Bioinformatics analysis of molecular mechanism of the expansion of hematopoietic stem cell transduced by HOXB4/HOXC4

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Objectives: We aimed to identify the potential HOXB4/HOXC4 downstream effectors and elucidate their regulatory mechanism in the expansion of hematopoietic stem cell (HSC).

Methods: The microarray data GSE24379 were downloaded from Gene Expression Omnibus database, including 12 human CD34⁺ hematopoietic cells with irradiated EGFP-, HOXB4-, or HOXC4-transduced MS-5 cells, respectively. Then common differentially expressed genes (DEGs) in HOXB4- and HOXC4-treated hematopoietic cells (HOXB4&HOXC4.DEGs) were screened out. Protein–protein interaction (PPI) network was constructed and functional modules analysis was performed. Pathway enrichment analysis was performed using the Database for Annotation Visualization and Integrated Discovery. Besides, transcription regulatory network (TRN) was constructed to screen transcription factors (TFs) corresponding to HOXB4&HOXC4.DEGs.

Results: A total of 408 HOXB4&HOXC4.DEGs (373 up- and 35 down-regulated) in hematopoietic cells were identified. Tumor protein p53 (TP53) had the highest degrees in PPI network. Cyclin B1 (CCNB1) was a hub node in Cluster 1. V-myc avian myelocytomatosis viral oncogene homolog (MYC) and MYC-associated factor X (MAX) were important TFs with higher degrees. Meanwhile, MYC, TP53, and CCNB1 were significantly enriched in cell cycle.

Conclusion: MYC, MAX, TP53, and CCNB1 may be crucial HOXB4/HOXC4 downstream molecules potentially involved in HSCs expansion, and HOXB4 and HOXC4 homeoprotein could display positive effects on expansion of human HSCs via regulating these genes.

Keywords: Bioinformatics analysis, Hematopoietic stem cell (HSC), Differentially expressed genes, HOXB4, HOXC4

Introduction
The multipotent hematopoietic stem cells (HSCs), the common ancestor of all types of blood cells, possess the ability of both self-renewal and differentiation according to body needs.¹,² Expansion of HSCs underlie the basis of gene therapy and various genetic diseases of blood cells have shown to be successfully treated with HSC transplantation (HSCT).³ Given the propensity of HSCs to remain quiescent, gene therapy applications and the translational use of them in transplantation biology remain a great challenge.⁴ Until now, the mechanisms that control self-renewal and hematopoietic differentiation are still ambiguous, a better understanding of the biological nature of HSCs and molecular mechanisms underlying HSC expansion will have significant impact on transplantation and current gene therapy strategies.

Recently, numerous studies have implicated that a variety of transcription factors (TFs) are important regulatory components of HSC expansion, especially Hox homeobox gene family.⁵,⁶ HOXB4 appears to be a strong positive regulator of HSCs self-renewal, and overexpression of HOXB4 significantly promotes HSCs regeneration and expansion.⁷-⁹ A degradation-resistant HOXB4 variant is also shown to markedly improve ex vivo expansion of adult HSCs.¹⁰ It has also been suggested that HOXB4 can induce stable gene expression changes in transplanted HSCs that...
are often involved in balanced self-renewal and differentiation divisions. Moreover, a previous study also confirms that HOXB4 confers definitive lymphoid-myeloid engraftment potential on HSCs derived from yolk sac hematopoietic progenitors or embryonic stem cell. Besides, genetic alterations of HSC caused by retrovirus-mediated constitutive expression of human HOXB4 is likely to be hazardous for therapeutic applications. In addition to HOXB4, HOXC4, a paralog of the HOXB4, has been shown to be able to induce an in vitro expansion of clonogenic and early progenitors after retroviral transduction of HOXC4 into human CD34+ cells. Also, the work of Auvray et al. demonstrated that HOXC4 homeoprotein efficiently expanded human HSCs and triggered similar molecular alterations as HOXB4, in which microarray GSE24379 was used. Progresses triggered similar molecular alterations as HOXB4, in homeoprotein efficiently expanded human HSCs and Omnisus database (http://www.ncbi.nlm.nih.gov). A total of 12 CD34+ treated CD34+ cells (HOXB4&HOXC4.DEGs) were used for subsequent analysis.

