HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4

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Online Supplementary Data to Design and Methods

Isolation and immuno-labeling of CD34⁺ cells

Normal cord blood was collected according to institutional guidelines and after informed consent of the mothers. Following Ficoll separation (Lymphoprep, Fresenius Kabi, Sèvres, France), CD34⁺ cells were enriched with the CD34 microBead kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. For isolation of the most immature hematopoietic progenitor cells, CD34⁺ cells were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated antibody to CD34 (clone 581, Beckman Coulter, Villepinte, France) and phycoerythrin-conjugated antibody to CD38 (clone T16, Beckman Coulter).

Co-cultures

CD34⁺ cells (1,000 cells/cm²) were co-cultured for 4-5 weeks with 25-Gy pre-irradiated MS-5/GFP, MS-5/HOXB4, or MS-5/HOXC4 cells in H5100 long-term culture medium (StemCell Technologies, Grenoble, France). Total cells and CD34⁺ cells were counted every week to evaluate expansion ratios. A cell sample was seeded every week into methylcellulose as described below for progenitor assays. For quantification of long-term culture-initiating cells (LTC-IC), CD34⁺ cells derived from 4- to 5-week-old primary co-cultures were sorted and plated again at limiting dilutions on pre-irradiated non-transduced MS-5 cells for 4-5 additional weeks, after which the number of LTC-IC was determined by counting the number of colony-containing wells.

Mouse assays

For *in vivo* experiments, expansion rates of CD34⁺ cells after 4 weeks of co-culture varied from x12 (in the presence of MS-5/GFP cells) to x30 (in the presence of MS-5/HOXB4 or /C4 cells). The mean total CD34⁺ cell numbers obtained from 5,000 initial CD34⁺ cells was, therefore, 60,000 with MS-5/GFP and 150,000 with MS-5/HOXB4 or /C4. Considering that about 50% of cells were retrieved after cell sorting, a mean of 30,000 and 75,000 CD34⁺ cells, respectively, were injected into animals.

Semi-solid cloning assays

Human clonogenic progenitors were tested by plating 500 cells in 35-mm duplicate dishes (Dutscher, Brumath, France), in 1 mL methylcellulose (StemCell Technologies) supplemented with 2 U/mL erythropoietin, 10 ng/mL interleukin-3, 15 ng/mL stem cell factor, 20 ng/mL granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA, USA), and 5 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; Schering-Plough, Heist-op-den-Berg, Belgium). Dishes were incubated at 37°C in a fully humidified atmosphere containing 5% CO_2. Burst-forming units-erythrocyte (BFU-E), colony-forming units-granulocyte/macrophage (CFU-GM), and colony-forming units-granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) were counted on day 15 under an inverted microscope.

Western blots

Cells were rinsed and then lysed in Ripa (Tris-HCl pH8, EDTA 4 mM, NaCl 150 mM, Triton 0.5%, SDS 0.2%) in the presence of a protease inhibitor cocktail (Roche, Rosny-sous-Bois, France). After denaturing polyacrylamide gel electrophoresis (SDS-PAGE) migration, proteins were transferred onto a HybondC Extra membrane (Amersham Bioscience, Munich, Germany). The membrane was blocked for 1 h at room temperature with 5% (w/v) skimmed milk in Tris-buffered saline-0.2% Tween (TBS-T), then hybridized in the same buffer with the I12 rat anti-human HOXB4 monoclonal antibody and/or the N-20 goat anti-human HOXC4 antibody (sc-49986, Santa Cruz Biotechnology, Santa Cruz, CA, USA), or a mouse monoclonal anti-actin antibody (clone AC-15, Abcam, Paris, France) as a control. Antibodies were diluted 1:1000 and incubated overnight at 4°C. Blots were visualized using a horseradish peroxidase-conjugated secondary goat anti-rat (sc-2006, Santa Cruz Biotech) anti-mouse antibodies, or a donkey anti-goat antibody (sc-2056, Santa Cruz Biotech). Chemiluminescence was detected (enhanced chemiluminescence, Amersham Bioscience, Villejuif, France). Images were captured using a CCD camera (LAS4000, Fujifilm, Tokyo, Japan) and quantification was performed using Image Reader Las-4000 software (Fujifilm).

