FOXF1 inhibits hematopoietic lineage commitment during early mesoderm specification

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ABSTRACT

The molecular mechanisms orchestrating early mesoderm specification are still poorly understood. In particular, how alternate cell fate decisions are regulated in nascent mesoderm remains mostly unknown. In the present study, we investigated both in vitro in differentiating embryonic stem cells, and in vivo in gastrulating embryos, the lineage specification of early mesodermal precursors expressing or not the Forkhead transcription factor FOXF1. Our data revealed that FOXF1-expressing mesoderm is derived from FLK1⁺ progenitors and that in vitro this transcription factor is expressed in smooth muscle and transiently in endothelial lineages, but not in hematopoietic cells. In gastrulating embryos, FOXF1 marks most extra-embryonic mesoderm derivatives including the chorion, the allantois, the amnion and a subset of endothelial cells. Similarly to the in vitro situation, FOXF1 expression is excluded from the blood islands and blood cells. Further analysis revealed an inverse correlation between hematopoietic potential and FOXF1 expression in vivo with increased commitment toward primitive erythropoiesis in Foxf1-deficient embryos, whereas FOXF1-enforced expression in vitro was shown to repress hematopoiesis. Altogether, our data establish that during gastrulation, FOXF1 marks all posterior primitive streak extra-embryonic mesoderm derivatives with the remarkable exception of the blood lineage. Our study further suggests that this transcription factor is implicated in actively restraining the specification of mesodermal progenitors to hematopoiesis.

KEY WORDS: Gastrulation, Hemangioblast, Mesoderm

INTRODUCTION

At the early stages of embryonic development, the multipotent epiblast gives rise to the three germ layers which are subsequently specified to form all tissues of the developing organism (Tam and Loebel, 2007). The initiation of germ layer formation occurs through a process known as gastrulation in which cells from the epiblast undergo an epithelial to mesenchymal transition and ingress through the primitive streak to generate mesoderm and endoderm (Tam and Behringer, 1997). Cells exiting the posterior part of the primitive streak give rise to the extra-embryonic and lateral plate mesoderm. In gastrulating embryos, the extra-embryonic mesoderm contributes to the formation of the yolk sac blood islands (Palis et al., 1995), the allantois, the amnion, the chorion and the mesothelium lining of the exocoelomic cavity (Tam et al., 1997). Fusion of the allantois and chorion later establishes the connection between the placenta and the embryo, forming the umbilical cord (Downs, 1998). Endothelial and smooth muscle lineages forming the vasculature and both the endocardium and myocardium of the heart are also derived from the mesoderm emerging from the posterior primitive streak (Kinder et al., 2001, 1999). Some of these early events of embryonic development can be recapitulated in vitro upon the differentiation of pluripotent embryonic stem cells (ESCs), providing an alternative model system to the in vivo settings for the study of cell fate specification during development (Keller, 2005; Lacaud et al., 2004). The molecular mechanisms controlling the specification of mesoderm are starting to be unraveled but the overall transcriptional network orchestrating this process is still poorly understood. In particular, how alternate cell fates are regulated in nascent mesoderm is mostly unknown.