Data preprocessing and DEGs screening
All the array data were preprocessed using LOESS normalization. We then identified DEGs in CD34+ cells co-cultured with MS-5/HOXB4 and co-cultured MS-5/HOXC4 using limma in R package, respectively. The significant P-value was adjusted by Benjamini and Hochberg (BH) method. Then the adjusted P-value < 0.05 and |log fold change (FC)| ≥ 0.5 were considered as the cutoff value.

In addition, the common DEGs in HOXB4- and HOXC4-treated CD34+ cells with the same expression pattern (high expression or low expression) were considered as HOXB4&HOXC4.DEGs. These HOXB4&HOXC4.DEGs were used for subsequent analysis.

Pathway enrichment analysis
Kyoto Encyclopedia of Genes and Genomes (KEGG) is a pathway-related database for classification of related gene sets into their respective pathways. The BiocCarta website is an interactive online resource which mainly divides into three categories: gene function, proteomic pathway, and reagent exchange. Database for Annotation Visualization and Integrated Discovery (DAVID) is a tool for providing functional annotation behind large-scale genomic or transcriptomic data.

In this study, KEGG and BiocCarta pathway enrichment analysis for HOXB4&HOXC4.DEGs were performed by DAVID online analytical tools. The P-value < 0.05 was set as the threshold value.

Protein–protein interaction (PPI) network construction and functional modules analysis
Search Tool for the Retrieval of Interacting Genes (STRING) database provides comprehensive information of both predicted and experimental interactions of proteins. The PPI pairs in STRING database were displayed with a combined score. The HOXB4&HOXC4.DEGs were mapped into PPIs with combined score ≥ 0.7 as the significant cutoff value. Then PPI network was then built using Cytoscape software. Besides, the significant modules of PPI network with score cutoff = 0.2 were screened out using Molecular Complex Detection (MCODE) plugin of Cytoscape. Then cluster analysis for assessing the function modules of PPI network
was performed by Biological Networks Gene Ontology (BiNGO) plugin of Cytoscape. The P-value was adjusted by false discovery rate (FDR), and FDR < 0.05 was defined as significant.

**TRN construction**

Encyclopedia of DNA Elements (ENCODE) database provides all functional elements in the human genome sequence and TF binding sites. In our study, we first extracted all the information of human TF binding sites based on the information of ENCODE database. Then TF binding site existed in at least two independent samples was identified and used for subsequent analysis. According to the annotation information of transcription region, we further identified the TFs located in promoter region, a region of 1.5 kb around transcriptional start sites (TSS) with 1 kb upstream and 0.5 kb downstream. Then the corresponding target genes regulated by these TFs were obtained. With the information of TFs-target genes pairs, the TRN of DEGs was constructed.

**Results**

**DEGs screening**

In CD34+/MS-5/HOXB4 vs. CD34+/MS-5/HOXC4 groups, only 28 DEGs (14 up- and 14 down-regulated) were identified. However, we identified a total of 547 DEGs (478 up- and 69 down-regulated) in CD34+/MS-5/HOXB4 vs. CD34+/MS-5/GFP group and 718 DEGs (642 up- and 76 down-regulated) in CD34+/MS-5/HOXC4 vs. CD34+/MS-5/GFP group. The results showed that the numbers of up-regulated genes were significantly more than down-regulated genes no matter in HOXB4-treated or HOXC4-treated CD34 cells.

