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Original Article

The effect of decitabine on human induced pluripotent stem cells (hiPSCs) derived CD34$^+$ cells expansion and the megakaryocytes generation and maturation

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Abstract: Background: Epigenetic modifiers play an important role in regulating the fate of hematopoietic stem cells (HSCs). The chromatin-modifying agents (CMA) have previously been shown to expand HSCs from cord blood (CB) and bone marrow (BM) CD34$^+$ cells. Meanwhile, DNA methylation maintains persistent cellular memories and is thought to be the primary epigenetic barrier to reprogramming. The DNA hypomethylation drug decitabine is one of the CMA that could alter gene expression and HSC self-renewal. It has been reported that decitabine could promote platelets generation in ITP patients. Objective: It's unknown if decitabine could affect CD34$^+$ cells and megakaryocytes generation and maturation from human induced pluripotent stem cells (hiPSCs). Methods: We utilized serum free, exon free and feeder free differentiation system to generate CD34$^+$ from hiPSCs and induced them differentiation into megakaryocytes. Different concentrations of decitabine were added at different stages and analyzed these cells by RT-PCR, flow cytometry analysis, cell counting and other regular experimental methods. Results: The proliferation and function of CD34$^+$ cells in vitro were significantly suspended after exposure to decitabine. Low concentration of decitabine could maintain the CD34$^+$ function. In addition, we found that decitabine did not have any effect on the megakaryocyte generation, but it prevented megakaryocyte maturation. The DNA methyltransferases (*DNMT*s) changed a lot not only in CD34$^+$ stage but also in the megakaryocyte generation and maturation due to decitabine addition. Conclusions: These results suggested that the effect of decitabine on CD34$^+$ cells from hiPSCs was very different from CB, PB and BM CD34$^+$ cells and the epigenetic changes may play an important role in the CD34$^+$ expansion and megakaryocytes maturation. It may provide a potential mechanism of studying hiPSCs derived HSCs and megakaryocytes maturation in the future.

Keywords: Human induced pluripotent stem cells (hiPSCs), CD34$^+$ cells, decitabine, megakaryocytes

Introduction

All blood cells originate from hematopoietic stem cells (HSCs), which represent the apex of a differentiation cascade of progenitor cell types that give rise to billions of new cells every day [1]. HSCs are rare cells, relatively quiescent under steady state conditions [2, 3], which express specific phenotypic markers and possess characteristic functional properties [4]. However, they could lose their characteristic function when they are exposed to cytokines in vitro. There are several sources of HSCs ex vivo: Umbilical cord blood (UCB), fetal liver, adult bone marrow (BM) and cytokines-mobilized peripheral blood (PB) [2]. With the emergence of the pluripotent stem cells in the past decades, human HSCs also could be acquired from all these differentiated human cells, including human embryonic stem cells (hESCs) and hiPSCs [5].

Epigenetic modifiers play an important role in regulating the fate of HSCs [6] and could modulate cell differentiation and proliferation. The chromatin-modifying agents (CMA) have previously been shown to expand HSCs from UCB, BM and PB CD34$^+$ cells [3, 6, 7]. Meanwhile, DNA methylation maintains persistent cellular memories and is thought to be the primary epi-

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genetic barrier to reprogramming [8-10]. DNA methylation map of HSCs demonstrate that HSCs are much more hypermethylated than their lineage cells [1]. Meanwhile, different mature megakaryocytes are much more highly methylated than the other megakaryocyte progenitor cell types [1].

DNA methyltransferases (*DNMTs*) are the group of enzymes responsible for establishment and maintenance of genomic DNA methylation. They include the de novo methyltransferases Dnmt3a and Dnmt3b, and the maintenance methyltransferase Dnmt1 [11, 12]. The cytosine analogue 5-aza-2'-deoxycytidine (decitabine), as a drug, could induce primary cells terminal differentiation for the treatment of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) by relieving aberrant epigenetic gene silencing [13, 14]. It has been reported that low dose of decitabine could induce megakaryocytes maturation and platelets generation in ITP patients [15]. Additionally, decitabine could deplete DNA methyltransferase 1 (*DNMT1*), which is an essential enzyme for regulating hematopoietic stem and progenitor cells (HSCs/HPCs) and can increase self-renewal of HSCs, which might play a role in ex vivo expansion of HSCs [16, 17]. Therefore, the mechanism underlying decitabine induced differentiation is poorly understood.

Because human $CD34^+$ cells from hiPSCs are much easier to obtain than other sources and it might be used for personalized treatment in the future. In this study, we assessed the effects of decitabine on $CD34^+$ cells expansion and their differentiation into megakaryocytes from hiPSCs in vitro. A unique system was used to generate the $CD34^+$ cells and megakaryocytes as previous published paper [5] and decitabine was added at different concentrations and different stages. Our results showed that the number of $CD34^+$ cells was decreased significantly after incubating with decitabine for 7 days. However, the percentage of $CD34^+$ cells was increased after decitabine treatment. Low concentrations of decitabine maintained the HSCs self-renewal by over-expressing the implicated sell-renewal genes. Meanwhile, the expression of CD42b was significantly decreased after adding decitabine and it had no effect on megakaryocyte generation in our experiments. The result was verified by analysis of megakaryocyte-associated genes. The expression of DNMTs changed a lot not only at $CD34^+$ stage but also during the megakaryocyte generation and maturation. Our results contributed to a better understanding of the role of decitabine on hiPSCs derived $CD34^+$ cells expansion and their differentiation and maturation into megakaryocytes.

Material and methods

Maintenance and expansion of hiPSC

Human iPSC line, BC1 cell, was gifted from Johns Hopkins and cultured in regular Essential 8 (E8) medium under feeder-free condition (Life Technologies). BC1 cells were kept in an undifferentiated state and routinely passaged as small clumps using 0.5 mM EDTA. To enhance the cell survival, 5 μM ROCK inhibitor Y27632 (Sigma) was added into the medium for the first 24 hours after seeding. The cell medium was changed every day to keep the undifferentiated condition.

$CD34^+$ cells generation and isolation from cultured hiPSCs

BC1 cells were differentiated into $CD34^+$ cells, using the "spin-embryoid body" (spin-EB) method in feeder-free and serum-free conditions from our previously published paper [5]. On day 14, the suspended cells were harvested and then filtered through 100-mm cell strainers (BD Biosciences) to remove the EBs. The harvested single cells from BC1 cells were sorted by $CD34^+$ and were immunomagnetically purified using a magnetic activated cell sorting system, $CD34^+$ MACS (Miltenyi Biotech), according to the manufacturer's instructions. The purity of the $CD34^+$ population was determined by immunolabeling with CD34-PE (eBioscience).