The specification of blood and endothelial lineages is tightly associated, depending on similar signaling pathways and transcription factors for their emergence (Costa et al., 2012a; Moignard et al., 2013). Soon after gastrulation, the hemangioblast, found within the primitive streak, gives rise to blood precursors through a specific population of endothelial cells with hemogenic properties, i.e. the hemogenic endothelium (Eilkem et al., 2009; Huber et al., 2004; Lancrin et al., 2009b). Whereas it is clear that not all endothelial cells are derived from hemangioblast precursors, it is still not understood how a specific subset of mesoderm becomes committed to the hematopoietic fate. The FLK1-VEGF signaling axis and the ETS transcription factor ETV2 have been shown to be essential very early in mesoderm for the generation of both endothelial and hematopoietic lineages (Kataoka et al., 2011; Lee et al., 2008; Shalaby et al., 1995; Wareing et al., 2012b). However, both FLK1 (KDR – Mouse Genome Informatics) and ETV2 are widely expressed in nascent mesoderm and are unlikely to be responsible for restricting mesoderm specification to blood and/or endothelium fate. Directly regulated by ETV2, the bHLH transcription factor SCL (TAL1 – Mouse Genome Informatics) is required for both primitive and definitive hematopoiesis through its control of hemogenic endothelium formation (Lancrin et al., 2009b; Porcher et al., 1996; Wareing et al., 2012b). Specification to the cardiac tissues was shown to depend on a distinct set of transcriptional regulators. The heart is composed of several lineages, including cardiomyocytes, endothelial and smooth muscle cells and is formed by two sources of progenitors derived from the first heart field formed by the cardiac crescent and the second heart fields derived from pharyngeal mesoderm (Olson, 2006). The transcription factor MESP1, a master regulator of cardiac development, is expressed in the primitive streak, and is the earliest marker of cardiogenesis (Saga et al., 1996, 2000). Upon enforced expression, MESP1 promotes the generation of all cardiac lineages, controlling the downstream cascade of transcriptional
Foxf1::venus ESCs and differentiated embryoid bodies (EBs) cells showed no significant alteration in hematopoietic output and expression of a panel of selected genes when compared to wild-type ESCs (supplementary material Figs S1C and S2). EB cells were next analyzed by flow cytometry for the presence of FLK1 and Foxf1::VENUS expression. Upon differentiation, Foxf1::VENUS was first detected within the mesodermal FLK1+ population (Fig. 1B), and within 12 h of culture a FLK1−FOXF1::VENUS+ population appeared and was maintained upon further differentiation (supplementary material Fig. S3, upper panels). To delineate how FOXF1::VENUS+ cells were generated, three subpopulations were sorted according to their relative expression of FLK1 and Foxf1::VENUS then reaggregated and further cultured for up to 48 h (Fig. 1C). The reaggregated cells were then analyzed by flow cytometry at different times during the culture. The sorted FLK1+ FOXF1::VENUS+ cells almost exclusively retained their expression of FOXF1::VENUS during the culture, while progressively downregulating FLK1 expression (Fig. 1C). A large fraction of FLK1+ FOXF1::VENUS− cells quickly gave rise to FOXF1::VENUS+ cells which then downregulated Foxf1::VENUS expression, hence resulting in a phenotypic subpopulation similar to that obtained from the FLK1+ FOXF1::VENUS−. Finally, the cell population negative for both markers first upregulated FLK1 expression followed by the upregulation of FOXF1::VENUS within the FLK1-expressing cells. Together these data suggest an in vitro progression in which mesoderm cells first express FLK1 and then upregulate FOXF1 expression.

We next assessed the expression of FOXF1::VENUS upon further commitment of the FLK1+ population toward the hematopoietic and endothelial lineages. To this end, sorted FLK1+ mesodermal cells were further differentiated for 4 days in VEGF-containing hemangioblast culture conditions which promote the differentiation to endothelium and hematopoiesis (Fig. 1D). Although FOXF1::VENUS− cells were detected from day 1 of the culture and remained present throughout the culture period, the vast majority of these cells did not express the hematopoietic marker CD41 (integrin α2b – Mouse Genome Informatics) (Ferkowicz et al., 2003; Mikkola et al., 2003) or the endothelial marker CD144 (cadherin 5 – Mouse Genome Informatics) (Fig. 1D; gating controls supplementary material Fig. S4A). Similar results were observed for CD41 expression when FOXF1::VENUS expression was followed in differentiating EBs for up to day 7 (supplementary material Fig. S3, lower panels). Altogether, these data reveal that, upon in vitro differentiation FOXF1::VENUS− cells are generated from the FLK1+ mesodermal population and that this transcription factor is not expressed in hematopoietic cells. Surprisingly, FOXF1::VENUS-expressing cells represent a large fraction of the differentiated cells within the hemangioblast culture or whole EB culture.

**RESULTS**

**FOXF1 expression is initiated within the early FLK1+ mesoderm population during in vitro differentiation**

To track the expression of FOXF1 at the earliest stage of mesoderm commitment, we generated an ESC line carrying the H2B-VENUS reporter protein under the control of Foxf1 regulatory sequences (hereafter referred to as FOXF1::VENUS), through knocking in a reporter cassette in the first exon of the Foxf1 allele (Fig. 1A; supplementary material Fig. S1A,B). This ESC line allowed monitoring the emergence of Foxf1 expression in conjunction with cell surface markers upon in vitro differentiation to hematopoietic, endothelial and smooth muscle lineages. The analysis of Foxf1::VENUS ESCs and differentiated embryoid bodies (EBs) cells showed no significant alteration in hematopoietic output and expression of a panel of selected genes when compared to wild-type ESCs (supplementary material Figs S1C and S2). EB cells were next analyzed by flow cytometry for the presence of FLK1 and Foxf1::VENUS expression. Upon differentiation, Foxf1::VENUS was first detected within the mesodermal FLK1+ population (Fig. 1B), and within 12 h of culture a FLK1−FOXF1::VENUS+ population appeared and was maintained upon further differentiation (supplementary material Fig. S3, upper panels). To delineate how FOXF1::VENUS+ cells were generated, three subpopulations were sorted according to their relative expression of FLK1 and Foxf1::VENUS then reaggregated and further cultured for up to 48 h (Fig. 1C). The reaggregated cells were then analyzed by flow cytometry at different times during the culture. The sorted FLK1+ FOXF1::VENUS+ cells almost exclusively retained their expression of FOXF1::VENUS during the culture, while progressively downregulating FLK1 expression (Fig. 1C). A large fraction of FLK1+ FOXF1::VENUS− cells quickly gave rise to FOXF1::VENUS+ cells which then downregulated Foxf1::VENUS expression, hence resulting in a phenotypic subpopulation similar to that obtained from the FLK1+ FOXF1::VENUS−. Finally, the cell population negative for both markers first upregulated FLK1 expression followed by the upregulation of FOXF1::VENUS within the FLK1-expressing cells. Together these data suggest an in vitro progression in which mesoderm cells first express FLK1 and then upregulate FOXF1 expression.

