Role of Long non-coding RNAs in Human Induced Pluripotent Stem Cells derived Megakaryocytes: A p53, HOTAIRM1 and miR-125b interaction study

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Abbreviations:

CSCR - Centre for Stem Cell Research;
hiPSCs-HSCs - Human induced pluripotent stem cells -derived CD34\(^+\) hematopoietic stem cells;
HOTAIRM1 - HOX antisense intergenic RNA myeloid 1;
IncRNAs - long non coding RNAs;
MKs - Megakaryocytes;
NAC - N-Acetyl-L-cysteine
PMA - phorbol 12-myristate 13-acetate
ROS - Reactive Oxygen Species
Abstract

Megakaryocytes (MKs) are rare polyploid cells found in the bone marrow and produce platelets. Platelets are small cell fragments that are essential during wound healing and vascular hemostasis. *In vitro* differentiation of MKs from hiPSCs-HSCs could provide an alternative treatment option for thrombocytopenic patients as a platelet source. In this approach, we developed a method to produce functional MKs from hiPSCs-HSCs using a xeno-free and feeder-free condition and minimize the variation and risk from animal-derived products in cell culture. We have also investigated the genome-wide expression as well as functional significance of long non-coding RNAs (lncRNAs) in hiPSCs-HSCs-derived MKs to get insight into MK biology. We have performed lncRNAs expression profiling by using the Human LncProfilers™ qPCR Array Kit and identified 26 differentially regulated lncRNAs in hiPSCs-HSCs-derived MKs as compared to those in hiPSCs-HSCs. HOTAIRM1 was the most highly upregulated lncRNA in hiPSCs-HSCs derived MKs and phorbol 12-myristate 13-acetate (PMA)-induced megakaryocytic differentiating K562 cells. Furthermore, we have studied the potential mechanism of HOTAIRM1 based on the interactions between HOTAIRM1, p53, and miR-125b in PMA-induced K562 cells. Our results demonstrated that during MKs maturation HOTAIRM1 might be associated with the transcriptional regulation of p53, via acting as a decoy for miR-125b. Thus, the interaction between HOTAIRM1, p53, and miR-125b is likely involved in controlling cell cycling (cyclin D1), ROS production, and apoptosis to support terminal maturation of MKs.

Significance Statement

*In vitro* generation of MKs from hiPSCs-HSCs could provide an alternative source of platelets for treating thrombocytopenic patients. We have investigated the functional significance of lncRNAs in hiPSCs-HSCs-derived MKs which remains unclear and their role is important in studying the megakaryocyte biology. Our findings suggest that the regulatory role of HOTAIRM1 in p53 mediated regulation of cyclin D1 during megakaryocytopenesis to promote MK maturation by decoying miR-125b.
INTRODUCTION

Human megakaryocytes (MKs) are large, polyploid (≥8N) cells that are derived from hematopoietic stem cells (HSCs). MKs are specialized precursor cells found in the bone marrow that give rise to platelets in the blood circulation (Patel et al., 2005; Richardson et al., 2005; Deutsch and Tomer, 2006; Machlus et al., 2014). The principal function of platelets in the vascular system is to prevent bleeding. Thrombocytopenia is characterized by a low platelet count (<1.5 x 10^6 cells/L) in the blood circulation. This condition can be caused by various blood disorders and factors as well as a number of medications. Human diseases related to MK development and blood platelets affect a significant portion of the population and constitute a serious health problem worldwide. Platelet transfusion is critical to maintaining normal platelet counts in patients suffering from thrombocytopenia. Ex vivo generation of MK/platelets from hiPSCs could offer an alternative treatment option for patients diagnosed with thrombocytopenia and allow the study of the molecular mechanisms involved in MK development and platelet production (Liu et al., 2015; Börger et al., 2016).

A variety of cytokines and transcription factors have been implicated in megakaryocytopoiesis, but there is also a new player involved in genetic regulation: ncRNAs, such as microRNAs (miRNAs) and long non-coding RNAs (IncRNAs). Recognizing the functional role of these molecules is an area of focus in the study of molecular regulatory mechanisms governed by different cytokines and transcription factors.

The human transcriptome consists of various RNA biotypes, including many non-protein-coding RNA transcripts that have regulatory, structural, or unidentified functions. MicroRNAs can be extensively dysregulated in multiple human diseases (Ambros, 2004; Bartel, 2004) and several miRNAs have also been implicated in the regulation of platelet biogenesis and MKs development (Raghuwanshi, Dahariya, et al., 2019). In general, IncRNAs are characterized as ncRNAs that stretch over 200 nucleotides and lack a known protein-coding function (Saxena and Carninci, 2011). Researchers are interested in IncRNAs because their functions have been defined in several biological processes (Tsai et al., 2010; Kaikkonen et al., 2011). Unlike miRNAs, however, the role of IncRNAs is not yet fully explored and much less is known about the function of IncRNAs.
in megakaryocytopoiesis (Tian et al., 2016). Only a few studies have shown that lncRNAs play a role in cancers associated with abnormal gene expression (Ji et al., 2003; Panzitt et al., 2007; Gupta et al., 2010). Although lncRNAs have been characterized in different cells, their role in megakaryocytopoiesis has not been investigated.

