# HOXB4's road map to stem cell expansion

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**Homeodomain-containing transcription factors are important regulators of stem cell behavior. HOXB4 mediates expansion of adult and embryo-derived hematopoietic stem cells (HSCs) when expressed ectopically. To define the underlying molecular mechanisms, we performed gene expression profiling in combination with subsequent functional analysis with enriched adult HSCs and embryonic derivatives expressing inducible HOXB4. Thereby, we identified a set of overlapping genes that likely represent "universal" targets of HOXB4. A substantial number of loci are involved in signaling pathways important for controlling self-renewal, maintenance, and differentiation of stem cells. Functional assays performed on selected pathways confirmed the biological coherence of the array results. HOXB4 activity protected adult HSCs from the detrimental effects mediated by the proinflammatory cytokine TNF-$\alpha$. This protection likely contributes to the competitive repopulation advantage of HOXB4-expressing HSCs observed *in vivo*. The concept of TNF-$\alpha$ inhibition may also prove beneficial for patients undergoing bone marrow transplantation. Furthermore, we demonstrate that HOXB4 activity and FGF signaling are intertwined. HOXB4-mediated expansion of adult and ES cell-derived HSCs was enhanced by specific and complete inhibition of FGF receptors. In contrast, the expanding activity of HOXB4 on hematopoietic progenitors in day 4–6 embryoid bodies was blunted in the presence of basic FGF (FGF2), indicating a dominant negative effect of FGF signaling on the earliest hematopoietic cells. In summary, our results strongly suggest that HOXB4 modulates the response of HSCs to multiple extrinsic signals in a concerted manner, thereby shifting the balance toward stem cell self-renewal.**

hematopoiesis | HOX genes | microarray | self-renewal | embryoid bodies

The lifelong production of blood cells by the hematopoietic system strictly depends on the maintenance of its central organizer, the hematopoietic stem cell (HSC). Because of its unique and characteristic ability to undergo self-renewal divisions and/or differentiate into mature blood cells, strategies to expand HSCs *ex vivo* to increase their clinical potential in cell and gene therapy have become of key interest.

Expansion of HSCs naturally occurs in the fetal liver during embryonic development and in the bone marrow (BM) of the adult organism as a response to extrinsic cues. The signaling pathways controlling this process and the molecular mechanisms specifying whether the self-renewal potential is lost or retained during HSC cell divisions remain largely ill-defined. However, accumulative evidence shows that HSC fate decisions are controlled by a delicate balance of the intrinsic genetic program of HSCs and extrinsic signals provided by their microenvironment, the stem cell niche. Some of the intrinsic regulatory genes (*Bmi1*, *Cdkn1a*, *Pten*, *Etv6*, *Mcl1*, *Hoxb4*) that control stem cell maintenance, expansion, and differentiation and some extrinsic signals (Wnt, Ang-1, Notch ligands, Gp130 ligands) that are exchanged between HSCs and niche cells have been identified recently (1).

Numerous members of the homeobox gene family are involved in the regulation of normal and leukemic hematopoiesis at various stages. In fact, the human homeodomain transcription factor HOXB4 was the first gene shown to lead to profound HSC expansion *in vitro* and *in vivo* when ectopically expressed in murine BM cells. These HSCs fully replenish the stem cell pool of lethally irradiated mice and maintain a normal supply of HSCs and mature blood cells for the duration of life (2, 3). Ectopic HOXB4 expression also enhances the *in vitro* development of definitive HSCs from differentiating mouse ES cells that are capable of reconstituting recipient mice (4, 5). However, ectopic expresssion of HOXB4 may eventually transform hematopoietic cells. Our group and others have described that the amount of ectopically expressed HOXB4 has an impact on myeloid, lymphoid, and erythroid differentiation of adult HSCs of mice and humans *in vitro* and *in vivo* and on ES cell-derived hematopoietic differentiation (6–8).

The molecular mechanism behind how this transcription factor acts in immature blood cells is poorly understood. Homeodomain-mediated DNA binding is essential for the increased self-renewal of HOXB4-expressing HSCs (9). However, only a few HOXB4 target genes have been identified so far. HOX proteins can form high-affinity DNA-binding complexes with other homeodomain-containing proteins such as PBX1 (10). However, direct HOXB4–PBX1 protein interactions are not required for stem cell expansion (9). In contrast, PBX1 limits HOXB4-induced HSC expansion *in vivo* (11).

To further characterize the molecular mechanisms underlying the HOXB4-induced expansion of HSCs, we analyzed the gene expression profiles of adult HSC/hematopoietic progenitor cells (HPCs) and differentiating ES cells expressing inducible forms of HOXB4. By identification of its target genes and through functional tests, *in vivo*, we demonstrate that HOXB4 changes the cellular response of stem cells to conserved signaling pathways known to affect cell fate decisions of HSCs in adult and differentiating ES cells. These results may also reflect general pathways of homeobox transcription factor activities that are relevant for developmental decisions.

