miR-125b inhibited by ROS. miR-125b expression levels were down regulated by PMA, whereas, up regulated in PMA with ROS scavenger NAC treatment (n=3).

Discussion

In present study, we explored the cross-talk between ROS and miR-125b during megakaryocyte differentiation. During megakaryocyte differentiation, the cells migrate to the vascular niche from the osteoblastic niche in the BM (bone marrow), leading to change in ROS dependent oxidation state in the microenvironment. This suggests that ROS can distinctly regulate platelet production and function in a microenvironment dependent manner [**Chen et al, 2013]**.

In order to understand ROS relationship with miRNA in MK development, we treated K562 cells with PMA (40nM) and analysed the intracellular ROS production by flow cytometry at different time points ((24h, 48h, 72h). We observed significant increase in ROS levels with increasing time in PMA treated K562 cells. During the last few years several reports suggested ROS acts as second messengers and participate in redox signalling. ROS can influence the activity and function of various molecules involved in the commitment, differentiation, proliferation of MKs, and platelet release.

ROS production has mainly been related to cell growth **[Zhu et al, 2006],** only a few reports showed their participation in the activation of particular differentiation programmes **[Buggisch et al, 2007; Tsatamil et al, 2006; Lee et al, 2005]**. In differentiation experiments, PMA induced cells showed higher megakaryocytic marker (CD41 and CD61) expression and decreased erythrocyte marker (Gly A) expression. NAC treated cells showed reduced expression of the the megakaryocyte specific markers such as CD 41 and CD 61. Increased expression of erythroid specific marker Gly A was observed in the presence of antioxidant. Earlier reports suggest that, Reactive oxygen species affect the fate of HSCs by regulating the ROS-phosphatidylinositol-3-kinase (PI3K)/Akt transcription factor forkhead box O (FOXO)-3 and ROS-p38 mitogen-activated protein kinase α (MAPKα) pathways. $P22^{phox}$-dependent nicotinamide-adenine dinucleotide phosphate oxidase (NOX)-1 may be responsible for ROS generation in the regulation of MK commitment and differentiation **[Sardina et al, 2010; Chen et al, 2013]**.

Our studies demonstrated that miR-125b might be regulated by intracellular ROS which is generated by PMA. Previous studies also show excessive ROS in cells inhibit expression of miR-199a and miR-125b particularly in ovarian cancer cells [**He et al, 2012**]. Based on these reports, changes in the anti oxidants super oxidase desmutases (SOD1 & SOD2) and catalase levels in K562 cells may also play possible role in differentiation and regulation of cell proliferation.

Objective 3

Objective 3: To establish murine model of megakaryocytopoiesis

(a)To understand the differential expression of miR-99a and miR-125b at neonatal and adult stages.

Mouse models, holding genetic changes modelling mutations that are related with human disease, have provided important insights into regulation and deregulation of megakaryopoiesis in disease. Together with the emerging knowledge of mouse models, this has provided a new contribution to the understanding of external and internal elements that organized the transition of one large MK gives rise to thousands of discoid platelets that are identical from one another. Taking advantage of the murine fetal liver as a source of MKs to provide enough cells for biochemical analyses and as well as many studies on single cell level analysis. Along with fetal liver other sources of MK progenitors are adult bone marrow and spleen, but the yields of MK progenitor cells are usually lower compared to fetal liver **[Schulze et al, 2012]**.

Before to the development of MK isolation techniques, a great deal had been discovered about MK size and morphology using electron microscopy and Wright staining as well as by using *in vivo* experimental studies. However, the development of isolation techniques comes up completely novel areas of research on megakaryocytes was started. But isolation of MKs has been a dispute due to the fragile nature of MKs and constituting > 1% of the nucleated cells in mammalian BM. Nevertheless, techniques including centrifugal elutriation, density gradient centrifugation, immunomagnetic bead isolation and selective aggregation have been described for MK isolation from mice, rat, guinea pig, and human tissue **[Leven RM, 2004]**.

Establishing a standard culture system of murine MKs from fetal liver and BM along with approaches to purify MKs using bovine serum albumin gradients (Fraction V BSA) and sorting gave us an edge to study these cells in detail. These culture systems let us to investigations on MK development (ploidy, surface markers, etc.) and platelet production, including effects of exogenous components such as structural proteins or feeder layers.

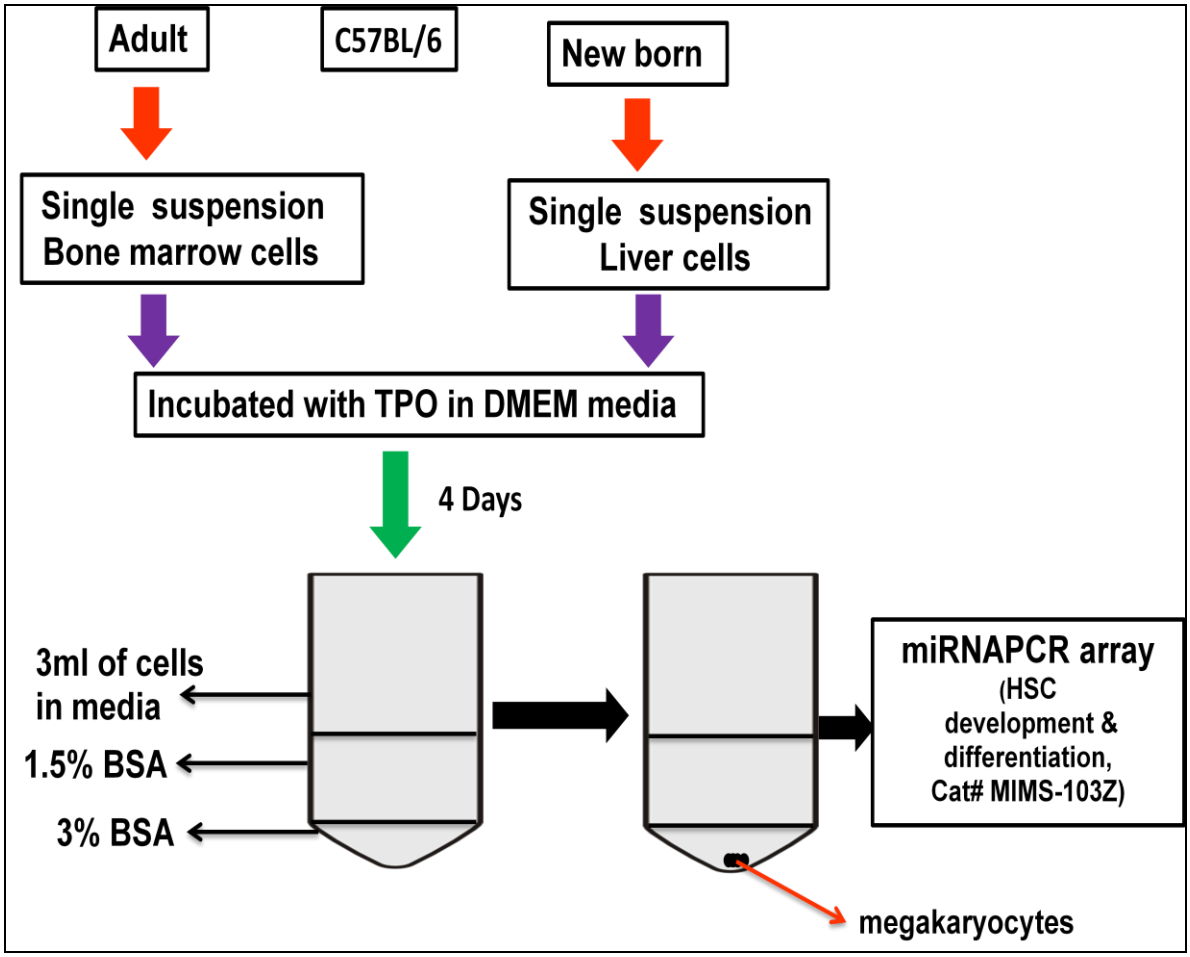


Figure 1. Schematic representation of megakaryocyte isolation from fetal liver and bone marrow of murine model. Single suspension cells were prepared from both fetal liver and Bone marrow cells. Cells were incubated with DMEM media supplemented with TPO. After 4 days of culture cells were subjected to gradient centrifugation with Fraction V BSA (3% and 1.5% BSA). Incubated at 37^0C for 1 hr and collected bottom cells of gradient tube to perform acetyl choline esterase staining to study the purity.