$CD34^+$ cells expansion and differentiation into megakaryocytes

The sorted $CD34^+$ cells were expanded for 7 days in SFM medium supplement with SCF (100 ng/ml, R&D), TPO (50 ng/ml, PeproTech), FLT-3 ligand (100 ng/ml, Pepro Tech). For MK differentiation and maturation, the sorted $CD34^+$ cells were seeded into 12-well plate (3×10^5/well) and then cultured in SFM with SCF (20 ng/ml, R&D), TPO (50 ng/ml, PeproTech), IL-11 (10 ng/ml, PeproTech) for additional 5 days.

Table 1. All the primers were used in this experiment

Primer	Forward	Reverse
GAPDH	AGC CAC ATC GCT CAG ACA C	GCC CAA TAC GAC CAA ATC C
GATA2	ACTGACGGAGAGCATGAAGAT	CCGGCACATAGGAGGGGTA
BMI-1	TGGCTCTAATGAAGATAGAGG	TTCCGATCCAATCTGTTCTG
HOXB4	TCCCACTCCGCGTGCAAAGA	GCCGGCGTAATTGGGGTTTA
MPO	ACCCTCATCCAACCCTTC	GTCAATGCCACCTTCCAG
GATA1	CAC TGA GCT TGC CAC ATC C	ATG GAG CCT CTG GGG ATT A
p21	GTCTTGTACCCTTGTGCCTC	GGTAGAAATCTGTCATGCTGG
p27	TTTAATTGGGTCTCAGGCAAACTCT	CCGTCTGAAACATTTTCTTCTGTTC
Notch1	GAGGCGTGGCAGACTATGC	CTTGTACTCCGTCAGCGTGA
DNMT1	AGGCGGCTCAAAGATTTGGAA	GCAGAAATTCGTGCAAGAGATTC
DNMT3A	CCGATGCTGGGGACAAGAAT	CCCGTCATCCACCAAGACAC
DNMT3B	AGGGAAGACTCGATCCTCGTC	GTGTGTAGCTTAGCAGACTGG
FOG1	GAG AGG AGG TGC AGT TGG TG	GTG GGG GTG AGT TAA CAT CTG
β1-tublin	GGA TGC GTG AAA TTG TCC AT	AGT CGA TCC CGT GTT CCT C
NFE2	GCAGGAACAGGGTGATACAGC	GCAGCTCGGTGATGGACAT

RNA extraction and real-time PCR

The cells were harvested at pointed time points and extracted RNeasy Mini kit (Qiagen). The cDNA was generated using oligo dT_{18} primers (Life Technologies) and M-MLV reverse transcriptase (Life Technologies). Real-time PCR was performed using SYBR green Master Mix (Roche Technologies) following the manufacture's guideline and amplified by Step one plus system (ABI). The relative expression was calculating by normalizing the levels of GAPDH. Measurements were performed in triplicate and negative controls without cDNA template were included in each assay. The primer sequences were shown in **Table 1**.

Cell counting and viability assay

Countess II (Invitrogen, Life technology) was used for both cell counting and assessment of cell viability as the manufacturer's instructions. Cell counting was validated using the trypan blue exclusion method in selected situations: Each experiment was repeated three independents and three times.

Colony forming unit (CFU) assays

The sorted $CD34^+$ cells were seeded into methylcellulose-enriched media (MethoCult H4434, STEMCELL Technologies). The mixture cells were plated in 35 mm culture dishes at densities of 6×10^4/dish. Colonies were counted after 14 days of incubation using a standard criterion under the inverted microscope.

Treatment cells with decitabine

Decitabine stock solution (10 mM) was generated by reconstituting lyophilized decitabine in water (Sigma). Stock solution was stored at -80°C for up to 6 months. Working solution was calculated before the addition to the cells. Cells were treated with different concentrations of decitabine at the indicated time points in this experiment.

Flow cytometry

The percentage of sorted and expansion $CD34^+$ were labeled with CD34-PE (eBioscience). Megakaryocytes were labeled with anti-CD41a-APC (BD Biosciences), CD42b-fluorescein isothiocyanate (FITC) (eBioscience). The cells were incubated with the antibody for 30 minutes on ice and then washed with PBE (PBS, 1%FBS or BSA, 0.5 mM EDTA) twice. The stained cells were filtered before FACS and analyzed on Beckman coulter cytometer. At least 20,000 live cells were acquired per analysis. Ig isotype controls were used as the control for flow cytometry in each experiment.

Statistical analysis

The results are presented as the mean ± standard deviation (SD), if not otherwise specified. A two-tailed Student's t test or two-way analysis of variance was performed using GraphPad Prism, version 7, software (GraphPad Software) for comparisons between two groups (with decitabine versus without decitabine). The significance level was set as $P<0.005$ or $P<0.0001$.

Results

Decitabine inhibits $CD34^+$ cells expansion and differentiation ability

To confirm whether the function of $CD34^+$ cells from hiPSCs were affected by decitabine in vitro, different concentrations of decitabine (1 nM, 10 nM, 100 nM, 500 nM and 1 μM) were added into the expansion medium $CD34^+$ cells and incubated in serum-free media (SFM) supplemented with the cytokines for 7 days. The

untreated CD34$^+$ cells were used as control. A schematic diagram of the strategy in this study was shown in **Figure 1A**. The number of decitabine treated CD34$^+$ cells were lower than untreated cells. The concentration and cell number were positively correlated (**Figure 1B**). However, decitabine treated CD34$^+$ cells contained a larger proportion of CD34$^+$ cells for 7 days culture compared to control (**Figure 1C**, **1D**). Whereas the number of CD34$^+$ cells was nearly the same as the control at low concentrations (1 nM, 10 nM) (**Figure 1D**). The CFC assay showed that the differentiation ability of CD34$^+$ cells was significantly inhibited after treating with decitabine even at low concentration (1 nM) (**Figure 1E**, **1F**). This result showed that decitabine inhibited CD34$^+$ differentiation while it could maintain CD34$^+$ population at low concentrations in vitro.

