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The expression of *Sox17* identifies and regulates hemogenic endothelium

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Although it is well recognized that hematopoietic stem cells (HSCs) develop from a specialized population of endothelial cells known as ‘hemogenic endothelium’ (HE), the regulatory pathways that control this transition are not well defined. Here we identify *Sox17* as a key regulator of HE development. Analysis of *Sox17*-GFP reporter mice revealed that *Sox17* is expressed in HE and emerging HSCs and that it is required for HSC development. Using the mouse embryonic stem cell (mESC) differentiation model, we show that *Sox17* is also expressed in HE generated in vitro and that it plays a pivotal role in the development and/or expansion of HE via the Notch signaling pathway. Taken together, these findings position *Sox17* as a key regulator of HE and hematopoietic development.

The embryonic hematopoietic system consists of two distinct programs that differ in their potential and temporal patterns of development. Primitive hematopoiesis emerges in the yolk sac and displays restricted potential, generating primitive erythroblasts, macrophages, megakaryocytes but no lymphoid lineage cells or HSCs^{1,2}. HSCs are generated during definitive hematopoiesis derived from HE that is specified at different sites, the best characterized being the region comprising the developing aorta, gonads and mesonephros (AGM)³⁻⁵. Histological analyses and imaging studies have shown that the hematopoietic cells “bud” from the HE through a process known as the endothelial to hematopoietic transition (EHT) and subsequently form distinct clusters within the lumen of the aorta⁶⁻⁸. Numerous studies have identified key regulators of this process including *HoxA3* that plays a role in the development of HE and *Notch1* and *Runx1* that function during the EHT⁹⁻¹¹. The *SoxF* family of transcription factors; *Sox7*, *Sox17* and *Sox18*, have also been shown to play a role in embryonic hematopoiesis¹²⁻¹⁴. *Sox17* is of particular interest in this context as

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AUTHOR CONTRIBUTIONS

R.C. and G.K. designed experiments and wrote the manuscript. G.K. supervised the project. R.C. performed all mESC and transplantation experiments. A.Y. performed confocal imaging. Y.Y. performed JASPER analysis and luciferase assays. A.B. generated the doxycycline-inducible mESC line.

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it is expressed in the arterial vasculature⁹, it is required for the generation and maintenance of HSCs in the mouse fetal liver¹⁵ and its expression marks progenitors that contribute to HE and adult definitive hematopoiesis¹⁶

We previously reported that expression of *Sox17* together with Flk-1 identifies a definitive hematopoietic progenitor in mESC differentiation cultures and distinguishes it from an earlier developing Flk-1⁺*Sox17*⁻ population that represents the primitive hematopoietic program¹⁷. To further characterize this *Sox17*⁺ population with respect to HE, we used a reporter mESC line carrying a targeted *Sox17*-mCherry fusion cDNA (*Sox17*-mC)¹⁸ to track its development. When differentiated in defined culture conditions (Fig 1a) the *Sox17*-mC mESC line generates a Flk-1⁺*Sox17*-mC⁻ population within 3.0 to 3.25 days (D) of culture (Fig 1b). Although these early progenitors do not express *Sox17* as they emerge, they do give rise to *Sox17*⁺ cells that co-express the endothelial markers Flk-1, VE-Cadherin (VEC) and CD31 following an additional 24 hours of culture (Supp Fig 1a). Whether this *Sox17*⁺ population represents the developing primitive erythroid lineage, definitive hematopoietic progenitors contaminating the D3.25 Flk-1⁺ population¹⁷ or emerging endothelial progenitors remains to be determined.

To examine the role of *Sox17* in the generation of the definitive hematopoietic program, D3.25 embryoid bodies (EBs) were dissociated and reaggregated for two days (D5.25) to induce a second population of Flk-1⁺ cells that does express *Sox17*-mC. FACS analysis revealed that the D5.25 *Sox17*-mC⁺ cells also expressed VEC, CD31 and AA4.1 (CD93). The majority of D5.25 *Sox17*-mC⁺ cells did not express CD41 nor CXCR4 and none expressed Sca-1 (*Ly6a*) (Fig 1b). qRT-PCR analysis confirmed *Sox17* expression at D5.25 of differentiation (Supp Fig 1b).