Besides, as shown in Fig. 1, totally 408 overlaps (HOXB4&HOXC4.DEGs) were identified. Among them, 373 DEGs, such as Heat Shock 70 kDa Protein 1A (HSPA1A), Arrestin, Beta 1 (ARRB1), ATM Serine/Threonine Kinase (ATM), Chromodomain Helicase DNA Binding Protein 4 (CHD4), Cullin 4A (CUL4A), and Prosaposin (PSAP) were up-regulated (Fig. 1A) and 35 DEGs, like HMG-Box Transcription Factor 1 (HBP1), Myocyte Enhancer Factor 2C (MEF2C), Dachshund Family Transcription Factor 1 (DACH1) and Cyclin-Dependent Kinase Inhibitor 1C (P57, Kip2) (CDKN1C), were down-regulated (Fig.1B). These HOXB4&HOXC4.DEGs were considered as key HOXB4/HOXC4 downstream molecules used for further analysis.

**Pathway enrichment analysis**

Based on the information of KEGG database and the BiocCarta website, five KEGG pathways and five Biocarta pathways were enriched by 408 HOXB4&HOXC4.DEGs respectively with P-value < 0.05 (Table 1). The enriched KEGG pathways were Cell cycle, Spliceosome, DNA replication, Citrate cycle (TCA cycle), and Progesterone-mediated oocyte maturation. The enriched Biocarta pathways were Regulation of eIF2, Cyclins and Cell Cycle Regulation, G1/S Check Point, CDK Regulation of DNA Replication, and ATM Signaling Pathway.

| Term | P-value |
|------|---------|
| hsa04110:Cell cycle | 3.94E-12 |
| hsa03040:Spliceosome | 2.48E-04 |
| hsa03030:DNA replication | 0.003449873 |
| hsa00020:Citrate cycle (TCA cycle) | 0.011494494 |
| hsa04914:Progesterone-mediated oocyte maturation | 0.037316052 |
| h_eif2Pathway:Regulation of elf2 | 0.011636837 |
| h_cellcyclePathway:Cyclins and Cell Cycle Regulation | 0.017997328 |
| h_g1Pathway:Cell Cycle: G1/S Check Point | 0.029838037 |
| h_mcmPathway:CDK Regulation of DNA Replication | 0.040823573 |
| h_atmPathway:ATM Signaling Pathway | 0.047567843 |

**Table 1** The KEGG and Biocarta pathways enriched by 408 common DEGs in HOXB4/HOXC4-treated cells (HOXB4&HOXC4.DEGs)

Term represents the name of pathway; **P-value** represents significantly P.

![Figure 1](image-url) Differentially expressed genes. (A) indicates highly expressed genes, (B) indicates lowly expressed genes. Orange and green represent DEGs in HOXB4-treated hematopoietic cells; pink and blue green represent DEGs in HOXB4-treated hematopoietic cells.
**PPI network construction and functional modules analysis of network**

Based on the information of STRING\textsuperscript{21} database, the HOXB4&HOXC4.DEGs were mapped into PPIs with combined score ≥ 0.7 as the significant cutoff value. Then PPI network constructed by HOXB4&HOXC4.DEGs contained 214 nodes and 664 edges (Fig. 2A). Thereinto, 201 nodes (red) were up-regulated genes and 13 nodes (green) were down-regulated gene. Based on nodes degrees, the top 10 nodes were tumor protein p53 (TP53), cyclin B1 (CCNB1), cell division cycle 6 (CDC6), cyclin A2 (CCNA2), RAD51 recombinase (RAD51), cyclin-dependent kinase 2 (CDK2), minichromosome maintenance complex component 4 (MCM4), cell division cycle 20 (CDC20), v-myc avian myelocytomatosis viral oncogene homolog (MYC), and minichromosome maintenance complex component 7 (MCM7). All these DEGs were up-regulated.