## Results

**Identification of HOXB4 Target Genes in Primary Murine HSCs and HPCs.** To understand the mechanisms of HOXB4 activity, we wanted to identify target genes of HOXB4 in adult HSCs and HPCs. We thus transduced murine HSC/HPCs with a retroviral vector that coexpresses EGFP and a 4-hydroxytamoxifen (TMX)-

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Abbreviations: BM, bone marrow; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; LSK, Lin$^-$ Sca1$^+$ c-kit$^+$; EB, embryoid body; TMX, 4-hydroxytamoxifen; qRT-PCR, quantitative RT-PCR; CHX, cycloheximide; Dox, doxycycline; ES-HC, ES cell-derived hematopoietic cell; GEMM, granulocyte, erythroid, macrophage, megakaryocyte.

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inducible form of HOXB4 (HOXB4-ER). Upon addition of TMX, the HOXB4-ER fusion protein translocates from the cytoplasm to the nucleus, consequently being capable of modulating gene expression. Transduced cell populations were expanded for 14 days in the presence of TMX. Thereafter, HOXB4-ER$^+$LSK (where LSK is Lin$^-$, Sca1$^+$, c-kit$^+$) cells were flow cytometrically isolated and cultivated either with or without TMX for 1 or 4 h. Inactivation of HOXB4 activity by TMX withdrawal was intended to mimic the naturally occurring down-regulation of HOXB4 in differentiating stem cells (12). RNA was prepared after the aforementioned times, and the transcriptional profiles of HOXB4-ER$^+$LSK ± TMX were analyzed with the Affimetrix platform. As a control, profiling was also performed with LSK cells expressing unmodified constitutively active HOXB4 (HOXB4$^{const}$) ± TMX, to exclude changes in gene expression caused by unknown effects of TMX itself [supporting information (SI) Fig. 5].

Genes whose level of expression consistently and significantly (*i*) changed >2.0 fold 4 h after withdrawal of TMX in one of the two independent experiments and (*ii*) changed < 1.5-fold after withdrawal of TMX in both HOXB4$^{const}$ LSK control group samples were considered differentially and specifically regulated by HOXB4. This analysis identified 156 HOXB4-regulated genes in proliferating LSK cells, of which 23 were uncharacterized transcripts (SI Table 1). All 23 uncharacterized target genes, and 103 of the 133 characterized genes, were also differentially regulated >1.5-fold in the second independent experiment. The 133 characterized genes identified by the above criteria belonged to different functional groups (SI Table 2). A comparison of our HOXB4 target gene list with molecular signatures shown to be specific for naturally (*in vivo*) proliferating fetal liver HSCs and their quiescent adult BM-derived counterparts revealed significant overlaps (SI Table 3).

**Validation of Microarray Results by Quantitative RT-PCR (qRT-PCR).** To verify the observed expression differences, we selected 49 of the identified HOXB4 target genes for qRT-PCR analysis (Fig. 1 and SI Fig. 6). For this purpose, RNA was prepared from independent samples of transduced and purified HOXB4-ER$^+$LSK cells that had been incubated with and without TMX for 4 h to induce HOXB4. To check whether these genes are likely direct target genes of HOXB4, samples of sorted HOXB4-ER$^+$LSK cells were treated with cycloheximide (CHX) 30 min before exposure to TMX. Thus, any change of RNA levels in response to HOXB4 activation is independent of new protein synthesis. The results of the qRT-PCR generally showed that the majority of the genes found down-regulated after inactivation of HOXB4 activity by TMX withdrawal (microarray results) are up-regulated when HOXB4 activity is induced (36/49 = 72%). With this approach, we identified 40 of the 50 tested genes as putative direct targets of HOXB4 activity (e.g., *Bambi*, *Bre*, *Catnb*, *Cdkn1a*, *Cyclin G2*, *Dll-1*, *Dusp6*, *Foxo3a*, *Klf3*, *Mad*, *Ptgs2*, *Socs2*, *Socs6*, *T1e1*, *Tnfrsf1b*, and *Zfx*). Interestingly, inhibiting translation before induction of HOXB4 activity evoked a contrary gene expression pattern of 14 analyzed targets after 4 h. This finding strongly suggests that the activating or repressing activity of HOXB4-containing transcription factor complexes depends on unstable protein cofactor(s).

**HOXB4 Targets Similar Signaling Pathways in Differentiating ES Cells and Adult HSCs.** To uncover the genetic program underlying the enhanced production of definitive HSCs from early embryonic cells enforced by HOXB4 (4–6), we also analyzed gene expression changes in differentiating mouse ES cells containing a tetracycline-inducible HOXB4 gene (4). We applied doxycycline (Dox) for 48 h from day 4 to day 6 of embryoid body (EB) differentiation, the period during which commitment to primitive HSCs occurs and the very first HSCs are detectable (14, 15). EBs were dissociated at day 6, and total RNA was isolated for subsequent microarray hybridization.

