DNA Replication-Repair In Megakaryopoiesis

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Saran Chattopadhyaya

List of Publications

- **In Thesis**:

1. **Saran Chattopadhyaya**, Srijan Haldar and Subrata Banerjee. **Microvesicles promote megakaryopoiesis by regulating DNA methyltransferase and methylation of Notch 1 promoter — *Journal of Cellular Physiology*, 2019. Article DOI: 10.1002/jcp.29166.**

2. **Saran Chattopadhyaya** and Subrata Banerjee. ***Regulation of Nucleolus activity via miRNA 146b in polyploid megakaryocytes*** — **Communicated.**

3. **Saran Chattopadhyaya** and Subrata Banerjee. **Polyploidy** – Review article (Manuscript under preparation).

- **Not In Thesis:**

1. Dr. Madhuparna Nandi , Ms. Sourina Pal , Dr. Sumantra Ghosh , Mr. Bidhan Chandra Chakraborty , Ms. Debangana Dey , Ms. Ayana Baidya , Mr. Achintya Shil , **Mr. Saran Chattopadhyaya** , Dr. Soma Banerjee , Prof. Amal Santra , Prof. Abhijit Chowdhury. Global CD8+CD28-T-cells: Key cytotoxic players impacting disease pathogenesis and viral persistence in chronic HBV infection – Accepted in **Clinical Science Journal, 2019, (133), 1917–1934.**

2. Zeaul Hoque Mazumdar, **Saran Chattopadhyaya** et al., Synthesis of Unsymmetrical Water-Soluble Cationic Pyridinium Mesoporphyrinic Free-Base Porphyrins and Its Zn (II) Complex: Photophysical and Photocytotoxicity Evaluation – Published in **IOSR Journal of Applied Chemistry (IOSR-JAC), 2018. Volume 10, Issue 7 Ver III (July 2017), PP 43-50.**

3. Devashish Sengupta, Subhojit Das, **Saran Chattopadhyaya**, Debdulal Sharma, Zeaul Hoque Mazumdar, Samita Basu and Subrata Banerjee. ‡Immunogenic Cancer Photodynamic Therapy (PDT)- induced Apoptosis by Two Supermagnetite Nanohybrids of Fe3O4 Nanoparticles Functionalized with Identical Porphyrin Photosensitizers Differentiated by Two Types of Linkers – **Communicated**.

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Conferences Attended

1. **Saran Chattopadhyaya** and Subrata Banerjee. International Congress of Cell Biology, Hyderabad, 27th January – 31st January, 2018. Chromatin Condensation and Megakaryopoiesis**.**
2. **Saran Chattopadhyaya** and Subrata Banerjee. SINP International Cancer Meeting. Cancer Biology Still A Challenge In 21st Century And SINP School on Epigenetics, 26th September – 28th September,2018. Microvesicles Promote Megakaryopoiesis.
3. **Saran Chattopadhyaya** and Subrata Banerjee. International Symposium on Frontiers in Development & Molecular Medicine (FDMM), Kolkata, 1st March to 3rd March, 2019. Nucleolus Activity During Maturation Of Polyploid Megakaryocytes.
4. **Saran Chattopadhyaya** and Subrata Banerjee. School and symposium on Advances in Biomedical Mass Spectrometry (SSABMS), Kolkata, 11th November to 17th November, 2019. Regulation of Nucleolus activity via miRNA 146b in polyploid megakaryocytes.

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Saran Chattopadhyaya

DEDICATIONS

To my parents….

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Chapter 5

Discussion

Megakaryopoiesis is highly orchestrated by the communication of various types of growth factors which promotes the progenitor cells to differentiate into mature MKs via the interaction of Fli1, Runx1, c-Myb etc[80]. During maturation, large polyploid MKs are formed through the changes in morphological and molecular level via the subsequent formation of sub-stages from the progenitor cells. Although polyploidy is a widespread occurrence in yeasts and plants, in human polyploidization needs to be removed through the interaction in molecular level. Other than these organisms, polyploidy also occurs in amphibians and fish resulting in the alteration of the gene`s expression. Alteration in gene`s expression is associated with the evolution which frequently is effected by the epigenetic silencing[263–266]. Although researchers previously assumed that platelet production is associated with cell enlargement and polyploidization, later on it was shown that platelet production is not dependent on those factors. On the contrary, it was also observed that without polyploidization, platelets could not be generated from matured MKs.Thus, it appears that megakaryocytic maturation is accompanied by platelet production which is supported by polyploidization – basically polyploidization aids in the genome amplification resulting in cellular growth[9]. A growing body of evidence has indicated that polyploidization also promotes chromosome instability or DNA damage, leading to the regulation of DNA repair processes. In the context of DNA repair, cell cycle check-points are responsible for halting the progression[150]. However, in MKs it is largely remain unknown about DNA replication and DNA repair.

Depending upon multiple morphogen and growth factors, cellular membrane releases out different submicron-sized vesicles. One of them is MVs and it is rich in phospholipids. During hematopoiesis, MVs are produced from various cells in the blood-stream such as macrophages, erythrocytes, platelets, B-cell, T-cell, dendritic cell etc. other than progenitor cells, HSCs also

release EVs. Those EVs help them to maintain self-renewal capacity, differentiation and also damage repairing. Maximum MVs that are circulating in the blood are generated from platelets. Recently, it has been delineated that a pool of EVs also are produced from MKs and they are totally different from PLMs in the context of surface expression markers and lamin expression. In contrast to PLMs, MVs that are derived from MKs contain $CD62^{-}LAMP1^{-}$ and also express full length filamin. In angiogenesis and cancer metastasis, EVs play an important role. Researchers have documented that the site of cancer metastasis are abundant in PLMs[267–272]. However, the exact role MVs that are derived from mature MKs is yet to be deciphered.

In the first study, we examined the role MVs in the differentiation of MKs and also in DNA damage repair process. Recent reports have indicated that DNMTs regulate various cellular processes along with differentiation as well as proliferation[43]. Still the regulation of megakaryopoiesis through the involvement of DNMTs is unknown. Therefore, we focused to examine the role and regulation of DNMTs in megakaryopoiesis.Additionally, the process of lineage specialisation upon incubation with Meg-MVs remains unknown. Firstly we analysed MVs and then observed that Meg-MVs are specific for their lineage and they are unable to transdifferentiate other lineage specific cells into megakaryopoiesis. In support of this observation, we found that unsorted MKs purified from in vitro cultured CB-$CD34^{+}$ stem and progenitor cells also showed an increase in megakaryopoiesis markers upon treatment with MVs. Additionally, we found that upon treatment with MVs, recipient quickly internalised the MVs through membrane fusion or endocytosis. We also measured the percentage of MVs that was engulfed by the recipient cells in terms of fluorescence intensity that corroborated the recent findings.

In order to examine the cellular methylation, we measured the expression of DNMT1, DNMT3A and DNMT3B for the maturation of MKs. Recently, researchers have found the presence of both

mRNA and non-coding RNA within the EVs. Multiple miRNAs were found to be involved in the progression of megakaryopoiesis such as miR 27a, miR 34a, miR 99a, miR 99b, miR 101, miR 107, miR 126, miR 149, miR 150, miR191, miR 198, miR 339, miR 500, miR 501[243,246,273–276]. However, each of these miRNAs is either up-regulated or plays a direct role in the progression of megakaryopoiesis. Recently, it has been identified that miR 17-92 cluster, miR 296 and miR 126 are involved in the regulation of the expression of endothelial genes[277,278]. In our study we observed that miR 125b and miR 99a can be possible regulator of DNMT3A and DNMT3B respectively. Consequently, methylation level within the cell is getting effected which subsequently is effecting various cellular genes expression involved in the progression of megakaryopoiesis. Previously, our lab documented that Notch1, Shh and Akt pathways crosstalk among each other for the development of MKs and erythrocytes. They also has reported that Notch1 plays a very crucial role in the lineage commitment of MKs. They has observed that Notch1 is getting significantly up-regulated during megakaryopoiesis[279]. This observation led us to find the reason behind the up-regulation of Notch1 during the maturation of MKs. We observed that promoter hypo-methylation of Notch1 through the down-regulation of DNMTs is responsible for megakaryopoiesis. Researchers have also suggested the involvement of Notch1 in the lineage commitment from HSCs in association with Jag1 and DII4. In conclusion, it may, therefore, be suggested that expression of DNMTs play a distinct role for the progression of megakaryopoiesis via the impairment of Notch1 promoter methylation while miR99a and miR125b concomitantly target the expression of DNA methyltransferases (Figure 5.1).

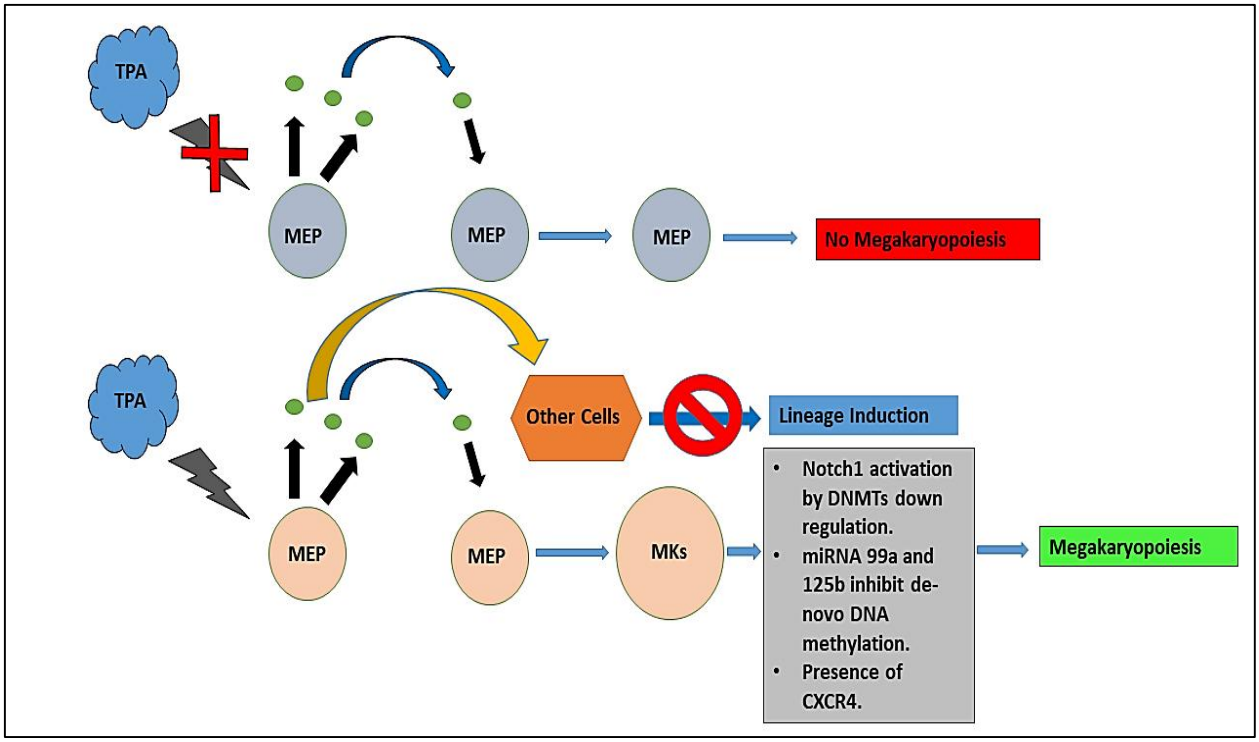


Figure 5.1. ***Schematic Representation of the study: Microvesicles promotes megakaryopoiesis by the regulation of DNMTs and methylation of Notch1 promoter.***

Moreover, researchers have also reported that down-regulation of DNMTs are involved with chromatin remodelling. Chromatin becomes more loosen after the down-regulation of DNMTs[280]. In this context, we checked the packaging of chromatin during megakaryopoiesis. During the development multiple cancer, chromatin condensation warrants the constant progression and maturation of cancer. On the contrary, although chromatin number increases during megakaryopoiesis, polyploid MKs loosens its chromatin compactness for the establishment and development of polyploid MKs which is also supported by several distinct features of MKs including increment in cell size *vis-a-vis* nuclei size. In this regard, researchers have observed the regulation of various genes. Some of them are getting up-regulated while others are down-regulated. As for example, GATA1, FOG1, Fli1, NF-E2, GAPDH etc. have been found to be

upregulated during megakaryopoiesis while Xist, Pu.1, c-Myb have been reported to be down-regulated[10,114,116,281–283]. We also observed the same for some of them (Figure 29B). In order to find the reason of chromatin decompactness during megakaryopoiesis, expression of MCM complex and histones were measured. Surprisingly, although chromatin number increases during the development of MKs, MCM2, MCM4 and MCM6 were found to be down-regulated while histones got up-regulated. This intriguing observation may assist in explaining why the megakaryocytic cells loosens it`s chromatin compactness during progression.

Since the discovery of lncRNAs, researchers have suggested the role of lncRNAs in the differentiation process of HSCs. It has been also indicated that chromatin compactness depends on the expression of lncRNAs. In hematopoiesis, B-cell and T-cell lineage commitment are regulated by lncRNAs[284]. Conceivably, MKs generate such distinct features to assist them to be polyploid, even though it is still unclear how decondensation cues result in polyploid MKs, especially through the involvement of long non-coding RNAs (lncRNAs). In this respect, profiling of lncRNAs in megakaryocytic cells shown that lncRNAs that are involved in ribosomal biogenesis are getting down-regulated. Importantly, a huge number of chromatin is needed for polyploidization of the cells accomplished through rapid cell cycle kinetics which are supported by various proteins[285]. As mentioned earlier, nucleolus acts as a hub for ribosomal biogenesis which was observed to be down-regulated during megakaryopoiesis. During proliferation of the cells, nucleolus activity and size positively regulate the cellular growth. Moreover, in cancer cells the size and activity of nucleolus are getting upregulated. Interestingly, although the cytoplasmic volume of megakaryocytic cells increase, the size of nucleolus decrease which concomitantly raise the question about the requirement of protein synthesis during megakaryopoiesis. Surprisingly, in this study, we also found an irregularity in size and activity of nucleolus during megakaryopoiesis. As

mentioned earlier nucleolus`s size and activity, can also be modulated by various proteins such as NOLC1, fibrillarin and nucleolin. NOLC1 gene locus is unique due to the presence of miR 146b within its promoter region. Therefore, we checked how this miRNA and nucleolus regulate megakaryopoiesis. Our study suggested that miR 146b not only regulate nucleolus size and activity but also regulate the progression of megakaryopoiesis.

Regeneration of MKs and thrombopoiesis are two important processes for the development of hematopoietic regenerative medicine. Numerous progress have been made for the cure of thrombocytopenia which occurs due to the low number of blood platelets. Notably, diseases like acute myeloid leukaemia (AML), AKML etc. occur in the body due to the impairment in the terminal maturation of committed MKs which are consequently characterised by thrombocytopenia. Therefore, researchers have tried to delineate the differentiation and maturation process of MKs along with the production process of platelets. Therefore, the prospect of developing artificially produced platelets is a logical and attractive goal. Conversely a parallel study indicated that enlarged nucleolar size are observed in fibroblast cells derived from Hutchinson-Gilford progeria syndrome[286], if so, then it would be interesting to investigate how platelet biogenesis are induced by nucleolar size. This study indeed demonstrated some important attributes of DNA repair associated factor DNMTs via the regulation of promoter of Notch1 for the maturation of MKs. This is the first report the not only establishes the role of Notch1 but also connects the involvement of DNMTs during EVs therapy during megakaryopoiesis not only in megakaryocytic cell lines but also in CB derived $CD34^{+}$ cells. Furthermore, the regulation of nucleolus through miR 146b during megakaryopoiesis has been also revealed. Patients suffering from thrombocytopenia also diagnosed with insufficient energy production. However, in contrast to megakaryopoiesis enlargement of nucleolus size is accompanied by the ribosomal biogenesis

which is consequently corroborated with high metabolic demands. Ultimately, this study also indicated how cells programme themselves so that they can get favourable environment for proliferation and differentiation. Indeed, our study suggests that chromatin decondensation not only supports gene activation, it may play a decisive role on cell fate also in a context-dependent manner. Finally, a better understanding of the role of the nucleolus in differentiation of MKs vis-à-vis polyploid giant cancer cells (PGCCs) could have far-reaching implications[287–291].

SYNOPSIS

In the classical model of haematopoiesis, megakaryocyte-erythrocyte progenitors (MEPs) are responsible for the production of unipotent megakaryocytes (MKs) which subsequently give rise to blood platelets through the developmental process. In higher vertebrates, platelets are essential for procedures such as blood coagulation during wound healing, angiogenesis etc. To become polyploid cells, the MKs follow a unique cell cycle replication process, commonly designated as endomitosis. Previously, our lab reported about the down regulation of MCM7 and the involvement of miR-106b-25 cluster during megakaryopoiesis. It is yet to be decided about the status of other replication and repair associated proteins during megakaryopoiesis.

In order to circumvent genomic insults, such as DNA mutation, DNA damages etc., researchers have established a link between DNA damage repair process and DNA methylation. In multiple cancers such as lung cancer, breast cancer etc. mounting lines of evidence have been suggested that the aberrant promoter hypermethylation leads to the transcriptional silencing of the DNA key repair genes. Generally, DNA methylation is executed by a family of proteins designated as DNA methyltransferases (DNMTs) including DNMT1, DNMT3A and DNMT3B. However, within the bone marrow niche it is still need to be elucidated how progenitor cells are induced for megakaryopoiesis in the over-crowded bone marrow microenvironment and subsequently regulate DNA methylation status during megakaryopoiesis. In this study, we explored the effects of megakaryocyte derived microvesicles in haematopoietic cell lines as well as in $CD34^+$ cells in the context of differentiation. Our study demonstrated that microvesicles isolated from the induced megakaryocytic cell lines have the ability to stimulate non induced cells specifically into that particular lineage. We showed that this lineage commencement comes from the change in the

methylation status of Notch 1 promoter which is regulated by DNA methyltransferases (DNMTs). Moreover, in order to reveal the status of double strand breaks (DSBs) during megakaryopoiesis, we measured the expression of H2A.X and γ-H2A.X which indicated that during this process repair process are getting elevated in expression.

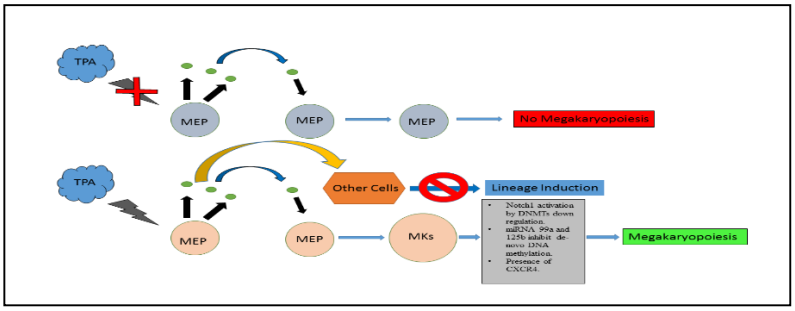


Figure1: ***<u>Schematic Representation of the study: Microvesicles promotes megakaryopoiesis by the regulation of DNMTs and methylation of Notch1 promoter.</u>***

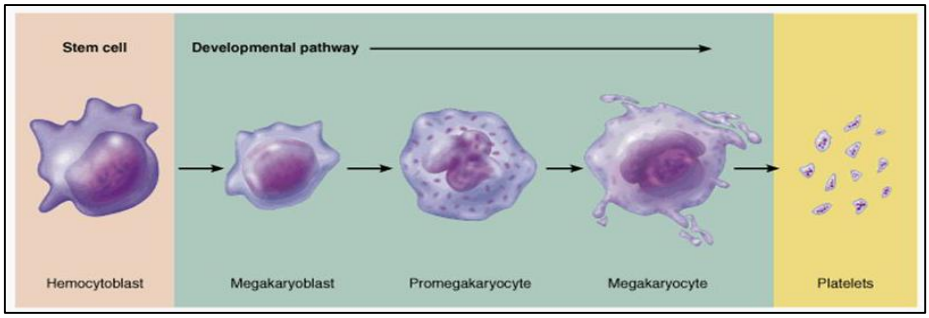
As we mentioned earlier, the MKs follow an unfamiliar cell cycle replication process, commonly designated as endomitosis. In another study, we checked the expression of other MCM proteins (MCM 2, 4, 6) and histones loaders to further elucidate the chromatin organisation in polyploid MKs. It was observed that MKs showed a chromatin decompactness which was aided by the expression of lncRNAs. Generally, nucleolus acts as a hub for the maturation of lncRNAs. We studied the nucleolar size and activity in polyploid megakaryocytic cell lines and *in vitro* cultured MKs obtained from human cord blood derived CD 34^{+} cells. Our investigation revealed the involvement of miRNA-146b in regulating the activity of NOLC1 which plays an integral role in nucleolus. Moreover, we also checked different epigenetic marks during megakaryopoiesis. This study clearly emphasized the role of chromatin organisation through the impairment of nucleolus`s activity in the process of megakaryopoiesis.

Chapter 1

Introduction

In the classical hierarchy model of hematopoiesis, hematopoietic stem cells (HSCs) produce all types of blood cells in the circulation of a living being. At the beginning of embryonic development, hematopoiesis occurs in placenta while in an adult, it takes place in bone marrow (BM). Depending upon different types of cues, HSCs at first differentiate into two types of progenitor cells – myeloid cells and lymphoid cells. Again under the differentiation, myeloid cells produce two types of progenitor cells and one of them is megakaryocytes-erythrocytes progenitor cells (MEPs). Eventually, megakaryocytes (MKs) are derived from this bipotent progenitor, MEPs. Depending upon different types of cues, such as SCF, TPO, IL3, GM-CSF etc., MEPs regularly commits for the megakaryopoiesis lineage[1–3]. MKs means "Large (Mega)", "Nucleus (Karyo)", "Cells (Cytes)" which has been coined by Howell in 1890 and those cells are usually derived from the bone marrow but liver, kidney and spleen also produce MKs. Recently, murine lung has also been reported as a site for platelet production. Average occurrence level of MKs is approximately 0.05% to 0.1% of all nucleated human bone marrow (BM) cells, but their number increases several folds as the demand for platelets increase. During its maturation, cell`s sizes increase, and this maturation process is typically defined as megakaryopoiesis. During this process, MKs undergo a stringent process to generate platelets which are indispensable for procedures such as blood coagulation, wound healing, angiogenesis etc. (Figure 1.1)[4–6]. MKs release platelets through a stringently regulated process, such as the formation of long, branching protrusions, referred to as proplatelets, consisted of platelet-sized swellings connected by cytoplasmic bridges. This biogenesis model of platelets has been validated both in vivo and in vitro. Yet, the signal behind the extension of proplatelet formation remains poorly understood. Normally, in lower vertebrates such as bird and fish, circulating blood cells, platelets are generated from diploid BM precursor

cells [3,7]. In those lower vertebrates, platelets are produced by a less stringent process than that of the higher vertebrates, but the reason is still evolutionary unclear. With the proposal of Ernest Neumann in 1868 about the renewal of blood cells, researchers have identified BM as the leading site for hematopoiesis other than spleen[8,9].



(Adapted and modified from *Basic Hematology, F. L. Garishah, 2015*)

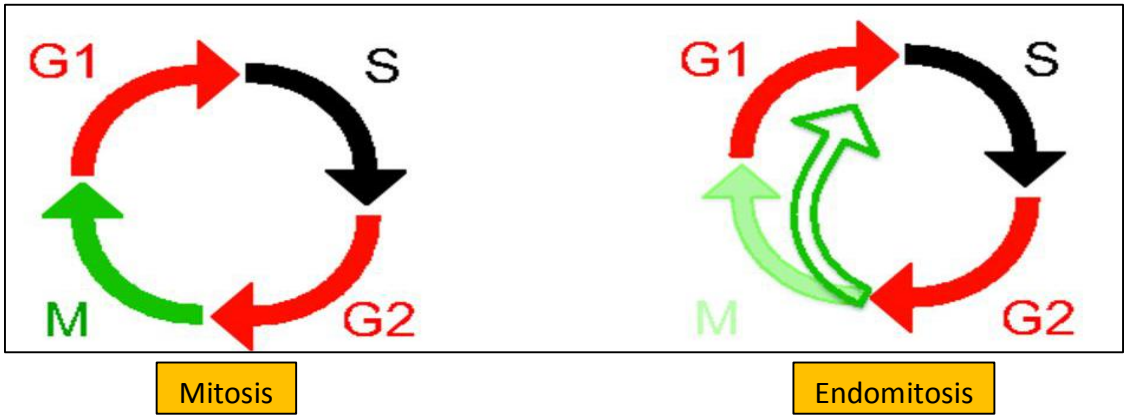
Figure 1.1 <u>Megakaryopoiesis</u>*. MKs are derived from the HSCs and produce platelets.*

Later on, it has been observed that both cytokines and adhesive interaction play a pivotal role in this developmental process. Eventually, MKs reside in the subendothelial layer of BM sinuses where different cytokines, released by several cells, such as endothelial or perivascular stromal or mesenchymal cells, regulate the maturation of MKs and the biogenesis of platelets, known as thrombopoiesis. Among cytokines, stromal-derived factor-1 (SDF-1), produced by both endosteal osteoblast and vascular endothelial cells, was identified as a first chemokine that helps to redirect MKs to their maturation through the involvement of specific receptor CXCR4. However, this developmental process is governed by thrombopoietin (TPO) upon synthesized by BM and liver. TPO induces the proliferation and maturation of MKs via the binding with the c-Mpl receptor and signalling induction. During this induction process, a subset of specific transcription factors become activated to drive the maturation of MKs. Other than TPO, cytokine stem cell factor (c-SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, FLT

ligand, IL-6, IL-11 and erythropoietin also can modulate the maturation of MKs but appear to function in cooperation with TPO. Experimental evidence has given the proof for the importance of TPO or its receptor c-Mpl in mice for megakaryopoiesis [10–14].

The most crucial characteristic of MKs is their cell cycle process. During maturation, typically MKs follow normal mitosis progression, but later MKs shift to a unique cell division which is termed as endomitosis. Endomitosis defines as a process where the cell cycle progresses without karyokinesis and cytokinesis. Usually, other blood cells can have two nuclei just before the cytoplasm division after which two identical daughter cells are formed with a single nucleus (Figure 1.2) [15]. For MKs, researchers have reported that during maturation MKs can go up to 64N in human and 256N in the mouse, which assists them in achieving polyploidy. It is still intriguing for megakaryopoiesis-study about the progression of MKs beyond 64N in human or 256N in the mouse [16,17]. In the case of MKs, the reasons behind the failure of karyokinesis and cytokinesis have been evaluated. Though previously it was assumed that endomitosis occurred due to the absence of the sub-stages of mitosis, after each round of DNA replication, Lordier *et al.*, showed that MKs indeed complete the processes of mitosis except late anaphase where sister chromatid normally separate from each other. Surprisingly, MKs cannot separate sister chromatid from each other which consequently leads to the failure of karyokinesis. In the case of cytokinesis, RhoA molecule plays a pivotal role. Recently, it has been reported that during megakaryopoiesis RhoA molecule is regulated differentially. Activated RhoA directs the actomyosin ring formation, ingression of the cleavage furrow to complete cytokinesis. Activation of RhoA critically depends on guanine exchange factors, GEF-H1 and ECT2. Recently, it has been reported that the initial progression of endomitotic cells requires the down-regulation of GEF-H1, whereas later cycles require ECT2 down-regulation[18,19]. Interestingly, this complex regulation drives the

development of matured MKs. Though DNA replication and cell division are a tightly coupled process, MKs somehow manage to bypass this process and reenter G1 stage as polyploid cells.



(Adapted and modified from *Fox et al., 2013*)

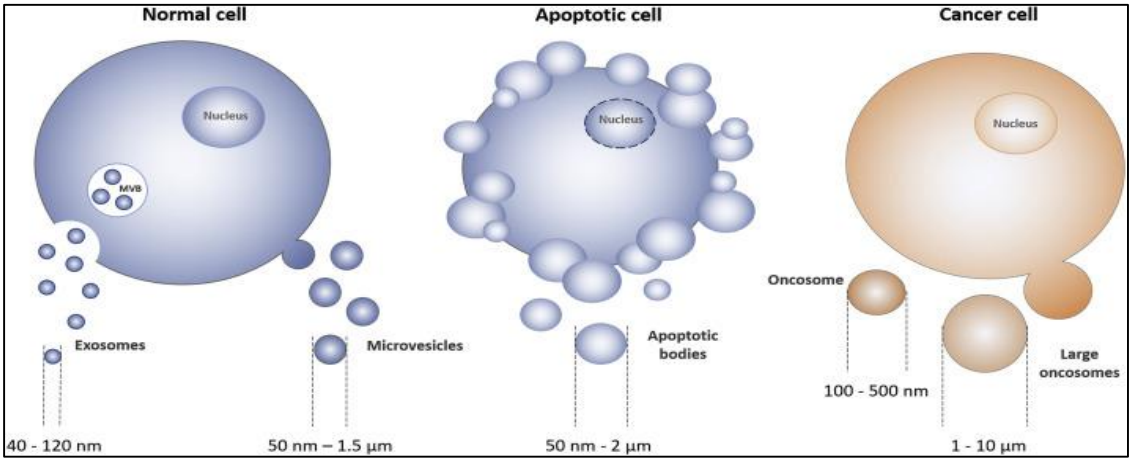
***Figure 1.2 <u>Sketch of mitosis and endomitosis</u>**. After the lineage commitment of MKs, emdomitosis are observed in those MKs specific cells.*

While progressing for the differentiation and maturation, HSCs are governed by the microenvironment of BM including cellular and extracellular compounds such as growth factors, extracellular matrices (ECM) etc[20–22]. Although very few studies focused on the BM microenvironment (BMM) for the lineage specificity, none of them outlined the relation between BMM and MKs. On one side, for differentiation HSCs get the cues for the induction from BM niche and on the other hand, they modify the microenvironment of the BM niche. In vitro and in vivo studies indicated that MKs interact with the different cellular components of mesenchymal stem/ stromal cells (MSCs) and progenitors of hematopoietic origin. MSCs not only express different types of cytokines and soluble factors, such as SDF-1, IL-6, IL-11, and SCF but also they secrete them in the concert to assist MKs maturation and proplatelet formation [23,24]. Indeed, evidence has suggested direct cell to cell interaction in between MSCs and MKs in the BM niche. Studying the crosstalk between MKs and BM niche, extracellular vesicles (EVs) turns out as an

emerging player in the intracellular communication[25,26]. For a while, researchers recognized the cellular release of the cell membrane-bound vesicles as a “Cell debris”. With the development of methodologies, it has become clear that in the context of the cell to cell communication cell releases these EVs as cellular messengers at a short distance in a paracrine or endocrine manner through the blood-stream. Reports have suggested that stem cells (SCs) produce these EVs in huge amount in order to maintain the hematopoietic cell`s expansion. On the contrary, studies have also reported that these SCs-EVs exhibit an inhibitory effect on the progression of fibrosis and immune system. Although various reports have suggested the role of SCs-EVs and MSCs-EVs, less is documented about the role of these EVs in megakaryopoiesis.

EVs are broadly categorized into four types – exosome, microvesicles (MVs), large vesicles and apoptotic bodies depending on their sizes (Figure 1.3). They are a heterogeneous group of cell-derived micron-sized vesicles. Exosomes have the diameter 40-100 nm whereas MVs can go up to 1000 nm. Exosomes formation start with the inward invasion of the plasma membrane (PM) including membrane receptors, transporters and other components of the PM and this vesicles are referred to as early endosomes. These early endosomes convert into late endosome vesicles which are subsequently released as exosomes. In contrast to exosome, MVs are formed by the outward budding of the PM[27]. Studies have shown that during MVs formation phospholipids of these vesicles redistribute itself, and it has been demonstrated that annexin V, phosphatidyl-serine (PS) can be used as markers of MVs[28,29]. However, for large vesicles and apoptotic bodies, sizes of the vesicles are more than 1000 nm. Normally, apoptotic bodies which are formed due to the hydrostatic pressure and cell contraction, contain DNA binding histones releasing from apoptotic cells while large vesicles are generated from the membrane protrusion of malignant cells carrying

components assisting in the progression of cancer cells. These micron-sized phospho-lipid-rich EVs carry cytoplasmic as well as membrane components from the producing cells.



(Adapted and modified from *Quezada et al., 2018*)

Figure 1.3 <u>Types of EVs</u>*. There are at least four types of EVs: Exosomes, microvesicles (MVs), apoptotic bodies and large vesicles. Larger vesicles can be of various sizes.*

These molecular cargos can transfer DNA, RNA, proteins, lipids molecules etc. from one cell to another as a part of cellular communication. Indeed, interest in the studies of EVs increased with the identification of both mRNA and non-coding RNA within the EVs as a previously unrecognized form of cellular communication. Recently, it has been observed that different cells release varying sizes of EVs with complex cargos, in parallel with cells specific cargos[30–32]. Since these EVs have found in the biological fluids such as blood, urine, sperm etc., now-a-days they are treated as identification markers for many biological disorders[33,34]. For instance, miRNA 155 has been observed to be upregulated in hematopoietic malignancy patients than in normal individuals[35]. Maturation of MKs occurs in specialised niches in the BM where MKs align adjacent to vascular endothelial cells. As platelets are the second most abundant cell type in the blood and play pivotal roles in hemostasis and thrombosis, understanding cellular

communication during megakaryopoiesis and platelet production have significant implications for human health. During this process growth factors, cytokines and transcription factors are essential to label the cell fate or destiny of HSCs[36,37]. With the identification of platelet-derived vesicles by electron microscopy (EM) [38], EVs become the main focus of interest in the development of hematopoiesis. In the early 1980s, researchers have reported about presence of transferrin receptors within the EVs of sheep reticulocytes[39,40]. Recent studies have given the proof for the participation of the EVs as a regulator of hematopoiesis, an activator of immune cells etc. Studies have given the evidence for the production of EVs from various hematopoietic cells such as monocytes, neutrophils, B and T lymphocytes, mast cells, reticulocytes, platelets etc. during differentiation. In this context, the EVs must able to deliver its cargo to the specific target within the BM to directly impact phenotypic and functional characteristics of recipient cells[41]. Till now platelets derived MVs have been studied substantially while EVs purified from megakaryocytic cells are poorly explored. It would be very fascinating to examine why MKs themselves secreted EVs when platelets derived MVs are present in huge number within our body. Moreover, though multiple delivery options are available for EVs such as endocytosis, direct fusion with the plasma membrane, thus it would also be interesting to probe the mechanism by which these EVs interact with cells and aid in the development of megakaryopoiesis within the BM.

In vertebrates, differentiation of HSCs into different types of blood cells is maintained by cell-type-specific factors residing in the downstream of the key signalling pathways. In addition to signalling pathways, epigenetic modification such as DNA modification especially DNA methylation (Figure 1.4), histone modification, small RNAs etc. have also now been reported as a regulator of gene expression in hematopoiesis in adult life. High throughput RNA sequencing and bisulfite modification assist in revealing the role of DNA methylation during hematopoiesis[42].

Generally, DNA methylation is executed by a family of proteins designated as DNA methyltransferases (DNMTs) including DNMT1, DNMT3A and DNMT3B. There is another member in this group, DNMT3L. Unlike other members of this group, DNMT3L does not possess any enzymatic activity while other DNMTs catalyse the transfer of methyl group at the 5th position of cytosine (C) from S-adenosyl methionine in the dinucleotide CpG called as CpG islands (CGIs). Basically, these methylations are governed by two processes – maintenance DNMTs and de novo DNMTs. Maintainance DNMT is involved in the process of methyltransferase activity during DNA replication, whereas de novo DNMT is responsible for the transfer of the methyl group to the unmethylated DNA during development. In vertebrates, DNMT 1 is the maintenance DNA methyltransferase. Unlike the single maintenance DNMT, vertebrates have multiple de novo DNMTs that are normally expressed in different cell types and tissues at different times and targets different but overlapping sets of CGIs. DNMT 3A and DNMT 3B are two de novo DNMTs that can modulate the cell fate by expressing DNA methylation at the new site. Recently, researchers have established a link between DNA methylation and activation of transcription[43]. In colon cancer cell lines, it has been reported that methylation of actively expressing genes by DNMT 3B have been executed[44]. This report has revealed a new regulation process of gene expression by DNA methylation. It is well established that DNMTs are required for chromosome instability and tumour progression. Now-a-days, a pile of evidence has suggested the link between DNA methylation and DNA repair process. Strong supports come from recent observation between promoter hypermethylation and inactivation of DNA repair system in various types of cancer. It has been reported that DNA mismatch repair gene`s promoters like MLH1 and MSH2 are observed to be hypermethylated, which leads to the inactivation of that gene in multiple types of cancer. Indeed like DNA mismatch repair, promoters of other genes from other DNA repair processes are

also found to be hypermethylated in cancer[45–47]. The discovery that DNA methylation is assisted in the expression of DNA repair process has opened new avenues for future research to understand the role of DNA methylation in gene expression in polyploid MKs and identify genes, specific for differentiation of megakaryopoiesis.