Online Supplementary Table S1. List of primers used for RT-qPCR analysis of genes from the transcriptomes of CD34$^+$ cells exposed to HOXB4 or HOXC4.

Gene Symbols	Pubmed accession numbers	Sequences 5' TO 3'
ABCE1 (F)	NM_002940.2	GCCAAACCTTGGAAAGTACG
ABCE1 (R)		TTGTAATTCAGATCCACGGAAA
BOP (F)	NM_015201.3	TCTGAGCCCGAACTGGAG
BOP (R)		TGCTGTGGCTGAGAGGAGA
BRCA2 (F)	NM_000059.3	GCGCGGTTTTTGTCAGCTTA
BRCA2 (R)		TGGTCCTAAATCTGCTTTGTTGC
CASP8 AP2 (F)	NM_012115.2	GAAATGAAAGCCCACCTCAA
CASP8 AP2 (R)		GCTGAATTTGGTAAAAACACATCTT
CCND2 (F)	NM_001759.2	GGACATCCAACCCTACATGC
CCND2 (R)		CGCACTTCTGTTCCTCACAG
C-MYC (F)	NM_002467.4	CACCAGCAGCGACTCTGA
C-MYC (R)		GATCCAGACTCTGACCTTTTGC
DACH1 (F)	NM_004392.4	AGCAGTTGGCTATGGAACAAA
DACH1 (R)		CCGTTTCGTCTCAAACTCAAG
DBF4 (F)	NM_006716.3	TGGAATATGAAAAGGACACACCT
DBF4 (R)		CAGTCTTTTTCAGGACACTTGC
DHX9 (F)	NM_001357.3	CTGCCTGTATCACTGGTC
DHX9 (R)		CTACACACAGGAACTGAC
EZH2 (F)	NM_004456.3	TGGTCTCCCCTACAGCAGAA
EZH2 (R)		CCGTTTCGTCTCAAACTCAAG
GNL3 (F)	NM_014366.4	CAGCAACGTCTATGACCTCCT
GNL3 (R)		CTTGAAGATCTGCCCAGTCC
HBP1(F)	NM_012257.3	AATATACTCAGATGTATCCAGGGAAAG
HBP1 (R)		TTCCACCTGTCACCAAGGA
HDAC2 (F)	NM_001527.2	CAGATCGTGTAATGACGGTATCA
HDAC2 (R)		CCTTTTCCAGCACCAATATCC
HBG1 (F)	NM_000184.2	TGGATCCTGAGAACTTCAAGC
HBG1 (R)		CACTGGCCACTCCAGTCAC
HBA1(F)	NM_000558.3	GACCCGGTCAACTTCAAGC
HBA1 (R)		AGAAGCCAGGAACTTGTCCA
HNRNPR (F)	NM_001102398.1	TGGAAAACTCGAAAGAGTAAAGAAG
HNRNPR (R)		CCATTCATTTCATCCATAGCC
HSP90AB1 (F)	NM_007355.2	AGCCTACGTTGCTCACTATTACG
HSP90AB1 (R)		GAAAGGCAAAAGTCTCCACCT
IFITM1 (F)	NM_003641.2	CACGCAGAAAACCACACTTC
IFITM1 (R)		TGTTCCTCCTTGTGCATCTTC
IGFBP2 (F)	NM_000597.2	GGTGGCAAGCATCACCTT
IGFBP2 (R)		TCCTGTTGGCAGGGAGTC
IKZF (F)	NM_006060.2	CCTTCCGGGCACACTGTA
IKZF (R)		TCTCTCTGATCCTATCTTGCACA
NOL5A (F)	NM_006392.2	AATTCCACAGCATCGTTCG
NOL5A (R)		GCGGAGGTCCTCATGAAC
PBXIP1 (F)	NM_020524.2	TCAGGGACCTCAGCAACTATG
PBXIP1 (R)		CTCCACTGGCAGGCTCTC
PPAN (F)	NM_020230.4	CCATCAACGTGCACAAGG
PPAN (R)		GAGTCGGGGTTGTAGTCGAT
PUM1 (F)	NM_014676.2	GTCCTGTCATTGGCACTACAGAT
PUM1 (R)		CCCGAACCATCTCATTCTG
PUM2 (F)	NM_015317.1	TGATCTGTGGATGAAATGAATC
PUM2 (R)		TTTACAGCATTCCATTTGGTG
RAD51 (F)	NM_002875.3	CAAAGGCGGTCAGAGATCAT
RAD51 (R)		TGATAGATCCAGTCTCAATTCCAC
RCF3 (F)	NM_181558.2	GGGACGGCTGGACTATCA
RCF3 (R)		GGAAAGTCACCACACTGCAC
TP53 (F)	NM_000546.3	AGGCCTTGGAACTCAAGGAT
TP53 (R)		CCCTTTTTGGACTTCAGGTG
YPEL5 (F)	NM_016061.1	CACATCTCCACTCGTTTCACAGG
YPEL5 (R)		CGCTGTACTGCAGGTTAACTACC

