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The contribution of the Tie2$^+$ lineage to primitive and definitive hematopoietic cells

Yuefeng Tang, M.S., **Anne Harrington, B.S.**, **Xuehui Yang, Ph.D.**, **Robert E. Friesel, Ph.D.**, and **Lucy Liaw, Ph.D.**
Maine Medical Center Research Institute, Scarborough, ME 04074 USA

Abstract

The regulatory elements of the *Tie2/Tek* promoter are commonly used in mouse models to direct transgene expression to endothelial cells. *Tie2* is also expressed in hematopoietic cells, although this has not been fully characterized. We determine the lineages of adult hematopoietic cells derived from *Tie2* expressing populations using *Tie2-Cre;Rosa26R-EYFP* mice. In *Tie2-Cre;Rosa26R-EYFP* mice, analysis of bone marrow cells showed Cre-mediated recombination in 85% of the population. In adult bone marrow and spleen, we analyzed sub-classes of early hematopoietic progenitors, T cells, monocytes, granulocytes, and B cells. We found that ~84% of each lineage was EYFP$^+$, and nearly all cells that come from Tie2 expressing lineages are CD45$^+$, confirming widespread contribution to definitive hematopoietic cells. In addition, more than 82% of blood cells within the embryonic yolk sac were of *Tie2*$^+$ origin. Our findings of high levels of Tie2-Cre recombination in the hematopoietic lineage have implications for the use of the *Tie2-Cre* mouse as a lineage restricted driver strain.

Keywords

Tie2; Cre recombinase; hematopoietic lineage; mouse transgenic; ROSA reporter

There are two types of embryonic hematopoietic cells, primitive and definitive. Primitive hematopoietic cells arise in the yolk sac from blood islands from Flk1$^+$ cells (Lugus *et al.*, 2009), and contain erythroid and megakaryocyte progenitors. Primitive erythroid progenitors appear in the murine yolk sac between E7.25 and E9.0, and produce erythroid cells expressing both embryonic and adult hemoglobins. Primitive erythroid progenitors express the endothelial cell markers VE-cadherin, CD31, Tie2, and endoglin (Ema *et al.*, 2006). Definitive hematopoiesis generates erythroid cells expressing adult hemoglobins (McGrath and Palis, 2005). Definitive hematopoietic cells expand in the yolk sac, and are in the embryo soon after E8.25 (Palis *et al.*, 2001). In the mouse, hematopoietic stem cells are generated at E10.5 in the aorta-gonad-mesonephros (AGM) region (Palis *et al.*, 2001), and migrate to the fetal liver, spleen, and bone marrow. All primitive and definitive hematopoietic cells emerging before E10 in the mouse embryo are from the yolk sac (Lux *et al.*, 2008), which also contributes to adult hematopoiesis (Samokhvalov *et al.*, 2007).

The contribution of endothelial cells to the adult hematopoietic lineage was shown using Cre/LoxP (Chen *et al.*, 2009; Eilken *et al.*, 2009; Lancrin *et al.*, 2009; Zovein *et al.*, 2008), and the VE-cadherin-Cre strain, but it is not clear whether endothelial cells contribute to primitive hematopoiesis. Endothelial and hematopoietic cells share molecular determinants,

Corresponding author: Lucy Liaw, Ph.D., Maine Medical Center Research Institute, 81 Research Drive, Scarborough, ME 04074, USA, Ph: 207-396-8142, Fax: 207-396-8179, liawl@mmc.org.

and are postulated to arise from a shared hemangioblast. Previous studies of VE-cadherin expression in the yolk sac, and analysis of single yolk sac cells from*VEC-Cre;Rosa26R-EYFP* mice at E9.5 indicated little contribution of VE-cadherin positive cells to primitive hematopoietic cells. A similar study has not been performed to test other endothelial lineage markers.

