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Etv2 rescues *Flk1* mutant embryoid bodies

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Abstract

Independent mouse knockouts of *Etv2* and *Flk1* are embryonic lethal and lack hematopoietic and endothelial lineages. We previously reported that *Flk1* activates *Etv2* in the initiation of hematopoiesis and vasculogenesis. However, *Flk1* and its ligand *VEGF* are expressed throughout development, from E7.0 to adulthood, whereas *Etv2* is expressed only transiently during embryogenesis. These observations suggest a complex regulatory interaction between *Flk1* and *Etv2*. To further examine the *Flk1* and *Etv2* regulatory interaction, we transduced *Etv2* and *Flk1* mutant ES cells with viral integrants that inducibly overexpress *Flk1* or *Etv2*. We demonstrated that forced expression of *Etv2* rescued the hematopoietic and endothelial potential of differentiating *Flk1* and *Etv2* mutant cells. We further discovered that forced expression of *Flk1* can rescue that of the *Flk1*, but not *Etv2* mutant cells. Therefore, we conclude that the requirement for *Flk1* can be bypassed by expressing *Etv2*, supporting the notion that disruption of *Etv2* expression is responsible for the early phenotypes of the *Etv2* and *Flk1* mutant embryos.

Keywords

vasculogenesis; hematopoiesis; development

Introduction

Flk1 is the primary receptor for Vascular Endothelial Growth Factor (VEGF). *Flk1* mutant embryos maintain *Flk1* reporter expression as demonstrated in LacZ knockin/knockout embryos, but fail to develop hematopoietic or endothelial lineages (Shalaby *et al.*, 1995). *Flk1* mutant embryonic stem cells (ESCs) that are differentiated on a stromal cell layer using OP9 co-culture techniques do not yield hematopoietic cells, although Brachyury positive mesoderm clusters form under these conditions (Hidaka *et al.*, 1999). Surprisingly, however, *Flk1* mutant ESCs can develop hematopoietic and endothelial lineages, albeit at a reduced level, when induced to differentiate using embryoid body (EB) formation (Hidaka *et al.*, 1999). Previous studies have revealed that hematopoietic progenitors are present in the E7.5 *Flk1* mutant embryos and that *Flk1* deficient cells cannot migrate from the posterior primitive streak to the yolk sac (Schuh *et al.*, 1999). These results support the notion that *Flk1*⁺ hematopoietic and endothelial progenitors encounter inductive signals that allow

further differentiation as the progenitors migrate into the yolk sac (Hidaka *et al.*, 1999). While there is a three dimensional component in EB differentiation, structures such as the primitive streak and yolk sac are not present and therefore migration may not be necessary to receive these inductive cues in the context of EB differentiation.

Mouse knockouts of *Etv2* and *Flk1* result in similar embryonic lethal phenotypes, which lack hematopoietic and endothelial lineages (Ferdous *et al.*, 2009; Koyano-Nakagawa *et al.*, 2012; Lee *et al.*, 2008; Shalaby *et al.*, 1995). Previous discrepant observations regarding the genetic hierarchy of *Etv2* and *Flk1* can be explained with a model in which *Etv2* and *Flk1* reciprocally regulate each other dependent on the developmental stages. At early stages of mesodermal induction and endothelial specification, *Flk1* signaling is required for *Etv2* expression, as *Flk1* mutant embryos lack *Etv2* expression (Rasmussen *et al.*, 2012). *Flk1* is expressed in the *Etv2* mutant embryos and in *Etv2* mutant ESCs after 4 days of differentiation with OP9 coculture techniques, indicating that *Flk1* is necessary for *Etv2* expression but not *vice versa*, at these developmental stages (Kataoka *et al.*, 2011; Rasmussen *et al.*, 2012). Further evidence that *Flk1*/VEGF signaling can activate the *Etv2* promoter demonstrates that this signaling cascade is operational (Rasmussen *et al.*, 2012). In contrast, after completion of gastrulation and specification of the endothelial lineage, *Etv2* may reinforce *Flk1* expression. Transcriptional assays and promoter analysis provide evidence that *Etv2* binds and activates the *Flk1* promoter (De Val *et al.*, 2008; Lee *et al.*, 2008). This notion is further supported as there is decreased expression of *Flk1* in *Etv2* mutant embryos (Ferdous *et al.*, 2009; Kataoka *et al.*, 2011; Lee *et al.*, 2008; Rasmussen *et al.*, 2011; Rasmussen *et al.*, 2012). However, these reports vary widely as to the degree of this effect, suggesting that while *Etv2* may reinforce *Flk1* expression, this could be a minor effect or could occur in a tissue or temporal specific pattern.

