Influence of neonatal gender on cord blood CD34+ cell amplification and gene expression

LIPING ZHOU[1,2], ZHE CHE[3], XIAOWEI ZHANG[1], PANPAN ZHOU[2], XUE LI[2], XUEJING XU[2], QING SHI[2], DONG LI[2,4] and XIULI JU[2,4]

[1]Department of Pediatrics, The Sixth People's Hospital of Ji'nan, Jinan, Shandong 250200;
[2]Cryomedicine Laboratory, Qilu Hospital of Shandong University, Jinan, Shandong 250012;
[3]Health and Family Planning Inspection Agency of Zhangqiu District, Jinan, Shandong 250200;
[4]Stem Cell and Regenerative Medicine Research Center, Shandong University, Jinan, Shandong 250012, P.R. China

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Abstract. The present study attempted to evaluate whether neonatal gender affects the hematopoietic potential of cord blood (CB) transplants and, if so, to determine the underlying molecular mechanisms. $CD34^+$ cells from CB were isolated and divided into male and female groups. $CD34^+CD38^-$ cell populations were then compared using fluorescence-assisted cell sorting (FACS) and a colony formation assay was performed. Next, a Genechip microarray analysis was used to identify differentially expressed genes (DEGs). Finally, the Genechip results were validated by FACS analysis. It was revealed that the male group had higher amplification efficiency. Gene ontology analysis indicated differences in the biological function of the DEGs between the two groups. Kyoto Encyclopedia of Genes and Genomes analysis suggested that the hematopoietic cell lineage signaling pathway was upregulated in the male group along with high expression levels of genes including interleukin (IL) 6 signal transducer (glycoprotein 130), IL-7 and IL-7 receptor. It was speculated that this may be partially due to numerous upregulated DEGs being involved in chromosomal segregation and hematopoietic cell lineage signaling pathways in $CD34^+$ cells from the male group.

Introduction

Hematopoietic stem cells (HSCs) are a specific type of cell with multi-potency and self-renewal ability (1). It has been demonstrated that bone marrow-reconstituting HSCs reside within a subpopulation of bone marrow or blood-derived mononuclear cells of a few percents which express the surface antigen CD34 (2). CD34 and CD38 surface antigens have long been used as surface molecules for the identification of primitive hematopoietic stem and progenitor cells. It has been reported that Severe combined immunodeficient-repopulating cells were present exclusively in the $CD34^+CD38^-$ cell fraction (3,4). Under the same conditions, recipients of cord blood (CB) transplants experience a lower incidence of acute and chronic graft-vs.-host disease than recipients of bone marrow transplants (5). However, widespread utilization of CB is limited by the relatively low number of HSCs per unit, and most CB units provide insufficient stem cells for adults (6). Multiple strategies for overcoming these limitations are now being investigated. One important strategy involves *ex vivo* expansion of the umbilical CB unit prior to transplantation (7). However, the selection of the best source of HSCs remains the first and most important challenge.

To date, the nucleated cell dose has been used to select more suitable CB units for transplantation for a given patient. However, Aroviita *et al* (8) reported that female infants have higher median nucleated cell concentrations than male infants (13.9 vs. $13.3x10^9$/l) after investigating 1,999 units of CB. By contrast, male infants had significantly higher median $CD34^+$ cell concentrations than female infants (31.8 vs. 30.2/μl), even after correcting for birth weight. Bijou *et al* (9) also reported that male neonatal CB was significantly richer in $CD34^+$ cells than female CB. From the above studies, it may be speculated that a correlation exists between neonatal gender and HSCs derived from CB. Therefore, in the present study, differences in the expansion of $CD34^+$ cells from male and female neonatal CB and their gene expression were compared, and the underlying molecular mechanisms were explored. The detailed experimental design of the study is provided in the flow chart in Fig. 1.

Materials and methods

Collection and purification of CB $CD34^+$ cells. Human CB samples (n=43; 20 males and 23 females) were obtained from mothers undergoing full-term deliveries between

Correspondence to: Professor Dong Li or Professor Xiuli Ju, Cryomedicine Laboratory, Qilu Hospital of Shandong University, 17 Wen Hua Xi Road, Jinan, Shandong 250012, P.R. China
E-mail: lidong73@sdu.edu.cn
E-mail: jxlqlyy@163.com

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January and May 2015 at the Department of Obstetrics at Qilu Hospital of Shandong University (Jinan, China) after informed written consent was obtained. The maternal age was between 20 and 40 years (mean, 26±2 years), and there was no history of acute, chronic or infectious disease, neonatal apnea, edema or jaundice. The use of CB was approved by the Ethics Committee of Shandong University Qilu Hospital (Jinan, China). The birth weight was 3,000-4,000 g for the males and females considered for inclusion. Among the CB units harvested, only those in which at least 70 ml CB was collected and the total nucleated cell count exceeded $8x10^8$ per unit were considered for processing.

Mononuclear cells (MNCs) of each single CB unit were separated using Ficollpaque medium (density, 1.077±0.001 g/ml; HaoYang Co.) and centrifuged at 1,726 x g at 12˚C for 25 min. CB MNCs were incubated with 100 μl CD34+ micro beads (Miltenyi Biotec GmbH) at 4˚C for 30 min. Cells were subsequently passed through an LS MACS column (Miltenyi Biotec GmbH) and enriched CD34+ cells were collected by flushing the column. Cells were subsequently suspended in 0.1 M PBS (pH 7.4). The purity of the CD34+ cells was detected by a fluorescence-assisted cell sorting (FACS) system (Guava easyCyte 6HT; EMD Millipore) and data were analyzed using Guava Incyte (version 2. 8; EMD Millipore).

CB CD34+ colony formation assay. CB CD34+ cells derived from male and female newborns were divided into two groups. CD34+ cells were seeded into 24-well plates for culture with semisolid medium (MethoCult GF H4434; Stem Cell Technologies) at a density of $1.0x10^4$ cells/well according to the manufacturer's protocols. Cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2 and 20% O_2 (HF240; Heal Force Tris-gas Incubator; Heal Force). After 2 weeks of culture, the numbers CFUs were determined under an inverted microscope (IX71; Olympus).

Amplification of CD34+ cells. CB CD34+ cells from CB from neonates of different gender were cultured in HSC expansion medium (Stem Cell Technologies, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 70 ng/ml stem cell factor, 30 ng/ml interleukin (IL)-3, 30 ng/ml FMS-like tyrosine kinase 3 ligand, 20 ng/ml IL-6, 20 ng/ml bone morphogenetic protein-2 and 20 ng/ml thrombopoietin at $1.1x10^5$ cells/ml (R&D Systems, Inc.). Cytokine concentrations were determined as described previously (10,11). The medium was changed every 3-4 days. On day 7, the total suspension of cells was harvested for further analysis.

Immunophenotypic analysis. On days 0 and 7, cells were suspended in 100 μl PBS containing FITC-conjugated anti-CD34 (1:10; cat. no. 343604; BioLegend) and phycoerythrin (PE)-conjugated anti-CD38 (1:10; cat. no. 560981; BD Biosciences) for 30 min at 4˚C, and were then washed with PBS. Cells were analyzed using Guava Incyte, as outlined above.

Microarray-based gene expression analysis. Total RNA was extracted from purified and untreated CD34+ cells from male and female UCB samples using TRIzol (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Agilent Whole Genome Oligo Microarrays (one-color; Agilent Technologies, Inc.) were used to analyze the samples. The RNA was hybridized to the microarray, which was washed and scanned. Finally, the data were extracted using Agilent Feature Extraction Software (version 11.0.1.1; Agilent Technologies, Inc.). Affymetrix GeneChip Human Genome U133 Plus 2.0 was used on an Affymetrix 3000 instrument (fluidics station and scanner; Thermo Fisher Scientific, Inc.) running Gene-Chip operating software (version 11.0; Gene Spring Software; Agilent Technologies, Inc.) to generate gene expression data. Through the Gene Ontology (GO; www.geneontology.gov) function and Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/pathway.html) pathway enrichment analysis, significantly enriched pathways associated with hematopoietic cell proliferation were identified in differentially expressed genes (DEGs) with a fold change ≥two.

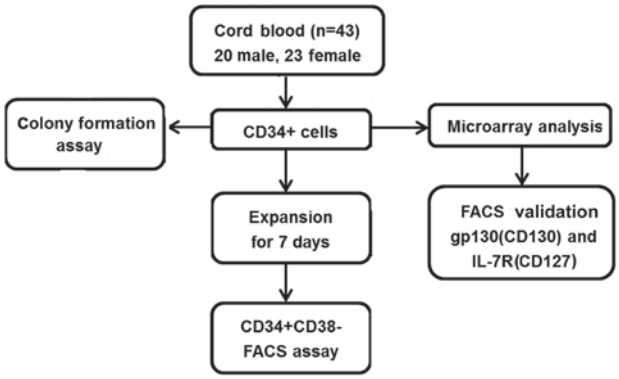

Figure 1. Flow chart depicting the detailed processes of the study. FACS, fluorescence-assisted cell sorting; CFU, colony-forming units; IL-7R, interleukin-7 receptor; gp, glycoprotein.