To determine if the Flk-1⁺*Sox17*-mC⁺ cells display HE potential the F⁺S⁺, F⁺S⁻ and F⁻S⁻ populations were isolated from D5.25 EBs (Fig 1c, Supp Fig 1b) and plated as a monolayer with cytokines known to promote the growth of endothelial cells. Molecular analyses showed that F⁺S⁺ cells expressed the highest levels of *Sox17*, verifying the fidelity of the mCherry reporter (Supp Fig 1b). Following two days of culture a significant proportion of the population derived from the F⁺S⁺ fraction downregulated expression of both VEC and *Sox17*-mC and upregulated the expression of CD45 suggesting these cells initiated the EHT (Fig 1c). The F⁺S⁻ fraction gave rise to a population with a similar profile, including cells that co-express VEC and *Sox17*-mC suggesting that the F⁺S⁻ fraction contains progenitors of the F⁺S⁺ population. The F⁻S⁻ fraction showed little capacity to generate HE.

To promote hematopoietic development, the monolayers were dissociated and cultured as aggregates in hematopoietic cytokines for two days (Fig 1a). During this time, the F⁺S⁺ and F⁺S⁻-derived cells downregulated *Sox17*-mC expression and generated CD45⁺VEC⁻ cells. The F⁻S⁻ fraction did not give rise to any CD45⁺ cells, indicating that hematopoietic potential was restricted to populations that expressed or acquired expression of F⁺*Sox17*-mC (Fig 1c). The population derived from the F⁺S⁺ fraction contained the highest frequency of myeloid and multipotent progenitors and was the only one that generated T cells (Fig 1d,e). qRT-PCR analyses revealed that cells generated from the F⁺S⁺ and F⁺S⁻ fractions also expressed the highest levels of *Aml1c* (Supp Fig 1c). Taken together, these findings

demonstrate that *Sox17* expression marks the emergence of HE in mESC differentiation cultures.

To investigate the role of *Sox17* in the generation of definitive hematopoiesis *in vivo*, we analyzed the AGM region of E11.5 *Sox17^{GFP/+}* embryos¹⁵ for expression of Sox17-GFP. As shown in Figure 2a, a distinct Sox17-GFP⁺ population was detected at this stage and a subset of these cells co-expressed the HE markers Flk-1, VEC, CD31, CD34 and Tie2. Only a small minority of Sox17-GFP⁺ cells expressed Sca-1, CD41 and CD11b. Intriguingly a small proportion of the Sox17-GFP population were CD45⁺ suggesting that *Sox17* expression marks the emerging HSCs¹⁹. Analyses of the YS of E7.5 embryos identified a Sox17-GFP⁺ population that co-expressed Flk-1 and CD31 (Supp Fig 2b,c). As with the early mESC-derived population, it is unclear if these cells represent the developing primitive erythroid lineage, the YS HE population recently described²⁰ or vascular progenitors.

Analyses of E10.5 embryos by whole mount immunostaining revealed that Sox17-GFP was present in the endothelial cells lining the arteries but not the veins (Supp Fig 2a) as well as in all cells of the emerging hematopoietic clusters found in the dorsal aorta (Fig 2b), vitelline and umbilical arteries (data not shown). These observations clearly show that *Sox17* is expressed in the newly forming hematopoietic cells and the HE from which they differentiate. To formally demonstrate that Sox17-GFP marks emerging HSCs the VEC⁺Sox17⁺, VEC⁺Sox17⁻, VEC⁻Sox17⁺ and VEC⁻Sox17⁻ fractions were isolated by FACS from E11.5 *Sox17^{GFP/+}* embryos (CD45.2⁺) and the cells transplanted into CD45.1⁺ recipients (Fig 2d). Seven out of ten recipients transplanted with VEC⁺GFP⁺ cells showed significant levels of donor-derived multilineage hematopoietic reconstitution (Supp Fig 2d). In contrast, no donor-derived hematopoietic cells were detected in the recipient mice transplanted with VEC⁺GFP⁻, VEC⁻ GFP⁺, or VEC⁻GFP⁻ cells indicating that all HSCs at this stage are Sox17-GFP⁺.