We identified >700 differentially regulated genes in HOXB4-induced EBs whose level of expression consistently changed >2.0-fold 48 h after addition of Dox (SI Table 4). A functional annotation of selected genes differentially regulated in EBs is provided in SI Table 5. Remarkably, HOXB4 engaged in the same signal transduction pathways during EB differentiation as in adult HSCs/HPCs, in part by targeting identical gene products. Fig. 2 summarizes the results obtained with ES cell-derived cells and adult HSCs/HPCs according to their affiliation to various signaling pathways already implicated in HSC self-renewal (among others, TNF-α, FGF, Wnt, and Notch). A common set of 52 gene loci was targeted by HOXB4 in adult HSC/HPCs and EBs (SI Table 6). qRT-PCR analysis of independent RNA populations from Dox-induced HOXB4EB day-6 cells confirmed 19 of 30 common gene loci examined as differentially expressed (SI Fig. 7). A similar analysis performed with ES cell-derived hematopoietic cells (ES-HCs) (6) expressing the TMX-inducible form of HOXB4 (SI Fig. 8) proved 31 of the 32 overlapping genes examined to be regulated by HOXB4; 25 of those

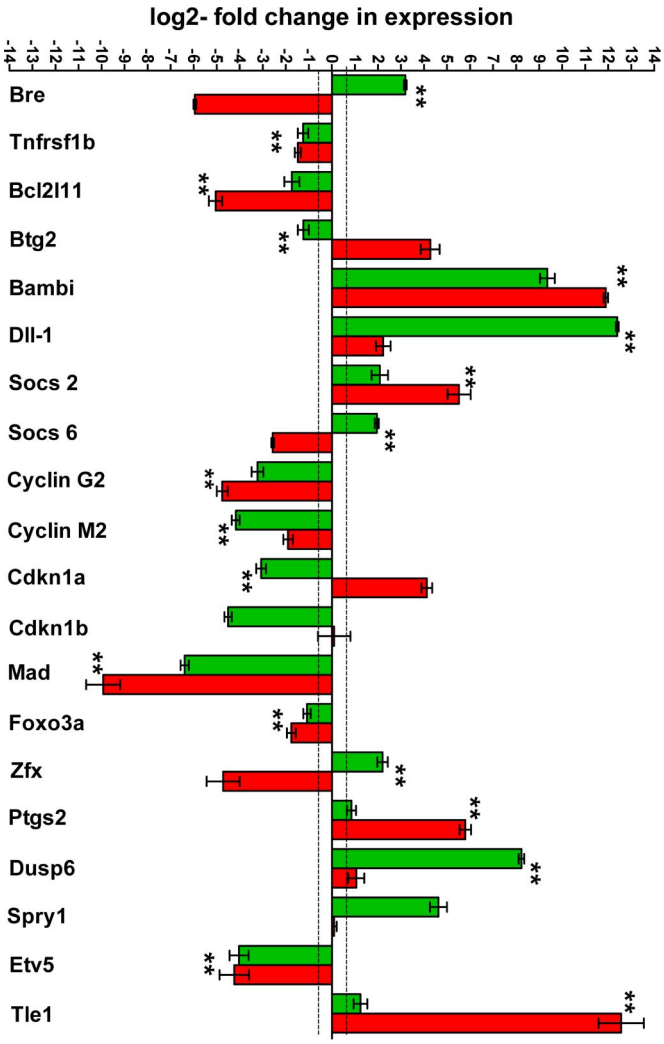


**Fig. 1.** qRT-PCR confirms changes in gene expression observed in the microarray study. Gene expression changes were measured in triplicate analysis of RNA samples from purified HOXB4ER$^+$LSK cells that had been incubated with and without TMX for 4 h to induce HOXB4 in the presence or absence of CHX. Relative differences in gene expression between TMX-induced and noninduced HOXB4ER samples were calculated by using the $2^{-\Delta\Delta CT}$ method (13) by normalizing the CT values for each gene to the CT values of the housekeeping gene, actin. Values are shown as log2-fold induction (mean ± SD) in the absence (green bars) and presence of CHX (red bars). ** indicates direct gene targets of HOXB4, displaying differential gene expression in the presence of CHX.

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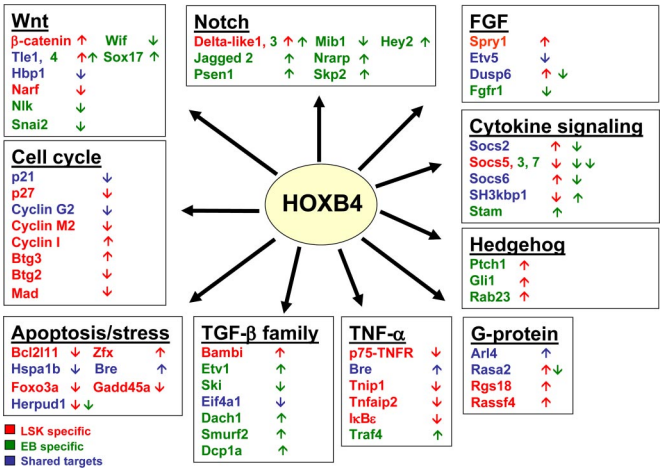