FACS validation of microarray expression data. CB samples (100 μl) from males and females were added to 2 ml red blood cell lysis buffer (BD Biosciences), followed by incubation for 15 min at room temperature as recommended by the manufacturer. Subsequently, samples were centrifuged at 300 x g for 5 min at room temperature and re-suspended in 100 μl PBS. Cells ($1x10^6$) were stained with FITC-conjugated anti-human CD34 10 μl (1:10; cat. no. 343604; BioLegend) and PE-conjugated anti-human CD127 (1:10; cat. no. 557938; BD Biosciences) or PE-conjugated anti-human CD130 (1:10; cat. no. 555757; BD Biosciences) for 15 min at room temperature, according to the manufacturer's protocol. Analyses were performed with Guava Incyte, as described above.

Statistical analysis. The data were analyzed using SPSS software (version 17.0; SPSS Inc.). Quantitative values are expressed as the mean ± standard deviation unless otherwise specified. One-way analysis of variance with Fisher's protected least-significant differences test as a post-hoc analysis was used for multi-group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Higher CD34+ cell populations in CB MNCs from male infants. The median birth weight of the male infants (3,508 g;

Table I. Birth weight, number of $CD34^+$ cells, CB MNCs, and the percentage of CB $CD34^+$ cells/CB MNC.

Sample	Sex	Weight (g)	CB MNC ($x10^6$)	CB $CD34^+$ cells ($x10^6$)	CB $CD34^+$ cells/CB MNC (%)
1	M	3,000	1.02	0.04	4.00
2	M	2,950	1.20	0.02	1.62
3	M	2,765	1.22	0.03	2.50
4	M	3,500	1.35	0.04	3.02
5	M	3,380	1.44	0.02	1.43
6	M	3,800	1.59	0.05	3.11
7	M	3,745	1.80	0.04	2.50
8	M	3,860	1.86	0.02	2.50
9	M	3,300	2.00	0.04	1.54
10	M	3,460	2.08	0.07	3.40
11	M	3,556	2.10	0.06	2.92
12	M	3,700	2.35	0.07	3.03
13	M	3,850	2.52	0.09	3.06
14	M	2,940	2.71	0.03	1.11
15	M	2,670	2.82	0.09	3.19
16	M	3,950	3.0	0.09	3.00
17	M	3,820	3.43	0.06	1.74
18	M	3,150	3.59	0.06	1.62
19	M	3,230	3.93	0.08	2.09
20	M	3,690	4.02	0.04	1.21
1	F	3,300	1.21	0.01	0.08
2	F	3,620	1.30	0.02	1.52
3	F	3,630	1.41	0.02	1.41
4	F	3,820	1.53	0.03	2.00
5	F	3,010	1.70	0.02	0.98
6	F	3,770	1.94	0.02	0.09
7	F	3,640	2.07	0.04	1.17
8	F	3,710	2.20	0.03	1.35
9	F	2,750	2.25	0.03	1.00
10	F	3,620	2.30	0.03	1.05
11	F	3,130	2.74	0.07	2.50
12	F	3,900	2.76	0.07	2.56
13	F	3,250	2.90	0.06	2.11
14	F	3,120	2.95	0.03	0.09
15	F	3,770	3.08	0.06	2.03
16	F	3,200	3.33	0.08	2.41
17	F	3,880	3.52	0.07	2.03
18	F	2,950	3.77	0.06	1.54
19	F	2,600	3.90	0.05	1.12
20	F	3,150	3.95	0.04	1.06
21	F	3,650	4.10	0.05	2.55
22	F	3,690	2.92	0.03	1.00
23	F	3,710	3.55	0.05	1.4
Mean ± SEM	M	3,416±88.72	2.30±0.21	0.052±0.005	2.43±0.19
	F	3,429±79.03	2.67±0.19	0.042±0.004	1.44±0.16
P-value		0.911	0.196	0.148	0.0002

M, male; F, female.

n=20) was similar to that of the female infants (3,460 g; n=23; P>0.05; Table I). However, the concentration of CB MNCs was lower in the male group than that in the female group (median, 2.04±0.20 vs. 2.52±0.38x10^6/ml; P<0.05; Table I).

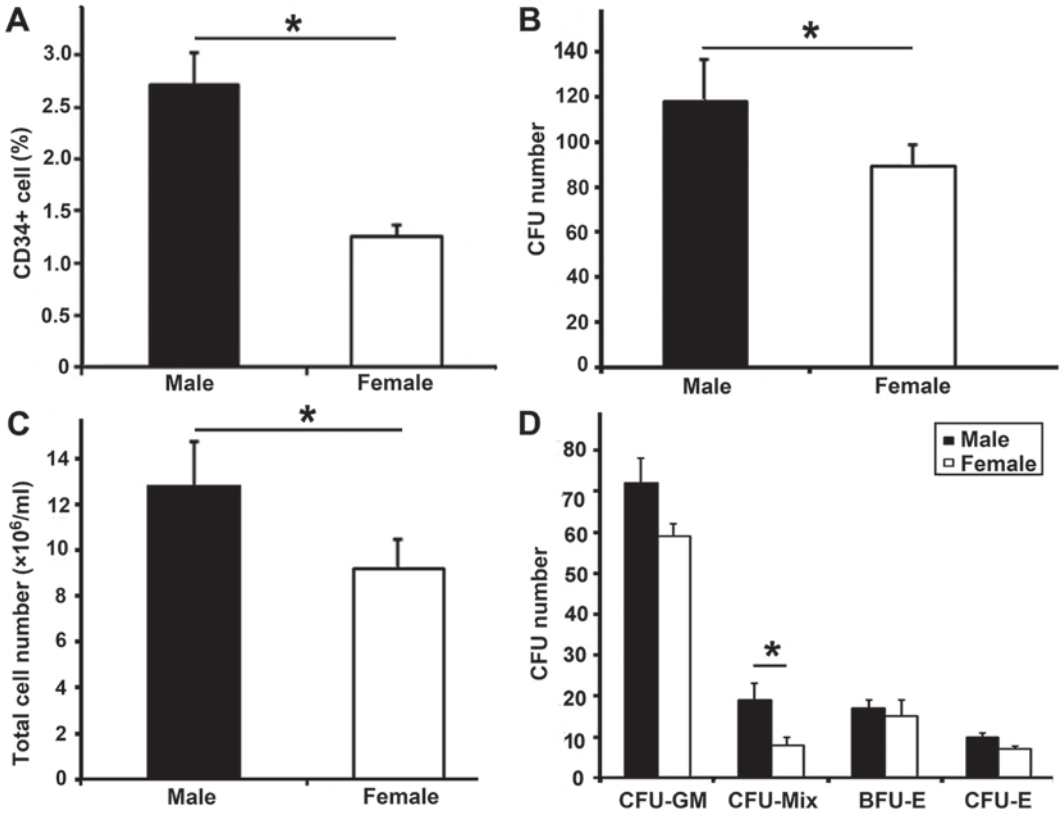

Figure 2. (A) Comparison of CD34+ cell populations among mononuclear cells in the male and female groups. (B) Comparison of CFU numbers between the two groups. (C) Comparison of total cell numbers between the two groups. (D) Comparison of CFU types between the two groups; the number of CFU-Mix colonies was higher in the male group. *P<0.05. Groups: CFU-GM, CFU-granulocyte, macrophage; CFU-Mix, CFU-mixed hematopoietic progenitor cells (>50 cells); BFU-E, burst-forming unit-erythroid; CFU-E, CFU-erythroid. CFU, colony-forming units.