Flow cytometric analyses of the AGM region of E11.5 *Sox17^{GFP/+}* embryos revealed that 23% of the VEC⁺CD45⁺ population, previously shown to contain all HSC activity¹⁹, expressed Sox17-GFP. These observations are consistent with the above transplantation studies and suggest that expression of Sox17 marks the HSCs within the VEC⁺CD45⁺ population (Fig 2c). In contrast to the heterozygous embryos, the AGM region of *Sox17^{GFP/GFP}* (null) embryos had no detectable VEC⁺CD45⁺ cells, suggesting that they are deficient in HSCs. Transplantation studies confirmed the deficiency in HSC development as none of the eight recipient mice transplanted with *Sox17^{GFP/GFP}* cells showed donor cell reconstitution (Fig 2d). These findings suggest that *Sox17* is required for the generation of HSCs. Alternatively, the lack of HSCs may be a secondary effect of the posterior patterning defects resulting from the deletion of *Sox17* in all lineages.

To determine if endothelial expression of *Sox17* is required for establishment of definitive hematopoiesis, we next conditionally deleted *Sox17* in VEC⁺ cells by crossing *Sox17^{fl/fl}* and *VEC-Cre* mice^{11,15}. *VEC-Cre⁺Sox17^{fl/fl}* embryos were observed at expected numbers at E11.5. However, by E13.5 no live Sox17 deleted embryos were detected. (Fig 2e, Supp Fig 2e). As observed in embryos in which Sox17 was deleted in Tie2⁺ endothelial cells⁸, the E11.5 *VEC-Cre⁺Sox17^{fl/fl}* embryos showed dramatic growth retardation compared to their

heterozygous littermates (Fig 2f). FACS analysis revealed that the AGM region of the *VEC-Cre⁺Sox17^{fl/fl}* embryos have reduced *VEC⁺CD45⁻*, *VEC⁺CD45⁺*, *VEC⁻CD45⁺* and *Sca-1⁺CD45⁺* populations, indicating a defect in HE and HSC development (Fig 2g, Supp Fig 2f). Collectively, the findings from these *in vivo* studies demonstrate that expression of *Sox17*-GFP marks the developing HE and HSCs in the AGM and that it is required for HSC development at this site.

To further investigate the role of *Sox17* in the establishment of hematopoiesis, we analyzed the effects of deleting its expression *in vitro* using a *Sox17^{-/-}* mESC line (Supp Fig 3a). Although cell proliferation appeared to be lower in the *Sox17^{-/-}* cultures compared to the *Sox17*-mC controls (Supp Fig 3b), *Sox17^{-/-}* Flk-1⁺ populations were detected at D3.25 and D5.25 (Fig 3a). The *Sox17^{-/-}* D3.25 Flk-1⁺ population displayed normal primitive erythroid and myeloid potential, indicating that *Sox17* is not required for establishment of the primitive hematopoietic program (Fig 3b). Analyses of the *Sox17^{-/-}* D5.25 Flk-1⁺ cells showed that they retained the capacity to generate adhesive populations that had HE cell surface marker profiles similar to those derived from the *Sox17*-mC control cells (Fig 3c, left). Both wild type and *Sox17* null D5.25 Flk-1⁺ cells also gave rise to *CD31⁺CXCR4⁻* and *CD31⁺CXCR4⁺* populations thought to represent venous and arterial endothelium respectively²¹. qRT-PCR analysis showed that the D7 *Sox17^{-/-}* cells expressed higher levels of *Sox7* than the controls, suggesting that its upregulation may compensate for the loss of *Sox17*. Expression levels of *Sox18*, as well as of genes associated with arterial (*EphrinB2*), venous (*COUP-TFII*), and HE (*HoxA3*, *Aml1c*) development, and notch signaling (*Notch1*, *Jagged1*) were unaffected by the loss of *Sox17* (Fig 3d, upper).

