HOXC4 up-regulates NF-κB signaling and promotes the cell proliferation to drive development of human hematopoiesis, especially CD43+ cells

Jiahui Zenga, Wencui Suna, Jing Changb, Danying Yia, Lijiao Zhua, Yonggang Zhanga, Xu Pana, Ya Zhoua, Mowen Lai, Guohui Biana, Qiongxiu Zhoub, Jiaxin Liua, Bo Chenb,c,∗

∗Institute of Blood Transfusion, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS & PUMC), Chengdu 610052, China; bState Key Laboratory of Biotherapy, Sichuan University, Chengdu 61006, China; cState Key Laboratory of Experimental Hematology, CAMS & PUMC, Tianjin 300020, China

Abstract
The hematopoietic function of HOXC4 has not been extensively investigated. Our research indicated that induction of HOXC4 in coculture system from D10 significantly promoted productions of most hematopoietic progenitor cells. CD34–CD43+ cells could be clearly classified into CD34–CD43low and CD34–CD43high sub-populations at D14. The former cells had greater myelogenic potential, and their production was not significantly influenced by induction of HOXC4. By contrast, the latter cells had greater potential to differentiate into megakaryocytes and erythroid cells, and thus had properties of erythroid–megakaryocyte common progenitors, which abundance was increased by ~2-fold when HOXC4 was induced from D10. HOXC4 level served as a natural index for the tendency to undergo hematopoiesis. Induction of HOXC4 from D10 caused more CD43+ cells sustain in S-phase with up-regulation of NF-κB signaling, which could be counteracted by inhibition of NF-κB signaling. These observations suggested that promotion of hematopoiesis by HOXC4 is closely related to NF-κB signaling and a change in cell-cycle status, which containing potential of clinical applications.

Keywords: CD43, Erythroid–megakaryocyte common progenitor (EMKP), Hematopoiesis, HOXC4, Human embryonic stem cells (hESCs), Inducible expression

1. INTRODUCTION

The class I homeobox (HOX) family of homeodomain-containing transcription factors has 39 members in mammals. These genes are distributed throughout the genome in four clusters: HOXA (7p15), HOXB (17q21), HOXC (12q13), and HOXD (2q31).1,2 These genes exhibit high conservation and redundancy of function.3

*Address correspondence: Bo Chen, Feng Ma, Institute of Blood Transfusion, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS & PUMC), No. 26, Huacai Road, Longtan Industry Park, Chenghua District, Chengdu, 610052, Sichuan, China. E-mail addresses: Bo_Chen@ibt.pumc.edu.cn, mafeng@ibt.pumc.edu.cn (B. Chen, F. Ma).

Conflicts of interest: The authors declare no conflicts of interest.

J.Z. and W.S. contributed equally to this work.

Author contributions statement: Conceived and designed the experiments: B. Chen, F. Ma. Performed the experiments: B. Chen, J. Zeng, W. Sun, J. Chang, D. Yi, L. Zhu, Y. Zhang, X. Pan, Y. Zhou, M. Lai, G. Bian, J. Liu. Analyzed and interpreted the data: J. Zeng, B. Chen, W. Sun. Contributed reagents, materials, analytical tools, or data: B. Chen, J. Zeng, W. Sun, F. Ma, J. Liu. Drafted and revisited the work: B. Chen, J. Zeng, W. Sun, F. Ma.

Received March 27, 2020; Accepted July 19, 2020.
http://dx.doi.org/10.1097/BS9.000000000000054
Copyright © 2020 The Authors. Published by Wolters Kluwer Health Inc., on behalf of the Chinese Association for Blood Sciences. This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. Blood Science © 2020 The Authors. Published by Wolters Kluwer Health Inc., on behalf of the Chinese Association for Blood Sciences. This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Blood Science

© 2020 The Authors. Published by Wolters Kluwer Health Inc., on behalf of the Chinese Association for Blood Sciences. This is an open access article distributed under the Creative Commons Attribution License 4.0 (CCBY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Blood Science
2. RESULTS

2.1. HOXB4 is expressed in a different pattern in cocultured H1 hESCs than HOXA4/1/ HOXC4/HOXD4, and shares low sequence similarity with the other genes

We monitored mRNA expression of HOXA4/HOXB4/ HOXC4/HOXD4 at various times in H1 hESCs co-cultured with AGM-S3. Expression of all four genes gradually decreased during hematopoietic development, and reached a minimum at D10 (Fig. S1, http://links.lww.com/BS/A14). HOXA4/HOXC4/HOXD4 were more highly expressed during the mesoderm induction (D0–D4) than during hematopoietic differentiation (D4–D14), although a significant increase was observed at D14 for HOXA4. HOXB4 was expressed differently during hematopoiesis than the other three members of the HOX family, including HOXC4. Amino acid sequence alignment revealed low sequence similarity between HOXB4 and HOXA4/HOXC4/HOXD4 (Fig. S2, http://links.lww.com/BS/A15), indicating that these proteins might serve different functions during hematopoiesis.