Tie2 (tunica intima endothelial kinase 2) is a receptor tyrosine kinase that binds angiopoietin-1 and angiopoietin-2. *Tie2* is expressed in all endothelial cells (Kisanuki *et al.*, 2001), and is also expressed in hematopoietic cells in the AGM region, fetal liver and adult bone marrow, as well as in several differentiated hematopoietic cells (Puri and Bernstein, 2003; Takakura *et al.*, 1998). *Tie2* null embryos die at E10.5 due to defects in the cardiovasculature and definitive hematopoiesis (Takakura *et al.*, 1998) (Dumont *et al.*, 1994; Sato *et al.*, 1995). Mesodermal precursors undergoing primitive erythropoiesis express endothelial cell markers including *Tie2* in the mouse embryo (Ema *et al.*, 2006), although the lineages that derive from *Tie2* expressing cells are not well defined. This is in part due to the transient *Tie2* expression in developing hematopoietic cells. We utilized the Cre-loxP system to trace the progeny of $Tie2^+$ cells in distinct lineages of the hematopoietic system, and found that almost all subtypes of hematopoietic cells are the progeny of $Tie2^+$ cells.

Because *Tie2* is expressed within both endothelium and hematopoietic cells (Dumont *et al.*, 1994; Kisanuki *et al.*, 2001; Takakura *et al.*, 1998), we investigated whether both primitive and definitive hematopoietic cells can be traced using *Tie2-Cre* mice. *Tie2-Cre* mice were crossed to flox-STOP-flox-EYFP (*Rosa26R-EYFP*) *Rosa* reporter mice. In this system, cells expressing *Tie2* will express Cre recombinase and recombine the floxed-STOP sequence. Such a cell and its progeny will permanently express EYFP after recombination based on the constitutively active nature of the Rosa26 locus.

To trace the contribution of $Tie2^+$ cells to definitive hematopoiesis, we analyzed the adult mouse hematopoietic tissues, bone marrow and spleen. In the bone marrow, about 85% (average, 85.9%± 5.1%; n = 8) cells were $EYFP^+$, and more than 98% (average, 98.3%± 0.4%; n = 8) of $EYFP^+$ cells were positive for CD45 (Fig. 1A–B). More importantly, approximately 85% (n = 8) of each lineage including CD4, CD8, B220, Mac1, CD41, and Gr1 positive cells are $EYFP^+$. In the spleen, we also observed that about 84% (average, 84.6 %± 4.7%; n = 8) cells were positive for EYFP, and nearly all (average, 98.7%± 0.4%; n = 8) $EYFP^+$ cells are positive for CD45 (Fig, 1C–D). Moreover, we also found about 84% (n = 8) of CD4, CD8, and B220 positive spleen cells were $EYFP^+$ (Fig. 1C–D). These data indicated that *Tie2* is expressed in the progeny of the majority of adult hematopoietic cells, within multiple lineages.

A previous study suggested that all adult hematopoietic cells developed from *Tie2* positive precursors (Liakhovitskaia *et al.*, 2009), although specific adult lineages were not tested. Indeed, this study focused primarily on Cre-mediated recombination in mid-gestation embryos between E9.5–11.5, whereas our study focuses on adult bone marrow and spleen populations. Although we had a high proportion of positive cells in each hematopoietic lineage (we observed ~84% $EYFP^+$ in adult hematopoietic cells), there was a significant population in each lineage that was not lineage marked. To test whether it is possible that we experienced incomplete recombination efficiency, single bone marrow cells from *Tie2-Cre;Rosa26R-EYFP* mice were stained with anti-Tie2 antibody and analyzed by FACS for Tie2 expression and EYFP expression (Fig. 1A). Out of the total cells positive for Tie2 based on anti-Tie2 staining, about 85% (average, 85.7%± 2.4%; n = 6) were also positive for EYFP. These data show that 10–15% of the *Tie-2* expressing cells were not marked by EYFP, suggesting a high, but not complete level of Cre-mediated recombination and activation of the EYFP transgene. However, we cannot disregard the potential that there are

small hematopoietic lineage subpopulations that derive from precursors independent of the *Tie2* lineage.