Therefore, a thorough exploration of the MKs transcriptome might offer an opportunity to uncover the functions of ncRNAs in the development of MKs and platelets. Hence, the purpose of this study was to standardize a protocol for the MKs generation in xeno-free and defined conditions, and to understand the lncRNAs profile of hiPSCs-HSCs-derived MKs. Furthermore, during megakaryocytic differentiation, we tried to explore the potential of the interactions between p53, HOTAIRM1, and miR-125b.

MATERIALS AND METHODS

Cell Culture

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

On the 5th day of culture, the hiPSCs-HSCs were collected and transferred to media used for differentiation of MKs: StemPro-34™ basal media with L-Glutamine (2 mM), PenStrep (1X), IL-3 (10 ng/mL), IL-11 (10 ng/mL), TPO (50 ng/mL), VEGF-2 (10 ng/mL), SCF (20 ng/mL), BMP4 (10 ng/mL), and FGF2 (10 ng/mL). This optimized differentiation media offers an easy solution to generate MKs in feeder free condition within 5–10 days. At regular intervals (i.e. every 2 days), half of the media was substituted.

For the functional study of lncRNAs, phorbol 12-myristate 13-acetate (PMA) induced-megakaryocytic differentiating K562 cells were used. RPMI-1640 (Invitrogen) media with 1% antibiotic antimycotic solution...
(Sigma) and 10% fetal bovine serum (FBS) was used followed by incubation at 37 °C in a 5% CO₂ incubator. K562 cells were treated with PMA (50 nM) (Sigma-Aldrich) for 72 h to initiate megakaryocytic differentiation. After 72 h, cells were collected to study their RNAs and proteins.

**Bright-field imaging**

On the 10th day, phase contrast micrographs (Confocal Microscope, Olympus with 405 LASER) were taken to evaluate morphological changes on hiPSCs-HSCs-derived MKs.

To observe the multilobed polyploid nucleus of differentiated MKs, harvested cells were rinsed with 1X PBS and fixated on glass slides. Wright-Giemsa (WG) (Baxter) staining was performed according to the manufacturer’s instructions. A confocal microscope (Zeiss LSM 510) was used to capture the images, in conjunction with the Zeiss ZEM imaging software.

**Flow cytometry analysis**

Cells (1 x 10⁶/mL) were prepared in 1X PBS with 0.1% FBS. Cells were harvested and labelled with PE (phycoerythrin)-tagged anti-CD34 and DAPI (4, 6-dimidino-2-phenylindole)-tagged anti-CD41 (BD Biosciences, and PE-tagged anti-CD61 (BD Biosciences). Ig isotype controls (DAPI and PE) were used as controls for flow cytometry. All samples were analyzed for CD34⁺, CD41⁺, and CD61⁺ expression using the FACS AriaTM III flow cytometer (BD Biosciences). iPSCs-HSCs were used as a control.

**Ploidy (DNA index) analysis**

To properly calculate the DNA Index (DI), the harvested cells were stained (1 x 10⁶ cells/mL) using reagents from the Muse Cell Cycle Assay Kit (Merck Millipore) according to the manufacturer’s instructions. The Millipore Muse Cell Cycle Assay kit comes with propidium iodide (PI) that will be detected by the Yellow detector in linear mode. PI is the most regularly used dye for quantification of DNA content, which could be assessed by employing a flow cytometer (Muse Cell Analyzer; Merck Millipore). The DNA profile of the cell
culture was determined by plotting a histogram between DNA content and cell number. The experiments contained a minimum of 5000 events per run.

**RNA isolation and quantitative RT-PCR analysis**

The miRNeasy mini kit (Qiagen) was used to extract the total RNA from the cell culture according to the manufacturer’s instructions. Total RNA concentration was estimated using Nanodrop (Thermo Scientific) and 1 µg RNA was used as input for cDNA synthesis using EasyScript™ cDNA Synthesis Kit (abm) according to the manufacturer's instructions. To amplify the first strand of cDNA, specific primers were used in real-time quantitative PCR (qRT-PCR; Applied Biosystems) with SYBR Green FAST qPCR Master Mix (Kappa Biosystems) according to the manufacturer’s instructions. The qRT-PCR was run under the following conditions: initial denaturation for 2 min at 52 °C followed by 8 min at 95 °C; 20 sec at 56 °C for 42 cycles; and 30 sec at 72 °C. GAPDH was used as the internal control to normalize the Ct-values. Analysis of miR-125b expression was performed by qRT-PCR using miScript Primer Assay kit (Qiagen). Data were normalized against the internal control U6. Relative quantification was determined by the comparative ΔΔCt method (2^(-ΔΔCt)). Table 1 shows the primer sets used for the qRT-PCR.

**RNA isolation and lncRNAs expression profiling**

For lncRNAs expression profiling, the miRNeasy mini kit (Qiagen) was employed to extract total RNA from the cultured cells and 1 µg of RNA was used as input for reverse transcription using the Human LncProfilers™ cDNA synthesis Kit (System Biosciences) according to the manufacturer’s instructions.

