Global gene expression analyses of hematopoietic stem cell lines with inducible \textit{Lhx2} expression

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**Abstract**

**Background:** Expression of the LIM-homeobox gene \textit{Lhx2} in murine hematopoietic cells allows for the generation of hematopoietic stem cell (HSC)-like cell lines. To address the molecular basis of \textit{Lhx2} function, we generated HSC-like cell lines where \textit{Lhx2} expression is regulated by a tet-on system and hence dependent on the presence of doxycyclin (dox). These cell lines efficiently down-regulate \textit{Lhx2} expression upon dox withdrawal leading to a rapid differentiation into various myeloid cell types.

**Results:** Global gene expression of these cell lines cultured in dox was compared to different time points after dox withdrawal using microarray technology. We identified 267 differentially expressed genes. The majority of the genes overlapping with HSC-specific databases were those down-regulated after turning off \textit{Lhx2} expression and a majority of the genes overlapping with those defined as late progenitor-specific genes were the up-regulated genes, suggesting that these cell lines represent a relevant model system for normal HSCs also at the level of global gene expression. Moreover, \textit{in situ} hybridisations of several genes down-regulated after dox withdrawal showed overlapping expression patterns with \textit{Lhx2} in various tissues during embryonic development.

**Conclusion:** Global gene expression analysis of HSC-like cell lines with inducible \textit{Lhx2} expression has identified genes putatively linked to self-renewal / differentiation of HSCs, and function of \textit{Lhx2} in organ development and stem / progenitor cells of non-hematopoietic origin.

**Background**

A small number of hematopoietic stem cells (HSCs) are responsible for the continuous production of mature blood cells throughout life. This process is based on the capability of the HSC to replenish itself through a process called self-renewal [1-3], and to differentiate into all hematopoietic lineages. Consequently, analysis of the mechanisms underlying HSC self-renewal and differentiation is fundamental for understanding the maintenance of the normal hematopoietic system. At present, our
knowledge of these processes on the molecular and cellular level is limited, since studies on HSCs are hampered by their low abundance in hematopoietic organs and are thus difficult to access in sufficiently large quantities for direct studies. An increase in the number of HSCs occurs under normal physiological conditions in the liver during embryonic development [4], indicating that the microenvironment in the fetal liver efficiently promotes self-renewal of HSCs. Elucidation of the mechanisms responsible for the expansion of the hematopoietic system during embryonic development might therefore offer insights into the mechanisms of self-renewal in the hematopoietic system.

The expansion of the hematopoietic system is intimately connected with the development of the liver, suggesting over-lapping molecular mechanisms of these processes. Liver development in the mouse is initiated at embryonic day 8 (E8) when a distinct region of the ventral foregut endoderm receives inductive signals from two adjacent tissues, the septum transversum mesenchyme and the pre-cardiac mesoderm [reviewed in [5]]. Ventral foregut endodermal cells committed to hepatic fate proliferate and form a liver bud from which hepatoblasts migrate and intermingle with cells of the septum transversum mesenchyme. The mesenchymal cells originating from the septum transversum thereby contribute to the mesenchymal part of the liver, and development into a functional organ relies on continuous interactions between the mesenchymal and endodermal portions of the liver [6-8]. At E10 the liver has become a distinct organ with discernible lobes and is infiltrated by numerous hematopoietic cells and cells with HSC properties can be detected in the liver by E11 [9].

Members of the LIM-homeodomain transcription factor family play critical roles during embryonic development in both vertebrates and invertebrates by controlling processes such as asymmetric cell division, tissue specification and differentiation of specific cell types [reviewed in [10]]. One member of this family, Lhx2, is of particular interest, based on its function in the development of several different tissues via mesenchymal-epithelial interactions and regulation of stem/progenitor cells [11-17]. Lhx2 is expressed in the liver-associated septum transversum mesenchyme that becomes an integral part of the liver and its expression is maintained during liver development until adult stage in hepatic stellate cells [12,16]. Lhx2-/- embryos display a decreased size of the liver manifested already at E10.5, suggesting that Lhx2 is required for expansion of the fetal liver [12,17]. The mutant phenotype is due to the presence of activated hepatic stellate cells causing a fibrotic and disorganized liver containing phenotypically abnormal endodermal cells [12,16]. The mesenchymal defect in the liver of Lhx2-/- mice cause a lethal anemia, which is cell non-autonomous since the Lhx2-/- hematopoietic cells appears to be normal [17], suggesting that the mutant microenvironment is unable to support hematopoietic development. These observations indicate that Lhx2 expression in hepatic stellate cells is involved in mesenchymal-epithelial cell interactions important for liver expansion, organization, differentiation and formation of the hematopoietic microenvironment in the fetal liver.

To further elucidate the putative role of Lhx2 in the hematopoietic system we ectopically expressed Lhx2 in hematopoietic progenitor/stem cells derived from embryonic stem (ES) cells differentiated *in vitro* and from adult bone marrow (BM) cells. This approach allowed for the generation of immortalized multipotent and Steel factor-dependent hematopoietic progenitor cell (HPC) lines [18,19]. The HPC lines share several characteristics with normal HSCs such as response to specific cytokines/growth factors, expression of transcription factors and interactions with stromal cells [18,20]. The pattern of cell surface markers expressed by HPC lines derived from ES cells and adult BM is similar to that of early fetal and adult HSCs, respectively. The HPC lines derived from adult BM can generate erythroid, myeloid and lymphoid cells following transplantation into lethally irradiated recipients, and can long-term engraft stem cell-deficient mice [19]. The cells engraving the stem cell-deficient mice maintain high level of expression of Lhx2 *in vivo*, which eventually leads to a chronic myeloproliferative disorder resembling human chronic myeloid leukemia [21]. Thus, ectopic Lhx2 expression in hematopoietic cells allows for the generation of HSC-like cell lines, and molecular analyses of these HSC-like cell lines would give information of the role of Lhx2-induced self-renewal of HSCs, and hence novel insights into stem cell physiology and pathology.

In order to elucidate the molecular basis of Lhx2-induced self-renewal of HSCs we generated HSC-like cell lines from ES cells differentiated *in vitro* with Lhx2 expression controlled by a tetracycline-responsive element, and hence dependent on the presence of the tetracycline-analogue doxycyclin (dox) in the culture media [22]. These dox-dependent hematopoietic progenitor cell (DoxHPC) lines down-regulate Lhx2 expression almost two orders of magnitude within 24 hrs after dox withdrawal, leading to rapid differentiation into various myeloid cell types. We used the DoxHPC lines to analyse Lhx2 function by comparing global gene expression in the presence of dox to different time points after dox withdrawal using cDNA array technology. This approach identified 267 genes differentially expressed at all time points, and thus putatively involved in Lhx2 function in stem cell self-renewal and/or differentiation, and during organ development.
Results