The alter expression of DNA methyltransferases (DNMTs) after decitabine addition

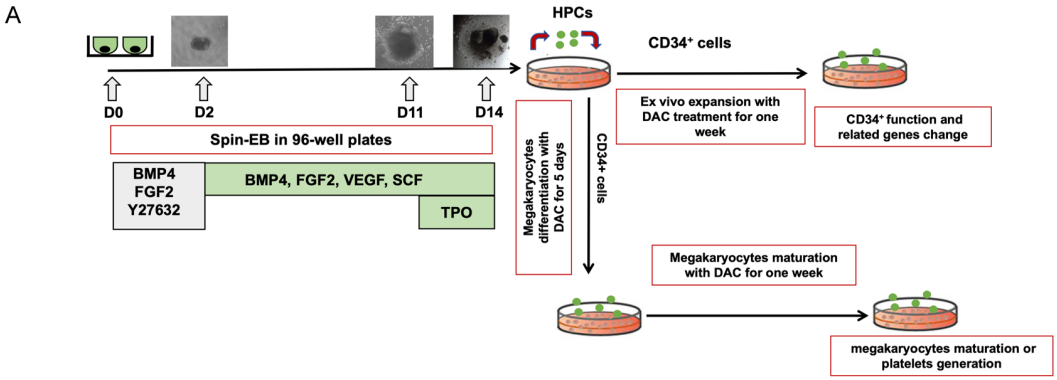
Previous study showed that decitabine could act on *DNMT1*, which was more essential for the HSC self-renewal [11]. Meanwhile, some studies also showed that the other two *DNMT*s, *DNMT3A* and *DNMT3B*, which were highly homologous, were essential for the HSCs differentiation [18, 19]. In our study, the expression level of the *DNMT*s expression was measured after different concentrations of decitabine for 7 days treatment. Real-time PCR results showed that *DNMT1*, *DNMT3A* and *DNMT3B* decreased significantly after 7 days culture compared to day 0 control cells (**Figure 2**). The *DNMT1* and *DNMT3A* expression increased after decitabine treatment on day 7. The transcriptional level of *DNMT1* was negatively correlated with the concentrations, while the *DNMT3A* expression was positively with the concentrations. On the contrary, low dose of decitabine had no effect on *DNMT3B* expression. However, the *DNMT3B* expression could not be detected with higher concentrations (500 nM, 1 μM) treatment on day 7 (**Figure 2**). The expression of *DNMT*s changed significantly after decitabine treatment, and this might be the reason behind the inhibition of CD34$^+$ function.

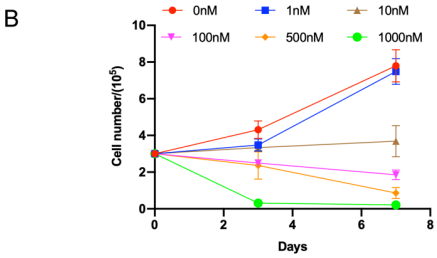
Treatment with decitabine alters the expression of self-renewal related genes in HSCs

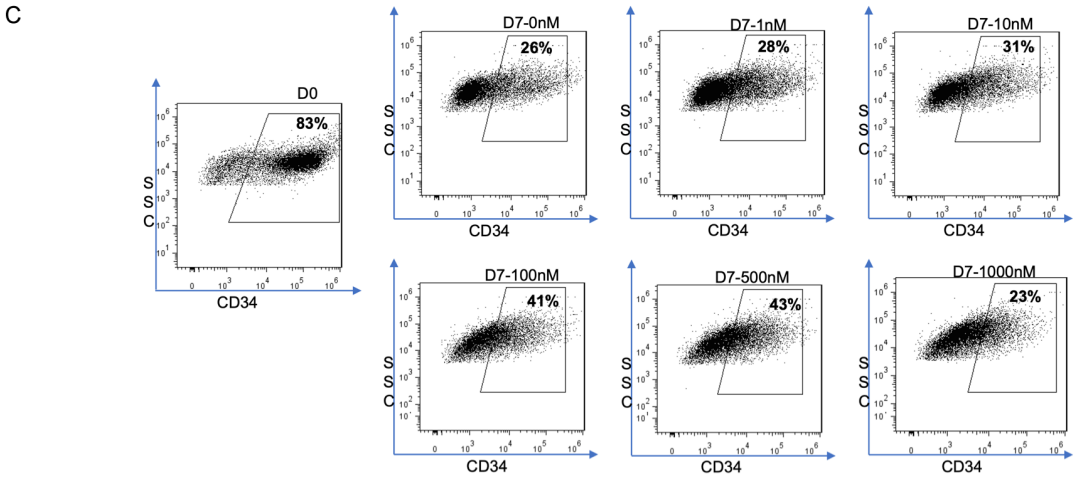
To understand the molecular mechanism of HSCs function after decitabine treatment, we detected the expression of several self-renewal related genes in HSCs using real-time PCR. Primers specific for *HOXB4*, *BMI-1*, *GATA 2*, *NOTCH1*, *P21*, *P27*, *GATA 1*, and myeloperoxidase (*MPO*) were used (**Table 1**). Total RNA from cells were extracted from different concentrations of decitabine and control cells (day 0 and day 7). The expressions of these genes were up regulated except *MPO* and *HOXB4* on day 7 compared to day 0. We observed relatively higher levels of transcripts for *BMI-1* and *GATA-2* genes in cells cultured with decitabine in the culture. The *HOXB4* gene was inhibited in decitabine treated cells except at 10 nM concentration. In addition, the transcriptional levels of *P21* and *P27* were increased to a greater degree in higher concentration of decitabine-treated cells after 7 days of culture (**Figure 3**), which could regulate cell cycle. The higher expression of *P21* and *P27* in decitabine treated cells is consistent with the proposed role of these gene products that could regulate the cycling behavior of HSCs [20, 21]. The *Notch1* expression, which was related with promoting stem cell expansion, was examined [22, 23]. Surprisingly, *Notch1* expression is increased in higher concentration (100 nM, 500 nM) decitabine-treated cells (**Figure 3**). The expression of lineage-specific genes such as *MPO* and *GATA-1* were also examined. *GATA1* is a transcriptional factor, which is important for commitment of HSCs to adult erythroid and megakaryocytic lineages [24], and increased transcription of *GATA1* was seen at lower concentrations of decitabine (1 nM, 10 nM, and 100 nM). The expression of *MPO* is associated with granulocytic differentiation [25]. The transcript level of *MPO* was strongly inhibited in decitabine treated cells.

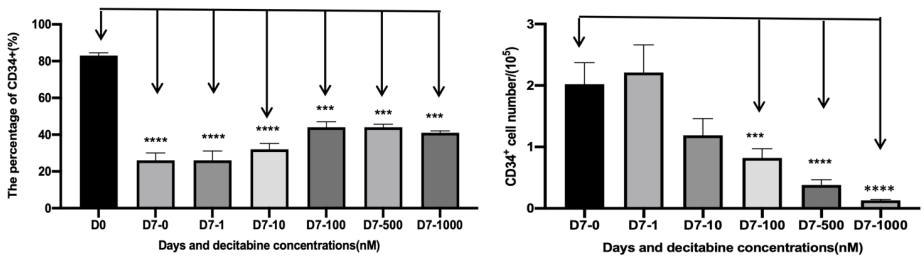
Megakaryocytes generation after decitabine treatment