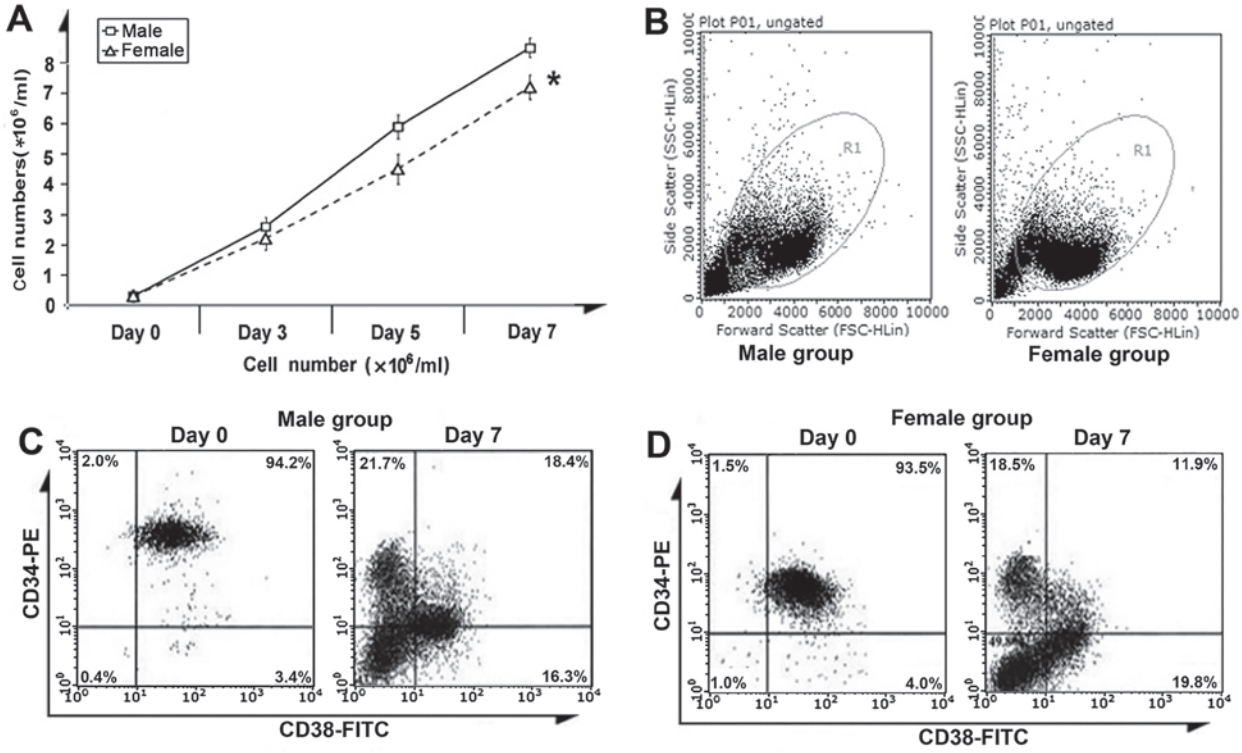

Figure 3. Comparison of CB CD34+ cells prior to and after amplification by FACS analysis in the male and female groups. (A) Comparison of CD34+ cell expansion curves in the two groups; the male group exhibited a higher amplification efficiency. *P<0.05. (B) The FSC/SSC scatter diagram of the male and female groups were gated on the cell population. FACS analysis of $CD34^+CD38^-$ surface antigen expression in CD34+ cells prior to and after *ex vivo* expansion for 7 days in the (C) male and (D) female groups. Day 0: 2.0±0.14 vs. 1.5±0.07%, P>0.05; Day 7: 21.7±2.0 vs. 18.5±1.1%; P>0.05). FACS, fluorescence-assisted cell sorting; PE, phycoerythrin; SSC, side scatter; FSC, forward scatter.

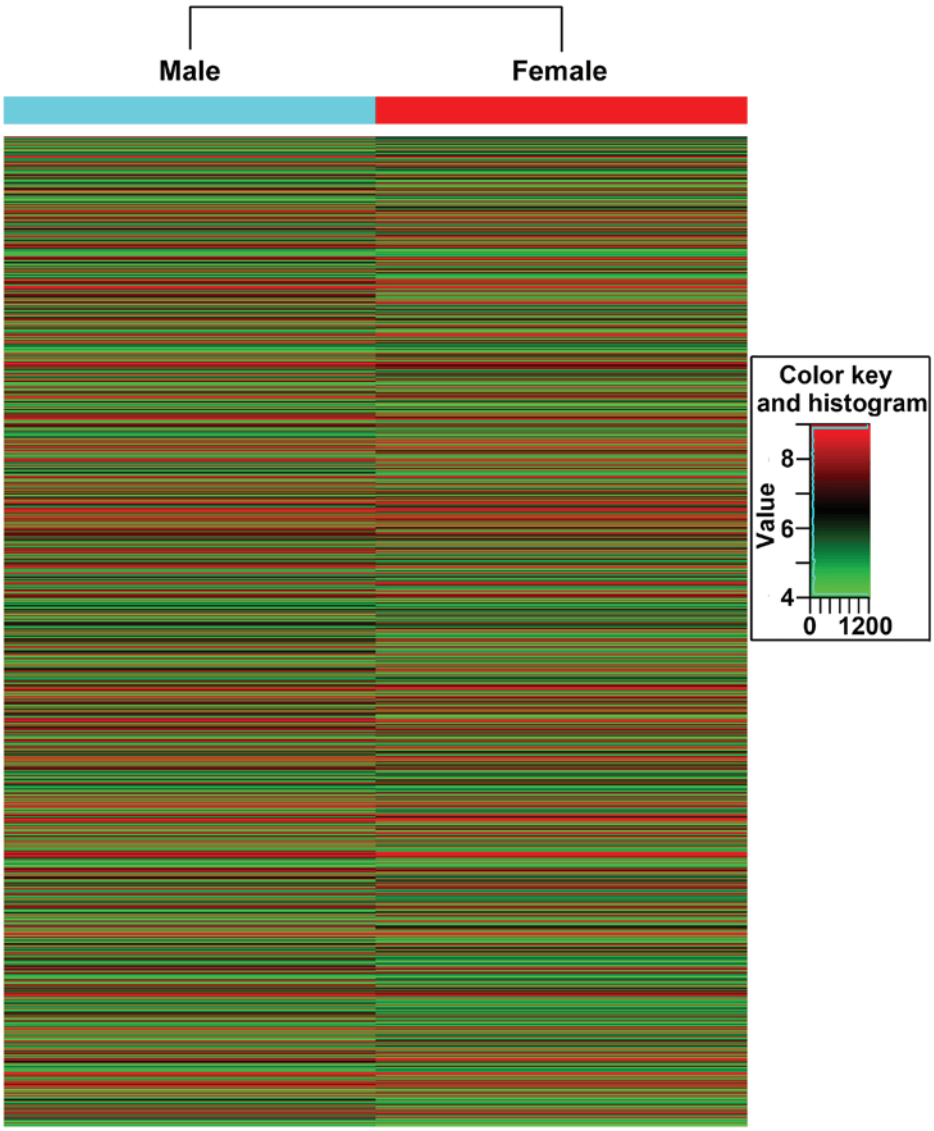

Figure 4. Heat map of the up- and downregulated DEGs in the male and female groups.

The population of CB CD34$^+$ cells in MNCs from the male group was higher than that in the female group (median, 2.72±0.17 vs. 1.26±0.09%; P<0.05; Fig. 2A). The concentration of CB CD34$^+$ cells in the male group was higher than that in the female group (median, 0.04±0.02 vs. 0.03±0.03x10^6/ml; P<0.05).

Enumeration of CFUs from sorted CD34$^+$ cells. For the 43 samples processed, the purity of CD34$^+$ cells after separation was >95%, as verified by FACS. The number of CFUs and total cells produced in the male group was higher than that in the female group (118±20 vs. 89±8 and 12.8±2.5 vs. 9.2±3.3x10^6/ml, respectively; P<0.05; Fig. 2B and C). It was observed that the number of CFU-Mix in the male group was higher than that in the female group (20±5 vs. 9±2; P<0.05). There were no significant differences in the number of CFU-GM (colony forming unit-granulocyte/ macrophage), BFU-E (burst forming unit-erythroid) or CFU-E (colony forming unit-erythroid) colonies formed between the two groups (P>0.05; Fig. 2D).

Male CB CD34 $^+$cells exhibit better amplification. CB CD34$^+$ cells in the male group exhibited a better amplification efficiency than those in the female group on day 7 (P<0.05; Fig. 3A). The forward vs. side scatter diagrams of the male and female group were gated on the cell population (Fig. 3B). According to FACS analysis, there were no significant differences in the fractions of CD34$^+$CD38$^-$ cells between the male and female groups on day 0 (2.0±0.14 vs.1.5±0.07%; P>0.05) and day 7 (21.7±2.0 vs. 18.5±1.1%; P>0.05; Fig. 3C and D).

Microarray-based gene expression analysis

GO enrichment analysis. Agilent Whole Genome Oligo Microarrays containing sequences from 13,594 genes were used. Overall, 64.9% of all genes were differentially expressed in both male and female groups. The up- and downregulated genes of the male and female groups were displayed as a heat map (Fig. 4). In the male group, a total of 1,205 genes (8.8% of total genes) were upregulated by at least 2.0-fold. Upregulated (>6 fold) DEGs are summarized in Table II, and >30% were associated with Y chromosome function. In the female group,

Table II. Upregulated DEGs in the male group (>6-fold).