There are two types of hematopoiesis during embryogenesis, primitive and definitive. Several studies indicate the contribution of VE-cadherin positive endothelial cells to definitive hematopoietic cells (Chen *et al.*, 2009; Eilken *et al.*, 2009; Lancrin *et al.*, 2009; Zovein *et al.*, 2008). To trace the contribution of VE-cadherin-expressing cells to primitive hematopoietic cells, yolk sacs from *VE-cadherin-Cre;Rosa26R-EYFP* embryos at E9.5 were collected and analyzed for $EYFP^+$ cells. Very few $EYFP^+$ cells could be identified (data not shown). Tie2 is expressed significantly earlier than VE-cadherin, starting at E6.5, and reporter activity from *Tie2-Cre;CAG-CAT-Z* double transgenic embryos at E7.5 and E8.5 indicated positive hematopoietic cells in yolk sacs and the dorsal aorta (Kisanuki *et al.*, 2001), suggesting that *Tie2-Cre* could be used to trace primitive hematopoiesis. To address this, we collected yolk sacs of *Tie2-Cre;Rosa26R-EYFP* at different embryonic stages. At E7.5, there was abundant expression of EYFP in cells of the extraembryonic mesoderm of the visceral yolk sac, corresponding to aggregations that are precursors of blood islands (Fig. 2A). We collected yolk sacs at E9.5 for single cell analysis, and found that about 88% (average, 88.7%± 5.7%; n = 10) of primitive hematopoietic progenitors (CD41) (Ferkowicz et al., 2003; Mitjavila-Garcia et al., 2002) from yolk sacs and 82% (average, 82.1%± 6.4%; n = 10) of the primitive erythroid cells (Ter119) were positive for EYFP. Notably, *Tie2-Cre* does not label all primitive hematopoietic cells, again suggesting a non-$Tie2^+$ hematopoietic source or incomplete recombination mediated by the Tie2-Cre transgene. Recent studies indicate that both definitive and primitive hematopoietic cells developed from $Flk1^+$ cells (Lugus *et al.*, 2009). The expression pattern of Flk1 and Tie2 in the yolk sac may support the idea that a very small population of primitive hematopoietic cells develop from $Flk1^+Tie2^-$ cells (Ema *et al.*, 2006; Lugus *et al.*, 2009). Our data indicate that almost all primitive hematopoietic cells can be traced to $Tie2^+$ cells.

Tie2 and other endothelial markers have been reported to be expressed in mesodermal progenitors that contribute to primitive erythropoiesis (Ema *et al.*, 2006). However, the activity of Tie2 promoter in these cells is unknown. To address this, circulating embryonic blood cells were isolated from *Tie2-ER-Cre;Rosa26R-EYFP* embryos, and then treated with 4OTH to induce Cre recombinase expression. No significant expression of EYFP was detected in this population (Fig. 3), suggesting that the *Tie2* promoter is no longer active in circulating embryonic hematopoietic cells.

Embryonic hematopoiesis is a dynamic process coordinated by multiple signals. Our study, using a highly utilized *Tie2-Cre* transgenic strain, demonstrates that the majority of adult blood cells derive from a $Tie2^+$ origin. Therefore, our study shows that *Tie2* promoter/enhancer elements are widely active in the hematopoietic lineage in addition to endothelial lineages during development.

Methods

Mouse Strains

Tie2-Cre mice (Jackson Laboratory, B6.Cg-Tg(Tek-Cre)12Flv, stock 004128), *Tie2-CreER* mice (Forde *et al.*, 2002) (provided by Bernd Arnold, German Cancer Research Center, Heidelberg, Germany) and VE-cadherin-Cre mice (Jackson Laboratory, B6.Cg-Tg(Cdh5-Cre)7Mlia/J, stock 006137) were crossed with *Rosa26R-EYFP* mice (Jackson Laboratory, B6.129X1-*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$/J, stock 006148) to generate *Tie2-Cre;Rosa26R-EYFP* mice, *Tie2-CreER;Rosa26R-EYFP* mice, or *VE-cadherin-Cre;Rosa26R-EYFP* mice. The utilization of the mouse model in these experiments was approval by the Institutional Animal Care and Use Committee of Maine Medical Center.