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**FOXF1 expression defines a non-hemogenic vascular progenitor population within the FLK1+ mesoderm population in vitro**

To explore the biological characteristics of FOXF1-expressing cells, FLK1+ FOXF1::VENUS− and FLK1+ FOXF1::VENUS+ populations were sorted from day 2.5 EBs and analyzed for the expression of genes known to be involved in lineage decisions during mesoderm differentiation. As shown in Fig. 2A, whilst as the expected expression of Foxf1 was enriched in the FOXF1::VENUS+ population, the expression of genes involved in hemogenic endothelial specification (Scl, Gata2, Sox7) (Costa et al., 2012b; Pimanda et al., 2007) were enriched in the FOXF1::VENUS− population.
VENUS\(^{-}\) population compared to FOXF1::VENUS\(^{+}\). Similarly, genes involved in cardiac lineage cell fate decision (Nkx2-5 and Mesp1) (Chen and Fishman, 1996; Saga et al., 1999) were more predominantly expressed in the cell population negative for FOXF1 expression. We also assessed the hematopoietic output of these populations through differentiation in hemangioblast culture followed by flow cytometry and clonogenic analysis. Upon differentiation, FLK1\(^{+}\) FOXF1::VENUS\(^{-}\) cells produced hematopoietic cells as demonstrated by the generation of a high frequency of CD41\(^{+}\) cells (Fig. 2B, upper panels; gating controls supplementary material Fig. S4B). This population also produced FOXF1::VENUS\(^{+}\) cells, but those FOXF1-expressing cells were negative for CD41 expression. In contrast, the sorted FLK1\(^{+}\) FOXF1::VENUS\(^{-}\) cells produced very few CD41\(^{+}\) cells and mostly maintained their expression of FOXF1::VENUS upon further differentiation (Fig. 2B, lower panels). To define the clonogenic potential of the cells generated during this culture period, cells were harvested after 2 days of culture and assessed for progenitor potential in semi-solid hematopoietic condition. Whereas cells derived from the FLK1\(^{+}\) FOXF1::VENUS\(^{-}\) culture generated hematopoietic colonies, cells derived from the FLK1\(^{+}\) FOXF1::VENUS\(^{+}\) culture produced very few colonies (Fig. 2C). During the hemangioblast culture, both populations gave rise to CD144\(^{+}\) endothelial cells and \(\alpha\)SMA\(^{+}\) smooth muscle cells (Fig. 2D), although the FLK1\(^{+}\) FOXF1::VENUS\(^{-}\) cells produced a higher frequency of \(\alpha\)SMA\(^{+}\) cells and a lower frequency of CD144\(^{+}\) cells than the FLK1\(^{+}\) FOXF1::VENUS\(^{-}\) population. Taken together, these results reveal that FOXF1 expression marks a non-hemogenic population within the FLK1\(^{+}\) mesodermal population generated during ESC differentiation in vitro. These FOXF1-expressing mesoderm cells display a vascular-restricted differentiation potential, generating both endothelial and smooth muscle cells but no or very few hematopoietic cells upon culture.

To also characterize the population of FOXF1::VENUS\(^{-}\) cells observed at later stage of EB differentiation, co-staining with various cell surface markers was performed at EB day 6 (Fig. 3A). This analysis revealed that most FOXF1::VENUS\(^{-}\) cells had downregulated FLK1 expression by day 6 of EB differentiation whereas CD140a (PDGRF\(\alpha\)) and TIE2 were highly expressed by FOXF1::VENUS\(^{-}\) cells. Gene expression analysis further revealed high levels of both smooth muscle markers Sma (Acta2) and Sm22 [transgelin (Tagln)] in the FOXF1::VENUS\(^{-}\) population whereas hematopoietic genes (Gata1, Gata2) were not expressed in this fraction (Fig. 3B). Finally, upon further culture FOXF1::VENUS\(^{-}\)
cells isolated from day 6 EBs rapidly gave rise to smooth muscle cells as demonstrated by extensive αSMA staining (Fig. 3C). Together, these data suggest that in late differentiating EBs Foxf1 expression marks cells fated to the smooth muscle lineage.