Online Supplementary Table S2. List of genes up- or down-regulated after CD34$^+$ cell exposure to HOXB4 and to HOXC4 (fold change threshold = 0.5; false discovery rate, i.e. corrected *P*-value = 5%). A total of 422 genes were up-regulated and 167 genes were down-regulated (see Table S2 xlsx).

Online Supplementary Table S3. Genes involved in the cell cycle, and whose expression was deregulated in hematopoietic cells after 24 h exposure to HOXB4/C4.

Positively regulated	Negatively regulated
Cyclins: A2, D2, E2	Cyclin D binding protein 1 (CCNDBP1)
CDCs: 2, 6, 20	
CDC associated-5	
CDC2-like	
CDC CDK2	CDKI-1C (= P57 or Kip2)
MCMs: 3, 4, 5, 6, 7, 8	MCM3APAS (anti-sense RNA)
Myc, Max	
Activator of S-phase kinase (ASK)	
MAD2	
MARK3	
HDAC2	
M-phase phosphoprotein 6	
Origin recognition complexes: 1, 6	
RCCs: 1, 2	
Thymidine kinase 1	

Online Supplementary Table S4. List of genes involved in various "Ingenuity" canonical pathways, whose expression was modified in hematopoietic cells exposed to HOXB4/C4.

Ingenuity Canonical Pathways	Molecules
Role of BRCA1 in DNA Damage Response	MLH1, RAD51, SMARCA4, RFC3, BRCA2, RBBP8, TP53, ATM, RFC2
Hypoxia Signaling in the Cardiovascular System	LDHA, NFKBIA, UBE2D3, UBE2N, UBE2M, HSP90AA1, UBE2I, HSP90AB1, TP53, ATM, PTEN
ATM Signaling	RAD51, NFKBIA, SMC1A, TP53, ATM, SMC2, CDK2, SMC3
Cell Cycle: G1/S Checkpoint Regulation	MAX, HDAC2, TFDP1, MYC, CCND2, CCNE2, TP53, ATM, CDK2
Role of CHK Proteins in Cell Cycle Checkpoint Control	RFC3, TP53, ATM, RFC2, CDK2
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	YWHAE, YWHAZ, PRKDC, TOP2B, TP53, ATM
EIF2 Signaling	GRB2, EIF2AK2, PDPK1, EIF3C, PIK3C2A, EIF3H, EIF3A, EIF4G1, EIF2B5, PPP1CA, EIF2S3, EIF3F, EIF2S1
RAN Signaling	RCC1 (includes EG:1104), RANBP1, CSE1L
Cell Cycle Regulation by BTG Family Proteins	PRMT1, CCNE2, PPP2R1A, CDK2
p53 Signaling	PRKDC, STAG1, PIK3C2A, CCND2, TP53, ATM, PTEN, CDK2, PLAGL1
Myc Mediated Apoptosis Signaling	GRB2, YWHAE, MYC, YWHAZ, PIK3C2A, TP53
Mitotic Roles of Polo-Like Kinase	KIF23, SMC1A, HSP90AA1, CDC20, HSP90AB1, PPP2R1A
IGF-1 Signaling	IGFBP2, GRB2, YWHAE, PRKCH, PDPK1, YWHAZ, PRKAR1A, PIK3C2A, CSNK2A1
14-3-3-mediated Signaling	TUBB, GRB2, YWHAE, PRKCH, YWHAZ, TUBA1A, PIK3C2A, TUBA4A, VIM, TUBB2A
Angiopoietin Signaling	GRB2, NFKBIA, CRK, TEK, PIK3C2A, ANGPT1
PI3K/AKT Signaling	GRB2, YWHAE, NFKBIA, PDPK1, YWHAZ, HSP90AA1, HSP90AB1, PPP2R1A, TP53, PTEN
Protein Ubiquitination Pathway	USP22, UBE2D3, UCHL5, CDC20, PSMD3, HSP90AA1, UBE2I, USP10, PSMD12, USP14, HSPA8, UBE2M, UBE2N, PSMD11