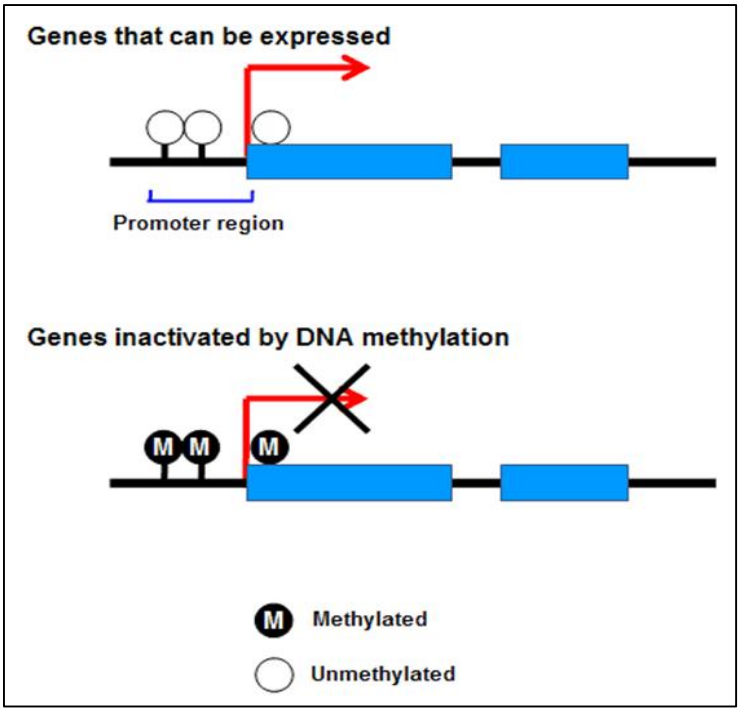


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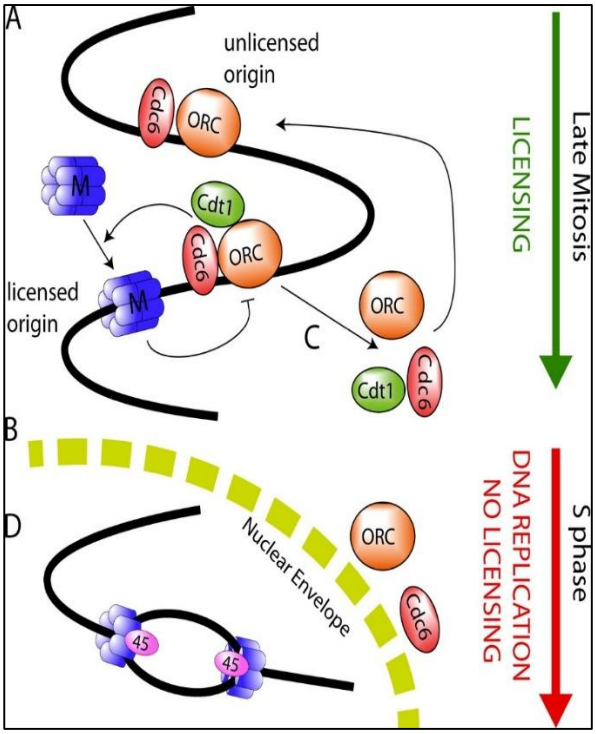
Figure 1.4 <u>Gene activation/inactivation process by DNA methylation</u>.

During hematopoiesis, Notch signalling is well known for the differentiation of HSCs. Normally, human contains four Notch single-pass trans-membrane receptors (Notch1-4) and five trans-membrane ligands (Delta-like1-3 and Jagged1-2). At the initiation of the Notch signalling, Notch receptors on the cell surface grab their respective ligands dispensed on the opposite cells. This interaction leads to the cleavage of the Notch receptor by two enzymes, referred to as ADAM family metalloprotease and γ-secretase leading to the production of Notch intracellular domain (NICD) which then translocates into the nucleus. Within the nucleus, NICD binds with RBP-Jκ

and its downstream co-activators like MAML-1, p300 or PCAF p300/CBP associated factor leading to the activation of multiple genes such as members of the basic helix-loop-helix Hairy enhancer of split (Hes) factors, Hes-related repressor protein (HERP), Gata-3 and Deltex. The direct translocation of a component of Notch signalling to the nucleus puts the pathway apart from other signalling cascades which normally depend on multiprotein phosphorylation cascades and second messengers[48,49]. This very direct mode of signalling helps in rapid transmission of cues from the cell surface to the nucleus in response to immediate cell to cell contact. Earlier, our lab has shown that Notch1 acts in equilibrium with sonic hedgehog (Shh) for the differentiation of megakaryopoiesis[50]. Additionally, Notch1 helps to commit for T cell over B cell lineage during lymphoid differentiation[51]. A large body of evidence has suggested the involvement of promoter methylation for gene`s activation. In humans, X chromosome inactivation is executed by the methylation of the gene loci suggesting the role of DNA methylation in the promoter region. Subsequently, like X chromosome inactivation several other genes were also reported whose expressions were depended on the promoter methylation level[52]. Yet it is still inconclusive about the mechanism by which Notch1 becomes activated during megakaryopoiesis.

Previously our lab showed that polyploidy in MKs is governed with changes in the expression of multiple genes associated with the various cellular pathways including DNA replication[53]. To maintain diploid chromatin number within the cell, multiples DNA replication-associated proteins ensure that each replication origin fires only once during each cell division cycle. At the initiation of DNA replication, licensing of replication origin must be accomplished. Once licensing is completed, copy of the DNA strand starts. Licensing of DNA requires the loading of minichromosome maintenance (MCM) 2-7 complexes onto the DNA origin (Figure 1.5). Licensing of DNA origin must be completed before cells enter the S phase. DNA origins act as

start site for the initiation of DNA replication. MCM 2-7 is a hetero-hexameric complex consisting of six proteins which form a ring-shaped structure after assembly. The process of origin licensing involves clamping of MCM 2-7 hexamers in an anti-parallel conformation around Origin DNA[54–57].



(Adapted and modified from *Sonneville et al., 2012*)

Figure 1.5 <u>Model for DNA replication origin licensing</u>. *DNA (solid black line) are licensed during anaphase while replicate during S phase. (A) ORC and Cdc6 at first bind to an unlicensed origin. (B) Then the origin is licensed by loading of MCM 2–7 double hexamer (M). (C) The binding of MCM 2–7 releases ORC, Cdc6, and Cdt1 at that particular site. (D) ORC and Cdc6 translocate into the cytoplasm, whereas Cdc45 binds to MCM 2–7 at the initiation of replication fork..*

It is an ATP dependent process. Additionally, the origin recognition complex (ORC), cell division cycle 6 protein (Cdc6), chromatin licensing and DNA replication factor (Cdt1) proteins assist in this licensing. At first, ORC which is consisted of six polypeptides (ORC 1-6) binds the

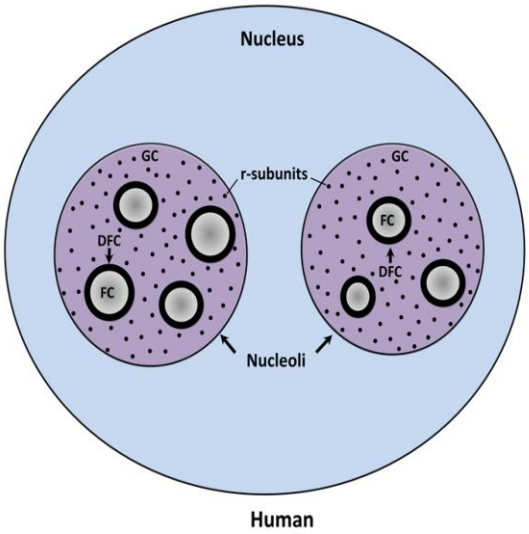
asymmetric A:T rich DNA in the presence of ATP. After binding with the DNA, Cdc6 is recruited in the origin to form a stable complex. Then Cdt1 binds the complex, and its C-terminal domain helps to recruit the MCM2-7 complex in the origin to form a pre-replication complex (pre-RC). After entering the S-phase, phosphorylation of the pre-RC complex is taken place by cyclin-dependent kinases resulting in dissociation of ORC, Cdc6, Cdt1 complex from the pre-RC complex which ensures that within the same cell division cycle the same origin of DNA must not be fired for the second times[58]. Several studies have suggested that various MCM components help in cellular proliferation *in-vitro* and *in-vivo,* especially in cancer cells by crediting its ability to increase DNA replication. Our lab earlier showed that during megakaryopoiesis the expression of MCM 7 was decreased due to the intronic miRNA cluster miR106b-25 though the expression of other MCM components during megakaryopoiesis still remains to be elucidated.

To maintain genomic function and integrity, DNA must be faithfully duplicated during cell division. In order to maintain the heterochromatin and euchromatin domains in dividing cells, chromatin duplication and chromatin organization are tightly linked. In this context, proliferating cell nuclear antigen (PCNA) and MCM2 play a very crucial role. PCNA helps to load DNA polymerases and histone chaperone, designated as chromatin assembly factor 1 (CAF-1) while apart from helicase activity MCM2 loads H3-H4 histones in association with the anti-silencing factor 1 (ASF1), a histone chaperone. During DNA replication fork movement, MCM2 helps to disassemble the nucleosome and histones are evicted from that nucleosome suggesting the modulation of chromatin organization. Apart from other processes, there are multiple forces at play in the chromatin organization that in turn affect cellular fate[59,60]. Moreover, the compaction of our large-sized flexible chromatin into the small nucleus is possible only due to the

histones and other non-histone proteins that implicate condensation of chromatin, yet chromatin organisation in megakaryopoiesis is still unknown.

Among the human genome, only 1.2% of the genome build the protein-coding exon while rest encode an ever-expanding array of non-coding RNAs (ncRNAs) whose function is yet to be deciphered. Depending on the sizes, ncRNAs are classified into two main categories – small ncRNAs having a length below 200 nucleotides (nts) and long ncRNAs (lncRNAs) whose sizes are above 200 nts. Normally, depending upon their biogenesis loci lncRNAs again are sub-divided into antisense, intronic, intergenic, bidirectional and enhancer transcripts which are transcribed by the RNA polymerases II (RNA Pol II). A mounting line of evidence has suggested the link between lncRNAs and orchestration of chromatin organization. Indeed, lncRNAs induce to load chromatin-modifying complexes to the specific genomic loci[61,62]. Indeed, several studies have identified multiple types of lncRNAs such as XIST, AIR etc. were also associated in heterochromatin formation which subsequently assists in the chromatin remodelling[63,64]. Additionally, lncRNAs are implicated in the regulation of nuclear architecture and consequently in gene expression[65]. Moreover, it is yet to be elucidated the relationship between the chromatin dynamics and accessibility with the lncRNAs in megakaryopoiesis. Moreover, nucleolar localization of lncRNAs suggests the role of lncRNAs as a regulator of ribosomal RNA (rRNA) transcription and maturation through the changes in the rDNA epigenetic status or the inhibition of transcription factor activity[66]. It is now well known that nucleolus acts as a hub for the maturation of lncRNAs. The nucleolus is the most prominent membrane-less structure of the interphase cell nucleus. Since 1962 nucleolus is considered as the site of rRNA transcription by RNA polymerase I (RNA Pol I). It is also the site for pre-rRNA processing and ribosomal subunit assembly. Like nuclear structure, nucleolus disassembles during initiation of mitosis. Due to the advancement in

microscopy techniques, the substructure of nucleolus was discovered referred to as fibrillar centre (FC) which is surrounded by dense fibrillar component (DFC) which consists of granules known as granular component (GC) (Figure 1.6)[67,68].



(Adapted and modified from *Martínez-Calvillo et al., 2019*)

Figure 1.6 <u>Schematic representation of the ultrastructural architecture of the nucleolus in humans.</u> *Human cells contain tripartite nucleolus with FC, DFC and GC region. The precursors of the r-subunits are represented by black spheres which are loosely distributed into the GCs.*

Since the last couple of years, researchers have reported a link between nucleolus`s size and cancer proliferation. Notably, enlarged nucleolus has been observed in multiple cancers. Indeed, it has been suggested that nucleolus`s size plays a very crucial role in protecting cells against infection. Enlarged nucleolus has been considered as an increment in ribosome biogenesis and chromatin number. Researchers have shown that cells containing enlarged nucleolus must contain larger volumes of the nucleus (N) and cytoplasm (C). The ratio of N/C determines the size of the nucleolus. Nucleolus activity and size is regulated by various factors. Fibrillarin, a nucleolus methyltransferase, regulates nucleolus size and activity by methylating H2A at the rDNA loci.

Nucleolin, coilin and NOLC1 were also observed to be a regulator of nucleolus size and activity. Regulatory networks governing nucleolus size are evolutionary conserved.

In this study, we explored the role of induced-MKs derived MVs (Meg-MVs) in the differentiation as well as the maturation process of MKs. Our results revealed that Meg-MVs, can induce not only HEL and K562 cell lines but also $CD34^{+}$ haematopoietic stem and progenitor cells into the megakaryocytic lineage. Induction of megakaryopoiesis through MVs was mediated by membrane-bound activated C-X-C chemokine receptor type 4 (CXCR4) present in the surface of these vesicles. We also showed that these MVs regulate the expression of cellular DNA methyltransferases- DNMT1, DNMT3a and DNMT3b which then induce the differentiation process of MKs. In particular, our study revealed a unique developmental phenomenon that of specificity, as these MVs were unable to induce other hematopoietic lineages.

Additionally, our study also revealed that unlike erythropoietic lineage where nuclear condensation occurs with cell size decrement, endomitosis proceeds oppositely. This is the first report about the nuclear decondensation which occurs during megakaryopoiesis along with a decrease in the size of the nucleolus. In contrast to polyploid giant cancer cells (PGCC), size decrement of nucleolus modulates the progression of megakaryopoiesis. Moreover, we provide evidence behind the size regulation of nucleolus during megakaryopoiesis. Our study thus reveals a vital aspect of megakaryopoiesis for their progression in terms of nucleolus activity.

Aims & Objectives

Aims and Objectives

It is evident that the process of megakaryopoiesis and platelet production is complex, with the potential for regulation at multiple stages. Functional platelets are very much crucial in the regulation of hemostasis and blood coagulation. Given the enormous importance of platelets, efforts are on to increase its production after bone marrow transplantation and chemotherapy. In this study, we have focused on the regulation of cellular interaction occurs between cells of different lineages, especially in MKs. MKs are located deep inside the bone marrow niche. The cell to cell communication via MVs and their role in different areas has been studied in various systems but significantly little in megakaryopoiesis.

Furthermore, in the context of polyploidy MKs modulate its gene expression pattern. In this regards, the importance of lncRNAs and nucleolus also have been explored.

With this information about MKs regulation, this dissertation was performed by addressing the followings:

1. In order to reveal the information regarding the lineage committment of stem and progenitor cells towards megakaryopoiesis in the presence of other blood cells of the BM, the role of MVs on the differentiation of MKs was investigated.
2. Furthermore, we wanted to check the promoter activity of Notch 1 during megakaryopoiesis. Additionally, in this context, regulation of DNA methyltransferases (DNMTs) in the differentiation process as well as in DNA repair pathways during endo-replication were explored.

3. To Probe the chromatin organisation during megakaryopoiesis and understand if this modulation were governed by the DNA replication-associated proteins and histone chaperones.
4. As chromatin organisation can be modulated by various long non-coding RNAs (lncRNAs), expression of various lncRNAs was checked in megakaryopoiesis. In this context, we tried to explore the involvement of nucleolus in the maturation of MKs.
5. To Inspect the role of miRNA in megakaryopoiesis and nucleolus size/activity.

Chapter 2

Scientific Background

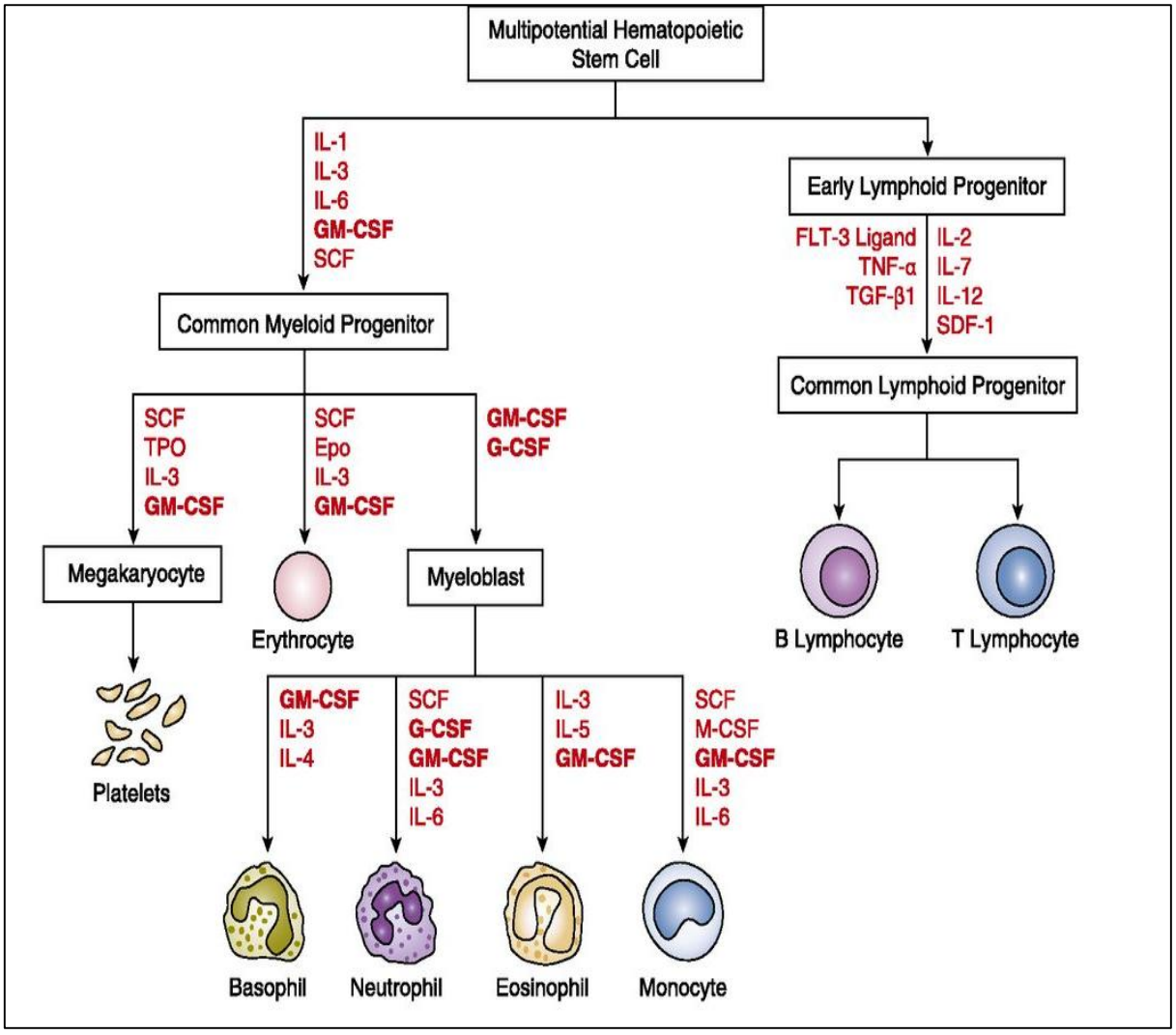
2.1 Hematopoiesis and production of MKs:

Normally, stem cells are responsible for the production of any types of cells that are required in our body. It acts as a production house of our cells. Two main sources act as an origin of stem cells – adult body tissues and embryos. During the embryo development, the pool of HSCs is produced through a complex, organised process. This developmental process includes various anatomical sites within the body such as yolk sac, the aorta-gonad mesonephros (AGM) region, the placenta and the fetal liver[69]. After birth, HSCs translocate into the BM. HSCs are produced either from cells that are bipotential for the development of both blood and endothelial cells or directly from specialised endothelium. Haematopoiesis is the production process of distinct blood cell types from a common mother cell type called HSCs[70,71]. During embryos development, the first and most specific marker that differs HSCs from other cell types is CD 41[72,73]. The first blood cells that are generated are erythrocytes[74]. However, although nascent HSCs, some lineage-committed progenitors and MKs maintain CD 41 as a surface expression marker, mature HSCs unburden this surface marker. Normally, immature or nascent HSCs do not express CD 45 though Sca1, a maker of mature HSCs, is found to be up-regulated in those nascent HSCs. In adults, adult or mature HSCs that are characterized as $SLAM^{+}CD34^{+}CD38^{-}CD91^{+}Lin^{-}$ in the human system generate primarily in the BM where HSCs reside as rare cells and sit atop a hierarchy of progenitors that become progressively restricted to several or single lineages in association with a supportive niche[75–77]. As with all other stem cells, HSCs are capable of self-renewal through asymmetric division in which specific cell fate determinants are distributed unequally into two daughter cells depending upon BMM. In this asymmetric division, one daughter cell retains the self-renewal capacity while the other one takes the path of lineage differentiation within the correct niche[78].

Haematopoiesis proceeds through a hierarchial differentiation whereby the cells differentiate and progressively lose their multi-potency abilities. HSCs may either self-renew or differentiate to produce multi-potent progenitors (MPP) which then differentiate further into the lineage-committed progenitor cell populations of common myeloid progenitor cells (CMPs) or common lymphoid progenitor cells (CLPs). CMPs under further differentiation process give rise to MEPs and granulocyte-macrophage progenitors (GMP) while CLPs produce the cells of the lymphoid lineage. MEPs are defined by their limited potential to give rise to erythroid and megakaryocytic cells[5]. Recently, it has been documented that HSCs can also directly produce MKs depending upon lineage induction[16] (Figure 2.1). Large MKs are (50–100μm) one of the rarest cells in the BM, they account for approximately 0.01% of the nucleated BM cells[2].

2.2 Megakaryopoiesis and generation of pro-platelets:

The wonderful path from HSC to the formation of mature MKs which ultimately lead to the production of functional platelets is termed as megakaryopoiesis. Thrombopoietin (TPO), the primary regulator of megakaryopoiesis, governs the induction for the maturation of MKs. Other than TPO, other cytokines such as IL-3, IL-6, IL-11 etc. are also involved in the regulation of MK`s maturation. The formation of platelets from matured MKs is known as thrombopoiesis [3]. Researchers have shown that MKs during maturation extend its internal membrane-like pseudopods from which platelets are released. This is termed as proplatelets formation. The production of platelets from matured MKs induces the cytoplasmic expulsion of those MKs through the formation of 100-500μm long branched proplatelets. This proplatelets formation appears as beads on a cytoplasmic string[79].

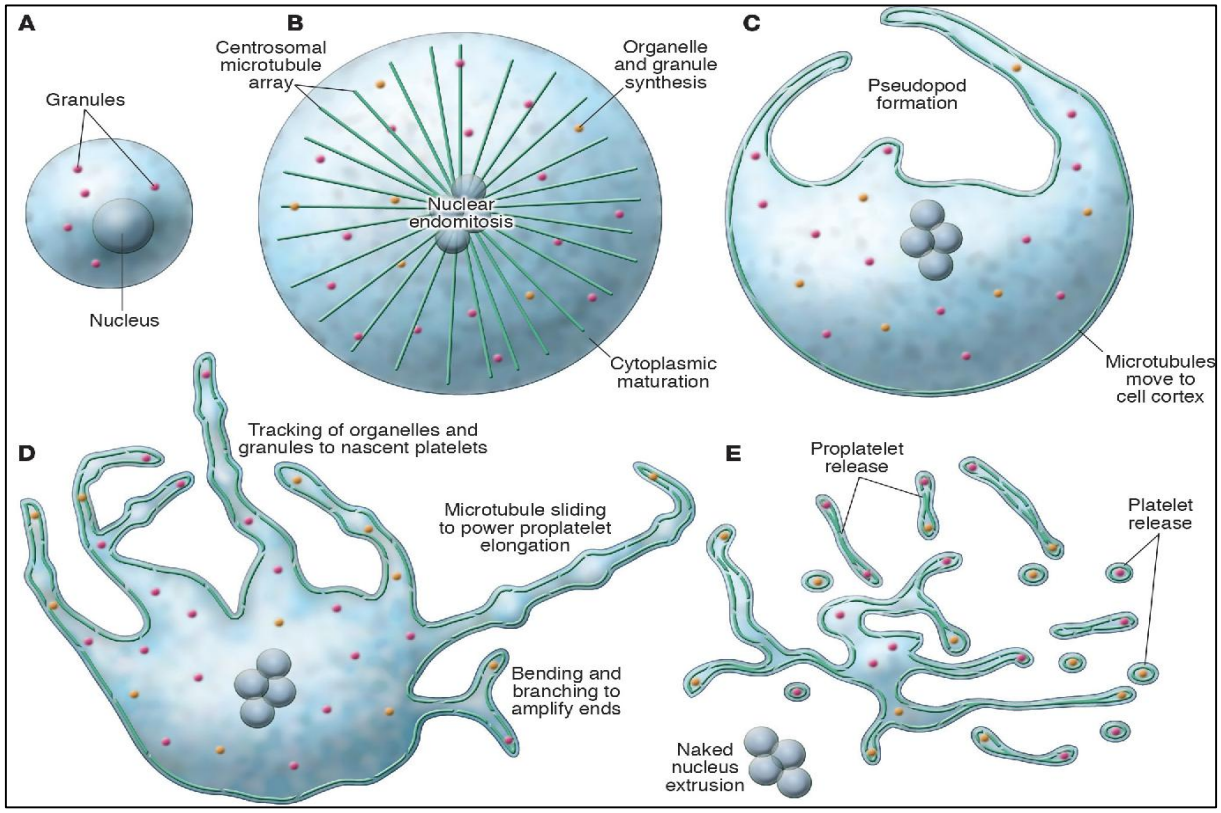


(Adapted and modified from *Mehta et al. 2015*)

Figure 2.1 <u>Schematic diagram of HSCs differentiation into different blood cells through cytokines induction</u>.

The generation of proplatelets continues until the MKs cytoplasm transformed into the complex network of interconnected proplatelets and subsequently they become extruded and degraded. The average growth rate of proplatelet formation from MKs is 0.85 μm/min[80]. Extensive studies have given the evidence that proplatelets growth are supported by microtubule polymerisation which helps to extend the size of this cytoplasmic strings in both the direction. Though inhibition

of microtubule polymerisation has given the proof that microtubule assembly in proplatelet elongation follows another mechanism for assemble, it is normally termed as the sliding mechanism[81]. However, in this extension process proplatelets are released into the BM vascular sinusoids from where they normally enter the circulation. Proplatelets are released as chains of platelet-sized particles from which individual platelets are produced[82,83]. Platelets are small anucleate cell fragments that have a characteristic discoid shape and range from 1 to 3μm in diameter and play an indispensable role in processes such as haemostasis, wound healing, angiogenesis, inflammation, and innate immunity [84–86](Figure 2.2).



(Adapted and modified from *Kleiman et al 2008*)

Figure 2.2 <u>Schematic diagram (A to E) of Megakaryopoiesis & platelet production via the transition from MEPs to platelets releasing</u>. (A). *MEPs ready to become lineage committment.* ***(B).*** *MEPs in the BM at first differentiate into MKs through endomitosis, organelle synthesis and*

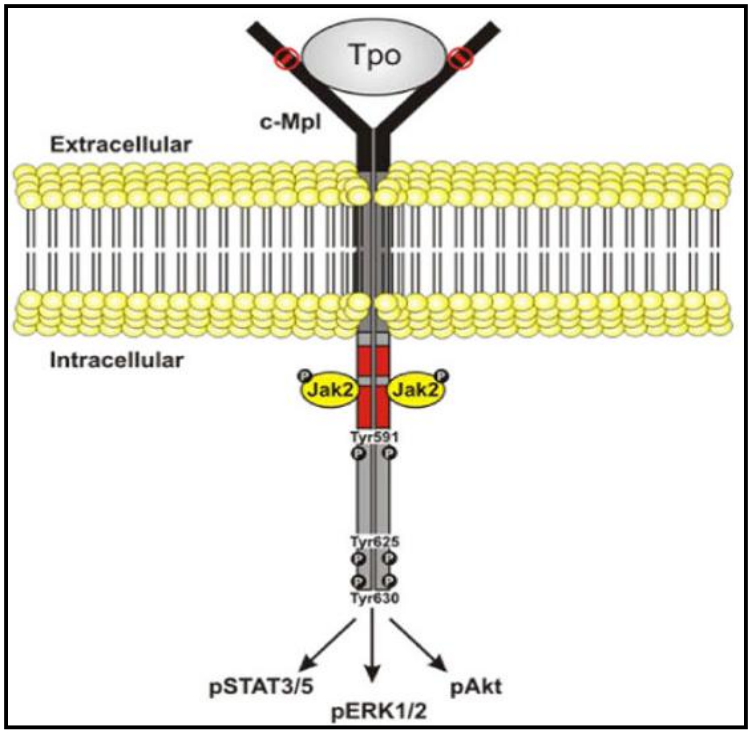
cytoplasm expansion-maturation with increasing DNA content. ***(C).*** *Proplates formation begins with the development of thick pseudopods after disassembling the centrosomes.* ***(D).*** *Proplatelets formation continues throughout the cell while branching of proplatelet ends amplify.* ***(E).*** *The entire MKs cytoplasm is converted into proplatelets while the nucleus is essentially extruded from the proplatelets. Platelets are produced from proplatelet ends*

2.3 Cellular signalling during MKs maturation:

Megakaryopoiesis is guided by a complex regulatory mechanism of hematopoietic growth factors that assist in expressing various transcription factors which govern the stage-specific maturation process of MKs[87,88]. Researchers have documented that HSCs and MKs share almost similar surface expression markers and transcription factors. Those transcription factors are involved in HSCs maintenance and self-renewal as well as in lineage commitment for MKs. In this maturation process, TPO or megakaryocyte growth and development factors (MGDFs) play a very crucial role by behaving as a ligand of c-Mpl which initiates the differentiation signal of MKs from the MEPs[1,89–91] (Figure 2.3).

Different signalling processes such as G-protein-coupled receptor signal transduction system, janus family of protein kinases (Jaks)/signal transducers and activators of transcription (STATs) pathway, Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, phosphoinositide-3 kinase (PI-3K)/AKT pathway, the nuclear factor kappa B (NF-κB) pathway are indispensable for the maturation of MKs. Previously it was reported that c-Mpl or TPO knockout mice successfully produced platelets which indicated the existence of an alternative pathway for the production of platelets[92–96]. Recently, it has been suggested that HSCs can directly produce MKs through the subsequent developmental process. Reports support the previous notion that other than TPO, our body might have alternative regulators for megakaryopoiesis which subsequently maintain

haemostasis of the blood cell production[97,98]. Additionally, it has also been observed that other important regulatory pathways such as SDF1/CXCR4 signalling, N-methyl-D-aspartate (NMDA) receptor-mediated pathway, Notch signalling, gp130-dependent signalling can also promote megakaryopoiesis even in the absence of TPO[99–104].



(Adapted and modified from *Geddies et al. 2010*)

Figure 2.3 <u>TPO initiates MKs lineage commitment through c-Mpl receptor, a cytokine receptor in association with Jak2 kinase</u>.

To understand the involvement of the specific genes in the maturation of megakaryopoiesis, two model system such as congenital and familial thrombocytopenia syndromes, have paved the pathways for researchers[105,106]. In the 1990s in independent research, the molecular and transcriptional understanding of HSCs differentiation was reported. Analysis of *cis*-regulation of cell-specific genes, isolation and gene targeting of lineage-restricted transcription factors and complementary studies in cell differentiation in vitro, all combined to provide a glimpse of how

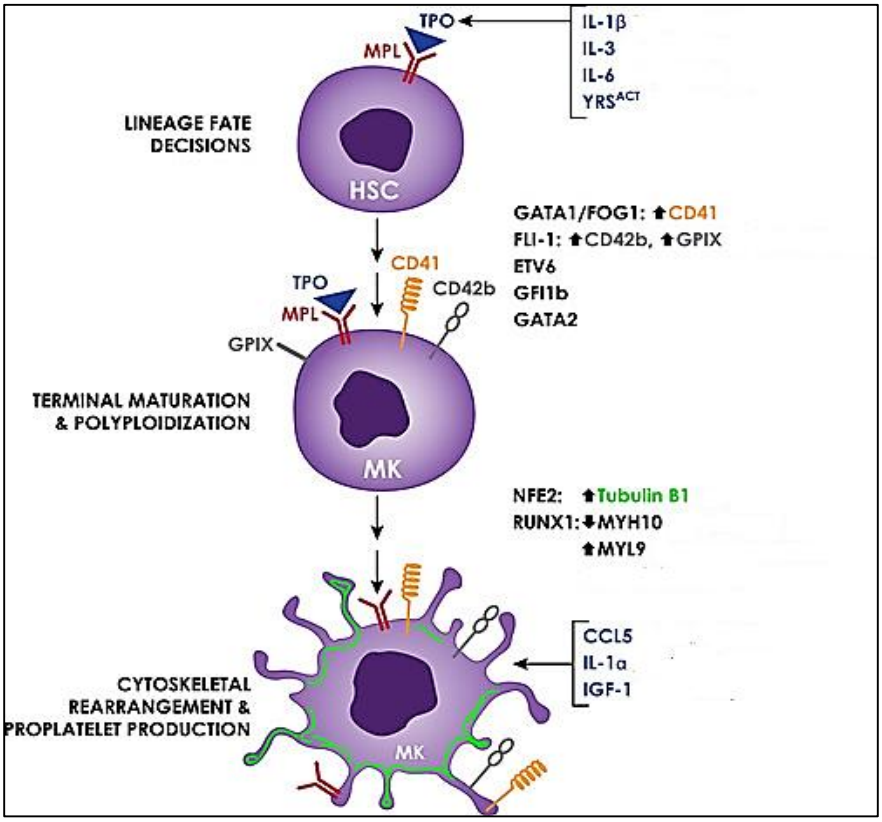
common progenitors produce vastly different cell types[107,108]. One phenomenon that became apparent from these studies that commitment for a particular lineage is achieved via the involvement of both widely expressed and lineage-restricted transcriptional regulators[109]. The potential complexity afforded by the combinatorial action of proteins probably allows cells considerable flexibility to regulate gene expression. Recently, researchers have also documented the importance of erythrocyte-megakaryocyte transcriptional regulators, such as GATA-1, FOG-1, NF-E2, Fli-1, Runx1 etc. in the maturation of MKs which subsequently leads to the production of platelets[110,111].

An Investigation into the transcriptional regulation of megakaryocytic genes has led to focus on the GATA family of zinc-finger proteins, which activate transcription by engaging the DNA sequence WGATAR in the *cis*-regulatory elements of many lineage-restricted genes[112]. Important early advances included identifying GATA-1 and GATA-2 as erythrocytic-megakaryocytic transcription factors and finding that the rat platelet factor 4 (PF4) and human glycoprotein (GP) IIB genes were regulated in part through isolated GATA sites or the combination of juxtaposed GATA and Ets-binding *cis*-elements[106,113].

For the differentiation of MEPs into MKs, multiple transcription factors regulate the lineage-commitment decision point[16]. The first commitment step is controlled by the expression of GATA-1 and its binding factor FOG-1 followed by PU.1 which induce hematopoietic skewing towards macrophage. Knockout GATA-1 mice characterised with thrombocytopenia followed by MKs maturation delay which was marked by reduced polyploidization, dorsal mesoderm and granular content. Indeed, mice lacking FOG-1 also showed abnormality in MKs maturation. However, the importance of GATA-1 for megakaryopoiesis is not exclusive. Researchers have reported that GATA-1 can also influence erythropoiesis while GATA-2 promotes

megakaryopoiesis at the expense of erythropoiesis[113–116]. The next factor that influences megakaryopoiesis is Fli-1 which also plays an important role in vasculogenesis. In fact, overexpression of Fli-1 in mice showed induction of megakaryopoiesis from HSCs while lacking Fli-1 in mice model was characterised with abnormal MKs and thrombocytopenia[117]. A conditional knockout of ETV6, an E26 transformation- specific (Ets) family protein variant, was also characterised with abnormal MKs and thrombocytopenia. Likewise, SCL (TAL BHLH transcription factor 1), is also essential for MKs differentiation by regulating the cytokine responses of MKs to stress. Mice lacking SCL expression develop thrombocytopenia when stressed and are shown abnormal MKs with increased ploidy, abnormal demarcation membranes and reduced granules[118]. Basically, the expression of those transcription factors in a time and dose-dependent manner regulates the differentiation and commitment process from MEPs[119]. Of these, nuclear factor erythroid 2 (NF-E2) and RUNX-1 have been widely studied and regulate the expression of many genes important for thrombopoiesis. NF-E2 knockout mice have severely dysfunctional MKs resulting in thrombocytopenia because NF-E2 regulates expression of the MKs specific microtubule component. However, Chen and colleagues recently used mRNA expression profiling in different developmental stages of MKs and found new targets for NF-E2, including the kinase adaptor protein LIMS1/PINCH1. Most recently, the AML/ RUNX-1 complex was shown to be involved in transcriptional regulation of megakaryopoiesis through down-regulation of KLF-1 (Kruppel-like Factor) which promotes erythropoiesis differentiation and subsequently promotes the proliferation of megakaryocytic progenitors[120–122]. Indeed, in the last stage of megakaryopoiesis, RUNX-1 regulates myosin expression differentially. RUNX-1 activates MYL9 (Myosin Light Chain) whereas represses MYH10 (Myosin Heavy Chain)[121,123] (Figure 2.4). This complex regulated expression of myosin are basically required for megakaryopoiesis.