We established the murine MK cultures from neonatal liver and adult bone marrow **(Fig 1)**. After confirming the purity of the samples, we extracted miRNA and prepared cDNA from these samples using standard methods. We screened 88 miRNAs which are expressed in hematopoietic stem cell development and differentiation. Both neonatal and adult murine megakaryocyte samples expressed detectable amounts of all 88 screened miRNAs. Nevertheless, the specific miRNA expression levels differed significantly between neonates and adults derived megakaryocytes **(Fig 2)**.

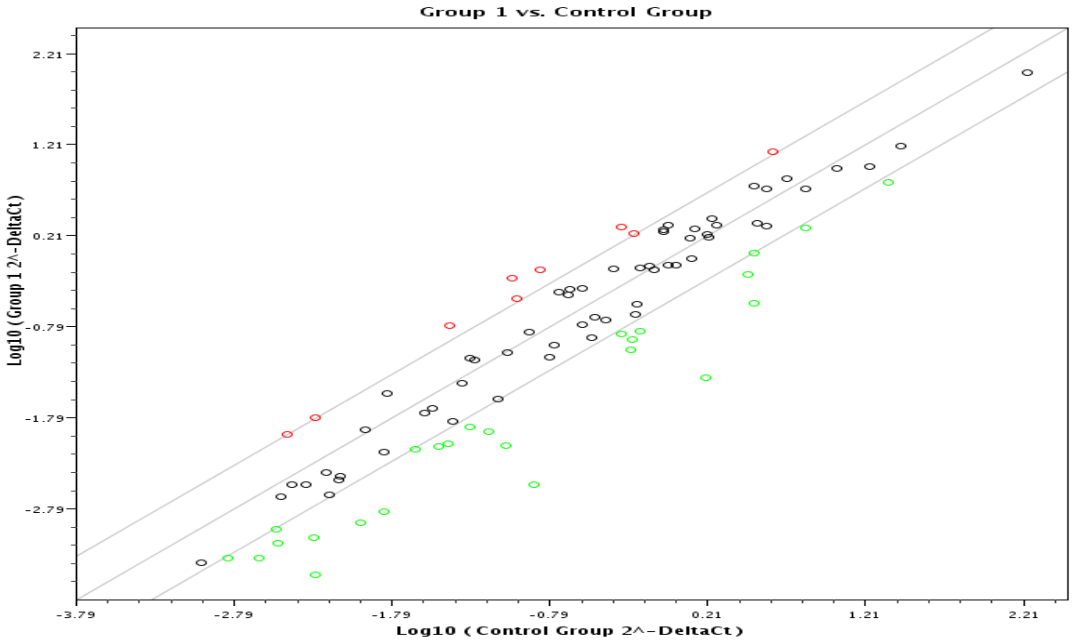


Figure 2. Scatter plot of differentially expressed miRNA in neonatal Vs adult MKs in mouse model.

Ten miRNAs were expressed at significantly higher levels, whereas, mmu-let-7b, mmu-miR-140b and mmu-miR-26b were down-regulated in neonatal MKs. All of these differences reached statistical significance ($p<0.05$, **Fig 3**).

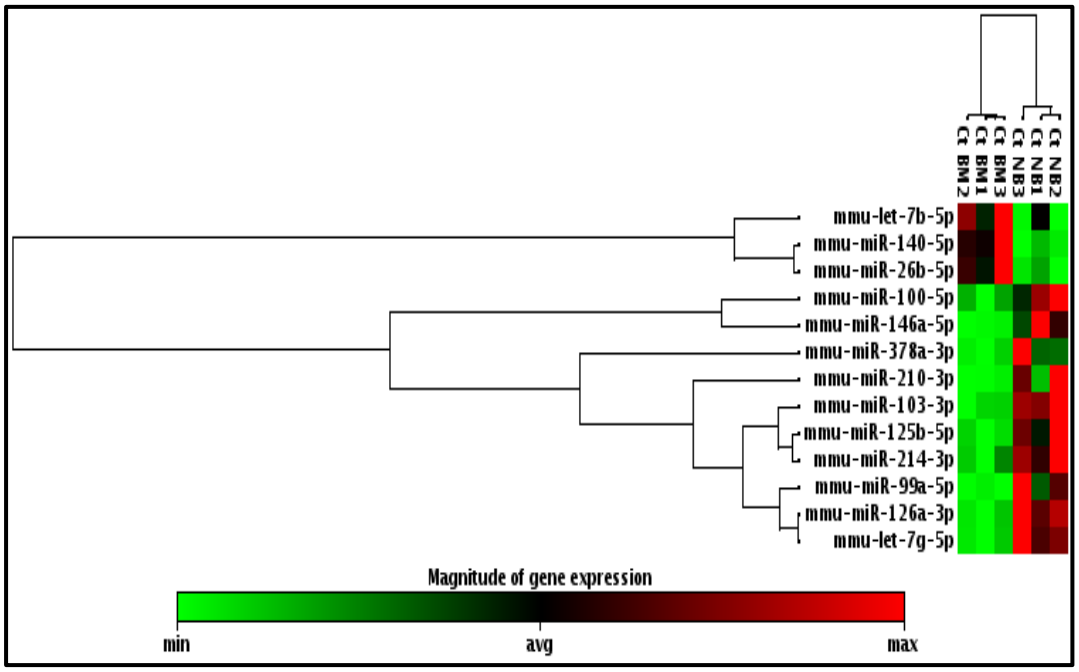


Figure 3: Cluster gram of differentially expressed miRNA in neonatal and adult murine MKs.