**FOXF1 is expressed in all extra-embryonic tissues in gastrulating embryos except in the blood islands**

To define the expression pattern of FOXF1 during gastrulation and to compare in vitro and in vivo data, the Foxf1::venus ESC line was used to generate a Foxf1::venus transgenic mouse line. At the early stage of embryonic development (primitive streak to headfold stage), FOXF1::VENUS was detected in the extra-embryonic mesoderm and the lateral plate mesoderm of the embryo proper (Fig. 4A) as previously documented using in situ hybridization (Mahlapuu et al., 2001b; Peterson et al., 1997). In particular, FOXF1::VENUS was homogenously expressed in the mesothelium lining the exocoelomic cavity and the amnion as well as in the allantois but FOXF1::VENUS was not detected in the nascent mesoderm of the primitive streak. To define the progressive changes in FOXF1 expression during gastrulation and to correlate this pattern with the in vitro data presented above, sequential stages of gastrulation, as previously defined (Downs and Davies, 1993), were analyzed by flow cytometry for FLK1 and FOXF1::VENUS expression (Fig. 4B and supplementary material Fig. S5A). At the early streak (ES) stage, the first FOXF1::VENUS-expressing cells were detected within the FLK1+ population, similar to the onset of FOXF1 expression during in vitro differentiation. From the mid-streak (MS) stage, FLK1−FOXF1::VENUS+ cells were detected and both FLK1+ FOXF1::VENUS+ and FLK1−FOXF1::VENUS+ populations increased progressively as gastrulation proceeded to late streak (LS) and neural plate (NP) stages. At the NP stage (OB: no allantoic bud, EB: early allantoic bud) and early headfold (EHF) stage, most of the FLK1+ cells co-expressed FOXF1::VENUS, whereas at the later headfold stage (LHF) a clear FLK1+ FOXF1::VENUS+ population was observed. To define the anatomical localization of these distinct populations, neural plate Foxf1::venus embryo sections were stained for FLK1 expression (Fig. 4C). The few double positive cells were found spread throughout the extra-embryonic mesoderm, allantois and embryonic mesoderm whereas most of the FOXF1::VENUS+ negative for FLK1 expression were detected in the mesothelium and allantois (Fig. 4Ci-iii).
Of interest, no cells within the developing blood islands expressed detectable levels of FOXF1::VENUS (Fig. 4Ciii,iv), whereas FLK1+ FOXF1::VENUS+ endothelial cells were detected at the endoderm interface of the blood islands in older embryos (Fig. 4Civ, arrowheads). Flow cytometry analysis showed that a very small frequency of CD41+ cells co-expressed FOXF1::VENUS+ (supplementary material Fig. S5B) raising the possibility that FOXF1 might be expressed in some of the mesoderm precursors giving rise to hematopoiesis, although the bulk of CD41+ cells did not express FOXF1::VENUS. To determine whether Foxf1-expressing mesoderm was under the same molecular control which specifies the mesodermal progenitors giving rise to the hematopoietic and vascular system, we next analyzed FOXF1::VENUS expression in the context of Etv2 deficiency. We and others have previously shown that the ETS transcription factor ET2 is expressed in mesodermal progenitors which give rise to both hematopoietic and vascular system, and that Etv2−/− embryos lack both lineages (Kataoka et al., 2011; Lee et al., 2008; Wareing et al., 2012b).Whilst FLK1+ cells are still detected at the expected frequency in Etv2−/− embryos, the TIE2+ population is drastically reduced (Wareing et al., 2012a); we therefore analyzed the co-expression of FOXF1::VENUS and TIE2 in wild-type, heterozygous or homozygous null Etv2 embryos (Fig. 4D). In ETV2-expressing embryos, both TIE2+ FOXF1::VENUS+ and TIE2− FOXF1::VENUS+ populations were detected. In contrast, in Etv2−/− embryos the TIE2+ FOXF1::VENUS+ population was strongly reduced whereas the TIE2− FOXF1::VENUS+ population was present at the expected frequency. Together these data reveal that most of the FOXF1-expressing mesoderm is specified independently of ETV2 and the hematopoietic and endothelial lineages. However, the small fraction of FOXF1::VENUS+ cells that co-express TIE2 is dependent on ET2, suggesting that some mesoderm progenitors giving rise to the hematopoietic and endothelial lineages do express FOXF1 during their ontogeny. As we observed in vitro that a large fraction of the FOXF1::VENUS+ population co-expressed the smooth muscle marker αSMA.
(Fig. 2D), we assessed *in vivo* the co-expression of FOXF1 and αSMA during gastrulation. At the early headfold stage, αSMA expression was mainly detected in the chorion where it overlapped with FOXF1::VENUS expression (Fig. 5A). At the late headfold and early somite stage, αSMA expression was now also observed in the mesothelium lining the exocoelomic cavity and the prospective heart field (Fig. 5B-D). At these stages, FOXF1::VENUS and αSMA were clearly coexpressed in the mesothelium; a few αSMA+FOXF1::VENUSlow were also observed in the presumptive cardiac mesoderm (Fig. 5C-D).