Identification of the downstream effectors of these transcription factors should continue to increase our understanding of the cell biological pathways that regulate MK`s development.



(Adapted and modified from *Noetzli et al., 2019*)

Figure 2.4 <u>Schematic representation of the established roles of individual factors that are involved in megakaryocytic differentiation</u>. *Role of interleukins (IL) in the commencement of megakaryopoiesis from the progenitors. Positive regulation of proliferation of megakaryocyte progenitors by GATA1, FOG1, FLI1 and NF-E2, and negative regulation by RUNX1. Fli-1 helps in cytoplasmic maturation while platelet release is regulated by NF-E2.*

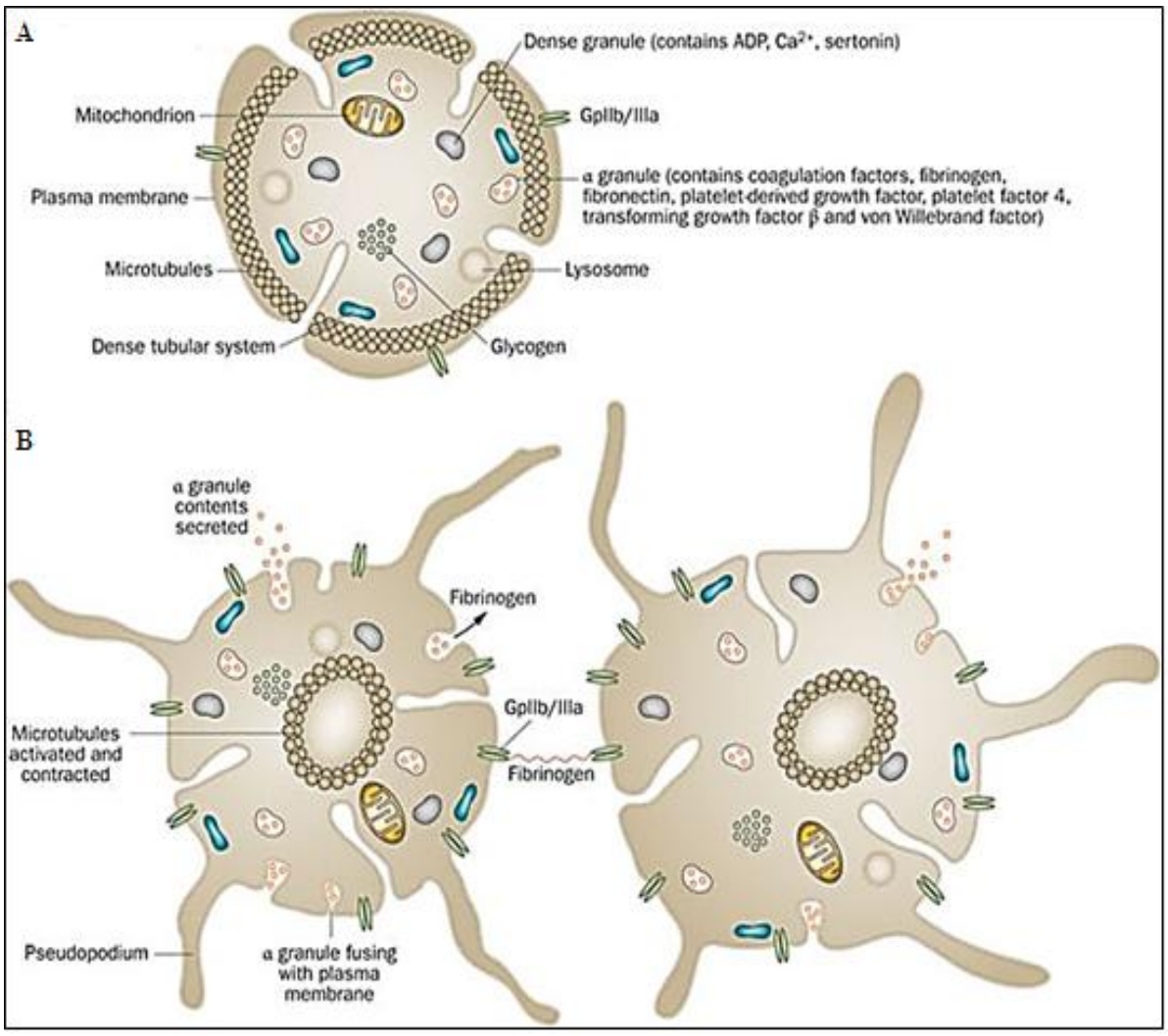
2.4 <u>Cytoplasmic maturation during megakaryopoiesis</u>:

The characteristic feature of matured MKs is the development of a unique and elongated membrane system designated as demarcation membrane system (DMS) along with the increment in dense

and alpha granules[124,125]. After nuclear polyploidization, MKs undergo cytoplasmic maturation involving the formation of an extensive DMS which divides the cytosol into different platelets territories. It has been indicated that DMS forms the periphery of the platelets though it is still inconclusive about the mechanism of formation of these elaborate membrane network. However, regarding the biogenesis of DMS, multiple subcellular origins have been suggested such as PM, endoplasmic reticulum (ER), golgi apparatus etc. Studies have also suggested about the role of DMS. Basically, it assists in the proplatelets formation[126–129]. Researchers have reported that before proplatelet formation, DMS contains a huge amount of poly-phosphatidyl inositol lipid, PI-4,5-P_2. This Incorporation helps DMS to interact with the actin cytoskeleton through lipid binding sites which normally are present in actin regulatory proteins such as cofilin, gelsolin etc. and other lipid-binding sites containing signalling proteins [130–132](Figure 2.5).

2.5 DNA replication during megakaryopoiesis:

Francois Jacob once told poetically that cells dream for becoming two identical cells with same number of genomes. In this context, DNA replication is the exact process. Molecular machinaries that assist in DNA replication to ensure DNA replication number per division do not differ in case of endo-reduplication which is associated with endomitosis. To convert normal cell division process into endomitotic cycle, developmental signals utilize this inherent plasticity of cell cycle[133,134].



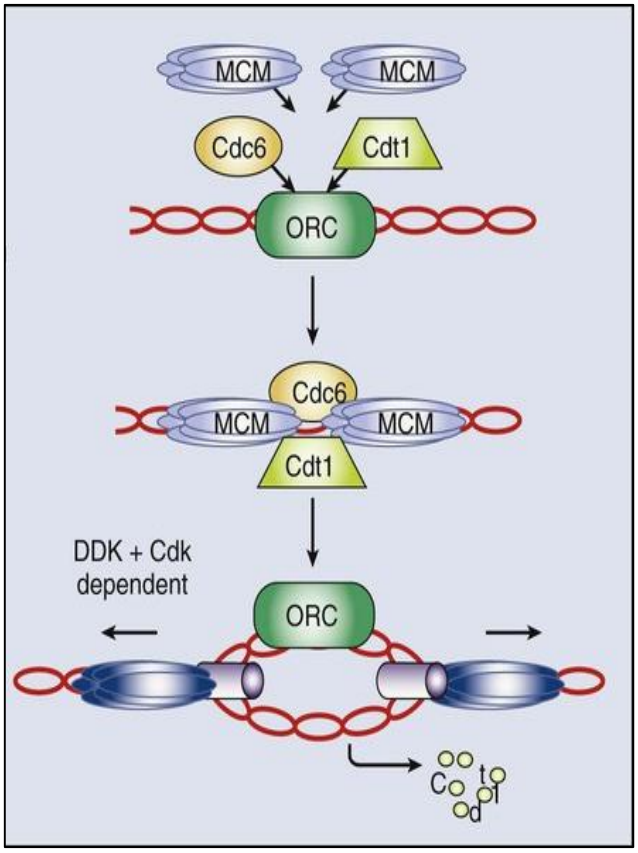
(Adapted and modified from *Martin et al., 2012*)

Figure 2.5 <u>Demarcation membrane system (DMS)</u>. (A). *A metabolically active membrane with a large surface area populated by the glycoprotein receptors on outer surface of the platelet acts as the adhesion molecules which lead to platelet aggregation and activation of other platelets.* ***(B).*** *The activated platelets with additional glycoprotein receptors have more stellate form and long pseudopodia. Platelet activation results in the expression of various factors which act in a feedback loop mechanisms such as, platelet factor 4 (PF4), platelet-derived growth factor (PDGF), Von Willebrand Factor, transforming growth factor-β (TGF-β), fibronectin, fibrinogen and coagulation factors—are secreted and further amplified platelet formation.*

A protein of serine/threonine kinase family, normally designated as cyclin-dependent kinases (CDKs), controls the progression of the canonical cell cycle process via G1-S-G2-M. After the completion G1, CDKs phosphorylate specific substrates which assist the major event of interphase, i.e. DNA replication that occurs only in the S-phase. DNA replication is strictly coupled with S-phase in such a way that without completion of replication cell division process cannot further enter into mitosis. These two events are tightly guided by CDKs. At the end of M-phase, Low CDKs activity with respect to S phase aid the cell to reset for replication while CDKs activity at G1/S permits the initiation signals. Surprisingly, the pool of mitotic CDKs prevent cells from re-replication in the same cell cycle. In eukaryotes, due to the length of the chromatin and time constriction for cell cycle, eukaryotes require multiple sites on chromatin which are normally referred as origins of replication. For the initiation of DNA replication, cells require two stages of cellular governance – first stage assists to form a multiprotein complex, known as pre-replicative complex (pre-RC) and second stage governs the completion of DNA replication in association with another multiprotein complex known as post-replicative complex (post-RC). At the beginning of G1 phase, the low activity of CDKs helps to bind pre-RC complex in the origin of replication while higher CDKs activity permits the conversion of pre-RC into post-RC. This high CDKs activity prevents cells to form pre-RC and subsequently inhibits the re-replication of DNA in a single cell cycle event[135–139].

This pre-RC formation during G1 phase of the cell cycle is normally known as “DNA licensing”. Studies have shown that this licensing factor becomes inactivated during S-phase of the cell cycle. After the completion of one round of cell cycle, these DNA licensing factors re-enter the nucleus from cytoplasm to re-initiate the DNA replication in the next cell cycle process. This licensing process starts with the binding of origin recognition complex (ORC) to double-stranded DNA

(dsDNA) in an ATP manner. As ORC binds to the chromatin, two additional DNA licensing factors, Cdc6 and Cdt1, are recruited to ORC complex in association with ATP. The ORC-Cdc6-Cdt1 complex then recruit another complex i.e. MCM complex to the origin of replication. Usually, mammalian cells require the loading of all six MCM units (MCM2.3, 4, 5, 6 and7) in order to complete the complex. This functional hexameric complex forms an important component of pre-RC complex. This is the transition period of a cell from G1 to S phase and pre-RC complex converts into post-RC complex with the recruitment of DDK into the complex. Interestingly, CDK activity at the initiation of S phase increases which prevents the reassembly of the pre-RC on the chromatin. With the conversion of pre-RC into post-RC, ORC component becomes phosphorylated and leaves the complex which indicates the initiation of elongation step for DNA replication[140–144]. Subsequently, two important events are required in order to convert a normal cell cycle process into an endoreplication so that the cell become polyploidy. The two events are – 1. Absence of karyokinesis and cytokinesis and 2. CDK activity alternate between low and high levels in order for the genome to be reduplicated in the absence of cell division. During megakaryopoiesis, CDKs are inhibited through direct binding to proteins called Cyclin Kinase Inhibitors (CKIs) to trigger endoreplication. Induction of CKI during MKs differentiation must be transient in order not to inhibit the CDK activity required for endoreplication during S phase. Cdk2 is an important kinase for endomitotic S phase in animal cells, although in the absence of Cdk2 in mammals, Cdk1 can act as a substitute. Cyclin E over expression increases the ploidy of MKs, suggesting that the Cyclin E/Cdk2 complex is the relevant kinase. Inhibiting cytokinesis is another mechanism that can promote endoreplication and polyploidy[15,139,145–148] (Figure 2.6).



(Adapted and modified from *Oncohemakey*)

Figure 2.6 <u>Model for Mcm2-7 Loading by the ORC and Cdc6.</u> *ORC first binds origin DNA while Cdc6 then binds ORC. Cdt1 and Mcm2-7 associate with ORC and Cdc6 at the origin. ATP hydrolysis by Cdc6 leads to the loading of Mcm2-7 complexes on DNA and then release of Cdt1 from the origin. Subsequently, DNA replication fork creates.*

2.6 <u>DNA Repair and DNA Methyltransferases (DNMTs)</u>:

Cells come across various types of potential DNA damages from either endogenous or exogenous sources which deliberately insults DNA bases. In this context, cells have evolved DNA repair process to circumvent genomic damage which loosens genomic integrity. DNA repair process works in association with cell cycle checkpoint and replication[149,150]. Evidence are growing to

reveal the putative genome-wide surveillance system in order to avoid these genomic lesion. In fact, if excessive DNA damage remain within the cell, cells triggers cellular death pathways. In this context, researchers have established a link between DNA damage repair system and DNA methylation[151]. Jacinto et al. at first shown that promoter hypermethylation and knock out of DNA repair genes are the responsible factors for multiple types of cancer progression such as colorectal, breast, lung cancer etc[152]. Aberrant promoter hypermethylation that occurs at the promoter CpG islands (CGIs) directs potent and heritable transcriptional silencing to the key repair genes. In fact, researchers also showed that DNMT1 in association with proliferating cell nuclear antigen (PCNA) directly recruits in the region of dsDNA breaks (DSBs). Malfunctioning in the DNA repair pathways cause cells to be repaired incorrectly[153,154].

Among DNA repair, DNA mismatch repair (MMR) plays a very significant role during DNA replication. Silencing of the MMR pathways produce microsatellite instability within the genome. Microsatellite region, a short tandem repeats of 1-4 bases, gives rise to the secondary structure which are required for expansion and contraction of the genome. Microsatellite basically responsible for frameshift mutation within the genes. MMR comprises of MutS and MutL complex. Researchers have identified that hypermethylation of the MutL promoter is associated with various cancers including oral squamous cell carcinoma, gastric cancer, acute myeloid leukemia, colorectal cancer etc. They showed that reduced protein expression of MutL component is correlated with hypermethylation of MutL promoter. In fact depending on the observation made in germ line cells, researchers have shown that hypermethylation in the promoter region of MutS give rise to hereditary non-polyposis colorectal cancer (HNPCC). Cellular genome is also challenged by environmental threats and endogenous metabolic by-products which damage DNA bases and nucleotides. Damaged bases and nucleotides are basically excised by specific

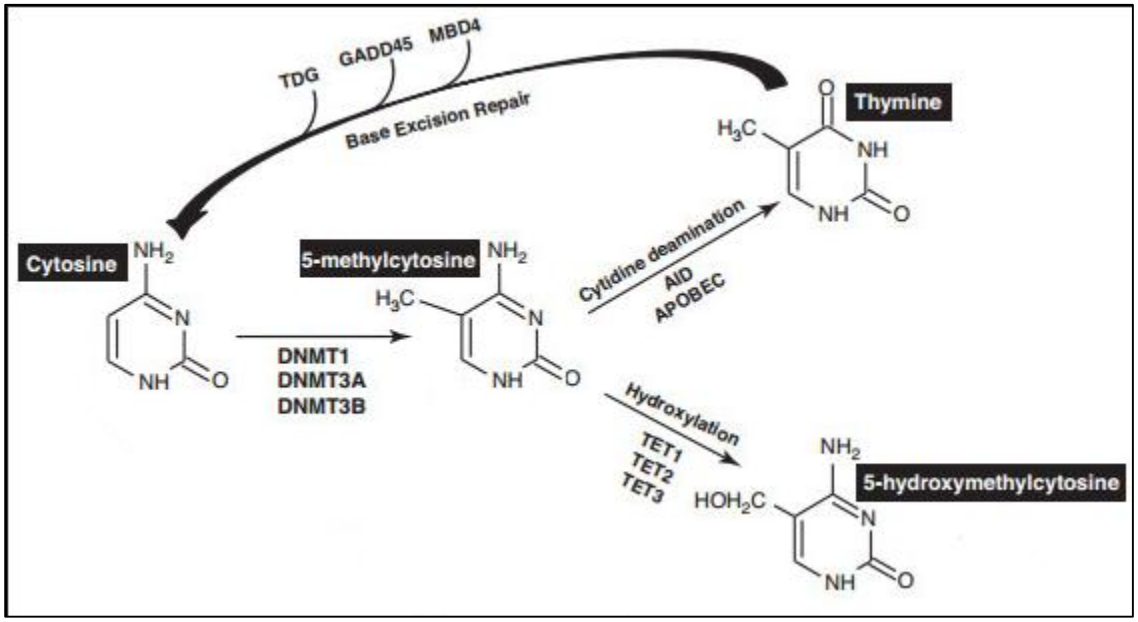
glycosylases followed by recruitment of other enzymes. It has been observed that hypermethylation of promoter region of DNA glycosylase leads to genomic instability which normally finds in cancer cells. Of these, patients of xeroderma pigmentosum, Cockayne syndrome etc. are associated with impairment of nucleotide excision repair process. It has been reported that one out of seven xeroderma pigmentosum group genes are significantly down-regulated in bladder cancer. It was observed that due to promoter methylation of the genome, mRNA levels of genes in xeroderma pigmentosum are showing down-regulation. Additionally, in rat lung cancer researchers have shown that promoter methylation of ERCC1 which is basically involved in nucleotide excision pathway is involved in the defect of the repair of platinum-DNA adduct. In fact, promoter of RAD23B, a major component for repair in nucleotide excision repair, is hypermethylated in multiple myeloma cancers[47,155–159]. During cell division different types of DNA lesions such as DNA interstrand cross links (ICLs), DNA double strand break (DSBs), DNA damage during replication pose a threat to the cellular genome integrity. In order to maintain the genome integrity, cells evolve different counter striking strategies. Homologus recombination (HR) is one of them which permits an important mechanism to repair those DNA lesions. Normally, HR uses the intact sister chromatids as a template to repair the DNA damage. In this context, BRCA1 and BRCA2 play a pivotal role in association with RAD51 and DNA nucleoprotein filament. Silencing of BRCA genes via promoter hypermethylation promotes progression of various cancers including breast, ovarian, gastric cancer etc. ICLs are resolved by the Fanconi Anemia (FA) pathway. Interestingly, it has been observed that in cervical and ovarian cancer, the silencing of FA pathway is acquired by the promoter hypermethylation of one of the thirteen genes of FA groups. HR basically provides error-free repair[160–167]. Unlike HR, non-homologus end joining (NHEJ) maintains genomic integrity by joining two DNA strands. It acts

as a SOS response. Subsequently, it is error prone repair process which leads to mutagenic consequences. KU complex participates in this repair process. Researchers have observed that defects in NHEJ process by the hypermethylation of KU promoter frequently results in the development of cancer. Alkylation of the DNA bases also lead to the damage of the DNA bases. O^6-methylguanine (O^6-mG) which produces due to the alkylation pairs with thymine (T) rather than cytosine (C). This conversion provides mutation in the DNA bases which is repaired by O^6-alkylguanine-DNA alkyltransferase (AGT). It has been suggested that defects in AGT sometimes occurs due to the abnormal promoter hypermethylation which results in loss of AGT protein expression and consequently leads to cancer development such as glioblastomas, anaplastic astrocytomas etc. it has been documented that deficiency in AGT expression results increment in mutation in KRAS and P53 genes. DSBs are also recognised during the transition from G1 to S phase by ATM/ATR signalling. This signalling process activates two effector kinases CHK1 and CHK2. In human colon cancer, researchers have observed aberrant promoter hypermethylation of ATM whereas lung cancer, glioma and Hodgkin`s lymphoma are associated with the promoter hypermethylation of CHK2 gene[45,162,168–172] (Figure 2.7).

2.7 Notch Signaling and Megakaryopoiesis:

Notch important pathway signalling is one of the key regulator of cell fate decisions[173]. During hematopoiesis system, Notch signalling is shown to be for the proper development of the lymphoid stages. In particular, Notch1 is important for T cell specification over B cell lineage while Notch2 is required for B cell development. Moreover, Notch mutations are common in T cell acute lymphoblastic leukemia[174–178]. However, the role of Notch for the myeloid compartment is unequivocally ascertained. Interestingly, Numb and Numblike, two endogenous antagonists of

Notch receptor were shown to be important for the development of erythroid lineage in Zebrafish, while some reports denied the involvement of Notch signalling in erythropoiesis.



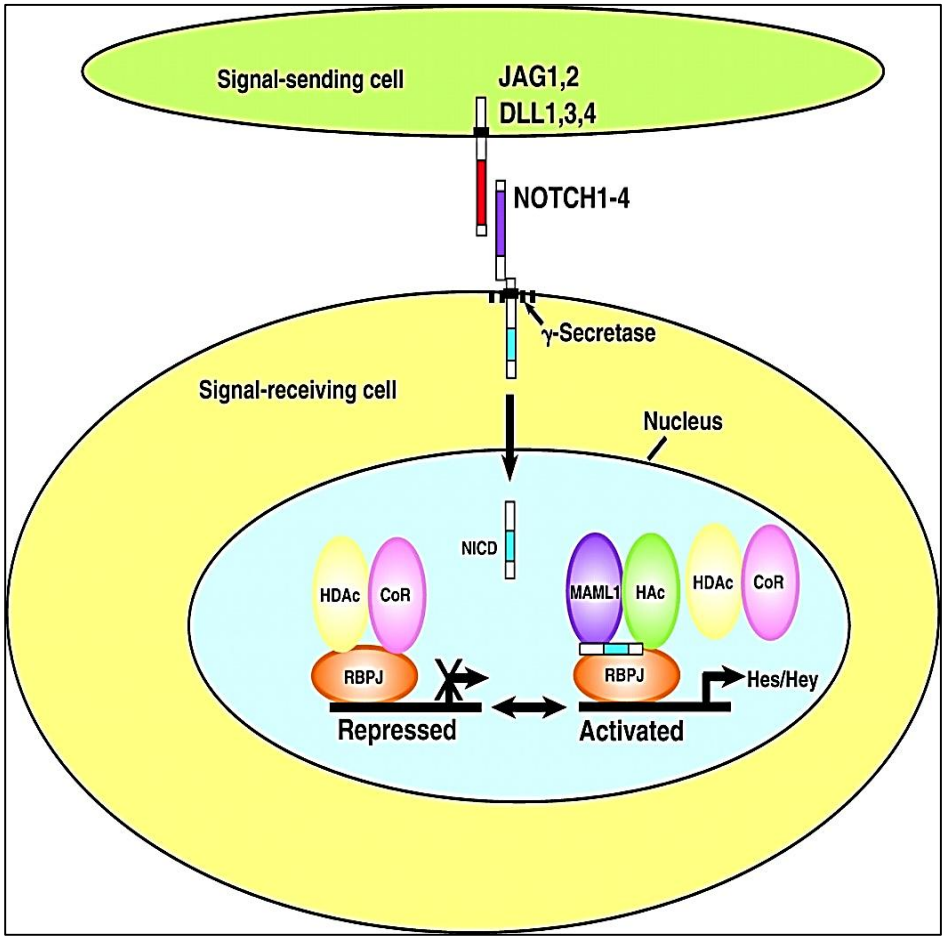
(Adapted and modified from *Carey et al., 2011*)

***Figure 2.7 <u>Schematic representation of cytosine methylation and active demethylation</u>**. BER refers to base excision repair. Gene names refer to proteins implicated in BER.*

Recently, it has been reported that Notch2 promotes erythroblast survival and inhibits erythroid differentiation in a SCF induced system. However, the importance of Notch signalling in megakaryopoiesis progression has been documented. Anita et al. has shown that Notch1 in association with Shh (Sonic-Hedgehog Pathway) and Akt pathway differentially regulate the progression of megakaryopoiesis and erythropoiesis[48,179,180]. Defects in Notch1 expression prevents the progression of megakaryopoiesis [50,181](Figure 2.8).

2.8 Microvesicles:

In order to maintain the intercellular communication, cells transfer signal to the nearby or distant cells by various mechanism such as they can communicate through (a) Cell-to-cell adhesion which are interposed by sets of specialized adhesion molecules, (b) Secreted cytokines, growth factors, chemokines and small molecules like bioactive lipids, nucleotides etc. and (c) Nano tunnelling tubules. Another mechanism for intercellular communication has evolved which involves the intercellular transfer of EVs[31,32,182–184]. This mode of transfer has been largely overlooked for many years. These circular small membrane fragments normally shed from the healthy or damaged cells. Researchers have documented that the membrane of EVs consists of membrane components like proteins and lipids which are present in the cellular membrane and cytoplasmic contents from the particular cells (RNA, proteins, lipids etc.). Shedding of these tiny cellular membrane derive EVs are a physiological phenomenon which guides cell for differentiation and proliferation[185–190]. Other than cell doubling time, shedding of EVs also depend on the degradation of membrane skeleton and increment in cytosolic Ca^{2+}. In normal steady state conditions, the abundance of plate derived MVs (PLMs) in our body are higher.

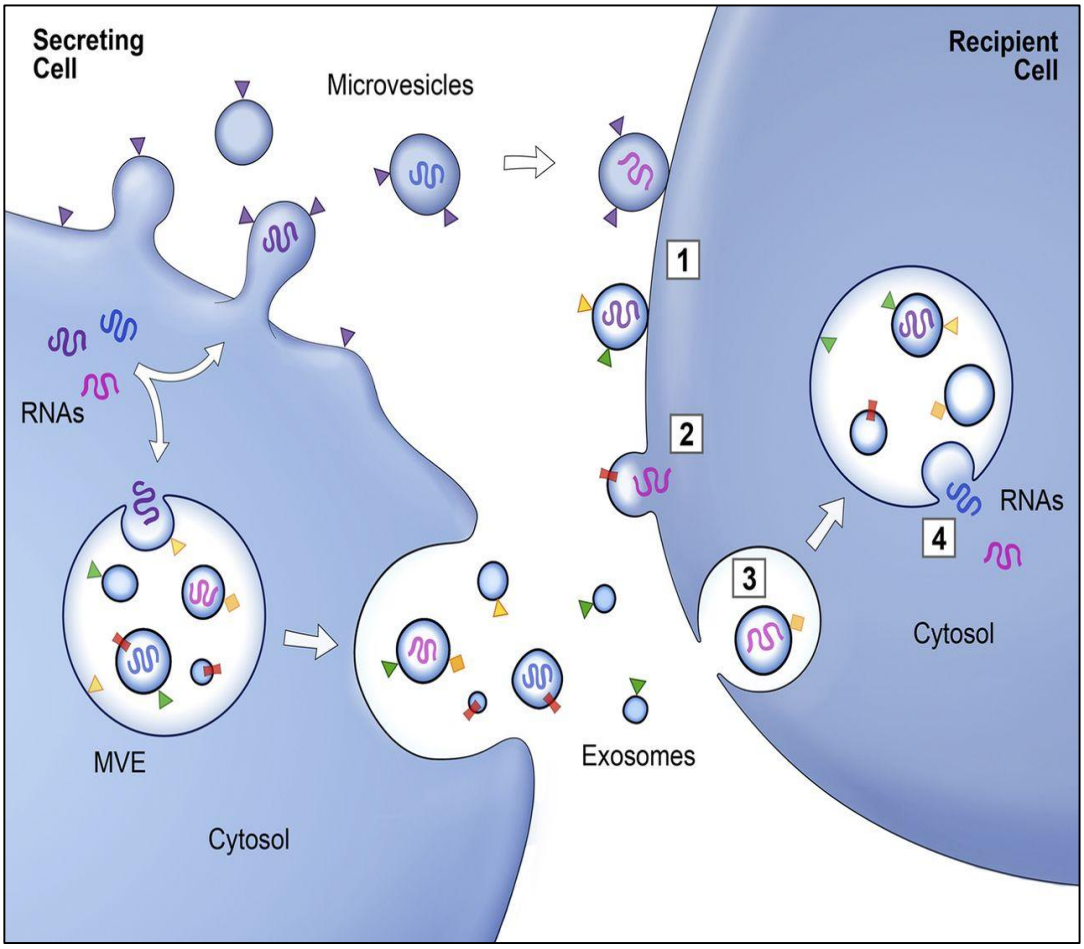


(Adapted from *Thomas Gridley, 2007*)

Figure 2.8 <u>Components of the canonical Notch signaling pathway</u>*. Ligands of Jagged (JAG1-2) and Delta-like (DLL1-4) families (upper cell) interact with Notch family receptors (NOTCH1-4) on nearby cell (lower cell). The receptor-ligand interaction starts with two proteolytic cleavages which release Notch intracellular domain (NICD) from PM. NICD translocates to the nucleus (blue) and forms a complex with the RBPJ protein. Components of the activation complex, such as histone acetyltransferases (HAc) interacts with NICD-RBPJ complex, leading to transcriptional activation of Notch target genes.*

Almost 75%-80% PLMs of total MVs population are found in our blood serum. Besides PLMs, researchers also documented the presence of endothelial MVs and other blood cells MVs[191–195]. Cellular injury, inflammation, thrombosis and platelet activation etc. promote the increment

in the number of circulating MVs in the peripheral blood (PB). Normally, the size and composition of MVs depend on the specific cell type. During the formation of MVs, the inner leaflets of the bilayer cell membrane translocate to the outer side which is then governed by the flippases, floppases and scramblases. Shedding of MVs is accompanied by the segregation and formation of the cellular membrane proteins in lipid raft. MVs also play a significant role in morphogenesis. It has been delineated that during tissue patterning, the gradient of Hedgehog (Hh), Wingless proteins are established by the secreted MVs. Even, argosomes (Morphogen-enriched MVs) are important for creating the morphogen gradient during proper tissue development [31,196–202](Figure 2.9).



(Adapted from *Raposo et al., 2013*)

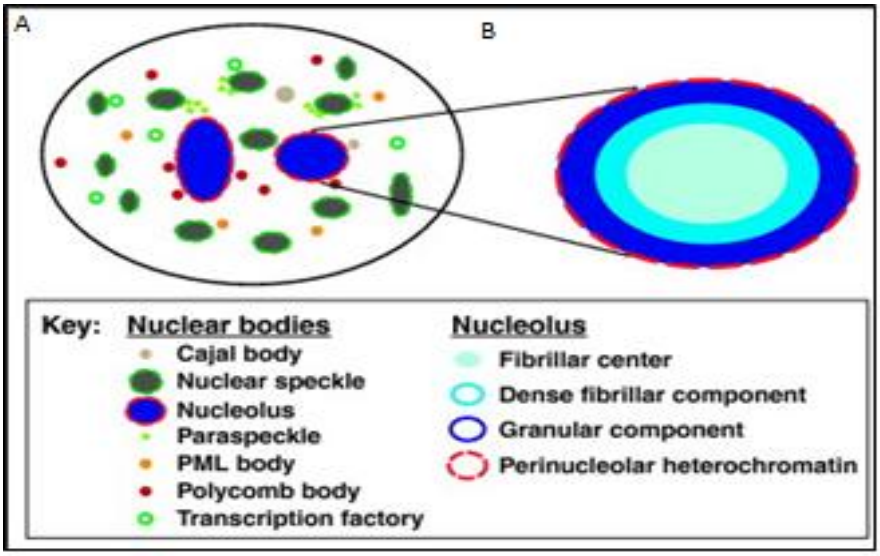
Figure 2.9 <u>Schematic diagram of crosstalk of EVs</u>. *Membrane-associated and transmembrane proteins and RNAs are selectively incorporated into the MVs shedding from the plasma membrane (PM). MVs fuse with the plasma membrane to release EVs into the extracellular milieu. EVs may dock at the surface of PM of a target cell* ***(1).*** *Bound vesicles may be either fused directly with PM* ***(2).*** *or endocytosed* ***(3).*** *Endocytosed EVs may then fuse with an endocytic vesicles* ***(4).*** *Both pathways leads to the delivery of cargos into membrane or cytosol of the target cell.*

2.9 <u>Nucleolus</u>:

Nucleolus, the primary site for ribosomal biogenesis, assembles around the rRNA genes during late telophase and disassembles at the initiation of mitosis[203]. Similar to all other intracellular organelles, nucleolus is membrane-less structure that is surrounded by nucleoplasm. Due to the difference in density, nucleolus is clearly visible in either phase contrast or differential interference contrast microscopy. Due to high throughput Mass-spectrometry (MS) based techniques, proteomes of nucleolus shown that not only rRNA biogenesis related proteins are present but also different proteins regulating various processes of the cell such as cell cycle, DNA damage repair, pre-mRNA processing etc. are also detected within the nucleolus[204–208]. Researchers also linked tRNA processing, RNA editing, long non-coding RNA maturation with nucleolus. Based on Immuno-EM analysis, different compartments of nucleolus consist of unique protein compositions and functions. Researchers have shown that nucleophosmin/B23 (NPM) and fibrillarin (FBRL) are responsible for the proteasomal degradation and or on contrary protein stability of rRNA processing machineries. Deficiency of proteasome activity leads to a defect in rRNA processing by affecting mobility and nuclear distribution of rRNA processing machineries. Normally it has also been delineated cancer cells, stem cells and progenitor cells show enlarged nucleolus which is considered as the signature of proliferation, stemness and pluripotency. In fact,

one nucleolar protein, called nucleolin, has been shown to be expressed in higher amount in embryonic stem cells (ESCs). Deficiency of nucleolin, FBRL have been associated with the maintenance of self-renewal of ESCs[209–217]. Nucleolus is also essential for the regulation of cell senescence. Senescent cells show enlarged nucleolus while presenescent cells are associated with multiple small-sized nucleolus[218,219]. Nucleophosmin also modulates cellular senescent by regulating the stability of p53. Accumulating evidence indicated that other than p53, other proteins such as HSP70, RDM1, PML also showed a distribution pattern between nucleolus and nucleus. Moreover, nucleolus is also suggested as a site for the biogenesis of signal recognition particle (SRP). This ribonucleo protein complex (RNP) is assisted in the translocation of the membrane and secretory proteins into the endoplasmic reticulum (ER). The growth-promoting transcription factor, c-Myc is essential for the regulation of ribosome biogenesis. It shows a positive correlation with the ribosome biogenesis. Actively growing and proliferating cells show upregulation in the ribosome biogenesis which is accompanied by a higher level of c-Myc. On the contrary, deficiency in the nucleolar GTPase, NOG-1 (Nucleolar GTP Binding Protein -1), express defect in the maturation of 60S ribosomal subunit subsequently affecting the ribosome biogenesis. Additionally, researchers found a link with Myokine for the size regulation of nucleolus. Overexpression of Mnt in muscle reduced the nucleolar size and simultaneously effect the life span. Mnt acts through a secreted myokine, known as myoglianin. Similar to Mnt, overexpression of myoglinin also reduced nucleolar size[220–224]. Nucleolar and coiled-body phosphoprotein 1 (NOLC1) which is at first described as a nuclear localization signal-binding protein, functions as a chaperone for shuttling between cytoplasm and nucleolus. The orthologues of NOLC1 in Xenopus, human and Drosophila share a common organisation. It has a conserved N-terminal, C-terminal domains and a central region which contains multiple interspersed basic and acidic amino

acid clusters repeat. NOLC1 is monoubiquitylated by BCR complex in association with TCOF1 in order to remodelling the differentiating cells. Localizing in the nucleolar DFC region, NOLC1 regulates the rRNA transcription by connecting with RNA polymerase 1 (RNA pol1). Additionally, doxorubicin, an anti-cancer drug, can target NOLC1 and inhibit the proliferation of cancer cells. NOLC1 has nucleolar intrinsic ATPase and GTPase activities and in nucleogenesis it is essential for maintaining the structure of nucleolar FC and DFC [219,225,226](Figure 2.10).