Fold-change	Gene symbol	Definition [GenBank accession no.]
594.2159004	RPS4Y2	Ribosomal protein S4, Y-linked 2 (RPS4Y2) [NM_001039567]
325.6161621	RPS4Y1	*S* ribosomal protein S4, Y-linked 1 (RPS4Y1) [NM_001008]
140.3517653	EIF1AY	*S* eukaryotic translation initiation factor 1A, Y-linked (EIF1AY) [NM_004681]
130.2768923	DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked (DDX3Y), transcript variant 1 [NM_001122665]
78.8031503	SIGLEC14	Sialic acid binding Ig-like lectin 14 (SIGLEC14) [NM_001098612]
76.6696878	USP9Y	Ubiquitin specific peptidase 9, Y-linked (USP9Y) [NM_004654]
35.4723966	CYorf15B	Chromosome Y open reading frame 15B (CYorf15B) [NM_032576]
30.3805272	UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked [NC_000024.10]
18.1032197	CYorf15A	*S* chromosome Y open reading frame 15A (CYorf15A) [NM_001005852]
14.4214818	UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked (UTY), transcript variant 1, [NM_182660]
14.2319062	HIPK2	Homeodomain interacting protein kinase 2 (HIPK2), transcript variant 1[NM_022740]
13.2519228	ZFY	Zinc finger protein, Y-linked (ZFY), transcript variant 1 [NM_003411]
12.8883619	CCL20	Chemokine (C-C motif) ligand 20 (CCL20), transcript variant 1 [NM_004591]
12.5203328	EIF1AY	Eukaryotic translation initiation factor 1A, Y-linked (EIF1AY) [NM_004681]
12.4420681	UTY	Ubiquitously transcribed tetratricopeptide repeat gene, Y-linked (UTY), transcript variant 3[NM_007125]
11.9361188	LOC728073	mRNA; cDNA DKFZp762K239 (from clone DKFZp762K239) [AL833395]
11.1046799	TTTY15	Testis-specific transcript, Y-linked 15 (non-protein coding) (TTTY15), non-coding RNA [NR_001545]
9.9518083	HLA-DRB6	Major histocompatibility complex, class II, DR beta 6 (pseudogene) (HLA-DRB6), non-coding RNA [NR_001298]
9.5174719	KIAA1751	Chromosome 1 open reading frame 222 [Source:HGNC Symbol;Acc:27917] [ENST00000378592]
9.2779971	OR2A5	*S* olfactory receptor, family 2, subfamily A, member 5 (OR2A5) [NM_012365]
8.8590285	WISP2	WNT1 inducible signaling pathway protein 2 (WISP2) [NM_003881]
8.7457057	NPTX1	Neuronal pentraxin I (NPTX1) [NM_002522]
8.719558	CEACAM8	Carcinoembryonic antigen-related cell adhesion molecule 8 (CEACAM8) [NM_001816]
8.601976	ZDBF2	*S* zinc finger, DBF-type containing 2 (ZDBF2) [NM_020923]
8.2684666	USP9Y	Ubiquitin specific peptidase 9, Y-linked (USP9Y) [NM_004654]
7.9132372	S100B	S100 calcium binding protein B (S100B) [NM_006272]
7.8571146	DAND5	DAN domain family, member 5 (DAND5) [NM_152654]
7.7909864	IFI27	Interferon, alpha-inducible protein 27 (IFI27), transcript variant 2 [NM_005532]
7.509532	ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit) [ENST00000397846]
7.5074346	IL7R	*S* interleukin 7 receptor (IL7R) [NM_002185]
7.4898327	CAMP	Cathelicidin antimicrobial peptide (CAMP) [NM_004345]
7.0189348	PDZRN4	PDZ domain containing ring finger 4 (PDZRN4), transcript variant 2[NM_013377]
6.9116334	HINT3	Histidine triad nucleotide binding protein 3 (HINT3) [NM_138571]
6.6545679	THEMIS	Thymocyte selection associated (THEMIS), transcript variant 1 [NM_001164685]
6.6351266	OLFM4	Olfactomedin 4 (OLFM4) [NM_006418]
6.620981	PDE4B	Phosphodiesterase 4B, cAMP-specific (PDE4B), transcript variant d [NM_001037341]
6.3871366	ASS1	Argininosuccinate synthase 1 (ASS1), transcript variant 1 [NM_000050]
6.2447192	LOC283174	Hypothetical LOC283174 (LOC283174), non-coding RNA [NR_024344]
6.204357	MORN4	MORN repeat containing 4, mRNA (cDNA clone IMAGE:4690584), complete cds. [BC022054]
6.1692183	CD8B	CD8b molecule (CD8B), transcript variant 4 [NM_172102]
6.1476516	NCRNA00282	Non-protein coding RNA 282 (NCRNA00282), transcript variant 1, non-coding RNA [NR_027047]
6.0312787	LOC254099	Hypothetical LOC254099 (LOC254099), non-coding RNA [NR_038869]

Table III. Upregulated DEGs in the female group (>6-fold).

Fold-change	Gene symbol	Definition [GenBank accession no.]
544.6738522	XIST	X (inactive)-specific transcript (non-protein coding) (XIST), non-coding RNA [NR_001564]
28.4003794	FFAR3	Free fatty acid receptor 3 (FFAR3) [NM_005304]
17.5307644	EGR2	Early growth response 2 (EGR2), transcript variant 1 [NM_000399]
12.1291479	CHRM4	Cholinergic receptor, muscarinic 4 (CHRM4) [NM_000741]
11.8541392	CCL3L3	Chemokine (C-C motif) ligand 3-like 3 (CCL3L3) [NM_001001437]
11.4339739	CCL4	Chemokine (C-C motif) ligand 4 (CCL4), transcript variant 1 [NM_002984]
10.4989686	NCRNA00238	Non-protein coding RNA 238 (NCRNA00238), transcript variant 1, non-coding RNA [NR_024338]
10.0601213	MXRA7	Matrix-remodelling associated 7 (MXRA7), transcript variant 1 [NM_001008528]
10.0576266	CCL4	Chemokine (C-C motif) ligand 4 (CCL4), transcript variant 1 [NM_002984]
9.4497247	H19	H19, imprinted maternally expressed transcript (non-protein coding) (H19), non-coding RNA [NR_002196]
8.8575678	TAF5L	TAF5-like RNA polymerase II, p300/CBP-associated factor (PCAF)-associated factor, 65kDa (TAF5L), transcript variant 1, [NM_014409]
8.5429208	HEMK1	HemK methyltransferase family member 1 (HEMK1) [NM_016173]
8.5307418	ZSCAN2	Zinc finger and SCAN domain containing 2 (ZSCAN2), transcript variant 1 [NM_181877]
8.320606	LIMK1	LIM domain kinase 1 (LIMK1), transcript variant 1 [NM_002314]
8.2361226	CCL3L3	Chemokine (C-C motif) ligand 3-like 3 (CCL3L3) [NM_001001437]
8.0555941	CCL3	Chemokine (C-C motif) ligand 3 (CCL3) [NM_002983]
7.9687299	XAGE5	X antigen family, member 5 (XAGE5) [NM_130775]
6.7899982	POU2F2	POU class 2 homeobox 2 (POU2F2), transcript variant 3 [NM_001207026]
6.7290741	PF4V1	Platelet factor 4 variant 1 (PF4V1) [NM_002620]
6.5002189	LOC285224	*S* hypothetical LOC285224 (LOC285224), non-coding RNA [NR_037890]
6.4555177	CXCL10	Chemokine (C-X-C motif) ligand 10 (CXCL10) [NM_001565]
6.4486358	BTG2	BTG family, member 2 (BTG2) [NM_006763]
6.2758219	KIAA0408	KIAA0408 (KIAA0408) [NM_014702]
6.2581186	SOCS3	Suppressor of cytokine signaling 3 (SOCS3) [NM_003955]
6.2558308	ARPC5	Actin related protein 2/3 complex, subunit 5, 16kDa [ENST00000367534]
6.1485137	ECSCR	*S* endothelial cell-specific chemotaxis regulator (ECSCR) [NM_001077693]
6.1442538	MEOX2	Mesenchyme homeobox 2 (MEOX2) [NM_005924]
6.0415968	FLJ40606	cDNA FLJ40606 fis, clone THYMU2011939. [AK097925]
6.0281884	MYL9	Myosin, light chain 9, regulatory (MYL9), transcript variant 2 [NM_181526]

1,313 genes (9.6% of total genes) were upregulated by at least 2.0-fold. Upregulated (>6 fold) DEGs are summarized in Table III. To examine the specific function and biological pathways of the DEGs identified, GO and KEGG pathway analysis was performed using microarray dataset using the GO (www.geneontology.gov) and KEGG pathway database (www.genome.jp/kegg/pathway.html). Among these DEGs, the top five significantly enriched biological processes in the male group were sister chromatid segregation, chromosome segregation, neural precursor cell proliferation, mitotic sister chromatid segregation and positive regulation of cell proliferation (Fig. 5A). The top five significantly enriched biological processes in the female group were platelet activation, response to wounding, wound healing, cell activation and blood coagulation (Fig. 5B).