We next sorted the D7 *VEC⁺* fraction cells and cultured them as a monolayer for 48 hours to determine if the *Sox17^{-/-}* cells could undergo the EHT. Both the *Sox17^{-/-}* and *Sox17*-mC control *VEC⁺* cells generated large hematopoietic *CD45⁺* populations demonstrating their capacity transition to a hematopoietic fate (Fig 3c, right). With this transition, the size of the *VEC⁺* and *CD31⁺* populations in the *Sox17^{-/-}* cultures decreased dramatically compared to controls, suggesting that *Sox17* is required for the maintenance of the hemogenic vasculature *in vitro*. qRT-PCR analyses supported this interpretation in demonstrating that expression of genes associated with endothelial development, including *Sox7*, *Sox18*, *EphrinB2* and *Notch1* was significantly downregulated in the *Sox17^{-/-}* population compared to controls (Fig 3d, lower). No differences were detected in the expression of *Runx1*, *HoxA3*, or *Coup-TFII*. CFC assays revealed that the *Sox17^{-/-}* *VEC* cells were able to generate myeloid and erythroid progenitors at numbers comparable to those from the control population. In contrast, the *Sox17^{-/-}* cells generated fewer multipotential progenitors than the control cells (Fig 3e) and showed a complete lack of T lymphoid potential (Fig 3f). Collectively, these findings indicate that *Sox17* is dispensable for erythroid and myeloid development from the D7 *VEC⁺* HE but is required for the generation of multipotential and T lymphoid progenitors from this population.

To gain further insight into the mechanism by which *Sox17* is regulating definitive hematopoiesis we investigated the consequences of enforced expression, using a mESC line containing a doxycycline (dox) inducible *Sox17* cDNA²². For these studies, *Sox17* expression was induced in the D5.25 Flk-1⁺ population during the 2 days of monolayer

culture that promotes the expansion of HE. During this culture period, untreated Flk-1⁺ cells gave rise to an adherent HE population that initiated the EHT, as demonstrated by the emergence of round hematopoietic cells (Supp Fig. 4a). In contrast, the HE population generated from the dox-induced Flk-1⁺ contained no budding hematopoietic cells. These differences were not due to an increase in cell death as total cell numbers were equal in both groups (Supp Fig 4b). The morphological changes induced by overexpression of *Sox17* were mirrored by a dramatic increase in the size of the VEC⁺ population, a significant decrease in the size of the CD45⁺VEC⁻ population and a reduction in the frequency of myeloid and T-cell progenitors at D7 (Fig 4a,b). Interestingly, enforced expression of *Sox17* also led to an increase in the size of the arterial-like CD31⁺CXCR4⁺ population. These observations suggest that enforced expression of *Sox17* results in an increase in the size of the HE population and an impairment in its ability to undergo the EHT.

To determine if the induced HE population can generate hematopoietic cells, VEC⁺CD45⁻ cells were isolated from the induced and non-induced D7 populations and cultured in the absence of doxycycline (Fig 4a, right panel). As expected, the non-induced VEC⁺ cells generated a substantial VEC⁻CD45⁺ population by D9. The VEC⁺ cells from the D7 induced monolayer also produced CD45⁺ hematopoietic cells, although the size of the population was considerably smaller than that generated by the non-induced cells. The frequency of myeloid and multipotent progenitors in the non-induced and induced D9 populations was comparable, consistent with the observation that both contained CD45⁺ cells (Fig. 4c). In contrast, the induced population showed a 3-fold higher frequency of T-lymphoid progenitors (Fig. 4c), indicating that it displays enhanced definitive hematopoietic potential. Molecular analyses revealed that expression of *EphrinB2*, *Notch1* and *Jagged1* were significantly upregulated whereas that of *COUP-TFII* was reduced in the induced population at D7 of culture (Fig 4d). Expression of *Aml1c* was lower in the induced compared to non-induced population at D7. By D9, the levels had increased in both populations. Enforced expression of *Sox17* had no effect on the expression of *HoxA3*. Collectively, these findings demonstrate that enforced expression of *Sox17* leads to an increase in the size of the HE population able to generate T lymphocytes and suggest that this effect may be mediated via the Notch pathway.