Human LncProfilers™ qPCR Array Kits were used for lncRNAs profiling. The differentially expressed lncRNAs between differentiated hiPSCs-HSCs-derived MKs and controls (iPSCs-HSCs) were identified using the ΔΔCT analysis software (fold change (FC) ±2 and p<0.05) available on the SBI website (https://www.systembio.com/lncrna-profiler-qpcr-array-kit-human).
**Protein extraction and Western blot assay**

Cells were rinsed twice with 1X PBS (ice cold) followed by 30 min of cell lysis in RIPA buffer (ice cold) (Sigma) with phosphatase arrest (g-Biosciences) and protease inhibitor cocktail (Sigma) under continuous shaking at 4 °C. Insoluble matter was separated and discarded from samples through centrifugation (12,000 g at 4 °C for 15 min). Proteins (50 µg) were separated using a 6–12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose (NC) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated overnight with specific primary antibodies (β-actin: Sigma; p53, Cyt C, Caspase 3, and Caspase 9: Biosciences; p21, PARP, Bcl2, BAX, and BAD: Santa Cruz). The membranes were rinsed twice with 1X TBS and 1X TBST buffers, followed by 1 h of incubation with suitable peroxidase-conjugated secondary antibodies. A chemiluminescent solution, Pierce™ ECL Substrate (Thermo Fisher Scientific), was employed for developing protein bands on NC membranes. For visualization, the Chemidoc Imaging System (BioRad) was used according to the manufacturer’s instructions. Relative protein levels were quantified by calculating the western blot band density using ImageJ software. For normalization, β-actin was employed.

**Determination of intracellular ROS**

To scavenge the ROS produced by PMA-stimulated K562 cells, the cells were treated with 5 µm N-Acetyl-L-cysteine (NAC; Sigma). In order to understand the effect of the intracellular ROS levels on miR-125b in the context of MK differentiation, a DCFDA assay was performed using NAC (a ROS quencher) that usually interacts with ROS to reduce intracellular ROS levels. To monitor the intracellular ROS levels, PMA-activated K562 cells (1 x 10⁶ cells/mL) were stained with a cell permeable dye (2,7-dichloro-dihydro-fluorescein-diacetate; H2DCFDA). Here, we measured ROS levels from different experimental time points such as control, 24 h, 48 h, and 72 h treated with PMA (40 ng/mL). In brief, cells were rinsed with PBS and incubated with dye (10 µM) in PBS for 15 min in the dark at room temperature. Green fluorescence emitted by 2,7-dichlorofluorescein (H2DCF) was quantified by a flow cytometry (BD LSR FORTESSA). Mean fluorescence
intensity (MFI) was graphed in scatter plots. Later, cells were harvested to analyze the miR-125b expression levels.

**Cell culture and small interfering RNA (siRNA)-HOTAIRM1 transfection**

K562 cells were cultured in RPMI-1640-GlutaMAX™ media (Gibco; Thermo Fisher Scientific) supplemented with 1% antibiotic–antimycotic solution (Sigma-Aldrich) and 10% FBS (Gibco; Thermo Fisher Scientific). Cells were maintained at 37 °C in a 5% CO₂ humidified incubator. Cells were cultured overnight in 24-well plates (1 × 10⁵ cells/well). To induce K562 differentiation into MKs, the culture media was supplemented with PMA (50 nM). The lncRNA HOTAIRM1-siRNAs were designed and synthesized using the Sigma-Aldrich website (sigma-aldrich.com) and transfected to PMA-induced K562 cells using the X-tremeGENE 360 Transfection Reagent (Sigma-Aldrich) and negative controls (non-targeting or nonsense siRNA control sequence). A defined volume of each si-HOTAIRM1 was added to the transfection samples to achieve a final concentration of 25 nM, according to the manufacturer’s instructions. The expression of the lncRNA HOTAIRM1 was determined via RT-PCR to verify the occurrence of any off-target effects after three transfections with the HOTAIRM1-siRNA mixture; the aim was to successfully avoid off-target effects. Following transfection of PMA-induced K562 cells with human siRNA-HOTAIRM1 for 48 h, cells were subjected to downstream experiments.

**Statistical analysis**

All experimental studies were conducted in triplicate. Results are represented as mean ± standard deviation. The significance of variance among the groups was calculated by the Student’s t-test. The biological significance threshold and statistical significance was set at ±2-fold-change and p<0.05, respectively. The statistical significance of the data is shown by the p-value (*p<0.05 and **p<0.002).
RESULTS

MKs differentiation from iPSCs-HSCs in defined and xeno-free conditions

The present study focuses on the generation of MKs from iPSCs-HSCs. Previous studies have reported the differentiation of MKs from iPSCs-HSCs under defined and xeno-free conditions (Liu et al., 2015; Börger et al., 2016; Hansen et al., 2018). In this culture system, we cultured iPSCs-HSCs for 5 days in HSCs expansion media. We further transfected these cells to MK differentiation media supplemented with cytokines and growth factors (FGF2, BMP4, SCF, VEGF-2, TPO, IL-11, IL-3), which are essential for MK differentiation and growth. At the 10th day of culture, we collected cells and analyzed them for morphological changes. We further analyzed the expression of MK-specific markers using FACS and qRT-PCR. We analyzed polyploidy using WG staining.