UniProtKB analysis. Among the DEGs, 2,368 genes in the male group and 3,011 genes in the female group were successfully mapped to the UniProtKB IDs. Differences between the male and female group were categorized into six common biological functions: Cell adhesion, growth, immunity, response to stimulus, proliferation and other. It is noteworthy that a number of genes were involved in the response to stimulus pathway in males and females (37 vs. 45%; Fig. 6), suggesting that hematopoietic stem/progenitor cells are likely to be resistant to unfavorable growth environments, which is consistent with recent studies (12-14).

Signaling pathways. The top three significantly enriched signaling pathways identified in the male group were arginine biosynthesis, hematopoietic cell lineage and cytokine-cytokine

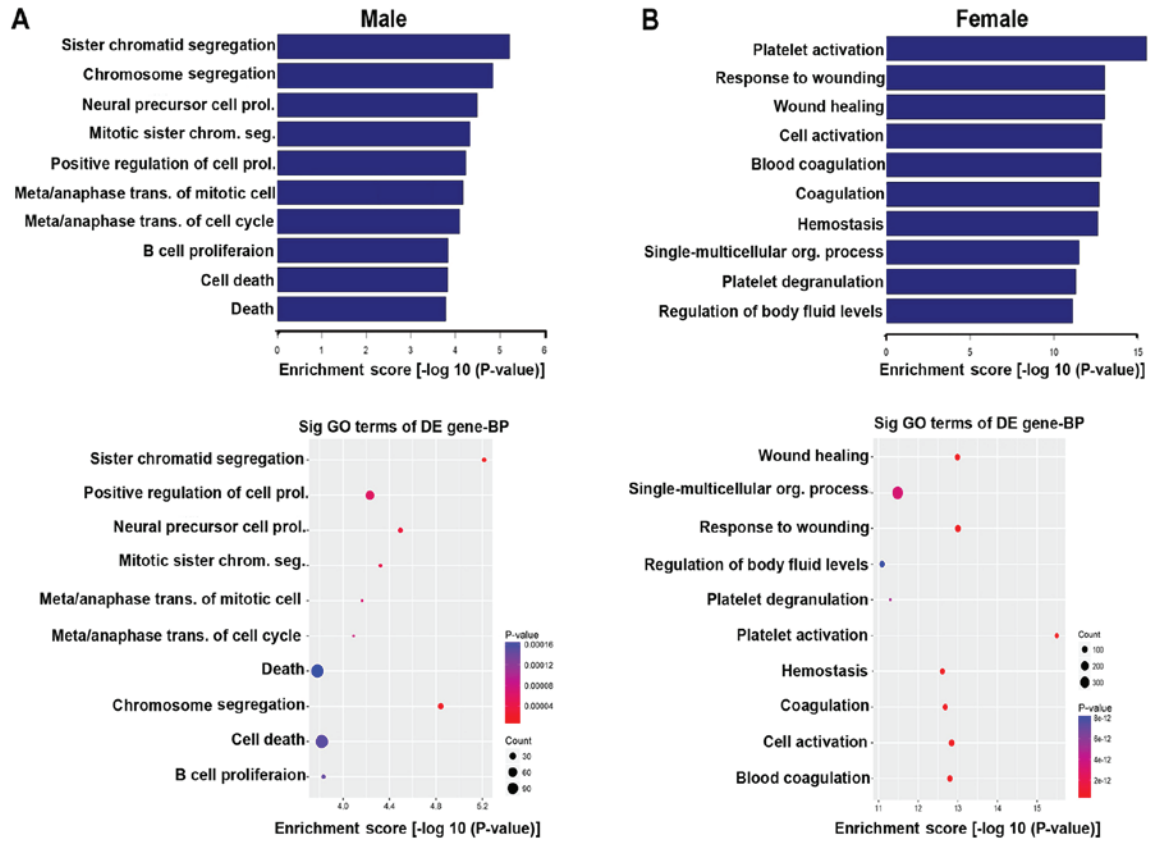

Figure 5. GO term enrichment analysis in DEGs. (A) Top 10 significantly enriched biological processes in upregulated DEGs in the male group. (B) Top 10 significantly enriched biological processes in upregulated DEGs in the female group. GO, Gene Ontology; BP, biological processes; DE, differentially expressed.

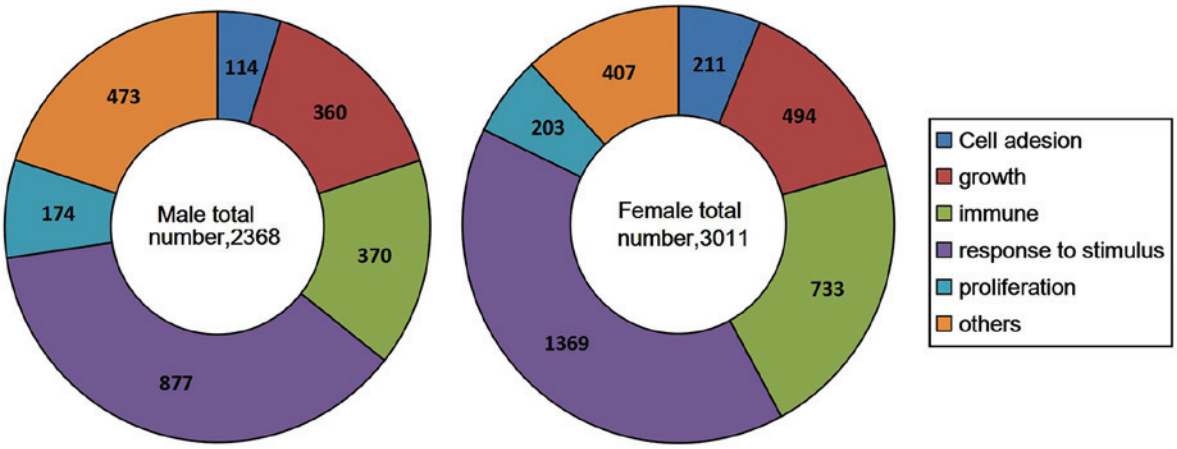

Figure 6. DEGs were successfully mapped to UniProtKB IDs. Among the DEGs, 2,368 genes in the male group and 3,011 genes in the female group were successfully mapped to UniProtKB IDs. Differences between the male and female groups were categorized into six common biological functions: Cell adhesion, growth, immunity, response to stimulus, proliferation and others. CD34+ cells from cord blood of males and females express a large number of genes involved in response to stimuli (37 vs. 45%, respectively).

receptor interaction, while the top three significantly enriched signaling pathways in the female group were graft-vs.-host disease, malaria and African trypanosomiasis (Fig. 7). The top 10 upregulated signaling pathway in the male and female group are listed in Tables IV and V, respectively. Signaling pathways associated with hematopoietic cell proliferation were screened and analyzed. Subsequent analysis focused on the hematopoietic cell lineage and cytokine-cytokine receptor interaction signaling pathways in the two groups.

In the hematopoietic cell lineage signaling pathway, the male group exhibited high expression of CD5, CD8B, CD20, CD21, CD24, CD126, CD127 and IL-7 (Fig. 8A), which are mainly associated with lymphocyte function. By contrast, the female group exhibited high expression of CD41, CD42, CD61 and thrombopoietin (TPO), which are mainly associated with platelet function (Fig. 8B).