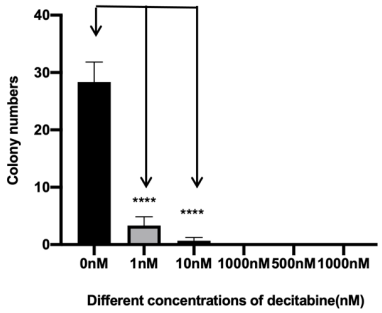
Treatment with decitabine reduced methylated CG levels in megakaryocyte progenitor cells (MKPs) and enhanced megakaryocyte production in mice [26]. Thus, we evaluated the effect of decitabine on megakaryocytes generation. A previous reported system to generate megakaryocytes from hiPSCs derived CD34$^+$ cells [5]. CD34$^+$ cells were sorted on day 14 and cultured in SFM medium containing cytokines (Day 0) with or without different concentrations of decitabine for 5 days (Day 5). The cell proliferation decreased dramatically after adding


A
HPCs
CD34+ cells
D0
D2
D11
D14
Spin-EB in 96-well plates
BMP4
FGF2
Y27632
BMP4, FGF2, VEGF, SCF
TPO
Megakaryocytes differentiation with DAC for 5 days
CD34+ cells
Ex vivo expansion with DAC treatment for one week
CD34+ function and related genes change
Megakaryocytes maturation with DAC for one week
megakaryocytes maturation or platelets generation


B
0nM
1nM
10nM
100nM
500nM
1000nM
Cell number/(10^5)
Days


C
D0
83%
D7-0nM
26%
D7-1nM
28%
D7-10nM
31%
D7-100nM
41%
D7-500nM
43%
D7-1000nM
23%
SSC
CD34


D
The percentage of CD34+(%)
D0
D7-0
D7-1
D7-10
D7-100
D7-500
D7-1000
Days and decitabine concentrations(nM)
CD34+ cell number/(10^5)


E
Colony numbers
0nM
1nM
10nM
1000nM
500nM
1000nM
Different concentrations of decitabine(nM)

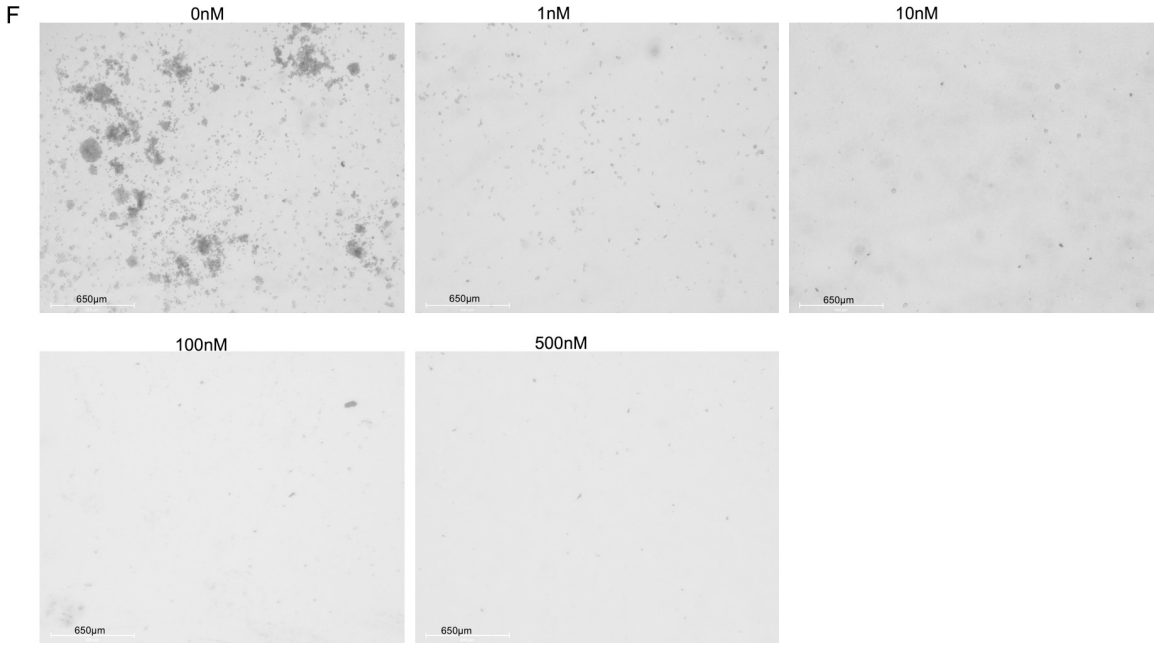


Figure 1. The function of hiPSCs derived CD34$^+$ cells expansion and differentiation ability after decitabine addition. A. The schematic diagram of the strategy used in this study for the CD34$^+$ function and differentiation into megakaryocyte. B. The curve of CD34$^+$ cell number after adding decitabine for one week, n=3. C. Flow cytometry analysis of CD34$^+$ population from hiPSCs. D. The percentage and number of CD34$^+$ cells from hiPSCs treated with decitabine for one week. E. The function of CD34$^+$ differentiation after adding different concentrations of decitabine by colony forming cell (CFC) in vitro, n=3. F. Photos of CFC at day 14 after adding different concentrations of decitabine (nM), scale bar, 650 μm. Note: Values are mean ± SD, initial cells number was 3×10^5, cytokines alone were set as 0 nM, ***P<0.005, ****P<0.0001.

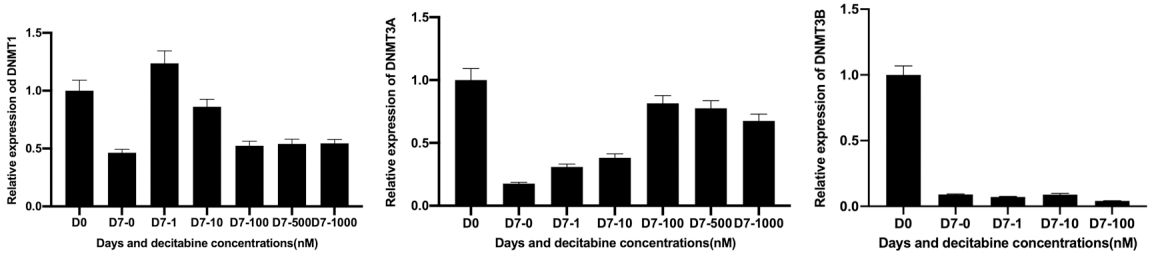


Figure 2. The DNA methyltransferases expression of CD34$^+$ cells after adding decitabine for one week in vitro. The relative transcription expression levels of DNA methyltransferases (*DNMT1*, *DNMT3A* and *DNMT3B*) were measured by real-time quantitative PCR (qRT-PCR).