2.2. Transgenic hESCs exhibit normal inducible expression and pluripotency

We constructed PB-Tet-on-GFP-T2A-hHOXC4 and established the corresponding H1-derived inducible HOXC4/hESC line (Fig. 1A). Monitoring of HOXC4/hESCs treated with or without DOX for 48 hours by fluorescence microscopy (Fig. 1B), qRT-PCR (Fig. 1C), and western blotting assays (Fig. 1D) revealed highly stringent and efficient induction of HOXC4. Pluripotency of induced or non-induced HOXC4/hESCs was confirmed by western blotting to detect SOX2, OCT4, and NANOG proteins (Fig. 1E).

2.3. HOXC4 overexpression from D6 or later broadly promotes hESC-derived hematopoiesis

HOXC4/hESCs co-cultured with AGM-S3 cells were induced to overexpress HOXC4 during different stages of hematopoiesis and then were subjected to FACS at D4, D8, and D14. Observations at D14 indicated that HOXC4 overexpression broadly promoted the production of hematopoietic populations, including CD34−CD43+, CD34+CD43+, CD34+CD45+, CD34−CD45+, GPA +CD71+, and particularly, CD34−CD43+ cells (Fig. 2C; Fig. S3C, http://links.lww.com/BS/A16). By contrast, no significant effects on hematopoietic populations, such as CD34+CD43+ and CD34−CD43+, were observed at D8 (Fig. 2B; Fig. S3B, http://links.lww.com/BS/A16). Together, these findings indicate that hematopoiesis at D14 was significantly promoted by overexpression of HOXC4 from D6, and especially from D10. Severe inhibitory effects on CD34+CD43+ and CD34−CD43+ populations, but not on CD34+CD43+ populations, were observed at D8 when induction began at D0 (Fig. 2B). Earlier detection (D4) revealed no obvious inhibitory effects on the KDR+CD34−, KDR+CD34+, and KDR−CD34+ populations (Fig. 2A; Fig. S3A, http://links.lww.com/BS/A16). Thus, overexpression of HOXC4 might only influence the production of hematopoietic cells, but not influence the induction of mesoderm and endothelium.

To clarify how the hematopoietic potentials of these populations were promoted by HOXC4 overexpression, the GFP+ fraction or non-induced cells were sorted from the D10-induced or non-induced co-culture cells at D14, and cultured to form colonies. Colony numbers (colony-forming unit-granulocyte/macrophage (CFU-GM), colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), colony-forming unit-mixed (CFU-Mix)) were significantly higher for induced cells than non-induced cells, indicating that DOX treatment increased hematopoietic potential (Fig. 3A). Typical colonies of CFU-GM, CFU-E, BFU-E, and CFU-GM were observed (Fig. 3B–d), and erythroid cells were confirmed by May–Grunwald–Giemsa staining (MGG) (Fig. 3B,e).

2.4. HOXC4 promotes hematopoiesis originating from HSC-independent routes

The FACS analysis at D14 revealed that both erythroid-megakaryocyte common progenitors (EMKp)-like cells (CD34−CD43,GPA+CD41+), the classic populations of HSC-independent, were more abundant when the cultures were treated with...
DOX from D6, especially from D10, indicating that overexpression of \textit{HOXC4} can promote hematopoiesis originating from HSC-independent routes (Fig. 2D; Fig. S5, http://links.lww.com/BS/A18).

2.5. \textit{HOXC4} significantly promotes the production of CD43+ cells and the potential of its sub-populations

FACS detection at D14 revealed that three sub-populations of CD43+ cells, including CD34+CD43+, CD34−CD43+h, and
CD34–CD43low cells, were promoted (about 2-, 3-, and 1.2-fold, respectively) by induction of HOXC4 from D10. Production of CD45+ cells and GPA+CD41a+ cells in these sub-populations was significantly elevated following DOX treatment (except that the number of GPA+CD41a+ cells decreased in the CD34+CD43low sub-population), indicating that overexpression of HOXC4 from D10 strongly stimulates the production of myeloid progenitors and erythroid progenitors in most CD43+ cells (Fig. 4).