Regarding cytokine-cytokine receptor interaction signaling pathways, the C-X-C motif chemokine ligand 12

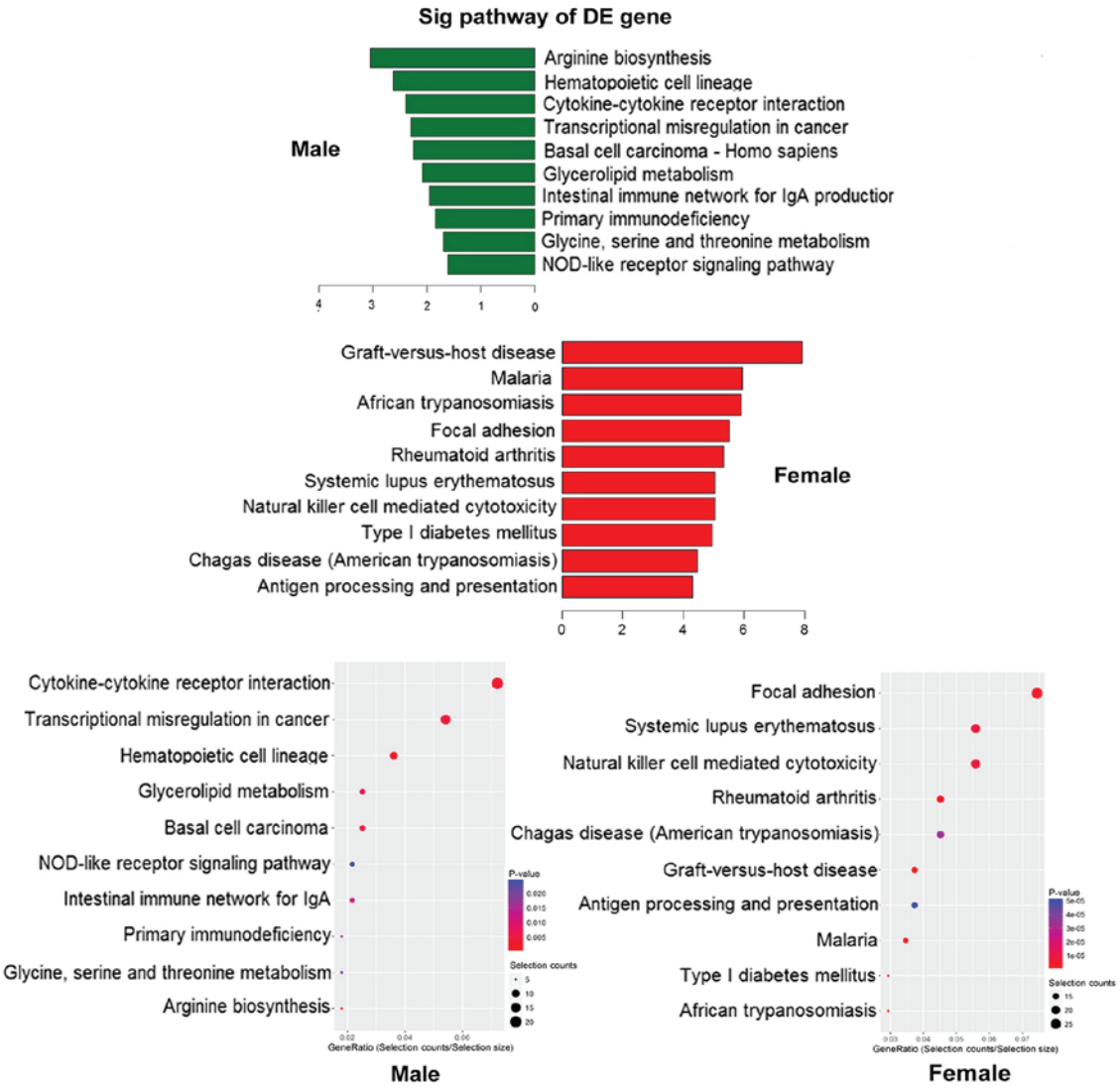

Figure 7. KEGG pathway analysis in DEGs. Comparison of significantly enriched KEGG pathways in DEGs in the male and female groups. DE, differentially expressed; Sig, signaling; Ig, immunoglobulin.

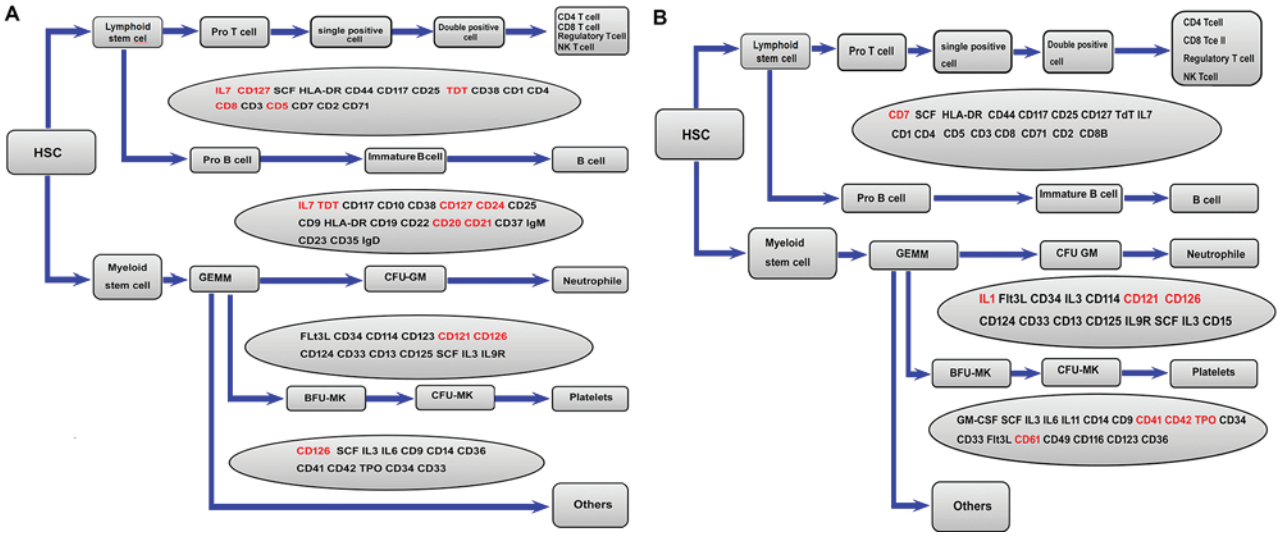

Figure 8. Hematopoietic cell lineage signaling pathway in the male and female groups. (A) Hematopoietic cell lineage signaling pathway map in the male group. Genes indicated in red were upregulated in the male group and those in gray were not differentially expressed. (B) Hematopoietic cell lineage signaling pathway map in the female group. Genes in red were upregulated in females and those in gray were not significantly differentially expressed. NK, natural killer; HSC, hematopoietic stem cell; CFU, colony-forming units; CFU-GM, CFU-granulocyte, macrophage; BFU, burst-forming unit; IL, interleukin; Ig, immunoglobulin, GEMM, granulocyte, erythrocyte, monocyte and megakaryocyte; CFU-MK, CFU-megakaryocyte.

Table IV. Top 10 significantly enriched signaling pathways in DEGs in the male group.

Signaling pathway	Fisher P-value	Genes
Arginine biosynthesis	0.0008948247	ARG1/ASS1/CPS1/GLS/GPT
Hematopoietic cell lineage	0.002383657	CD24/CD5/CD8B/CR2/DNTT/IL1R2/IL6R/IL7/IL7R/MS4A1
Cytokine-cytokine receptor interaction	0.004093339	CCL20/CCR7/CCR9/CD40LG/CXCL12/CXCR4/IL13RA1/IL1R2/IL23A/IL6R/IL6ST/IL7/IL7R/INHBB/PRL/TGFBR2/TNFRSF10D/TNFRSF25/TNFSF15/VEGFA
Transcriptional misregulation in cancer	0.005049407	ARNT2/ATM/BIRC3/CCR7/CEBPE/DEFA3/IL1R2/MMP9/NR4A3/RUNX1/SMAD1/TGFBR2/TMPRSS2/UTY/ZEB1
Basal cell carcinoma	0.005654648	APC/APC2/GLI1/HHIP/LEF1/TCF7/TCF7L2
Glycerolipid metabolism	0.008313989	AGPAT5/ALDH7A1/GPAM/GPAT2/LCLAT1/LPL/MOGAT1
Intestinal immune network for IgA production	0.001110992	CCR9/CD28/CD40LG/CXCL12/CXCR4/MADCAM1
Primary immunodeficiency	0.001453322	CD40LG/CD8B/IL7R/PTPRC/RAG1
Glycine, serine and threonine metabolism	0.001993447	ALDH7A1/CTH/GLDC/PSAT1/SARDH
NOD-like receptor signaling pathway	0.002447901	BIRC3/CARD8/NAIP/NLRP3/RIPK2/TAB1

DEGs, differentially expressed genes; Ig, Immunoglobulin.

Table V. Top 10 significantly enriched signaling pathways in DEGs in the female group.