Generation of HPC lines from the ES cells with inducible Lhx2 expression

Previously, when generating HPC lines, we used ES cells transduced with retroviral vectors containing Lhx2 cDNA that were subsequently differentiated in vitro into embryoid bodies (EBs). The HPC lines were established by expanding progenitor cells expressing Lhx2 present in colonies showing a distinct morphology that appeared in the clonal assays of EB cells [18,23]. A similar approach was used here, however, since we were unable to identify distinct colonies in the clonal assays containing dox (probably due to the efficient up-regulation of Lhx2 expression in all cells), 96 colonies generated in the factor combination supplemented with dox were randomly picked and expanded individually in medium containing SF, IL-6 and dox. Stable doxycyclin-dependent hematopoietic progenitor cell (DoxHPC) lines could be established from 66
(69%) of these 96 colonies. The pattern of cell surface marker expression was similar to that previously reported for the HPC lines generated from day 6 EBs [18,23], e.g. c-kit+/CD45+/CD41+/Sca-1-/Lin- (data not shown). All DoxHPC lines expressed Lhx2 and transcription of Lhx2 was efficiently down-regulated (>90%) within 24 hours after dox withdrawal and was undetectable after 72 hours (Figure 1A). In the DoxHPC7 line where GFP was linked
to Lhx2 expression, flow cytometry analyses revealed similar instantaneous and efficient down-regulation of GFP expression after dox withdrawal (Figure 1B). Rapid cellular changes appeared immediately after dox withdrawal with respect to differentiation status and fraction of apoptotic cells. The fraction of apoptotic cells increased from a background level of 5% (cells in the presence of dox) to a maximum of 35% five days after dox withdrawal. The fraction of apoptotic cells decreased thereafter to background level with ten days after dox withdrawal (data not shown). Under these culture conditions (SF+IL-6 without dox), differentiation into various myeloid cells such as neutrophilic granulocytes, macrophages and megakaryocytes, could usually be observed after 4–5 days (Figure 1E), with concomitant up-regulation of lineage specific cell surface markers (data not shown). Although the distribution of the various myeloid cells differed between different DoxHPC lines after dox withdrawal, all DoxHPC lines maintained in SF+IL-6 without dox generated mast cells and beyond eight days this cell type dominated the cultures (Figure 1E). Immature hematopoietic progenitor cells can functionally interact with stromal cells in vitro by generating cobblestone areas [24] in long-term bone marrow culture) and are referred to as cobblestone area forming cells (CAFCs) [25]. We have previously shown that the HPC lines derived from ES cells differentiated in vitro efficiently generated cobblestone areas when seeded onto stromal cells [20]. The DoxHPC lines only formed cobblestone areas when seeded onto stromal cells in the presence of dox (Figure 1C), whereas rapid differentiation into macrophages occurred in the absence of dox (Figure 1D). Thus, this is a reproducible and efficient system amenable for global gene expression analyses comparing Lhx2+ stem cells to their immediate Lhx2+ progeny.

**Analysis of gene expression changes caused by down-regulation of Lhx2**

We used cDNA microarray technology to investigate the genome-wide transcriptional effects of down-regulation of Lhx2 expression in the DoxHPC1 and DoxHPC7 lines. Gene expression at 36, 72 and 96 hours after dox withdrawal were analysed to elucidate differential gene expression early after Lhx2 down-regulation, but prior to the time point when the majority of the cells are committed myeloid cells. The hybridizations were carried out using the design depicted in Figure 2A. The number of features that remained on each array after filtering was high (82–92%) and the majority of the excluded features were removed by the first filtering step (i.e. GenePix and manual flagging).

We used an empirical Bayes moderated t-test to identify differential gene expression. Genes with a false-discovery rate adjusted p-value lower than 0.01 were considered differentially expressed. However, several of these showed small fold-changes, and therefore only genes with an absolute M-value higher than 0.4 (corresponds to a 1.3-fold difference) were included in further analysis. The results are summarized in Table 1, 2 and 3, and Figure 2 and 4A. Complete results are available as supplementary data. Comparison of the two independent cultures within each Lhx2-expressing cell line at time-point 0 (D-comparisons) indicated no differential expression (data not shown). On the other hand, the comparison between DoxHPC1 and DoxHPC7 lines (E-comparison) indicated that a large number of genes differed in their expression levels (Figure 2B). In total, 646 differentially expressed array probes were identified (see Additional file 2), which could be further divided into 265 probes (206 genes) with higher expression in DoxHPC1 and 381 probes (295 genes) with higher expression in DoxHPC7. Interestingly, the expression of Lhx2 is 2.7-fold higher in DoxHPC1 as compared to DoxHPC7, which may at least partly explain the difference between the cell lines. To identify genes differentially expressed as a consequence of Lhx2 down-regulation, we combined the data for DoxHPC1 and DoxHPC7 cell lines. This analysis revealed 463 probes (365 genes) at 36 hours, 695 probes (525 genes) at 72 hours and 699 probes (528 genes) at 96 hours to be differentially expressed (Figure 2C–H). A large proportion of these genes were differentially expressed at all three time-points (Figure 4A and Additional file 1). This shared set of 267 genes included 141 down-regulated and 126 up-regulated genes. The array data confirms the dox-dependent regulation of Lhx2, as Lhx2 is the gene with the largest decrease in expression (down by ~90%) after dox with-

| Comparison             | p < 0.01 (fdr) probes | p < 0.01 (fdr) genes | p < 0.01 (fdr) and |M| > 0.4 probes | p < 0.01 (fdr) and |M| > 0.4 genes |
|------------------------|-----------------------|----------------------|-------------------|----------------|----------------|----------------|
| A (36 vs. 0 h)         | 1718                  | 1325                 | 463               | 365            |
| B (72 vs. 0 h)         | 2572                  | 1936                 | 695               | 525            |
| C (96 vs. 0 h)         | 2562                  | 1947                 | 699               | 528            |
| D (0 h vs. 0 h)        | 0                     | 0                    | 0                 | 0              |
| HPC1 vs. HPC7 (0 h)    | 2580                  | 1995                 | 646               | 498            |

**Table 1: Number of array features and corresponding genes that are differentially expressed in the various comparisons. A false-discovery rate of 0.01 is used, with and without a M-value cut-off of 0.4.**
Table 2: The top 25 down-regulated genes in the DoxHPC lines after dox withdrawal. The columns 36 h, 72 h and 96 h refer to hours after dox withdrawal and include the M-values (=log2 [expression in differentiated/expression in undifferentiated]). The M-value of Lhx2 down-regulation is equivalent to a fold-change of approximately 8.

| Symbol     | Gene name                          | Genbank | Unigene ID | Gene ID | 36 h         | 72 h         | 96 h         |
|------------|------------------------------------|---------|------------|---------|--------------|--------------|--------------|
| Lhx2       | LIM homeobox protein 2             | CX208629| Mm.142856  | 16870   | -2.95        | -2.71        | -3.06        |
| SerpinA3g  | Serine (or cysteine) proteinase inhibitor, clade A, member 3G | CX234493| Mm.264709  | 20715   | -2.02        | -2.41        | -2.53        |
| Bpgm       | 2.3-bisphosphoglycerate mutase     | CX200054| Mm.282863  | 12183   | -1.63        | -2.07        | -2.06        |
| Cerp2      | Cysteine and glycine-rich protein 2 | CX241650| Mm.2020    | 13008   | -1.29        | -1.28        | -1.31        |
| Pscr1      | Phospholipid scramblase I          | CX230405| Mm.14627   | 22038   | -1.21        | -1.48        | -1.64        |
| Mal        | Myelin and lymphocyte protein, T-cell differentiation protein | CX219564| Mm.39040   | 17153   | -1.16        | -1.14        | -1.12        |
|            | Unknown                            | CX220013|            |         | -1.13        | 1.2          | -1.12        |
| Sdh1       | Sorbitol dehydrogenase I           | CX204086| Mm.371580  | 20322   | -1.1         | -1           | -1           |
| Upp1       | Uridine phosphorylase I            | CX218174| Mm.4610    | 22271   | -1.09        | -1.12        | -1.33        |
| Syne2      | Synaptic nuclear envelope 2        | CX218234| Mm.26652   | 319565  | -1.02        | -1.12        | -1.12        |
| Catnd2     | DNA segment, KIST 4               | CX237590| Mm.321648  | 18163   | 1           | -0.95        | -1.02        |
| Galnt2     | UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl transferase 2 | CX228653| Mm.33808   | 108148  | 1           | -1.1         | -1.22        |
| Etv5       | Ets variant gene 5                | CX240212| Mm.155708  | 104156  | 0.99         | -1.06        | -1.16        |
| Myo1b      | Myosin IB                         | CX206461| Mm.3390    | 17912   | -0.99        | -1.17        | -1.23        |
| Porp8      | RIKEN cDNA 5930433N17 gene         | CX230711| Mm.274466  | 52552   | 0.96         | -0.83        | -0.66        |
| Laptm4b    | Lyosomal-associated protein transmembrane 4B | CX240664| Mm.197518  | 114128  | -0.92        | -0.91        | -0.95        |
| Nrgn       | Neurogranin                       | CX204939| Mm.33505   | 64011   | 0.91         | 0.9         | -1.18        |
| B230104P22Rik | RIKEN cDNA B230104P22 gene         | CX241478| Mm.23587   | 77976   | -0.89        | -0.98        | -0.99        |
| 1810009M01Rik | RIKEN cDNA 1810009M01 gene        | CX239344| Mm.234385  | 65963   | -0.89        | -0.93        | -1.07        |
| Sax4       | SRY-box containing gene 4         | CX240226| Mm.240627  | 20677   | 0.88         | 1.04         | 1.06         |
| 2810408E1Rik | RIKEN cDNA 2810408E1 gene         | CX242247| Mm.291015  | 106200  | -0.86        | -0.88        | -0.97        |
| Mfge8      | Milk fat globule-EGF factor 8 protein | CX239348| Mm.1451    | 17304   | 0.86         | -0.88        | -1.01        |
| SerpinA3n  | Serine (or cysteine) proteinase inhibitor, clade A, member 3N | CX200330| Mm.22650   | 20716   | -0.86        | -1.07        | -1.15        |
| Vim        | Vimentin                          | CX235232| Mm.268000  | 22352   | 0.85         | -0.83        | -0.81        |
| Cklf4      | Chemokine-like factor super family 4 | CX206907| Mm.29658   | 97487   | 0.85         | -1.08        | -1.16        |

drawal (indicated by the arrows in Figure 2B–H). Agreement in the M and p-values was observed in cases where several independent probes represented different regions of the same gene (data not shown), further validating the accuracy of the method. Differential expression of 10 down-regulated and 10 up-regulated selected genes was confirmed by using real-time PCR to compare gene expression in the DoxHPC1 line in the presence of dox to three days after dox withdrawal (Figure 3).