Although ample evidence demonstrates that *Flk1* is upstream of *Etv2* at the onset of vasculogenesis and hematopoiesis (see above), the expression patterns of these genes are significantly different; *Etv2* is expressed transiently during development whereas *Flk1* and its ligand VEGF are expressed throughout development, from E7.0 into adulthood (Dumont *et al.*, 1995). Thus, it is possible that the *Flk1* signal activates targets other than *Etv2* to initiate vascular and hematopoietic development. To definitively address whether *Etv2* is the *Flk1* target necessary for hematopoietic and endothelial differentiation, we undertook a rescue strategy of the *Flk1* and *Etv2* mutant EB phenotypes with forced expression of *Flk1* or *Etv2*. We demonstrated using the ES/EB system that *Etv2* could rescue the *Flk1* mutant phenotype, but *Flk1* could not rescue the *Etv2* mutant phenotype, further supporting the notion that the *Flk1*-*Etv2* cascade is operational during hemato-endothelial development.

Results

In order to perform the rescue experiments, we chose to use *Etv2* mutant ES cells derived in our laboratory and *Flk1* mutant ES cells, generously provided by the Stanford laboratory (Hidaka *et al.*, 1999). We initially characterized these cells by analyzing transcript levels throughout differentiation. A wildtype 2.1 line that was derived from a littermate blastocyst of the *Etv2* mutant ESCs and a wildtype R1 line, the line genetically engineered to generate the *Flk1* mutant were used as controls. We differentiated the wildtype 2.1 ESCs, the *Etv2*

mutant ESCs, the R1 ESCs, and the *Flk1* mutant ESCs side by side using a hanging-drop method and harvested cells every 24 hours from embryoid body day (EB D) 2 through EB D6. *Etv2* expression peaks at EB D3 in the 2.1 EBs and is absent in the *Etv2* mutant EBs (Fig. S1 A). *Flk1* expression is minimally affected or unaffected in the *Etv2* mutant cell line (Fig. S1B), which supports our previous observation that *Etv2* plays a minor, if any, role upstream of *Flk1* signaling. Brachyury (Bry) was used as a marker of early mesoderm and *Fgf5* was used as a marker of embryonic ectoderm and the epiblast state (Leahy *et al.*, 1999). Both of these undifferentiated germ layer markers are increased in the *Etv2* mutant compared to wildtype cell lines (Fig S1 C-D). This may be due to impaired differentiation and therefore, more cells may be committed to the germ layers, but not fully differentiated. Finally, we observed that in the *Etv2* mutant cells, markers of the endothelial and hematopoietic lineages are absent, such as *Lmo2* (Fig. S1E), *Scl* (Fig. S1F), *Gata1* (Fig. S1G), and Embryonic Globin (Fig. S1H).

The wildtype R1 line showed slightly different expression patterns from the wildtype 2.1 line. For example, the expression of *Etv2* was detectable as early as EB D2 and was more broadly expressed, peaking at EB D4 (Fig. S1I). In the *Flk1* mutant, *Etv2* expression was significantly delayed and was undetectable until EB D4 (Fig. S1I). This supports the notion that *Flk1* signaling is one of the early regulators of *Etv2* expression, but other factors can compensate, at least at later time points during EB development. The presence of *Etv2* may also explain why *Flk1* mutant EBs are capable of generating some endothelial and hematopoietic cells. As expected, the *Flk1* transcript was undetectable in the *Flk1* mutant compared to the R1 wildtype (Fig. S1J). We also observed that Bry and *Fgf5* are expressed at similar levels in the mutant compared to the wildtype, although the timing is delayed by a day (Fig. S1K-L). Furthermore, markers of endothelial and hematopoietic lineages are reduced or delayed in the *Flk1* mutant compared to R1 control EBs (Fig. S1 M-P).