Signaling pathway	Fisher P-value	Genes
Graft-vs.-host disease	0.001244344	FASLG/HLA-A/HLA-DPA1/HLA-DQA1/HLA-DQA2/IFNG/IL1A/IL1B/IL6/KIR2DL2/KLRC1/KLRD1/PRF1/TNF-
Malaria	0.001148487	CCL2/HBA2/IFNG/IL1B/IL6/ITGAL/KLRK1/SELE/SELP/TGFB1/THBS2/TNF/VCAM1
African trypanosomiasis	0.001221394	FASLG/HBA2/IDO1/IDO2/IFNG/IL1B/IL6/LAMA4/SELE/TNF/VCAM1
Focal adhesion	0.003092215	ACTN3/AKT1/BCAR1/CAV1/CAV2/COL2A1/EGF/FLNA/ILK/ITGA2B/ITGB3/ITGB5/JUN/LAMA4/LAMB1/MYL9/MYLK/PAK6/PARVB/PDGFD/PDGFRB/RASGRF1/RELN/THBS2/TLN1/TNXB/VCL/VWF
Rheumatoid arthritis	0.004613411	CCL2/CCL3/CCL3L3/CCL5/CXCL6/FOS/HLA-DPA1/HLA-DQA1/HLA-DQA2/IFNG/IL1A/IL1B/IL6/ITGAL/JUN/TGFB1/TNF
Natural killer cell-mediated cytotoxicity	0.009071911	CD247/FASLG/FCER1G/FCGR3A/HLA-A/IFNG/ITGAL/KIR2DL2/KIR2DS2/KIR2DS3/KIR2DS4/KLRC1/KLRC3/KLRD1/KLRK1/NCR3/NFATC2/PPP3R1/PRF1/SH2D1B/TNF
Systemic lupus erythematosus	0.009071911	ACTN3/C1QA/C1QB/FCGR3A/HIST1H2AC/HIST1H2AD/HIST1H2AE/HIST1H2AM/HIST1H2BM/HIST1H3B/HIST1H3H/HIST1H4A/HIST1H4E/HIST2H2AA4/HIST2H2AB/HIST2H2BE/HLA-DPA1/HLA-DQA1/HLA-DQA2/IFNG/TNF
Type I diabetes mellitus	0.001139852	CPE/FASLG/HLA-A/HLA-DPA1/HLA-DQA1/HLA-DQA2/IFNG/IL1A/IL1B/PRF1/TNF
Chagas disease	0.003387479	AKT1/C1QA/C1QB/CCL2/CCL3/CCL3L3/CCL5/CD247/FASLG/FOS/GNAO1/IFNG/IL1B/IL6/JUN/TGFB1/TNF
Antigen processing and presentation	0.005018711	HLA-A/HLA-DPA1/HLA-DQA1/HLA-DQA2/HSPA6/IFNG/KIR2DL2/KIR2DS2/KIR2DS3/KIR2DS4/KLRC1/KLRC3/KLRD1/TNF

DEGs, differentially expressed genes.

(CXCL12)/C-X-C motif chemokine receptor 4 (CXCR4) pathway was upregulated in the male group and the expression levels of IL-6 signal transducer (IL6ST) [glycoprotein (gp)130] and IL-7 receptor (IL-7R) were significantly higher in cells from the male than in the female group (Fig. 9A). However, in the female group, more cytokines were expressed in CD34+

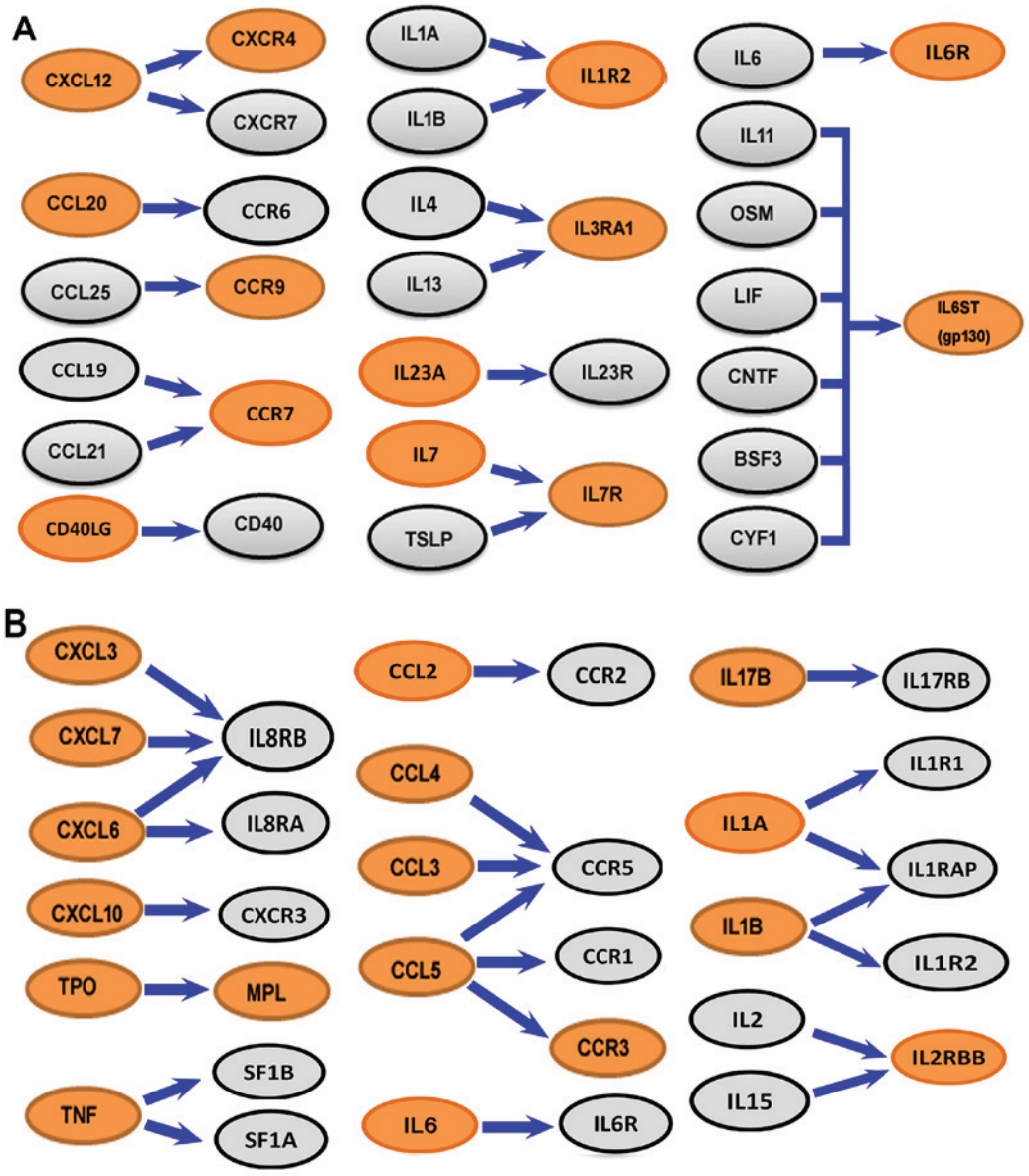

Figure 9. (A) Cytokine-cytokine receptor interaction signaling pathway map in the male group. Genes in orange were upregulated in males and those in gray were not significantly differentially expressed. (B) Cytokine-cytokine receptor interaction signaling pathway map in the female group. Genes in orange were upregulated in females and those in gray were not significantly different.

cells, including C-C motif chemokine ligand 2 (CCL2), CCL3, CCL4, CCL5, CXCL3, CXCL6, CXCL7, CXCL10, IL1A, IL1B, IL6, TPO and tumor necrosis factor (TNF) (Fig. 9B).

Higher expression of gp130 (CD130) and IL-7R (CD127) in male CD34⁺ cells. In order to verify the results of the gene chip analysis, FACS was used to validate the expression of two key molecules in the male group, namely IL-7R (CD127) and gp130 (CD130). FSC/SSC scatter diagrams for the male and female groups were gated on the overall cell population (Fig. 10A). The proportion of $CD34^+CD127^+$ cells and $CD34^+CD130^+$ cells were higher in the male group compared with the female group. $P<0.05$; Fig. 10B-D). Statistical analysis of the expression of $CD34^+CD130^+$ or $CD34^+CD127^+$cells in the male and female groups was detected by flow cytometry (Fig. 10E and F). These results are consistent with those following gene chip analysis.

Discussion

The present study attempted to determine whether neonatal gender affects the hematopoietic potential of CB transplants and, if so, to determine the underlying molecular mechanisms. The experimental results revealed a difference in the concentration of $CD34^+$ cells among CB MNCs between the male and female groups prior to amplification. In the colony formation assay, the number of CFUs and total cells in the male group were higher than those in the female group. Furthermore, the male group exhibited a higher number of CFU-Mix, and this result is consistent with that of a previous study (8). Therefore, it may be hypothesized that gender is a factor influencing HSC amplification.