decitabine even at low concentration (1 nM) (**Figure 4A**). The percentage of CD41a and CD42b on day 5 were higher than on day 0. After decitabine treatment for 5 days, the proportion of CD41a had no big difference between different concentrations of decitabine treated cells and the control cells (**Figure 4B**, **4C**). However, the population of CD42b was significantly decreased, which was a crucial marker for the megakaryocytes and platelet function (**Figure 4B**). However, the number of CD41a and CD42b were much lower than the cytokines only on day 5 (**Figure 4C**). And the number of CD41a and CD42b were negatively correlated with the concentrations (**Figure 4C**). The status and morphology of the cells at different concentrations of decitabine were also showed that there were less cells after treating with higher concentrations of decitabine (**Figure 4D**). The maturation genes associated with megakaryocytes, including *GATA1*, *FOG1*, *NFE2*, and *β1-tubulin*, underwent different changes after adding different concentrations of decitabine (**Figure 4E**). The results

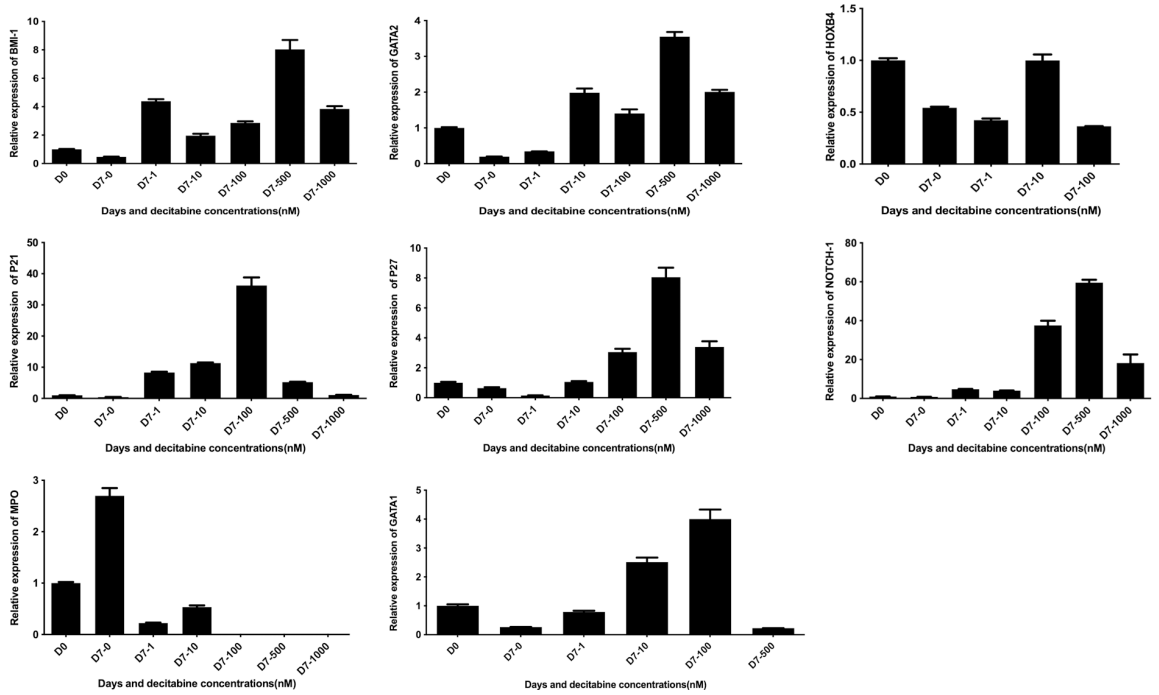
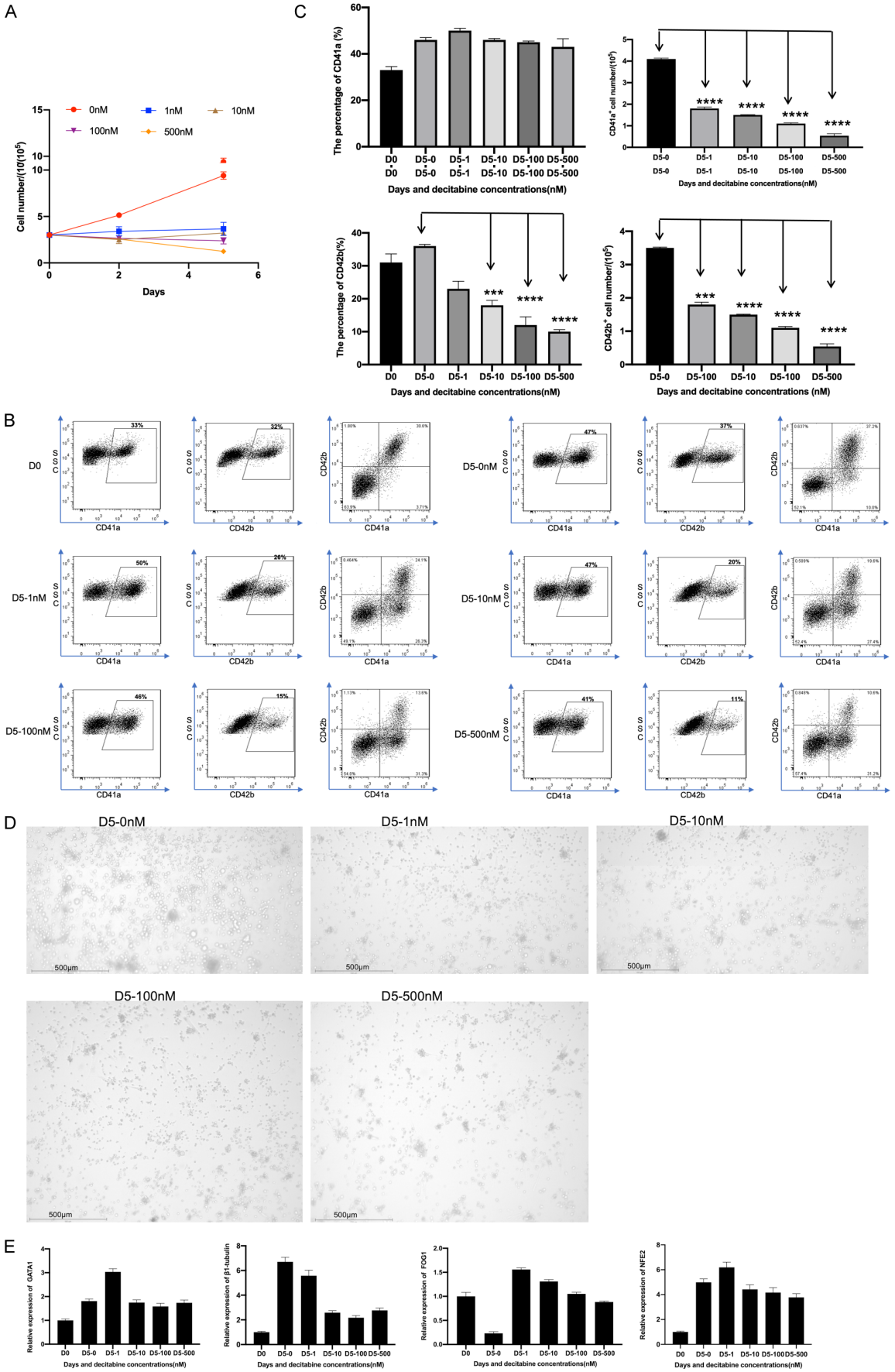