After the 10th day, cell mass had increased compared to that on day 0 (Figure 1B). About 80.3% of non-stimulated cells (day 0) expressed CD34 surface antigens specific to iPSCs-HSCs (Figure 1C). Interestingly, after 10 days of culture with cytokines, >90% of the cells were MKs (CD41+/CD61+; Figures 1C and 1D), as determined using FACS. The MK surface antigens, CD41 and CD61, are commonly used to define MK differentiation and maturational stages (Raghuwanshi, Sharma, et al., 2019; Raghuwanshi et al., 2020). Cell morphology and nuclear size was identified in WG-stained smears of cells and compared the results between day 0 and day 10. In this study, after the 10th day, we have observed MKs with large sizes having polyploid multilobed nucleus (Figure 1E). However, when measuring the ploidy of the MKs, DNA content is presented as DI, which is the ratio of DNA content of the experimental sample to the corresponding control (normal diploid) population. In general, cells in G0 or G1 phases contain normal diploid chromosomal content (2n DNA) whereas cells in G2, and just prior to mitosis, consists double (4n) the normal quantity. This analysis was performed to determine the content of DNA in the range of 2n, 4n, and so on, as DNA synthesis occurs in S-phase. Hence, we have focused our interest to diploid cells which produce polyploid progeny since this is a...
distinctive feature of MKs. Increased ploidy levels as evaluated by cell cycle analysis was detected in hiPSCs-HSCs derived MKs as compared with hiPSCs-HSCs on the 10th day (Figure 1G).

**Differential expression analysis of IncRNAs in iPSCs-HSCs vs MKs**

Following the confirmation of the MK differentiation from hiPSCs-HSCs, we have identified the expression profile of IncRNAs in control (hiPSCs-HSCs) and experiment (MKs) groups using Human LncProfilers™ qPCR Array (SBI). The differential expression of IncRNAs between hiPSCs-HSCs and MKs was analyzed via ΔΔCt analysis software (https://www.systembio.com/lncrna-profiler-qpcr-array-kit-human).

Figure 2A shows a Heat Map with the Ct values of 90 IncRNAs which were profiled in hiPSCs-HSCs and MKs groups. Of the 90 IncRNAs analyzed in MKs, only 26 (28.88%) IncRNAs showed statistically significant difference with respect to hiPSCs-HSCs (p<0.05; FC ±2.0; Figure 2B). Of these 26 differentially regulated IncRNAs, 24 (26.66%) genes were up-regulated, whereas only 2 (2.22%) genes were down-regulated (Figure 2B). HOTAIRM1 IncRNA was identified as the highest upregulated in MKs in comparison with hiPSCs-HSCs. Furthermore, by using IncRNA specific primers through qRT-PCR, Array-Based Gene Expression Profile was validated. To confirm these significant IncRNAs signature performance in MKs, we validated the expression of randomly selected top three IncRNAs (HOTAIRM1, LUST, and TncRNA) out of 24 upregulated IncRNAs by qRT-PCR. We quantified expression of the highest upregulated IncRNA HOTAIRM1, LUST, and TncRNA and similar distinctions between hiPSCs-HSCs and MKs were observed in qRT-PCR result (p<0.02; Figure 2C). In both experiments more than 2-fold difference in the expression levels of IncRNAs was observed. These results support the use of qPCR array as a screening tool and emphasize the need for the validation of the array results.

**HOTAIRM1, a natural sponge, likely to be associated with the p53 mRNA regulation and protein expression by competing with miR-125b during megakaryocytic differentiation**

In this study, we used a well-defined PMA-induced megakaryocytic differentiation of a K562 cell line model to explore the HOTAIRM1-regulated mechanism in MKs. Interestingly, validation results confirmed that
HOTAIRM1 expression was significantly increased (~20-fold; p<0.002; Figure 2C) in hiPSCs-HSCs-derived MKs as compared to control iPSCs-HSCs. In addition, we observed that the expression pattern of HOTAIRM1 in PMA induced megakaryocytic K562 cells was closely related with its expression in iPSCs-HSCs derived MKs (~4.7-fold; p<0.002; Figure 3A). In the present study, it was observed that during the megakaryocytic differentiation initiation using PMA in K562 cell line, p53 protein levels were significantly increased (~7-fold; **p<0.02, *p<0.01 Figures 3D and 3E). It has been demonstrated that the lncRNA HOTAIRM1 serves as a decoy for miR-125b as well as for other miRNAs, keeping them away from p53 mRNAs (Ng et al., 2019). We have also found significant interactions between lncRNA HOTAIRM1 and miR-125b (Figure 3B) by employing IntaRNA tool (http://rna.informatik.uni-freiburg.de/IntaRNA). miR-125b is a well-known oncomiR which negatively regulates p53 (Le et al., 2009; Shaham et al., 2012). We have also found that the potential target gene of miR-125b is p53 (Figure 3C) by using TargetScan Human (http://www.targetscan.org/vert_72/). Furthermore, it was observed that miR-125b expression level was significantly low in both iPSCs-HSCs derived MKs and PMA induced MKs in comparison to control groups (iPSCs-HSCs and untreated K562 cells) (p<0.002; Figures 2D and 3A).