Online Supplementary Table S5. Genes of the heat shock protein (HSP) family up-regulated by HOXB4/C4 in hematopoietic cells.

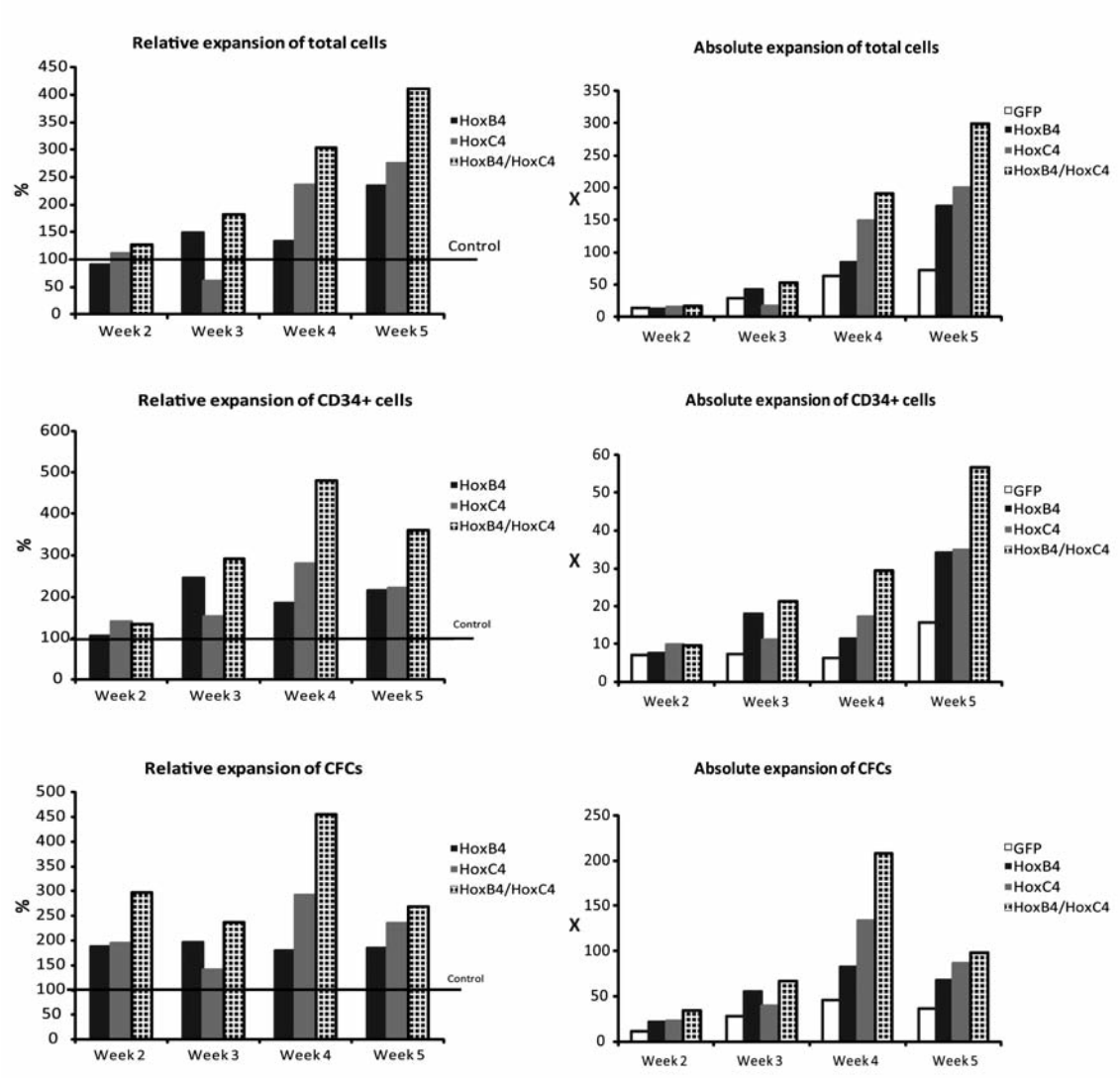
HSP40 A member 1
A member 2
B member 11
C member 7
HSP70 protein 1A (= HSPA1A, see Table 2)
protein 8
HSP70 homolog
HSP90 αA1
αB1
β1 (TRA1)
HSP105-110 (HSP H1)
HSP70/97 organizing protein (STIP1)
HSP60 pseudogene1
pseudogene3