2.6. Sub-populations of CD43+ cells have different hematopoietic potential

Colony culture assays of CD34+CD43+, CD34−CD43low, and CD34−CD43high sorted from non-induced co-cultures at D14 revealed that these sub-populations had distinct differentiation potentials. Colonies formed from CD34+CD43+ cells were mainly CFU-GM colonies, and also contained fewer CFU-E, CFU-MIX, and BFU-E colonies. CD34−CD43low cells had the lowest overall hematopoietic potential, but the colonies they formed contained the highest proportion of CFU-GM colonies and a much lower proportion of CFU-E colonies; they lacked BFU-E and CFU-MIX colonies altogether. The colonies formed by CD34−CD43high cells contained the highest proportion of CFU-E colonies and much lower proportions of CFU-GM, BFU-E, and CFU-MIX colonies (Fig. 5A). Classic BFU-E colonies were tested by MGG staining (Fig. 5B,a) and immunofluorescence staining (Fig. 5B,b) to confirm that they were erythroid cells.

2.7. HOXC4 promoted expansion of myeloid progenitors and EMKP-like populations

CD34+CD43+, CD34−CD43low, and CD34−CD43high sub-populations at D14 were further cultured in myeloid, megakaryocyte, or erythroid expansion medium (for 14, 7, or 14 days, respectively), with or without DOX. The results of flow analysis indicated that CD34+CD43+, CD34−CD43low, and CD34−CD43high cells could differentiate into CD41a+CD42b+ cells (15%, 7%, and 82%, respectively), GPA+CD71+ cells (23%, 15.9%, and 61.2%, respectively), and CD45−CD15+ cells (7%, 44.6%, and 7.4%, respectively). Among them, CD34−CD43high sub-populations had the highest differentiation potentials of megakaryocyte and erythroid, reflecting classic traits of EMKP-like populations, and HOXC4 induction significantly promoted their erythroid potential. The CD34−CD43low sub-population had the highest myeloid potential, which could be significantly increased by HOXC4 induction. The differentiation potential of the CD34+CD43+ sub-population was intermediate between the two (Fig. 6).

2.8. The level of CD43 protein serves as an index of erythroid or myeloid differentiation potential

GPA and CD45 at the late stage of hematopoiesis (e.g., D14) are regarded as the main surface markers for erythroid and myeloid progenitors. FACS analysis indicated that GPA+ cells (especially GPA+CD71+ cells, as well as GPA+CD71− cells) contain a much higher proportion of CD43high cells than GPA− (GPA−CD71+ and GPA−CD71− cells) (81.3% and
59.5% vs 10.7% and 10.5%) (Fig. 7A), whereas CD45+ cells (especially CD34+/CD45+ cells, as well as CD34+/CD45+ cells) contain a much higher proportion of CD43low cells than CD45− (CD34+/CD45− and CD34−/CD45−) cells (81% and 72.4% vs 23.6% and 20.5%) (Fig. 7B). These results implied that at least in the AGM-S3 co-culture system, CD43high is an index of erythrogenic potential, whereas CD43low is an index of myelogenic potential, during hematopoiesis.

**Figure 4.** Sub-populations of CD43+ cells have different components, and their production is promoted by induction of HOXC4. (A) HOXC4/hESCs co-cultured with AGM-S3 were induced to overexpress HOXC4 from D10, and were subjected to FACS analysis at D14. CD34+/CD43+ sub-populations mainly contained CD34+/CD45+, CD43low mainly contained CD45+ cells; and CD43high contained a high proportion of GPA+CD41a+ cells. (B) Production of these three sub-populations and of the hematopoietic populations they contained was promoted by treatment with DOX, indicating that overexpression of HOXC4 from D10 strongly promoted hematopoiesis from CD43+ cells.
2.9. HOXC4 overexpression up-regulates NF-κB signaling to promote hematopoiesis

When HOXC4/hESCs co-cultured with AGM-S3 cells were induced from D10, qRT-PCR at D14 revealed that NF-κB expression was up-regulated following induction which could be completely counteracted by addition of 20nM QNZ (NF-κB transcription inhibitor) or NF-κB1 siRNA from D10 (Fig. 8A). In addition, cell-cycle assays revealed significant promotion of S-phase in CD43+ cells in co-cultures induced from D10. The positive effects of HOXC4 on hematopoiesis and change in cell-cycle status were partially or completely counteracted by addition of 20nM QNZ or NF-κB1 siRNA (Fig. 8B–C, Fig. S4, http://links.lww.com/BS/A17). This indicated that the increase in proliferation is the main cellular mechanism underlying the increased abundance of CD43+ cells, which is closely related to NF-κB signaling.