Twenty-six of the 337 probes that were differentially expressed at all three time points were not included in UniGene build 144. We used Blast sequence similarity searches [26] to annotate the corresponding ESTs and the results are provided in the (see Additional file 1). Several of the unknowns map to either 5' or 3' UTRs, but also partly to known genes such as Wbcr5, Map3k7, and Eifay/Eifax. Also, one of the probes [GenBank:CX207886] has no matches in any public sequence repository. Whether these transcripts correspond to novel genes or novel splice variants of already defined genes remains to be determined.

Gene Ontology functional analysis of differentially expressed genes

We used the Gene Ontology classification of the 267 differentially expressed genes for a two-step functional analysis to define the overall Gene Ontology category representation and to identify themes that were over-represented compared to the complete mouse transcriptome [27]. A Gene Ontology annotation was assigned to approximately 50% of the 141 down-regulated and 126 up-regulated genes. Within the 'Biological processes' branch of Gene Ontology, the largest groups that changed their expression upon Lhx2 down-regulation were 'macromolecule metabolism' (20 down-regulated / 42 up-regulated), 'signal transduction (19/20), 'cell growth and/or maintenance' (18/19), 'nucleobase, nucleoside, nucleotide and nucleic acid metabolism' (18/17) and 'biosynthesis' (8/20). A complete listing, which also includes the 'Molecular function' and 'Cellular component' branches of the Gene Ontology classification, is available as Supplementary data (see Additional file 3). The themes 'regulation of signal transduction', 'organogenesis' and 'cell death' were significantly over-represented among the down-regulated genes (i.e. self-renewal-specific) (Table 4). Several metabolism-related genes were significantly enriched among the up-regulated genes (i.e. differentiation-specific), which was also evident when the data was
analysed using the KEGG metabolic pathways [28] (Table 4). Of note is that ten genes encoding components of the glycolytic pathway, including a glucose transporter (Slc2a3) and glycogen synthase, are among the genes up-regulated after dox withdrawal (see Additional file 1 and 3), suggesting that this metabolic pathway is important when stem cells are initiating differentiation.

**Comparison with HSC gene expression signatures**

Global gene expression in murine HSCs has previously been studied using Affymetrix arrays [29,30]. Both studies analysed the transcriptome of HSCs by comparing gene expression in stem cell-enriched populations to that of more differentiated cells (late progenitors and Lin-positive mature blood cells in Ivanova et al., and the main cell population of the bone marrow in Ramalho-Santos et al.). We compared the genes enriched in these HSCs populations to our list of differentially expressed genes (at UniGene transcript level 275 UniGene clusters correlated to the previously defined 267 differentially expressed genes, where 144 UniGene clusters were down-regulated and 131 were up-regulated). In the comparison with the data from Ivanova et al. (Signature 1 in Figure 4B), we divided their dataset into two groups: "HSC and early progenitor-enriched" (groups i-iv in Ivanova et al.) or "Late progenitor and mature blood cell-enriched" (groups v-vii). Of our differentially expressed Unigene transcripts 46 overlapped with those in the "HSC and early progenitor-enriched" transcripts and 25 overlapped with those in the "Late progenitor and mature blood cell-enriched" transcripts (Figure 4B). A majority (30 or 65%) of the 46 Unigene transcripts overlapping with the "HSC and early progenitor-enriched" signature were down-regulated after dox withdrawal, and a majority of the transcripts overlapping with the late progenitors (20 genes or 80%) were up-regulated after dox withdrawal. The comparison between the HSC-enriched transcripts from the study by Ramalho-Santos et al. (Signature 2 in Figure 4C) and our set of differentially expressed transcripts showed an overlap of 57 genes. Also in this comparison many transcripts (33, or 58%) were found to be down-regulated after dox withdrawal, and a majority of the transcripts overlapping with the "HSC and early progenitor-enriched" signature were down-regulated after dox withdrawal (Figure 4C). Thirteen genes were found in common between all three studies (Smo, Tes, Upp1, Laptm4b, Slco3a1, Fkbp1a, Itga6, A530057M15Rik, 6430596G11Rik, 1810009M01Rik, 1110004D19Rik, D13Ertd275e, and AA536749). Collectively these results show that Lhx2 expression partly maintains the HSC signature and hence, the DoxHPC lines with inducible Lhx2 expression is a relevant model.

### Table 3: The top 25 up-regulated genes in the DoxHPC lines after dox withdrawal. The columns 36 h, 72 h and 96 h refer to hours after dox withdrawal and include the M-values (=log2 [expression in differentiated/expression in undifferentiated]).

| Symbol | Gene name | Genbank | Unigene ID | Gene ID | 36 h | 72 h | 96 h |
|--------|-----------|---------|------------|---------|------|------|------|
| Slc2a3 | Solute carrier family 2 (facilitated glucose transporter), member 3 | CX234108 | Mm.269857 | 20527 | 2.04 | 1.5 | 1 |
| Bnip3  | BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3 | CX229381 | Mm.2159 | 12176 | 1.8 | 1.69 | 1.53 |
| Car2   | Carbonic anhydrase 2 | CX241425 | Mm.1186 | 12349 | 1.49 | 1.25 | 1.07 |
| Eno1   | Enolase 1, alpha non-neuron | CX240534 | Mm.70666 | 13906 | 1.39 | 1.27 | 1.15 |
| Aldoa  | Aldolase A, A isoform | CX237582 | Mm.275831 | 11674 | 1.37 | 1.52 | 1.27 |
| 2410129H14Rik | RIKEN cDNA 2410129H14 gene | CX231735 | Mm.38912 | 76789 | 1.36 | 1.19 | 1.05 |
| Ndgfl  | N-mycol downstream regulated gene 1 | CX218239 | Mm.30837 | 17988 | 1.35 | 0.86 | 0.71 |
| Ncf1   | Neutrophil cytosolic factor 1 | CX225706 | Mm.4149 | 17969 | 1.13 | 1.48 | 1.62 |
| -      | Unknown | CX635138 | - | - | 1.29 | 1.49 | 1.64 |
| Perp   | PERP, TP53 apoptosis effector | CX231659 | Mm.28209 | 64058 | 1.29 | 1.29 | 1.08 |
| Pkg1   | Phosphoglycerate kinase 1 | CX242304 | Mm.336205 | 18655 | 1.27 | 1.32 | 1.22 |
| -      | Unknown | CX207798 | - | - | - | 1.19 | 1.66 |
| -      | Unknown | CX241476 | - | - | - | 1.18 | 1.08 |
| -      | Unknown | CX200988 | - | - | - | 1.18 | 1.09 |
| Tpi1   | Triosephosphate isomerase 1 | CX242931 | Mm.4222 | 21991 | 1.17 | 1.07 | 1.03 |
| -      | Unknown | CX237601 | Mm.278512 | 69239 | 1.11 | 1.31 | 1.26 |
| Mcm10  | Minichromosome maintenance deficient 10 (S. cerevisiae) | CX214265 | Mm.23824 | 70024 | 1.15 | 1.34 | 1.2 |
| Tnixip | Thioredoxin interacting protein | CX201093 | Mm.271877 | 56338 | 1.14 | 1.32 | 1.15 |
| P4ha1  | Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide | CX231610 | Mm.2212 | 18451 | 1.13 | 0.82 | 0.81 |
| 2610034M16Rik | RIKEN cDNA 2610034M16 gene | CX201694 | Mm.278512 | 69239 | 1.11 | 1.31 | 1.26 |
| 9830134C10Rik | RIKEN cDNA 9830134C10 gene | CX233114 | Mm.328927 | 442827 | 1.11 | 1.43 | 1.37 |
| 2410034H20Rik | RIKEN cDNA 2410034H20 gene | CX225894 | Mm.250541 | 76375 | 1.09 | 1.05 | 0.99 |
| 6430596G11Rik | Lactate dehydrogenase 1, A chain | CX239308 | Mm.29324 | 16828 | 1.08 | 0.92 | 0.83 |
| A830010M20Rik | RIKEN cDNA A830010M20 gene | CX233114 | Mm.328927 | 442827 | 1.11 | 1.43 | 1.37 |
| Tec    | Cytoplasmic tyrosine kinase, Dscr28C related (Drosophila) | CX229381 | Mm.2159 | 12176 | 1.8 | 1.69 | 1.53 |
system for normal HSCs also at the level of global gene expression.