**Fig. 2.** Selected regulatory pathways affected by HOXB4 in adult HSCs/HPCs and EBs. HOXB4 influences the expression of genes involved in pivotal cell-intrinsic pathways such as regulation of cell cycle, differentiation, and apoptosis. It also modulates the response to multiple conserved extrinsic signals provided by the microenvironment. Affiliations of the genes with the indicated pathways are based on published reports in various mammalian systems. Arrows indicate up-/down-regulation of gene expression as a consequence of HOXB4 induction.

turned out to be putative direct target genes. Some of the shared direct target genes were regulated in opposite directions in adult HSCs/HPCs, EBs, and ES-HCs, again demonstrating that HOXB4-containing transcription factor complexes may either act as repressors or activators of one and the same gene locus. The results presented so far suggest that the overlapping genes represent "universal" targets of HOXB4 and affirm the idea that HOXB4 affects similar molecular circuits during the commitment of hematopoietic progenitors in differentiating ES cells (EBs) and during the expansion of ES-HCs and adult HSCs.

**HOXB4 Protects Long-Term Repopulating HSCs from the Negative Effects of TNF-α.** Gene profiling results *per se* are not sufficient to predict biological effects. Therefore, we grouped target genes based on their known involvement in defined pathways and tested their collective relevance for HSCs/HPCs' self-renewal in functional assays.

In adult HSCs/HPCs, HOXB4 mediated the down-regulation of *Tnfrsf1b* (also known as p75/TNFR) and concomitantly up-regulated *Bre* (brain and reproductive organ-expressed protein), an inhibitor of TNF receptor 1 (p55/TNFR) and Fas signaling. TNF-α negatively regulates self-renewal of cycling murine and human HSCs by enforcing myeloid differentiation (16, 17). Our results suggested that interference with this signaling pathway would contribute to continued HSC self-renewal. To test this hypothesis, LSK cells expressing the inducible HOXB4-ER vector or a truncated form of human CD34 (tCD34) that does not affect multilineage differentiation *in vivo* (18) were mixed in a 1:1 ratio and cultivated for 7 days in serum-free medium with or without TNF-α ± HOXB4 induction by TMX (Fig. 3*A*). Thereafter, we determined the proportion of LSK cells in the HOXB4-ER/tCD34 cell mixes and tested their ability to reconstitute the hematopoietic system of lethally irradiated mice. In the absence of TNF-α and TMX, cultured HOXB4ER$^+$ and tCD34$^+$ cells both contained similar proportions of LSK cells (SI Fig. 9) and had equal potentials of repopulation (Fig. 3*B*). However, in the presence of TNF-α without HOXB4 induction, the reconstitution ability of both cell populations was almost completely lost. After induction of HOXB4 and in the presence of TNF-α, multilineage reconstitution was achieved with HOXB4-ER cells, but not with tCD34$^+$ control cells.