Online Supplementary Table S6. Main functions of the genes listed in Table 1A and Table 2 (underlined genes) (from the Gene Database of The National Center for Biotechnology Information (NCBI).

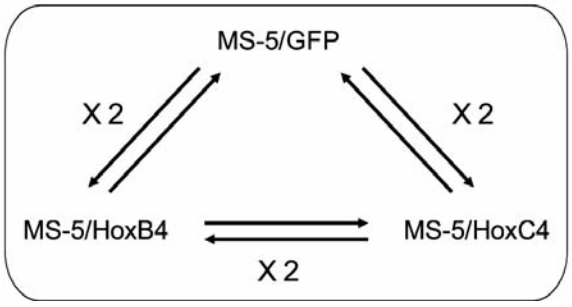
EGR1 is an early growth response gene that displays FOS-like functions in several cell types, particularly in hematopoietic cells. It acts as a cell cycle and transcriptional regulator following mitogenic stimulation and as a tumor suppressor gene.
MEF2C encodes a transcription factor mainly involved in muscle differentiation, but also promotes myeloid progenitor proliferation as a target of the myeloid-specific miRNA-223 known to negatively regulate progenitor proliferation and granulocytic differentiation.
NCL (Nucleolin) is a nucleolar phosphoprotein involved in the synthesis and maturation of ribosomes.
TRIM28 mediates transcriptional control by interacting with the Krüppel-associated box repression domain found in many transcription factors. It acts as a retroviral silencer in embryonic stem cells.
BPTF is thought to be a transcription regulator. The protein contains a C-terminal bromodomain characteristic of proteins that regulate transcription during proliferation. It is necessary for the chromatin remodeling that occurs during gene transcription.
TES is similar to mouse Testin, a testosterone-responsive gene containing 3 LIM domains that mediate protein interactions between transcription factors, cytoskeletal proteins, and signaling proteins.
KLF10 (Krüppel-like factor 10) acts as an inducer or repressor of gene transcription to enhance the TGFβ/Smad pathway and other signaling pathways to regulate cell proliferation, differentiation, and apoptosis.
GATA3 encodes an enhancer-binding zinc-finger protein that plays important roles during fetal liver hematopoiesis; GATA-3 is also a regulator of T-cell development.
HNRPDL binds to pre-mRNAs and plays a role in mRNA splicing and nuclear export. Its expression is down-regulated in leukemic cells induced to proliferate and differentiate with interleukin-4 plus phorbol ester.
HNRPK encodes a nuclear RNA-binding protein that complexes with heterogeneous nuclear RNAs (hnRNAs). It influences pre-mRNA processing, metabolism, and transport, and strongly binds to poly(C). This protein is thought to have a role during cell cycle progression.
HOXA9 is a member of the *Hox* complex gene family alike *Hoxb4* and *Hoxc4*, and seems to be involved in hematopoietic cell differentiation. It is frequently over-expressed in human myeloid leukemias; *Hoxa9-NUP98* gene fusions are observed in recurrent t(7;11) translocations in some acute myeloid leukemias of the FAB M2 and M4 types.
RNPS1 encodes a protein that is part of a postsplicing multiprotein complex involved in mRNA nuclear export and surveillance and that acts by triggering the degradation of mRNAs containing premature stop codons. This protein remains bound to mRNAs after nuclear export, thus acting a nucleo-cytoplasmic shuttling protein.
CEBPB encodes a transcription factor involved in regeneration and metabolism of stem cells of numerous tissues, particularly the hematopoietic tissue.
TSC22D1 encodes a transcription factor that belongs to the early response gene family and has a transcriptional repressor activity.
ZMYND11 encodes a nuclear zinc-finger protein first identified by its ability to bind the adenovirus E1A protein. It is a transcriptional repressor whose activity is inhibited by E1A.
HBP1 encodes a transcriptional repressor and cell cycle inhibitor. It is involved in the development of thymocytes, and acts as a tumor suppressor and differentiation inducer of myeloid cells. It also regulates the cell cycle during liver regeneration and has a role in tissue maintenance. Finally, it is known to be a suppressor of Wnt signaling.
IKZF1 (*ikaros*) encodes a key factor for overall lymphocyte development. Mutations of this gene can lead to lymphoid malignancies. ikaros-null mice display pleiotropic hematopoietic cell defects, including stem cell and lymphoid defects besides abnormalities of red cell and megakaryocyte progenitors.
LYAR encodes a nucleolar zinc-finger protein that plays a critical role in maintaining embryonic stem (ES) cell identity and growth, and in preventing their apoptosis. The protein forms a complex with nucleolin (NCL, see above) and prevents self-cleavage of that factor which is crucial for ES cell growth. Thus, LYAR controls the stability of NCL which in turn, acts by maintaining ES cell self-renewal.
RNF10 encodes a protein containing a ring-finger motif known to be involved in protein-protein interactions. It regulates the expression of myelin-associated glycoprotein, but further specific functions of this protein remain to be determined.
SUPT16H is a suppressor of Ty16 element homologs (*S. cerevisiae*). This actor could play a role through interactions with histones.
SYF2 encodes a nuclear protein that interacts with cyclin-D-type binding-protein 1 which is involved in cell cycle regulation at the G1/S transition.