Additionally, using MCODE plugin of Cytoscape, a total of nine functional modules were identified with FDR < 0.05 (Table 2). The score of Cluster 1 and Cluster 2 were more than 10. Cluster 1 contains 16 nodes and 115 edges (Fig. 2B), and Cluster 2 contains...
10 nodes and 45 edges (Fig. 2C). The hub nodes in Cluster 1 were CCNB1, CDC6, and CCNA2, while the hub nodes in Cluster 2 were nuclear cap binding protein subunit 2, 20 kDa (NCBP2), splicing factor 3b, subunit 3, 130 kDa (SF3B3) and fused in sarcoma (FUS). All these DEGs were also up-regulated genes.

TRN construction of HOXB4&HOXC4.DEGs

As shown in Fig. 3, TRN constructed by HOXB4&HOXC4.DEGs contained 329 edges and 236 nodes based on the information of ENCODE database. According to the annotation information of transcription region, the TFs located in promoter region, a region of 1.5 kb around TSS with 1 kb upstream and 0.5 kb downstream, 223 up-regulated nodes and 13 down-regulated ones were identified. Thereinto, five nodes were TFs (4 up- and 1 down-regulated). Up-regulated TFs were MYC, MYC-associated factor X (MAX), histone deacetylase 2 (HDAC2), and tripartite motif containing 28. The only down-regulated TF was nuclear receptor subfamily 3, group C, member 1 (NR3C1).

Discussion

Expansion of HSCs represents an important objective for gene therapy strategies. In the current study, we used bioinformatics approach to explore the potential molecular mechanism involved in HOXB4/HOXC4-treated HSCs expansion. Consistent with the work of Auvray et al., in which microarray GSE24379 was used, we also identified same up-regulated DEGs, such as HSPA1A, ARRBI, ATM, CHD4, CUL4A, and PSAP, and down-regulated DEGs, like HBP1, MEF2C, DACH1, and CDKN1C. Moreover, as a result of CD34+ cell exposure to HOXB4/C4 using different cell models, HSPA1A and HBP1 were also identified by Schiedlemeier et al., ARRBI and MEF2C were also identified by Palmqvist, Pineault, et al., CDKN1C and DACH1 were also identified by Forsberg et al., and ATM, CHD4, CUL4A, and PSAP were also identified by Kent et al. In contrast to these previous findings, our results also identified other different DEGs. Among them, MYC and MAX were important TFs involved in HSCs expansion, and MYC interacted with MAX, TP53, and CCNB1 directly in TRN network. Meanwhile, MYC, TP53, and CCNB1 were significantly enriched in cell cycle and were regarded to be strongly associated with HOX homeoprotein in HSCs expansion.

MYC and MAX are members of basic helix-loop-helix leucine zipper TFs. c-MYC is shown to control the balance between HSCs self-renewal and differentiation via regulating the interaction between HSCs and their niche including N-cadherin and a number of adhesion receptors. The work of Laurenti et al. also confirmed that MYC activity (c-MYC and N-MYC) regulated crucial aspects of HSC function, such as proliferation and differentiation, and survival. Moreover, c-MYC can promote self-renewal of HSCs as a downstream critical effector molecule of HOXB4.33 In addition, increasing evidences suggest that the functions of the MYC/MAX/MAD network play roles in cell proliferation, differentiation, and death. Auvray et al. have shown that c-MYC and its partner MAX are up-regulated in HSC exposed to HOXB4 or to HOXC4. Because HOXB4/HOXC4 protein transfer is operative for expanding HSCs, thereby, we speculate that MYC and MAX may be key common downstream effectors of HOXB4/HOXC4 and may play a crucial role in controlling the balance between HSCs self-renewal and differentiation.