These mutant mouse ES cells were further genetically modified using a stably-integrating, doxycycline inducible lenti-viral vector. cDNAs encoding *Flk1* and *Etv2* with an HA tag (HA-*Etv2*) were subcloned into the pSAM2 vector (Darabi *et al.*, 2012), where GFP was replaced with the mCherry reporter gene, and will be referred to as *Flk1*-pSam2 and *Etv2*-pSam2, respectively. The reporter gene was expressed in response to doxycycline along with the subcloned gene, and allowed monitoring of the response of the transduced cells to doxycycline. After enrichment, all four resulting transduced cell stocks, including *Etv2* mutant:*Flk1*-pSam2, *Flk1* mutant:*Flk1*-pSam2, *Etv2* mutant:*Etv2*-pSam2, and *Flk1* mutant:*Etv2*-pSam2, responded to doxycycline by expressing mCherry in 60-80% of the cell population (Fig. 1 A,B,D,E). We confirmed that this inducible expression strategy resulted in an increase in the appropriate transcript levels using qPCR (data not shown). Furthermore, we confirmed that the respective protein was induced by performing western blots of the cell extracts for *Flk1* and the HA tag. Neither the *Etv2* nor the *Flk1* mutant cell lines transduced with *Flk1*-pSam2 had *Flk1* expression in the absence of doxycycline. However, in the presence of doxycycline, both cell lines showed robust *Flk1* expression (Fig. 1C). Likewise, the *Etv2* and *Flk1* mutant ES/EB cell lines transduced with *Etv2*-pSam2 had no detectable levels of HA expression in the absence of doxycycline. In the presence of doxycycline, the *Flk1* mutant ES/EBs transduced with *Etv2*-pSam2 displayed a robust HA-*Etv2* band, and the

Etv2 mutant ES/EBs transduced with the same virus displayed a clear induction, albeit at a lower level (Fig. 1F). Therefore, we established four ESC stocks that expressed the transduced proteins only in the presence of doxycycline.

First, we examined whether overexpression of *Etv2* or *Flk1* could rescue the *Etv2* mutant phenotype. We differentiated the wildtype 2.1 ES cells, the *Etv2* mutant: *Etv2*-pSam2 ES cells, and the *Etv2* mutant: *Flk1*-pSam2 ES cells side by side using a hanging-drop method, and cultured without doxycycline or with 1 µg/ml of doxycycline from days (D) 2-4, or D4-6 and analyzed the EBs at day 6 (EB D6). As expected, the wildtype cells were unaffected using any doxycycline treatments (data not shown). At EB D6, these wildtype cells contained approximately 3% endothelial cells ($\text{Flk1}^+/\text{CD31}^+$) (Fig. 2A, upper panel; Fig. 2D, red bar), 20% hematopoietic progenitors ($\text{CD41}^+/\text{CD45}^-$) (Fig. 2A, lower panel; Fig. 2E, red bar), and a small number of hematopoietic cells ($\text{CD41}^+/\text{CD45}^+$) (Fig. 2A, lower panel; Fig. 2F, red bar, Table S1). As expected the *Etv2* mutant: *Etv2*-pSam2 cells with no doxycycline treatment had essentially no (0.3%) endothelial cells ($\text{Flk1}^+/\text{CD31}^+$) (Fig. 2B, upper panel; Fig. 2D, Table S1) and essentially no hematopoietic progenitors ($\text{CD41}^+/\text{CD45}^-$) or hematopoietic cells ($\text{CD41}^+/\text{CD45}^+$) (Fig. 2B, lower panel; Fig. 2E and 2F, Table S1). Doxycycline treatment from EB D2-4 increased the $\text{Flk1}^+/\text{CD31}^+$ endothelial population to normal levels (Fig. 2B, upper panel; Fig. 2D, Table S1); treatment from EB D4-6 further increased the same population. The lack of *Flk1* protein on the cell surface of the *Etv2* mutant EBs and the rescue of *Flk1* protein expression after doxycycline induction of *Etv2* supports the notion that *Etv2* may activate *Flk1* through positive feedback. Due to the similar expression levels of *Flk1* in *Etv2* wildtype and mutant EBs (Fig S1B), *Etv2* may induce pathways that affect *Flk1* translation or localization instead of directly regulating *Flk1* transcription. Doxycycline treatment also had a strong positive effect on the hematopoietic compartment. Treatment from EB D2-4 rescued both the $\text{CD41}^+/\text{CD45}^-$ and $\text{CD41}^+/\text{CD45}^+$ cell populations (Fig. 2B, lower panel; Fig. 2E and 2F, Table S1). Treatment from EB D4-6 yielded an increase of the $\text{CD41}^+/\text{CD45}^-$ population, but with minimal contribution to the $\text{CD41}^+/\text{CD45}^+$ cell population (Fig. 2B, lower panel; Fig. 2E and 2F, Table S1). These data demonstrate that forced expression of *Etv2* could, at least partially, rescue the defect in endothelial and hematopoietic differentiation of *Etv2* mutant EBs.