**Genes most affected by Lhx2 expression**

The fifty genes that show largest fold-change (up- or down-regulated) after dox withdrawal are presented in Table 2 and 3. The list of down-regulated genes contains two members of the serpin family of protease inhibitors, Serpina3g and Serpina3n, where the former is the most highly differentially expressed gene. In general, serpins are involved in diverse processes such as coagulation, extracellular matrix degradation, complement activation, fibrinolysis and apoptosis [31]. The expression pattern of Serpina3n has not been well characterized, but expression of Serpina3g is highly enriched in normal HSCs [32], and in the hematopoietic precursor cell line FDCP-Mix [33]. The exact biological function of Serpina3g is not known, but has been shown to involve inhibition of caspase-independent cell death [34]. This is in agreement with the observed increase in apoptosis after turning Lhx2 expression off, which is also reflected by the observation that two pro-apoptotic genes, Bnip3 and Perp [35,36], are among the 25 most up-regulated genes (Table 3). Overexpression of Serpina3g in FDCP-Mix cells caused delay in differentiation and increased clonogenic potential of these cells [33], suggesting similar function in the Lhx2 expressing HPC lines and normal HSCs.

Most genes encoding enzymes within the glycolytic pathway were up-regulated after Lhx2 expression was turned off, but one highly differentially expressed gene within this pathway, Bpgm (2,3-bisphosphoglycerate mutase), was an exception as it showed a decrease by 75%. This enzyme is known to be present in erythrocytes where it is involved in the reaction generating the metabolite 2,3-bisphosphoglycerate, which is important for the oxygen-hemoglobin interaction. However, Bpgm is also expressed during early developmental stages (egg and pre-implantation stages) [37], suggesting that it may affect other cellular processes.

Genes linked to both normal and abnormal hematopoietic progenitor cell function were identified among the top 50 most differentially expressed genes. For example, among the most down-regulated genes Sox4 and Plscr1 (Phospholipid scramblase 1), have both been reported to be enriched in HSCs [29,30]. Although the normal function of Sox4 in HSCs is not known, over-expression of Sox4 in mouse HSCs has recently been shown to cause myeloid leukemia and Sox4 is also a frequent insertional mutagenesis target in murine myeloid leukemias [38,39]. Plscr1-deficient mice have no major hematopoietic defects, but Plscr1−/− hematopoietic progenitor cells show decreased colony formation and impaired differentiation to neutrophils in response to Steel factor and G-CSF [40]. Examples of the most up-regulated genes were Ndrg1 (N-myc downstream regulated gene 1), and Ncf1 (Neutrophil cytosolic factor 1). Up-regulation of these genes would be expected since the DoxHPC lines under the culture conditions used herein differentiate towards both neutrophil and mast cell lineages, and these genes are linked to the development and/or function of these cell types [41,42].

Etv5 and Csrp2 (cysteine and glycine-rich protein 2), which also showed significant decrease in expression after
turning Lhx2 expression off, have been implicated in mesenchyme-epithelia interactions during embryogenesis [43,44], similar to Lhx2. Interestingly, both Csrp2 and Lhx2 are specifically expressed in hepatic stellate cells of the liver [12,45]. Chronic liver injury causes activation of hepatic stellate cells leading to hepatic fibrosis [46], and both Lhx2 and Csrp2 have been negatively implicated in this process [16,45], suggesting overlapping function of these genes also in non-hematopoietic cells. In this respect it is interesting to note that one of the top 25 up-regulated genes is P4ha1 (proline-4-hydroxylase), which encodes an enzyme important for post-translational modifications of collagen, leading to increased stability of collagen fibrils. Expression of P4ha1 is also up-regulated in the fibrotic Lhx2−/− fetal liver as compared to the normal fetal liver [16]. Thus, analyses of differentially expressed genes identified in this hematopoietic-based system might also give insights into Lhx2 function (and lack of function) in non-hematopoietic cells.

Overlapping gene expression patterns in mouse embryos of Lhx2 and genes down-regulated in DoxHPC lines upon dox removal

Lhx2 plays an important role during embryonic development in the formation of several different tissues via mesenchymal-epithelial interactions and/or regulation of stem/progenitor cells [11-17]. We therefore wanted to elucidate whether any of the differentially expressed genes, particularly genes that were down-regulated upon dox withdrawal, also were expressed in tissues/organs where Lhx2 is expressed during embryonic development. Although we were comparing differential expression in hematopoietic cells, 6 of 13 (46 %) of the genes down-regulated after dox withdrawal that we have analysed thus far, Nuak1, Tmem2, Etv5, Enc1, Csrp2, and Tgfb1i1 (Transforming growth factor β1 induced transcript 4), showed partly overlapping expression pattern with Lhx2 in different tissues. Lhx2 is expressed by cells in the liver, the hair follicles, the developing cerebral cortex of the forebrain and the olfactory epithelium, and examples of genes showing overlapping expression with Lhx2 in two or more of these tissues are shown in Figure 5. Tmem2 is expressed in all four tissues, Nuak1 and Enc1 are expressed in olfactory epithelium, hair follicles, cortex area of the forebrain but not within the liver lobes, whereas Etv5 is expressed hair follicles, cortex area of the forebrain, weakly within the liver lobes but not in olfactory epithelium (Figure 5). These results suggest that the mechanism whereby Lhx2 immortalizes hematopoietic stem/progeni-

Table 4: The most regulated groups genes. Enriched biological processes (level 3), molecular function and chromosome distribution as defined by Gene Ontology and pathways as defined by Kegg of the differentially expressed genes.

| Categorial system | Differential Expression | Term | Genes | p-value |
|-------------------|------------------------|------|-------|---------|
| Biological processes | Down-regulated | REGULATION OF SIGNAL TRANSDUCTION | 4 | 0.015 |
| | | ORGANOGENESIS | 10 | 0.070 |
| | | CELL DEATH | 6 | 0.077 |
| | Up-regulated | ALCOHOL METABOLISM | 16 | 0.00000000000087 |
| | | MACROMOLECULE METABOLISM | 38 | 0.0000080 |
| | | BIOSYNTHESIS | 19 | 0.000086 |
| | | CATABOLISM | 16 | 0.0064 |
| | | COENZYME AND PROSTHETIC GROUP METABOLISM | 6 | 0.0028 |
| | | VITAMIN METABOLISM | 3 | 0.035 |
| | | AMINE METABOLISM | 5 | 0.063 |
| Molecular function | Down-regulated | TRANSFERASE ACTIVITY, TRANSFERRING GLYCOSYL GROUPS | 6 | 0.013 |
| | | OXIDOREDUCTASE ACTIVITY, ACTING ON PEROXIDE AS ACCEPTOR | 3 | 0.030 |
| | Up-regulated | PROTEASE INHIBITOR ACTIVITY | 4 | 0.087 |
| | | CARBOHYDRATE KINASE ACTIVITY | 3 | 0.0096 |
| | | TRANSFERASE ACTIVITY, TRANSFERRING PHOSPHORUS-CONTAINING GROUPS | 12 | 0.013 |
| | | CARBON-OXYGEN LYASE ACTIVITY | 3 | 0.054 |
| | | PURINE NUCLEOTIDE BINDING | 15 | 0.061 |
| Chromosome | Down-regulated | 2 | 17 | 0.053 |
| | Up-regulated | 8 | 12 | 0.021 |
| Kegg pathways | Up-regulated | PENTOSE PHOSPHATE PATHWAY | 6 | 0.0000029 |
| | | GLYCOLYSIS / GLUCONEOGENESIS | 10 | 0.0011 |
| | | GALACTOSE METABOLISM | 5 | 0.0018 |
| | | FRUCTOSE AND MANNOSE METABOLISM | 5 | 0.0061 |
| | | CARBON FIXATION | 4 | 0.0018 |
| | | STARCH AND SUCROSE METABOLISM | 4 | 0.026 |
Comparisons between previously published stem cell signatures. 