To investigate the role of Notch signaling in the *Sox17*-induced expansion of HE, induced and non-induced D5 Flk-1⁺ cells were cultured (48 hours) in the presence and absence of the pathway inhibitor γ -secretase inhibitor (γ SI). The addition of γ SI reduced total cell numbers as well as the size of the CD31⁺CXCR4⁺ arterial-like endothelial population generated from both the *Sox17*-induced and non-induced populations (Fig 5a, Supp Fig 5a). Manipulation of the Notch pathway did not, however, impact the size of the induced VEC⁺ population. The addition of γ SI did prevent the *Sox17* induced changes in *EphrinB2* and *COUP-TFII* expression (Fig. 5b). D7 induced and non-induced VEC⁺CD45⁻ cells from γ SI-treated or control cultures were next isolated, cultured as aggregates for 2 days and the resulting populations assayed for the presence of CD45⁺ cells, for *Aml1c* expression and for myeloid/erythroid and T cell potential. The addition of γ SI to the non-induced progenitors had no effect on the CD45 profile, the levels of *Aml1c* expression or the T cell potential of the day 9 population (Fig. 5c,d). In contrast, Notch inhibition in *Sox17*-induced cultures

dramatically reduced the size of the CD45⁺ population, the expression levels of *Aml1c* and the frequency of T cell progenitors (Fig 5c,d and Supp Fig 5b). The addition of γ SI did not, however impact the generation of myeloid and erythroid progenitors (Supp Fig 5c),

To determine if Sox17 can directly activate *Notch1*, we analyzed the *Notch1* promoter region for motifs that can be recognized by Sox17. The JASPER database identified four Sox17-binding sites in the mouse *Notch1* promoter, two (BS1 and BS2) of which are evolutionarily conserved (Fig 5e and Supp Fig 5d)²³. To determine whether Sox17 can activate *Notch1*, U2OS cells were transfected with a Sox17 expression plasmid together with a reporter construct containing a 660bp region of the *Notch1* promoter including the BS1 and BS2 sites upstream of luciferase. Sox17 induced luciferase activity in a dose dependent fashion, demonstrating that Sox17 can activate the *Notch1* promoter (Fig 5f). Mutations of the Sox-binding motif of BS1 and BS2 reduced luciferase activity, demonstrating that these sites are necessary for the Sox17-mediated activation of the *Notch1* promoter.

To determine if the loss of *Sox17* also affected the levels of *Notch1* expression in vivo, the AGM regions from E11.5 *VEC-Cre⁺Sox17^{fl/fl}* and *VEC-Cre⁺Sox17^{fl/fl}* embryos were isolated and analyzed for gene expression by qRT-PCR (Supp Fig 5e). The expression of *Notch1* but not other members of the Notch family (*Notch4*, *Jagged1*) was significantly decreased in the *VEC-Cre⁺Sox17^{fl/fl}* cells suggesting that *Notch1* is also a target of Sox17 during embryogenesis.