We next tested whether overexpression of *Flk1* could rescue the *Etv2* mutant phenotype. Similar to the *Etv2* mutant: *Etv2*-pSam2 cells, the *Etv2* mutant: *Flk1*-pSam2 cells without doxycycline treatment also showed a minimal contribution to the $\text{CD31}^+/\text{Flk1}^+$, $\text{CD41}^+/\text{CD45}^-$, or $\text{CD41}^+/\text{CD45}^+$ populations (Fig. 2C-F, Table S1). While the doxycycline treatment from EB D2-4 did not have an increase in *Flk1* expression at EB D6, doxycycline treatment from EB D4-6 showed an increase in Flk1^+ cells (Fig. 2C), as expected with forced overexpression of *Flk1*. However, forced expression of *Flk1* did not allow these cells to re-express $\text{CD31}^+/\text{Flk1}^+$, $\text{CD41}^+/\text{CD45}^-$, or $\text{CD41}^+/\text{CD45}^+$ (Fig 2C-F, Table S1). Therefore, *Etv2* mutant EBs could not be rescued by forced expression of *Flk1*.

In parallel experiments, we examined whether overexpression of *Etv2* or *Flk1* could rescue the phenotype of *Flk1* mutant ESCs. We differentiated the wildtype R1 ES cells, the *Flk1* mutant: *Flk1*-pSam2 ES cells, and the *Flk1* mutant: *Etv2*-pSam2 ES cells side by side and cultured with or without doxycycline from EB D2-4, or EB D4-6. The wildtype cells were

minimally affected by the doxycycline treatment, with a slight reduction in CD31⁺/Flk1⁺ cells (data not shown). The *Flk1* mutant: Flk1-pSam2 cells with no doxycycline treatment had CD31⁺/Flk1⁺ endothelial cells present, albeit at a much reduced level than the wildtype control (Fig. 3A, 3B, 3D, Table S2). The presence of a Flk1⁺ population is likely due to leaky expression of the Flk1-pSam2 vector as this was almost entirely absent in the *Flk1* mutant: Etv2-pSam2 cells (Fig. 3C, 3D). While the *Flk1* mutant: Flk1-pSam2 cells without doxycycline induction had nearly as many CD31⁺/Flk1⁻ and CD41⁺/CD45⁻ cells as the wildtype control (Fig. 3A, 3B, 3D, 3E, Table S2), there was a striking reduction of CD41⁺/CD45⁺ hematopoietic cells (Fig. 3B, 3F, Table S2) consistent with the previously reported phenotype (Hidaka *et al.*, 1999). Forced expression of Flk1 from EB D2-4 or EB D4-6 nearly doubled the CD31⁺/Flk1⁻ population, and induction from EB D4-6 led to a robust increase of Flk1⁺/CD31⁺ population (Fig. 3B, 3D, Table S2). We also observed a 2-3 fold increase in CD41⁺/CD45⁻ cells when induced from either EB D2-4 or EB D4-6 and a rescue of the CD41⁺/CD45⁺ population when induced from EB D2-4 (Fig. 3B, 3E, 3F, Table S2). Similar to the observation that D4-6 induction of *Etv2* mutant: Etv2-pSam2 cells was unable to rescue the CD41⁺/CD45⁺ population (Fig. 2C, 2F, Table S2), D4-6 induction of *Flk1* mutant: Flk1-pSam2 cells did not rescue this population (Fig. 3B, 3F, Table S2). This may be due to the ability of Flk1 to rescue during this time period and a longer chase period may be needed to observe the CD45⁺ cells. Nevertheless, our data clearly demonstrate that Flk1 can rescue the *Flk1* mutant EB phenotype, indicating that the cells, timing of doxycycline treatment, and the viral construct are functional and appropriate to evaluate the rescue of the respective cell populations.