(Adapted from *Nemeth et al, 2011*)

Figure 2.10 Schematic representation of mammalian nucleus. *. **(A).** Schematic overview of the nuclear bodies of the mammalian nucleus. **(B).** High-resolution structure of the nucleolus.*

2.10 miRNA biogenesis and hematopoietic differentiation

Differentiation and proliferation of HSCs are carefully regulated through a complex interconnected network. This network consists of niche, signalling molecules and specialised transcription factors. Both transcriptional and post-translational regulation are required for proper maintenance of HSCs. Among these regulatory processes, micro RNAs (miRNAs) play a pivotal role in the

differentiation of HSCs. Each stage of the hematopoietic system is tightly regulated by different families of this non-coding RNAs i.e. miRNAs[227–229].

The small sized miRNA is approximately 21-25 nucleotides (nts) long and found in animal, plants and other organisms. Thousands of miRNAs are encoded by human genome as categorised by miR Base. In order to produce primary miRNA transcripts (pri-miRNAs), RNA polymerase II (RNA pol II) or occasionally RNA polymerase III (RNA pol III) transcribes miRNA genes. The resulting transcripts are usually long and modified in 5` and 3` terminus similar to the modification which are normally observed in post-transcriptional modification of mRNAs. Interestingly, several miRNAs are encoded from the genes of many RNAs which are frequently designated as the host genes for many miRNAs. Similar to the genes of mRNAs, genes of several pri-miRNAs and miRNAs are not characterised well till now. It is assumed that transcripts of the mature miRNAs are encoded from the nearby known genes or may be they are transcripted from their own promoters that have no connection with those genes. It is also possible that miRNAs produce from independent transcription units[230–233]. Mammalian miRNAs are categorized into two broad classes – canonical and non-canonical miRNAs[234]. This division in categories totally depends on the process that lead to produce mature miRNAs from pri-miRNAs.

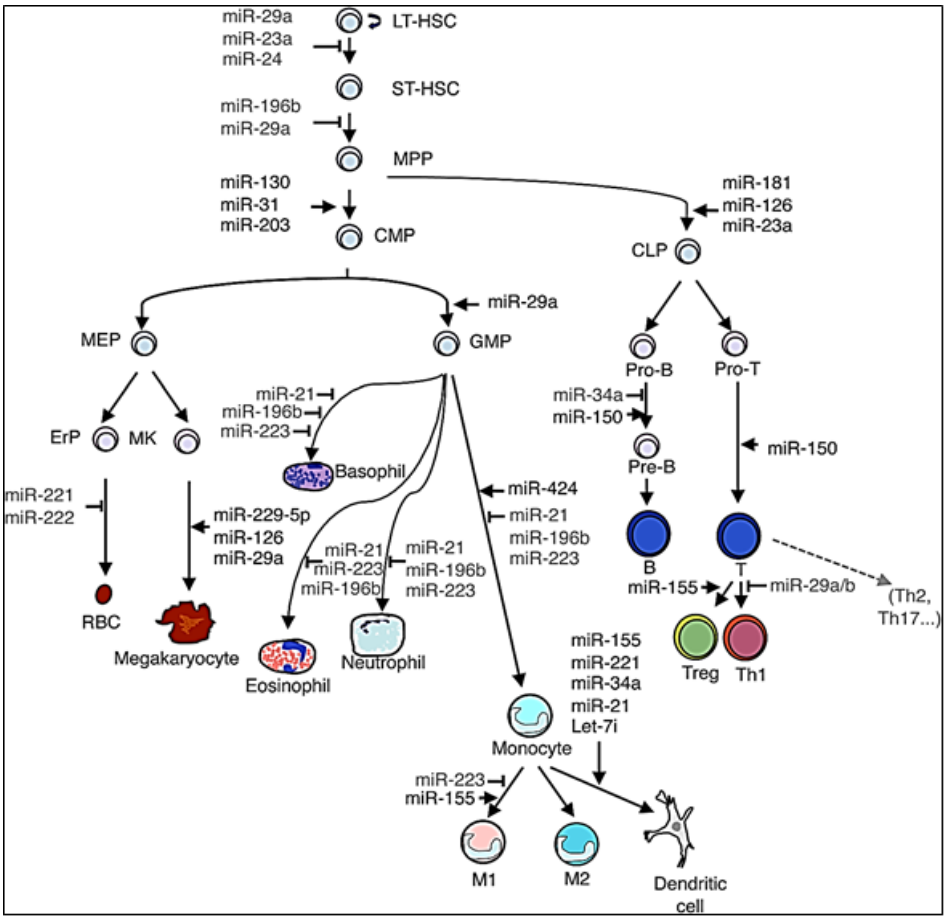
In the canonical pathway, at first pre-miRNAs are produced from the pri-miRNAs. Drosha cleaves these pri-miRNAs by binding to the regulatory subunit DGCR8. Pre-miRNAs are 60-70 nts long which is produced within the nucleus and are then translocated to the cytoplasm by exportin5 which is associated with Ran cofactor coupled to GTP. Due to the Drosha`s RNase III activity, pre-miRNAs usually contain 3` overhang with 2 nts. The pre-miRNAs are cleaved by another RNase, known as Dicer which leads to the production of pre-mature miRNA duplex. This duplex is 22 base pairs (bp) long. Then an Argonaute (Ago) proteins interact with the duplex and form the

Ago/miRNA complex which produces the mature, single stranded miRNA. The other strand of the duplex miRNAs is discarded. However, the choice of retained strand depends on the relative thermodynamic stability of the two ends of the duplex miRNAs. TRBP and PACT, Dicer and RNA-binding proteins may accompany in the mature miRNAs to Ago complex[235–238].

In non-canonical pathway, the aforementioned protein factors do not participate in the process of mature miRNA production. In this pathway, pre-miRNAs of Mitron genes are cleaved by splicing rather not by Drosha. Additionally, Ago2 cleaves pre-miRNA451 where as in canonical pathway this process is achieved by Dicer. Normally, these RNAs are designated as small endogenous interfering RNAs (siRNAs). Mature miRNAs are also produced after the editing. During this editing process, adenosine (A) changes into inosine (I) residues by RNA adenosine deaminases. It can pair with the cytosine (C) or uracils (U). It is assumed that the deaminases can modify those pri-miRNAs, pre-miRNA and mature miRNAs which eventually modulate miRNA processing and target modification[239–241].

For the differentiation of HSCs, several miRNAs are involved. It can regulate the self-renewal, differentiation and proliferation capabilities. As, long term HSCs (LT-HSCs) are positively regulated by many miRNAs, such as miRNA155, miRNA125, miRNA99a, miRNA126 etc. Deficiency in dicer activity in hematopoiesis leads to severe effect on embryos and the production of HSCs, especially the lymphoid progenitors. Lack of dicer activity block not only the transition from pro-B cells to pre-B cells but also the production of $CD8^{+}$T cells and $CD4^{+}$T cells in the thymus[242]. Recently, the role of several miRNAs in megakaryopoiesis and thrombopoiesis have been evaluated. Opalinska et al. showed that among 435 miRNAs that are expressed in murine system 13 miRNAs and 81 miRNAs were upregulated and downregulated respectively. Among them, miRNA146a plays a pivotal role for the differentiation and proliferation of MKs[243–245].

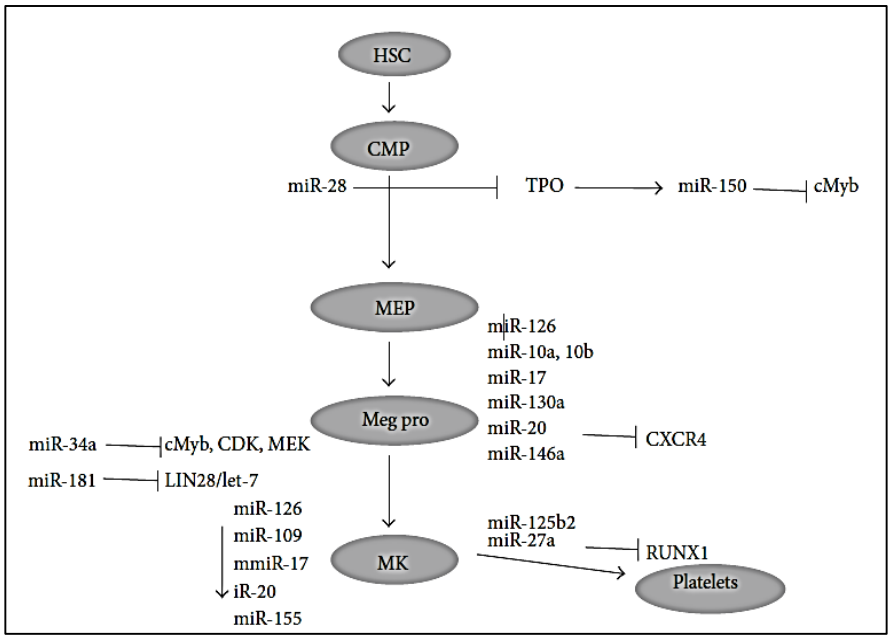
It promotes differentiation of the progenitor cells into MKs while miRNA 155 inhibits this differentiation process by targeting Meis-1 and Ets-1. miRNA 145 contributes in megakaryopoiesis by targeting TRAF6 which are associated with 5q syndrome phenotype[246,247].



(Adapted from *Montagner et al., 2014*)

Figure 2.11 <u>Schematic diagram of the hematopoiesis with miRNAs involvement</u>. *miRNAs are involved in blocking as well as in proliferation during hematopoiesis. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; CMP: common myeloid progenitor; MPP: multi-potent progenitors; CLP: common lymphoid progenitor;GMP: granulocyte-macrophage progenitor; ErP: erythroid progenitor; RBC: red blood cells.*

Addition to these, miRNA150 was found to promote the differentiation of progenitor cells into MKs rather than in erythroid lineage by targeting c-Myb expression in megakaryopoiesis induced cells[248]. In fact, miRNA34a also induces megakaryopoiesis by targeting c-Myb in association with CDKs and MEK-1[249,250]. In myeloproliferative neoplasm patients, overexpression of miRNA28 which blocks the differentiation of $CD34^{+}$ cells into MKs by targeting TPO receptor has been observed[251]. miRNA181a which represses LIN28, prevents the Ca^{2+}-dependent differentiation. Normally, LIN28 inhibits Let-7 expression and subsequently prevents differentiation of MKs[252]. Finally, miRNA130 and miRNA10a expression induce the differentiation of MKs. Irrespective of the up-gradation of different types of techniques, still the list of miRNAs that are responsible for megakaryopoiesis is increasing[253].



(Adapted from *Undi et al., 2013*)

Figure 2.12: <u>Involvement of miRNA in megakaryopoiesis</u>. *miRNAs playing a pivotal role in the maturation of MKs (MegPro-megakaryocyte progenitor, CMP-common myeloid progenitor).*

Chapter 3

Materials & Methods

3.1 Isolation of $CD34^+$ cells from cord blood:

After taking the donor`s consent CD 34^+ blood cells were collected from the cord blood (CB) samples. All clinical samples were collected under strict institutional ethical biosafety guidelines. For isolation of $CD34^+$ cells, percoll (GE Amersham, UK) were layered over CB and then centrifuged. In order to enrich $CD34^+$ cells in the purified samples, magnetic separation (Miltenyi Biotech, Germany) were used next and purity was confirmed by Anti-CD34 antibody (BD Biosciences, California, USA)[254]. Isolated fresh $CD34^+$ cells were cultured in IMDM supplemented with 10% fetal bovine serum (FBS) Serum (Stem cell technologies Inc., MA, USA) including 100 μg/ml streptomycin, 100 μg/ml penicillin and 2 mM GlutaMAX (Invitrogen/Life Technologies, California, USA). Cytokines including TPO (10mg/ml) and SCF (50mg/ml) were used for first 2 days and then cells were maintained in 10ηg/ml TPO for subsequent 48 hrs or 10 days for MKs lineage commitment. Percentage of MKs committed cells were determined using $CD61^+CD42b^+$ (BD Bioscience, California, USA) in BD FACS system. Cytokines were purchased from R&D systems Inc. (Minnesota, USA). Cytokines were dissolved in Dulbecco PBS (pH 7.4) including 0.1% Bovine serum albumin (BSA) and filtered in 0.22μ filtered before use.

3.2 Cell lines and cell culture conditions:

K562, HEL and CMK cell lines were cultured in RPMI1640 (Gibco, California, USA) with 10% FBS (Gibco, CA, USA). To induced megakaryopoiesis lineage differentiation K562 and CMK, HEL cells were subjected to 50nM and 10nM of 12-O-Tetradecanoylphorbol 13-acetate (TPA)

respectively. Cells were incubated with MVs for 48 hours (hrs), washed and subsequently used for experimental studies[255].

MCF 7 were cultured in DMEM (Gibco, CA, USA) with 10% FBS (Gibco, CA, USA). MDA-MB-231 were grown in DMEM (Gibco, CA, USA) supplemented with 10% FBS (Gibco, CA, USA) and high glucose (Sigma-Aldrich, Missouri, USA). All cells were kept at 37^{o} C with 5% CO_2. For hypoxia induction $CoCl_2$ were used[256].

3.3 Transfection:

Lipofectamine 3000 (Invitrogen, California, USA) were used for all the transfections according to the manufacturer`s protocol. Briefly, when plates were 70-90% confluent with cells, those plates were taken for transfection. At first, Lipofectamine 3000 were diluted in serum free medium and then added to master mix prepared with P-3000 reagents, DNA or Plasmids and serum free medium. Then, that mixture were incubated for 15-20 minutes (mins) before adding them into the plates.

3.4 Extracellular vesicles isolation (EVs):

HEL, K562 and CMK cells were induced for megakaryocytic lineage by TPA for consecutive 3 days. EVs were isolated from the cultured medium by using ultra-centrifugation at 1, 00,000g for 1 hr. The isolated EVs were then washed with PBS to remove contaminating proteins and medium components and used for experimental purpose.

3.5 Transmission electron microscopy (TEM):

EVs were laid on formvar carbon-coated 400 mesh copper grids. It was stained with 1% (w / v) phosphotungstic acid with incubation time of 10-15 seconds. The grids were dried before viewing under the Tecnai S Twin, FEI electron microscope (Eindhoven, the Netherlands) operating at 200 kV accelerating voltage.

3.6 Dynamic light scattering:

The hydrodynamic sizes of EVs were examined by Dynamic light scattering (DLS). The measurements were prformed on a Zetasizer Nano S particle analyzer from Malvern Instruments, UK, whereby a He–Ne laser (633 nm) utilizing 4 mW power at 632.8 nm was used as the light source. The intensity autocorrelation function generates a correlation curve. Cumulants analysis of the correlation curve gives the intensity weighted mean hydrodynamic diameter or Z_{av} diameter of the sample.

3.7 Confocal microscopic analysis:

MVs isolated from megakaryocytic lineage K562 cells were treated with phospholipid membrane dye (Invitrogen, California, USA). After 30 mins of incubation at 37°C, the vesicles were washed twice with media containing no serum and co-cultured with recipient cells. After washing with EDTA, co-cultured cells were then imaged using the NIKON Inverted Research Microscope ECLIPSE TiE with Plan Apo VC 100X oil DIC N2 objective/1.40 NA/1.515 RI with a digital 4X

zoom. The images were captured with Ixon DU-897 EM CCD camera (Galvano mode) mounted on Ti-E inverted microscope. Images were processed using Ni Elements AR Ver 4.13.

For nucleolus visualisation and analysis, SytoSelect Nucleolus dye (Abcam, Cambridge, UK) was used according to the manufacturer`s protocol. Briefly, prepared cells were incubated for 15-20 minutes at 37^0C after the addition of nucleolus staining dye. Then washings were done twice with PBS and next samples were analysed with fluorescence detection system. For chromatin staining, cells were then stained with DAPI or DRAQ5 (Abcam, Cambridge, UK) and imaged under confocal mode in a Nikon Instruments Inc., USA or in ZEISS Microscope, Germany. For in-situ protein staining, cells were blocked at first and then were stained with primary antibodies (Abcam, Cambridge, UK) followed by secondary antibodies (Invitrogen, California, USA). 4% paraformaldehyde were used for fixation.

3.8 <u>Flow cytometric analysis</u>:

For the analysis of MKs specific surface markers, cells were incubated at room temperature for 30 mins according to the manufacturer`s protocol after staining with fluorescent (FITC/PE) tagged antibody (BD bioscience, California, USA) in BD FACS Calibur platform (CA, USA). FITC conjugated CD42b, FITC conjugated CD41a, PE-conjugated CD61, PE-conjugated CD235a and FITC conjugated Annexin V obtained from BD Science, USA.

For cell-cycle analysis cells were stained with Propidium iodide (PI) (Sigma) or vibrant orange (Invitrogen, California, USA) and experiments were performed on a BD FACS Calibur platform (CA, USA) according to the manufacturer`s protocol. Cells stained with vibrant orange were sorted based on BDFACS Aria II platform on the basis of their DNA content. For fixation, cells were

dissolved in 70% pre-chilled ethanol and kept overnight at 4^0C. After centrifugation at 1000g, cells were washed twice with pre-chilled PBS and incubated at 37°C for 1hr in PBS containing 10µg/ml RNase A (SRL, India). Finally, cells were incubated with PI (BD Biosciences, CA, USA) to a final concentration of 20-40µg/ml for 15-30 mins in dark at room temperature. Progression through the cell cycle was followed by Cell Quest Pro software in FACS Calibur (Becton Dickinson, CA, USA). A total of 10,000 gated events were acquired for analysis.

3.9 <u>Western blotting</u>:

For western blotting, cells were harvested and lysed in cold RIPA buffer consisting of [20 mM Tris-HCl (pH 8.0), 1% NonidtP-40, 10% glycerol, 137 mM NaCl , 2mM EDTA, 200µM Na_3VO_4, 100mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (Roche, Mannheim, Germany) and 50m phosphatase inhibitor (Roche, Mannheim, Germany)]. Whole cell protein lysates were purified using freeze-thaw system. After Bradford protein assay, equal amounts of protein from different samples were run on parallel lanes of SDS PAGE and blotted on PVDF membrane (GE Amersham, UK). Blockings were done by non-fat dry milk or with 5% BSA in TBS containing 0.1% Tween 20 for 1.30-2 hrs at room temperature and then washed with TBST. After incubation with primary and HRP conjugated secondary antibodies, the blots were developed using Enhanced Chemiluminescent substrate (Thermo Fischer Scientific Pierce, Massachusetts, USA). Anti CXCR4, anti phospho-CXCR4, anti phospho-moesin, integrin αIIb-β3, DNMT1, DNMT3a, DNMT3b, NOLC1, Nucleolin, Fibrillarin, H2A.Z, $H3K27me^3$, γ-H2A.X and NOTCH1 antibodies were obtained from Abcam whereas anti ERK1/2, anti phospho ERK1/2, MCM 2 and anti phospho MEK antibodies were obtained from Cell signalling technology, Massachusetts, USA. For whole cell lysate Beta Actin (Abcam, Cambridge, UK) was used as the loading control.

3.10 MSP and bisulfite sequencing of Notch 1 promoter:

DNA was extracted using the Wizard Genomic DNA Purification Kit according to the manufacturer's protocol (Promega, Wisconsin, USA). Briefly, cells were lysed using Cell Lysis solution and then centrifuged. The pellet were dissolved in Nuclei Lysis solution including RNaseA and incubated for 30 mins at 37^0C. Then into the mixture Protein Precipitate solution were added and centrifuged for 3mins. The supernatant was collected into a new tube containing isopropanol. After the centrifugation, pellet was washed and centrifuged with pre-chilled 70% ethanol. Pellet was dissolved in water. The methylation status of the Notch1 promoter in MKs cell line was analysed by bisulfite sequencing followed by methylation specific-PCR (MSP) after converting 1 μg of DNA with sodium bisulfite using Epitect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's protocol. Modified DNA was then used as a template for PCR reactions. Methylation-specific PCR (MSP) was performed for methylation analysis of the Notch1 promoter, the primers were within a CpG island region, for the unmethylated reaction were 5`-GTTTTGTTTTTTTTATTTTGTTTTG-3` and 5`-CAAACACC TAAAACTACTTCTCATT-3` which amplify a 168-bp product (positions 630 to 463) and the primers for the methylated reaction were 5`-GTTTCGTTTTTTTTATTTCGTTTC-3` and 5`-GAACGCCTAAAACTACTTC TCGTT-3` which amplify a 167-bp product (positions 630 to 464)[257]. PCR amplification was performed for 5 cycles with extension time 2 minutes and an additional 25 cycles with an extension time 1.30 minutes. Each cycle started with denaturation at 95°C and ended with extension at 72°C. To confirm the methylation results, methylated PCR product was purified by GenElute Gel Extraction Kit (Sigma-Aldrich, Missouri, USA), then purified DNA was sequenced.

3.11 Plasmids construct and transfection:

Both DNMT1 (HG11494-ACG) and DNMT 3A (HG11320-ACG) were purchased from Sinobiological, Beijing, China. DNMT 3B cDNA (a generous gift from Prof. Che-Kun James Shen) tagged with pEGFP were used for expression in transfected cells. Lipofectamine 3000 (Invitrogen, California, USA) was used for cell transfection according to the manufacturer`s protocol. After 24 hrs of transfection, cells were treated with the purified MVs and then incubated for 48 hrs. Subsequently, cells were collected for downstream experiments.

3.12 MNase assay:

For micrococcal nuclease assay, we simply used SimpleChip Enzymatic Chromatin IP Kit Magnetic Beads) (CST, Massachusetts, USA). Briefly, both HEL and K562 cell lines were at first fixed with formaldehyde and then the cells were incubated with glycine. Cells were sonicated using standard methods followed by MNase incubation at 37^{o} C for 20 min and RNA was removed from the samples by treating them with RNase A for 30 min at 37^{o} C. Then the fragmented chromatin were purified and run on 1% agarose gel with TAE buffer.

3.13 SUnSET assay:

Cells were incubated with 150 µl of 10 µg/ml puromycin solution (SIGMA, min. 98 % TLC, cell culture tested, P8833, diluted in PBS) for 15 min at 37 °C and 5 % CO2. Cells were then centrifuged and washed for two times with pre-warmed media. Then, the pellet were resuspended

in 1ml pre-warmed complete media and incubated for 1 hour at 37 ℃ and 5 % CO2. Cells were then fixed with 4% formaldehyde and washed two times with cold PBS containing 0.1 % BSA (PBS/BSA). Cells were incubated with Triton X-100 for 15 minutes at room temperature and again washed with PBS/BSA. After that, anti-puromycin antibody (12D10) was added followed by secondary antibody. Cells were washed with PBS/ BSA and stored at 4 ℃ until FACS analysis[258].

3.14 Genomic DNA purification and construction of expression plasmids:

Genomic DNA was isolated from HEL cells using a Genomic DNA Purification kit (Promega, Wisconsin, USA) as mentioned earlier. A DNA fragments upstream of the transcription initiation site of NOLC1 was amplified using Phusion High Fidelity DNA Polymerase (Thermo Fischer Scientific, Massachusetts, USA). PCR amplification was initiated with initial denaturation step at 98^0C for 20sec. Then 35 cycles with an extension time 45sec. were performed. Each cycle started with a denaturation at 98°C and ended with an extension at 72°C. The product was then inserted into Nhe I and Hind III restriction sites of a pGL4.10 Basic vector (Promega, Wisconsin, USA) after agarose gel purification.

3.15 Dual-luciferase reporter assay for detection of promoter activity:

Empty or NOLC1 promoter coded with luciferase reporter containing vector were transfected into HEL and K562 cells, followed by TPA treatment. Expression of the firefly luciferase was quantified with a Dual-Luciferase Reporter assay system (Promega, Wisconsin, USA) according to the manufacturer's protocol. In short, samples were washed with PBS twice. Then cell lysis

buffer was added and incubated for with gentle shaking. Cell lysates were transferred into a new tube containing LARII reagent. Immediately first reading was measured and then Stop & Glo reagent was added and subsequently second measure was taken. All transfections were performed in triplicate. The level of the firefly luciferase activity was normalized by the corresponding level of the Renilla luciferase activity and the values for the negative control were normalized. Luminescence was measured using a Sirius Luminometer, Berthold detection systems (USA).

3.16 Statistical analysis:

Results of experiments were performed three times and expressed as mean ± S.D. Student's two-tailed t-test was used to compare the mean of test and control samples. Significance was set at *P*-value ≤ 0.05.

3.17 RNA interference:

The sequence of anti-miRNA-146b for inhibiting the expression of miRNA-146b was similar as previously reported. A controlled scrambled siRNA was also used (Sigma, Missouri, USA). The anti-miRNA-146b siRNAs both were transfected into cells using Lipofectamine 3000 (Invitrogen, California, USA).

3.18 Reagents:

Antibodies such as phospho p44/42 MAPK, PTEN, AKT and phosphorylated-AKT were purchased from Cell Signaling Technology (CST) (Massachusetts, USA). Antibodies including

MCM2, MCM4, MCM6, H2A.X, H1, H2A, H2B, H3 and H4 were obtained from Abcam (Cambridge, UK). PE-conjugated CD235a, APC conjugated CD11b, APC conjugated CD33 were purchased from BD Biosciences (California, USA).

3.19 Flow cytometric sorting:

Megakaryocytic cells were stained with vibrant orange and then sorted based on their DNA content (ploidy level) on BDFACS Aria II platform.

3.20 Cloning:

3.20(a) Competent cell preparation:

Cells from frozen glycerol stock of bacterial cells were incubated overnight at 37^0C in Luria Broth (LB) containing no antibiotics. Next day, a secondary culture was given from the primary culture and measured the OD_{600} of the secondary culture till it reached 0.35-0.4. Immediately, cells were transferred and kept for 20-30 mins into the ice. Then, the secondary culture was pellet down in a cold centrifuge and supernatant was discarded. Pellet was washed with cold $MgCl_2$ for once and then with $CaCl_2$ for at least 3-4 times. Lastly, pellet was resuspended with $CaCl_2$ and glycerol mixture and after snap freezing with liquid nitrogen, cells were kept at -80^0C.

3.20(b) Transformation:

DNA or plasmid construct were added into the tube containing competent cells and incubated in ice for 20-30 mins. Cells were then heat shocked into the water bath at 42^0C for 90sec and then tube was put back into the ice for 2 minutes. LB or SOC without any antibiotic was added into the

tube and incubated for 1 hour at 37^0C. After that, cells were plated in the agar LB plate and also inoculated into the LB containing specific antibiotic.

3.20(c) Plasmid purification, Restriction digestion, Ligation and Gel purification:

DNA construct or plasmid at first purified according to the manufacturer`s protocol using Qiagen Plasmid Purification Kit. Then, plasmid`s quality and quantity was measured. For the specific digestion of the required sized plasmid, several restriction enzymes were used and the reaction was carried out in the water bath at 37^0C according to the New England Biology (NEB) protocols. Ligation reaction is carried out with NEB quick ligation kit. Then, the products were purified from agarose gel by using Qiagen Gel Purification Kit.

3.21 RNA extraction and real-time PCR:

Total cellular RNA were extracted from cells by using Tripure isolation reagent (TRIZOL, Roche, Germany). Quality and quantity of RNA were measured using Nanodrop (Thermo Scientific, USA). Subsequently, 200ng RNA for miRNA and 1500ng RNA for mRNA were then reverse-transcribed using AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, California, USA) according to the manufacturer`s protocol. Expression level of genes were quantified by quantitative RT-PCR using SYBR Green core PCR reagents (Thermo Scientific, Massachusetts, USA). HPRT1 was used to normalise the expression of the genes (Primers in Supplementary Figure Table 1 and Table 2). The reactions were parformed on 7500 Sequence Detection Systems (Applied Biosystems).

For $CD34^+$ cells, total RNA was extracted using Tripure isolation reagent (TRIZOL, Roche, GmbH, Mannheim, Germany) and subsequently treated with DNAse I (Roche, Germany) and then

quality, quantity were analysed using Nanodrop. Total 3000 ng of total RNA were used for the cDNA using reverse transcriptase reagents (Agilent Technologies, California, USA). Quantitative PCR was subsequently performed using SYBR Green core PCR reagents (Thermo Scientific, Massachusetts, USA) and for endogenous control, HPRT1 was used. Details of the primer sequences are given in the below tables.

- **Table3.1**: ***Forward and reverse primer sequence with corresponding genes names*.**

Genes	Accession Number	Forward primer	Reverse Primer
HPRT	NM_000194	5'GACACTGGCAAAACAATGCAGAC3'	5'TGGCTTATATCCAACACTTCGTGG3'
DNMT1	AF180682	5`GTGGGGGACTGTGTCTCTGT3`	5`TGAAAGCTGCATGTCCTCAC3`
DNMT 3a	AF331856	5`CCGGAACATTGAGGACATCT3`	5`CAGCAGATGGTGCAGTAGGA3`
DNMT3b	NM_006892	5`AATGTGAATCCAGCCAGGAAAGGC3`	5`ACTGGATTACACTCCAGGAACCGT3`
Notch1	NM_017617	5`ACTGTGAGGACCTGGTGGAC3`	5`TTGTAGGTGTTGGGGAGGTC3`
Hes1	NM_005524	5'GAAGGCGGACATTCTGGAAA3'	5'GTTCATGCACTCGCTGAAGC3'
Hey1	NM_012258	5'CTGCAGATGACCGTGGATCA3'	5'CAACTTCTGCCAGGCATTCC3'
FOG1	AF488691	5'AGACGAGAAGCCCAAAGAGACC3'	5'TAGATCTCACCCTTGGAGCCAG3'
FLI 1	NM_002017	5'CAGGAGTGGATCAATCAGCCA3'	5'CATTTGCTAACGCTGCAGTCC3'
NF-E2	NM_001136023	5'AAAGGTACTCCCCAACCCTGAG3'	5'TGAACACACCTTGGAACTTCCA3'
MCM 2	ENSG00000073111	5`TGTGCAAAGAGAACCGTGAG3`	5`GACATGGATGTGGTTGGTGA 3`
MCM 4	ENSG00000125816	5`TGTGATCTGGGGAACAGATG3`	5`TAATCTCCCCAAGTCGTTGC 3`
MCM 6	ENSG00000076003	5`ATCCCCCGCAGTTTAGAAGT3`	5`TCCTCGAATGCCTTCTGTCT3`

NOLC 1 Promoter	ENSG00000166197	5`-CCA CTG GAT TTG AAC CTT GG-3`	5`-TAC GCG TCA CTA CCG TTG TC-3`
NOLC 1	ENSG00000166197	5`GGCTCCTCAGGGTAGTAGGG-3`	5`GCCCTTGAGCTTCTCTTCCT-3`
STAT 3	ENSG00000168610	5`TAGCAGGATGGCCCAATGGAATCA-3`	5`AGCTGTCACTGTAGAGCTGATGGA-3`
STAT 1	ENSG00000115415	5`CTAGTGGAGTGGAAGCGGAG-3`	5`CACCACAAACGAGCTCTGAA-3‘
28S rRNA	Reference [259]	5`AGAGGTAAACGGGTGGGGTC-3`	5`-GGGGTCGGGAGGAACGG-3`
18S rRNA	Reference [259]	5`GATGGTAGTCGCCGTGCC-3	5`-GCCTGCTGCCTTCCTTGG-3`
GAPDH	ENSG00000111640	5`AGAAGGCTGGGGCTCATTTG 3`	5`AGGGGCCATCCACAGTCTTC 3`

Table3.2: ***Sequence of primers that are used for corresponding miRNAs detection*.**

miRNA	**Sequence**
Universal RV	5’GTGCAGGGTCCGAGGT3’
miRNA 99a Forward Primer	5`AACCCGTAGATCCGATCTTGTG3`
miRNA 99a Stem Loop Primer	5`GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCACAAGA3`
miRNA 125b Forward Primer	5`TCCCTGAGACCCTAACTTGTGA3`
miRNA 125b Stem Loop Primer	5`GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAAG3`
miRNA 150 Forward Primer	5`GCGCGCTCTCCCAACCCTTGTACCAGTG3`
miRNA 150 Stem Loop Primer	5'GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACTGGT3'
miRNA 500 Forward Primer	5`AATGCACCCGGGCAAGGATTCT3`
miRNA 500 Stem Loop Primer	5`GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAATC3`
miRNA 551 Forward Primer	5`GCGACCCATACTTGGTTTCAG3`

miRNA 551 Stem Loop Primer	5`GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGAAA3`
146b RT Primer	5`GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTATGG-3`
146b Up Primer	5`-ATGCGCTGCTGAGAACTGAATT-3`
146b Down Primer	5`-CAGTGCAGGGTCCGAGGT-3`

Chapter 4

Results

4.1 Characterisation of EVs produced from cells:

In order to characterise the size distribution of EVs, EVs were analysed with dynamic light scattering. With respect to the control, mean hydrodynamic diameter (Z average value) was of 119.42nm with a range between 35 - 450 nm; approximately 80% EVs population, were in the size range 75 - 250 nm (Figure 4.1A). Based on size distribution of isolated heterogeneous population of the EVs, they were referred as MVs. TEM micrograph analysis was further used to detect shape of MVs and the heterogeneity of MVs population was confirmed (Figure 4.1B). To further characterise MVs, we measured the presence of integrin, CD41a and annexin V – well-known markers for MVs derived from the MKs[28]. While, the presence of integrin-αIIb chain was revealed by the western blot analysis in the MVs derived from both HEL and K562 cells (Figure 4.1C), flow cytometry analysis confirmed the presence of annexin V (Figure 4.1D, Figure 4.1E & Table 4.1) and that of CD41a (Figure 4.2A) in the isolated MVs derived from both cell lines.

4.2 Interaction of MVs with the recipient cells:

To investigate time scale interaction between MVs and recipient cells, we purified the MVs and then stained them with the plasma membrane staining dye. Stained MVs were then co-cultured with recipient cells (Figure 4.2B). Flow cytometry analysis indicated that within 20 minutes of the co-culture, the fluorescence intensity of the dye increased and showed highest value within 1 hour. Surprisingly, though the intensity value remained almost similar after 2 hours, after 4 hours of co-culture it started decreasing. To analyse whether the MVs were getting internalised within cells or were just attaching to the cell surface, imaging analysis for the continuous time-dependent study

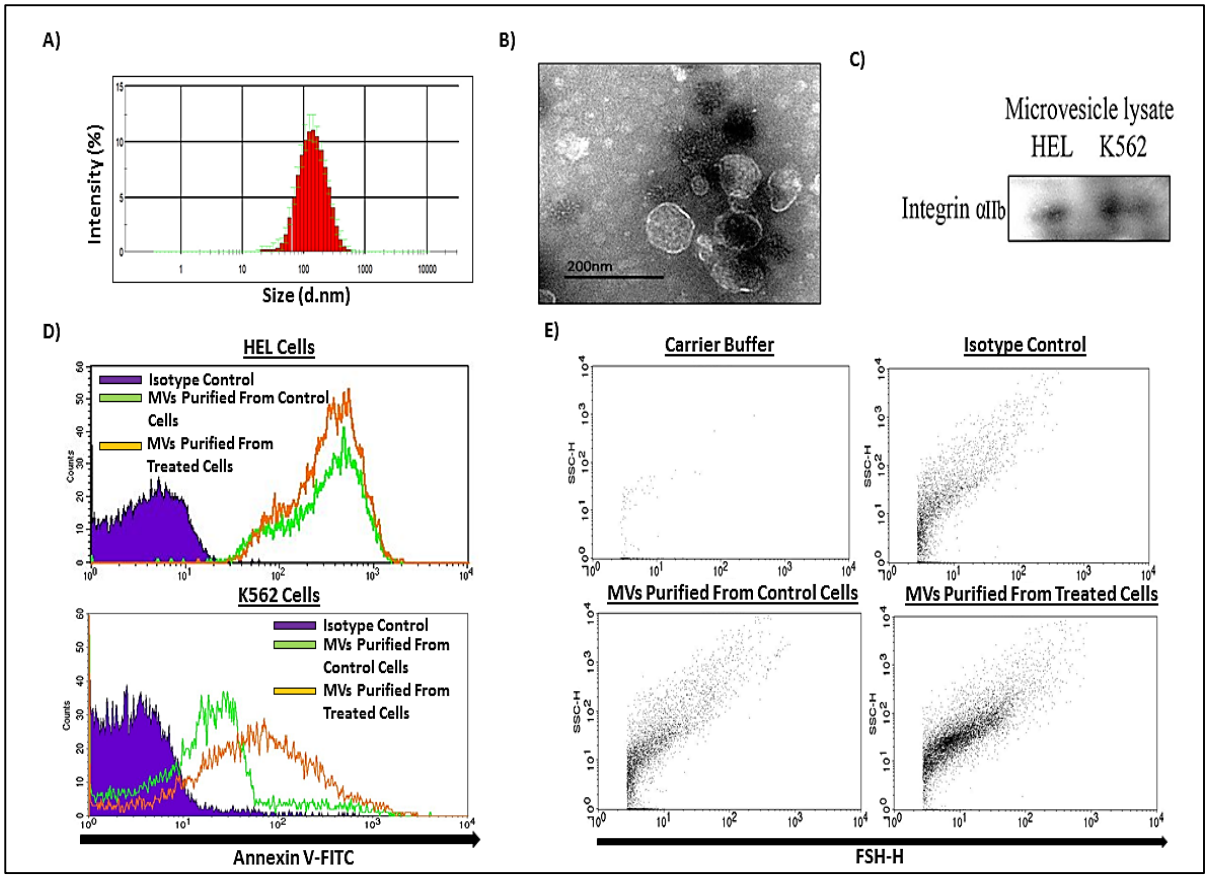


Figure 4.1. ***<u>Characterisation of megakaryocyte derived MVs.</u>*** ***(A).*** *Intensity vs. size plot of microvesicles.* ***(B).*** *TEM picture of microvesicles.* ***(C).*** *Detection of integrin-αIIb in megakaryocyte derived MVs lysate by western blot.* ***(D).*** *Detection of Annexin –V in MVs purified from both induced and non-induced cells. Median intensity value (MIV) in Table 4.1.* ***(E).*** *Dot plot analysis for Annexin V-FITC.*

Table 4.1: <u>Flow cytometric analysis of the expression of Annexin V-FITC</u>.