**HOTAIRM1 upregulates p53 expression and is involved in MK maturation via regulating downstream target genes p21 and cyclin D1, apoptosis regulators, and ROS.**

To understand the functional role of p53 in MKs, expression levels of three known transcriptional targets of p53 (p21, BAD, and Bcl-2) were studied. In present study, p21 and BAD were upregulated and Bcl-2 was down regulated in protein expression analysis by western blotting in PMA induced MKs in comparison with control (**p<0.02, *p<0.01; Figures 3D and 3E). Furthermore, we have also observed the increased production of ROS by flow cytometry and MK-specific late marker CD61 expression by qRT-PCR at different time points during megakaryocytic maturation (**p<0.02; Figures 4A and 4B). It was observed that, p53, p21, BAX, and BAD were upregulated in PMA induced megakaryocytic cells (**p<0.02, *p<0.01; Figures 3D and 3E). In contrast, Bcl-2 expression was down-regulated, and it is known to be negatively affected by p53.
Our results also demonstrated the activation of PARP cleavage and of caspase cascade known for markers for the components of classical intrinsic apoptosis pathway during terminal maturation of MKs (\(p<0.02, p<0.01\); Figures 3D and 3E) (Ravid et al., 2002). Our results also demonstrated the activation of PARP cleavage and of caspase cascade known for markers for the components of classical intrinsic apoptosis pathway during terminal maturation of MKs (\(p<0.02, p<0.01\); Figures 3D and 3E) (Josefsson et al., 2011; Kile, 2014; McArthur et al., 2018; Kovuru et al., 2020).

To further understand whether ROS generation is important for megakaryocyte maturation, we performed PMA-induced cell differentiation experiments employing NAC (an ROS quencher). NAC associates directly with ROS to function as a scavenger of oxygen free radicals, thereby, intracellularly reducing the ROS levels. During differentiation experiments, PMA-induced cells showed that ROS down regulates miR-125b expression in matured MKs which shows higher megakaryocytic marker CD61 expression in comparison with control (\(p<0.05\); Figures 4C and 4D). Cells treated with NAC showed no significant change in CD61 marker expression in comparison with control (\(p<0.05\); Figure 4C). However, upon addition of PMA and NAC together, cells showed elevated levels of CD61 expression compared with NAC treated cells (\(p<0.05\); Figure 4C); however, the expression was lower as compared to PMA induced cells. Taken together, this data supports the notion that ROS production is required for megakaryocytic maturation which associated with the p53 expression depended on miR-125b status (\(p<0.05\); Figures 4C and 4D).

**Suppression of lncRNA HOTAIRM1 expression induces impairment in the differentiation and maturation of PMA-induced K562 cells.**

To determine the function of lncRNA HOTAIRM1 in MKs differentiation and maturation, we used siRNA-HOTAIRM1 to down regulate the lncRNA HOTAIRM1 expression and detected its effects on cell cycle regulators (p53, p21, and cyclin D1) and MK-specific cell surface markers including miR-125b expression. After 48 h of transfection, cells were collected and observed under Bright field microscope. For both the control group and the group transfected with siRNA-HOTAIRM1, no large sized cells like MKs were observed (Figure 5A). Next, we investigated gene expression for cyclin D1, p53, and p21 by RT-PCR analysis. This revealed that inhibition of lncRNA HOTAIRM1 expression in si-RNA transfected PMA-induced K562 cells could not
generate cyclin D1, p53, and p21 respective mRNAs, and as a result, their protein levels were decreased considerably (\(**p<0.02, *p<0.01\); Figures 5B, 5C, and 5D). Furthermore, significant upregulation of miR-125b level was observed in the si-HOTAIRM1 treated PMA-induced megakaryocytic K562 cells in comparison with control (\(**p<0.02, *p<0.01\); Figure 5B). To determine the lncRNA HOTAIRM1 role in MK maturation, impact of HOTAIRM1 downregulation on MK-specific cell surface markers (CD41 and CD61) was studied. RT-PCR results demonstrated that CD41 and CD61 expressions were considerably decreased in the si-HOTAIRM1 treated group (\(**p<0.02, *p<0.01\); Figure 5B). Therefore, these results might imply that downregulation of lncRNA HOTAIRM1 expression might affect MK differentiation and maturation by influencing the expression of miR-125b, which might have an impact on p53 and p21 expression to stimulate MK maturation.

**DISCUSSION**

In the present study, we differentiated MKs from iPSCs-HSCs under defined and xeno-free conditions. For efficient production of MKs, we modified culture conditions such as culture media composition (Table 2 and Figure 1A) from previously reported protocols (Liu et al., 2015; Börger et al., 2016; Hansen et al., 2018). In contrast to previous studies, the present work uses the same media composition throughout the MK differentiation phase. In addition, the MK differentiation had been achieved on day 11 (Hansen et al., 2018) and day 19 (Liu et al., 2015; Börger et al., 2016), whereas we report the MK differentiation on day 10, which was attributed to the modifications made to the media composition.