Similar to humans, we found miR-99a and miR-125b were significantly up regulated in mouse neonatal MKs. In order further confirm we evaluated fold change expression of miR-125b and miR-99a by Real time PCR analysis. We found ~11-fold up regulation of miR-125b and ~3.5 fold higher miR-99a levels in murine neonatal MKs compared to adult MKs **(Fig 4).**

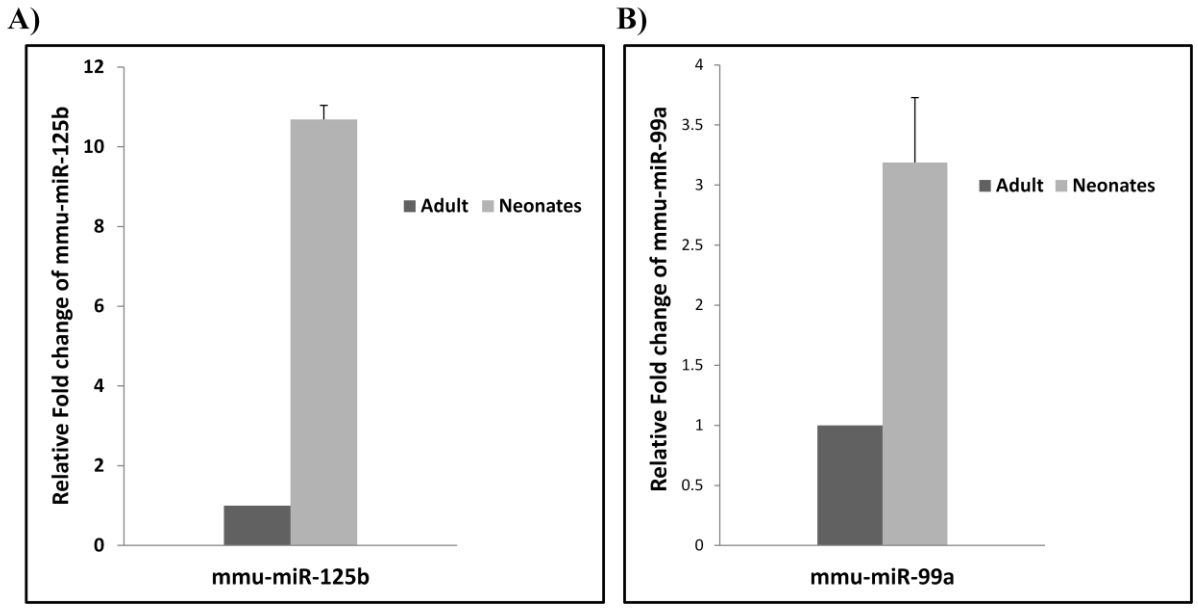


Figure 4: Expression of miR-125b and miR-99a in neonatal vs adult MKs. (A) Real time PCR quantification of the miR-125b in neonatal Vs adult MKs (day 6), (*p<0.05, n=3). (B) Real time PCR quantification of the miR-99a in neonatal Vs adult MKs (day 6), (*p<0.05, n=3).

(b) *In silico analysis of miR-99a and miR-125b regulated signaling pathways.*

The clarification of miRNA targets remains a major issue in functional examination of miRNAs. All the putative targets of miR-99a were explored using TargetScan, PicTar, miRBase and RNAhybrid. These tools suggest that there are 1024 targets of mmu-miR-99a, whereas, 665 predictable targets of mmu-miR-125b. To interpret the roles of miRNA in complex systems such as megakaryocyte development, it is important to determine relevant molecular pathways that are regulated by these miRNA taking into consideration their target genes. All targets of mmu-miR-99a (1024 genes) and mmu-miR-125b (665 genes) were predicted by web-based computational approaches and were submitted to David database to identify functional signaling pathways **(Fig 5).** Bioinformatics analysis (target prediction and pathway analysis) revealed that a number of biological pathways are targeted by these

miRNAs, miR-99a and miR-125b that are differentially-expressed in neonates and adult MKs.

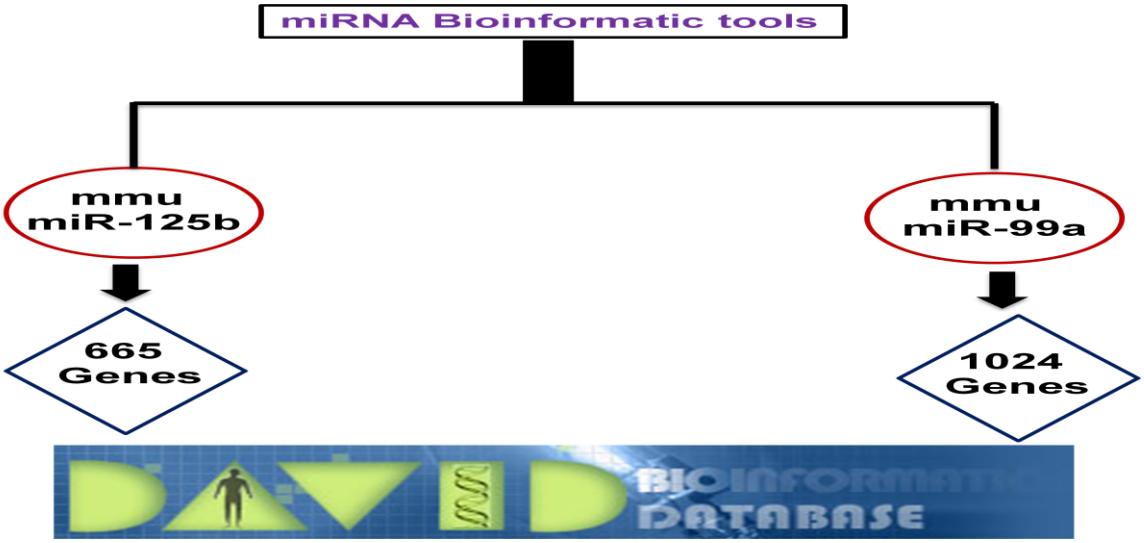


Figure 5. Flow chart of miRNA functional pathways predictions by *in silico* approach.

In silico analysis showed that mmu-miR-125b was regulating 19 signalling pathways, particularly, hematopoietic cell lineage MAPK signaling and cytokine-cytokine receptor interaction pathways **(Fig 6).** These pathways have previously been known to be regulators of MK development.

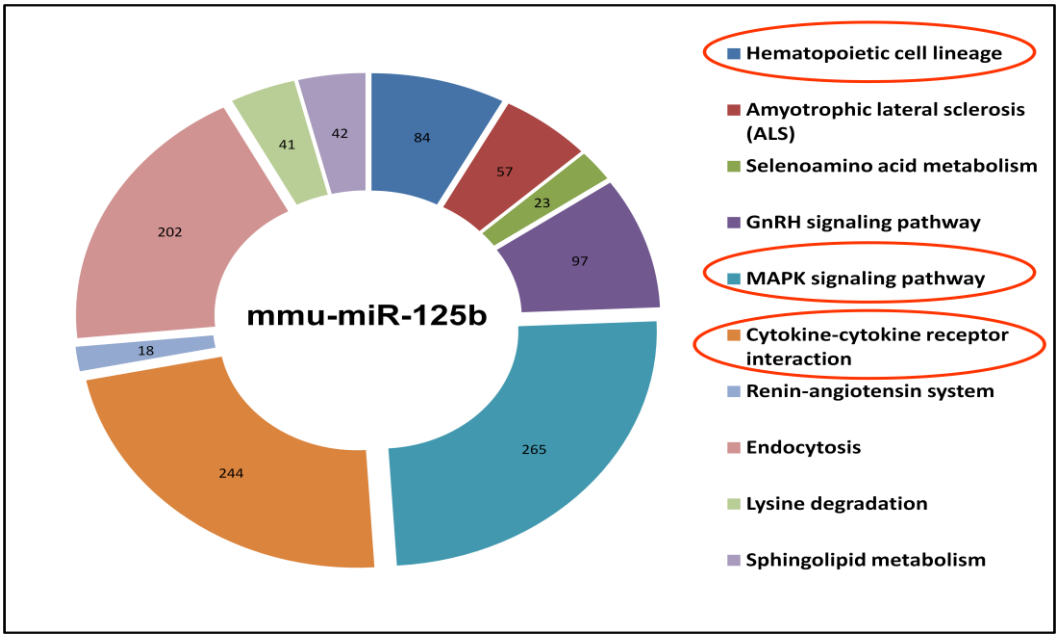


Figure 6. mmu-miR-125b regulated signaling pathways by *in silico* analysis.