Next, we examined whether overexpression of Etv2 could rescue the *Flk1* mutant phenotype. The *Flk1* mutant Etv2-pSam2 cells without doxycycline treatment had a significant defect in CD31⁺/Flk1⁻ and CD31⁺/Flk1⁺ cells as well as a two fold reduction in the CD41⁺/CD45⁻ population and an almost complete loss of CD41⁺/CD45⁺ cells (Fig. 3C-3F, Table S2). To quantify the endothelial cells, we analyzed the CD31⁺/Flk1⁻ population, because the *Flk1* mutant cells are not able to express Flk1. Doxycycline induction at EB D2-4 and EB D4-6 resulted in an increase of CD31⁺ cells to approximately the level observed in the wildtype (Fig. 3C, 3D, Table S2). Doxycycline induction at EB D2-4 and EB D4-6 also resulted in an increase in CD41⁺/CD45⁻ cells and induction of EB D2-4 but not EB D4-6 rescued the CD41⁺/CD45⁺ population (Fig. 3C, 3E, 3F, Table S2), similar to the observation with Flk1 overexpression. Therefore, Etv2 can rescue the *Flk1* mutant endothelial and hematopoietic phenotypes.

To further ensure that these phenomena were not just limited to expression of the selected cell surface markers, we performed qPCR to analyze the transcript levels of known lineage markers. Representative hematopoietic (*CD41*) and endothelial (*vWF* and *Cdh5*) genes were examined. *Etv2* mutant cells without doxycycline treatment had an almost complete loss of *Cdh5* (Fig. 4B), and *CD41* (Fig. 4C), and a significant decrease in *vWF* (Fig. 4A) compared to the wildtype cells. Forced expression of Etv2 from EB D2-4 or EB D4-6 rescued or partially rescued expression of all 3 of these genes. However forced expression of Flk1 from EB D2-4 or EB D4-6 had no effect on these genes. These results further support the previous observations that Flk1 cannot rescue the defects of *Etv2* mutant EBs.

Flk1 mutant cells without doxycycline treatment had a reduction in expression of *vWF*, *Cdh5*, and *CD41*. Forced expression of Flk1 rescued (and increased above wildtype level) expression of *vWF*, *Cdh5*, and *CD41* (Fig. 4D, 4E, 4F). Furthermore, forced expression of Etv2 in *Flk1* mutant cells rescued the expression levels of *vWF*, *Cdh5*, and *CD41* (Fig. 4D-4F). These data further support the finding that Etv2 expression can bypass the need for Flk1 in developing EBs.

Finally, to examine whether the rescue of cell surface markers and gene expression leads to functional recovery of the hematopoietic lineages and to address whether treatments from EB D2-4 and/or EB D4-6 can rescue the hematopoietic lineage, we performed colony forming assays by plating dissociated cells from D6 EBs in methylcellulose. *Etv2* mutant cells without doxycycline treatment (Fig. 5A-5C) displayed no hematopoietic colonies (Fig. 5A-5C). Forced expression of Etv2 in *Etv2* mutants from EB D2-4 partially rescued the number of Granulocyte-macrophage (GM) colonies (Fig. 5A), but had little effect on erythroid burst forming units (BFUs) (Fig. 5B) and GM, erythrocyte, and megakaryocyte mixed colonies (GEMMs) (Fig. 5C). Forced expression of Etv2 from EB D4-6 completely rescued the number of observed GM, BFU, and GEMM colonies (Fig. 5A-5C). In contrast, forced expression of Flk1 in the *Etv2* mutant EBs from EB D2-4 or EB D4-6 had no effect on any colony types (Fig. 5A-5C). Therefore, Etv2 can rescue the hematopoietic colony formation of the *Etv2* mutant ESCs, but Flk1 is unable to rescue the hematopoietic colony formation of the *Etv2* mutant EBs.

Flk1 mutant cells with no doxycycline (Fig. 5D-5F) produced roughly 2 fold fewer GM, BFU, and GEMM colonies than the wildtype R1 control as described previously (Fig. 5D-5F) (Hidaka *et al.*, 1999). Forced expression of Etv2 or Flk1 from EB D2-4 did not increase the number of any colony types (Fig. 5D-5F). However, forced expression of Etv2 or Flk1 from EB D4-6 completely restored or increased the number of GM (Fig. 5D), BFU (Fig. 5E), and GEMM (Fig. 5F) colonies observed. Therefore, forced expression of Etv2 in the *Flk1* mutant cells from EB D4-6 rescues the phenotype of the *Flk1* mutant equally as well as Flk1 itself.