MVs Type	Median Intensity ± S.D.
Isotype Control	0.65 ± 0.25
MVs Purified From Control Cells	21.7 ± 5.82
MVs Purified From Treated Cells	72.99 ± 9.88

revealed after 30 minutes maximum population of the MVs were either already bound to the cell surface or getting internalised by the recipient cells (Figure 4.2C). Intact MVs were appeared in the micrographs as fluorescence dots. It was observed that the maximum population of MVs were internalised and only a few remained at the boundaries of cell periphery after 1 hour of co-culture (Figure 4.2C). Interestingly, within 2 hours of co-culture, fluorescence dots from the internalised MVs were coalescing together while no signal was found at the cellular periphery (Figure 4.2C).

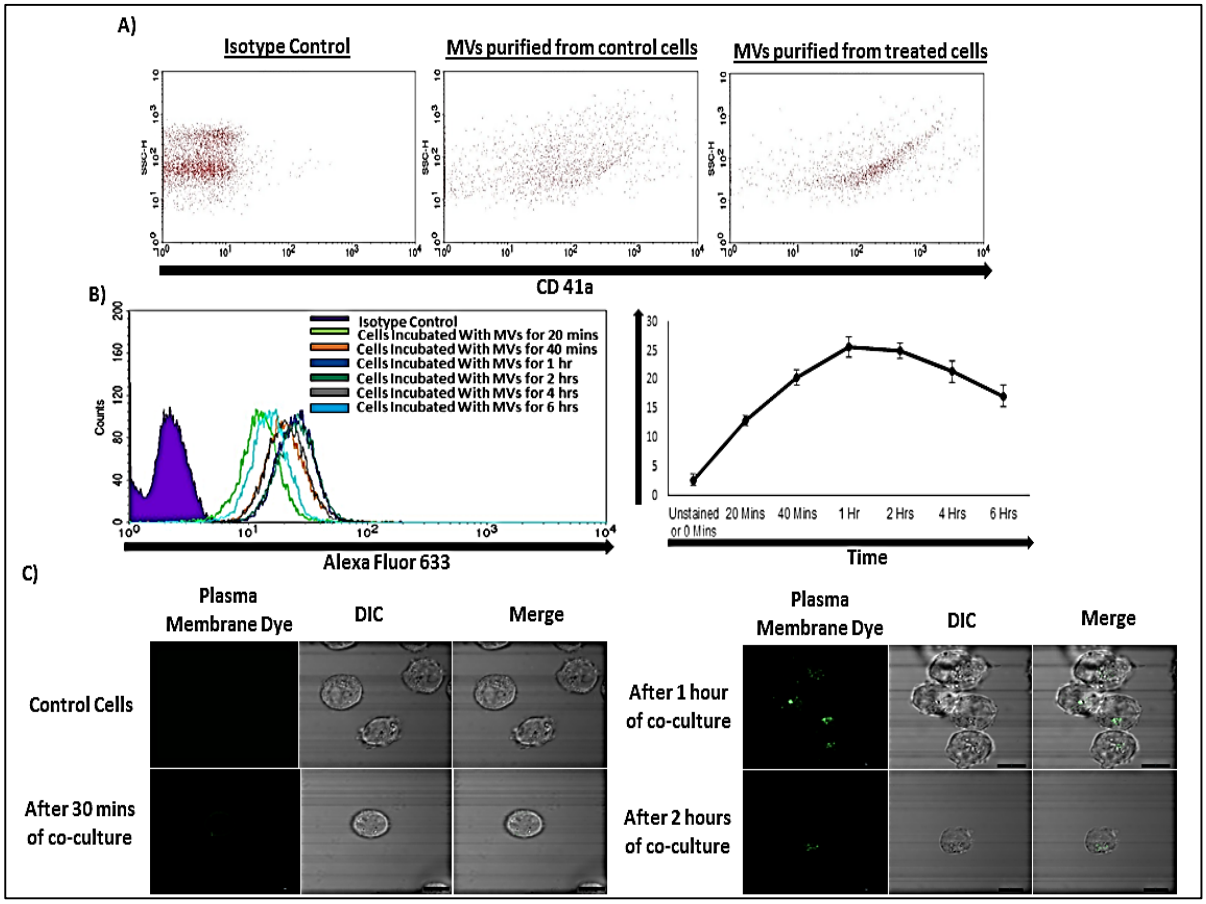


Figure 4.2. <u>Uptake timing for MVs.</u> (A). *CD 41a analysis for MVs purified from K562 cells.* ***(B).*** *Histogram analysis of MVs internalisation within the recipient cells after staining the MVs with plasma membrane dye. Plot Time vs Intensity of the fluor (Right Side). Experiments were repeated for three times (n=3).* ***(C).*** *Confocal images of stained MVs after co-culturing for different times.*

Notably, image slice analysis with 0.250µm apart at different confocal planes showed that some of the MVs fluorescence dots disappeared (Blue Arrow) while some of them were showing up (White Arrow) (Figure 4.3A). This observation suggested the MVs were internalised via endocytosis. Moreover, when we executed a continuous-time course study to track the MVs internalisation with 1-second delay, we observed that after binding on the cell boundaries some of the MVs had disappeared (White Arrow) (Figure 4.3B) suggesting cell membrane fusion as a mode of interaction between cells and MVs.

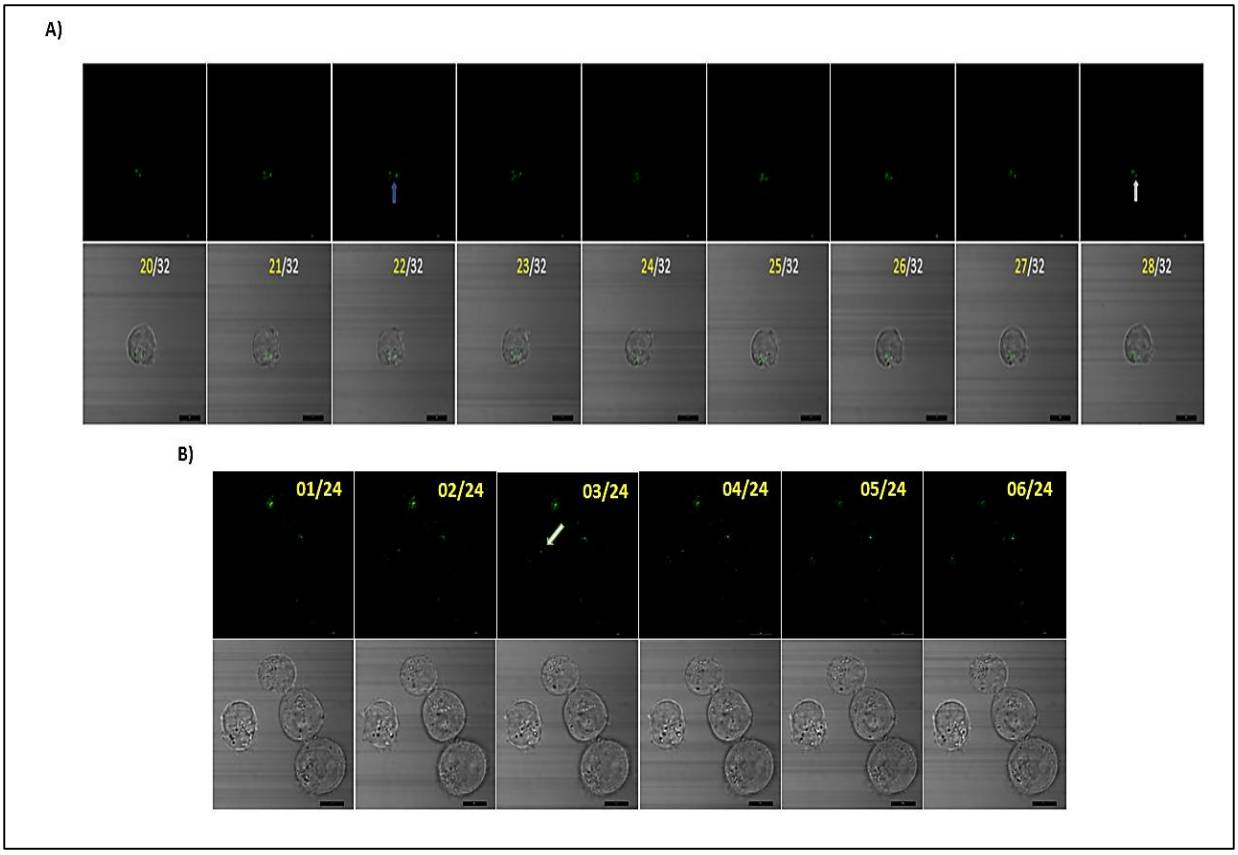
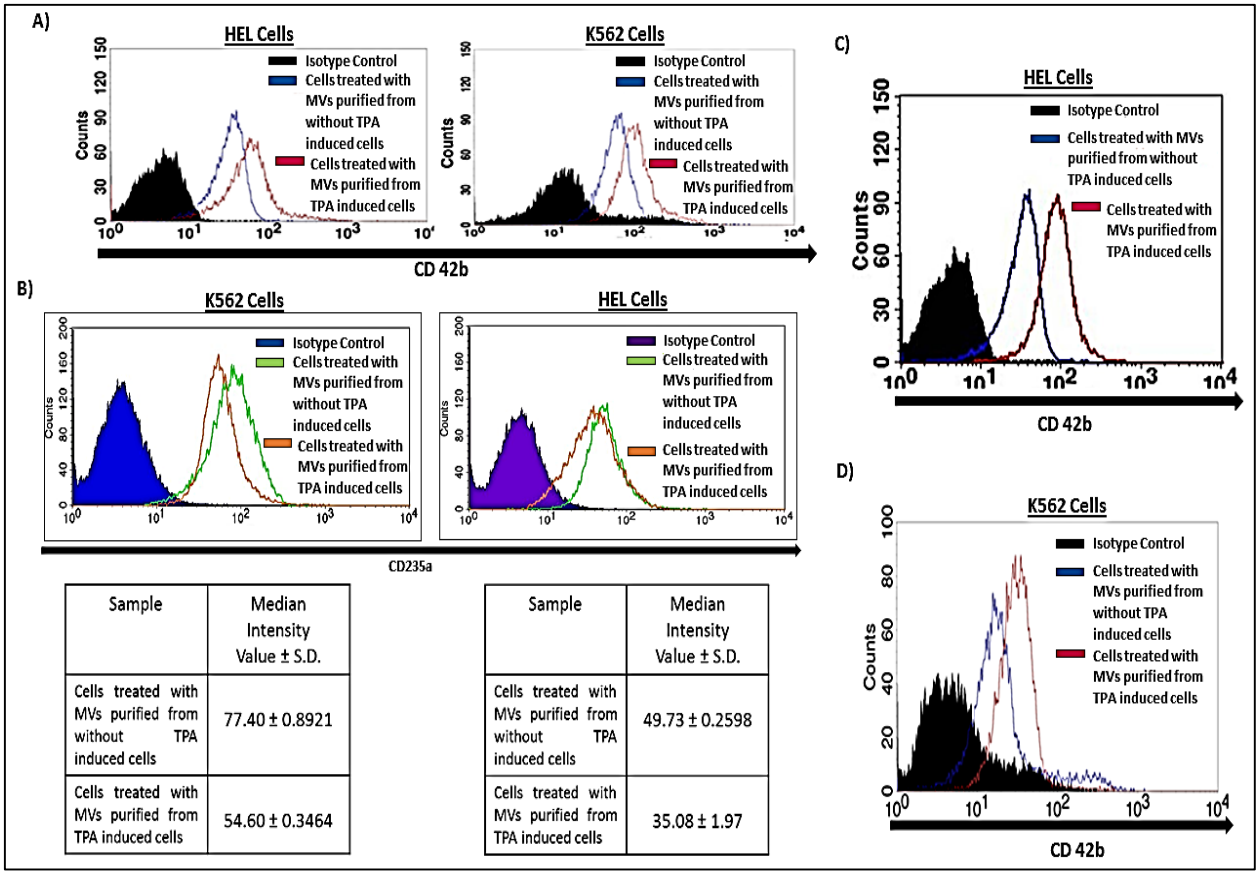


Figure 4.3. <u>Process of Internalisation of MVs.</u> (A). *Z-stack image analysis in confocal after co-culturing them. The numbers on the top right of each image represent (image slice number)/(total number of slices).* ***(B).*** *Representation of MVs fusion with the recipient cells.*

4.3 <u>Microvesicle treatment induces differentiation in recipient cells</u>:

To examine the role of induced-MKs derived MVs (Meg-MVs) in the differentiation as well as the maturation process of MKs and consequently in the endomitosis process, they were co-cultured with both uninduced K562 and HEL cells respectively. Then, cell surface marker (CD42B) for megakaryopoiesis was checked by using flow-cytometer. Surprisingly, we observed that those Meg-MVs have the ability to induce megakaryopoiesis (Figure 4.4A and Table 4.2). In order to

confirm the lineage commitment, erythrocyte surface expression marker was also analysed after co-culturing Meg-MVs with uninduced cells. We found decrement in the expression of CD235a



Sample	Median Intensity Value ± S.D.
Cells treated with MVs purified from without TPA induced cells	77.40 ± 0.8921
Cells treated with MVs purified from TPA induced cells	54.60 ± 0.3464

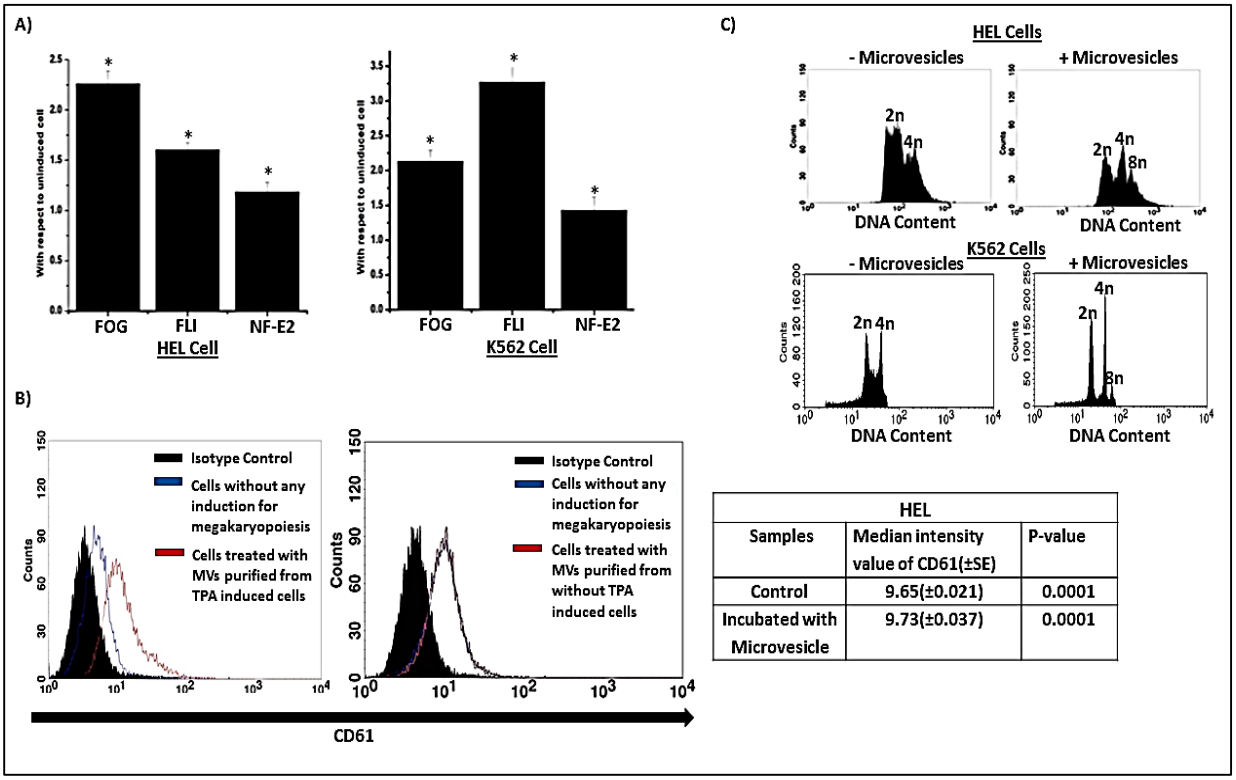
Sample	Median Intensity Value ± S.D.
Cells treated with MVs purified from without TPA induced cells	49.73 ± 0.2598
Cells treated with MVs purified from TPA induced cells	35.08 ± 1.97

Figure 4.4. <u>Lineage commitment of MEPs in megakaryopoiesis by Meg-MVs.</u> (A). *Flow cytometric analysis of the expression of CD42b in uninduced and Meg-MVs induced HEL, K562 cells. Median value in Table 4.2.* ***(B).*** *Flow cytometric analysis of the expression of CD235a in uninduced and microvesicle (megakaryocytic HEL derived) induced HEL cells and K562 cells. Meidan value for CD235a in table form (Down Side).* ***(C).*** *Flow cytometric analysis of the expression of CD42b in uninduced and microvesicle (megakaryocytic K562 derived) induced HEL cells and vice versa. Experiments were repeated for three times (n=3).*

Table 4.2: <u>Flow cytometric analysis of the expression of CD42b in cell lines.</u>

Cell Lines	Sample	Median Intensity value of Cd42b (±SE)	p-Value
K562	Control	74.47 (±0.2356)	0.0001
	Incubated with MVs	131.15 (±0.554)	0.0001
HEL	Control	34.57 (±0.8443)	0.0006
	Incubated with MVs	75.56 (±0.3238)	0.0003

(Figure 4.4B). Noticeably, to check the effect of Meg-MVs isolated from induced K562 cell line over other MgEr progenitors, Meg-MVs derived from K562 cells were co-cultured with HEL cells to study the differentiation and vice versa. We observed higher expression of megakaryocytic surface expression marker, CD42B (Figure 4.4C and 4.4D). From this flow cytometric data, it was assured that Meg-MVs carry lineage induction factor which subsequently is responsible for the induction of MEPs cells into a particular lineage. Additionally, we measured the transcript levels of some megakaryopoiesis specific transcription factors such as FOG, FLI, NF-E2 and they were observed to be up-regulated significantly after co-culturing Meg-MVs with the uninduced cells (Figure 4.5A).



HEL		
Samples	Median intensity value of CD61(±SE)	P-value
Control	9.65(±0.021)	0.0001
Incubated with Microvesicle	9.73(±0.037)	0.0001

Figure 4.5. <u>Analysis of megakaryopoiesis specific features</u> (A). *Relative gene expression of megakaryopoiesis lineage-specific transcription factors in Meg-MVs induced HEL, K562 as compared to uninduced cells. Data represent the mean ± s.e.m of three independent experiments (*p<0.05, n=3) normalised against HPRT1. Flow cytometric analysis of the expression of CD42b in uninduced and Meg-MVs induced HEL, K562 cells.* ***(B).*** *Flow cytometric analysis of the expression of CD61 in HEL cell when treated with MMVs isolated from uninduced and induced HEL cell. Median intensity value of CD61 in tabulated form (Right Side).* ***(C).*** *Polyploidization of HEL and K562 cells when induced with Meg-MVs. Experiments were repeated for three times (n=3).*

However, MVs derived from uninduced progenitors (NMeg-MVs) showed no effect on CD61 marker expression of HEL cells (Figure 4.5B). Our data thus clearly indicates that these Meg-MVs

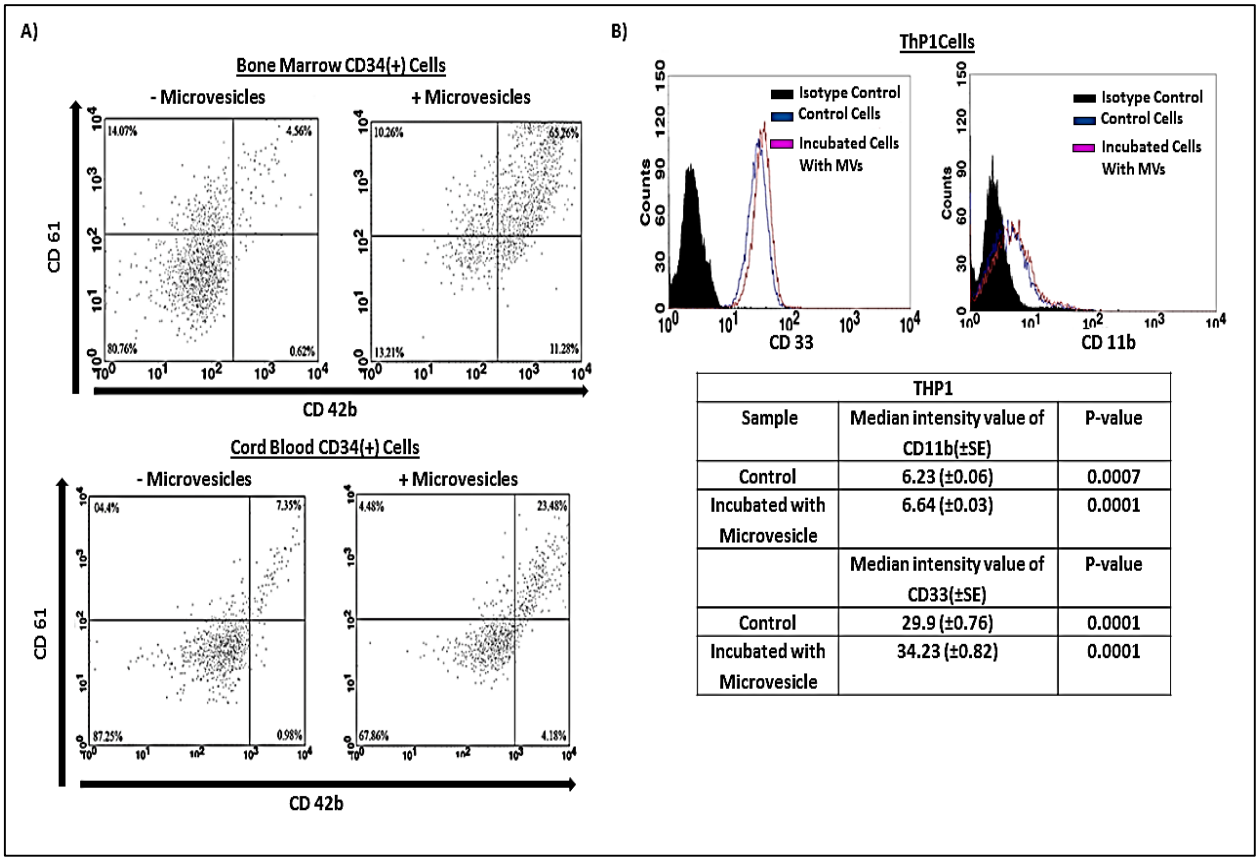
had the enough potential to induce megakaryopoiesis in the progenitors. Polyploidization of cellular DNA vis-à-vis increment in nuclear size due to endomitosis is distinct characteristics of megakaryopoiesis. We, therefore, aimed to examine the DNA content and nuclear size of all the cell lines (HEL and K562) before and after co-culturing with both NMeg-MVs and Meg-MVs. Interestingly, with respect to the NMeg-MVs, Meg-MVs has the ability to increase the ploidy numbers of all the cell lines when compared to uninduced cells (Figure 4.5C). Additionally, we also observed increment in the size of nucleus of HEL and K562 cells after co-culturing with Meg-MVs.

4.4 Effect of microvesicles on primary megakaryocytic culture and on the monocytic cell line:

To further verify whether Meg-MVs can induce normal hematopoietic stem cells into lineage specificity, Meg-MVs were incubated with human $CD34^+$ cells isolated from cord blood or bone marrow. Our data clearly revealed that megakaryocytic surface marker CD42b increased significantly due to the incubation (Figure 4.6A). To investigate the specificity of those Meg-MVs for megakaryopoiesis, we co-cultured them with monocytic cell line Thp1 and then checked for macrophage differentiation by measuring the surface expression of CD33 and CD11b in flow cytometry. Interestingly, no significant changes were found in the expression of both the surface markers after co-cultured with Meg-MVs (Figure 4.6B) suggesting Meg-MVs contain lineage-specific morphogen for megakaryopoiesis.

4.5 Involvement of Notch1 and its promoter methylation in endomitosis:

As we mentioned previously, our lab had shown that Notch1 is involved in the induction of megakaryopoiesis. In this context, after co-culturing Meg-MVs with the progenitors, we observed upregulation in both mRNA and protein level for Notch 1 in the recipient cells. Additionally, the



THP1		
Sample	Median intensity value of CD11b(±SE)	P-value
Control	6.23 (±0.06)	0.0007
Incubated with Microvesicle	6.64 (±0.03)	0.0001
	Median intensity value of CD33(±SE)	P-value
Control	29.9 (±0.76)	0.0001
Incubated with Microvesicle	34.23 (±0.82)	0.0001

Figure 4.6. <u>MVs contain lineage specificity</u>. (A). *In vitro megakaryocyte culture from bone marrow and cord blood-derived CD34$^+$ cells were characterized before and after incubation with Meg-MVs with anti CD61 and anti CD42b antibodies.* ***(B).*** *Flow cytometric analysis of Thp1 cells with uninduced control and MVs treatment indicating the expression of CD33 and CD11b. Median intensity value in of CD11b and CD33 in tabulated form (Down Side). Experiments were repeated for three times (n=3).*

expression of Notch1 effector genes Hey1 and Hes1 were also measured by qPCR assuring the activation of Notch1 and its downstream effector molecules during MKs differentiation (Figure 4.7A, B & C). As we mentioned, promoter methylation plays a crucial role for activation/inactivation of genes, we checked the promoter methylation status by meth-specific PCR for Notch1 promoter. Our result indicated that after induction of megakaryopoiesis through Meg-MVs, Notch 1 promoter contains less methylation in induced recipient cells with respect to the control cells. Consequently, the existence of methylation in promoter region of the Notch1 was examined by using bisulfite modification of the DNA samples followed by the MS-PCR. The primer sequences which are designed for the unmethylated and methylated alleles of the Notch1 promoter region are described in the method section. As expected Meg-MVs treated cells showed an unmethylated Notch1 promoter region (Figure 4.7D & E) which confirmed the activation mechanism of Notch1 during megakaryopoiesis. This observation was confirmed by the sequencing results of the methylated product which showed 93% homology with promoter sequence of Notch1 in the UCSC genome browser while the unmethylated product in the reverse stand showed only 11% homology.

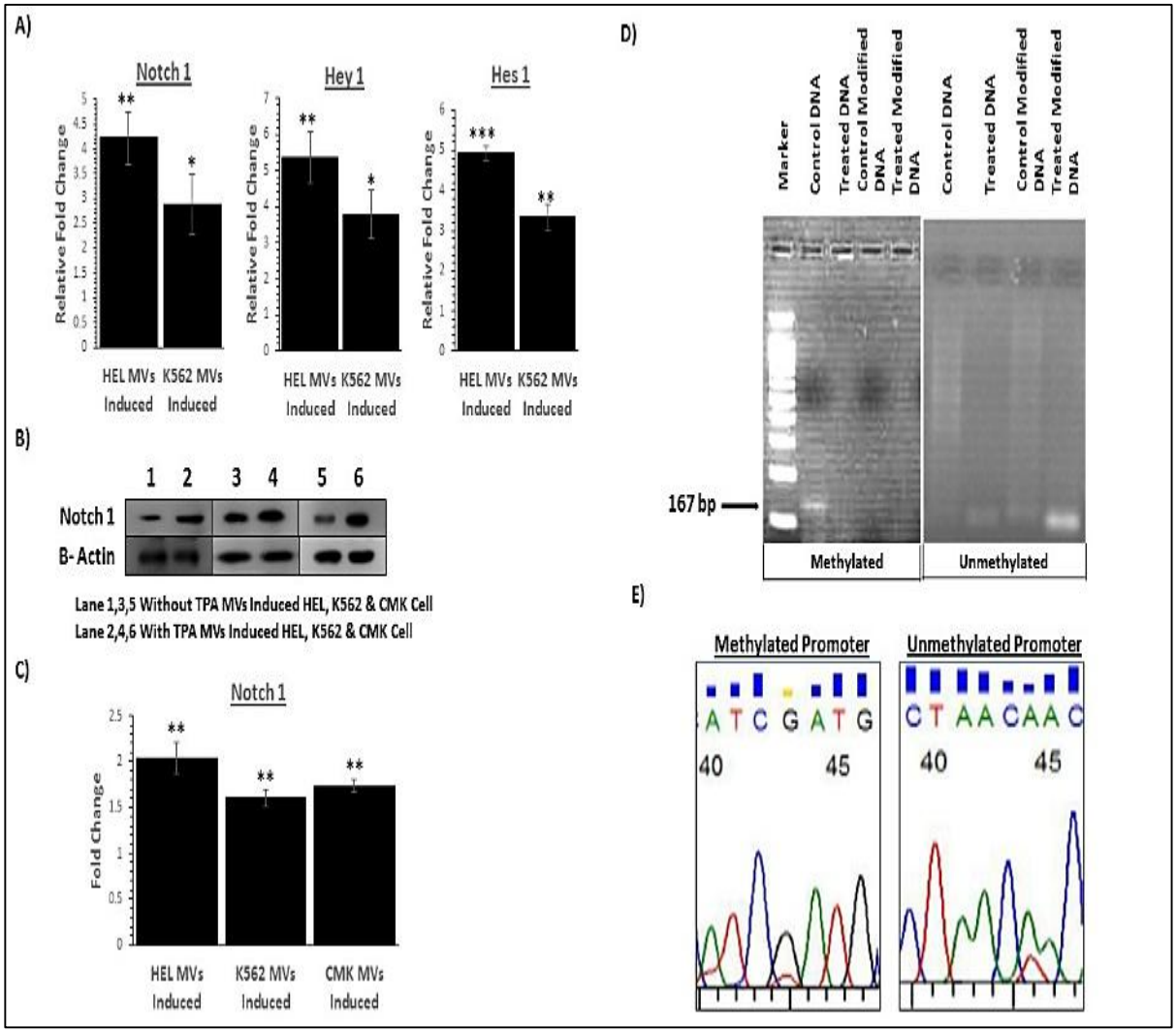


Figure 4.7. <u>Notch1 expression regulation</u>. (A). *Effect on Notch 1 and its downstream effectors. Relative gene expression for Notch 1, Hes 1 and Hey 1. Data normalized against HPRT. Experiments were repeated for three times (n=3).* ***(B).*** *Western Blot of HEL, K562 and CMK cells with and without TPA induced MVs for Notch1. Beta-actin act as a loading control. Experiments were repeated for three times (n=3).* ***(C).*** *Densitometric analysis of western blot for Notch1.* ***(D).*** *MSP analysis for Notch 1 promoter-specific PCR product* ***(E).*** *Sequence of the PCR product.*

4.6 Measurements of DNMTs in both NMeg-MVs and Meg-MVs induced cell lines:

In order to get the status of DNMTs expression upon incubating with both NMeg-MVs and Meg-MVs with progenitors, qPCR and western blotting for DNMTs were performed. Our results clearly showed that upon treatment with the Meg-MVs, the expression of DNMTs were down-regulated in recipient cells undergoing endomitosis (Figure 4.8A, B & C). Moreover, overexpression of DNMTs inhibited the activation of Notch1 as well as the megakaryocytic specific surface expression markers (Figure 4.9, Figure 4.10, Table 4.3, Table 4.4 & Figure 4.11A). To verify this inhibition of induction is not due to the cellular death, apoptosis assays were carried out (Figure 4.11B & Table 4.5). These observations indicated the involvement of Notch1 promoter`s hypomethylation through the downregulation of DNMTs during megakaryopoiesis.