**A.** Our study. Overlap between differentially expressed genes at 36, 72 and 96 hours after Lhx2 expression was down-regulated. The intersections between groups of differentially expressed genes are visualized using Venn-diagrams (for clarity not drawn to proportions). The numbers refer to unique genes.

**B-C.** Comparison between the transcripts differentially expressed in our study and two previously published hematopoietic stem cell-enriched gene expression signatures. Signature 1 (B) is derived from the [30] study and Signature 2 (C) from the [29] study. Transcripts that are differentially expressed in our study at all three time-points are included. The overlapping transcripts are further split into transcripts that are up- and down-regulated in our study. Comparison between our data set and the two HSC signatures was carried out on the UniGene transcript level (UniGene build 147).
Gene expression analyses in mouse embryos of genes with down-regulated expression when \textit{Lhx2} expression is turned off. \textit{In situ} hybridization using probes of the indicated genes on sagital sections on E12.5-13.5 embryos of the indicated tissues. The border between the olfactory epithelium and the underlying mesenchyme is outlined in each section by a broken line. Examples of individual hair (whisker) follicles are indicated by arrows in each section. The part of the developing forebrain that gives rise to the cerebral cortex is indicated by \textit{ctx} in each section. Arrowheads indicate the border between individual liver lobes in each section. Scale bars sections of Olfactory epithelium, Hair follicles and Liver; 100 µm, Fore brain; 200 µm.
tor cells might partly overlap with the function of Lhx2 in the development of a variety of organs.

Discussion
We have generated HSC-like cell lines by differentiating ES cells with inducible Lhx2 expression in vitro. Self-renewal of these DoxHPC lines, is strictly dependent on Lhx2 expression, since down-regulation of Lhx2 expression leads to loss of CAFCs and rapid and efficient differentiation into a variety of myeloid cells. To elucidate the putative function of Lhx2 in stem cells, we used microarray technology to compare global gene expression in these cell lines in the presence of dox (Lhx2 expression on) to different time points after dox withdrawal (Lhx2 expression off). We identified 267 genes (141 down-regulated and 126 up-regulated) that showed differential expression at all time points (36, 72 and 96 hours) after Lhx2 expression was turned off. Gene Ontology classification revealed that genes related to ‘regulation of signal transduction’, ‘organogenesis’ and ‘cell death’ were over-represented among the down-regulated genes (i.e. “stem cell-specific”), and that metabolism-related genes were over-represented among the up-regulated genes (i.e. “differentiation specific”).

We have previously shown that the HPC lines generated by Lhx2 expression resemble normal HSCs in many basic molecular, cellular and biochemical aspects [18-20,23,47]. Moreover, the HPC lines derived from bone marrow that engrafted stem cell-deficient mice maintained Lhx2 expression in vivo and caused myeloproliferation [21]. We therefore anticipated identifying genes involved in both physiological and pathological processes affecting HSCs by analyses of global gene expression of the DoxHPC lines. A comparison of the differentially expressed genes to previously defined HSC gene expression signatures revealed that a majority of the overlapping genes expressed in HSC-containing populations were those down-regulated after Lhx2 expression was turned off. Thus, although we have used a different microarray platform, the genes defined as stem cell-specific in our system (e.g. the 144 UniGene clusters that belonged to group of genes down-regulated after dox withdrawal), reveal a 21 and 23 % overlap with genes defined as HSC-specific in the two different stem cell databases, respectively (see Additional file 4). Furthermore, thirteen of the 144 UniGene clusters (9 %) defined as stem cell-specific in our study overlapped with both HSC databases (see Additional file 4). In interpreting the results from the comparison between our stem cell-specific genes and the HSC-enriched genes in Ivanova et al. [30] and Ramalho-Santos et al. [29], it should be noted that the probes on the arrays in those studies only has an overlap in our array of 50 and 57%, respectively. The overlap from our model system for HSCs could be further viewed in relation to a study showing that the overlap between three different HSC-specific datasets varied between 19 and 32 % [48]. It should be noted that, despite the fact that these studies were carried out using the same Affymetrix GeneChips, identical analysis approaches and essentially the same cell populations, there is still considerable variability between the different HSC-specific datasets. Moreover, a majority of the genes that were up-regulated after Lhx2 expression was turned off were preferentially expressed in differentiated cell populations. These results suggest that there is molecular overlap in the self-renewal and differentiation process between the DoxHPC lines and normal HSCs also at global gene expression level. We could also identify genes linked to malignant transformation of hematopoietic cells and one such example is Sox4, which was recently shown to cause acute leukemia if over-expressed in HSCs [38]. Sox4 has also been identified as a frequent insertional mutagenesis target in murine myeloid leukemias [39], further supporting a role of this gene in malignant transformation. Since Lhx2 is always integrated into the same position, close to the Hprt1 gene in the DoxHPC lines, expression of insertional mutagenesis target genes is not due to insertional mutation, as has been suggested for retroviral integration [49], hence, these genes are more likely to be linked to Lhx2 function and/or stem cell function. Human chronic myeloid leukemia cells have been reported to express Lhx2 although its function in the disease is not clear [50]. Another gene down-regulated after Lhx2 expression is turned off is Prkd2 (Protein kinase D2, see Additional file 1), which has also been shown to be a substrate for the BCR-ABL fusion protein present in chronic myeloid leukemia cells [51]. These results reveal a putative functional link between Lhx2 and the BCR-ABL fusion protein. However, both Sox4 and Prkd2 have been shown to be preferentially expressed in HSCs [29,30], which suggests overlapping molecular mechanism regulating both HSC physiology and pathology. By using our inducible system it would be possible to dissect these processes on the molecular, cellular and biochemical level.

It has been suggested that the Notch pathway and pathways activated by different morphogens such as Wnt, Bone morphogenetic proteins (BMP) and hedgehog, are important regulators of stem cell function [52-55]. We have previously shown that the Lhx2-induced HPC lines self-renew by a cell nonautonomous mechanism [20], and the anemia in Lhx2-/- mouse embryos is due to a cell nonautonomous mechanism, suggesting that Lhx2 regulates genes encoding proteins involved in cell-cell interactions [17]. However, we have not found any evidence for differential expression of major mediators of the Notch or Wnt pathways in the DoxHPC lines after turning Lhx2 expression off. Although the microarrays we used do not contain all mediators of these pathways, the results sug-
gest that the mechanism whereby Lhx2 expression generates HPC lines is partly or completely independent of at least the Wnt and Notch pathways. We did find consistent down-regulated expression of the hedgehog receptor Smo (Smoothened) after Lhx2 expression was turned off (see Additional file 1), and Smo has also been shown to be preferentially expressed in normal HSCs (see Additional file 4) [29,30]. However, expression of the ligands (Sonic, Indian and Desert hedgehog) was very low to undetectable by real-time PCR analyses of the cell lines (data not shown). Thus, the functional role, if any, of hedgehog signalling in these cells remains to be determined. Furthermore, Tsg (Twisted gastrulation), which is a soluble modulator of BMP-signalling, was identified among the genes down-regulated after Lhx2 expression was turned off (see Additional file 1), and is also defined as an HSC-specific gene (see Additional file 4) [29,30]. The functional relevance of this observation remains to be determined as Tsg protein has been shown to act as a BMP antagonist as well as a BMP agonist [56,57].