Discussion

It is interesting to note that Etv2 is capable of rescuing endothelial and hematopoietic progenitor population ($CD41^{+}/CD45^{-}$) of both the *Flk1* and *Etv2* mutant ESCs when induced from either EB D2-4 or EB D4-6 using flow cytometric analysis. However, at EB D6, forced expression of Etv2 in *Etv2* mutant EBs rescues the hematopoietic compartment ($CD41^{+}/CD45^{+}$) cells only when expressed from EB D2-4. In contrast, in methylcellulose assays, we observed a much more pronounced increase of GM colonies when Etv2 is overexpressed from EB D4-6 than from EB D2-4 and only see rescue of BFU and GEMM colonies when Etv2 is overexpressed from EB D4-6. These apparently discordant observations can be explained in light of the notion that hematopoietic progenitors, but not hematopoietic cells, form colonies in methylcellulose culture. Thus, the cells that were rescued from EB D2-4 have further differentiated to hematopoietic cells by D6, the time of analysis, and have retained limited progenitor capabilities compared to wildtype controls, whereas cells that were rescued from EB D4-6 are still progenitors at D6 and are capable of giving rise to GM,

BFU, and GEMM colonies as efficiently, if not more efficiently than wildtype controls. This supports the notion that *Etv2* expression is important for the initiation of differentiation.

Furthermore, the observation that *Etv2* induction from EB D4-6 generates hematopoietic progenitors raises a possibility that either *Etv2* is capable of inducing trans-differentiation of progenitors of one or more non-hematopoietic lineage to the hematopoietic lineage, or that in the *Etv2* and *Flk1* mutant EB cultures, there are still a significant number of mesodermal progenitors with hematopoietic potential at EB D4. Either of these possibilities would be unexpected because it has been shown that 1) at least a subset of *Etv2*-mutant progenitors in embryos and EBs trans-differentiate to the cardiac lineage in the absence of *Etv2*, suggesting that in the absence of *Etv2*, these progenitors will not just stall and retain their progenitor characteristics (Liu *et al.*, 2012; Palencia-Desai *et al.*, 2011; Rasmussen *et al.*, 2011) and 2) cells with non-hematopoietic or endothelial potential are unaffected by forced (and persistent) expression of *Etv2* (Hayashi *et al.*, 2012). While it has been shown that some of the progenitors can differentiate to the cardiac lineage (Liu *et al.*, 2012; Palencia-Desai *et al.*, 2011; Rasmussen *et al.*, 2011), it is unknown to what degree this occurs or if there are other differentiation pathways that these cells could follow. The limitations of the previous lineage tracing analyses in the *Etv2* mutant and the lack of an effect when *Etv2* is expressed in non-hematopoietic or vascular cells *in vivo* leads us to believe the second hypothesis is more likely that, in the *Etv2* and *Flk1* mutant EBs, the differentiation of a subset of hematopoietic and endothelial progenitors are simply arrested, and that a progenitor cell population is maintained without trans-differentiating down other pathways or undergoing cell death, such that, upon introduction of *Etv2*, some cells are still capable of resuming differentiation towards the hematopoietic and endothelial lineages. If this were not the case, we would expect that rescue could only occur within the narrow window that *Etv2* functions during wildtype development.

In summary, we have shown that forced expression of *Etv2* rescues both the *Etv2* and the *Flk1* mutant EB phenotype. In contrast, forced overexpression of *Flk1* rescues the *Flk1* mutant embryoid body phenotype but is unable to rescue the *Etv2* mutant EB phenotype. These data further support the notion that *Etv2* is genetically downstream of *Flk1* and that, at least in early hematopoietic development (EB differentiation parallels primitive hematopoiesis), *Etv2* induction by the *Flk1* signaling cascade is the critical pathway regulating the initiation of hematopoiesis and vasculogenesis. Future studies are warranted to analyze the extent of which forced expression of *Etv2* can rescue the *Flk1* mutant phenotype *in vivo*.

Methods

Cell lines

Etv2 mutant and wildtype mouse embryonic stem (ES) cells were derived using standard techniques (Bryja *et al.*, 2006; Nagy and Nichols, 2011) by harvesting blastocysts from *Etv2* heterozygous female mice bred with *Etv2* heterozygous males (Ferdous *et al.*, 2009). *Flk1* mutant cells were provided by William L. Stanford (Hidaka *et al.*, 1999) and were compared to unmodified wildtype R1 mouse ES cells. HA-*Etv2* cDNA or *Flk1* cDNA were cloned into pSAM2-IRES-mCherry (Darabi *et al.*, 2012), a lentiviral construct with a doxycycline