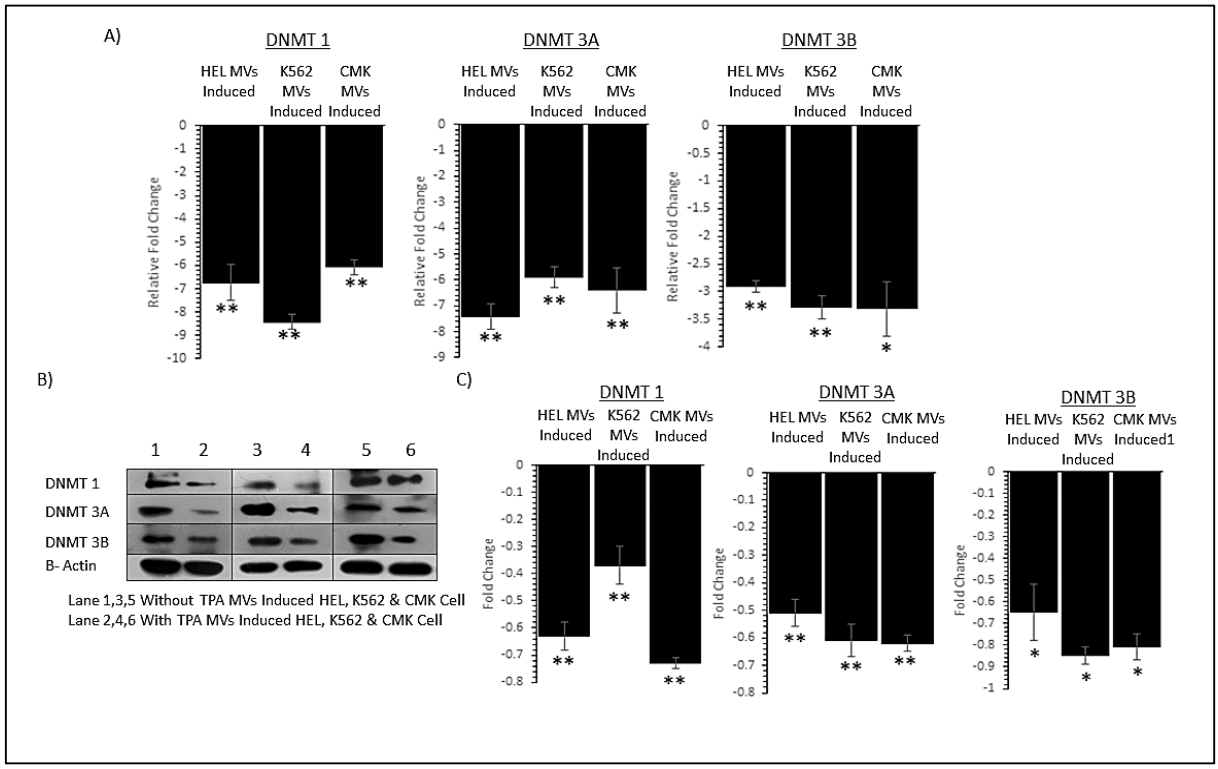


Figure 4.8. ***<u>DNMTs expression during megakaryopoiesis.</u>*** ***(A).*** *mRNA level measurement for DNMTs. Data normalized against HPRT. Experiments were repeated for three times (n=3).* ***(B).*** *Western Blot of HEL, K562 and CMK cells with and without TPA induced MVs for DNMT1, DNMT3A & DNMT3B. Beta-actin act as loading control. Experiments were repeated for three times (n=3).* ***(C).*** *Densitometric analysis of western blot for DNMTs.*

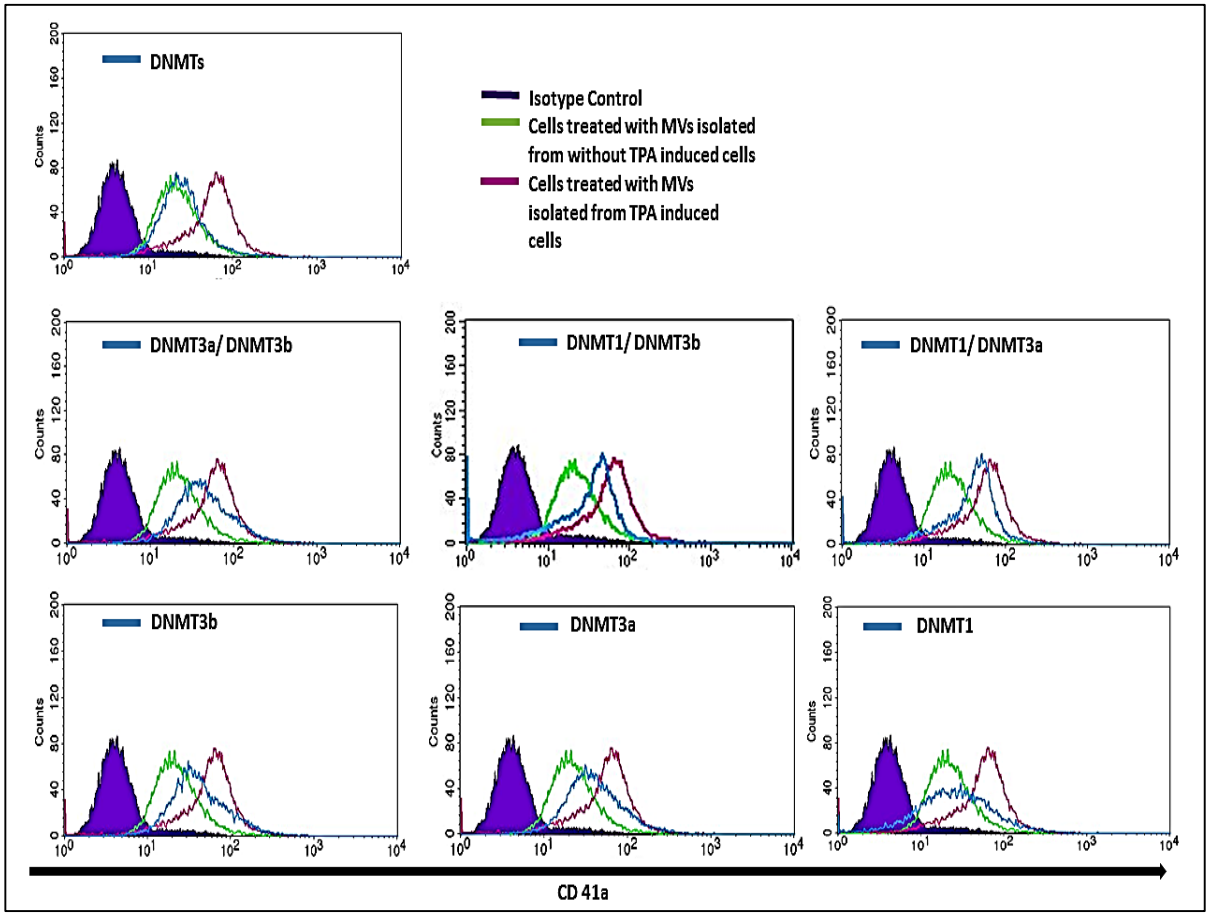


Figure 4.9. ***<u>Flow cytometric analysis of CD41a in HEL cells by overexpressing DNMT1, DNMT3A & DNMT3B</u>.*** *Median value in Table 4.3.*

Table 4.3. ***Calculation of Median Intensity Value (MIV) of CD41a***.

HEL Cells	
Sample	MIV CD41a (± S.D.)
Cells treated with MVs purified from uninduced cells	23.89 ± 1.68
Cells treated with MVs purified from induced cells	50.11 ± 4.87
Cells treated with MVs purified from induced cells + Transfected with three DNMTs	27.33 ± 1.40
Cells treated with MVs purified from induced cells + Transfected with DNMT1 & DNMT 3a	31.36 ± 2.82
Cells treated with MVs purified from induced cells + Transfected with DNMT1 & DNMT 3b	30.93 ± 1.83
Cells treated with MVs purified from induced cells + Transfected with DNMT3a & DNMT 3b	38.33 ± 2.40
Cells treated with MVs purified from induced cells + Transfected with DNMT1	37.84 ± 3.56
Cells treated with MVs purified from induced cells + Transfected with DNMT3a	35.10 ± 2.46
Cells treated with MVs purified from induced cells + Transfected with DNMT3b	36.42 ± 1.55

4.7 miRNAs involvement in DNMT3A and DNMT3B regulation:

In order to investigate the reason behind the down-regulation of DNMTs, we browsed many databases and in miRbase Target Scan we found that miR 99a and miR 125b can possibly target

DNMT 3B and DNMT 3A respectively (Figure 4.11C). To examine the effect of those miRNA on megakaryocytic differentiation, we, therefore, transfected anti-miRNAs into K562 cells. Fluorescence-activated cell sorting (FACS) analysis confirmed the decrease of the major surface markers of megakaryopoiesis (CD41a & CD42b) against the anti-miR 125b, confirming the role of miRNA 125b in megakaryopoiesis. However, for anti-miR 99a, the surface markers expression remained almost the same (Figure 4.12, Figure 4.13A, Table 4.6 & Table 4.7). To further verify this inhibition of proliferation was not due to cell death, the percentage of the healthy population was checked for apoptosis assay (Figure 4.13B & Table 4.8).

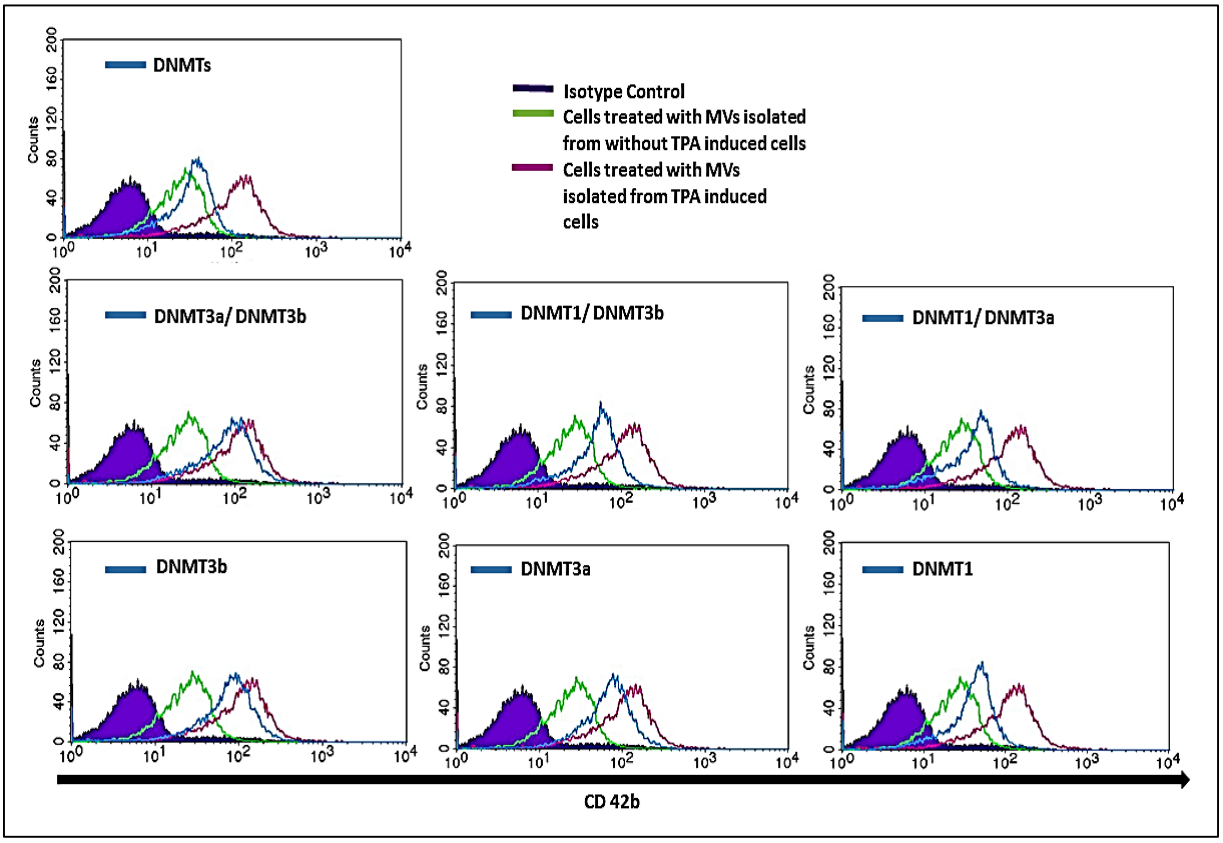


Figure 4.10. <u>Flow cytometric analysis of CD42b in K562 cells by overexpressing DNMT1, DNMT3A & DNMT3B</u>. *Median value in Table 4.4.*

Table 4.4. ***Calculation of Median Intensity Value (MIV) of CD42b.***

K562 Cells	
Sample	**MIV for CD42b (± S.D.)**
Cells treated with MVs purified from uninduced cells	26.49 ± 2.52
Cells treated with MVs purified from induced cells	93.37 ± 2.93
Cells treated with MVs purified from induced cells + Transfected with three DNMTs	36 ± 3.74
Cells treated with MVs purified from induced cells + Transfected with DNMT1 & DNMT 3a	41.64 ± 1.51
Cells treated with MVs purified from induced cells + Transfected with DNMT1 & DNMT 3b	39.87 ± 4.35
Cells treated with MVs purified from induced cells + Transfected with DNMT3a & DNMT 3b	54.9 ± 4.67
Cells treated with MVs purified from induced cells + Transfected with DNMT1	59.33 ± 3.60
Cells treated with MVs purified from induced cells + Transfected with DNMT3a	70.07 ± 1.41
Cells treated with MVs purified from induced cells + Transfected with DNMT3b	65.33 ± 1.99

4.8 Involvement of CXCR4 in megakaryocytic lineage induction:

As, CXCR4 has been suggested to be involved in the megakaryopoiesis, we checked its presence in MVs shedded from K562 and HEL cells (Figure 4.14A). Presence of the phosphorylated moesin which also plays a significant role in CXCR4 activation was detected in lysate of the MVs.

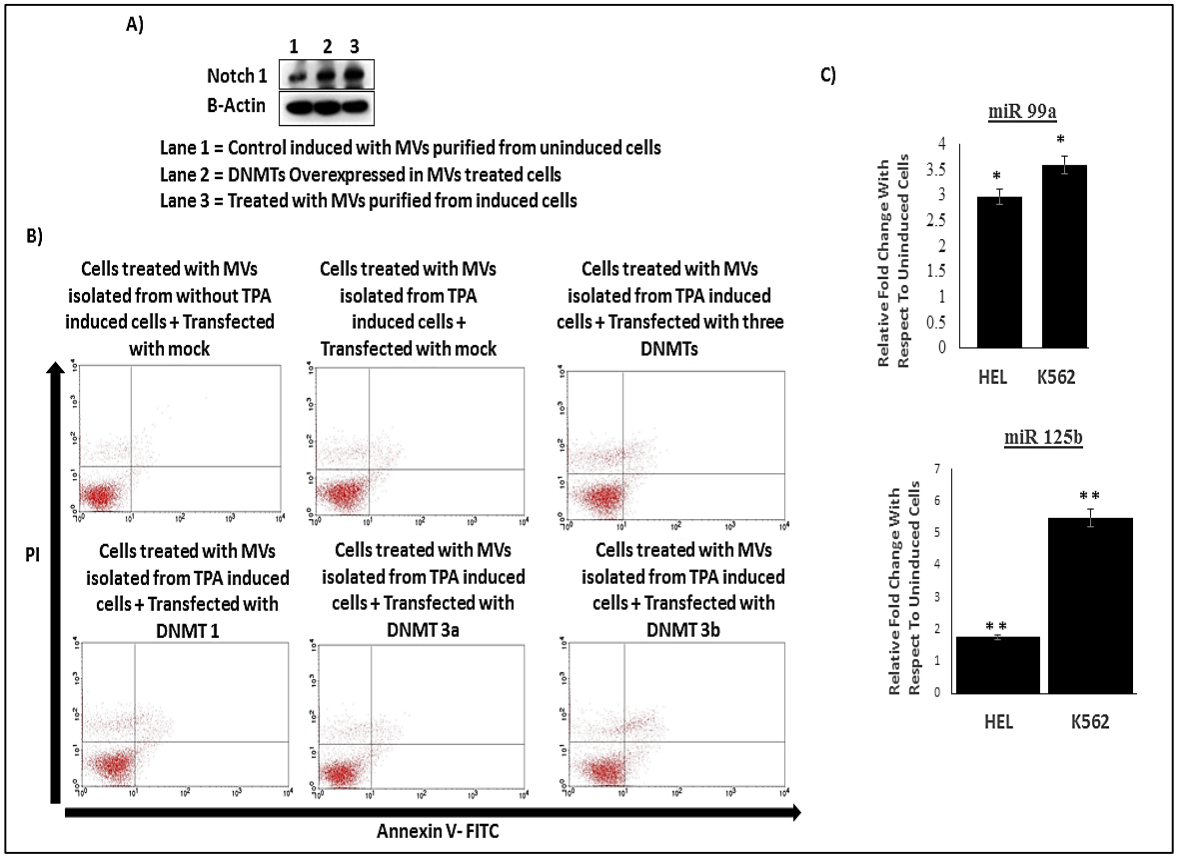


Figure 4.11. <u>Expression of miRNA99a and miRNA125b.</u> (A). *Western blot of whole cell lysates of HEL cells.* ***(B).*** *Measurement of Apoptotic Marker. Plots are divided into 4 quadrants. LL (Lower Left) shows healthy population, LR (Lower Right) presents lower apoptosis induced population, UR (Upper Right) shows higher apoptosis induced population and UL (Upper Left) indicates necrotic population. Viable population percentage in Table 4.5* ***(C).*** *Relative fold change of miR99a and miR125b level. Data represent the mean ± s.d. of three independent experiments (*p<0.05, n=3) normalised against GAPDH.*

Table 4.5. ***Calculation of viable population in transfected cell***.

K562 Cells	
Samples	**Percentage of viable population (%)**
Cells treated with MVs isolated from without TPA induced cells + Transfected with mock	74.32
Cells treated with MVs isolated from TPA induced cells + Transfected with mock	77.13
Cells treated with MVs isolated from TPA induced cells + Transfected with three DNMTs	68.40
Cells treated with MVs isolated from TPA induced cells + Transfected with DNMT 1	69.12
Cells treated with MVs isolated from TPA induced cells + Transfected with DNMT 3a	72.72
Cells treated with MVs isolated from TPA induced cells + Transfected with DNMT 3b	67.87

For further confirmation we induced HEL and CMK cells with the Meg- MVs in the presence of AMD3100, the well-known CXCR4 inhibitor and checked CD42b marker expression. Our data revealed in the presence of inhibitor, Meg-MVs were unable to induce megakaryocytic differentiation in both the cells (Figure 4.14B).

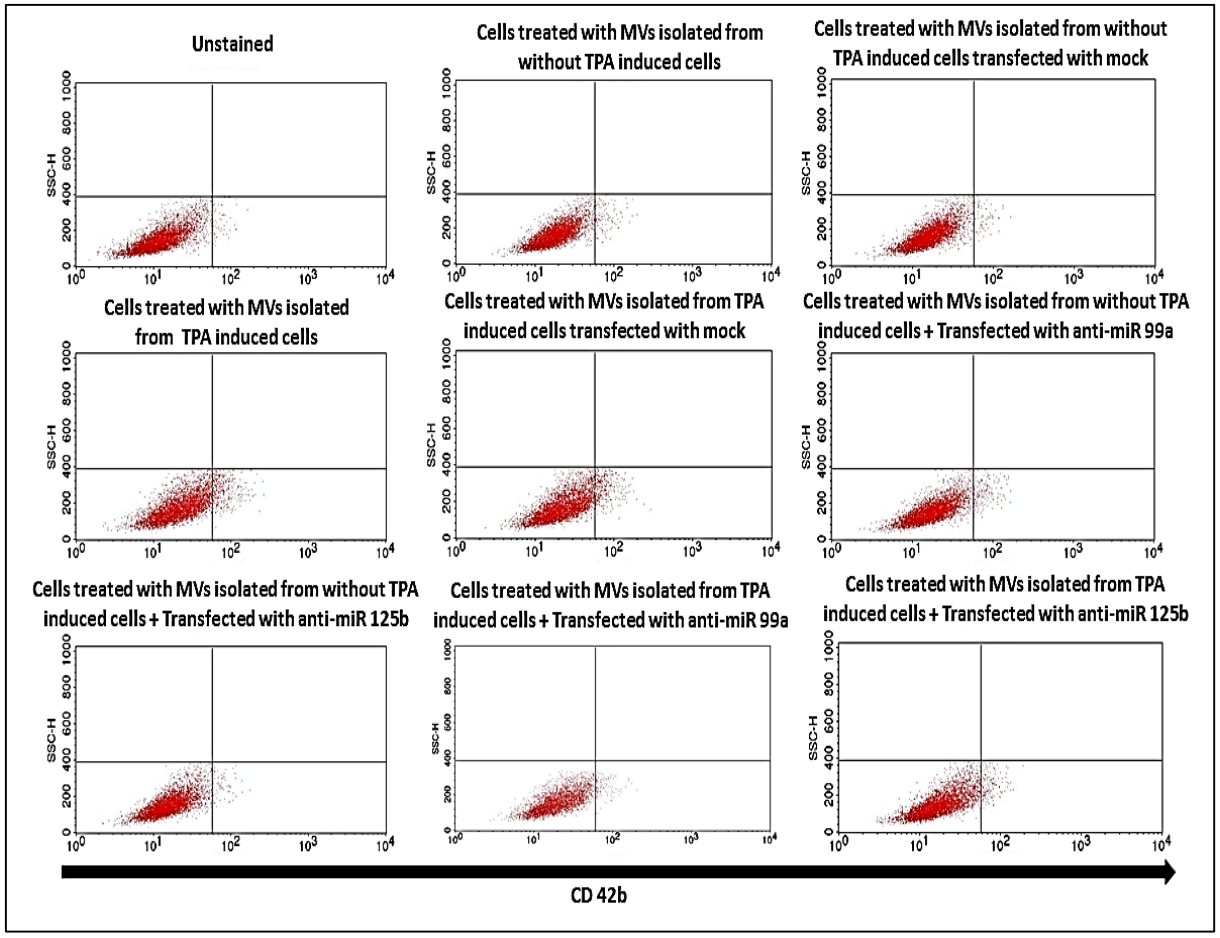


Figure 4.12. <u>Surface expression markers CD42b was checked against anti-miR 99a and miR 125b in K562 cells</u>. *Percentage of mean population of CD42b in Table 4.6.*

Table 4.6. ***Percentage of mean population of CD42b.***

K562 Cells	
Samples	**Percentage of Mean Population of CD42b (± S.D.)**
Unstained	13.48
Cells treated with MVs purified from without TPA induced cells	18.53 ± 0.56
Cells treated with MVs purified from TPA induced cells	23.79 ± 3.83
Cells treated with MVs purified from TPA induced cells + Transfected with mock	22.70 ± 1.20
Cells treated with MVs purified from TPA induced cells + Transfected negative control of si-RNA	23.41 ± 3.11
Cells treated with MVs purified from without TPA induced cells + Transfected with mock	17.01 ± 1.45
Cells treated with MVs purified from without TPA induced cells + Transfected with negative control of si-RNA	17.81 ± 0.89
Cells treated with MVs purified from without TPA induced cells + Transfected with anti-miR 99a	18.70 ± 3.54
Cells treated with MVs purified from TPA induced cells + Transfected with anti-miR 99a	22.05 ± 3.61
Cells treated with MVs purified from without TPA induced cells + Transfected with anti-miR 125b	16.52 ± 2.78
Cells treated with MVs purified from TPA induced cells + Transfected with anti-miR 125b	18.34 ± 2.47

We then checked downstream regulatory proteins in the HEL cells that were incubated with the Meg-MVs. Result showed the increased phosphorylation of MEK, pERK1/2 and ERK1/2 (Figure 4.14C & 4.14D). Additionally, we measured the expression of γH2A.X. However, there was no change in phosphorylation level of AKT which is the downstream regulatory protein of CXCR4 (Figure 4.14E). Our data thus suggest in recipient cells CXCR4 receptor protein induces megakaryocytic lineage through the activation of ERK1/2 pathway proteins.

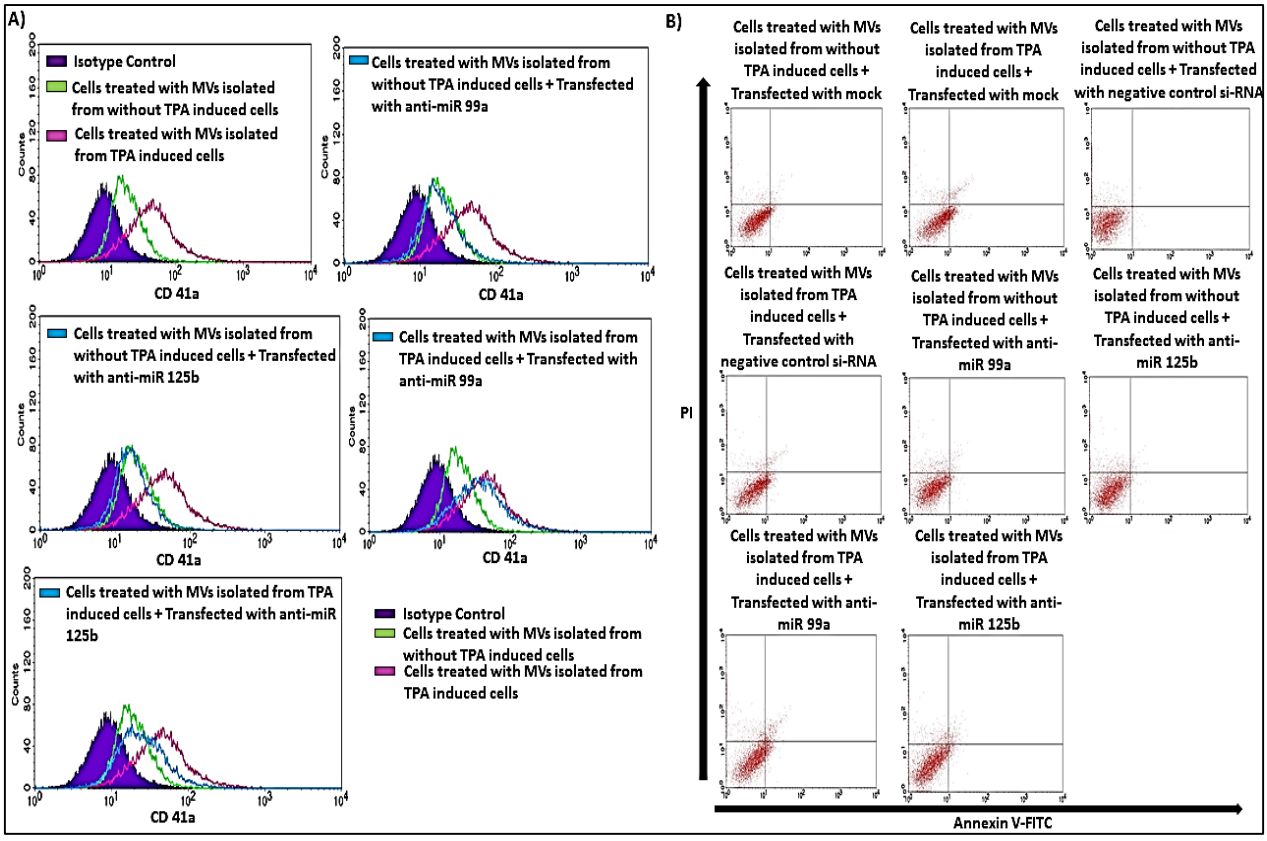


Figure 4.13. <u>Analysis of surface expression markers and apoptosis.</u> (A). *Expression of CD 41a was checked against anti-miR 99a and miR 125b in K562 cells. Median value in Table 4.7.* ***(B).***

Measurement of Apoptotic Marker Against anti-miR 99a & 125b. Plots are divided into 4 quadrants. LL (Lower Left) shows healthy population, LR (Lower Right) presents lower apoptosis induced population, UR (Upper Right) shows higher apoptosis induced population and UL (Upper Left) indicates necrotic population. Percentage of viable population in Table 4.8.

Table 4.7. *Median intensity value for CD41a*.

K562 Cells	
Sample	**Median Intensity Value for CD41a (± S.D.)**
Cells treated with MVs purified from without TPA induced cells	8.73 ± 0.56
Cells treated with MVs purified from TPA induced cells	31.25 ± 3.83
Cells treated with MVs purified from TPA induced cells + Transfected with mock	32.26 ± 1.20
Cells treated with MVs purified from TPA induced cells + Transfected negative control of si-RNA	30.74 ± 3.11
Cells treated with MVs purified from without TPA induced cells + Transfected with mock	10.16 ± 1.45
Cells treated with MVs purified from without TPA induced cells + Transfected with negative control of si-RNA	8.5 ± 0.89
Cells treated with MVs purified from without TPA induced cells + Transfected with anti-miR 99a	10.38 ± 3.54
Cells treated with MVs purified from TPA induced cells + Transfected with anti-miR 99a	29.63 ± 3.61
Cells treated with MVs purified from without TPA induced cells + Transfected with anti-miR 125b	9.82 ± 2.78
Cells treated with MVs purified from TPA induced cells + Transfected with anti-miR 125b	19.90 ± 2.47

Table 4.8. ***Calculation of viable population in transfected cell***.

K562 Cells	
Sample	**Percentage of viable population (%) (± S.D.)**
Cells treated with MVs isolated from without TPA induced cells + Transfected with mock	77.34 ± 1.03
Cells treated with MVs isolated from TPA induced cells + Transfected with mock	67.48 ± 2.34
Cells treated with MVs isolated from without TPA induced cells + Transfected with negative control si-RNA	75.26 ± 0.65
Cells treated with MVs isolated from TPA induced cells + Transfected with negative control si-RNA	66.95 ± 1.68
Cells treated with MVs isolated from without TPA induced cells + Transfected with anti-miR 99a	77.58 ± 0.80
Cells treated with MVs isolated from without TPA induced cells + Transfected with anti-miR 125b	82.25 ± 2.53
Cells treated with MVs isolated from TPA induced cells + Transfected with anti-miR 99a	65.61 ± 1.93
Cells treated with MVs isolated from TPA induced cells + Transfected with anti-miR 125b	74.35 ± 2.71

4.9 Change in chromatin condensation during late megakaryopoiesis:

As the down-regulation of DNMTs is associated with the remodelling of chromatin organisation, TPA induced HEL and K562 cells, showing megakaryopoiesis specific characteristics, were sorted according to their DNA content in order to study the dynamics of chromatin compactness during megakaryopoiesis. Cells, containing ≤ 4N DNA, were assigned as a lower ploidy (LP) while cells containing ≥ 8N DNA were referred as higher ploidy (HP) (Figure 4.15A, 4.15B & 4.15C).

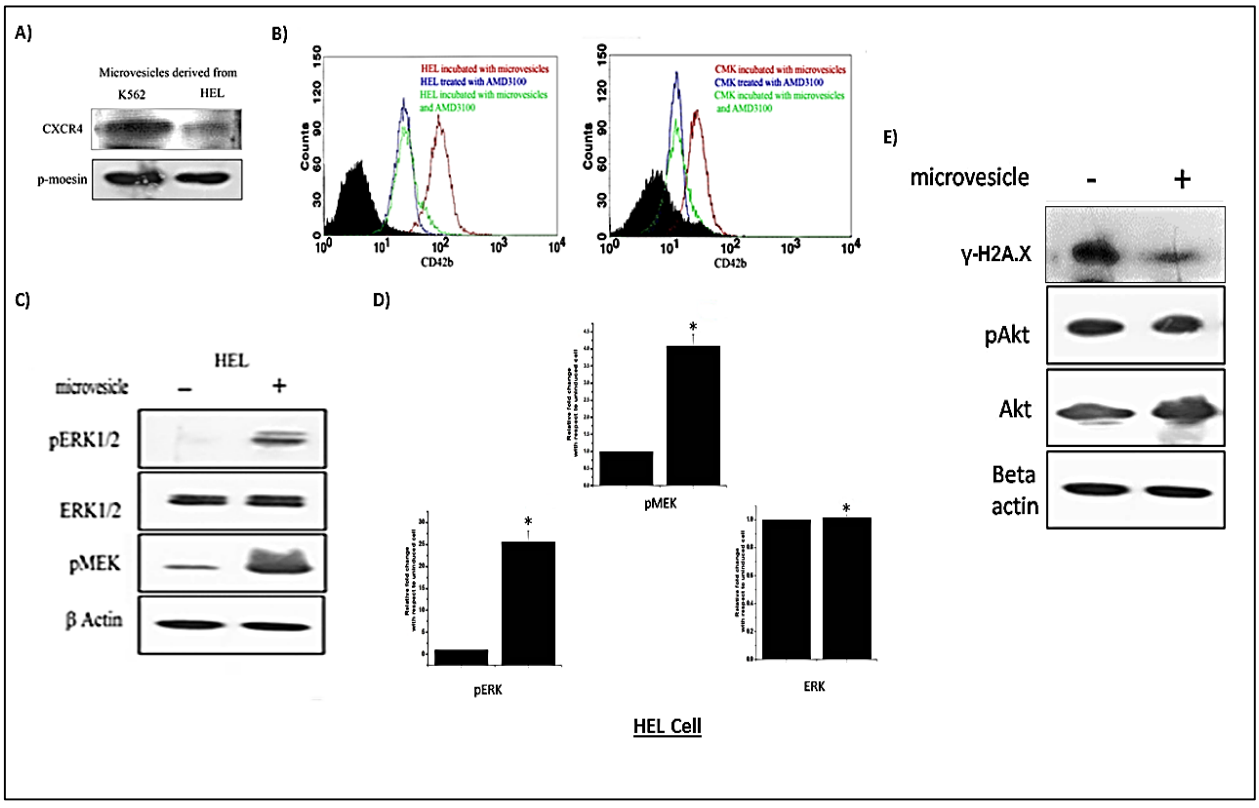
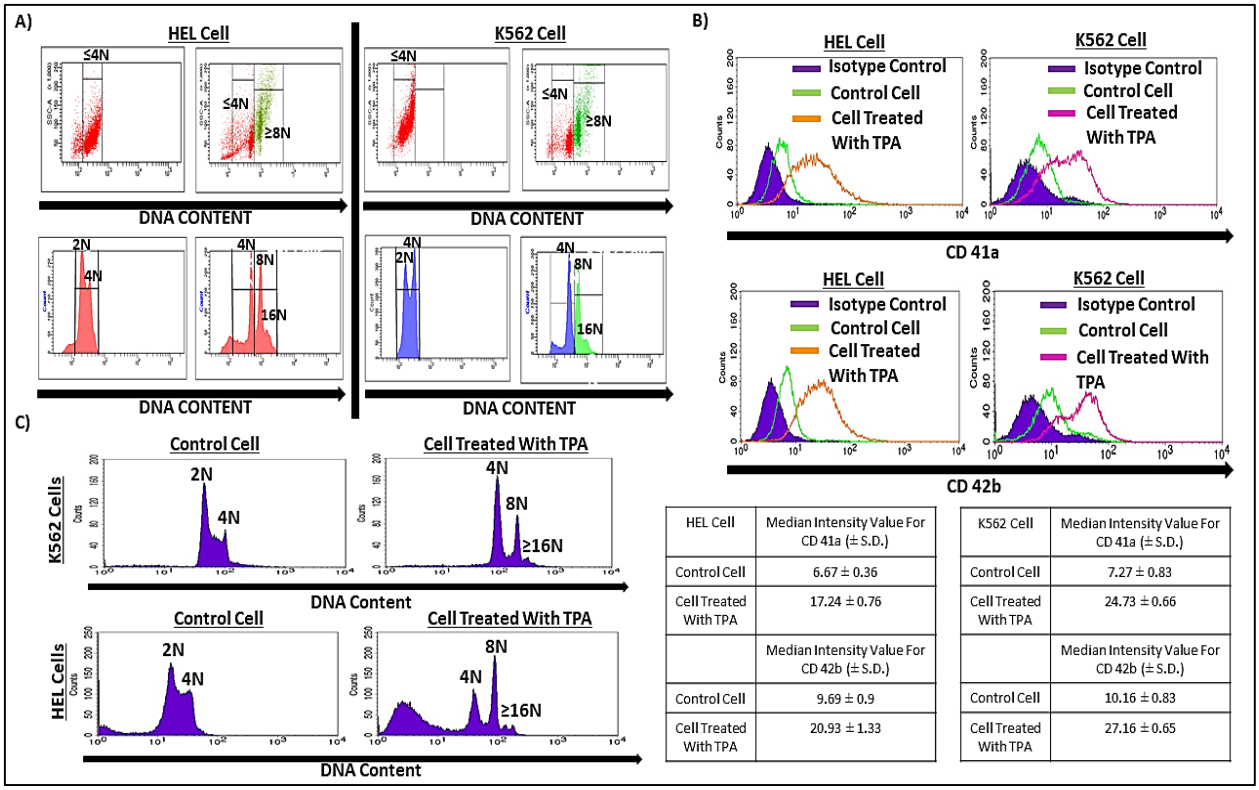


Figure 4.14. Role of CXCR4 in megakaryocytic induction. (A). *Detection of CXCR4 and phospho-moesin in megakaryocyte derived MV lysate by western blot.* ***(B).*** *Flow cytometric analysis of the expression of CD42b incubated with MVs only, AMD3100 only and both MVs and*

AMD3100 in HEL and CMK cell lines. ***(C).*** *Western blot of whole cell lysates of HEL cells before and after incubation with MVs.* ***(D).*** *Densitometric analysis of phospho-MEK, phospho-ERK, ERK protein expression in uninduced and MMVs induced HEL cell line .****(E).*** *Western blot of whole cell lysates of HEL cells before and after incubation with MVs. Beta-actin used as a loading control. shows the mean ± s.e.m of three individual experiments ($p<0.05$ for HEL).*

After sorting, cells were immediately assessed for nucleosome occupancy in the chromatin by partial MNase digestion. When compared to control cell, polyploid cells showed smaller DNA fragments, indicating that linker regions were more accessible to MNase and the inter band distance correspondence to a population average spacing between adjacent nucleosomes suggesting an alteration in chromatin architecture (Figure 4.16A & 4.16B). Furthermore, we checked the expression of $H3K27me^3$, a signature of chromatin condensation, in both K562 and HEL cells and observed a decrease in expression in HP population which indicate the activation of genes during megakaryopoiesis (Figure 4.16C). Moreover, the stained chromatin of HP population almost filled up maximum spaces of the nuclei supporting the MNase digestion data (Figure 4.16D). Additionally, we checked the expression of nucleosomal depletion mark, H2A.Z which showed a significant upregulation (Figure 4.17A), further confirming decondensation state of chromatin during megakaryopoiesis.



HEL Cell	Median Intensity Value For CD 41a (± S.D.)
Control Cell	6.67 ± 0.36
Cell Treated With TPA	17.24 ± 0.76
	Median Intensity Value For CD 42b (± S.D.)
Control Cell	9.69 ± 0.9
Cell Treated With TPA	20.93 ± 1.33

K562 Cell	Median Intensity Value For CD 41a (± S.D.)
Control Cell	7.27 ± 0.83
Cell Treated With TPA	24.73 ± 0.66
	Median Intensity Value For CD 42b (± S.D.)
Control Cell	10.16 ± 0.83
Cell Treated With TPA	27.16 ± 0.65

Figure 4.15. <u>Analysis of MKs specific surface expression markers and DNA content</u>. (A). Cell Sorting*. Both HEL and K562 cells subjected to TPA for 6 days and then stained with vibrant orange and sorted under flow cytometer. The left panels show uninduced HEL and K562 cell which only contain 2N-4N DNA while the right panels content TPA induced cells containing >8N DNA. The right panels were then sorted.* ***(B).*** *After TPA induction both HEL and K562 cells were checked for megakaryocyte specific surface expression markers. Median value in tabulated form.* ***(C).*** *The same were used for DNA content analysis.*

4.10 <u>Differential expression of MCM complex and histones (MCM 2, MCM 4 and MCM 6) during megakaryopoiesis</u>:

Our earlier study has shown that during megakaryopoiesis MCM 7 is down-regulated along with an up-regulation in the miRNA cluster 106b-25. In order to, find out the reason behind the

chromatin decompactness during megakaryopoiesis, sorted population were then examined for the expression of MCM 2, MCM 4 and MCM 6 and it was observed that during megakaryopoiesis MCM proteins were down-regulated in HP (Figure 4.17B, 4.17C & 4.17D). Both transcriptional and translational data showed similar phenomenon. However, in contrast to the chromatin decondensation total histone pools were observed to be up-regulated in the HP population indicating that the observed process is not dependent on the histone pools (Figure 4.17E).