Online Supplementary Table S7. Main functions of the tint genes listed in Table 2 (from the Gene Database of The National Center for Biotechnology Information (NCBI).

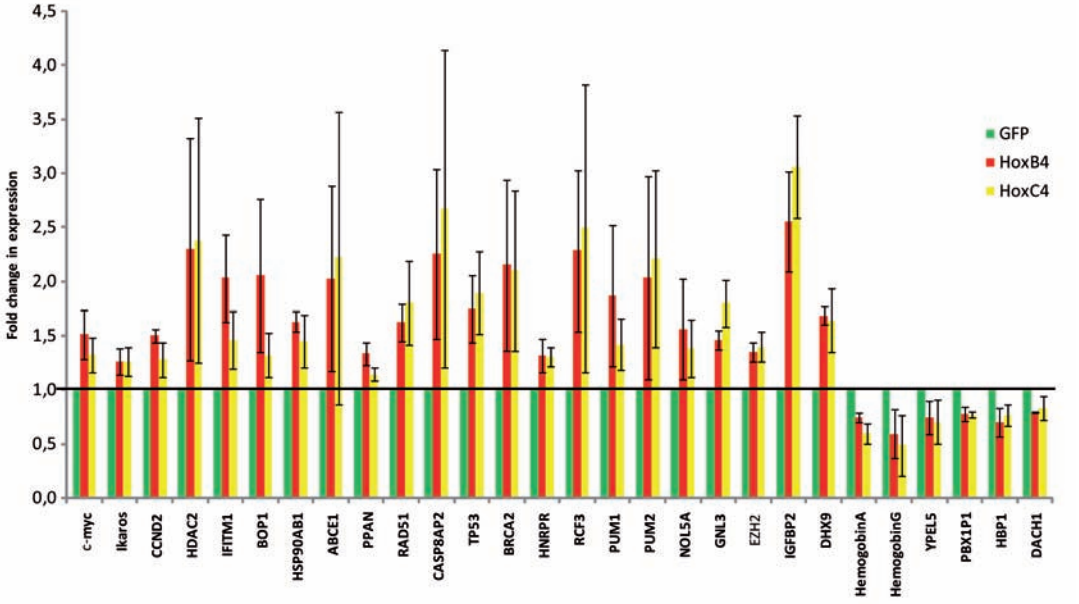
HSPA1A encodes the Hsp70 heat-shock chaperone protein that, among several functions, acts during erythroid differentiation. It interacts with GATA1 in the nucleus of erythroid precursors undergoing terminal differentiation and protects GATA1 from caspase-3 mediated cleavage and proteolysis.
DACH1 encodes an ubiquist protein that regulates early progenitor cell proliferation during retinogenesis and pituitary development by directly repressing cyclin-dependent kinase inhibitors. It could also play a role in other tissue stem cell expansion; loss of *DACH1* is associated with some poor-prognosis breast cancers and oncogene-dependent tumor metastasis.
CDKN1C encodes the cyclin-dependent kinase inhibitor KIP2 that is involved in cell proliferation and differentiation of various tissues. Several mutations of this gene can cause the Beckwith-Wiedemann syndrome, characterized by excessive growth, organomegaly, and tumors.
CD53 encodes a protein belonging to the tetraspanin family. This proteins plays a role in the regulation of cell development, activation, growth and motility. This cell surface glycoprotein is known to complex with integrins. Familial deficiency of this gene has been linked to an immunodeficiency associated with recurrent infectious diseases caused by bacteria, fungi and viruses.
LGALS3 encodes a member of the galectin family of carbohydrate binding proteins. This protein plays a role in numerous cellular functions including apoptosis, innate immunity, cell adhesion and T-cell regulation.