3. DISCUSSION

HOX genes that clustered at the same locus in the genome are likely to share similar traits, providing some key clues for studies of their functions.18–20 The group 4 HOX genes are HOXA4, HOXB4, HOXC4, and HOXD.21–23 Our qPCR analysis revealed distinctive profiles of mRNA expression among HOXB4 and three other group members during hematopoiesis in H1 hESCs co-cultured with AGM-S3 cells (Fig. S1, http://links.lww.com/BS/A14), and amino acid sequence alignment showed that except for the homeobox domains, the encoded proteins do not share a high degree of similarity (Fig. S2A, http://links.lww.com/BS/A15), suggesting divergence of their functions. Among them, only the function of HOXB4 has been intensively researched in the context of hematopoiesis.24–26 Previously, we showed that when RUNX1b is overexpressed at the early stage of mesoderm induction, hematopoiesis is strongly blocked,9 and many genes of the HOX family are down-regulated. HOXC4 was one of the most down-regulated HOX genes (unpublished data). A highly conserved gene (Fig. S2B, http://links.lww.com/BS/A15), HOXC4 has positive effects on HSC expansion similar to those of HOXB4,6,8 but its function during human hematopoiesis has not been examined in an in vitro hematopoiesis system derived from hESCs.

In our experiments, HOXC4/hESCs co-cultured with AGM-S3 cells were induced to overexpress HOXC4 on different days after the initiation of hematopoiesis, and were subsequently monitored by FACS analysis and colony culture assay. FACS analysis at D14
revealed that for multiple hematopoietic populations, including CD34+CD43+, CD34/C0CD43+, CD34+CD45+, CD34/C0CD45+, and GPA+CD71+, overexpression of HOXC4 had similar influence at different stages. When induction began in the earliest stage (D0–D2), especially at D0, production was dramatically reduced at D14, whereas D4 KDR+ cells and D8 CD34+/CD43− cells were not significantly affected (Fig. 2A–B, Fig. S3A–B, http://links.lww.com/BS/A16), indicating that HOXC4 induction from D0 can decrease production of the hematopoietic progenitors, but cannot influence induction of mesoderm or precursors of hematopoietic progenitors. When induced from D6 or later, production of all cell types was significantly increased, with effects growing larger when induction was started later (Fig. 2C). To facilitate detection, D10 was chosen as the day to start induction of HOXC4, which resulted in very strong positive effects on hematopoiesis, while at the same time yielding a higher ratio of GFP+ cells. Colony culture assays also demonstrated that colony numbers of CFU-GM, CFU-E, BFU-E, and CFU-MIX originating from D14 GFP+ co-cultures induced from D10 were significantly higher than in non-induced controls (Fig. 3). All results were consistent with a strong positive effect of HOXC4 on hematopoiesis (including myelogenesis and erythrogenesis) at the late stage.
CD43+ cells include the main blood progenitors. In the AGM-S3 system, the CD43+ cells of the D14 co-culture could be obviously divided into three separated sub-populations: CD34+CD43+, CD34−CD45−, and CD34−CD43low. Their compositions of hematopoietic cells are distinct from each other. The CD34−CD43high sub-population contained the highest proportion of GPA+CD41a+ cells (83.6%), whereas the CD34+CD43+ and CD34−CD43low sub-populations contained much lower proportions of such cells (16.3% and 14.9%) (Fig. 4). The colony culture assay revealed that the CD34−CD43high sub-population had very strong erythrogenic potential (Fig. 5). The results of further hematopoietic culture demonstrated that the CD34−CD43high sub-population could produce a much higher proportion of erythroid and megakaryocyte progenitors, indicating that these cells have the classical traits of EMkP-like progenitors, with strong potentials for erythrogenesis and plateletogenesis (Fig. 6). The CD34+CD43+ and CD34−CD43low sub-populations contained a higher proportion of CD45+ cells than the CD34−CD43high sub-population (Fig. 4). They had similar CFU-GM numbers, whereas the CD34−CD43high sub-population lacked CFU-E and CFU-MIX (Fig. 5A,i), and on the whole its colony-forming ability was much lower than that of the other two sub-populations (Fig. 5A,ii). The results of further hematopoietic culture demonstrated that the CD34−CD43low sub-population could produce a much higher ratio of myeloid cells, indicating stronger myelogenic potential (Fig. 6).