Figure 3. The transcription expression levels of HSCs self-renewal related genes after 7 days decitabine treatment in CD34$^+$ expansion procedure. The relative transcription expression levels of genes (*HOXB4*, *BMI-1*, *GATA 2*, *NOTCH 1*, *P21*, *P27*, *GATA 1*, and *MPO*) were measured by qRT-PCR.

manifested that maturation related genes were much higher expression on day 5 than day 0 except for *FOG1*. During maturation, megakaryocytes expressed *β1-tubulin* and rearranged their microtubule components to enlarge, form proplatelets, and eventually release platelets [27]. The transcriptional level of *β1-tubulin* was inhibited by decitabine, and it was negatively correlated with the concentration (**Figure 4E**). The expression levels of *DNMT*s changes were also detected with or without the decitabine treatment and we found that different concentrations of decitabine had different effects on *DNMT*s expression. *DNMT1* expression was not affected by decitabine treatment, but it promoted increased expression of *DNMT3A* and *DNMT3B* at low concentrations compared to control cells on day 5 cells (**Figure 4F**). It seemed that decitabine did play an important part in the megakaryocyte generation.

Effects of megakaryocyte maturation with decitabine treatment

It has been reported that decitabine could promote CB CD34$^+$ derived megakaryocytes maturation and platelets generation [15]. To determine the function of decitabine on hiPSCs derived megakaryocytes maturation, different concentrations of decitabine were added into day 5 megakaryocytes and cultured for one week. The cell proliferation was suspended after adding decitabine (**Figure 5A**). The number of control cells were increased almost up to 1.5-fold (**Figure 5A**) than decitabine treated cells. On day 12 the population of CD41a$^+$ and CD42b$^+$ were decreased dramatically, especially the percentage of CD42b$^+$ compared to day 5 in the control group (**Figure 5B**, **5C**). The percentage of CD41a$^+$ cells increased after adding decitabine compared to day 5, and higher concentration of decitabine got relatively higher percentage of CD41a$^+$. The CD42b expression was decreased compared to day 12 control (**Figure 5B**, **5C**). The number of CD41a and CD42b cells were significantly reduced compared to control cells on day 12 (**Figure 5C**). The concentration of decitabine had little effect on the numbers of CD41a$^+$ and CD42b$^+$ cells. The *DNMT*s expression were also detected after decitabine treatment. The *DNMT1* and *DNMT3A* expression of control cells on day 12 were higher than on day 5 control cells (**Figure 5D**). Whereas the *DNMT3B*


A
0nM
1nM
10nM
100nM
500nM
Cell number/(10(10^5)
Days
C
The percentage of CD41a (%)
Days and decitabine concentrations(nM)
CD41a+ cell number/(10^5)
The percentage of CD42b(%)
CD42b+ cell number/(10^5)
D0
D5-0
D5-1
D5-10
D5-100
D5-500
B
D0
D5-0nM
D5-1nM
D5-10nM
D5-100nM
D5-500nM
SSC
CD41a
CD42b
D
D5-0nM
D5-1nM
D5-10nM
D5-100nM
D5-500nM
500μm
E
Relative expression of GATA1
Relative expression of β1-tubulin
Relative expression of FOG1
Relative expression of NFE2
Days and decitabine concentrations(nM)

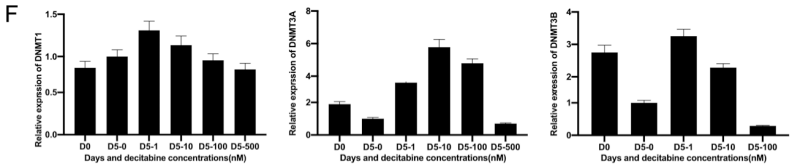
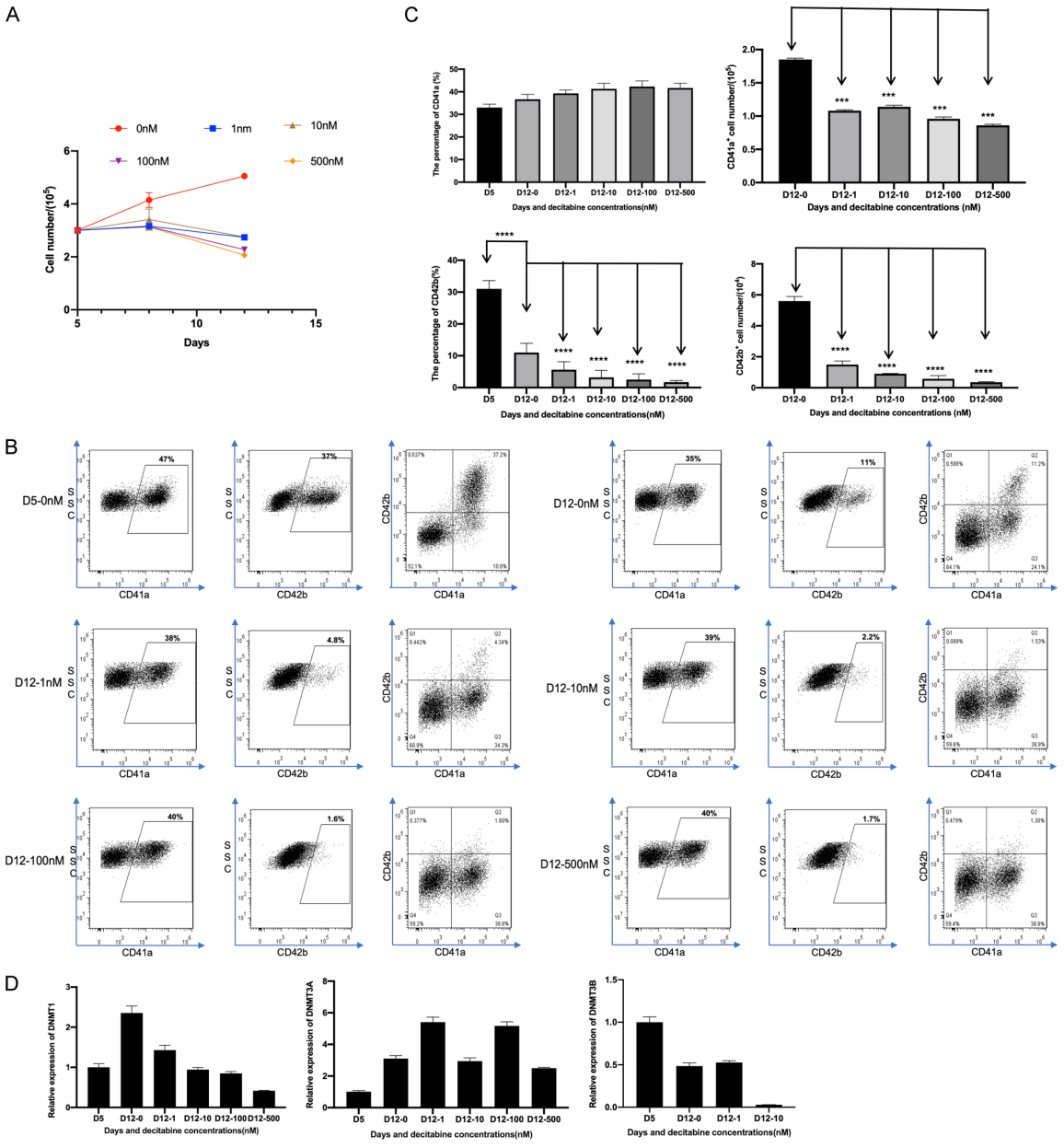