In silico analysis showed that mmu-miR-99a was regulating 25 signalling pathways, particularly, cancer associated pathways, MAPK signalling pathways **(Fig7).** These pathways have previously been known to be regulators of MK development. Association with cancer related pathways suggests its involvement in cell proliferation.

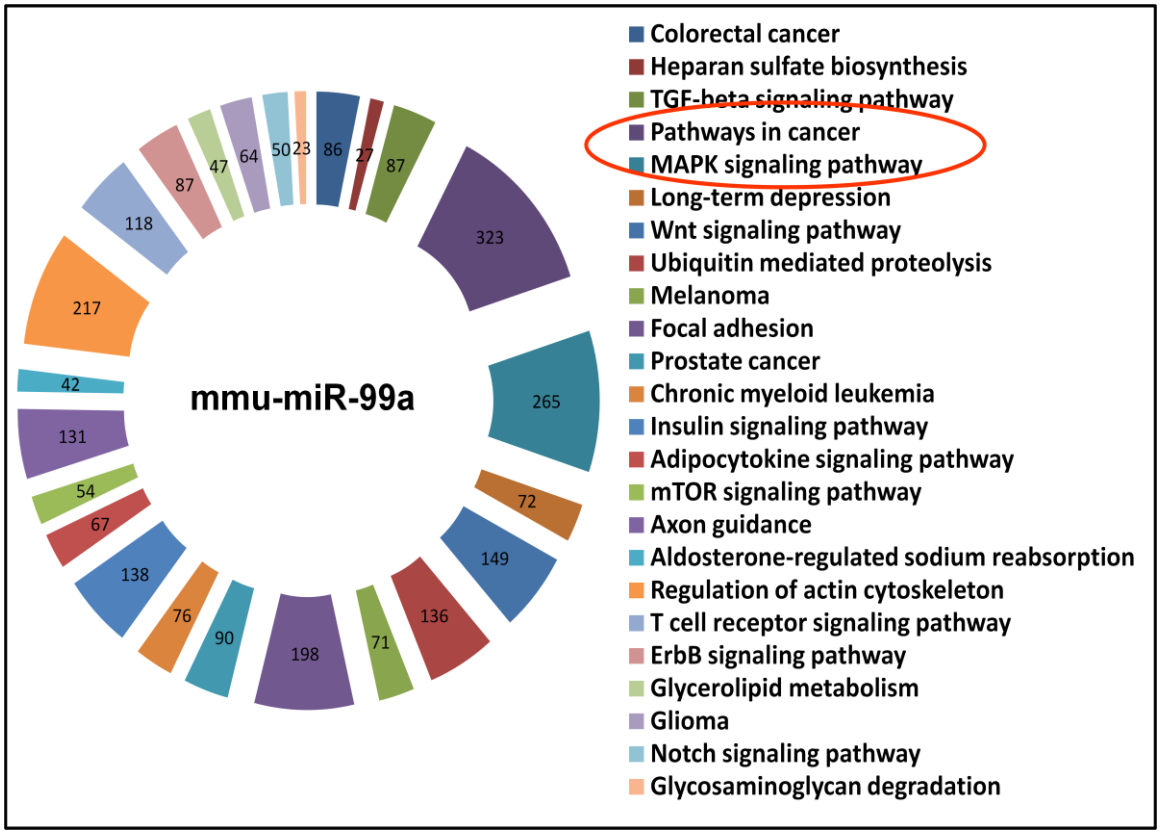


Figure 7. mmu-miR-99a regulated signaling pathways by *in silico* approach.

Disscussion

Several reports suggest that there are substantial developmental differences in neonates and adults megakaryocytopoiesis **[Liu et al, 2010],** involving several transcriptional factors and signaling pathways **[Liu & Sola- Visner, 2011; Klusmann et al, 2010]**. The molecular mechanisms behind these developmental differences are not known. miRNAs have been discovered as important players in various developmental systems and their differential expressions are reported in megakaryocytes. In current study, we have studied 88 miRNA expression profiles which are associated with hematopoietic stem cell development and differentiation and their involvement in regulation of the megakaryocyte development and elucidated its signaling pathways regulation by *in silico* approach.

miRNAs are small non-coding RNA molecules regulates gene expression by repressing protein coding genes thereby regulate physiological and pathological processes. Even though heterogeneity found within MK development miRNA and gene expression profiles, our studies were specific to differential miRNA expression profile in neonates and adult megakaryocytopoiesis. We found that ten miRNAs were expressed at significantly higher levels, whereas, mmu-let-7b, mmu-miR-140b and mmu-miR-26b were down-regulated in neonatal MKs. Similar to humans, we observed that miR-99a and miR-125b up regulated in neonates compared to adults MKs. These two miRNAs regulating mostly cancer associate pathways which suggesting its involvement in proliferation.

These studies will help us know the molecular basis for the specific phenotype of MKs and to elucidate the pathogenesis several MK disorders that predominantly associated with neonatal and other hematopoietic disorders.

SUMMARY

In conclusion our preliminary data suggest that miRNAs would be differentially expressed in neonatal and adult MKs, and that these differences would contribute to their biological differences. The expression levels of 88 miRNAs known to be involved in human stem cell differentiation and development were measured using a quantitative PCR-based array kit. All samples (n=3) expressed detectable amounts of all 88 screened miRNAs. However, ten miRNAs were expressed at significantly higher levels in CB compared to PB MKs (2 to 21 fold, $p<0.05$), while only one, miRNA, was down-regulated in CB (10% of PB). Among these up regulated miRNAs, interestingly we found that miR-99a and miR-125b located on same chromosome 21. We further investigated miR-99a and miR-125b role in neonatal megakaryocyte proliferation.

In the conclusion of the objective: 1 We compared miR-99a levels in primary MKs derived from cord blood (CB) and peripheral blood (PB) by quantitative PCR and found ~12-fold up-regulation (on day 14) in neonatal megakaryocytes compared to adults (n=3, $p<0.05$).To explain the potential roles of miR-99a in megakaryocyte development, we predicted the targets of miR-99a via the algorithms: TargetScan, PicTar, and miRanda and CTDSPL was found to be a predicted target by bioinformatic analysis. We showed that CTDSPL expression can be dramatically reduced by elevated levels of miR-99a, after which the activity of CTDSPL-downstream-proteins such as pRB, E2F1 Cyclin D1, D2 and D3 proteins known to be over expressed in CB-MKs compared with PB- MKs. Herein, we showed that miR-99a causes an arrest of the cell cycle at the G1/S stage and decreases cell proliferation probably in a CTDSPL-directed manner.

In the conclusion of the objective: 2 we compared miR-125b levels in primary MKs derived from cord blood (CB) and peripheral blood (PB) by quantitative PCR and found ~4-fold up-regulation (on day 14) in neonatal megakaryocytes compared to adults (n=3, $p<0.05$). Taken together, this data provide compelling evidence that miR-125b is differentially expressed in developmentally different CB- and PB-derived MKs. We harvested CB and PB cells on days 0, 7, 11, and 14 of MK culture, isolated miRNA, and determined miR-125b expression levels by quantitative PCR. Values in each sample were normalized against U6 (miRNA internal control), and CB miR-125b levels were expressed as relative values to the PB levels at the same stages of culture. This analysis revealed that neonatal MKs expressed higher miR-125b at all levels. A putative target of miR-125b was explored using Target Scan, PicTar and

miRBase. Three classes (one from each class) of targets viz. Tumor Suppressor, Apoptosis and Cell cycle regulator were selected. P53, BAK1 and CDK6 were most potential target among several putative targets. We confirmed the results using western blot analysis and found P53, BAK1 and CDK6 protein levels were higher in PB compared to CB MKs.