Furthermore, MYC interacted with TP53 and CCNB1 directly in TRN network. TP53 was also identified from PPI network with the highest degrees compared to others. Numerous studies have identified critical roles of TP53 in HSCs behavior, such as self-renewal, differentiation, apoptosis, and aging.
study of Mysm1-deficient mice have shown that p53 is essential for HSC function and lymphopoiesis.\textsuperscript{36} Moreover, TP53 plays an important role in steady-state hematopoiesis and is essential for the enhanced HSC quiescence seen in the absence of MEF.\textsuperscript{37} Besides, TP53 is involved in stress hematopoiesis and regulates its target genes, variably inducing cell cycle arrest, apoptosis, or senescence, in response to diverse stresses.\textsuperscript{38} During normal and stress hematopoiesis, the pathways that HOXB4 regulates HSC self-renewal also can be affected by TP53.\textsuperscript{8,39} Therefore, our results are in line with previous findings and suggest that TP53 may play a crucial role in hematopoiesis and thus regulate HOXB4/HOXC4-mediated HSCs expansion. In addition, CCNB1 was also identified as a hub node in Cluster 1 in our study. CCNB1 is likely to be privileged mediators of HSC differentiation-associated proliferation during the various phases of cytokine-mediated mobilization.\textsuperscript{40} CCNB1 is also found to be strongly associated with the fate of HSCs and progenitor cell.\textsuperscript{40} Thus, we speculate that CCNB1 may play crucial roles in the proliferation of HSCs following by exposure to HOXB4 or HOXC4.

Strikingly, MYC, TP53, and CCNB1 were significantly enriched in cell cycle. MYC protein is confirmed to induce cell cycle progression and apoptosis via dimerization with MAX.\textsuperscript{41} TP53 participates in multiple cell cycle checkpoints and controls HSCs self-renewal.\textsuperscript{42,43} CCNB1 is a regulatory protein which is essential for the control of the cell cycle at the G2/M (mitosis) transition.\textsuperscript{44} Furthermore,
previous study demonstrated that HSCs proliferation is likely determined by the function and/or regulation of components and regulators of the cell cycle machinery. Appropriate cell cycle control is required for maintaining normal hematopoiesis, especially at the early stage of HSCs/progenitor cells. Therefore, we further speculate that these proteins may play important roles in HSCs expansion via cell cycle.

Only 1 dataset with relatively small sample size was the limitation of our study. Moreover, there was no experimental validation, like shRNA approach, to validate the roles of key DEGs in HSCs expansion. Further study with more datasets and experiments are still needed to verify the findings of our study. Besides, chromatin immunoprecipitation followed by sequencing (ChIP-seq) is increasingly being used for mapping protein–DNA interactions in vivo on a genome scale. If ChIP-seq is performed to extract direct target genes of HOXB4/HOXC4 from DEGs lists, the obtained results will be more convincing.

In conclusion, MYC, MAX, TP53, and CCNB1 may be crucial HOXB4/HOXC4 downstream molecules potentially involved in HSCs expansion, and HOXB4 and HOXC4 homeoprotein may display positive effects on expansion of human HSCs by targeting these proteins. MYC may play important roles in controlling the balance between HSCs self-renewal and differentiation following by exposure to HOXB4 or HOXC4 via dimerization with MAX. TP53 may play important roles in regulating hematopoiesis and thus is involved in HOXB4- or HOXC4-mediated HSCs expansion. CCNB1 may play crucial roles in the proliferation of HOXB4/HOXC4-treated HSCs. Additionally, all these molecules may play important roles in HOXB4/ HOXC4-mediated HSCs expansion via cell cycle. Our findings would aid in a better understanding of the molecular mechanism of HSCs expansion and provide broader perspective for the development of transplantation and gene therapy strategies.

Disclaimer statements
Contributors Chunlei Xin and Chunting Zhao conceived and designed the study. Xiangcong Yin and Shaoling Wu were responsible for data collection and analysis. Zhan Su was responsible for data interpreting. Chunlei Xin wrote the article. Chunting Zhao guaranteed the integrity of the data on this article.

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Conflict of interest All authors declare that they have no conflict of interests.

Ethics approval These data were downloaded from common data base. So, the ethics approval is unnecessary for our study.

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