Overall, we observed a similar pattern of FOXF1 emergence *in vivo and in vitro* with FOXF1::VENUS expression first detected in emerging FLK1+ mesodermal cells followed by a progressive increase in the frequency of FOXF1::VENUS+ cells that do not express FLK1. Most cells of the allantois and all the mesothelium were FLK1−FOXF1::VENUS+. Interestingly, although FOXF1 was not detected within the blood islands giving rise to hematopoiesis, a few CD41+ cells expressed FOXF1::VENUS.

**Increasing Foxf1 expression levels correlate with decreasing hematopoietic potential *in vivo***

To further characterize the FOXF1::VENUS expressing and nonexpressing populations *in vivo*, cells from pooled E7.5 NP stage embryos were sorted according to their relative expression...
of FLK1 and FOXF1::VENUS (Fig. 6A) and processed for transcriptomic analysis. Interestingly, despite VENUS detection by flow cytometry, Foxf1 transcripts in the FLK1+ FOXF1::VENUS+ population were detected by Affymetrix array at the same background level as in the FLK1+ FOXF1::VENUS− population, whereas the Foxf1 transcripts were detected at a high level in the FLK1− FOXF1::VENUS+ population (Fig. 6B). To investigate this discrepancy in Foxf1 transcript levels, qPCR analysis was performed on single cells sorted from primitive streak (PS), neural plate (NP) and headfold (HF) embryos. Although FLK1+ FOXF1::VENUS− cells expressed very low or no detectable Foxf1 transcripts (Fig. 6C), the expression of Foxf1 in FLK1+ FOXF1::VENUS+ cells was highly heterogeneous across the gastrulating stages analyzed. At the PS stage, Foxf1 transcripts were detected at similar levels in FOXF1::VENUS cells expressing or not FLK1 but at the later NP and HF stages, an increasing frequency of FLK1+ FOXF1::VENUS+ cells expressed low or no detectable levels of Foxf1 transcripts (Fig. 6C). In contrast, Foxf1 transcripts remained overall at a higher level in FLK1− FOXF1::VENUS+ cells when compared to FLK1+ FOXF1::VENUS+ cells in both NP and HF stages. Together these data demonstrate that FLK1+ FOXF1::VENUS+ cells are only transiently expressing Foxf1. As expected, single cell analysis is more sensitive than global transcriptomic arrays in detecting small differences and heterogeneity in cell populations.

Fig. 5. Co-expression of FOXF1::VENUS and αSMA in gastrulating Foxf1::venus embryos. Whole mount staining of VENUS (green) and αSMA (red) in embryos ranging from early headfold stage (A), late headfold stage (B,C), to early somite stage (D). Left panel shows overlay of 5 consecutive images. z-stack is 5 µm in total. Scale bars: 100 µm. The right panels show higher magnification of the boxed areas in the left panels. Scale bars: 25 µm. Blue is DAPI counterstaining. Abbreviations: al, allantois; am, amnion; BI, blood island; ch, chorion; h, heart; xc, exocoelomic cavity.
In the transcriptomic analysis, differentially expressed genes with 2-fold or more changes in expression levels between at least two of the sorted populations were clustered and submitted to Gene Ontology annotation to highlight enriched biological functions within each population (Fig. 6D; supplementary material Table S1). This analysis identified three main clusters of differentially
expressed genes: cluster i containing genes with increased expression in FLK1+ FOXF1:VENUS<sup>+</sup> cells, cluster ii containing genes with increased expression in FLK1<sup>+</sup> FOXF1:VENUS<sup>+</sup> and FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> cells and cluster iii containing genes with increased expression in FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> cells. However, the Gene Ontology analysis revealed important overlap within the biological functions attributed to each of these clusters (Fig. 6D). Most notably, genes found in clusters i and ii were enriched for ‘hematopoiesis’ function whereas genes found in all three clusters were enriched for ‘development of blood vessel’ function. Results of this analysis highlighted the close relationship and common mesodermal origin between the populations analyzed. Nevertheless, a close inspection of specific genes differentially expressed within each cluster revealed important insights into the molecular identity of these three embryonic populations. Cluster i, which contained genes with the highest expression in the FLK1<sup>+</sup> FOXF1:VENUS<sup>+</sup> population, included many genes directly implicated in erythropoiesis (Fig. 6Ei) such as Hbb-bh1, Hbb-y, Hbb-b2, Klf1, Gata1, Nfe2, all expressed in primitive erythroid cells, the first blood cells to be specified from the mesoderm (Kingsley et al., 2013; Palis, 2008). In contrast, cluster ii genes, enriched in both FLK1<sup>+</sup> populations, comprised many genes encoding known regulators of endothelial and hematopoietic development (Fig. 6Eii) such as Egfl7, Etv2, Fil1, Foxc2, Gata2, Hhex or Sox17 (De Val et al., 2008; Kubo et al., 2005; Pimanda et al., 2007; Waring et al., 2012a), suggesting a common hematopoietic and endothelial potential in these two populations. Finally, genes found in cluster iii, for which expression was highest in the FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> population, included many Hox genes (Fig. 6Eiii), suggesting that this population contained mesoderm fated to other developmental pathways as suggested by the Gene Ontology analysis. This cluster was also enriched for genes related to muscle and contractile function (Acta1, Smc, Cnn2, Myl9, Tagln, Tnn1, Tnni2), most likely linked to the αSMA-expressing mesothelial subset of the cells within this population.