In the conclusion of the objective: 3 we established the murine MK cultures from neonatal liver and adult bone marrow. After confirming the purity of the samples, we extracted miRNA and prepared cDNA from these samples. Both neonatal and adult murine megakaryocyte samples expressed detectable amounts of all 88 screened miRNAs. Nevertheless, the specific miRNA expression levels differed significantly between neonates and adults derived megakaryocytes. Ten miRNAs were expressed at significantly higher levels, whereas, mmu-let-7b, mmu-miR-140b and mmu-miR-26b were down-regulated in neonatal MKs. All of these differences reached statistical significance ($p<0.05$). Similar to human miRNA expression profiles, we found miR-99a and miR-125b were significantly up regulated in mouse neonatal liver derived MKs. All targets of mmu-miR-99a (1024 genes) and mmu-miR-125b (665 genes) were predicted by web-based computational approaches (Target Scan, PicTar and MiRanda) and submitted to David database to identify functional signaling pathways. *In silico* analysis showed that mmu-miR-99a and mmu-miR-125b were regulating 25 and 10 signaling pathways respectively. The pathways identified were previously reported to be involved in MK development.

These findings mainly suggest that miR-99a and miR-125b play significant role in proliferation of megakaryocyte and its development, mediated by repressing the CTDSPL and p53 and this may be possible therapeutic target for treatment of several leukaemias and other blood disorders.

BIBLIOGRAPHY

- Metcalf D. (2007). Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. *Stem Cells*, 25(10): 2390-5.
- Akashi K, Traver D, Miyamoto T et al. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*, 404(6774):193-197.
- Seita J & Weissman I.L. (2010). Hematopoietic stem cell: Self- renewal versus differentiation. *Wiley Interdisciplinary Reviews systems Biology and Medicine*, 2(6): 640-53.
- Sanjuan-Pla A, Macaulay I. C, Jensen C. T. et al. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*, 502(7470):232-236.
- Svoboda, O & Bartunek P. (2015). Origins of the Vertebrate Erythro/Megakaryocytic System. *Biomed Research International*, 632171.
- Yamamoto R, Morita Y, Ooehara J. et al. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*, 154(5):1112-1126.
- Ogawa D. (1993). Differentiation and proliferation of hematopoietic stem cells. *Blood*, 81:2844-2853.
- Briddell R, Brandt J, Stravena J et al. (1989). Characterization of the human burst-forming unit-megakaryocyte. *Blood*, 59:145-151.
- Kaushansky K. (2005). The molecular mechanisms that control thrombopoiesis. *Journal of Clinical Investigation*, 115(12):3339-47.
- Shinjo, K. Takeshita A, Nakamura S, et al. (1998). Serum thrombopoietin levels in patients correlate inversely with platelet counts during chemotherapy-induced thrombocytopenia. *Leukemia*, 12 (3):295-300.
- Liu ZJ, Italiano J Jr, Ferrer-Marin F et al. (2011). Developmental differences in megakaryocytopoiesis are associated with up-regulated TPO signaling through mTOR and elevated GATA-1 levels in neonatal megakaryocytes. *Blood*, 117(15): 4106-4117.
- Pisciotta AV, Stefanini M, Dameshek W. (1953). Studies on platelets: morphologic characteristics of megakaryocytes by phase contrast microscopy in normals and in patients with idiopathic thrombocytopenic purpura. *Blood*, 8(8): 703- 723.
- Levine RF, Hazzard KC, Lamberg JD. (1982).The significance of megakaryocyte size. *Blood*, 60(5):1122-1131.

- Gewirtz AM & Schick B. (1994). Hemostasis and Thrombosis: Basic Principles and Clinical Practice edited by Colman RW, Marder VJ, Clowes AW, George JN, Goldhaber SZ. 5th ed. Philadelphia: *Lippincott Williams & Wilkins* pp 359-361.
- Cramer EM, Vainchenker W. (2006). Platelet Production: Cellular and Molecular Regulation Ch 25 in Hemostasis and Thrombosis: Basic Principles and Clinical Practice. *Lippincott Williams & Wilkins. USA*.
- Kaushansky K. (2009). Molecular mechanisms of thrombopoietin signaling. *Journal of Thrombosis Haemostasis*, 7: 235–238.
- Patel SR, Hartwig JH, Italiano JE. (2005).The biogenesis of platelets from megakaryocyte proplatelets. *Journal of Clinical Investigation*, 115(12): 3348-3354.
- Tavassoli M. (1980). Megakaryocyte-platelet axis and the process of platelet formation and release. *Blood*, 55: 537-545.
- Kleiman NS, Freedman JE, Tracy PB et al. (2008). Platelets: Developmental biology, physiology, and translatable platforms for preclinical investigation and drug development. *Platelets*, 19 (4):239-251.
- Jennings L. (2009). Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. *Thrombosis Haemostasis*, 102: 248–257.
- Johnson M, Ramey E, Ramwell P. (1977). Androgen-mediated sensitivity in platelet aggregation. *American Journal of Physiology*, 232(4):H381-H385.
- Sola-Visner MC, Christensen RD, Hutson AD, Rimsza LM. (2007) Megakaryocyte size and concentration in the bone marrow of thrombocytopenic and nonthrombocytopenic neonates. *Pediatric Research*, 61: 479-84.
- Pastos KM, Slayton WB, Rimsza LM et al. (2006). Differential effects of recombinant thrombopoietin and bone marrow stromal-conditioned media on neonatal versus adult megakaryocytes. *Blood*, 108:3360-2.

- Pastos KM, Slayton WB, Rimsza LM et al. (2006). Differential effects of recombinant thrombopoietin and bone marrow stromal-conditioned media on neonatal versus adult megakaryocytes. *Blood*, 108:3360-2.

- Sallmon H, Gutti RK, Ferrer-Marin F et al. (2010). Increasing platelets without transfusion: is it time to introduce novel thrombopoietic agents in neonatal care? *Journal of Perinatology*, 30:765-9.

- Mattia G, Vulcano F, Milazzo L. (2002). Different ploidy levels of megakaryocytes generated from peripheral or cord blood $CD34^+$ cells are correlated with different levels of platelet release. *Blood*, 99:888-897.
- Ambros V. (2004). The functions of animal microRNAs. *Nature*, 431: 350-355.
- Bartel DP. (2004). MicroRNAs: Genomics Biogenesis, Mechanism, and Function. *Cell*, 116: 281–297.
- Marcucci G, Radmacher MD, Maharry K et al. (2008). MicroRNA expression in cytogenetically normal acute myeloid leukemia. *New England Journal of Medicine*, 358:1919-1928.
- Lim LP, Lau NC, Weinstein EG et al. (2003). The microRNAs of Caenorhabditis elegans. *Genes& Development*, 17(8):991-1008.
- Chiang HR, Schoenfeld LW, Ruby JG et al. (2010). Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. *Genes& Development*, 24(10):992-1009.
- Engel, C., Loeffler, M., Franke, H. & Schmitz, S. (1999). Endogenous thrombopoietin serum levels during multicycle chemotherapy. *British Journal of Haematology*. 105:832–838.
- Cardier JE, Dempsey J. (1998). Thrombopoietin and its receptor, c-mpl, are constitutively expressed by mouse liver endothelial cells: evidence of thrombopoietin as a growth factor for liver endothelial cells. *Blood.* 91:923–929.
- He L. & Hannon G.J. (2004). MicroRNAs: Small RNAs with a big role in gene regulation. *Nature Reviews Genetics*. 5:522.
- Lee R.C., Feinbaum R.L. & Ambros V. (1993). The C. elegans heterochronic gene lin-4encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75:843-854.
- Olsen P.H. & Ambros V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Developmental Biology*, 216:671-680.
- Wightman, B, Ha I. & Ruvkun G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell*, 75:855-862.
- Bartel D.P. & Chen, C.Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan micro RNAs. *Nature Reviews Genetics*, 5:396-400.