The polyploidization is the crucial event of megakaryocytogenesis, and the large, polyploid-multilobed nucleus is considered as the hallmark of matured MKs (Odell et al., 1970). Also, the expression of MK-specific markers (CD41/CD61) was further confirmed by quantifying their mRNA levels using qRT-PCR. We observed a steadily increasing expression of both the CD41 (~6 fold) and CD61 (~15 fold) MK-marker mRNAs after the 10th day of the treatment with cytokines in comparison with day 0 (\(p<0.05\); Figure 1F). We developed a proficient differentiation system for MKs generation from hiPSCs-HSCs under feeder-free and serum-free conditions. These results indicate the feasibility to generate MKs from hiPSCs-HSCs. We used a defined and
basic differentiation system to generate MKs from hiPSCs-HSCs and further studied their role in molecular regulation of megakaryocytopoiesis.

After achieving MKs differentiation from hiPSCs-HSCs, we studied the lncRNAs expression profile in both the experiment (MKs) and control (hiPSCs-HSCs) groups, thereby defining the role of lncRNAs during MKs differentiation. Most recent studies have shown the functional significance of different lncRNAs in the regulation of major biological processes, such as cancer development and progression, chromatin remodeling, and cell pluripotency (Dinger et al., 2008; Gupta et al., 2010; Mizutani et al., 2012; Klattenhoff et al., 2013; Fang et al., 2014). Although a fair number of lncRNAs have been recognized in different lncRNA profiling studies, only a few have been experimentally validated in human hematopoiesis (Garzon et al., 2014). Recent studies indicate that HOTAIRM1 is involved in myeloid differentiation. However, the underlying mechanism of HOTAIRM1-mediated regulation in megakaryocytic maturation has not been determined.

Induction of MK differentiation in K562 cells using PMA is a classical and well-understood model to study megakaryocytic differentiation. However, this progression is accompanied by cell morphological changes, increased MK-specific marker expression, cell growth arrest, etc. (Huang et al., 2014; Raghuwanshi et al., 2020). Interestingly, HOTAIRM1 was significantly upregulated in both model systems. Under ordinary circumstances, the expression of HOTAIRM1 is constrained only to the myeloid lineage and it is associated with myeloid cell differentiation (Zhang et al., 2009). In addition, recent literature has shown the regulatory interactions amongst p53 and HOTAIRM1 (Jain et al., 2016; Jain and Barton, 2018). p53-dependent alterations in chromatin state regulate the lncRNA HOTAIRM1 during the human embryonic stem cell (hESCs) differentiation. The lncRNA HOTAIRM1 is induced by p53 only when hESCs were differentiated toward definitive ectoderm and mesoderm, but not endoderm lineages (Jain et al., 2016). The expression of miR-125b is increased in acute megakaryocytic leukemia associated with Down’s syndrome (DS) (Shaham et al., 2012). A possible explanation may be that the potential role of p53-dependent expression of differentiation-specific
lncRNA HOTAIRM1 can be able to protect p53 mRNA from miR-125b-mediated translation during MK maturation (Figure 4E).

p21 has been extensively studied in MKs, and the expression of p53 and p21 is increased during MK maturation (Ravid et al., 2002; Fuhrken et al., 2008). BAD and BAX belong to the Bcl-2 family as pro-apoptotic proteins, which inhibit the anti-apoptotic function of Bcl-2 (Jiang et al., 2007). Members of the Bcl-2 family are directly associated with MKs maturation and apoptosis (Avanzi et al., 2015; Kovuru et al., 2020).

In the context of megakaryocytopoiesis, ROS levels are elevated during megakaryocytic differentiation and maturation (Chen et al., 2013; Raghuwanshi et al., 2020). Furthermore, ROS production was associated with p53 expression, which depended on miR-125b expression status (Macip et al., 2003). ROS are crucial for MKs differentiation and overall development (Chen et al., 2013), as well as for the complete megakaryocytopoiesis process. Therefore, the H2DCFDA assay was performed to measure ROS levels at different days of MKs differentiation. A continuous induction of ROS levels during the MKs differentiation process was clearly evident from our results (Figure 4A).

Our results are consistent with previous reports, which identified the functional role of p53 in the regulation of endomitosis and polyploidization by decelerating cell cycling and promoting apoptosis to initiate terminal maturation of MKs (Fuhrken et al., 2008). PMA activates NADPH oxidase and increases ROS production. ROS are involved directly or indirectly in every step of the epigenetic alterations of miRNAs and p53 gene expression (Kuwabara et al., 2015). Furthermore, existing literature highlights a reciprocal association between microRNAs pathway and ROS signaling, that results in diverse biological effects in differentiating cells. In the context of MKs differentiation and development, increased levels of ROS were found to suppress miR-125 expression and induce p53 expression in MKs to regulate megakaryopoiesis. Thus, we report that the interactions among HOTAIRM1, p53, and miR-125b could be associated with cell cycle regulation, ROS production, and activation of intrinsic apoptosis during megakaryocytopoiesis (Figure 4E).
These data suggest that transfection of PMA-induced megakaryocytic K562 cells with HOTAIRM1-siRNA results in increased miR-125 expression. This is due to a decrease in the expression of its putative target, p53, as well as downstream targets. Alternatively, in both the control group and the group transfected with si-HOTAIRM1, no visual changes were observed. Next, the expressions of MK-specific markers were not significantly altered after the si-HOTAIRM1 transfection in PMA-induced megakaryocytic K562 cells, which verified the absence or differentiation of MK-like cells. These findings suggest that the HOTAIRM1 lncRNA may promote p53 gene expression by decoying miR-125b, thereby influencing differentiation and maturation of MKs. Thus, our findings revealed the functional role of HOTAIRM1 lncRNA in p53-mediated regulation of cyclin D1 as well as ROS production: to trigger the intrinsic apoptosis pathway during megakaryocytogenesis by decoying miR-125b (Figure 4E).