Lhx2 appears to play an important role during embryonic development in the formation of several different tissues via mesenchymal-epithelial interactions and/or regulation of stem/progenitor cells [11-17]. One of our aims was therefore to identify gene(s) directly or indirectly regulated by Lhx2, possibly providing insights into the function of Lhx2 in these processes. A caveat to this assumption is that it is not known whether Lhx2 functions as a transcriptional activator, repressor, or both. The only reported target genes putatively regulated by Lhx2 in a direct manner are those encoding olfactory receptors [58] and these receptors are not expressed in Lhx2/- olfactory epithelia [11,13]. These observations suggest that genes activated by Lhx2 are down-regulated in Lhx2/- animals, and conversely, genes repressed by Lhx2 are up-regulated in Lhx2/- animals. A putative example of the latter is P4ha1 as this gene was up-regulated after Lhx2 is turned off in DoxHPC lines, and the expression of this gene was also up-regulated in the liver of Lhx2/- embryos compared to wild type embryos [16]. Although our analysis was made in hematopoietic cells, almost 50% of the genes analysed among those down-regulated after dox withdrawal (Lhx2 expression turned off), showed similar expression pattern as Lhx2 in different non-hematopoietic organs/cells during embryonic development. Preliminary data suggests, however, that several of these genes show maintained expression pattern in Lhx2/- embryos, suggesting that these genes are not direct transcriptional targets for Lhx2. A plausible explanation for this observation is that different genes exert their function via parallel pathways in the regulation, formation or differentiation of a certain tissue but are not dependent on each other for their expression in that particular tissue. An illustrative example of such parallel but equally important pathways during organ for-

mation can be observed during eye development, where both Lhx2 and Pax6 null mutant (Sey) embryos show a developmental arrest at the optic vesicle stage [17,59]. However, the expression of these genes appear to be independent of each other since Pax6 is expressed in the arrested optic vesicle in the Lhx2/- embryos and Lhx2 is expressed in the arrested optic vesicle in Pax6/- embryos [17]. Whether the genes identified in this study play an equally important role as Lhx2 in the development of the respective organ remains to be determined. Our approach may therefore have identified both genes that are directly or indirectly regulated by Lhx2, and genes that play an important role together with Lhx2 via parallel pathways but are independent of Lhx2 expression, similar to the Lhx2/Pax6 example during eye formation. These results suggest that elucidation of Lhx2 function in various organs would give further insights into Lhx2 function in the DoxHPC lines and hence both physiological and pathological processes regulating normal HSCs. Conversely, elucidation of Lhx2 function in DoxHPC would give insights into Lhx2 function in various basic processes during embryonic development, such as mesenchymal-epithelial interactions and regulation of stem/progenitor cells in non-hematopoietic tissues. We are at present time exploring all alternatives of Lhx2 function presented above.

Conclusion

Expression of the LIM-homeobox gene Lhx2 in hematopoietic cells derived from both ES cells differentiated in vitro and from bone marrow, allows for the generation of HSC-like cell lines. To address the molecular basis of Lhx2 function, we have analysed global gene expression in HSC-like cell lines with inducible Lhx2 expression. This approach identified 267 differentially expressed genes where 141 (144 Unigene clusters) were down-regulated and 126 (131 Unigene clusters) were up-regulated when Lhx2 expression was turned off. The relevance of this model system for normal HSC function was revealed by that the majority of the differentially expressed genes overlapping with HSC-specific datasets were defined as stem cell-specific in our system, and the majority of the differentially expressed genes overlapping with more differentiated hematopoietic cell population were defined as differentiation- or commitment-specific in our system. Moreover, gene expression analyses in various tissues during embryonic development of the down-regulated genes revealed that almost half of these genes showed overlapping expression patterns with Lhx2, suggesting a functional link to the development and regulation of various tissues and stem/progenitor cells. Thus, this approach has identified genes putatively linked to self-renewal / differentiation of HSCs, and function of Lhx2 in organ development and stem / progenitor cells of non-hematopoietic origin.
Methods

Generation of ES cells with inducible Lhx2 expression
The ES cell line Ainv15 was maintained on irradiated mouse embryonic feeder (MEF) cells in Dulbecco’s modified Eagle medium (DMEM) (Gibco-BRL, United Kingdom) supplemented with 15% fetal calf serum (FCS) (Boehringer, Germany), 1.5 × 10^{-4} M monothioglycerol (MTG) (Sigma, Germany) and leukaemia inhibitory factor (LIF) (Chemicon, Ca, USA). Lhx2 cDNA or Lhx2 cDNA linked to a green fluorescent protein cDNA preceded by an internal ribosomal entry site (Lhx2-ires-GFP), was inserted into the plox vector and transfected into the Ainv15 ES cells together with Cre recombinase cDNA as previously described [22], and subsequently cultured in 200 µg/ml G418 (Gibco-BRL). Clones of ES cells resistant to G418 were isolated, pooled and expanded.

Differentiation of ES cell in vitro
ES cells used for in vitro differentiation were made feeder-independent in serum-free medium as previously described [60,61]. Briefly, ES cells cultured on MEF cells were trypsinized and transferred to gelatinized culture flasks in N2B27 medium (Gibco-BRL) supplemented with 10^4 U/ml LIF and 10 ng/ml BMP4 (R&D-systems, United Kingdom). The ES cells were trypsinized and transferred into Iscove’s modified Dulbecco’s media (IMDM) (Gibco-BRL) supplemented with 15% FCS (Integro Inc., The Netherlands), 4.5 × 10^{-4} M MTG and 25 µg/ml ascorbic acid (Sigma) at 10^3 cells/ml. Embryoid bodies (EBs) were collected after five days of differentiation, resuspended in trypsin-EDTA and incubated for three minutes. Two ml of FCS was added and the cells were gently passaged through a syringe with a 20-gauge needle. Ten ml of IMDM medium was added; the cells were spun down and resuspended in fresh IMDM medium.

Progenitor (clonal) assays of EB cells
The progenitor assays were carried out in IMDM containing 1% methylcellulose (Fluka, Switzerland) and supplemented with L-glutamine, 300 g/ml iron-saturated transferrin (Boehringer), 5% protein-free hydridoma medium II (Gibco-BRL), 10% plasma-derived serum (Antech Inc., Tx, USA), 100 ng/ml murine Steel factor (SF) R&D-systems), 10 ng/ml human IL-6 (R&D-systems), 4 IU/ml erythropoietin (Eprex Janssen-Cilag, Sweden) and with or without 2 µg/ml doxycyclin (dox) (Sigma). 10^3 or 2 × 10^5 EB cells were plated in triplicates in a final volume of 1.25 ml in 35-mm Petri dishes (Falcon 1008). Primitive erythroid colonies were scored after 4–6 days of incubation and definitive hematopoietic colonies (e.g. SF/IL-6-responsive) were scored after 10–12 days of incubation.

Generation and maintenance of DoxHPC lines with Lhx2 expression regulated by the tet-on system
Individual colonies were randomly picked from the clonal assays after 12 days of incubation, transferred to 96-well plates and expanded in IMDM supplemented with 5% FCS, 1.5 × 10^{-4} M MTG, 100 ng/ml SF, 10 ng/ml IL-6 and 2 µg/ml dox. Stable cell lines could be established from all cultures containing cells with blast-like morphology after three weeks in liquid culture. These cell lines were subsequently maintained in this media at cell densities between 5 × 10^5 and 2 × 10^6 cells/ml as previously described [20]. The efficiency of the system was verified by removing dox and analysing Lhx2 transcription by real-time PCR or GFP expression by flow cytometry at different time points after dox withdrawal (Figure 1A and 1B). No cell lines could be established from the control cell line expressing GFP alone or from colonies expanded in the absence of dox, as such cells differentiated into mast cells under these culture conditions. Two cell lines called DoxHPC1 and DoxHPC7 were selected for further gene expression analysis. The DoxHPC7 line was established from the ES cells where GFP was linked to Lhx2 expression.

Design of the microarray experiment
The gene expression changes in DoxHPC1 and DoxHPC7 cell lines were analysed using the hybridization scheme in Figure 2A. Total RNA prepared from Lhx2 expressing cells was used as reference and compared to total RNA prepared from three time-points (36, 72 and 96 hours) after dox withdrawal. Two independent cell cultures were carried out for both DoxHPC1 and DoxHPC7 lines, providing a first level of replication. Each comparison was further analysed using two replicated hybridizations with the dye assignments reversed, providing a second level of replication. Finally, each probe was printed in duplicate on the arrays, which for each comparison results in eight measurements using three different levels of replication. Additional hybridizations carried out included replicated comparisons between the two separate cultures of each cell line, and a direct replicated comparison between Lhx2-expressing DoxHPC1 and DoxHPC7 cell lines.