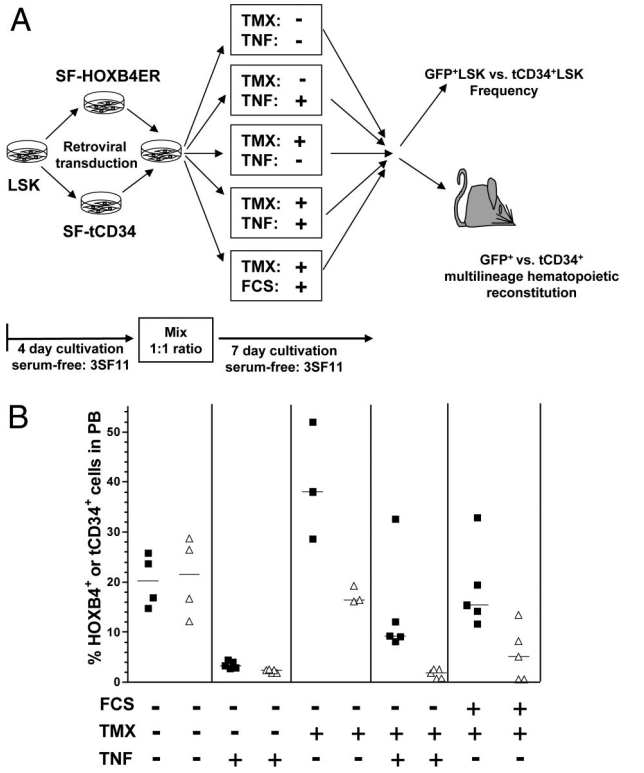


**Fig. 3.** Induction of HOXB4 activity protects cultivated HSCs from the negative effects TNF-α on stem cell self-renewal. (*A*) Experimental design to test the effect of TNF-α on the multilineage hematopoietic reconstitution capacity of HOXB4ER-expressing LSK cells in a competitive transplant setting. The progeny (expansion equivalent) of 2,000 HOXB4ER-transduced and 2,000 control vector (tCD34)-transduced LSK cells were mixed and cocultured, as indicated, in serum-free cytokine-supplemented medium in the presence or absence of TNF-α or FCS ± HOXB4 induction with TMX. After 7 days, the frequency of HOXB4ER-expressing and tCD34-expressing LSK cells in the resulting cell populations was determined by flow cytometry, and the cell samples were transplanted into cohorts of lethally irradiated recipient mice. (*B*) Reconstituting activity of HOXB4ER-expressing (■) and tCD34-expressing (△) cells cultivated for 7 days, as indicated. Small horizontal lines indicate the median percentage of HOXB4ER$^+$ or tCD34$^+$ cells in the peripheral blood of the mice 11 weeks after transplantation.

Hence, HOXB4 protects long-term repopulating HSCs from the negative effects of TNF-α.

**HOXB4 Modulates FGF Signaling.** In EBs, HOXB4 regulated the expression of FGFs (*Fgf8*, *Fgf15*), Fgf receptor 1 (*Fgfr1*), and Ets variant gene 5 (*Etv5*). Transcription factor *Etv5* is a key downstream player in the transcriptional activation of FGF target genes, which is commonly phosphorylated in response to FGF signaling. In line, qRT-PCR analysis of HOXB4-induced adult HSCs/HPCs showed a down-regulation of *Etv5* and an up-regulation of *Dusp6* and *Spry1*, two members of the negative feedback loop of FGF signaling (19). These findings prompted us to ask whether HOXB4 activity functionally interferes with FGF signaling during hematopoiesis. Thus, we measured colony formation by HOXB4-expressing differentiating ES cells that had been exposed to an FGF receptor tyrosine kinase inhibitor (SU5402) from day 4 to day 6 of EB differentiation. In parallel, we also analyzed the expression of *Dusp6*, *Spry2*, *Etv5*, and *Fgf1* by qRT-PCR. Without HOXB4 induction, inhibition of FGF signaling did not significantly affect the formation of the most primitive hematopoietic colonies, CFU-GEMM (granulocyte, erythroid, macrophage, megakaryocyte) (Fig. 4*A*; $P = 0.093$). The expression levels of the aforementioned FGF targets (Fig. 4*B*) decreased. In contrast, induced ectopic expression of HOXB4 without SU5402 stimulated an increase of

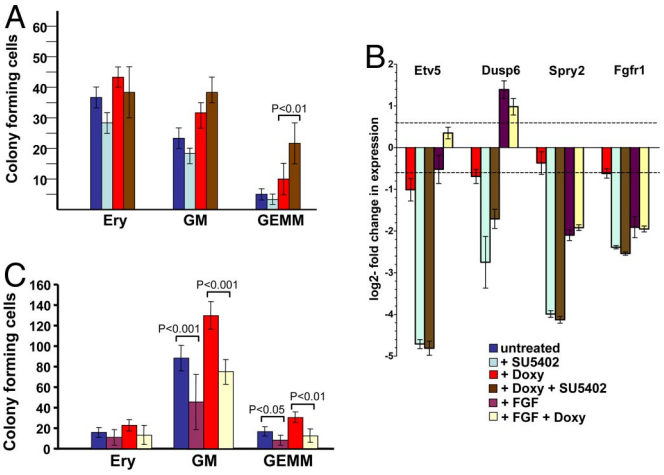


**Fig. 4.** HOXB4 and FGF signaling are intertwined. Inducible HOXB4 ES cells were treated from days 4 to 6 of EB formation with and without Dox in the absence or presence of either the FGF receptor inhibitor, SU5402, or bFGF (FGF2) as indicated. EBs were collected on day 6 and processed for methylcellulose-CFU assays or for RNA extraction and subsequent semiquantitative RT-PCR analysis. (*A*) Inhibition of FGF signaling enhances HOXB4-mediated expansion early HPCs. (*B*) HOXB4 down-regulates the expression of *Dusp6* and *Etv5*, a key player in the activation of FGF target genes. (*C*) bFGF inhibits HOXB4-mediated expansion of ES cell-derived early HPCs. Ery, definitive erythroid; GM, granulocyte macrophage. Results are presented as mean ± SD ($n = 3$). *P* values were determined by Student's *t* test.

CFU-GEMM numbers (Fig. 4*A*). HOXB4 activity down-regulated expression of *Etv5*, *Dusp6*, and *Fgfr1*, providing evidence that HOXB4 attenuates activation of these FGF target genes. HOXB4 induction plus additional chemical inhibition of FGF signaling at its uppermost level, the FGF receptor, significantly further increased the frequency of CFU-GEMMs ($P = 0.0012$). Consistently, we observed further reduced expression levels of *Dusp6* and *Etv5* (Fig. 4 *A* and *B*). Addition of bFGF (FGF2) inhibited the HOXB4-induced increase of CFU-GEMMs within differentiating EBs ($P = 0.0013$; Fig. 4*C*) and congruently up-regulated the expression of *Dusp6* (Fig. 4*B*). These results suggest that the biological outcome of HOXB4 activity is controlled by FGF signaling, which appears to limit the extent of expansion of early progenitor cells and possibly HSCs.