CONCLUSION

In the present work, we report a differential lncRNAs expression profile in MKs derived from hiPSCs-HSCs. This will provide the foundation for future studies of the biological functions of lncRNAs in the development of MKs. We found that most of the differentially expressed lncRNAs were upregulated in MKs compared to those in hiPSCs-HSCs. Furthermore, to confirm these significant lncRNAs signatures performances, we validated the expression of three lncRNAs (HOTAIRM1, LUST, and TncRNA) out of 24 upregulated lncRNAs by qRT-PCR. We observed similar distinctions between hiPSCs-HSCs and MKs. LncRNAs play a vital role in the regulation of gene expression during development as well as differentiation. The upregulated lncRNAs identified may have functional roles in gene regulation during MKs differentiation/maturation. Specifically, HOTAIRM1 was identified and confirmed as the highly upregulated lncRNA in hiPSCs-HSCs-derived MKs. In addition to interacting with proteins as a scaffold, the lncRNA HOTAIRM1 could exert its functional effects on several gene expressions by acting as a miRNA sponge/decoy for a number of miRNAs. We also found that the treatment of K562 cells with PMA induces HOTAIRM1 expression, similar to hiPSCs-HSC-derived MKs. Moreover, we noticed that p53-dependent HOTAIRM1
unlike miR-125b, a positive regulator of p53 was significantly upregulated in PMA-induced MKs. Importantly, we demonstrate that during MK maturation, the lncRNA HOTAIRM1 might be associated with the transcriptional regulation of p53, which in turn could act as a decoy for miR-125b, keeping them away from p53 mRNA. In downstream steps, p53 can also regulate the expression of apoptotic genes and promote ROS production, which is essential during megakaryocytopoiesis, by regulating miRNAs expression. Thus, p53, along with HOTAIRM1, via regulating p21, components of BAK/Bax pathway expression, and ROS production, enhances maturation and apoptosis in MKs development. However, further experiments are necessary to validate the associations between HOTAIRM1, p53, and miR-125b, as well as the functional effects of these associations in MKs development.

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Authorship Contributions

Participated in research design: Dahariya S, Gutti R

Conducted experiments: Dahariya S

Performed data analysis: Dahariya S, Raghuwanshi S, Thamodaran V, Velayudhan S, Gutti R

Contributed to the manuscript preparation: Dahariya S, Raghuwanshi S, Gutti R
References


Footnotes

Funding Information

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Disclosure of conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

All experiments were conducted in accord with the local regulations and laws as well as with the approval from the Institutional Ethics Committee.
Legends for Figures

Figure 1. Efficient differentiation of MKs from iPSCs-HSCs. A. Schematic representation of the iPSCs-HSCs differentiation protocol toward MKs. B. Phase contrast cell images on day 0 and day 10. C. Flow cytometry histograms of the cultured suspension cells on day 0 and day 10. The suspension cells on day 0 expressed CD34 marker and on day 10 maximum cells showed MK markers CD41 and CD61. D. FACS analysis for double staining of MK cell surface markers (CD41/CD61) in MKs was evaluated. E. Photomicrograph of cells stained with Giemsa showing the cell and nuclear size were increased for MKs on day 10. F. qRT-PCR results showing fold induction of CD41 and CD61 expressions in cells of day 10 as compared to the cells of day 0 (*p<0.05). G. DNA content (polyploidy status) was increased in MKs compared to iPSCs-HSCs which was analyzed by MUSE analyzer.

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

Figure 3. p53, HOTAIRM1 and miR-125b interaction study in PMA induced megakaryocytic differentiating cell line model. A. qRT-PCR results show the differential expression of HOTAIRM1 and miR-125b in PMA-induced MKs in comparison with control (uninduced) K562 cells (**p<0.002). B. HOTAIRM1 and miR-125b sequence interaction. Minimum free energy (mfe) duplex of miR-125b and HOTAIRM1 was determined by using IntaRNA tool. C. RNAhybrid prediction analysis of miR-125b with its target gene p53 by using TargetScan tool. D. The protein levels of p53, p21 and components of intrinsic apoptosis pathway were increased in PMA induced MKs in comparison with control (**p<0.02). E. Quantification of protein expression w.r.t. β-actin (**p<0.02, *p<0.01).
Figure 4. PMA-induced MK differentiation involves ROS production during maturation. A. For measurement of intracellular ROS production in cells, the DCFH-DA assay was performed and analyzed by flow cytometry. B. qRT-PCR analysis shows the increased expression of MK marker CD61 during PMA-induced MKs maturation of K562 (**p<0.02). C. In qRT-PCR analysis, MK marker CD61 expression was increased in PMA treated cells in comparison with control, whereas, it decreased in NAC-treated cells (**p<0.05). D. miR-125b expression was higher in ROS scavenger NAC-treated cells compared to untreated controls, but lower in PMA-treated cells (**p<0.05). E. The pictorial representation of the crosstalk between lncRNA (HOTAIRM1), miRNA (miR-125b) and p53 during MK development.