Deciphering the regulatory pathways that control the establishment of definitive hematopoiesis is essential for modeling this program in ESC differentiation cultures and ultimately to generate HSCs in vitro. Our findings have provided new insights into this process and position *Sox17* as a key regulator of HE. Specifically, our mESC data show that Sox17 is required for the development and/or maintenance of HE able to generate T cells. Interestingly, the *Sox17^{-/-}* *VEC⁺* HE did retain myeloid and erythroid potential, indicating that the specification of a subset of these progenitors is Sox17 independent. These observations suggest that Sox17 specifically regulates the subpopulation of HE that gives rise to the T lymphoid lineage, a measure of the definitive hematopoiesis and that dependence on Sox17 distinguishes it from the HE that generates myeloid and erythroid progenitors. The existence of distinct definitive progenitors populations in the mESC model is consistent with in vivo studies which showed that the formation of erythroid-myeloid progenitors and HSCs can be uncoupled based on the temporal and spatial expression of CBF β in *Ly6a*-expressing cells²⁴.

Our enforced expression studies indicate that sustained levels of Sox17 expression leads to an increase in the size of the HE population able to generate T lymphocytes. A recent study using human ESCs has also provided evidence that enforced expression of Sox17 results in expansion of a population with properties of HE.²⁵ However, as only erythroid and myeloid potential was analyzed, it is unclear if the observed effect was on HE with definitive hematopoietic potential. The demonstration that regulation of HE by *Sox17* may be mediated by Notch signaling is consistent with the known requirement of this pathway for definitive hematopoietic development¹⁰ and position the requirement for Notch signaling at the specification stage of HE. Previous studies have identified *HoxA3* as a transcriptional

regulator of HE and provided evidence that it functions upstream of *Sox17*⁹. The observation that *HoxA3* expression was not impacted following overexpression or deletion of *Sox17* in our mESCs-derived populations is consistent with this interpretation.

The findings in our study support the emerging body of work demonstrating that SoxF transcription factors are regulators of different stages of embryonic hematopoiesis. At E8.0 *Sox7* and *Sox18* are detected in the developing paired dorsal aortae whereas expression of *Sox17* emerges in the posterior regions of the dorsal aorta at E9-9.5⁷. Studies using mESCs have shown that *Sox7* and *Sox18* play a role in the regulation of primitive hematopoiesis^{12,13}. Our observation that primitive hematopoiesis was not perturbed in *Sox17*^{-/-} cultures suggest that the function of *Sox17* differs from that of *Sox7* and *Sox18*. The differential function of these factors is supported by studies showing that the knockdown of *Sox7* and *Sox18* expression has no impact on the development of HSCs in zebrafish²⁶ suggesting that *Sox17* is the sole SoxF member involved in definitive hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Expression of *Sox17* identifies the emergence of HE and hematopoietic progenitors *in vitro*. **(a)** Schematic representation of differentiation protocol for murine ESCs. **(b)** Left: Representative experiment depicting the expression of Flk-1 and Sox17-mCherry (Sox17-mC) in days (D) 3.25 and 5.25 EBs by flow cytometry. Right: Analysis of D5.25 EBs for co-expression of endothelial (CD31, AA4.1, CXCR4), HE (VEC, Sca-1), hematopoietic (CD41) markers with Sox17-mC **(c)** D5.25 EBs were dissociated and isolated for the following fractions F⁺S⁺, F⁺S⁻ and F⁻S⁻. The cells were cultured for 2 or 4 days as indicated in **(a)** and analyzed for the expression of Sox17-mC, VEC and CD45. Flow cytometry plots of D7 and D9 cells showing the 3 fractions isolated for hematopoietic analyses demonstrate that CD45⁺ only emerge from fractions that acquired expression of

Sox17-mC. **(d)** Hematopoietic progenitor potential of D5.25 sorted fractions depicted in (c) cultured for 4 days. D9 cells were dissociated and plated in methylcellulose cultures and scored for definitive erythroid (D-Ery), macrophage/monocyte (Mac) or granulocyte/erythrocyte/macrophage/megakaryocyte (Mixed) colonies after 8 days in culture. Bars represent standard deviation of the mean of 3 independent experiments; $P=0.003$. **(e)** T lymphoid potential of the D5.25 sorted fractions cultures for 4 days.