Overexpression of HOXC4 from D10 greatly increased the production of the CD34+CD43+ and CD34−CD43high sub-populations, but not the CD34−CD43low sub-population. The proportion of CD34+CD43+ cells that were GPA+CD41a+, and the proportion of CD34+CD43+ and CD34−CD43low cells that were CD45+, were significantly increased by induction (Fig. 4A). Except for GPA+CD41a+ cells in the CD34−CD43low sub-population, induction of HOXC4 from D10 could promote
production of GPA+CD41a+ and CD45+ cells in all three sub-populations (Fig. 4B). After the corresponding subsequent hematopoietic cultures, the erythrogenic potential of CD34+CD43+ and the myelogenic potential of CD34+/CD43low were significantly higher (Fig. 6). Together, these results indicated that HOXC4 induction increases the production and potentials of all sub-populations of CD43+ cells in the late stage of co-culture, as well as in suspension cultures.

Now that the new conceptions of HSC-independent and HSC-dependent hematopoiesis routes have been widely accepted,14 it is reasonable to apply these models to evaluation of the hematopoietic populations produced from the AGM-S3 system. EMkPs develop via a route of hematopoiesis parallel to the one originating from HSCs.33 The CD34+CD43high sub-population exhibited classic traits of EMkPs, which have strong potential for erythrogenesis and plateletogenesis, and might represent an HSC-independent population.32,34–37 On the other hand, CD34+CD43+ and CD34+CD45+ cells at D14 are a classic HSC-dependent population.10,38,39 Therefore, we tried to directly detect the effects of HOXC4 induction on the representative populations of these two routes according to the classic definition of surface markers.10,32,37,40–44

Besides classic HSC-dependent populations, FACS also revealed that the induction of HOXC4 from D10 significantly increased the proportion of cells in S-phase in CD43+ cells of co-cultures, and that these effects were eliminated by treatment with QNZ or NF-κB1 siRNA.

Figure 8. Promotion of hematopoiesis by HOXC4 overexpression from D10 is closely related to NF-κB signaling and might be caused by a change in cell-cycle status. (A) qRT-PCR at D14 showed that NF-κB1 was up-regulated when HOXC4 was induced from D10, which could be counteracted by treatment with QNZ or NF-κB1 siRNA. (B) If 20 nM QNZ or NF-κB1 siRNA was added together with DOX from D10, FACS analysis at D14 showed that the positive effects of HOXC4 overexpression on the CD34+CD43+, CD34+CD43+, CD34+CD45+, and GPA+CD71+ populations were weakened or even abolished. (C) Analyses of cell-cycle status revealed that HOXC4 induction from D10 increased the proportion of cells in S-phase in CD43+ cells of co-cultures, and that these effects were eliminated by treatment with QNZ or NF-κB1 siRNA.
To elucidate the molecular/cellular mechanism of HOXC4 on hematopoiesis, we checked the cell-cycle status of CD43+ cells and observed a significant increase in the proportion of cells in S-phase when HOXC4 was induced from D10. At the same time, NF-κB signaling was up-regulated, and an inhibitor of NF-κB, QNZ (NF-κB1 transcription inhibitor), counteracted the positive effects on production and cell-cycle status. The results of corresponding RNA inference against NF-κB1 also proved that the knockdown for key gene of NF-κB signaling had similar effects as its inhibitor did (Fig. 8). These observations indicate that activation of NF-κB signaling, stimulation of S-phase, and the positive effects of HOXC4 on hematopoiesis (including myelogenesis and erythropoiesis) are closely related (Fig. 8). Moreover, this could explain how HOXC4 promotes the proliferation of both HSC-dependent and -independent populations by up-regulating NF-κB signaling and accelerating their cell-cycle, with no obvious bias between these two routes or among any hematopoietic progenitors.

HOXC4 induction in the late stage can significantly promote both routes of hematopoiesis, and the production of most key hematopoietic progenitors, by accelerating cell proliferation without obvious bias; this phenomenon is closely related to NF-κB signaling. In addition, the differentiation tendency toward erythropoiesis or myelogenesis, and the degree to which HOXC4 promotes this tendency are strongly related to the expression level of CD43 protein in the target populations (Figs. 4 and 7). The hematopoietic population in that the higher CD43 protein was expressed could be stronger promoted production, and has stronger erythropoiesis potential but weaker myelogenesis (Fig. 4), which indicated that at least in the AGM-S3 system, the expression level of CD43 protein could serve as an index to assess the differentiation tendencies of target populations and the strength of the positive effect of HOXC4 on these populations. The detailed molecular and cellular mechanism by which HOXC4 promotes hematopoiesis still needs to be further explored, but it is clear that this knowledge will have clinical applications in the future.