Figure 5. Suppression of lncRNA HOTAIRM1 expression induces impairment the differentiation and maturation of PMA-induced K562 cells. A. si-HOTAIRM1 transfected cells show similar morphology to control as compared to PMA-induced megakaryocytic cells. (20X; 50 μm). B. qRT-PCR results shows the si-HOTAIRM1 transfection elevated the level of miR-125b which further reduced the expression of its respective target genes (**p<0.02, *p<0.01). C. si-HOTAIRM1 transfection significantly inhibits the protein expression of p53 and Cyclin D1 as analyzed by western blot. D. A plot showing the actual mean band intensity of the blots (*p<0.01).
Table 1: Primers used in the current study

<table>
<thead>
<tr>
<th>Gene/IncRNA</th>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Amplicon size (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>ACCACAGTCCATGCCATCAC</td>
<td>420</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCACCACCCTGTTGCTGTA</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>Forward</td>
<td>CTCGGCAACGGATATCTCG</td>
<td>400 – 900</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCCTCAACCTAATGGCTTC</td>
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</tr>
<tr>
<td>CD61</td>
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<td>260</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAGCTGCCAATAAGGCAAA</td>
<td></td>
</tr>
<tr>
<td>CD41</td>
<td>Forward</td>
<td>TCAACCCTCTCAAGGTGGAC</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCAGCACAAACTGATCCAGA</td>
<td></td>
</tr>
<tr>
<td>HOTAIRM1</td>
<td>Forward</td>
<td>AGGGGGTTGAAATGTGGGTG</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTGAAAGTGAGAAATAAAGTGCC</td>
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<tr>
<td>miRNA</td>
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<td>Length</td>
</tr>
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<td>--------------------------------</td>
<td>--------------------------------</td>
<td>--------</td>
</tr>
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<td>TCAACCGAGGCGCCTGTCTC</td>
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<td>LUST</td>
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<td>CGTGGAATCAATGGAGTGGA</td>
<td>745</td>
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<tr>
<td>miR-125b</td>
<td>CTTGCCAGAAACGTCAATGGA</td>
<td>GTGCAACTACGTGTCATAGCCTG</td>
<td>20</td>
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Table 2: Concentration of growth factors used for MKs differentiation

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Growth Factors</th>
<th>Stock Conc. (µg/mL)</th>
<th>Working Conc. (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FGF2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>BMP4</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>SCF</td>
<td>100</td>
<td>20 (2.0 µL/3 mL)</td>
</tr>
<tr>
<td>4</td>
<td>VEGF-2</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>TPO</td>
<td>50</td>
<td>50 (3.0 µL/3 mL)</td>
</tr>
<tr>
<td>6</td>
<td>IL-11</td>
<td>2</td>
<td>10 (15.0 µL/3 mL)</td>
</tr>
<tr>
<td>7</td>
<td>IL-6</td>
<td>10</td>
<td>10 (3.0 µL/3 mL)</td>
</tr>
</tbody>
</table>
Figure 1

[A] Analysis of CD34

- hiPSCs-derived CD34* cells (hiPSCs-HSCs)
- Media change
- Day 5
- Media change
- Day 7
- Phase II: MKs Differentiation Media
- Day 10

- FGF2, BMP4, SCF, VEGF-2, TPO, IL-11 and IL-6 (Growth Factors and Cytokines)

[B] IPSC-HSCs on Day 0

- MKs on Day 10

[C] ISO DAPI

- IPSC-HSCs
- P1
- P2

- Day 0

ISO PE

- Day 10 (Fold of Day 0)

[D] P1

- CD41
- CD61

- Q1
- Q2

- Day 0

[E] Day 10

[F] Relative fold change

- CD41
- CD61

- Day 0
- Day 10

[G] iPSC-HSCs

- MKs

- DNA CONTENT PROFILE

- DNA CONTENT PROFILE

- DNA CONTENT PROFILE

- DNA CONTENT PROFILE
Figure 5

[A] Control  PMA  si-HOTAIRM1

[B] Relative fold change

- ** Control
- Blue dots: MKs
- Purple dots: si-HOTAIRM1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>MKs</th>
<th>si-HOTAIRM1</th>
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<tbody>
<tr>
<td>HOTAIRM1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-125b</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD 41</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CD 61</td>
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</table>

ncRNAs  cell cycle regulators  MK markers

[C]  [D]

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>si-HOTAIRM1</th>
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</thead>
<tbody>
<tr>
<td>53 KDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 KDa</td>
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<td></td>
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<tr>
<td>42 KDa</td>
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</tbody>
</table>

Relative protein expression corrected by β-actin

- Red dots: Control
- Purple dots: si-HOTAIRM1

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>si-HOTAIRM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

0.0  0.5  1.0  1.5  2.0  2.5

p53  Cyclin D1