- Friedman R.C., Farh K.K., Burge C.B. & Bartel D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, 19:92-105.
- Concepcion CP, Bonetti C &Ventura A. (2012). The microRNA-17-92 family of microRNA clusters in development and disease. *Journal of Cancer*, 18(3):262-267.
- Houbaviy HB, Dennis L, Jaenisch R, Sharp PA. (2005). Characterization of a highly variable eutherian microRNA gene. *RNA*, 11(8):1245-1257.
- Lee Y, Kim M, Han J et al. (2004). MicroRNA genes are transcribed by RNA polymerase II. *EMBO Journal*, 23(20):4051-4060.
- Borchert GM, Lanier W, Davidson BL. (2006). RNA polymerase III transcribes humanmicroRNAs. *Nature structural & Molecular Biology* 13(12):097-1101.
- Kim VN, Han J, Siomi MC. (2009). Biogenesis of small RNAs in animals. *Nature Reviews Molecular Cell Biology*, 10(2):126-139.
- Lee Y, Ahn C, Han J et al. (2003).The nuclear RNase III Drosha initiates microRNA processing. *Nature*, 425(6956):415-419.
- Yi R, Qin Y, Macara IG & Cullen BR. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes& Development*, 17(24):3011-3016.
- Ballarino, M., F. Pagano, et al. (2009). Coupled RNA processing and transcription of intergenic primary microRNAs. *Molecular and Cell Biology*, 29(20): 5632-8.
- Nilsen T. W. (2007). Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends in Genetics*, 23(5):243-9.
- Ha M & Kim VN. (2015). Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology*, 15(8):509-24.
- Winter J, Jung S, Keller S et al. (2009). Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature Cell Biology*. 11(3):228-34.
- Undi R, Kandi R & Gutti RK. (2013). Micro RNAs as hematopoiesis regulators Regulators. *Advance in Hematology*, 2013:695754.
- S. Montagner, L.Deho & S. Monticelli. (2014). Micro RNAs in hematopoietic development. *BMC immunology***.** 15:14.
- Chen CZ, Li L, Lodish HF, Bartel DP: MicroRNAs modulate hematopoietic lineage differentiation. *Science*. 2004, 303 (5654): 83-86.

- Zhou B, Wang S, Mayr C et al. (2007). MiR-150, a microRNA expressed in mature Band T cells, blocks early B cell development when expressed prematurely. *Proceedings of the National Academy of Sciences U.S.A.,* 104 (17), 7080-5.
- Xiao C, Calado D.P, Galler G et al. (2007). miR-150 controls B cell differentiation by targeting the transcription factor c-myb. *Cell,* 131(1), 146-59.
- Tsai CY, Allie SR, Zhang W & Usherwood EJ. (2013). MicroRNA miR-155 affects antiviral effector and effector Memory CD8 T cell differentiation. *Journal of Virology*. 87(4):2348-51.
- Wu T, Wieland A, Araki K et al. (2012). Temporal expression of microRNA cluster miR-17-92 regulates effector and memory CD8+ T- cell differentiation. *Proceedings of the National Academy of Sciences U.S.A.,* 109(25):9965-70.
- Wu, H., Neilson, J.R., Kumar, P., et al. (2007). MiRNA profiling of naïve, effector and memory CD8 T cells. *PLoS One,* 2(10), 1020.
- Raghuwanshi S, Karnati HK, Sarvothaman S et al. (2015). MicroRNAs: Key players in Hematopoiesis. *Advance in Experimental Medicine Biology*. 2015; 887:171-211.
- Wakiyama M, Takimoto K, Ohara O & Yokoyama S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes & Development*. 21(15):1857-1862.
- Mathonnet G, Fabian MR, Svitkin YV, et al. (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science,* 317(5845):1764-1767.
- Kiriakidou M, Tan GS, Lamprinaki S et al. (2007). An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell,* 129 (6):1141-1151.
- Petersen CP, Bordeleau ME, Pelletier J & Sharp PA. (2006) Short RNAs repress translation after initiation in mammalian cells. *Molecular Cell,* 21(4):533-542.
- Petriv O.I, Kuchenbauer F, Delaney A.D et al. (2010). Comprehensive microRNA expression profiling of the hematopoietic hierarchy. *Proceedings of the National Academy of Sciences U.S.A.,* 107(35), 15443-8.
- Johnnidis J.B, Harris M.H, Wheeler R.T et al. (2008). Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature,* 451(7182), 1125-1129.

- Forrest A.R, Kanamori-Katayama M, Tomaru Y et al. (2010). Induction of micro RNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. *Leukemia*, 24(2):460-6.
- Wang J, Xiang G, Mitchelson K & Zhou Y. (2011). Microarray profiling of monocytic differentiation reveals miRNA mRNA intrinsic correlation. *Journal of Cellular Biochemistry*, 112(9):2443-53.
- Hornstein E. (2010). MiRNA activity directs stem cell commitment to a particular fate. *Cell Cycle*, 9(20):4041-2.
- Forntana L, Pelosi E, Greco P et al. (2007). MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor up regulation. *Nature Cell Biology*, 9(7):775-87.
- Velu, C.S., Baktula, A.M., & Grimes, H.L. (2009). Gfi1 regulates miR-21 and miR-196b to control Myelopoiesis. *Blood*, 113(19):4720-8.
- Lagrange B, Martin R.Z, Droin N et al. (2013). A role for miR-142-3p in colony-stimulating factor 1- induced monocyte differentiation into macrophages. *Biochimica et Biophysica Acta*, 1833(8):1936-46.
- Felli N, Fontana L, Pelosi E et al. (2005). MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proceedings of the National Academy of Sciences U.S.A.*, 102(50):18081-18086.
- Gabbianelli M, Testa U, Morsilli O et al. (2010). Mechanism of human Hb switching: A possible role of the kit receptor/miR 221–222 complex. *Haematologica*, 95:1253-1260.
- Felli N, Pedini F, Romania P et al. (2009). MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis. *Haematologica*, 94 (4):479-86.
- Mittal S.P, Mathai J, Kulkarni A.P, Pal J.K, & Chattopadhyay S. (2013). MiR-320a regulates erythroid differentiation through MAR binding protein SMAR1. *International Journal of Biochemistry & Cell Biology*, 45(11):2529-29.
- Wang Q, Huang Z, Xue H et al. (2008). MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. *Blood*, 11(2):588-95.
- Georgantas R.W, Hildreth R, Morisot S, Alder J, & Civin C.I. (2006). MicroRNA hsa-mir-155 Blocks Myeloid and Erythroid Differentiation of Human $CD34^{+}$ Cells. Blood (*ASH annual meeting abstract*).