The cDNA microarray
The cDNA arrays were produced at the KTH Microarray Center and contain 14,121 in-house sequenced cDNA clones originating from a mouse brain lateral ventricle wall library, a normalized neurosphere library, an adult bone-marrow derived hematopoietic stem cell line expressing Lhx2 and additional control features including the Lucidea Universal ScoreCard probes (Amersham Biosciences, Sweden). A detailed description of the libraries is available elsewhere [62]. Details regarding the probes and cDNA amplification, purification and printing are available through the ArrayExpress microarray data repository using the array accession number A-MEXP-175. In brief,
the cDNA inserts were amplified using Platinum Taq DNA polymerase (Invitrogen, Sweden), purified using the MultiV N-PCR 384-well plates (Millipore, Sweden) automated on the Biorobot 8000 platform (Qiagen, Germany) and printed in either 30 or 50% DMSO. Probes were printed with a feature-to-feature distance of 175 µm into two identical fields consisting of 24 blocks each. The printed DNA was attached to the reactive surface of the Ultra-GAPS slides (Corning B.V., The Netherlands) using 250 mJ/cm² UV light. All cDNA sequences on the chip are printed with a feature-to-feature distance of 175 and printed in either 30 or 50% DMSO. Probes were handled on the Biorobot 8000 platform (Qiagen, Germany) for tisscreen-PCR 384-well plates (Millipore, Sweden) automatically polymerase (Invitrogen, Sweden), purified using the RNA Nano LabChip kit on the Agilent 2100 bioanalyzer (Agilent Technologies, Ca, USA) and Nanodrop technology (Qiagen) and quality and quantity determined using the RNA Nano LabChip kit on the Agilent 2100 bioanalyzer (Agilent Technologies, Ca, USA) and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, De, USA), respectively. For each cDNA synthesis reaction 20 µg of total RNA was mixed with 5 µg of random hexamer primer (Invitrogen), incubated at 70°C for 10 minutes, and finally on ice for at least 5 minutes. Reverse-transcription reaction mixture and 400 units of SuperScript II RT-polymerase were added to yield a final volume of 30 µl containing first-strand buffer (Invitrogen), 10 mM DDT (Invitrogen) and 0.5 mM dNTPs (Sigma). The ratio of aminoallyl-modified dUTP to dTTP was 4:1 in the dNTP mixture. The reaction was carried out at 42°C for two hours, followed by 15 minutes hydrolysis of the RNA strand at 70°C in the presence of 16 mM EDTA (Sigma) and 150 mM NaOH. The reaction was neutralized using 150 mM HCl and purified using the MinElute Reaction Cleanup system (Qiagen) with the provided wash and elution buffers replaced by 80% ethanol and 100 mM NaHCO₃, pH 9.0, respectively. A repeated elution from the column was carried out, generating a total volume of 20 µl. This was transferred to an aliquot containing one tenth of the monofunctional NHS-ester Cy3 or Cy5 dye tubes (Amersham Biotech), which had been dissolved in DMSO and subsequently dried in a vacuum centrifuge. After a 30-minute incubation in darkness at room temperature, the samples to be co-hybridized on a slide were dried using a slide centrifuge. The labeled, pooled and denatured (3 minutes at 95°C) samples in a hybridization mixture containing 25 µg mouse Cot-1 DNA (Invitrogen), 40 µg poly-(dA) DNA (Operon), 25% formamide (Sigma), 5xSSC and 0.1% SDS were applied under a LifterSlip cover (Erie Scientific Company, Nh, USA) and hybridized for 16–20 hours at 42°C in a water bath. After hybridisation the slides were washed with increasing stringency using 2xSSC and 0.1% SDS at 42°C, followed by 0.1xSSC and 0.1% SDS at room temperature and finally five times with 0.1xSSC at room temperature. Scanning was carried out at 10-µm resolution using the G2565BA DNA microarray scanner (Agilent Technologies) for which the photo multiplier tube was set to 100. The obtained TIFF-images were analysed using the GenePix Pro 5.1 software (Axon Instruments, Ca, USA). For each slide the foreground and background signal intensities were separated using the irregular feature-finding algorithm implemented in the software. A manual inspection was carried out to verify the results. The raw data, including several quality parameters, is available from the ArrayExpress data repository using the experiment accession number E-MEXP-431.

**Target preparation**

Total RNA extraction from eight DoxHPC1 and eight DoxHPC7 samples was carried out using the RNeasy technology (Qiagen) and quality and quantity determined using the RNA Nano LabChip kit on the Agilent 2100 bioanalyzer (Agilent Technologies, Ca, USA) and Nanodrop technology (Qiagen) and quality and quantity determined using the RNA Nano LabChip kit on the Agilent 2100 bioanalyzer (Agilent Technologies, Ca, USA) and Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, De, USA), respectively. For each cDNA synthesis reaction 20 µg of total RNA was mixed with 5 µg of random hexamer primer (Invitrogen), incubated at 70°C for 10 minutes, and finally on ice for at least 5 minutes. Reverse-transcription reaction mixture and 400 units of SuperScript II RT-polymerase were added to yield a final volume of 30 µl containing first-strand buffer (Invitrogen), 10 mM DDT (Invitrogen) and 0.5 mM dNTPs (Sigma). The ratio of aminoallyl-modified dUTP to dTTP was 4:1 in the dNTP mixture. The reaction was carried out at 42°C for two hours, followed by 15 minutes hydrolysis of the RNA strand at 70°C in the presence of 16 mM EDTA (Sigma) and 150 mM NaOH. The reaction was neutralized using 150 mM HCl and purified using the MinElute Reaction Cleanup system (Qiagen) with the provided wash and elution buffers replaced by 80% ethanol and 100 mM NaHCO₃, pH 9.0, respectively. A repeated elution from the column was carried out, generating a total volume of 20 µl. This was transferred to an aliquot containing one tenth of the monofunctional NHS-ester Cy3 or Cy5 dye tubes (Amersham Biotech), which had been dissolved in DMSO and subsequently dried in a vacuum centrifuge. After a 30-minute incubation in darkness at room temperature, the samples to be co-hybridized on a slide were dried using a slide centrifuge. The labled, pooled and denatured (3 minutes at 95°C) samples in a hybridization mixture containing 25 µg mouse Cot-1 DNA (Invitrogen), 40 µg poly-(dA) DNA (Operon), 25% formamide (Sigma), 5xSSC and 0.1% SDS were applied under a LifterSlip cover (Erie Scientific Company, Nh, USA) and hybridized for 16–20 hours at 42°C in a water bath. After hybridisation the slides were washed with increasing stringency using 2xSSC and 0.1% SDS at 42°C, followed by 0.1xSSC and 0.1% SDS at room temperature and finally five times with 0.1xSSC at room temperature. Scanning was carried out at 10-µm resolution using the G2565BA DNA microarray scanner (Agilent Technologies) for which the photo multiplier tube was set to 100. The obtained TIFF-images were analysed using the GenePix Pro 5.1 software (Axon Instruments, Ca, USA). For each slide the foreground and background signal intensities were separated using the irregular feature-finding algorithm implemented in the software. A manual inspection was carried out to verify the results. The raw data, including several quality parameters, is available from the ArrayExpress data repository using the experiment accession number E-MEXP-431.