In HOXB4-expressing EBs, we had also observed transcriptional up-regulation of *T* (Brachyury) and *Kdr* (*Flk1*) markers that are hallmarks of mesoderm and hemangioblast development. Thus, to further clarify whether FGF signaling affects the commitment to definitive hematopoietic cells and/or their subsequent expansion/differentiation, HOXB4-ER-expressing ES-HCs (21 days after EB dissociation) were cultured with or without either bFGF or the inhibitor SU5402 for 14 days and colony assays were performed. HOXB4 induction plus inhibition of FGF signaling significantly increased the frequency of CFU-GEMMs compared with those treated with FGF2 ($P = 0.023$). In contrast to EBs, addition of bFGF did not apparently decrease the frequency of CFU-GEMMs in cultured ES-HCs (SI Fig. 10). Thus, HOXB4 and FGF signaling seem to act antagonistically during hematopoietic commitment within EBs but not during expansion of ES-HCs, which is in agreement with the known suppression of hematopoiesis by FGF signaling during embryonic development (20).

Finally, we investigated the relationship between HOXB4 and FGF signaling in the context of adult HSCs *in vivo*. HOXB4-ER/tCD34 cell mixes were tested for their ability to reconstitute lethally irradiated mice after expanding them for 7 days in serum-free medium with and without SU5402 in the presence of TMX. In the absence of SU5402, TMX-induced HOXB4-ER$^+$ and tCD34$^+$ cells exhibited long-term repopulating activity (SI Fig. 11). tCD34$^+$ cells provided a slightly higher, but not significant ($P = 0.10$), donor cell chimerism than induced HOXB4-ER$^+$ cells did in the recipient mice. However, in the presence of SU5402, the proportion of HOXB4-ER$^+$ donor cells was significantly higher than the proportion of tCD34$^+$ donor cells (1.6-fold; $P = 0.043$). Particularly, the proportion of HOXB4-ER$^+$ donor cells increased 1.95-fold, whereas the proportion of tCD34$^+$ donor cells decreased 2.6-fold compared with the chimerism generated without SU5402. Thus, inhibition of FGF signaling stimulated the expansion of HOXB4-ER$^+$ HSCs, whereas it suppressed the expansion of the tCD34$^+$ control HSCs. Hence, as our inhibitor studies clearly indicate, FGF signaling limits the extent of HSC expansion but only in the context of HOXB4 activity, whereas it stimulates the expansion of normal HSCs. This finding is congruent with the positive role of FGF signaling in self-renewal of normal HSCs previously reported by de Haan *et al.* (21).

## Discussion

To elucidate the molecular machinery responsible for HOXB4-induced HSC expansion, we identified gene targets of HOXB4 in adult HSCs/HPCs and ES cell-derived EB. We link HOXB4 to genes that are known to be expressed in adult stem cells. qRT-PCR assays confirmed the observed changes and suggest that the majority are likely direct targets of HOXB4. Some of these direct target genes like *Cnkn1b*, *Mad*, *Foxo3a*, *Ptgs2*, and *Zfx* have recently been shown to be crucial for self-renewal, survival, and maintenance of adult HSCs (22–28), an observation that fortifies the validity of our results. Fifty-two gene loci were regulated by this transcription factor in both adult HSC/HPC and EBs. This set of overlapping genes, also validated by real-time PCR, likely represent universal targets of HOXB4. The target genes are involved in a wide range of cellular processes, such as signal transduction, cell cycle, apoptosis, and response to stress and transcription. Our results also demonstrate that HOXB4 can act as a transcriptional activator or repressor of a given responsive gene locus, depending on the cellular context.

Interestingly, differentiating ES cells revealed far more genes selectively regulated by HOXB4 than enriched adult HSCs. This difference may be explained by the prolonged induction of HOXB4 expression, suggesting that many of the targets are indirectly regulated. Moreover, EBs contain a mixture of cells from all three germ layers. Thus, many of the EB-specific targets may be unrelated to the hematopoietic system.

A major finding of this study is that a large fraction of the HOXB4 target genes are components or essential regulators of signaling pathways, such as cytokine, Wnt/β-catenin, Notch, FGF, TGF-β/Activin/BMP, and TNF-α signaling (Fig. 2). All of those have a major impact on self-renewal, differentiation, and survival of adult HSCs and are pivotal in setting diverse developmental courses during embryonic development. Interestingly, HOXB4 orchestrated the same signaling pathways in EBs. The results support our proposed concept that HOXB4 modulates multiple inputs of distinct extrinsic signaling pathways, consequently leading to discrete biological outcomes (29).