- Romania P, Lulli V, Pelosi E et al. (2008). MicroRNA 155 modulates megakaryopoiesis at progenitor and precursor level by targeting Ets-1 and Meis1 transcription factors. *British Journal of Haematology*, 143:570-580.
- Lu J, Guo S, Ebert B.L et al. (2008). MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. *Developmental Cell*, 14(6):843-853.
- Barroga CF, Pham H & Kaushansky K. (2008). Thrombopoietin regulates c-myb expression by modulating microRNA150 expressions. *Experimental Hematology*, 36(12):1585-1592.
- Garzon R, Pichiorri F, Palumbo T et al. (2006). MicroRNA fingerprints during human megakaryocytopoiesis. *Proceedings of the National Academy of Sciences U.S.A.*, 103(13):5078-5083.
- Zhang L, Sankaran VG & Lodish HF. (2012). microRNAs in erythroid and megakaryocytic differentiation and megakaryocyte- erythroid progenitor lineage commitment. *Leukemia*, 26(11):2310-6.
- Bartel D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell*, 136(2):215-33.
- Chen CZ, Li L, Lodish HF, & Bartel D.P. (2004). MicroRNAs Modulate Hematopoietic Lineage Differentiation. *Science*, 303(5654):83-6.
- Li QJ, Chau J, Ebert PJ et al. (2007). miR-181a is an intrinsic modulator of T-cell sensitivity and selection. *Cell*, 129(1):147-61.
- Neilson JR, Zheng, GX, Bruge, CB, & Sharp, PA (2007). Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes & Development*, 21(5):578-89.
- Bousquet M, Nguyen D, Chen C, et al. (2012). MicroRNA-125b transforms myeloid cell lines by repressing multiple mRNA. *Haematologica*, 97:1713-1721.
- Lee RC, Feinbaum RL & Ambros V. (1993). The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75: 843-854.
- O'Connell RM, Chaudhuri AA, Rao DS et al. (2010). MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. *Proceedings of the National Academy of Sciences U.S.A.*, 107:14235-14240.
- Kirito K, Fox N, Komatsu N & Kaushansky K. (2005). Thrombopoietin enhances expression of vascular endothelial growth factor (VEGF) in primitive hematopoietic cell through induction of HIF-1alpha. *Blood*, 105(11):4258-4263.

- Umemoto T, Yamato M, Ishihara J et al. (2012). Integrin-αvβ3 regulates thrombopoietin-mediated maintenance of hematopoietic stem cells. *Blood*, 119(1):83-94.
- Atsushi Hirao. (2014). TPO signal for stem cell genomic integrity. *Blood*, 123:(4).
- Li Z, Lu J, Sun M, et al. (2008). Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proceedings of the National Academy of Sciences U.S.A.*, 105(40):15535-40.
- Francesca F, Ivan V & Muller F. (2009). MicroRNAs: tiny players with a big role in the pathogenesis of leukemias and lymphomas. *Hematology reports*, 1(1):8.
- Zanette D.L, Rivadavia F, Molfetta G.A et al. (2007) miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Brazilian Journal of Medical and Biological Research*, 40(11): 1435-40.
- Chen K, Rajewsky N. (2006). Natural selection on human microRNA binding sites inferred from SNP data. *Nature Genetics*, 38 (159):1452–6.
- Yu Z, Li Z, Jolicoeur N, Zhang L et al. (2007). Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers. *Nucleic Acids Research*, 35(160):4535–41.
- Saunders MA, Liang H, Li WH. (2007). Human polymorphism at microRNAs and microRNA target sites. *Proceedings of the National Academy of Sciences U.S.A.*, 104 (9):3300-5.
- Calin GA, Sevignani C, Dumitru CD et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences U.S.A.*, 101(9):2999-3004.
- Chapiro E, Russell LJ, Struski S et al. (2010). A new recurrent translocation t(11;14) q24;q32) involving IGH@ and miR-125b-1 in B-cell progenitor acute lymphoblastic leukemia. *Leukemia*, 24 (7):1362-1364.
- Enomoto Y, Kitaura J, Hatakeyama K et al. (2011). Emu/miR-125b transgenic mice develop lethal B-cell malignancies. *Leukemia*, 25(12):1849-1856.
- Tassano E, Acquila M, Tavella E, et al. (2010). MicroRNA-125b-1 and BLID upregulation resulting from a novel IGH translocation in childhood B-Cell precursor acute lymphoblastic leukemia. *Genes Chromosome & Cancer*, 49(8):682-687.
- Bousquet M, Quelen C, Rosati R, et al. (2008). Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the

t(2;11)(p21;q23) translocation. *Journal of Experimental Medicine,* 205(11):2499-2506.

- Hussein K, Theophile K, Büsche G et al. (2010). Aberrant microRNA expression pattern in myelodysplastic bone marrow cells. *Leukemia Research,* 34(9):1169–1174.
- Votavova H, Grmanova M, Dostalova Merkerova M et al. (2011). Differential expression of microRNAs in $CD34^{+}$ cells of 5q- syndrome. *Journal of Hematology & Oncology*, 4:1.
- Cammarata G, Augugliaro L, Salemi D et al. (2010). Differential expression of specific microRNA and their targets in acute myeloid leukemia. *American Journal of Hematology*, 85(5):331– 339.
- Zhang H, Luo XQ, Zhang P et al. (2009). MicroRNA patterns associated with clinical prognostic parameters and CNS relapse prediction in pediatric acute leukemia. *Plos one*, 4(11):e7826.
- Torres A, Torres K, Pesci A et al. (2012). Deregulation of miR-100, miR-99a and miR-199b in tissues and plasma coexists with increased expression of mTOR kinase in endometrial endometrial carcinoma. *BMC cancer*, 12:369.
- Emmrich S, Rasche M, Schoning J et al. (2014). MiR-99a/100-125b tricistrons regulate hematopoietic stem and progenitor cell homeostasis by shifting the balance between TGF β and Wnt signaling. *Genes & Development,* 28(8):858-74.
- Ooi AG, Sahoo D, Adorno M et al. (2010). MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. *Proceedings of the National Academy of Sciences U.S.A.,* 107(50):21505-21510.
- Guan Y, Yao H, Zheng Z et al. (2010). MiR-125b targets BCL3 and suppresses ovarian cancer proliferation. *International Journal of Cancer,* 128(10):2274-2283.
- Huang L, Luo J, Cai Q et al. (2010). MicroRNA-125b suppresses the development of bladder cancer by targeting E2F3. *International Journal of Cancer,* 128(8):1758-1769.
- Hui AB, Lenarduzzi M, Krushel T et al. (2010). Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. *Clinical Cancer Research,* 16(4):1129-1139.
- Baffa R, Fassan M, Volinia S et al. (2009). MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *Journal of Pathology*, 219(2):214-221.