In the next expansion experiment, it was observed that the cells in the male group exhibited consistently higher amplification efficiencies. However, the percentage of $CD34^+CD38^-$ cells

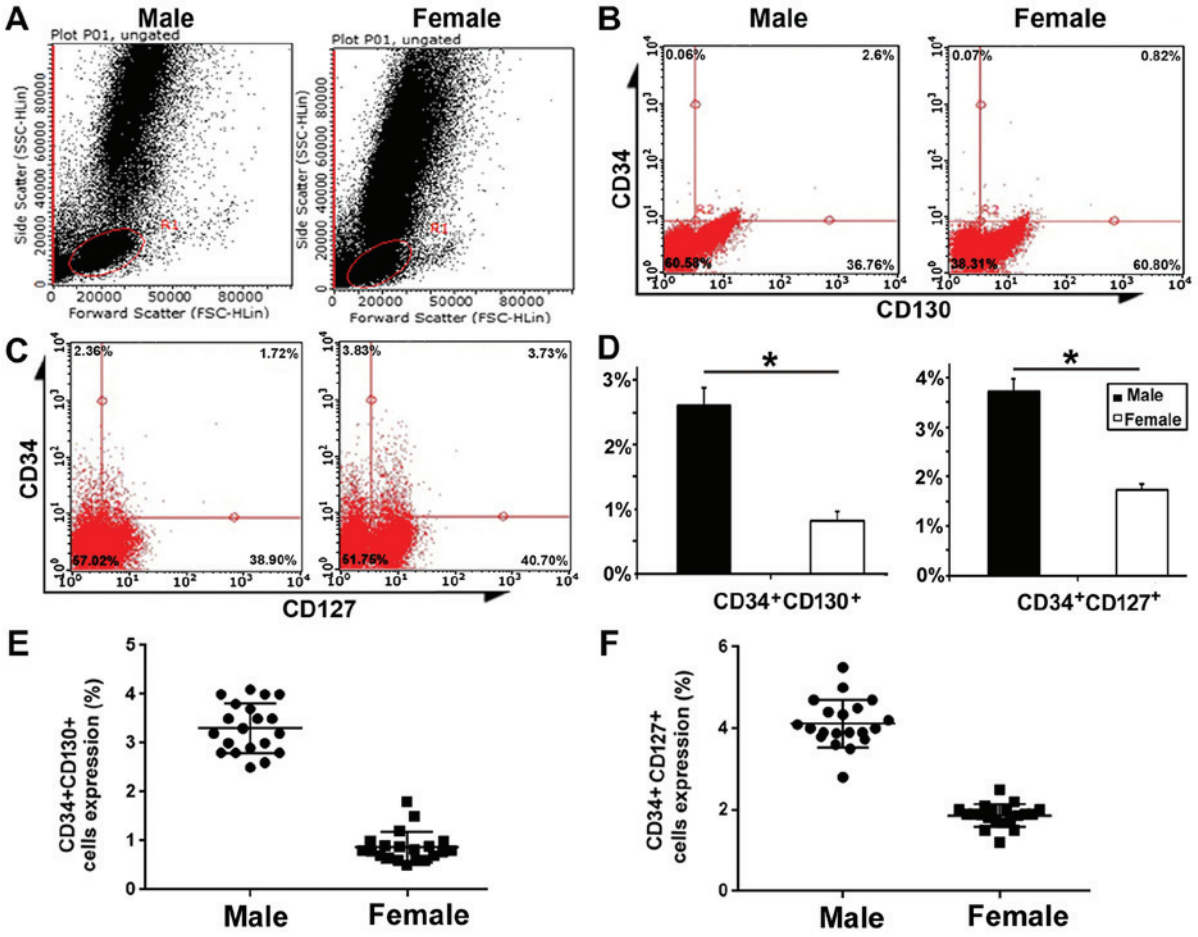

Figure 10. Validation of microarray expression data by fluorescence-assisted cell sorting. (A) FSC/SSC scatter diagrams for the male and female groups were gated on the cell population. (B) Representative FACS dot plot of $CD34^+CD130^+$ cells in male and female groups. (C) Representative FACS dot plot of $CD34^+CD127^+$ cells in male and female groups. (D) The proportions of $CD34^+CD130^+$ and $CD34^+CD127^+$ cells in the male and female groups. $^*P<0.05$. (E) Comparison of the $CD34^+CD130^+$ cells in the male and female groups. (F) Comparison of the $CD34^+CD127^+$ cells in the male and female groups. SSC, side scatter; FSC, forward scatter.

was not significantly different between the male and female groups.

The gene chip assay indicated that neonatal gender affected the gene expression in $CD34^+$ cells. In terms of the underlying mechanisms, the experimental results indicated that a large number of the upregulated DEGs were involved in chromosomal segregation in the male group. Further study focused on the analysis of signaling pathways associated with hematopoietic cell proliferation. The hematopoietic cell lineages and cytokine-cytokine receptor interaction signaling pathways were upregulated in the male group. Further analysis indicated that the genes CD5, CD8B, CD20, CD21, CD24, CD126, CD127 and IL-7 were highly expressed in the male group. CD5 and CD8B belong to the T-cell antigen phenotype, and CD20, CD21 and CD24 belong to the B-cell antigen phenotype. Regarding cytokine-cytokine receptor interaction signaling pathways, DEGs were upregulated in the CXCL12/CXCR4, IL6ST (gp130), IL-7 and IL-7R pathways in the male group. Based on the above analysis, it was hypothesized that the reasons for the better expansion of $CD34^+$ cells in the male group may be as follows: i) The male group had a higher expression of genes associated with chromosome segregation. Combined with the fact that DEGs involved in the positive regulation of cell proliferation were upregulated, it was speculated that male CB $CD34^+$ cells have more opportunities to enter the mitosis period, which is conducive to cell proliferation. This may explain for the observation that, under the same culture conditions, CB $CD34^+$ cells from males exhibited higher amplification efficiencies than those from females. ii) DEGs associated with lymphocyte proliferation, activation and other functions, including CD5, CD8B, CD20, CD21, CD24, CD126, CD127, IL-7 and IL-7R, were highly expressed in the male group. The major physiological role of IL-7 is to promote T/B lymphocyte development. IL-7 may promote the growth of B-progenitor cells, thymocytes and peripheral mature T cells derived from human or mouse bone marrow (15). According to FACS, CD127 antigens were more highly expressed in male CB $CD34^+$ cells. These results were consistent with those of the gene chip assay. iii) The expression of IL6ST (gp130) was obviously higher in the male group. According to previous studies (16-18), peripheral blood (PB)-derived CD34+ cells ubiquitously express gp130 (19). Gp130 forms the β subunit of IL-6R, which is composed of the IL-6 α-receptor and the β-subunit glycoprotein 130. It is

well documented that signals activated by IL-6 are transmitted through the signal-transducing gp130 (20). The physiological role of IL-6 is to stimulate B and T cell proliferation and secretion of antibodies (21). The expression of CD5, CD8B, CD20, CD21, CD24, CD126, CD127, IL-7, IL-7R and IL6ST were significantly higher in male CB $CD34^+$ cells. According to FACS, CD130 membrane surface antigens were more highly expressed in $CD34^+$ cells from male CB. These results were consistent with those of the gene chip. They suggest that there may be more lymphoid progenitor cells in the $CD34^+$ HSCs and hematopoietic progenitor cells (HSP) population, or that $CD34^+$ cells of the male group more easily differentiated into lymphoid progenitor cells. iv) Finally, the CXCL12/CXCR4 pathway was upregulated in the male group. Mice lacking either CXCL12 or CXCR4 exhibit similar embryonic lethal defects, including impaired myeloid and B-cell hematopoiesis (22-24). CXCL12 is a crucial factor involved in the migration, retention, and mobilization of HSCs during homeostasis and after injury, and its corresponding receptor is CXCR4. Collectively, the genetic and functional data indicate that the CXCL12/CXCR4 pathway is crucial for the retention and maintenance of adult HSCs (25). Therefore, it may be speculated that the upregulation of this signaling pathway in the male group is conducive to the maintenance of HSC characteristics and functions. Together, these four points may partially explain why the $CD34^+$ cells from CB of male neonates exhibit an increased amplification capacity.

In the female group, the results revealed an increased expression of platelet phenotype-associated genes, including CD41, CD42, TPO and CD61, suggesting that the $CD34^+$ cells from CB of female neonates may exhibit increased platelet activation and coagulation functions. In addition, female cells expressed more chemokines, including CCL2, CCL3, CCL4, CCL5, CXCL3, CXCL6, CXCL7, CXCL10, 1L1A, 1L1B, IL6, TPO and TNF. This may indicate that the $CD34^+$ cells in the female group have a better migration/chemotaxis ability than those in the male group.

In conclusion, the present study indicated that gender may affect the hematopoietic potential of CB. Whether this difference has any clinical consequences remains to be determined. Careful selection of the best CB units for transplantation may improve the efficiency of this source of HSCs and HSPs in adult transplantation settings and reduce the cost of processing (26). The experimental results of the present study may therefore provide a reference for the clinical selection of CB and insight into potential treatments for certain types of hematological disorder.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DL and XJ contributed to the study design. PZ, XL, XX and QS performed the experiments. LZ, ZC and XZ performed the statistical analysis and interpreted the data. LZ prepared the manuscript. All authors read and approved the final manuscript.

Ethical approval and consent to participate

The current study was approved by the Ethics Committee of Shandong University Qilu Hospital (Jinan, China). Written informed consent was obtained from all donors.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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