D9 cells were dissociated and plated on irradiated OP9-DL1 stromal cells in limiting dilution for 21 days. Positive wells were those that contained more than 500 cells of which more than 5% expressed the T cell signature $CD45^{+}TCR\beta^{+}CD4^{+}/CD8^{+}$. Bars represent standard deviation of the mean of 3 independent experiments $P=0.0001$.

Figure 2.

Sox17 expression marks HE *in vivo* and is required for the generation of long-term repopulating HSCs. **(a)** Representative flow cytometric profiles of AGM regions isolated from E11.5 *Sox17*^{GFP/+} embryos stained with endothelial (Flk-1, CD31, Tie2) HE (VEC, CD34, Sca-1) and hematopoietic (CD41, CD45, CD11b) markers. **(b)** Whole-mount immunofluorescence of an intra-arterial hematopoietic cluster from the dorsal aorta of a 36-39 somite pair *Sox17*^{GFP/+} embryo (20x) following staining with antibodies recognizing GFP (*Sox17*), cKIT and CD31. Scale bars = 50µm. **(c)** Representative flow cytometric profiles of AGM regions isolated from E11.5 *Sox17*^{GFP/+} heterozygote and *Sox17*^{GFP/GFP} null embryos demonstrates the absence of the VEC⁺CD45⁺ population that contains HSCs. **(d)** Proportion of donor-derived hematopoietic cells detected in the peripheral

blood of recipients 4 months following transplantation of the indicated populations. Three embryo equivalents were used for transplantation. Each dot represents an individual transplant recipient. Error bars represent SEM. **(e)** Progeny derived from mating $VEC-Cre^{+}Sox17^{fl/+}$ males with $Sox17^{fl/fl}$ females: conditional deletion of *Sox17* using *VEC-Cre* was lethal by E13.5. **(f)** Compared to control embryos the $VEC-Cre^{+}Sox17^{fl/fl}$ embryos were growth retarded, pale and lacked visible hematopoiesis. **(g)** Representative flow cytometric profiles of AGM regions isolated from $VEC-Cre^{+}Sox17^{fl/+}$ and $VEC-Cre^{+}Sox17^{fl/fl}$ embryos depicting the total decrease in VEC and CD45 staining and the absence of the $VEC^{+}CD45^{+}$ population that contains HSCs.

Figure 3.

Expression of *Sox17* is required for the endothelial to hematopoietic transition and definitive hematopoiesis in mESC differentiation cultures. (a) Flow cytometric analyses showing the proportion of Flk-1⁺ cells in D3.23 and D5.25 EBs generated from Sox17-mC and Sox17^{-/-} ESCs. (b) Primitive erythroid (EryP) and myeloid (Myeloid) progenitor potential of D3.25 Flk-1⁺ cells that were aggregated for 24 hours. There is no significant change in primitive hematopoietic output with the loss of *Sox17* expression. Bars represent standard deviation of the mean of 3 independent experiments. (c) Flow cytometric analyses showing the proportion of VEC⁺, CD45⁺, CD31⁺ and CXCR4⁺ cells in D7 from Sox17-mC and Sox17^{-/-} EBs. To generate D9 cultures the D7 VEC⁺CD45⁻ population was isolated by FACS and reaggregated as depicted in Fig 1a (d) qRT-PCR based analyses

showing expression of indicate genes in D7 and D9 aggregates generated from SOX17-mC and Sox17^{-/-} EBs. Values shown are relative to *Actβ*. For comparison of the 2 populations, the values for the expression levels in the Sox17-mC cells are set to 1.

Bars represent standard deviation of the mean of 3 independent experiments. D7: *Sox7* $P=0.01$. D9: *Sox7* $P=0.003$, *Sox18* $P=0.014$, *EphrinB2* $P=0.0004$, *Notch1* $P=0.01$, *Jagged1* $P=0.003$. (e) Hematopoietic progenitor potential of the Sox17-mC and

Sox17^{-/-} derived D9 aggregates. Bars represent standard deviation of the mean of 3 independent experiments. For mixed colonies $P=0.001$. (f) T-lymphoid progenitors measured on OP9-DL1 stromal cells. Bars represent standard deviation of the mean of 3 independent experiments. $P=0.0001$.