4. MATERIALS AND METHODS

4.1. Establishment of a HOXC4-inducible transgenic hESC lines

The coding region of HOXC4 was inserted between the SeuI and EcoRI sites of PB-Tet-on-OE to construct PB-Tet-on-GFP-T2A-hHOXC4, which was co-transfected into H1 hESCs along with the helper vector PB200PA-1 using Lipofectamine 3000 (Invitrogen, USA). Positive colonies were selected with 1 μg/ml puromycin, and then passaged using ReleSR (STEMCELL Technologies, Canada) to establish an inducible hESC line (HOXC4/hESCs) that co-expresses GFP. Induction by DOX was confirmed by quantitative reverse-transcription PCR (qRT-PCR) and western blot analyses. Pluripotency of induced or non-induced HOXC4/hESCs was confirmed by western blotting to detect SOX2, OCT4, and NANOG proteins.

4.2. Co-culture of hESCs with AGM-S3 cells

To induce hematopoietic differentiation, the H1 hESC line (generously provided by Prof. Tao Cheng) was co-cultured with AGM-S3 (a mouse stroma-derived cell line) as reported previously. This study was approved by the institutional ethics committee of the Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College (CAMS & PUMC). Briefly, undifferentiated H1 hESCs were seeded on irradiated AGM-S3 cells, cultured in hPSC maintenance medium for 3 days, and then switched to hematopoiesis-inducing medium (referred to as Day 0 [D0]). The co-cultured cells were dissociated with 0.05%-0.25% trypsin/EDTA (ethylenediaminetetraacetic acid) solution (Invitrogen, USA) at the indicated times after D0, and then subjected to FACS or other manipulations.

4.3. Monitoring the antagonistic effects of NF-κB signaling inhibitor on HOXC4 overexpression from D10

D10-induced HOXC4/hESCs co-cultured with AGM-S3 cells were treated with 20nM QNZ (Selleck, USA) starting on D10, and then subjected to analysis of qRT-PCR, FACS, and cell-cycle status at D14. Untreated D10-induced or non-induced cells were used as controls. The qPCR primers are listed in Table S1, http://links.lww.com/BS/A19.

4.4. Knockdown of NF-κB1

Two NF-κB1 siRNAs were purchased from Sangon Biotech, China, which detail information of RNA sequences were listed in Table S3, http://links.lww.com/BS/A21. Both of them were proved to efficiently knockdown the expression of NF-κB1 in 293T cells. Then co-cultures were transfected with both two NF-κB1 siRNAs using Lipotectamine RNAiMAX kit (Invitrogen, USA) from D10, and replaced medium every day. The co-culture cells treated with NF-κB1 siRNAs or control RNA were subjected to analysis of qRT-PCR, FACS, and cell-cycle status at D14. The qPCR primers are listed in Table S1, http://links.lww.com/BS/A19.

4.5. Cell-cycle analysis

HOXC4/hESC co-cultures at D14 were treated with 10 μM BrdU for 12 hours, dissociated by treatment with 0.25% trypsin solution, stained with anti-CD43 antibody, and then subjected to analysis of cell-cycle status using the APC-BrdU Flow Kit (BD Biosciences, USA), which was visualized by FACS analysis.

4.6. Flow cytometry and cell sorting

Co-cultured cells were dissociated with 0.25% trypsin-EDTA solution (Invitrogen, USA), filtered through a 40 μm nylon mesh to obtain a single-cell suspension, stained with the corresponding antibodies, and then subjected to flow cytometry on a FACSJazz Sorter (BD Biosciences, USA). All FACS data were analyzed using FlowJo 10. The antibodies used in FACS analyses are listed in Table S2, http://links.lww.com/BS/A20.

4.7. Colony culture assay

The hematopoietic potentials of D10-induced or non-induced HOXC4/hESC co-culture cells were assessed by culture on methylcellulose (Cat# H4320; STEMCELL Technologies, Canada) supplemented with 100ng/ml stem cell factor (SCF), 100ng/ml interleukin-6 (IL-6), 10ng/ml interleukin-3 (IL-3), 10ng/ml Fms-related tyrosine kinase 3 ligand (FL), 10ng/ml thrombopoietin (TPO), 10ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF), 4units/ml erythropoietin (EPO), and 1% penicillin/streptomycin, and then incubated in 5% CO2 at 37°C for 14 days. CFU-E was calculated at 7-10 days, and BFU-E, CFU-Mix, and CFU-GM were calculated at 14 days.