To evaluate the hematopoietic and endothelial potential of these three populations, sorted cells from E7.5 embryos (NP stage) were cultured on OP9 stroma to allow differentiation toward these two lineages. Flow cytometry analysis revealed that to some extent all sorted populations could generate CD41<sup>+</sup> cells during the culture, although only the FLK1<sup>+</sup> FOXF1:VENUS<sup>+</sup> cells displayed robust hematopoiesis producing high frequencies of CD41<sup>+</sup>/CD45<sup>+</sup> cells. The FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> cells could produce some CD41<sup>+</sup> CD45<sup>+</sup> cells and FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> cells produced very limited frequencies of CD41<sup>+</sup> cells only (Fig. 6F, upper panel). This differential hematopoietic potential was further confirmed using clonogenic assay which showed that hematopoietic progenitors were enriched in the FLK1<sup>+</sup> FOXF1:VENUS<sup>+</sup>-derived cells (mean±s.e.m.=23.4±9.2 colonies per cultured equivalent embryo) compared with the FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> cells (7.1±3.5), whereas the FLK1<sup>+</sup> FOXF1::VENUS<sup>+</sup> population was devoid of hematopoietic progenitors (0.2±0.1) (Fig. 6G). No significant differences in lineage output were observed between the two FLK1<sup>+</sup> populations (Fig. 6H). Similarly, all populations displayed endothelial potential as shown by CD144 staining (Fig. 6F, lower panel), although the frequency of CD144<sup>+</sup> cells generated was inversely correlated with the expression of FOXF1:VENUS.

Altogether, these data revealed that at this early stage of embryonic development the relative expression of FLK1 and FOXF1 delineates specific subsets of mesoderm cells. The absence of FOXF1 expression in FLK1<sup>+</sup> cells marks a population of cells highly enriched for both endothelium and hematopoietic potential and also contains the population of mesodermal progenitors already committed to primitive erythropoiesis. The transient expression of FOXF1 marks a population of FLK1<sup>+</sup> cells that contain both endothelium and hematopoietic potential but it is not clear if and how they differ from the FLK1<sup>+</sup> FOXF1:VENUS<sup>+</sup> population. Finally, the FOXF1<sup>+</sup> population that does not express FLK1 is enriched for mesothelial cells and mesodermal progenitors fated to other developmental pathways.

**FOXF1 expression impairs the hematopoietic potential of mesodermal precursors**

To establish whether FOXF1 might participate in lineage choices during mesoderm differentiation, we generated an ESC line in which FOXF1 linked to the GFP reporter protein via a 2A peptide can be expressed upon doxycycline addition using a Tet-on inducible system (thereafter referred to as iFoxf1), as previously described (Gandillet et al., 2009; Kyba et al., 2002). Upon EB differentiation, FLK1<sup>+</sup> mesodermal cells were sorted and cultured in hemangioblast condition to allow differentiation to hematopoietic, endothelial and smooth muscle lineages (Stefanska et al., 2014). The doxycycline-mediated induction of FOXF1 expression from day 0 or day 1 of the culture dramatically decreased the hematopoietic output as shown by flow cytometry analysis of CD41 and CD45 expression (Fig. 7A, upper panel), clonogenic repainting assay (Fig. 7B) and the absence of morphogen endothelial clusters giving rise to hematopoietic cells (supplementary material Fig. S6A) as previously shown in this culture system (Lancrn et al., 2009b). Interestingly, this block in hematopoietic differentiation was irreversible as removal of doxycycline even after only 12 h of induction was unable to restore the generation of hematopoietic cells (Fig. 7A, lower panel). Similar results were also obtained when the induction of FOXF1 expression was performed during EB culture with doxycycline added at day 3, 4 or 5 (supplementary material Fig. S6B). Induction of FOXF1 expression from day 0 of the culture also impaired endothelial differentiation as shown by a decrease in the frequency of CD144<sup>+</sup> cells produced in the culture (Fig. 7C, upper panel, Dox d0-3). In contrast, an increase in the frequency of αSMA<sup>+</sup> cells was observed (Fig. 7C lower panel). When FOXF1 expression was induced at day 1 of the culture (Dox, d1-3), the endothelial potential was similar when compared to the non-induced culture condition, but the frequency of αSMA<sup>+</sup> cells remained high. These results suggest that the induction of FOXF1 expression in mesodermal progenitors impairs hematopoietic differentiation potential, whereas vascular differentiation potential toward endothelial and smooth muscle lineages is maintained or enhanced.