Figure 4. The effect of decitabine on megakaryocytes generation from hiPSCs derived CD34$^+$ cells. A. The cell curve of megakaryocytes from megakaryocyte day 0 to day 5 for 5 days decitabine treatment, n=3. B. Flow cytometry analysis of megakaryocyte marker: CD41a and CD42b expression on day 5 megakaryocyte with decitabine treatment. C. The percentage and cell number of CD41a and CD42b on day 5 megakaryocyte, n=3. D. Photos of day 5 megakaryocyte with decitabine treatment. Scale bar, 500 μm. E. The maturation related genes expression on day 5 megakaryocyte. F. The DNA methyltransferases expression on day 5 megakaryocyte after decitabine treatment. Note: Initial cells number was 3×10^5. Starting time point was megakaryocyte day 0 that was also called EB day 14. Without decitabine treatment was 0 nM. Values are mean ± SD. ***P<0.005, ****P<0.0001.



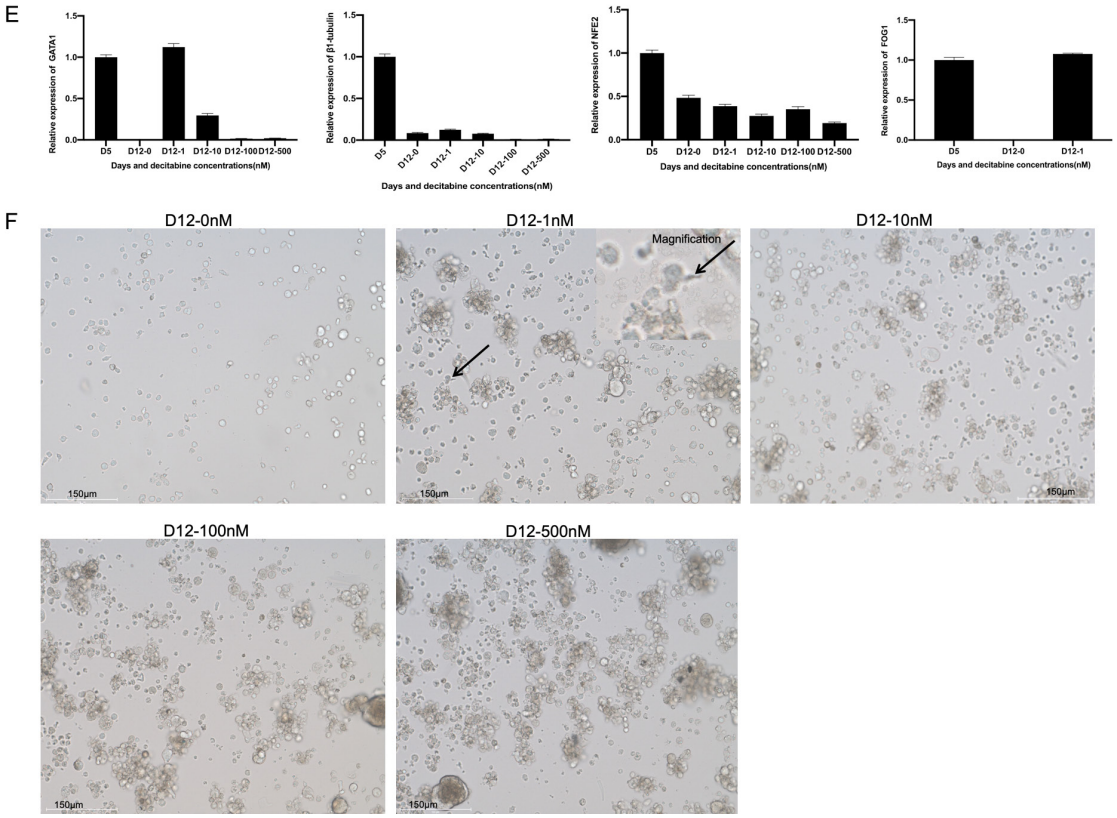


Figure 5. The maturation of megakaryocytes on day 12 after adding decitabine for one week. A. The cell curve of megakaryocytes from megakaryocyte day 5 to day 12 after adding decitabine for one week, n=3. B. Flow cytometry analysis of CD41a and CD42b expression on day 12 megakaryocyte after 7 days treatment with decitabine. C. The CD41a and CD42b population and cell number on day 12 megakaryocyte after 7 days treatment, n=3. D. The DNA methyltransferases expression on megakaryocytes day 12 after different decitabine treatment for one week. E. The maturation related genes expression on day 12 megakaryocytes day 12 after adding decitabine for one week. F. Photos of day 12 megakaryocyte at different concentrations of decitabine scale bar, 150 μm, the arrow showed proplatelet-bearing megakaryocytes. Note: Initial cells number was 3×10^5. Starting day was megakaryocyte day 5. Without decitabine treatment was 0 nM. Values are mean ± SD. ***P<0.005, ****P<0.0001.

expression was much lower. After treated with decitabine, the *DNMT1* and *DNMT3B* expression were inhibited, but the expression of *DNMT3A* did not change a lot with decitabine treatment compared to control (**Figure 5D**). All the maturation associated genes were down-regulated on day 12 in control cells and *FOG1* expression could not be detected (**Figure 5E**). The expression of *GATA1*, *FOG1*, and *β1-tubulin* were increased at 1 nM decitabine. At all the other concentrations, transcription was strongly inhibited or showed no significant change in treated cells (**Figure 5E**). Surprisingly, there were fewer proplatelet-bearing megakaryocytes at 1 nM concentration (**Figure 5F**). Our results demonstrated that longer treatment of decitabine could increase the CD41a expression, but still prevented megakaryocytes maturation.