4.11 Dysregulated expression profiles of Long Non Coding RNA during megakaryopoiesis:

Several studies reported about the involvement of long non-coding RNAs (lncRNAs) in many biological process such as integrity of the nuclear and cytoplasmic structure, cellular differentiation-proliferation and mRNA processing. Recently, involvement of long non-coding RNAs (lncRNAs) in the regulation of chromatin arrangement has been explicated. It can influence the gene expression by tuning the chromatin compactness via the regulation of nucleosome positioning. For instance, human SChLAP1 lncRNA interacts with the component of SWI/SNF complex and effect the gene expression profiling widespread in many cancer cells [61,62,260–262]. To study the involvement of lncRNAs during megakaryopoiesis, comparative studies between LP and HP were carried out. Out of the collection of 196,501 transcripts, the expressions of 33 non-coding transcripts with a minimal length 200bp were found to be significantly altered (coding potential ≤ 0.364) (Figure 4.18A). The hierarchical clustering analysis in scattered and volcano plots showed distinct expression signature (Figure 4.18B & 4.18C). Several studies have reported that certain lncRNAs are responsible for the alteration of chromatin compactness. However, none of them were among the 33 transcripts observed in megakaryopoiesis. On the contrary, we observed alteration in expression of 4 lncRNAs (Highlighted with red star) involving in ribosome biogenesis that were significantly downregulated. As mentioned earlier, these

lncRNAs production are controlled by nucleolus. Based on this report we then investigated whether the dysregulation in the expression of these lncRNAs were governed by the activity of the nucleolus.

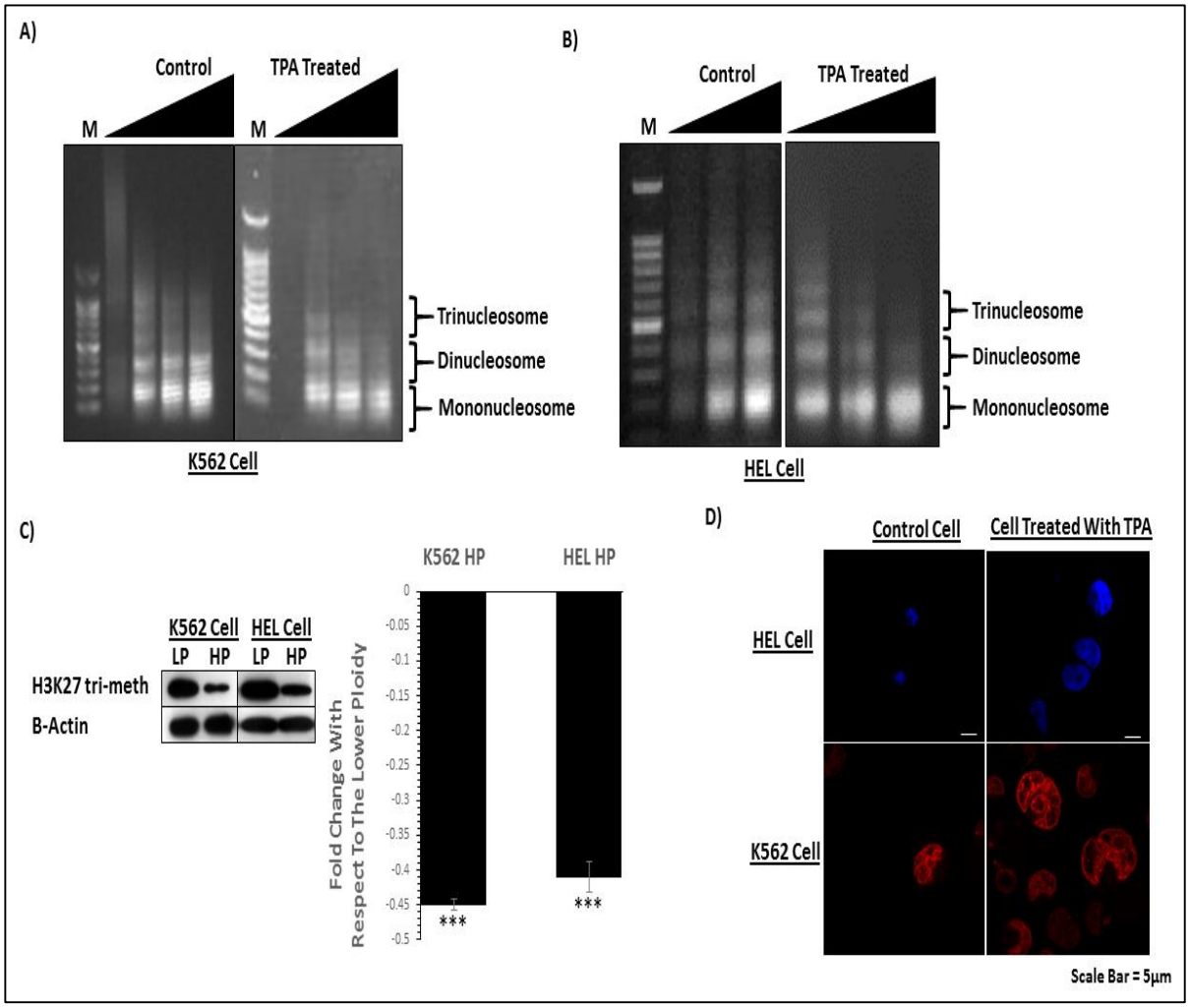


Figure 4.16. <u>Analysis of chromatin organisation</u>. (A). & (B). *Analysis of MNase assay in K562 and HEL Cells.* ***(C).*** *Western blot of whole cell lysate of HEL and K562 cells showing the expression of H3K27me^3 with densitometric analysis of the blot that shows the mean ± s.d. of 3 individual experiments.* ***(D).*** *Chromatin staining for measuring nucleus size and stainer intensity which indirectly correlates with chromatin condensation. Blue = DAPI Staining, Red = DRAQ5^{TM}.*

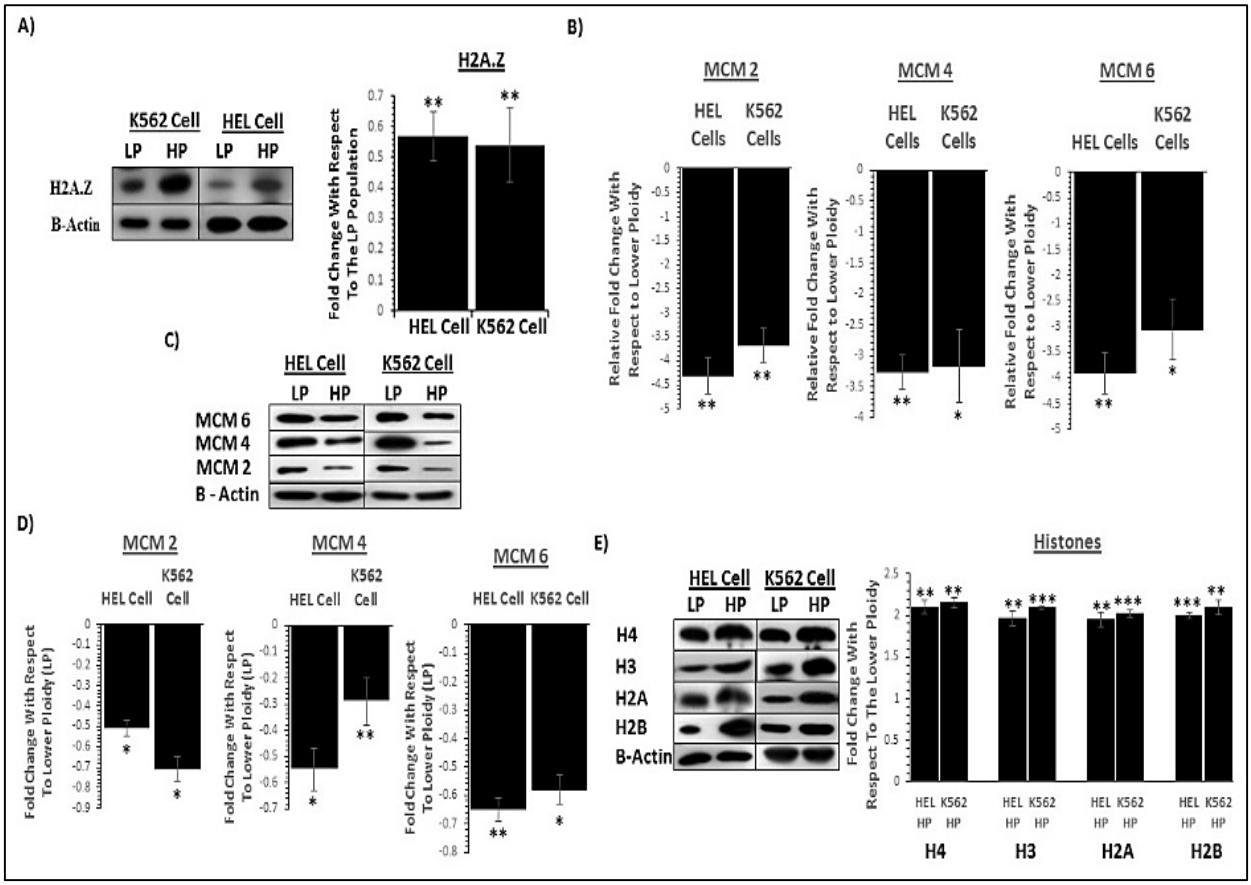


Figure 4.17. <u>Measurement of MCM Complex</u>. (A). *Western blot of whole cell lysate of HEL and K562 cells showing the expression of H2A.Z with densitometric analysis of the blot that shows the mean ± s.d. of 3 individual experiments.* ***(B).*** *Relative gene expression of MCM 2, MCM 4 and MCM 6 normalized against HPRT1 as seen by qRT-PCR. Data represents the mean ± s.d. of 3 independent experiments (*P< 0.05, **P< 0.01, ***P< 0.001, n = 3)* ***(C).*** *Western blot of whole cell lysate of cells showing the expression of MCM 2, MCM 4 and MCM 6.* ***(D).*** *Densitometric analysis of the blot shows the mean ± s.d. of 3 individual experiments. Beta Actin was used as a loading control.* ***(E).*** *Western blot of whole cell lysate of cells with densitometric analysis of the blot showing the mean ± s.d. of 3 individual experiments. Beta Actin was used as a loading control.*

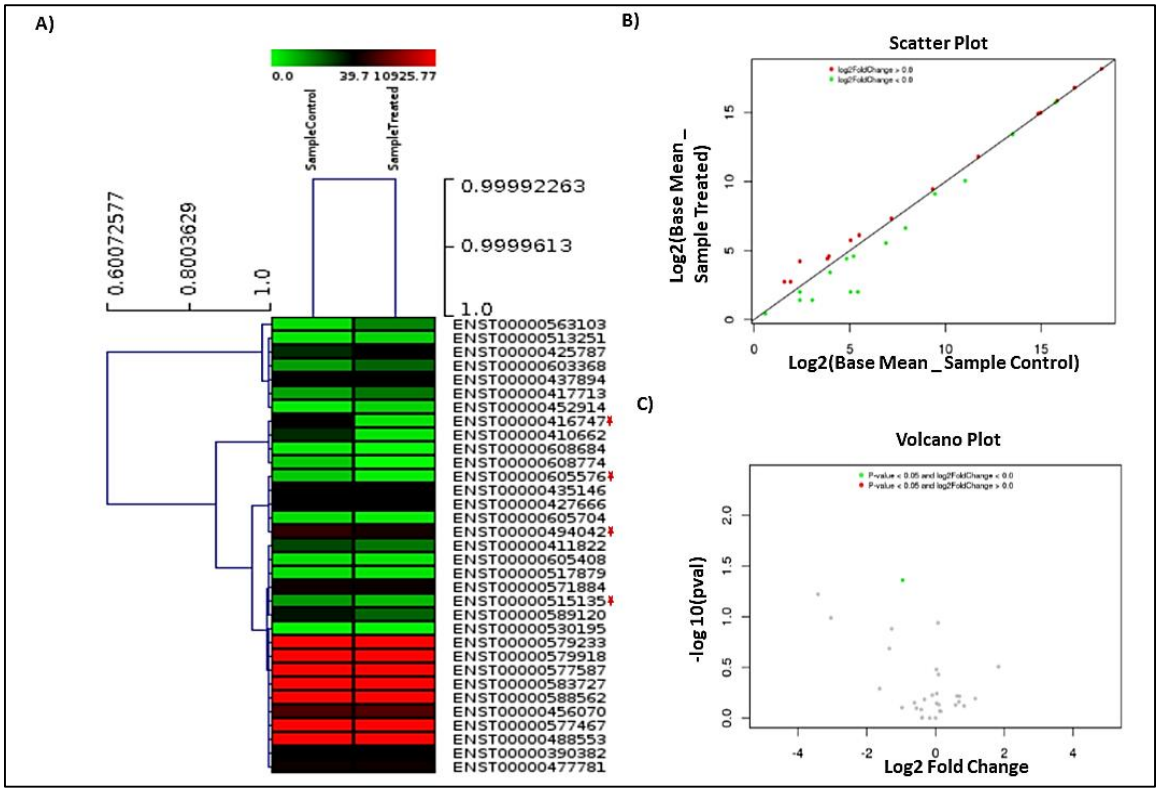


Figure 4.18. <u>Long Non-coding RNA profiling</u>. (A). *Long non-coding RNA profiling and subsequently* ***(B).*** *Scatter plot and* ***(C).*** *Volcano plot.*

4.12 <u>Change in nucleolus size and activity</u>:

As nucleolar activity directly depends on the size of the nucleolus, we next investigated if the nucleolar size changes in megakaryopoiesis. To address this issue, control and TPA induced population were stained with SytoSelect dye and DRAQ5 ™ dye specific for nucleolus and the chromatin, respectively. Surprisingly, we observed that control uninduced cells showed an enlarged nucleolus compared to TPA induced ones. To estimate nucleolus to nucleus ratio we considered them as spheres (Figure 4.19). Our observation were validated and compared with

actinomycin D treated populations (Figure 4.20A). To further verify the decrement in nucleolar size distribution in megakaryopoiesis, CD 34^+ cells isolated from CB were subjected to thrombopoietin (TPO) and then stained for MKs lineage specific surface markers (CD $41a^+$ /CD $42b^+$ / CD 61^+) (Figure 4.19). *In vitro* cultured primary polyploid MKs showed a significant decrease in the size of nucleolus with respect to the control uninduced CD 34^+ cells (Figure 4.19) in agreement to our previous observation on HEL and K562 cells. To get a quantitative aspect, we studied the mean fluorescence intensity (MFI) of the polyploid and control cells using flow cytometry.

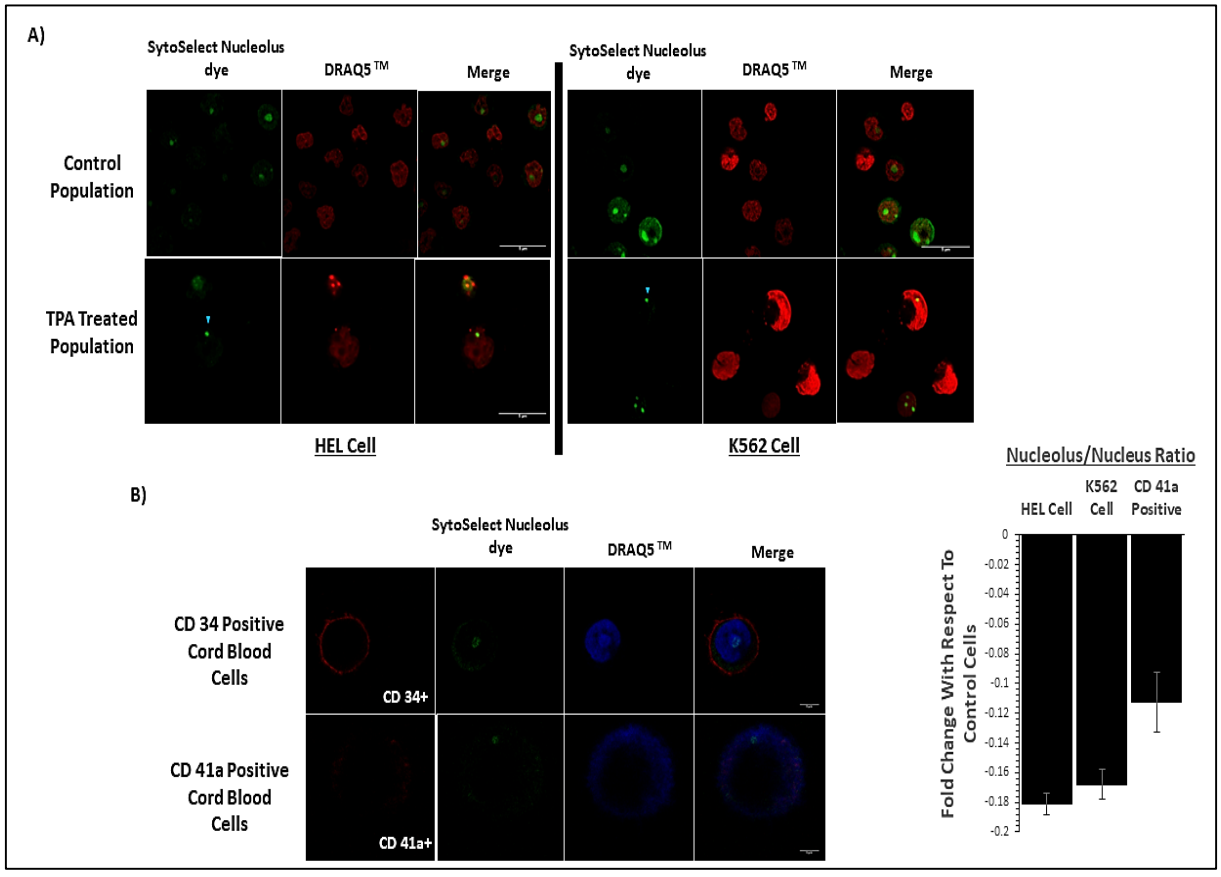


Figure 4.19. <u>Nucleolus size measurement in MKs</u>. *(A) First row of the panel is the control population for both HEL and K562 cells. TPA treated population is in the second row of the panel.*

(B) Nucleolar size measurement in in vitro cultured unilineage MKs from CD 34$^+$ cells in comparison with the megakaryocytic population. Bar diagram for nucleolus and Nucleus ratio.

When TPA induced cells were subjected to nucleolus and chromatin staining (with respect to ploidy) and assayed, they showed a lower increment in flourescent intensity compared to untreated control (Figure 4.20B and Table 4.9). The same was measured for actinomycin D treated population (Figure 4.20C and Table 4.10). In order to check the same phenomenon in primary polyploid MKs, cells were stained and analysed in a similar manner. CD 61$^+$ sorted population showed a decrement in the intensity with respect to the control uninduced CD 34$^+$cells (Figure 4.21A & 4.21B, Table 4.9). In fact Dot plot supported this observation (Figure 4.21C & Table 4.11). To further verify the activity of nucleolus, rRNA (28S rRNA & 18S rRNA) levels were examined and significant down-regulation was observed in induced MKs obtained from *in vitro* cultured CD 34$^+$cells and TPA induced K562 cells (Figure 4.21D). Interestingly, in order to, monitor protein synthesis during megakaryopoiesis, we performed SUnSET assay which demonstrated in a corroborative manner that in HP population with excess DNA content, protein synthesis rate decreased during megakaryopoiesis (Figure 4.22A and Table 4.12).

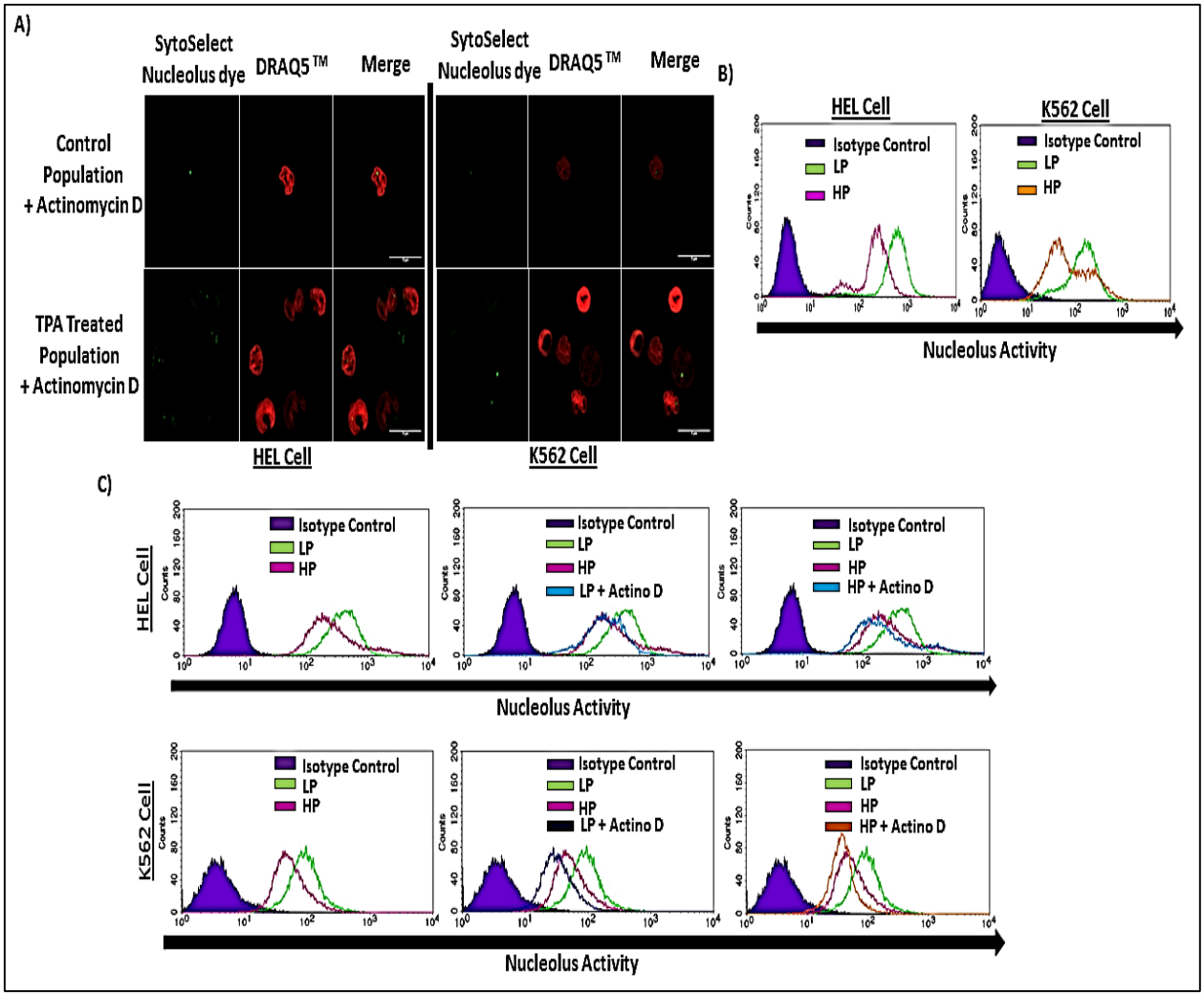


Figure 4.20. <u>Nucleolus activity measurement in MKs</u>. (A). *Controls incubated with Actinomycin D are showing in the first row of the panel whereas TPA treated population incubated with Actinomycin D are showing in the second row of the panel.* ***(B).*** *Histogram analysis for nucleolus activity measurement in both HEL and K562 cells (LP, HP). Median intensity value of nucleolar activity in Table 4.9.* ***(C).*** *Histogram analysis for nucleolus activity measurement in both HEL and K562 cells (LP, HP and Actinomycin D treated population of LP & HP). Actino D represents Actinomycin D. Median intensity value of nucleolar activity in Table 4.10.*

Table 4.9: ***Median intensity value of nucleolar activity***.

Cell Line	Ploidy	Median Intensity Value (± S.D.)
HEL Cell	LP	367.45 ± 2.53
	HP	245.16 ± 1.63
K562 Cell	LP	134.93 ± 1.30
	HP	40.94 ± 1.44
Cord Blood	CD 34^{+} Uninduced Control Cells	1910.95 ± 5.35
	CD 61^{+} Megakaryopoiesis Induced Cells	858.21 ± 8.50

Table 4.10: ***Median intensity value***.

Cell Line	Ploidy	Median Intensity value (± S.D.)
HEL	LP	319.64 ± 2.35
	HP	103.22 ± 1.89
	LP + Actinomycin D	105.37 ± 2.15
	HP + Actinomycin D	96.44 ± 2.13
K562	LP	93.9 ± 2.33
	HP	43.51 ± 0.77
	LP + Actinomycin D	26.9 ± 1.19
	HP + Actinomycin D	39.83 ± 1.23

As mentioned in the earlier section, it has been reported cancer cell lines show cancer stem cell characteristics through formation of polyploid giant cancer cells (PGCCs). MDA-MB-231 and MCF 7 breast cancer cells were incubated with cobalt chloride as reported and then checked for the size of nucleolus in PGCC like cells (Figure 4.22B). Interestingly, it was observed nucleolus`s size either increased or remained almost the same for the *in vitro* generated PGCC. Earlier reports have suggested that NOLC1, fibrillarin and nucleolin were important factors for regulating the activity and size of nucleolus. Therefore, expressions of these proteins were checked in MKs cells and it was observed that they were getting down-regulated in HP population with respect to the LP population (Figure 4.22C & 4.22D). In contrast to megakaryopoiesis, when the expression of NOLC 1 was examined in *in-vitro* generated PGCC cells, a clear increment in its expression was observed (Figure 4.23A). These intriguing observation led us to enquire whether megakaryocytic endomitosis process has any correlation with nucleolar size.

4.13 Involvement of miRNA 146b in megakaryopoiesis development via the regulation of NOLC 1 expression:

It is now well established that micro RNAs (miRNAs) play a pivotal role in megakaryopoiesis. To study the role by which megakaryocytic polyploidy controls the size variability and activity of nucleolus, the nucleolus size regulatory proteins were scanned in the several computational prediction programs such as TargetScan, miRGen and miRanda (mircorna.org) for putative internal short RNA i.e. miRNA binding sites. Several such were observed in those browsers. However, none of the putative targets were involved in megakaryopoiesis, except that of miRNA 146b. Therefore, we checked the expression of miRNA 146b in TPA induced cells and observed them to be up-regulated (Figure 4.23B). Curiously, ENSEMBL human genome browser also

predicts a target site for miRNA 146b to be in the NOLC1 promoter region. To elucidate the role of miRNA 146b in NOLC1 expression, inhibitor of miRNA 146b was used and it showed an increase in size of nucleolus (Figure 4.23C). The same were verified with flow cytometry (Figure 4.24A & 4.24B). This was further confirmed by a decrement in nucleolus activity, when mimic of miRNA 146b was transfected into the cells (Figure 4.25A). Furthermore, our western blot analysis confirmed the involvement of miRNA 146b in NOLC 1 expression`s regulation (Figure 4.25B).

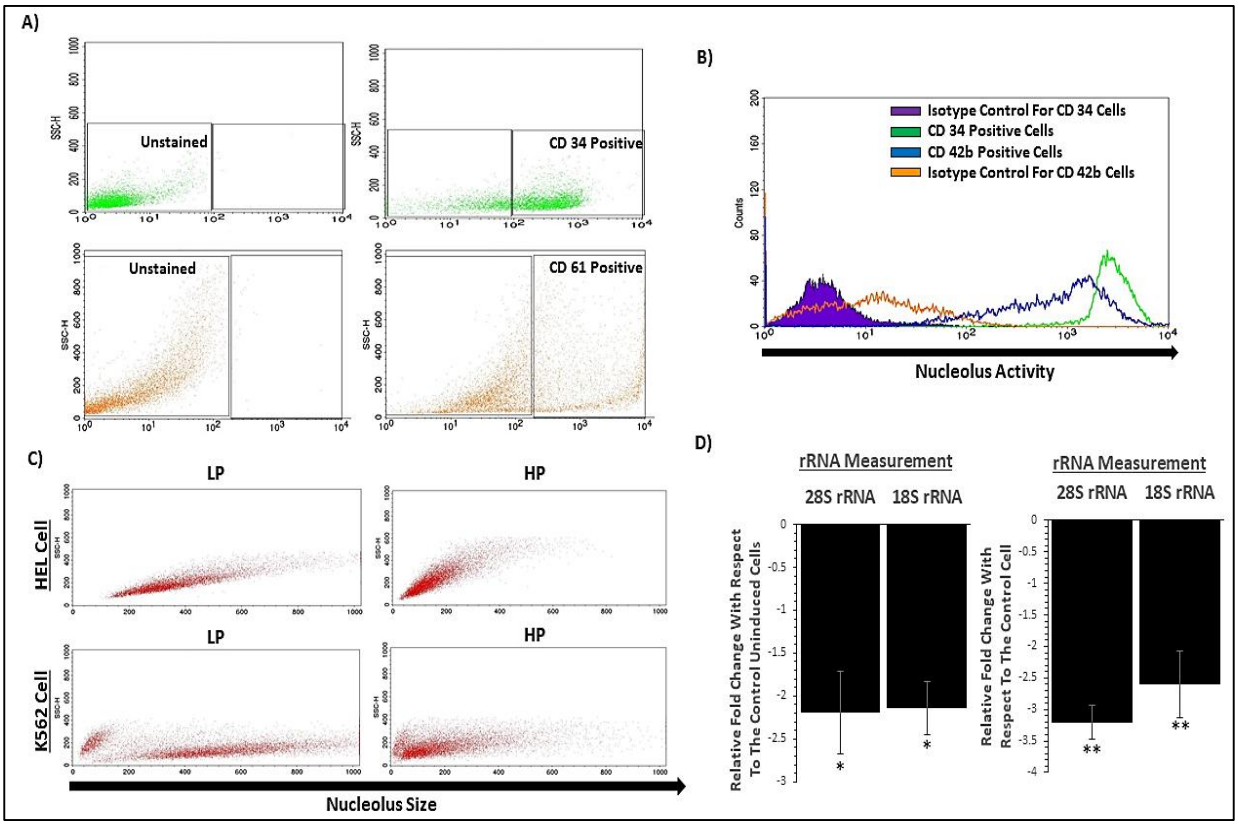


Figure 4.21. ***<u>Nucleolus activity measurement in in-vitro cultured primary MKs and rRNA expression in megakaryopoiesis</u>.*** ***(A).*** *Cell sorted for nucleolar size measurement in both CB derived CD 34⁺ cells and CD 61⁺ cells.* ***(B).*** *Histogram analysis of nucleolus activity in in vitro unilineage MKs culture from CD 34⁺ cells in comparison with the megakaryocytic population.*

Median intensity value of nucleolar activity in Table 4.9. ***(C).*** *Dot plot analysis of nucleolus size. Median intensity value of nucleolar activity in Table 4.10.* ***(D).*** *Relative gene expression of 28S rRNA and 18S rRNA normalized against HPRT1 as seen by qRT-PCR. Data represents the mean ± s.d. of 3 independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3) in CB derived megakaryocytic induced cells and TPA induced K562 cells.*

Table 4.11: ***Median intensity value of Dot Plot***.

Cell Line	**Ploidy**	**Median Intensity Value (± S.D.)**
HEL Cell	LP	1662.92 ± 10.61
	HP	491.59 ± 9.67
K562 Cell	LP	784.52 ± 8.56
	HP	350.25 ± 9.69

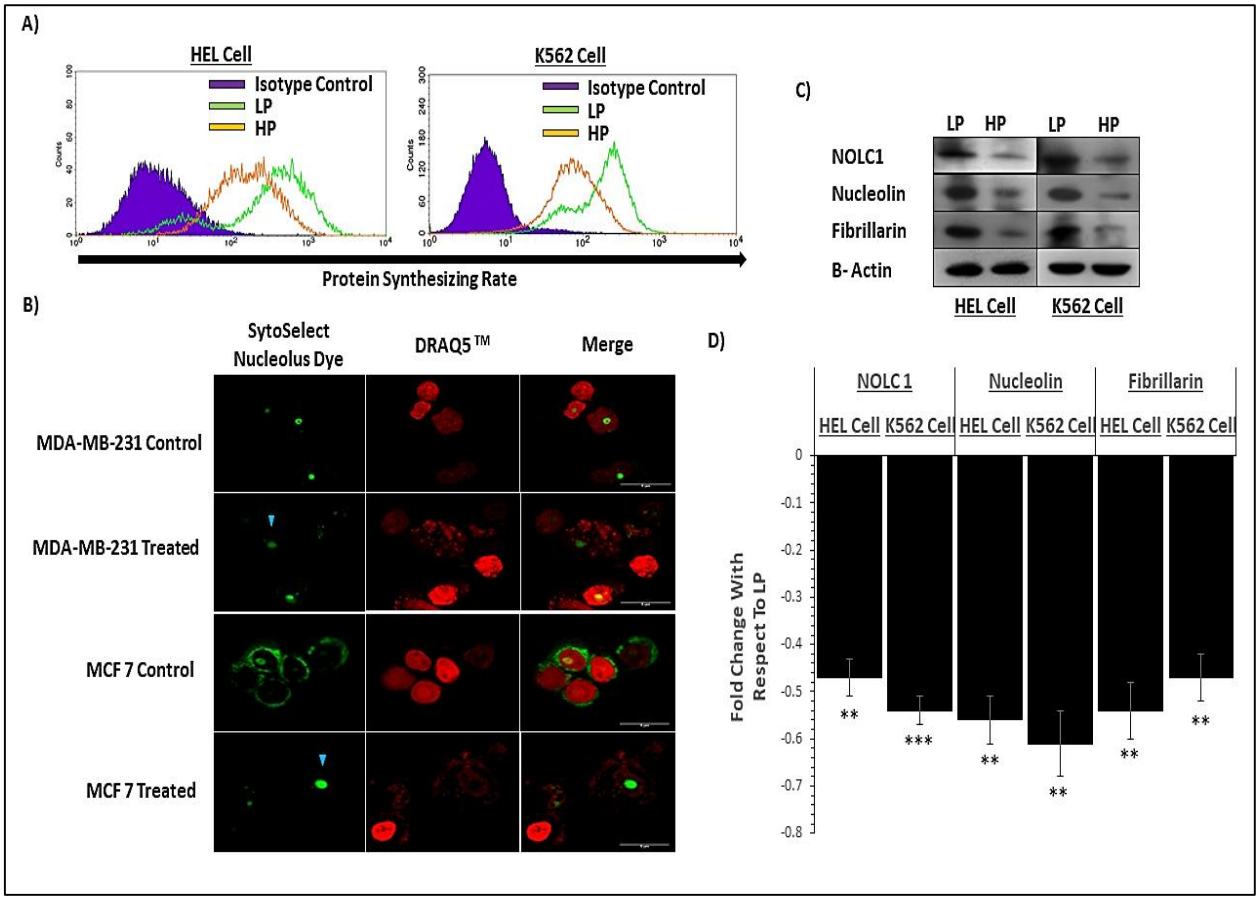


Figure 4.22. <u>Protein synthesizing rate in MKs and nucleolus size measurement in PGCCs</u>. (A). *Protein synthesizing rate measurement in polyploid MKs with respect to the lower polyploid cells. Median value in Table 4.11.* ***(B). Nucleolus size measurement****: MDA-MB-231 control cells (First Row of the upper panel). MDA-MB-231 incubated with cobalt chloride (Second Row of the upper panel). MCF 7 control cells (First Row of the lower panel). MCF 7 incubated with cobalt chloride (Second Row of the lower panel). All the imaging were done with scale bar 5µm.* ***(C).*** *&* ***(D).*** *Western Blot and densitometric analysis of different proteins in K562 and HEL cells. Beta Actin was used as a loading control.*

Table 4.12: ***Median intensity value of Protein synthesizing rate***.