inducible promoter and the internal ribosome entry site (IRES) sequence followed by mCherry at the 3' end. Viral particles were prepared and cells were infected as previously described (Darabi *et al.*, 2012). Infected cells were enriched by doxycycline induction followed by FACS for mCherry positive cells. The sorted cells were amplified by growing for three passages and the enrichment process was repeated twice. During the last enrichment, cells were sorted by FACS for mCherry and SSEA-APC (1:1000, eBiosciences 51-8813-71) expression to enrich for virally integrated, undifferentiated ES cells. Expression was confirmed by flow cytometric analysis of m-Cherry, qRT-PCR using FAM-labeled Gapdh (4352339E) or VIC-labeled Flk1 (mm00440085_m1) and Etv2 (mm01176581_g1) Taqman probes (Applied Biosystems) and western blot using the following antibodies: rat-anti-HA serum (1:2000, Roche 3F10 Roche), rabbit anti-Flk1 serum (1:1000, Cell Signaling 55B11), mouse-anti- α -tubulin serum (1:500, Sigma B-5-1-2), anti-rabbit-HRP serum (1:2000, Pierce), anti-mouse-HRP serum (1:2000, eBiosciences), and anti-rat-HRP serum (1:2000, Pierce). The resulting ES cells will be available to the research community upon acceptance of this manuscript.

EB differentiation

ES cells were preplated for 20 minutes on gelatin coated flasks to remove the mouse embryonic fibroblasts (MEFs). Cells were resuspended at a concentration of 10,000 cells/ml in mouse EB differentiation medium (IMDM, 15% Fetal Bovine Serum, 2mM Glutamax, 1 $\times$ Penicillin and Streptomycin, 450 μ M 1-thioglycerol (MTG), 200 μ g/ml Holo-Transferrin, and 50 μ g/ml Ascorbic acid). 10 μ l hanging drops were plated and cultured at 37°C with 5% CO₂ to induce differentiation. After 48 hours (at EB D2), hanging drops were washed from plates and further incubated in petri dishes on a rotating platform (80 rpm) at 37°C with 5% CO₂. Cells were fed with fresh medium with or without doxycycline every 48 hours, as previously described (Koyano-Nakagawa *et al.*, 2012).

Flow Cytometry

ES cells or EBs were dissociated using 0.25% trypsin-EDTA and filtered through 50 μ m cell strainers. Cells were incubated for 30 minutes on ice in the dark with combinations of the following antisera, SSEA-APC (eBiosciences 51-8813), CD31-PECy7 (eBiosciences 25-0311) and Flk1 APC (eBiosciences 17-5821), or CD41-PECy7 (eBiosciences 25-0311) and CD45-PE (BD Pharmingen 553081) and analyzed on a BD FACS Aria.

qPCR

D6 EBs were washed with PBS and resuspended in Trizol (Life Technologies). RNA was isolated using standard techniques and reverse transcribed using SuperScript Vilo cDNA Synthesis Kit (Life Technologies). Expression was analyzed in triplicate by qRT-PCR using FAM-labeled Gapdh (4352339E) or VIC-labeled vWF (Mm00550376_m1), Cdh5 (Mm00486938_m1), CD41 (Mm11439741_m1), Etv2 (Mm00468389_m1), Flk1 (Mm00440085_m1), Bry (Mm01318252_m1), Fgf5 (Mm 00438918_m1), Lmo2 (Mm00493153_m1), Scl (Mm01187033_m1), and Gata1 (Mm00484678_m1) Taqman probes (Life Technologies) and a VIC labeled Embryonic Globin (IDT 51863328) probe.

Methylcellulose assays

D6 EBs were dissociated and resuspended at 220,000cells/ml. Cells were further diluted 1:10 in 3ml methylcellulose (Methocult M3434, Stem Cell Technologies). 0.9ml of the resulting cells were plated in 35mm petri dishes in triplicate and allowed to differentiate for 7 days. Resulting colonies were counted and identified as GM (granulocyte and/or macrophage containing colonies), BFU (burst forming units or erythrocytes), and GEMM (GM, megakaryocyte and BFU mixed colonies) as previously described (Koyano-Nakagawa *et al.*, 2012).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Transduced cell lines overexpress Etv2 or Flk1
Etv2 mutant (A) or *Flk1* mutant (B) ESCs stably transduced with *Flk1*-pSam2 express mCherry in response to 1μg/ml doxycycline as detected by flow cytometric analysis. Both cell lines transduced with *Flk1*-pSam2 express *Flk1* protein only in the presence of doxycycline as shown by western blot analysis (C). *Etv2* mutant (D) or *Flk1* mutant (E) embryonic stem cells stably transduced with *Etv2*-pSam2 express mCherry in response to 1μg/ml doxycycline by flow cytometric analysis. Both cell lines transduced with *Etv2*-pSam2 express HA-tagged *Etv2* protein only in the presence of doxycycline as shown by western blot analysis (F). The control lanes contain cell extract from 293T cells transiently transfected with either a *Flk1* expression construct (C) or an *Etv2*-HA expression construct (F).