**HOXB4 Inhibits Negative Regulators of Adult HSC Self-Renewal.** HOXB4 mediated the down-regulation of *Tnfrsf1b* and up-regulated the expression of *Bre*, which has been shown to inhibit apoptosis induced by TNF-α, Fas ligand, and various other stress-related stimuli (30). TNF-α negatively affects the self-renewal of cycling murine and human HSCs (16, 17). In this study, we showed that both *Tnfrsf1b and Bre* are directly regulated by HOXB4. Moreover, we showed in a functional assay that HOXB4 activity protected adult HSCs from the compromising effects of TNF-α on HSC self-renewal. TNF-α levels quickly increase in irradiated or fluorouracil–treated recipients (31, 32). Thus, it is likely that HOXB4 activity protects long-term HSCs under these conditions *in*

*vivo*. This observation has potential clinical relevance, as licensed drugs for targeted suppression of TNF-$\alpha$ signaling are available.

*In vivo*, HSC expansion is also promoted by Smad7 overexpression, which blocks all TGF-$\beta$/Activin and BMP signaling (33). HOXB4 directly up-regulated the expression of *Bambi*, a pseudoreceptor that also acts as a pan inhibitor of this signaling pathway and therefore presumably facilitates *in vivo* HSC expansion. In fact, murine BaF3 cells expressing HOXB4–ER were resistant to TGF-$\beta$-mediated growth inhibition of the parental BaF3 cells (B.S., unpublished data). In contrast to adult HSCs/HPCs, HOXB4 apparently modulated signaling of the TGF-$\beta$ family at multiple levels in differentiating ES cells. Increased expression levels of the Smad-coactivator *Dcp1a* together with elevated expression of attenuators of this signaling pathway (*Smurf2*, *Dach1*, *Etv1*) suggest that HOXB4 presumably specifies and fine-tunes the duration of the cellular responses elicited in differentiating ES cells.

**HOXB4 and FGF Signaling Are Intertwined.** The role of the FGF pathway in embryonic hematopoietic development and regulation of adult HSCs is still controversial. Here, we showed that FGF signaling is a target of HOXB4 activity and also negatively regulates HOXB4-mediated expansion of adult and ES cell-derived HSCs. Chemical blockage of FGF signaling augmented the long-term repopulation activity of HOXB4-expressing HSCs/HPCs expanded *in vitro*, whereas it suppressed the repopulation activity of control HSCs/HPCs. Similarly, inhibition of FGF signaling in differentiating EBs between days 4 and 6 further expanded progenitor numbers, which were already increased by ectopic HOXB4 expression. However, without HOXB4 expression blocking, FGF signaling did not alter the frequency of early progenitors. Between days 4 and 6 EBs already contain mesodermal Brachyury$^+$ (Bry$^+$) cells. Our results fit well to the fact that a conditional knockout of *Fgfr1* or *Fgfr1/2* in already formed FLK1$^+$ mesodermal cells, *in vivo*, does not affect the hematopoietic progenitor content of fetal livers (34). Loss of *Fgfr1* at earlier stages, however, allows differentiation of knockout ES cells toward Bry$^+$ mesodermal cells *in vitro*, but they fail to express normal levels of FLK1 (35). Moreover, inhibition of FGF signaling in chicken embryos has been shown to promote blood cell differentiation and inhibit endothelial cell differentiation, presuambly at the hemangioblast stage (20). That finding supports our conclusions.

HOXB4 led to the down-regulation of *Dusp6* in EBs, a negative feedback regulator of FGF signaling, which also cross-talks with other signaling pathways (36, 37). For example, expansion of Flk1$^+$/SCL$^+$ cells depends on VEGF-mediated activation of Erk1/2 (38), a component of the MAP signaling pathway, which can be inhibited by Dusp6. Down-regulation of Dusp6 by HOXB4, thus, may enhance VEGF-mediated expansion of Flk1$^+$/SCL$^+$ precursor cells in EBs. Blockage of FGF signaling in HOXB4$^+$ EBs would lead to a further down-regulation of *Dusp6*, which, in turn, should boost VEGF-induced progenitor expansion. Consistent with this interpretation, we were able to abolish the synergistic HOXB4/SU5402 effect on progenitor expansion by a complete chemical blockage of ERK1/2 activation (SI Fig. 12).

Taken together, we demonstrate that modulation of FGF signaling is an essential feature of HOXB4 activity in the context of embryonic and adult hematopoiesis. Bobola *et al.* (39) recently showed that *Hoxa2* blocks FGF-dependent gene activation during mesenchymal patterning of the embryo, thus providing supporting evidence for a general role of HOX genes in the modulation of FGF signaling (39).