TPM4 encodes a member of the tropomyosin family of actin-binding proteins involved in the contractile system of striated and smooth muscles and the cytoskeleton of non-muscle cells.
SOD2 encodes a member of the iron/manganese superoxide dismutase family. This mitochondrial protein binds to the superoxide byproducts of oxidative phosphorylation and converts them to hydrogen peroxide and diatomic oxygen. Mutations in this gene have been associated with idiopathic cardiomyopathy (IDC), premature aging, sporadic motor neuron disease, and cancer.
WDR77 encodes a protein of the 20S PRMT5 containing methyltransferase complex, which modifies specific arginines to dimethylarginines in several spliceosomal Sm proteins. This modification targets Sm proteins to the survival of motor neurons (SMN) complex for assembly into small nuclear ribonucleoprotein core particles.



Online Supplementary Figure S1. Expansion of human CD34$^+$ cells cultured in the presence of HOXB4, HOXC4, or both HOXB4 and HOXC4 proteins. Left-side panels represent relative expansion rates of cells exposed to either HOXB4, or HOXC4, or HOXB4 and HOXC4; the 100% reference line corresponds to control expansion in the presence of MS-5/GFP. Right-side panels represent absolute cell expansion levels, relative to the day-0 cell input.

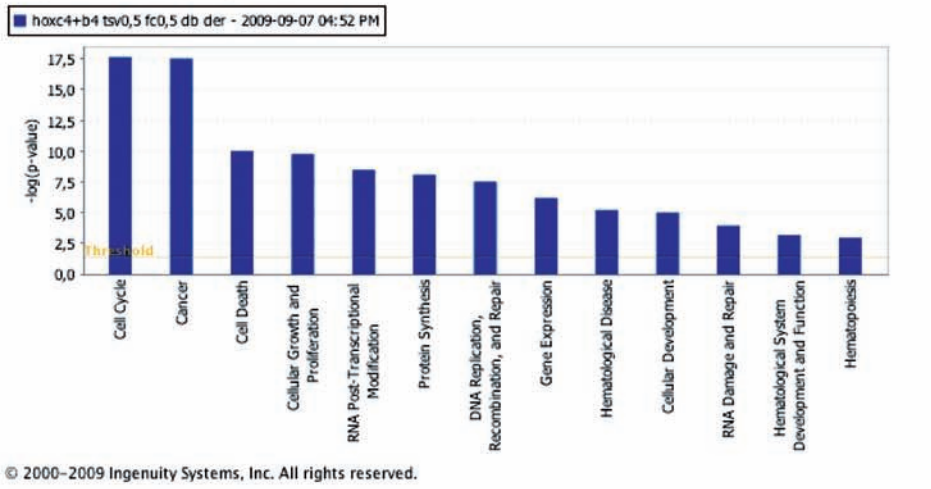


Online Supplementary Figure S2. Strategy used for transcriptome analysis. Duplicate, both-sense experiments were performed for either direct (MS-5/HOXB4 *versus* MS-5/HOXC4) or indirect (MS-5/HOXB4 *versus* MS-5/GFP + MS-5/HOXC4 *versus* MS-5/GFP) comparative transcriptome analysis.