Given the profound and irreversible block in hematopoiesis induced by FOXF1, we next investigated whether FOXF1 acts in a cell-autonomous manner to shut down hematopoiesis or through the production of soluble factors, as FOXF1 has been shown in vivo to control BMP4 transcription (Astorga and Carlsson, 2007). For this purpose, FLK1<sup>+</sup> Tomato-labeled cells (iTomato cells) were differentiated together with FLK1<sup>+</sup> iFoxf1 cells in hemangioblast culture condition (Fig. 7D). Upon addition of doxycycline, iTOMATO<sup>+</sup> cells were able to differentiate toward hematopoiesis at similar frequencies in the single or co-culture (Fig. 7E,F) whereas GFP<sup>+</sup> (iFoxf1) cells were not able to generate CD41<sup>+</sup> nor CD45<sup>+</sup> cells, supporting a cell-autonomous block of hematopoietic potential upon the induction of FOXF1 expression. Because FOXF1 appeared to inhibit hematopoietic specification, embryos deficient for FOXF1 expression might harbor increased frequency of mesoderm progenitors committed to hematopoiesis. To test this
hypothesis, embryos from Foxf1::venus mice intercrosses were replated in clonogenic assays. Neural plate stage was chosen for this analysis as this stage marks the onset of primitive hematopoiesis and developmental defects are not yet observed in Foxf1 null embryos.

Although wild-type and heterozygous Foxf1::venus embryos had similar frequencies of primitive erythroid progenitors, Foxf1::venus homozygous null embryos had on average 6-fold more primitive erythroid progenitors (Fig. 7G). Altogether, these data suggest that during gastrulation, mesoderm specification toward hematopoiesis is negatively regulated by FOXF1.

**DISCUSSION**

The use of a knock-in VENUS reporter system allowed us for the first time to track, isolate and analyze mesodermal progenitors and their derivatives expressing the transcription factor FOXF1. Both in differentiating ESCs and in gastrulating embryos, we observed that FLK1+ mesodermal cells emerge prior to FOXF1-expressing cells whilst later a population of FOXF1+ FLK1− was observed and became prominent over time. Additionally, in vitro sorted FLK1+ mesoderm gave rise to FOXF1-expressing cells that progressively lost FLK1 expression. Consistent with these findings, cell tracing experiments using a Flk1-LacZ reporter allele have shown that all extra-embryonic mesoderm was derived from FLK1-expressing cells (Ema et al., 2006). Altogether, these findings allow us to further dissect and understand the sequential commitment steps taking place during early mesoderm specification (Fig. 8). One of the most striking and surprising observations of our study is that of all the extra-embryonic mesoderm derivatives, hematopoiesis...
is the only one that does not express FOXF1. During the \textit{in vitro} differentiation of ESCs, we observed a clear dichotomy between the expression of FOXF1 and CD41; this mutually exclusive pattern of expression was observed in FLK1\textsuperscript{+} sorted cells differentiated in hemangioblast culture as well as in differentiating EB culture over time. Furthermore, when FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} cells were sorted and further cultured, they gave rise to very few CD41\textsuperscript{+} hematopoietic cells that did not express FOXF1::VENUS. In gastrulating embryos, we observed a similar trend, blood islands were devoid of FOXF1::VENUS expression and by flow cytometry the vast majority of CD41\textsuperscript{+} cells did not express FOXF1::VENUS. However, we did observe \textit{in vivo} a few CD41\textsuperscript{+} cells expressing FOXF1::VENUS and the FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} population gave rise to low hematopoietic output when cultured \textit{ex vivo}. In line with this observation, analysis of FOXF1::VENUS expression in \textit{Env2}-deficient embryos revealed that a subset of FOXF1::VENUS\textsuperscript{+} mesodermal progenitors co-expressing TIE2 was under the control of ETV2 expression, a master regulator of blood and endothelium specification (Kataoka et al., 2011; Lee et al., 2008; Wareing et al., 2012b). Finally, global transcriptomic analysis revealed the expression of many transcriptional regulators of hematopoietic and/or endothelial fate including \textit{Env2} in the FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} population. Given that the allantois is the main mesodermal tissue where both FOXF1 and ETV2 are expressed at the E7.5 stage of development (Kataoka et al., 2011; Wareing et al., 2012a), these FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} ETV2\textsuperscript{+} cells are most likely localized to this mesodermal derivative which has been shown previously to give rise to hematopoiesis autonomously (Zeigler et al., 2006). Altogether, these findings reveal that in gastrulating embryos a subset of FOXF1-expressing cells can give rise to hematopoietic cells. Interestingly, this was not observed \textit{in vitro} in differentiating \textit{Foxf1}::\textit{venus} ESCs and could be due to the culture conditions not being permissive for the generation of all mesodermal subsets.