Discussion

Numerous investigators have tried to find some optimal ex vivo conditions that favor HSCs self-renewal using DNA transferase [28, 29]. Several groups have hypothesized that short-term exposure of primary human or murine HSCs to low doses of CMA might be useful for reprogramming cells generated during the ex vivo expansion of primary HSCs [31, 32]. However, compared with the results from UB, PB and BM derived $CD34^+$ cells, hiPSCs derived $CD34^+$ cells were totally different in our experiments. With the long treatment and different concentrations of decitabine, the total cell number and $CD34^+$ cells were decreased dramatically. The differentiation ability of $CD34^+$ was inhibited in terms of both megakaryocyte generation and maturation, and

decitabine promoted a modest increase in CD41a expression, although megakaryocyte maturation was still prevented.

DNA hypomethylation drug decitabine is one of the CMA that could alter gene expression and HSCs self-renewal and expansion in CB or PB [25]. *DNMT1* is essential for HSCs self-renewal [11]. It is reported that decitabine could deplete *DNMT1* that affects HSC function in vitro [25]. Our results showed that the expression of *DNMT1* was not depleted after adding decitabine during $CD34^+$ cells expansion stage. On the contrary, *DNMT1* expression was increased especially at low concentration (1 nM), suggesting that *DNMT1* is not a crucial factor for hiPSC-derived $CD34^+$ cell self-renewal. A published paper reported that *DNMT3A* negatively regulated megakaryogenesis in megakaryocyte lineages derived from primary HSCs in mice [26]. In our experiment, *DNMT3A* expression was up regulated, and it was positively correlated with the concentrations. The result was opposite with the published paper. However, *DNMT3B* expression was not detected at 500 nM and 1 μM. Altogether, these results suggest that *DNMT3A* and *DNMT3B* might play an important role in inhibiting hiPSC-derived $CD34^+$ cell function.

Several transcriptional genes that have been implicated in determining the function and fate of HSCs are detected in this experiment as shown in published paper [3]. These genes include *HOXB4*, *BMI-1*, *GATA-2*, *Notch-1*, *P21*, *P27*, *GATA-2* and possibly other no unidentified genes. *BMI-1* gene is for the self-renewal not for the differentiation [30] and GATA-2 is important for the maintenance of HSCs and primitive HSCs [31]. *HOXB4* requires a functional *BMI* as HSCs activator and execute its function [30] and previous studies have revealed that over expressing *HOXB4* and *BMI-1* induce self-renewal of long-term multilineage repopulating HSCs without causing leukemia [30, 32]. Our results showed that the expression of *HOXB4* and *BMI-1* transcripts present in $CD34^+$ cells treated with lower concentration of decitabine may be consistent with the HSCs self-renewal.

The cell cyclin related genes *P21* and *P27* were highly expressed after 7 days treatment of decitabine compared to the control cells. Previous reported showed that absence of *P21* leaded to expansion of the stem cell pool, more active HSCs cycling [33] and greater sensitivity of the HSCs pool to exhaustion in response to a variety of challenges [33]. By contrast, *P27* has been shown not affect stem cell kinetics but rather to determine progenitor cells proliferation and the size of HSCs pool [20]. These findings could explain the expansion and exhaustion of $CD34^+$ cells after higher concentrations of decitabine treatment. *NOTCH 1* was highly expressed in our experiment, which was consistent with the previously published paper [34]. *GATA1* and *MPO*, two HSCs differentiation related gene, were also analyzed in this experiment. Transcription gene *GATA1* was increased at the low concentration after 7 days treatment, while *MPO* expression was decreased after 7 days treatment. The *GATA1* expression is associated with HSC commitment to erythropoiesis and megakaryopoiesis [35, 36]. Whereas *MPO* expression is associated with terminal myeloid maturation [25]. It suggested that the proliferation was inhibited by the exhaustion of $CD34^+$ cells.

In clinical ITP patients, low dose of decitabine could increase the platelets generation [15]. However, whether megakaryocytes generation or maturation are affected by decitabine still unclear. A modified unique system was used to explore the effect of decitabine on megakaryocytes. Different concentrations of decitabine were added at different time points to detect the megakaryocytes generation and maturation. At the megakaryocyte generation stage, we found that decitabine can suspend the megakaryocytes proliferation and the population of $CD42b^+$ decreased dramatically. CD42b is related with megakaryocytes maturation and platelet function [37]. The maturation associated genes, *GATA1*, *NFE2*, *FOG1*, *β1-tubulin* were detected with or without decitabine treatment. *GATA1* activates erythroid and megakaryocytes-specific genes [38]. *NFE2* encodes transcription factors is critical for megakaryocytes development [39] and *FOG1* controls erythrocyte and megakaryocyte differentiation [40]. There was modest higher expression of *GATA1*, *NFE2* and *FOG1* after decitabine treatment for the megakaryocyte generation, while *β1-tubulin* was inhibited after decitabine treatment, which was for the proplatelets formation from megakaryocytes during maturation. It showed that decitabine could promote megakaryocytes differentiation. However, its matura-

tion was prevented during megakaryocytes generation. The expression of *DNMT*s were complicated. The expression was no correlation with the concentrations. Decitabine did not deplete *DNMT1*, but it promoted the higher expression of *DNMT3A* and *DNMT3B* at low concentrations compared to control cells on day 5. However, their transcript expression level was decreased at the higher concentrations.

During the maturation stage, the results are nearly the same as day 5. The population of $CD41a^+$ cells were not affected by concentrations, while the cell number of $CD41a^+$ cells were decreased significantly. The percentage and number of $CD42b^+$ were decreased dramatically. All the maturation associated genes were lowly expressed on day 12 indicating that decitabine had no effect on the megakaryocyte maturation at late stage. Fewer proplatelet-bearing megakaryocytes came out at 1 nM decitabine treated cells. Unfortunately, we didn't get enough platelets for the further study. After treated with decitabine, the expression of *DNMT*s changed significantly and this observation was consistent with previously published results is consistent with the previous published papers [1]. During megakaryocytes maturation, it may not need too much methylation changes. During the hiPSCs generation, there might be too many epigenetic changes, and this might be exacerbated by decitabine treatment. Thus, the mechanism of chromatin changes in hiPSCs derived $CD34^+$ cells appear to be different from other sources of $CD34^+$ cells. Altogether, decitabine inhibited hiPSCs derived $CD34^+$ cells expansion and differentiation. Low concentration of decitabine maintained the $CD34^+$ function in vitro and the megakaryocytes maturation and platelet generation were also prevented by decitabine. It suggested that $CD34^+$ cells from hiPSCs were far different from CB, PB, BM $CD34^+$ cells and HSCs from mice. We speculated that it may be correlated with epigenetic changes caused by decitabine in hiPSCs and it should need to be further explored in future.

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Disclosure of conflict of interest

None.

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