Figure 2. Etv2, but not Flk1, rescues the hematopoietic and endothelial compartments of Etv2 mutant embryoid bodies

(A-C) Flow cytometric analysis of a representative differentiation experiment comparing Wildtype 2.1 EBs (A), Etv2 mutant:Etv2-pSam2 (B) and Etv2 mutant:Flk1-pSam2 (C) at EB D6. Wildtype and mutant cells were analyzed for endothelial (CD31, Flk1) (upper panel) and hematopoietic markers (CD41, CD45) (lower panel) in multiple doxycycline induction schemes: no doxycycline (A, B left panels, C left panels), 1 μ g/ml doxycycline from EB D2-4 (B middle panels, C middle panels), and 1 μ g/ml doxycycline from EB D4-6 (B right panels, C right panels). (D-F) Quantification of the FACS profiles is graphically displayed for CD31⁺/Flk1⁺ endothelial cells (D) CD41⁺/CD45⁻ hematopoietic progenitor cells (E), and CD41⁺/CD45⁺ hematopoietic cells (F). Results from the wildtype control are shown in red and results from Etv2 mutant cells are shown in black. Error bars indicate SEM, (n equals at least 3, see Table S1).

Figure 3. Etv2 or Flk1 rescues the phenotype of Flk1 mutant embryoid bodies

(A-C) Flow cytometric analysis of a representative differentiation experiment comparing Flk1 wildtype (R1) EBs (A), Flk1 mutant:Flk1-pSam2 (B) and Flk1 mutant:Etv2-pSam2 (C) at EB D6. Wildtype and mutant cells were analyzed for endothelial (CD31, Flk1) (upper panels) and hematopoietic markers (CD41, CD45) (lower panels) in multiple doxycycline induction schemes: no doxycycline (A, B left panels, C left panels), 1 μ g/ml doxycycline from D2-4 (B middle panels, C middle panels), and 1 μ g/ml doxycycline from D4-6 (B right panels, C right panels). (D-F) Data from all experiments are graphically displayed for CD31⁺/Flk1⁺ (D, left panel) cells and CD31⁺/Flk1⁺ endothelial cells (D, right panel), CD41⁺/CD45⁻ hematopoietic progenitor cells (E), and CD41⁺/CD45⁺ hematopoietic cells (F). Results from the wildtype control are shown in red and results from mutant cells are shown in black. Error bars indicate SEM (n equals at least 3, see Table S2).

Figure 4. Etv2 expression rescues the expression of hematopoietic and endothelial transcripts in Etv2 and Flk1 mutant embryoid bodies

(A-C) Expression levels vWF (A), Cdh5 (B), and CD41 (C) in Wildtype 2.1 (grey) and transduced Etv2 mutant D6 EBs (black). (D-F) Expression levels of vWF (D), Cdh5 (E), and CD41 (F) in Wildtype R1 (grey) and transduced Flk1 mutant D6 EBs (black). All results were normalized to the respective wildtype cells. The doxycycline induction scheme, transduced virus, and cell lines are indicated under each graph. (n= 3), Error bars represent SEM.

Figure 5. *Etv2* rescues hematopoietic progenitors in the *Etv2* mutant and *Flk1* mutant EBs
Cells were dissociated from Wildtype 2.1 (red bar) and *Etv2* mutant D6 EBs (A-C) transduced with *Etv2*-pSam2 (white bars) or *Flk1*-pSam2 (grey bars) or Wildtype R1 and *Flk1* mutant EBs (D-F) transduced with *Etv2*-pSam2 (white bars) or *Flk1*-pSam2 (grey bars). 20,000 cells were plated in methocult M3434 in triplicate and cultured for 7 days. Colonies were counted and identified as either GM (granulocyte or macrophage containing) (A,D), BFU (erythrocyte burst forming units) (B,E), or GEMM (granulocyte, erythrocyte and macrophage and megakaryocyte mixed) colonies (C,F). n=3, error bars represent SEM.