**HOXB4 Modulates Wnt and Notch Signaling.** The Wnt/$\beta$-catenin and Notch pathway are potent regulators of HSC function (40, 41). Continuous expression of stabilized $\beta$-catenin in HSCs *in vitro* and *in vivo* has shown that the balance between HSC self-renewal and differentiation requires a fine-tuned control of Wnt signaling. In adult HSCs/HPCs and EBs, HOXB4 induced the down-regulation of *Hbp1*, which acts as a transcriptional repressor of Wnt target genes and as a suppressor of cell cycle progression (42). Furthermore, enhanced expression of *Nrarp* together with down-regulation of *Narf* and *Nlk* indicate that TCF/LEF protein stability is enhanced (43, 44). Moreover, HOXB4 also increased in EBs the expression of *Pitx2* and *Sox17*, two proteins that mediate TCF/LEF-independent $\beta$-catenin target gene expression (45, 46). The results suggest that HOXB4 influences Wnt signaling at multiple stages. It may also govern which target genes become activated in response to Wnt signaling.

The enhanced expression of Notch ligands (Dll1, Dll3, Jagged2) and Notch target genes (*Hey2*, *Nrarp*, *Skp2*) support the idea that HOXB4 may stimulate Notch signaling and thereby would prevent the loss of HSCs caused by accelerated differentiation. In good agreement, low doses of immobilized Delta-like1 (Dll1) have been shown to enhance the expansion of human stem cells capable of repopulating SCID mice (47).

Based on the presented results, we propose that HOXB4 governs pivotal cell-intrinsic pathways involved in the regulation of cell cycle, differentiation, and apoptosis. Moreover, it modulates the response of adult HSC and EB cells to multiple conserved extrinsic signals provided by the microenvironment. HOXB4 activity fine-tunes negative and positive regulatory cues, which results in HSC expansion.

## Experimental Procedures

**Mice and Transplantation.** C57BL/6J (CD45.2) mice were used as transplant donors and recipients. Details of the BM transplantation procedure are described in *SI Text*.

**Isolation of Lin$^-$ Sca1$^+$ cells.** Mouse BM cells were depleted from lineage-committed cells (Gr-1, CD11b, CD45R/B220, CD5, TER-119; lineage depletion kit; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's recommendation. The lineage-depleted cells were selected for Sca-1$^+$ cells (Sca-1 selction kit; Miltenyi Biotech).

**Flow Cytometry and Cell Sorting.** Flow cytometry procedures and sorting of LSK subpopulations are described in detail in *SI Text*.

**Retroviral Constructs and Transduction of HSCs/HPCs.** The retroviral vectors, SF91-EGFP2AHAHOXB4-wPRE, expressing constitutively active HOXB4 (HOX$^{const}$), and vector SF11-tCD34, expressing a truncated form of human CD34 (tCD34), have been described (7, 48). The generation of the retroviral vector, SF91-EGFP2AHAHOXB4ER-wPRE, allowing coexpression of EGFP and a TMX-inducible form of HOXB4 (HOXB4ER), and procedures for transduction of Lin$^-$Sca1$^+$ and sorted LSK cells are described in detail in *SI Text*.

***Ex Vivo* Expansion of HOXB4-Transduced HSCs/HPCs.** Procedures and culture conditions of short-term *ex vivo* expansion of HOXB4-transduced HSCs/HPCs for microarray studies and *in vivo* reconstitution experiments are described in detail in *SI Text*.

**ES Cells and HOXB4 Induction.** Cultivation of the Hoxb4i ES cell line (4), Dox-mediated HOXB4 induction, and chemical inhibition of FGF receptor signaling are described in detail in *SI Text*. Details of the cultivation of the CCE ES cell line (6) and the generation and retroviral transduction of ES-HCs are also provided in *SI Text*.

**Microarray Expression Profiling.** RNAs from EBs and adult LSK cells were processed for use on Affymetrix GeneChips Mouse Genome 430 2.0 (Affymetrix, Santa Clara, CA). All quality

parameters for the arrays were confirmed to be in the range recommended by the manufacturer. A more detailed description of RNA isolation, target synthesis, and hybridization to Affymetrix GeneChips is provided in *SI Text*.

**GeneChip Data Analysis.** For experiments with differentiated ES cells, expression data were analyzed with dChip 1.3 by using a perfect match-only model (49). Replicate data for the same sample type were weighted genewise by using inverse squared standard error as weights. All genes compared were considered to be differentially expressed if the 90% lower confidence bound of the fold change between experiment and baseline was >1.4. For experiments with adult HSCs/HPCs, log2 expression estimates were calculated by RMAExpress using the RMA algorithm (50). Probe sets are reported as differentially expressed if at least one set of experiments showed absolute log2 difference >1 between HOXB4ER$^+$LSK minus TMX treatment and control cells (HOXB4ER$^+$LSK plus TMX treatment).

**Microarray Data.** Microarray data are available at the GEO web side (www.ncbi.nlm.nih.gov/geo) with GEO accession nos., GSE9010 (adult HSC/HPCs) and GSE9044 (differentiating ES cells).

**Gene Expression Analysis.** qRT-PCR analysis of RNA from sorted LSK cells and semiquantitative RT-PCR analysis of RNA from EB cells is described in *SI Text*.

**Statistical Analysis.** Statistical significance was determined with the two-tailed, paired Student's $t$ test with the $\alpha$-level set at 0.05.

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