**Microarray data analysis**

Data processing and identification of differentially expressed genes was carried out in the R environment for statistical computing and programming [64] using the Bioconductor package bundle [65], Limma [66], aroma package [67] and the kth-package [68]. As a first step the Cy3 and Cy5 intensities without background subtraction were converted to M (\(\log_2[\text{red channel}/\text{green channel}]\)) and A (\(\log_2[\text{red channel}^\ast\text{green channel}]/2\)) format using the median intensity values for both channels. A feature was considered unreliable and removed from further analysis if GenePix flagged it as "Not Found" or if it was manually flagged as "Bad" during the image analysis step. Furthermore, if a feature satisfied one of the following criteria it was considered unreliable: a) both channels were saturated (above 65190 intensity units), b) the percentage of foreground pixels above the local background plus two of its standard deviations were below 70 for both channels of a feature, or c) the signal-to-noise ratio (defined as [mean foreground-mean background]/background standard deviation) for both channels was below 3. Remaining data was normalized using the intensity-dependent print-tip lowess method [69] and differentially expressed genes identified using the empirical Bayes moderated t-test implemented in the Limma package. As a first step the signal intensities for duplicate features on each array were averaged. Replicated hybridizations are expected to have a higher correlation than the repeated cell cultures for each cell line. This was considered in the linear model by first estimating the between-replicates correlation by using restricted maximum likelihood methods to estimate a common correlation for all probes.
[70]. Secondly, a linear model was fit for each probe using generalized least squares that takes into account the between-replicates correlation. In the third step an empirical Bayes approach was used to shrink the gene-wise standard errors towards a common value and a moderated t-statistic was calculated [66,71]. To compensate for multiple testing, the p-values associated with this t-statistics were adjusted using a false-discovery rate approach [72] implemented in R. Finally, probabilities for differential expression (B-values) were calculated for each gene. For these calculations the a priori assumption of differentially expressed genes was set to 0.01.

Classification into Gene Ontology functional groups [27] and analysis of over-represented themes was carried out using the EASE-package available at [73,74]. The complete mouse transcriptome was used for calculation of the expected frequencies in the over-representation analysis, and a Gene Ontology theme (detail level 3) was considered over-represented if the calculated EASE score was below 0.1.

### Analysis of differentially expressed genes by real-time PCR

Total RNA was extracted from cell pellets with Trizol reagent (Invitrogen). cDNA was synthesized by using the First-strand cDNA synthesis kit (Amersham Biosciences). Real-time PCR reactions were carried out in triplicates using SYBR green PCR master mix (Applied Biosystems, CA, USA) and PCR products were detected with an ABI prism 7000 instrument (Applied Biosystems, CA, USA). PCR products were detected with an ABI 7000 instrument (Applied Biosystems). The expression levels of Gapdh and confirmed with two additional housekeeping genes: Hprt1 (hypoxanthine phosphoribosyltransferase 1) and Tbp (TATA box binding protein) (data not shown). The following primers were used in the PCR analyses: Lhx2 Forward (F)- GCC GAG AAA GCG CAA GAG T and Reverse (R)- TGT TCA GCA TCG TTC TCG TTA CA; Plscr1 F- AGC TGC TGT TTC GAC ATT GA and R- GGA ACT GGA TCC CAA AAT TGT CT; Bpzm F- CIT AAA GGG CAA AAG CAT TCT GAT and R- TGG GCA GAG TGA TGT TGA TAA TAT C; Galnt2 F- TGC GGG GCC TCA GAA ATG A and R- TCA GCA ACC GGC TCC AA; Serpina3g F- CCT ACA GAT CCT GGC AGA GTT CA and R- GAT CIT CCC CTG GGT CTG ATT; Syne2 F- GGA GGT GIT CGG CAG AGT GT and R- TCT TTC ATG TCC TTC TCA TCA G; Csrp2 F- CCG TGT ATG CTG CGG AGA A and R- TTG GCA CAC CGG AAA CAG T; Upp1 F- TGA CCG CTA CGC CAT GFA TAA A and R- CAT GAT GCC GAT GGA AGG A; Myolb F- ATC AGG TCA AGG AAC AGC TTC TG and R- TCA TTC TCT ACA GTC TGT GCA TT; Etv5 F- GAG CCG CTC TCT CCG CTA T and R- CCG GGT CAC ACA CAA ATTTG; Perp F- CIT TGT TTC CTG AGA GTC ATT GGA and R- GTG TAT CGT GAA GCC TGA AGG T; 2610034M16Rik F- GAG GAC TCA TCT AGG TTT TGT GAA and R- AAT GTC AAC TTC TGC TCC TTC TAA TTT TA; Ncf1 F- CAA AGA TGG CAA GAA TAA CGT AGC T and R- AGT CAG CAA TGG CCC GAT AG; Slc2a3 F- AGC ATC GGC TCT TCT CAG TTT and R- TCT TAA CCG CTT TTC CAA AGT GTA; Aldoa F- CCC TCC TTC CCA AGT GTA; 7294496D10Rik F- CCA GAG TAC TAA CGT CAG AGT TGT AAA GTA and R- GGC ACC ACC TAA TCT ACC T; Bnip3 F- GGT TTT CCT TCC ATC TCT GTT AGT GT and R- GTT GTC AGA CGC CTT CCA ATG; Tpi1 F- ACC GAG AAT CGT GTC GTG TAC GA and R- GGC GAC GAC CAC CIT GCT; Ndrg1 F- CAT CGG CAT GAA CCA CAA GA and R- AAA ATG TTT TGT GAT CTC CTG CAT; Pgk1 F- GGA AGC GGG TCG TGA TGA and R- GCC TTC ATC TCT TGG TTG TTT G; Eno1 F- GGC ACC CTC TTT CCT TGC TT and R- GCC GTG GAT CCT CCT GAT AAT AGA C; Gapdh F- CGT GGT TCT ACC CCC AAT GT and R- TGT CAT CAT ACT TGG CAG GTT TCT; Hprt1 F- GCA GTA CAG CCC CAA AAT GG CCT ACC CCC AAT GT and R- AAG AAA GTG TTC GTA TCC AA; Tbp F- GAA TTG TAC CGC AGC TTC AAA A and R- AGT GCA ATG GTC TTC ATG TCA ATG T.

### In situ hybridizations

Embryos were fixed in 4% paraformaldehyde, cryoprotected (30% sucrose in PBS), and embedded in Tissue-Tek OCT compound and cryosectioned (8 µm). In situ hybridization using digoxygenin (Dig)-labeled probes was performed as described [75], with some modifications. Briefly, sections from E13.5 embryos were treated with 5 µg/ml proteinase K (Roche) in 0.1 M PBS for 15 minutes at room temperature prior to hybridization. The DIG signal was visualized with NBT/BCIP (Roche). The following probes were used: Lhx2 ([GenBank:NM_010710]), probe spanning the region 460–1750, Nuak1 ([GenBank:NM_001004363], 2382–3143), Tmem2 ([GenBank:BC076570], 296–1262), Enc1 ([GenBank:NM_0027930], 920–1793), and Etv5 ([GenBank:NM_023794], 661–1609).

### Authors’ contributions

KR carried out generation and collection of material, confirmative analysis, in situ hybridizations and helped to draft the manuscript. LD carried out confirmative analysis, in situ hybridisations and helped to draft the manuscript. VW coordinated and carried out the manufacturing of the microarrays, carried out data analysis and statistical analysis of expression data and helped to draft the manuscript. SB performed the labelling and hybridisation procedures, participated in the analysis and helped to draft the manuscript. JL participated in the supervision and commented on the project, was responsible for funding at the KTH site. LC conceived of the study and helped to draft the manuscript. CW participated in the design of the study, its coordination, expression analyses and drafted the manuscript. All authors read and approved the final manuscript.
Additional material

Additional File 1
Microarray expression data. Values and annotations for genes differentially expressed at time points 36 h, 72 h and 96 h after dox withdrawal. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-7-75-S1.xls]

Additional File 2
Cell line expression data. Differential expression between the cell lines DoxHPC1 and DoxHPC7 at time-point 0. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-7-75-S2.xls]

Additional File 3
Biological classification of data. Complete listing of Gene Ontology classifications groups differentially expressed in this study. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-7-75-S3.xls]

Additional File 4
Overlap between genes defined as stem cell-specific in this study and HSC-specific datasets. Listing of genes in our study overlapping with other studies of hematopoietic stem cells using Affymetrix technology (refs 29,30). Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-7-75-S4.xls]

Acknowledgements
We thank Annelle Waldén for valuable assistance with the microarray printing and Peter Nilsson for discussions. This work was supported by grants from the Knut and Alice Wallenberg Foundation, the Wallenberg Consortium North, the Swedish Scientific Research Council, the Foundation for Strategic Research, the Swedish Cancer Society and the Västerbotten County. LC is supported by the Tobias Foundation and by a grant from the European Union Regional Fund (Objective 1).

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