Figure 4.

Effects of enforced *Sox17* expression on HE and hematopoietic development. **(a)** Flow cytometric analyses showing the proportion of CD31⁺CXCR4⁺ and VEC⁺ CD45⁺ cells in D5.25 Flk-1⁺ derived monolayers cultured for 2 days in the presence (+dox) or absence (-dox) of 1µg/ml doxycycline. Far right: Flow cytometric analysis showing the proportion of VEC⁺ and CD45⁺ cells in aggregates generated from the D7 induced and non-induced VEC⁺CD45⁻ population cultured for 2 days without doxycycline in the presence of hematopoietic cytokines. **(b)** Myeloid/erythroid and (top) and T-lymphoid (bottom) progenitor potential of the D7 induced and non-induced monolayers. Bars represent standard deviation of the mean of 3 independent experiments, for myeloid/erythroid $P=0.003$ and for T lymphoid $P=0.0006$ **(c)** Myeloid/erythroid and (top) and T-lymphoid

(bottom) progenitor potential of aggregates generated from VEC⁺CD45⁻ cells isolated by FACS from the day 7 induced and non-induced monolayer populations. Cells were cultured as aggregates for 2 days (D9) in the absence of doxycycline prior to analyses. Bars represent standard deviation of the mean of 3 independent experiments, For T lymphoid progenitors $P=0.0004$ (d) qRT-PCR analyses of indicated genes in D7 induced and non-induced monolayer and in D9 aggregates generated from them, VEC⁺ cells isolated from the AGM of an E11.5 wildtype embryo is shown as a control. Values shown are relative to *Actβ*. Bars represent standard deviation of the mean of 3 independent experiments *Aml1c* $P=0.012$, *Notch1* $P=0.03$, *Jagged1* $P=0.038$, *EphrinB2* $P=0.006$, *COUP-TFII* $P=0.006$.

Figure 5.

The effects of enforced Sox17 expression are mediated through Notch signaling. **(a)** Flow cytometric analyses of D5.25 Flk-1⁺ derived monolayer cultures for 2 days (D7) with (+dox) or without (-dox) 1 μ g/ml doxycycline in the presence or absence of γ -secretase inhibitor (γ SI, L-685458; 10 μ M, Tocris). Cells cultured in the vehicle DMSO represent the control. **(b)** qRT-PCR-based analysis of *EphrinB2* and *COUP-TFII* expression in the different D7 populations described in (a). Values shown are relative to *Actb*. Bars represent standard deviation of the mean of 3 independent experiments, *COUP-TFII* $P=0.002$, *EphrinB2* $P=0.003$. **(c)** Flow cytometric analyses showing the proportion of VEC⁺ and CD45⁺ in aggregates generated from VEC⁺CD45⁻ cells isolated by FACS from the different D7 monolayer populations indicated in (a). The sorted cells were cultured for 2 days

as aggregates in the absence of doxycycline and γ -secretase inhibitor. **(d)** T-lymphoid progenitor potential in the D9 aggregates generated from the D7 monolayer populations grown under the indicated conditions. Bars represent standard deviation of the mean of 3 independent experiments, $P=0.0008$ **(e)** Schematic diagram depicting the wildtype and mutated version of the BS1 and BS2 Sox17 binding sites in the *Notch1* promoter. Numbers indicate the position relative to the transcriptional start site. **(f)** Luciferase assays using U2OS cells co-transfected with pGL3 control vector or the indicated Notch1 promoter constructs and increasing concentrations of the pCAG expression plasmids. Bars represent mean luciferase intensity relative to pGL3-empty $\pm$ s.d., $n=3$, $P=0.001$.