As \textit{in vitro} FOXF1 expression and hematopoiesis appeared mutually exclusive, we investigated the outcome of FOXF1-enforced expression during ESC differentiation and observed that the generation of hematopoietic cells was inhibited by FOXF1 expression. Furthermore, short-term FOXF1 exposure was sufficient to block blood cell production and even previously specified CD41\textsuperscript{+} cells were lost upon FOXF1-enforced expression. These findings seem in opposition with the detection of a FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} population generating hematopoiesis in gastrulating embryos. However, at the time of isolation, these FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} cells expressed highly heterogeneous levels of \textit{Foxf1} transcripts, with many cells no longer expressing \textit{Foxf1} transcript. This suggests that this cell population did express \textit{Foxf1} earlier during ontogeny but that \textit{Foxf1} transcription had already been shut down at the time of isolation. Most likely, VENUS was still detected in these cells due to differential stability or half-life of this protein. One most likely interpretation is that FLK1\textsuperscript{+} FOXF1::VENUS\textsuperscript{+} progenitors are able to generate blood cells because FOXF1 is no longer expressed. The fact that FOXF1 blocks hematopoietic specification could explain the previously published observation of ectopic hematopoiesis in the amnion of \textit{Foxf1}-deficient embryos in which lack of FOXF1 expression resulted in the inappropriate specification of mesoderm progenitors to blood derivatives (Mahlapuu et al., 2001b). This is in line with our observation that \textit{Foxf1}-deficient embryos have a higher frequency of primitive erythroid progenitors than wild-type embryos.

\textit{In vitro}, we observe that FOXF1::VENUS is expressed by a high frequency of cells in hemangioblast culture and differentiating EBs; by day 6 of differentiation EB cells either express CD41 and are hence committed to hematopoiesis or they express FOXF1 and might represent mesenchyme or mesothelium progenitors which rapidly give rise to smooth muscle upon further culture. It is interesting to note that in the adult organism, the mesothelium retains remarkable progenitor characteristics with the ability to differentiate into myofibroblast, smooth muscle and endothelium upon injury or specific signals in organs such as lung (Que et al., 2008), heart (Zhou et al., 2008) or liver (Li et al., 2013; Rinkevich et al., 2012). In line with these observations, FOXF1 has been shown to be implicated later in life in the regulation of visceral smooth muscle cells (Hoggatt et al., 2013) and the formation of embryonic vasculature (Ren et al., 2014). The \textit{Foxf1}::\textit{venus} ESC line will be helpful in future studies to dissect how \textit{Foxf1} expression is regulated during mesoderm specification. It was shown \textit{in vivo} that Hedgehog signaling from the extra-embryonic endoderm is responsible for \textit{Foxf1} activation in the yolk sac mesoderm but not in the allantois (Astorga and Carlsson, 2007); it will be interesting to define \textit{in vitro} whether hedgehog inactivation prevents \textit{Foxf1} transcription in all or only a subset of mesodermal cells, possibly defining different types of mesoderm derivatives \textit{in vitro}. During embryonic development, cell fate specification is orchestrated by tightly regulated molecular and cellular processes. Understanding how these processes occur \textit{in vivo} is important from a fundamental perspective but it is also crucial in helping us devise optimal protocols for the \textit{in vitro} derivation of specific lineages from ESC to be used in the clinic for therapeutic applications.
MATERIALS AND METHODS
ESC and mouse lines
An inducible iFoxf1-2aGFP ESC line was established as previously described (Gandillet et al., 2009; Kyba et al., 2002). Briefly, a plasmid containing Foxf1 cDNA fused to 2a-GFP was inserted into the modified HPRT locus of Ainv18 ESC by Cre-mediated recombination. ESC clones with a restored functional Neomycin gene were selected using G418. The same protocol was used to establish an iTomato ESC line, with introduction of Tomato cDNA in the modified HPRT locus. For generation of the Foxf1::venus ESC line, homology arms were amplified with Phusion DNA Polymerase (NEB) and cloned into a vector containing a H2B-venus reporter gene and an FRT-flanked PGK/EM7-NeoR cassette. Ainv18 mouse ES cells were electroporated with linearized targeting vector and selected using G418. Correctly targeted clones were identified by PCR, confirmed by Southern blot analysis, and in FRT-PGK-Neo/R-FRT cassette was removed by transiently expressed flippase recombinase. To generate a corresponding mouse line, Foxf1::venus ESCs were injected into blastocysts and animals in subsequent generations were backcrossed with C57BL/6 mice. Double transgenic Foxf1::venus Foxf1::venus mice were obtained by mating Foxf1::venus and En^2^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^