Online Supplementary Figure S3. Validation of the transcriptome. RT-qPCR was used to confirm the changes in gene expression observed in the micro-array study. Gene expression changes were measured by duplicate analysis of four RNA samples from purified CD34$^+$ cells co-cultured with MS-5/GFP, MS-5/HOXB4, or MS-5/HOXC4 for 24 h. Relative differences in gene expression were calculated by using the $2^{-\Delta\Delta CT}$ method, which involves normalizing the CT value for each gene to the CT value of the β2-microglobulin housekeeping gene. Values are shown as the fold induction relative to GFP (mean ± standard deviation).

A



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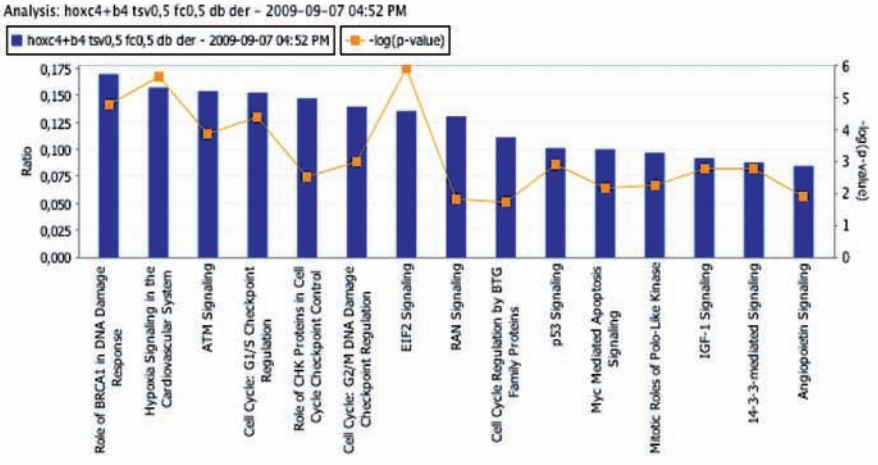

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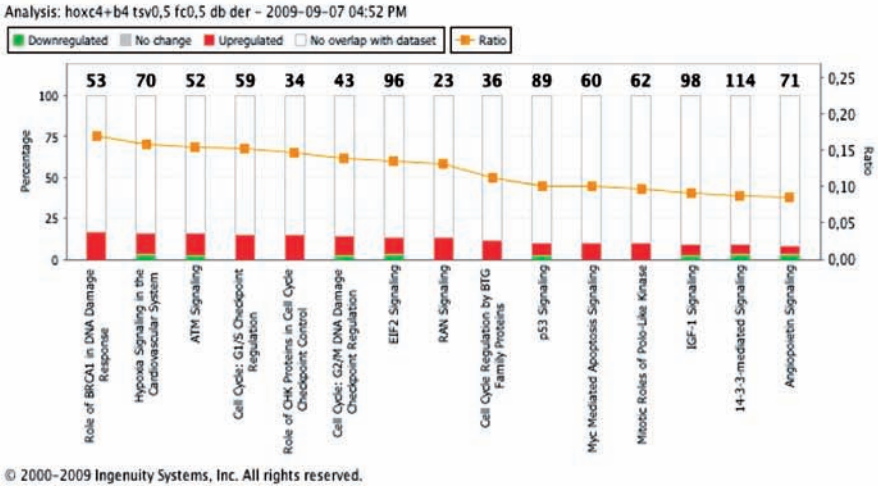

Online Supplementary Figure S4. Main cardinal functions and pathways altered after cell exposure to HOXB4/C4. (A) log(*P*-value) variation of the expression of genes involved in cardinal cell functions. (B) log(*P*-value) variation of the expression of main gene pathways deregulated by HOXB4/C4. (C) Proportion of the number of deregulated genes relative to the total number of genes reported for every pathway. All data are derived from Ingenuity software analysis.