Cells	Ploidy	Median Intensity ± S.D.
HEL Cells	LP	376.71 ± 7.43
	HP	56.31 ± 4.29
K562 Cells	LP	358.88 ± 5.35
	HP	53.28 ± 6.05

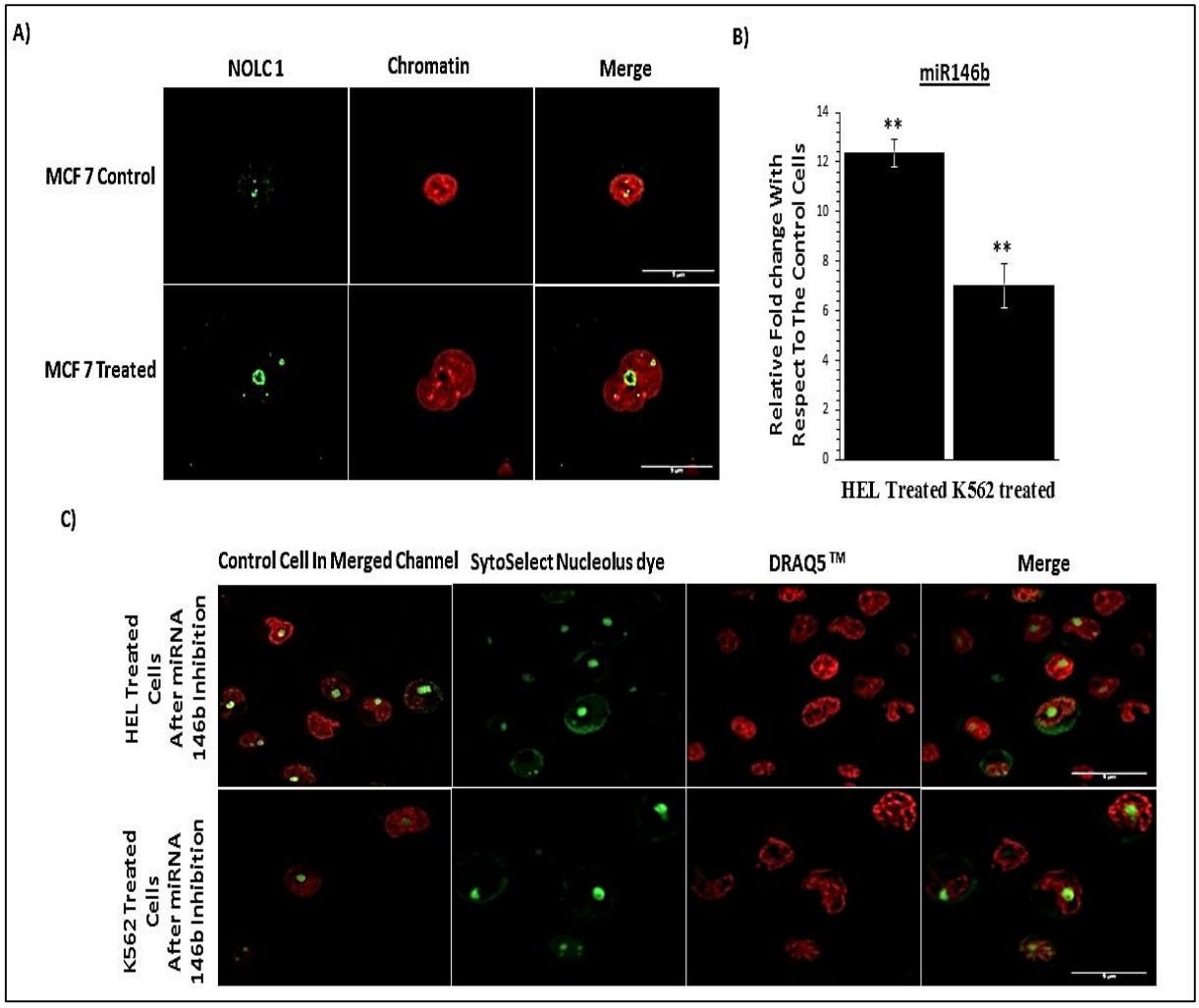
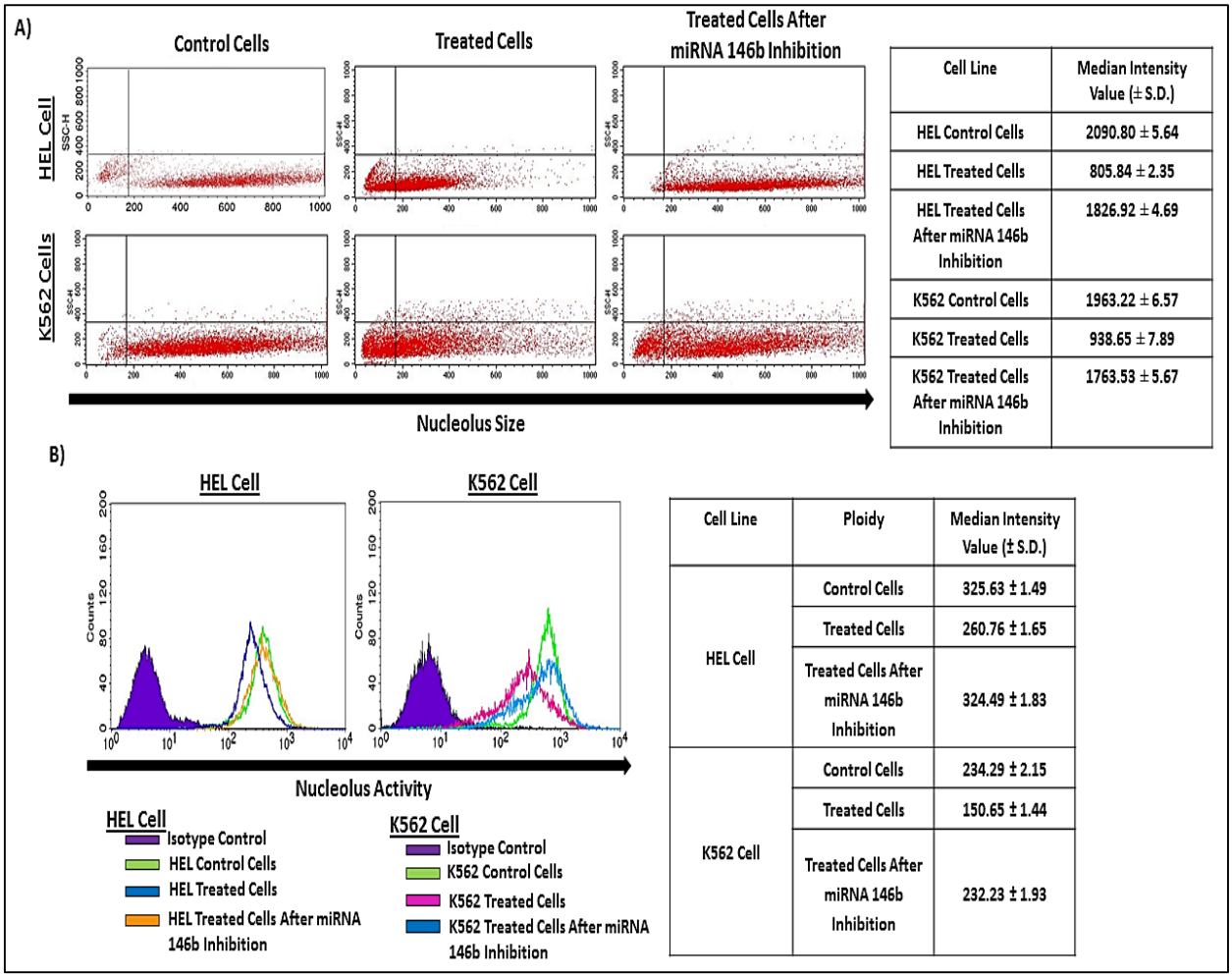


Figure 4.23. <u>Involvement of miRNA146b</u>. (A). *NOLC 1 expression in both MCF 7 untreated and treated population.* ***(B).*** *Relative gene expression of miRNA 146b normalized against HPRT1 as seen by qRT-PCR. Data represents the mean ± s.d. of 3 independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3) in K562 and HEL cells.* ***(C).*** *Nucleolus size measurement. HEL and K562 cells incubated with TPA after miRNA146b inhibition. All the imaging were done with scale bar 5µm.*



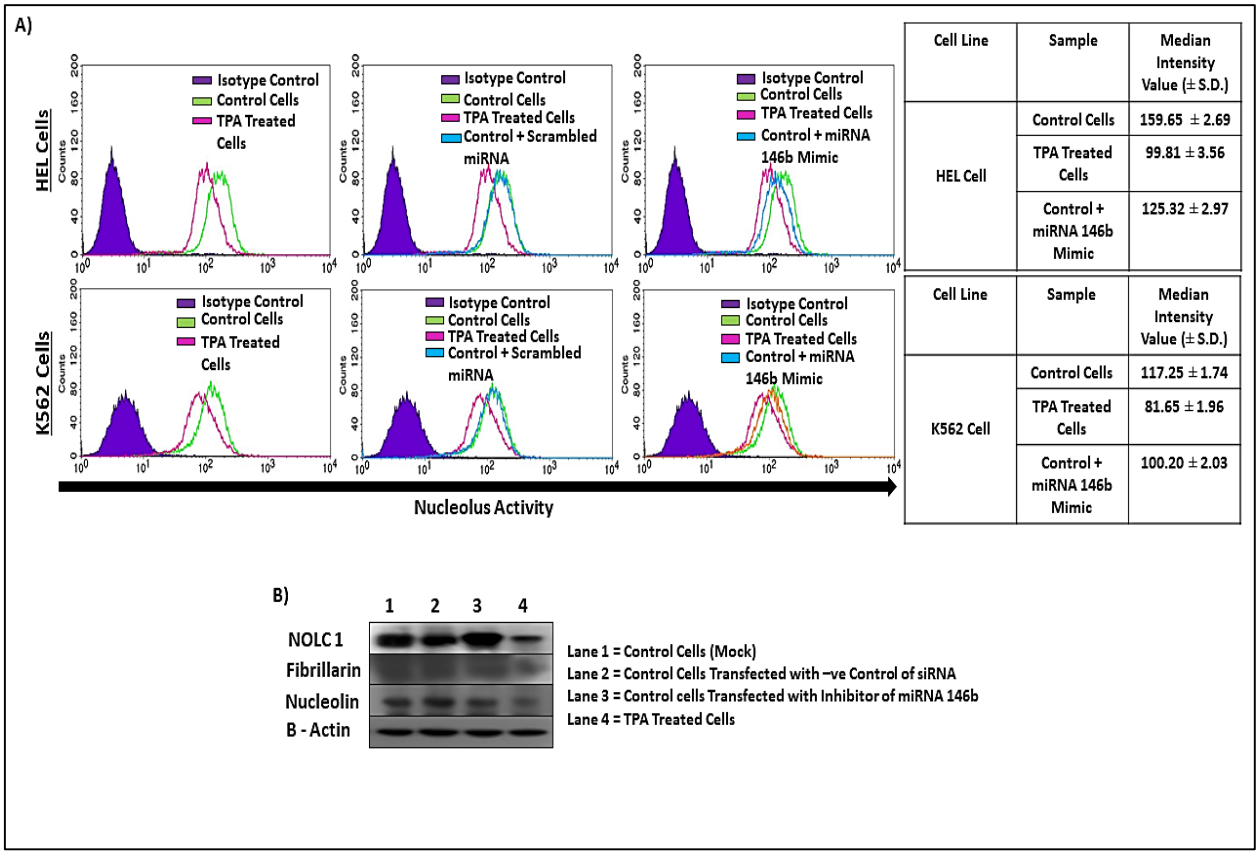
Cell Line	Median Intensity Value (± S.D.)
HEL Control Cells	2090.80 ± 5.64
HEL Treated Cells	805.84 ± 2.35
HEL Treated Cells After miRNA 146b Inhibition	1826.92 ± 4.69
K562 Control Cells	1963.22 ± 6.57
K562 Treated Cells	938.65 ± 7.89
K562 Treated Cells After miRNA 146b Inhibition	1763.53 ± 5.67

Cell Line	Ploidy	Median Intensity Value (± S.D.)
HEL Cell	Control Cells	325.63 ± 1.49
	Treated Cells	260.76 ± 1.65
	Treated Cells After miRNA 146b Inhibition	324.49 ± 1.83
K562 Cell	Control Cells	234.29 ± 2.15
	Treated Cells	150.65 ± 1.44
	Treated Cells After miRNA 146b Inhibition	232.23 ± 1.93

Figure 4.24. <u>Nucleolus size and activity measurement after miRNA146b inhibition in megakaryopoiesis induced cells.</u> (A). *Dot plot analysis of nucleolus`s size after miRNA 146b inhibition followed by TPA induction in k562 and HEL cells. Median intensity value (Right Side).* ***(B).*** *Histogram analysis of nucleolus activity for both HEL and K562 cells after miRNA146b inhibition. Median intensity value (Right Side).*

To verify whether miRNA 146b targets NOLC 1, promoter activity of NOLC 1 was examined during TPA induction in HEL and K562 cells. Dual-luciferase assay also showed a decrease in the NOLC 1 promoter activity after megakaryocytic induction with miRNA 146b mimic transfection

(Figure 4.26A). To further elucidate whether miRNA 146b was indeed involved in regulating MKs differentiation, we transiently transfected miRNA 146b inhibitor into cells and it showed significant decrement in the megakaryopoiesis markers (Figure 4.26B & 4.26C).



Cell Line	Sample	Median Intensity Value (± S.D.)
HEL Cell	Control Cells	159.65 ± 2.69
	TPA Treated Cells	99.81 ± 3.56
	Control + miRNA 146b Mimic	125.32 ± 2.97

Cell Line	Sample	Median Intensity Value (± S.D.)
K562 Cell	Control Cells	117.25 ± 1.74
	TPA Treated Cells	81.65 ± 1.96
	Control + miRNA 146b Mimic	100.20 ± 2.03

Figure 4.25. <u>miRNA146b mimic regulates nucleolus activity</u>. (A). *Histogram analysis of nucleolus activity for miRNA 146b mimic.Median intensity value(Right Side).* ***(B).*** *Western blot analysis for NOLC 1 and other nucleolus size and activity regulator proteins expression in different condition.*

Moreover, we observed that maximum cells could not follow the endomitosis process and could not get beyond 4N after the miRNA inhibition (Figure 4.27). It may be noted induction with

miRNA 146b mimic showed a lineage induction of megakaryocytic cells (Figure 4.28). To rule out the possibility of apoptosis for the inhibition of differentiation, apoptosis marker was checked (Figure 4.29A). Our experimental data clearly demonstrated that miRNA 146b not only is responsible for MKs induction but it also reduces the promoter activity of NOLC 1 and thereby decrease the size and activity of nucleolus.

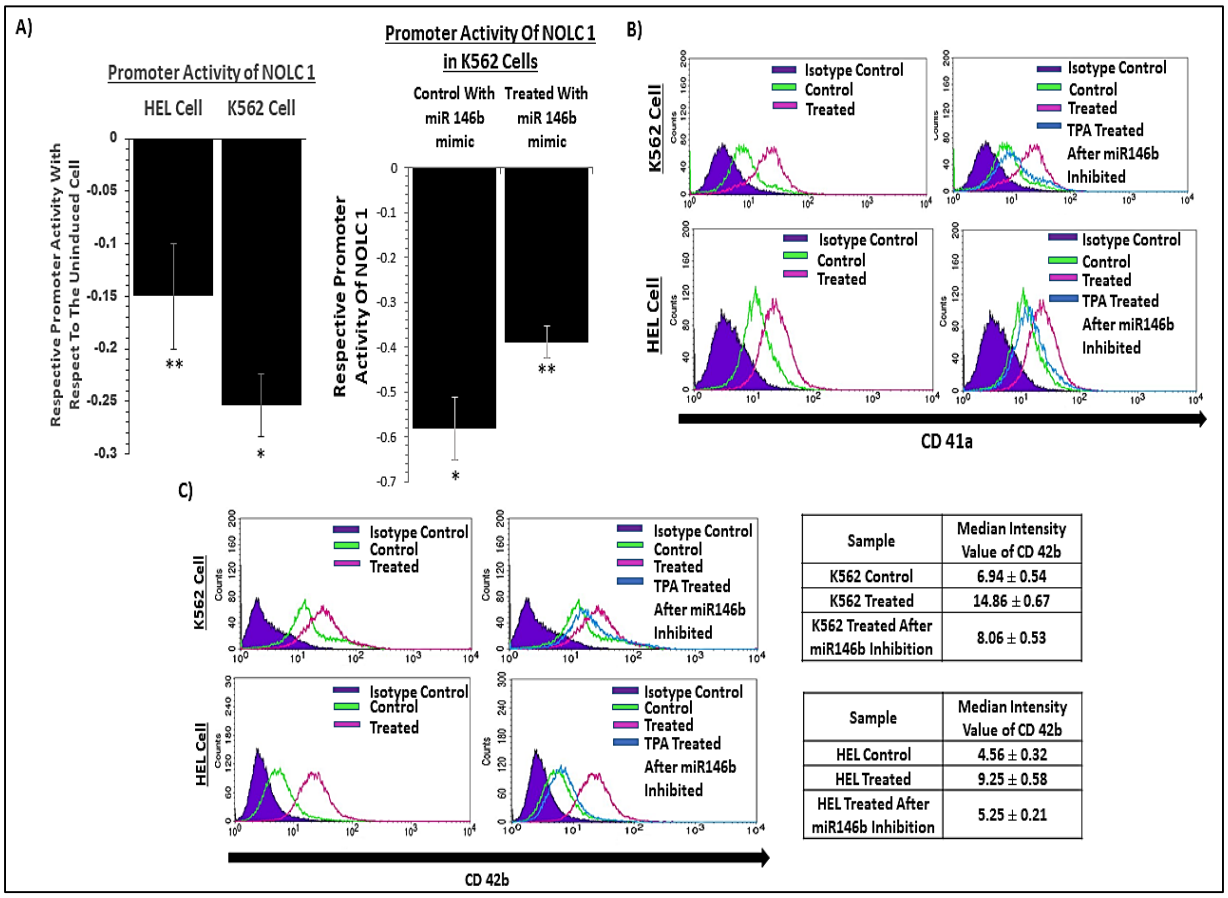
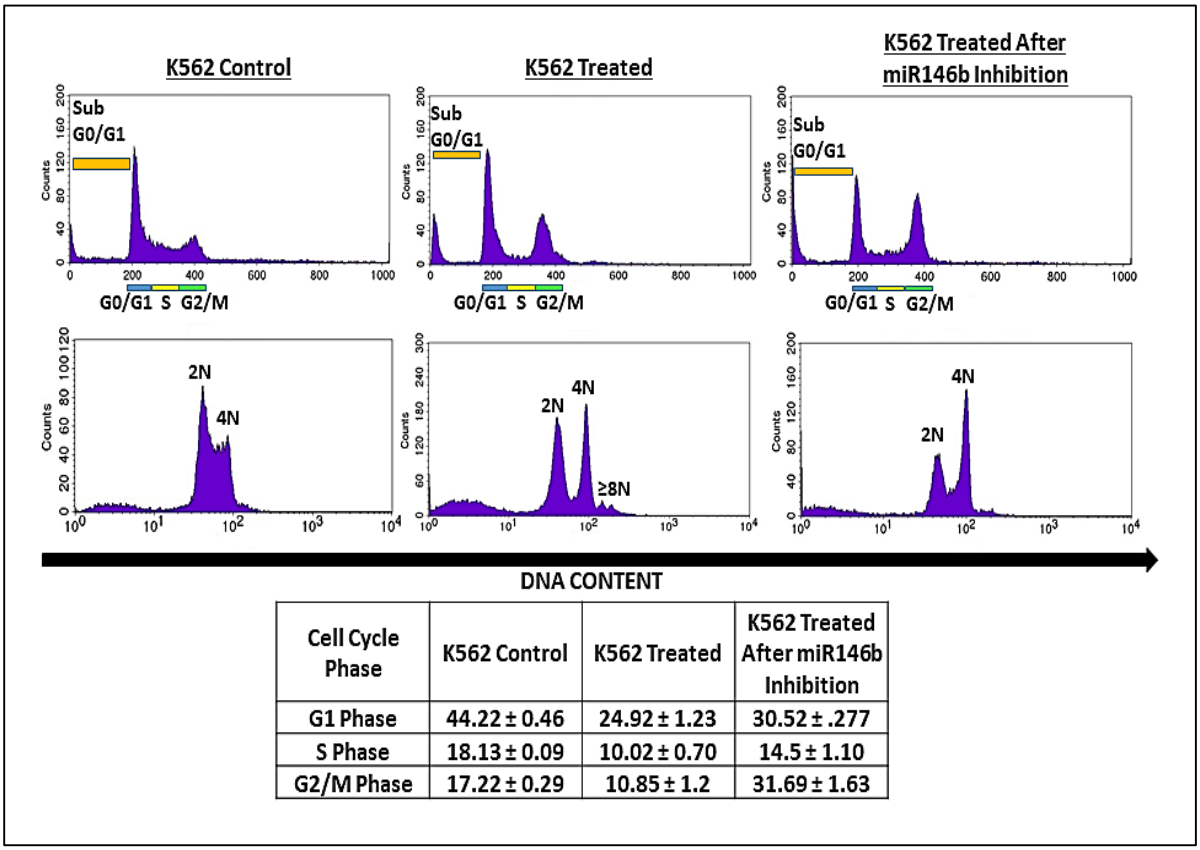


Figure 4.26. <u>miRNA146b regulates megakaryopoiesis maturation</u>. (A). *Dual-luciferase activity measurement for NOLC1 promoter.* ***(B).*** *Analysis of megakaryocyte specific surface expression marker in K562 and HEL cells after inhibiting cells with anti- miRNA 146b* ***(C).*** *Analysis of*

megakaryocyte specific surface expression marker in K562 and HEL cells after inhibiting cells with anti- miRNA 146b. Median intensity value (Right Side).



Cell Cycle Phase	K562 Control	K562 Treated	K562 Treated After miR146b Inhibition
G1 Phase	44.22 ± 0.46	24.92 ± 1.23	30.52 ± .277
S Phase	18.13 ± 0.09	10.02 ± 0.70	14.5 ± 1.10
G2/M Phase	17.22 ± 0.29	10.85 ± 1.2	31.69 ± 1.63

Figure 4.27. <u>Cell ploidy and cell cycle analysis after miRNA 146b inhibition followed by TPA induction in k562 cells.</u> *Percentage of cell in respective cell cycle stages in tabulated form(Down Side).*

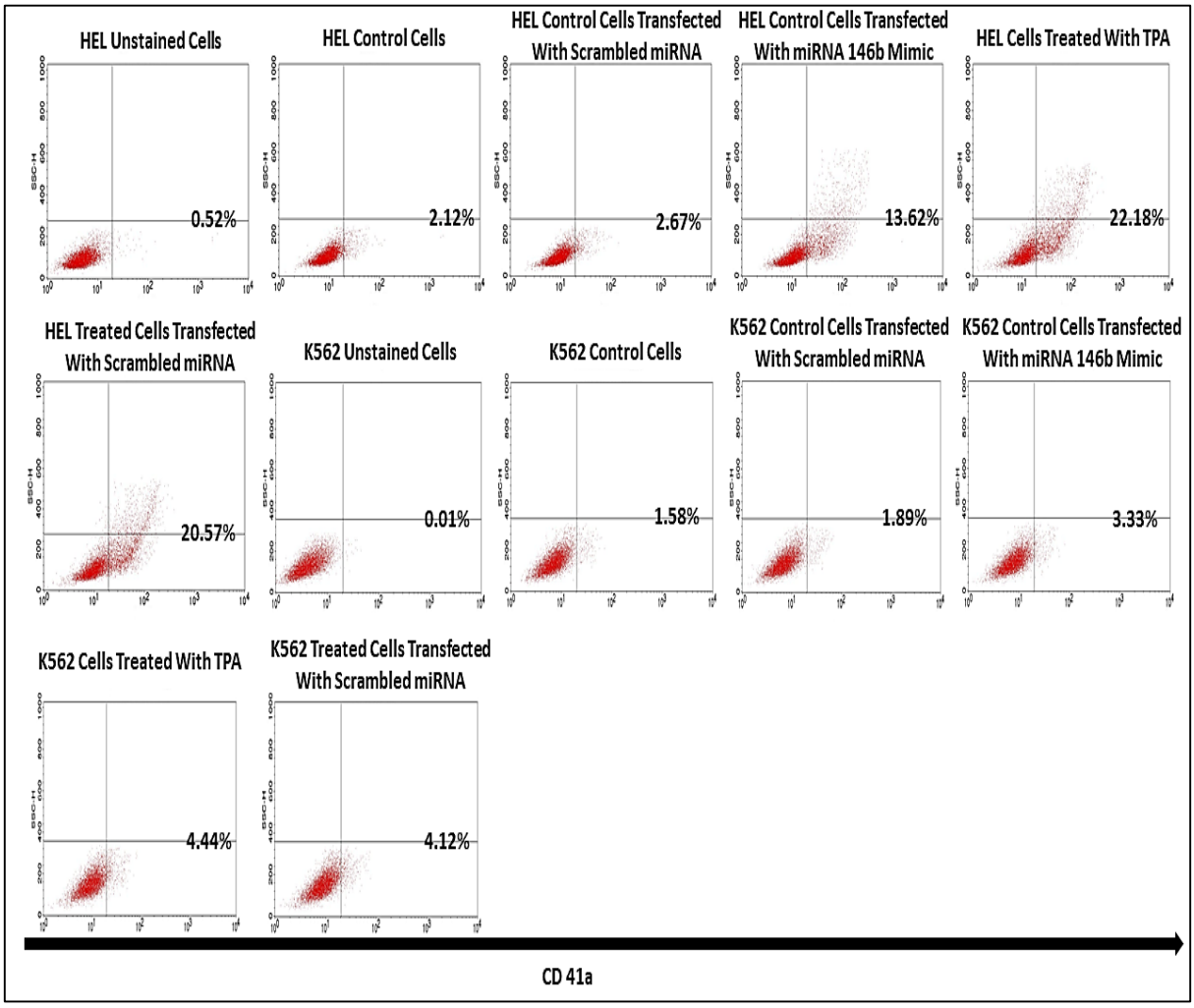
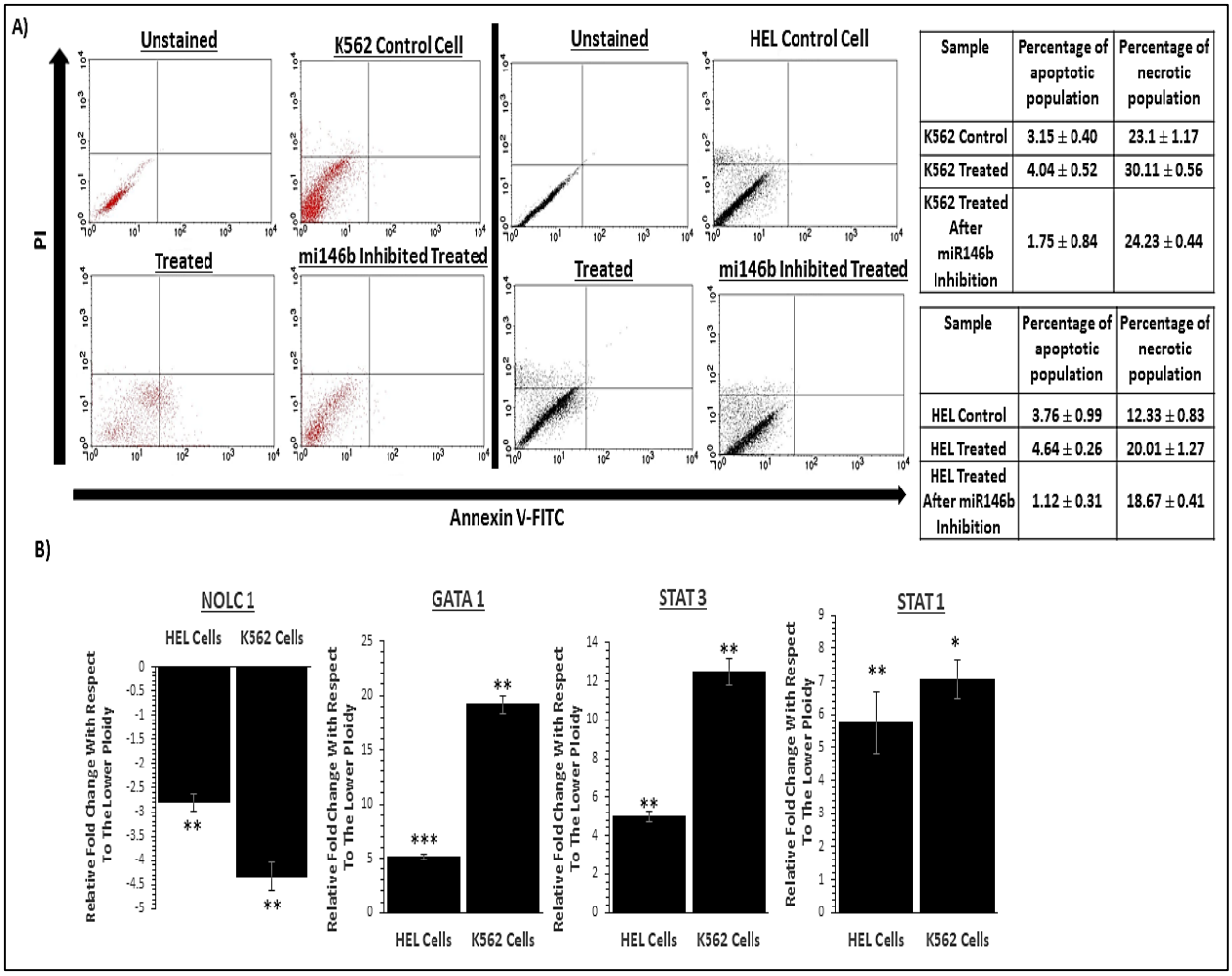


Figure 4.28. <u>Analysis of megakaryocyte specific surface expression marker in K562 cells after transfecting with miRNA 146b mimic.</u>



Sample	Percentage of apoptotic population	Percentage of necrotic population
K562 Control	3.15 ± 0.40	23.1 ± 1.17
K562 Treated	4.04 ± 0.52	30.11 ± 0.56
K562 Treated After miR146b Inhibition	1.75 ± 0.84	24.23 ± 0.44

Sample	Percentage of apoptotic population	Percentage of necrotic population
HEL Control	3.76 ± 0.99	12.33 ± 0.83
HEL Treated	4.64 ± 0.26	20.01 ± 1.27
HEL Treated After miR146b Inhibition	1.12 ± 0.31	18.67 ± 0.41

Figure 4.29. ***<u>Apoptosis assay and genes expression</u>.*** ***(A).*** *Apoptosis analysis in both HEL and K562 cells after miRNA146b inhibition. Percentage of apoptotic and necrotic population in table form (Right Side).* ***(B).*** *Relative gene expression of NOLC1, GATA1, STAT3 and STAT1 normalized against HPRT1 as seen by qRT-PCR. Data represents the mean ± s.d. of 3 independent experiments (*P< 0.05, **P< 0.01, ***P< 0.001, n = 3) in K562 and HEL cells.*

Chapter 6

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LIST OF ABBREVIATIONS

AKT	Protein Kinase B (PKB)
AMKL	Acute Megakaryoblastic Leukemia
APC	Allophycocyanin
CD	Cluster Of Differentiation
Cdt	Chromatin Licensing And DNA Replication Factor 1
cMPL	Thrombopoietin Receptor
CXCR4	C-X-C Chemokine Receptor Type 4
DMS	Demarcation Membrane System
FLI1	Friend Leukemia Integration 1 Transcription Factor
FOG1	Friend of GATA Protein 1
GATA1	GATA-Binding Factor 1
GTP	Guanosine Tri-Phosphate
HPRT1	Hypoxanthine Phosphoribosyl Transferase-1
HSCs	Haematopoietic Stem Cells
JAK	Janus Kinase
MAPK	Mitogen Activated Protein Kinase
miRNA	micro RNA
MP	Microparticles
MVs	Microvesicles

NFE2	Transcription Factor NF-E2
NFκβ	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells)
ORC	Origin Recognition Complex
PE	Phycoerythrin
PI	Propidium Iodide
pre RC	Pre Replicationg Complex
PTEN	Phosphatase and Tensin Homolog
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RIPA-buffer	Radioimmunoprecipitation Assay Buffer
RUNX1	Runt-Related Transcription Factor 1
SCF	Stem Cell Factor
SDF1	Stromal Cell-Derived Factor 1
STAT	Signal Transducer And Activator Of Transcription
TGFβ	Transforming Growth Factor Beta
TPA	12-*O*-Tetradecanoylphorbol 13-Acetate
TPO	Thombopoietin
MKs	Megakaryocytes
LP	Lower Ploidy
HP	Higher Ploidy
PGCCs	Polyploid Giant Cancer Cells
DNMTs	DNA Methyltransferase

Thesis Highlight

Name of the Student: Saran Chattopadhyaya

Name of the CI/OCC: Saha Institute of Nuclear Physics (SINP)

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Thesis Title: DNA Replication-Repair In Megakaryopoiesis

Discipline: Life Sciences **Sub-Area of Discipline: Hematopoiesis-Polyploidy**

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In the classical model of haematopoiesis, megakaryocyte-erythrocyte progenitors (MEPs) are responsible for the production of unipotent megakaryocytes (MKs) which subsequently give rise to blood platelets through the developmental process. In higher vertebrates, platelets are essential for procedures such as blood coagulation during wound healing, angiogenesis etc. To become polyploid cells, the MKs follow a unique cell cycle replication process, commonly designated as endomitosis. Previously, our lab reported about the down regulation of MCM7 and the involvement of miR-106b-25 cluster during megakaryopoiesis. It is yet to be decided about the status of other replication and repair associated proteins during megakaryopoiesis.

In order to circumvent genomic insults, such as DNA mutation, DNA damages etc., researchers have established a link between DNA damage repair process and DNA methylation. In multiple cancers such as lung cancer, breast cancer etc. mounting lines of evidence have been suggested that the aberrant promoter hypermethylation leads to the transcriptional silencing of the DNA key repair genes. Generally, DNA methylation is executed by a family of proteins designated as DNA methyltransferases (DNMTs) including DNMT1, DNMT3A and DNMT3B. However, within the bone marrow niche it still needs to be elucidated how progenitor cells are induced for megakaryopoiesis in the over-crowded bone marrow microenvironment and subsequently regulate DNA methylation status during megakaryopoiesis. In this study, we explored the effects of megakaryocyte derived microvesicles in haematopoietic cell lines as well as in $CD34^+$ cells in the context of differentiation. Our study demonstrated that microvesicles isolated from the induced megakaryocytic cell lines have the ability to stimulate non induced cells specifically into that particular lineage. We showed that this lineage commencement comes from the change in the methylation status of Notch 1 promoter which is regulated by DNA methyltransferases (DNMTs). Moreover, in order to reveal the status of double strand breaks (DSBs) during megakaryopoiesis, we measured the expression of H2A.X and γ-H2A.X which indicated that during this process repair process are getting elevated in expression.

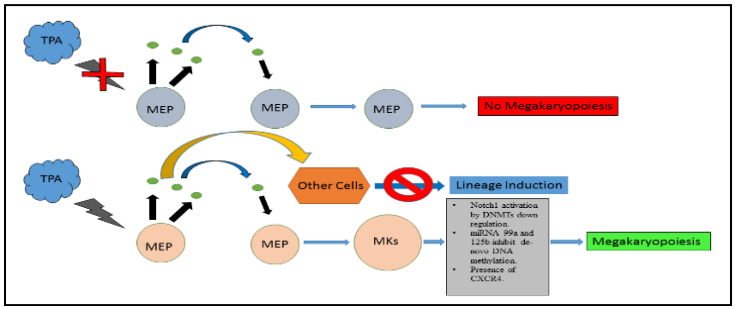

Figure1: Schematic Representation of the study: Microvesicles promotes megakaryopoiesis by the regulation of DNMTs and methylation of Notch1 promoter.

As we mentioned earlier, the MKs follow an unfamiliar cell cycle replication process, commonly designated as endomitosis. In another study, we checked the expression of other MCM proteins (MCM 2, 4, 6) and histones loaders to further elucidate the chromatin organisation in polyploid MKs. It was observed that MKs showed a chromatin decompactness which was aided by the expression of lncRNAs. Generally, nucleolus acts as a hub for the maturation of lncRNAs. We studied the nucleolar size and activity in polyploid megakaryocytic cell lines and *in vitro* cultured MKs obtained from human cord blood derived CD 34^+ cells. Our investigation revealed the involvement of miRNA-146b in regulating the activity of NOLC1 which plays an integral role in

nucleolus. Moreover, we also checked different epigenetic marks during megakaryopoiesis. This study clearly emphasized the role of chromatin organisation through the impairment of nucleolus`s activity in the process of megakaryopoiesis.