- Schaefer A, Jung M, Mollenkopf H. J. et al. (2010). Diagnostic and prognostic implications of microRNA profiling in prostate carcinoma**.** *International Journal of Cancer*, 126(5):1166-1176.
- Veerla S, Lindgren D, Kvist A et al. (2009). MiRNA expression in urothelial carcinomas: important roles of miR-10a, miR-222, miR-125b, miR-7 and miR-452 for tumor stage and metastasis, and frequent homozygous losses of miR-31. *International Journal of Cancer*, 124(9):2236-2242.
- Calin GA, Sevignani C, Dumitru CD et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proceedings of the National Academy of Sciences U.S.A.*, 101(9):2999-3004.
- Hu H, Zhang Y, Cai X et al. (2012). Changes in microRNA expression in the MG-63 osteo sarcoma cell line compared with osteoblasts. *Oncology Letters* 4(5):1037-1042.
- Li XJ, Luo XQ, Han BW et al. (2013). MicroRNA-100/99a deregulated in acute lymphoblastic leukaemia suppresses proliferation and promote apoptosis by regulating the FKBP51 and IGF1R/mTOR signalling pathways. *British Journal of Cancer*, 109(8):2189-98.
- Akbari Moqadam F, Lange-Turenhout EA, Aries IM et al. (2013). MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood acute lymphoblastic leukemia. *Leukemia research*, 37(10):1315-21.
- Zhang L, Li X, Ke Z, et al. (2013). MiR-99a may serve as a potential oncogene in pediatric myeloid leukemia. *Cancer Cell International*, 13(1):110.
- Ferrer-Marin F, Liu ZJ, Gutti R & Sola-Visner M. (2010). Neonatal thrombocytopenia and megakaryocytopoiesis. *Seminars in Hematology* 47(3): 281-8.
- Amir S, Ma AH, Shi XB et al. (2013). Oncomir miR-125b suppresses p14 (ARF) to modulate p53-dependent and p53- independent apoptosis in prostate cancer. *PLoS One*, 8 (4):e61064.
- Klusmann JH, Li Z, Böhmer K et al. (2010) miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. *Genes & Development*, 24(5):478-90.
- V Solozobova & C Blattner. (2011). P53 in stem cells. *Journal of Biological Chemistry*, 2(9):202–214.
- Roy A, Roberts I, Norton A & Vyas P. (2009). Acute megakaryoblastic leukaemia (AMKL) and transient myeloproliferative disorder (TMD) in Down syndrome: a

multi-step model of myeloid leukaemogenesis. *British Journal of Haematology*, 147 (1):3-12.

- Lin T, Chao C, Saito S et al. (2004). P53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nature Cell Biology*. 7(2):165-71.
- Bousquest M, Harris MH, Zhou B, Lodish HF. (2010). Micro RNA miR-125b causes leukemia. *Proceedings of the National Academy of Sciences U.S.A.,* 107(50):21558-63.
- So AY, Zhao JL, & Baltimore D. (2013). The Yin and Yang of micro RNAs: leukemia and immunity. *Immunological reviews*, 253 (1):129-45.
- Li Y, Jenkins CW, Nichols MA & Xiong Y. (1994). Cell Cycle expression and p53 regulation of the cyclin- dependent kinase inhibitor p21.*oncogene*. 9(8): 2261-8.
- Kashuba V.I, Li J, Wang F et al. (2004). RBSP3 (HYA22) is a tumor suppressor gene implicated in major epithelial malignancies. *Proceedings of the National Academy of Sciences U. S. A*, 101(14):4906-4911.
- Kandi R, Gutti U, Saladi R.G., & Gutti R.K. (2014). MiR-125b and miR-99a encoded on chromosome 21 co-regulate vincristine resistance in childhood acute megakaryoblastic leukemia. *Hematology Oncology and Stem Cell Therapy,* 8(2):95-7.
- He J, Xu Q, Jing Y et al. (2012). Reactive oxygen species regulate ERBB2 and ERBB3 expression via miR-199a/125b and DNA methylation. *EMBO Reports*, 13(12):1116-22.
- Chen S, SU Y & Wang J et al. (2013). ROS mediated platelet generation: a microenvironment dependent manner for megakaryocyte proliferation, differentiation and maturation. *Cell Death & Disease*, 4:e722.
- Sardina JL, Lopez-Ruano G, Sanchez-Abarca LI et al. (2010). P22phox-dependent NADPH oxidase activity is required for megakaryocytic differentiation. *Cell Death & Differentiation*, 17(12):1842-54.
- Zhu QS, Xia L, Mills GB et al. (2006). G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. *Blood*, 107(5): 1847-1856.
- Buggisch M, Ateghang B, Ruhe C et al. (2007). Stimulation of ES-cell-derived cardiomyogenesis and neonatal cardiac cell proliferation by reactive oxygen species and NADPH oxidase. *Journal of Cell Sciences,* 120(5): 885-894.

- Tsatmali M, Walcott EC, Makarenkova H & Crossin KL. (2006). Reactive oxygen species modulate the differentiation of neurons in clonal cortical cultures. *Molecular and Cellular Neurosciences,* 33(4): 345–357.
- Lee NK, Choi YG, Baik JY et al. (2005). A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. *Blood,* 106(3): 852–859.
- Schulze H. (2012). Culture of murine megakaryocytes and platelets from fetal liver and bone marrow. *Methods in molecular biology,* 788:193-203**.**
- Leven RM. (2004). Isolation of primary megakaryocyte and studies of proplatelet formation. *Methods in molecular Biology,* 272: 281-91.
- Sonkoly E, Stahle M, Pivarcsi A (2008). Micro RNAs and immunity: Novel players in the regulation of normal immune function and inflammation. *Seminars in Cancer Biology,* 18(2):131-40.

PUBLICATIONS

List of published articles

1) **Kandi R,** Gutti U, Undi R, Sahu I, Gutti RK. Understanding Thrombocytopenia: Physiological Role of microRNA in Survival of Neonatal Megakaryocytes. ***Journal of thrombosis and thrombolysis*****.** 2015; 40(3):310-6.

2) **Kandi. R,** Undi R, Gutti RK.MiR-125b regulates cell proliferation and survival in neonatal megakaryocytes. ***Annals of Hematology*****.** 2014; 93(6):1065-6.

3) **Kandi R,** Usha Gutti, Raja Gopal Venkata Saladi and Gutti RK. MiR-125b and miR-99a encoded on chromosome 21 co-regulate Vincristine resistance in childhood acute megakaryoblastic leukemia. ***Hematology Oncology and Stem Cell Therapy*****.** 2015 Jun; 8(2):95-7.

4) Sahu I, Mishra S, Undi R, **Kandi R,** Gutti U, Gutti RK. Sequence and structural difference favors a distinct preference of Wnt3a binding with co-receptor LRP6. ***Journal of biomolecular Structure & Dynamics.*** 2015, 1-12.

5) Karnati HK, Pasupuleti SR, **Kandi R,** Undi RB, Sahu I, Kannaki TR, Subbiah M, Gutti RK. TLR-4 signaling pathway: MyD88 independent pathway up-regulation in chicken breeds upon LPS treatment. ***Veterinary Research Communications*****.** 2014, 39(1):73-8.

6) Undi RB, **Kandi R,** Gutti RK. MicroRNAs as Haematopoiesis Regulators. ***Advances in Hematology*****.** 2013:695754.

7) Undi R, **Kandi R,** Tummula PR and Gutti RK. Cord Blood Banking: Current Developments and Future Regenerative Transplant Medicine. ***International Journal of General Medicine and Pharmacy (IJGMP*****).** 2012, 1, 36-52.

8) Undi R, Gutti U, Sahu I, Sarvothaman S, Pasupuleti SR, **Kandi R** and Gutti RK. Wnt Signaling: Role in Regulation of Haematopoiesis. ***Indian Journal of Hematology and Blood Transfusion.*** Doi: 10.1007/s12288-015-0585-3.

REPRINTS OF PUBLICATIONS