4.8. Myeloid/erythroid/megakaryocyte differentiation

Non-induced HOXC4/hESC co-cultures at D14 were dissociated by treatment with 0.25% trypsin solution and stained with
anti-CD34/CD43 antibodies. CD34+CD43+, CD34–CD43low, and CD34–CD43high populations were sorted from the corresponding co-culture cells. Next, ~5 × 10^4 sorted cells of each type were re-suspended in 250 μl myeloid expansion medium (Cat# 02693, STEMCELL Technologies, Canada), megakaryocyte expansion medium (Cat# 02696, STEMCELL Technologies, Canada), or erythroid expansion medium (containing 10^{-8} M Dexamethasone, 4 μM EPO, 100 ng/ml IL-6, and 100 ng/ml SCF), and seeded in 48-well plates. The cells were then cultured with or without DOX induction for 14, 7, or 14 days, respectively, with medium replaced every other day, and then were finally subjected to FACS analysis.

4.9. Statistical analysis

All data are presented as means ± SD; statistical analyses were performed using Student’s t test. P < 0.05 was considered significant.

ACKNOWLEDGMENTS

This work was supported by awards from the CAMS Initiatives for Innovative Medicine (2016-I2M-1-018 to F. Ma and 2017-I2M-3-021 to J. X. Liu, Sichuan Provincial Science and Technology Department Key R&D projects (020YSF0023 to B. Chen), and the Chengdu Science and Technology Project-Technology Innovation R&D (2018-YF05-01341-SN to B. Chen).

We thank Professor Tao Cheng from the State Key Laboratory of Experimental Hematology, CAMS & PUMC for generously providing the H1 hESC line.

Supplementary material: Supplementary material is available at Blood Science online, http://links.lww.com/BS/A22.

REFERENCES

[1] Amores A, Force A, Yan Y, et al. Zebrafish hox clusters and vertebrate genome evolution. Science 1998;282(5394):1711–1714.
[2] Rice KL, Licht JD. HOX deregulation in acute myeloid leukemia. J Clin Invest 2007;117(4):865–868.
[3] He H, Hua X, Yan J. Epigenetic regulations in hematopoietic Hox code. Oncogene 2011;30(4):379–388.
[4] Bijl JJ, Rieger E, van Oostveen JW, Walboomers JMM, Meijer CJLM. HOXCA, HOXCG, and HOXCH expression in primary human acute lymphoid leukemias: High expression of HOXCH5 in anaplastic large cell lymphomas. Am J Pathol 1997;151(4):1067–1074.
[5] Mezza R, Faella A, Corsetti MT, et al. Expression of HOXC4 homeoprotein in the nucleus of activated human lymphocytes. Blood 1995;85(8):2084–2090.
[6] Avruay C, Delahaye A, Pflumio F, et al. HOXCG homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations to HOXBG4. Haematologica 2012;97(2):168–178.
[7] Daga A, Podesta M, Capra MC, et al. The retroval translation of HOX4 into human CD34+ cells induces an in vitro expansion of clonogenic and early progenitors. Exp Hematol 2000;28(5):569–574.
[8] Xin C, Zhao C, Yin X, Wu S, Su Z. Bioinformatics analysis of molecular mechanism of the expansion of hematopoietic stem cell transduced by HOX4B4/HOX4C. Hematology 2016;21(4):462–469.
[9] Chen B, Teng J, Liu H, et al. Inducible overexpression of RUNX1b is required in human embryonic stem cells in human embryonic stem cells and inducible pluripotent stem cells. Blood 2013;121(15):2882–2890.
[10] Sun W, Teng J, Zeng J, et al. The piggyBac-based double-inducible binary vector system: a novel universal platform for studying gene functions and interactions. PloSPL 2013;10:12420–12425.

[11] Chang J, Sun W, Zeng J, et al. Establishment of an in vitro system based on AGM-SF co-culture for screening traditional herbal medicines that stimulate hematopoiesis. J Eubonpharmacol 2019;240:11938–11944.
[12] Dzierek E, Bigas A. Blood development: hematopoietic stem cell dependence and independence. Cell Stem Cell 2018;22(5):639–651.
[13] Nandakumar SK, Ulrich JC, Sankaran VG. Advances in understanding erythropoiesis: evolving perspectives. Br J Haematol 2016;173(2):206–218.
[14] An X, Schulz VP, Mohandas N, Gallagher PG. Human and murine erythropoiesis. Curr Opin Hematol 2015;22(3):206–211.
[15] Choi K, Vodyani MA, Shuklin II. Generation of mature human myelomonocytic cells through expansion and differentiation of pluripotent stem cell-derived lin-CD34+CD43+CD45+ progenitors. J Clin Invest 2009;119(9):2818–2829.
[16] Kawagoe H, Humphries RK, Blair A, Sutherland HJ, Hogge DE. Expression of HOX genes, HOX cofactors, and MLL in phenotypically and functionally defined subpopulations of leukemic and normal human hematopoietic cells. Leukemia 1999;13(5):687–698.
[17] Fongang B, Kong P, Negi S, Braun W, Kudlacki A. A conserved structural signature of the homeobox coding DNA in HOX genes. Sci Rep 2016;6:35413–35425.
[18] Rezahzahy R, Saurin AJ, Maaraz-Zaffran C, Grabi Y. Cellular and molecular insights into Hox protein action. Development 2015;142(7):1212–1227.
[19] Rubin MR, Toth LE, Patel MD, D’Eustachio P, Nguyen-Huu MC. A mouse homeo box gene is expressed in spermatozoa and embryos. Science 1986;233(4764):663–667.
[20] Featherstone MS, Baron A, Gasclo SJ, Mattei MG, Duboule D. Hox-5.1 defines a homeobox-containing gene locus on mouse chromosome 2. Proc Natl Acad Sci USA 1988;85(13):4760–4764.
[21] Geada AM, Gaunt SJ, Azzawi M, et al. Sequence and embryonic expression of the murine Hox-3.5 gene. Development 1992;116(2):497–506.
[22] Sauvageau G, Thorsteinsdottir U, Eaves CJ, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Gene Dev 1995;9(14):1733–1745.
[23] Kyba M, Perlingeiro RCR, Daley GQ. HOXB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 2002;109(1):29–37.
[24] Wang L, Menendez P, Shosphate F, et al. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. J Exp Med 2005;201(10):1603–1614.
[25] Kessel KU, Bluemke A, Scholer H, et al. Emergence of CD43-expressing hematopoietic progenitors from human induced pluripotent stem cells. Transfus Med Hemother 2017;44(3):143–150.
[26] Bazil V, Brandt JE, Hoffman R. Resistance of human hematopoietic stem cells to a monoclonal antibody recognizing CD43. Stem Cells 1997;15(S2):13–19.
[27] Brav-Caramele ME, Vera-Estrella R, Barkela BJ, et al. An alternative mode of CD43 signal transduction activates pro-survival pathways of T lymphocytes. Immunology 2016;150(1):87–99.
[28] Choi K, Vodyani MA, Togarrati P, et al. Identification of the homogenc endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. Cell Rep 2012;2(3):553–567.
[29] Vodyani M, Shuklin II, Choi K. Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells. Nat Protoc 2011;6(3):296–313.
[30] Vodyani MA, Thomson JA, Shuklin II. Leukosalam (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood 2006;108(6):2093–2103.
[31] Wang H, Liu Z, Li C, et al. High-level protein production in erythroid cells derived from in vivo transduced hematopoietic stem cells. Blood Adv 2019;3(19):2883–2894.
[32] Paalma B, Mead AJ. Single-cell approaches reveal novel cellular pathways for megakaryocyte and erythroid differentiation. Blood 2019;133(13):1427–1435.
[33] Li W, Wang Y, Zhao H, et al. Identification and transcriptome analysis of erythroidic island macrophages. Blood 2019;134(5):480–491.
[34] Palis J. Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo. FEBS Lett 2016;590(22):3965–3974.
[35] Paluru P, Huidock KM, Cheng X, et al. The negative impact of Wnt signaling on megakaryocyte and primitive erythroid progenitors derived from human embryonic stem cells. Stem Cell Res 2014;12(2):441–451.
[38] Zeng Y, He J, Bai Z, et al. Tracing the first hematopoietic stem cell generation in human embryo by single-cell RNA sequencing. *Cell Res* 2019;29(11):881–894.

[39] Mayani H. The regulation of hematopoietic stem cell populations. *F1000Research* 2016;5:1524–1529.

[40] Notta F, Doulatov S, Lauretti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* 2011;333(6039):218–221.

[41] Bereshchenko O, Mancini E, Moore S, Bilbao D, Nerlov C. Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPa mutant AML. *Cancer Cell* 2009;16(5):390–400.

[42] Ivanovs A, Rybtsov S, Anderson RA, Medvinsky A. CD43 but Not CD41 marks the first hematopoietic stem cells in the human embryo. *Blood* 2014;124(21):4330–4330.

[43] McKenzie JL, Takenaka K, Gan OI, Doedens M, Dick JE. Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38- population. *Blood* 2007;109(2):543–545.

[44] Notta F, Doulatov S, Dick JE. Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgnull recipients. *Blood* 2010;115(18):3704–3707.

[45] Park S, Kim P, Lee K, et al. APRIL stimulates NF-κB-mediated HoxC4 induction for AID expression in mouse B cells. *Cytokine* 2013;61(2):608–613.