The following primers were used for genotyping of the *Tie2-Cre, VE-cadherin-Cre, and Tie2-CreER strains*, and yields a ~200bp product:

5'-GCATTTCTGGGGATTGCTTA-3' and 5'-CCCGGCAAAACAGGTAGTTA-3'

The *Rosa26R-EYFP* strain was genotyped using the following primers, which will amplify a 320bp transgenic product:

5'-AAGACCGCGAAGACTTTGTC-3', 5'-AAAGTCGCTCTGAGTTGTTAT-3', and 5'-GGAGCGGGAGGAATGGATATG-3'

Cell Staining and Sorting

The bone marrow and spleen were isolated from *Tie2-Cre;Rosa26R-EYFP* mice at two months of age and mechanically dissociated into a single-cell suspension, and treated with red blood cell lysis buffer. Cells were analyzed on a FACSCalibur with the following monoclonal antibodies: CD45-APC, CD4-PE-Cy7, CD8-APC, CD41-PE-Cy7, B220-APC, Gr1-APC, Mac1-PE-Cy7, and Ter119-APC. For Tie2 staining, the purified Tie2 antibody was used, and then stained with APC-conjugated secondary antibodies before performing FACS.

E9.5 yolk sacs of *Tie2-Cre;Rosa26R-EYFP* mice were visualized for EYFP+ cells under fluorescence microscopy, and then confirmed by genotyping. The yolk sacs were dissected and digested at 37°C for 60 minutes in 0.2% collagenase with 20% fetal bovine serum in phosphate-buffered saline. After digestion, yolk sacs were separated into single-cell suspension by passing through a 20-gauge needle. Single cell suspensions were stained with CD41 and Ter119 antibodies (eBioscience, San Diego, CA), and analyzed by FACS.

Embryos from*Tie2-CreER; Rosa26R-EYFP* crosses at E12.5 were bled in warm PBS after removal from their yolk sacs and placentas. The peripheral blood cells were centrifuged and resuspended in myelocult media (Stem Cell Technologies), supplemented with 10^{-6} M hydrocortisone (Stem Cell Technologies), and 5 μM 4OTH (Sigma) for 24 hours in suspension. Flow cytometry was then performed to detect the expression of EYFP and Ter119. All monoclonal antibodies and their appropriate controls were purchased from eBioscience. The double positive cell populations were normalized to the total single positive EYFP or respective antibody to get percentage of double positive cells.

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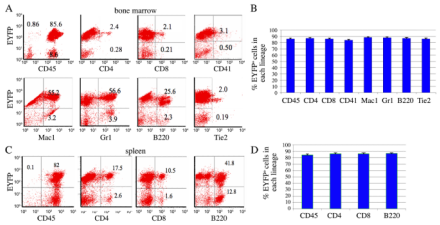

Figure 1. The contribution of Tie2^{+} lineage cells to adult blood cells

A) Representative FACS plots of staining of bone marrow cells for the hematopoietic markers CD45, CD4, CD8, CD41, Gr1, Mac1, B220, and Tie2 (x-axis) and EYFP (y-axis) from *Tie2-Cre;Rosa26R-EYFP* mice at 2 months of age. B) Summary data of multiple experiments (n=8) analyzing bone marrow cells from *Tie2-Cre;Rosa26R-EYFP* mice. Data are presented as the percentage of each lineage that was EYFP^{+} ± S.D. of the mean percentage of each lineage. C) Representative FACS plots of staining of spleen cells for the hematopoietic markers CD45, CD4, CD8, and B220 (x-axis) and EYFP (y-axis). Hematopoietic lineages analyzed represent T cells (CD4 and CD8), B cells (B220), granulocytes (Gr1), megakaryocytes (CD41), and macrophages (Mac1). D) Summary data of multiple experiments (n = 8) analyzing spleens from *Tie2-Cre;Rosa26R-EYFP* mice. Data are presented as the percentage of each lineage that are EYFP^{+} ± S.D. of the mean percentage of each lineage.

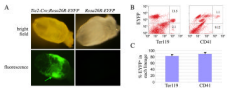

Figure 2. A Tie2^{+} origin for embryonic blood cells from *Tie2-Cre;Rosa26R-EYFP* embryos

A) *Tie2-Cre;Rosa26R-EYFP or* control *Rosa26R-EYFP* embryos were collected at E7.5. Arrow shows EYFP^{+} mesodermal aggregations in visceral yolk sac in regions of forming blood islands. B) Representative FACS plots of E9.5 *Tie2-Cre;Rosa26R-EYFP* yolk sacs stained for the primitive hematopoietic progenitor marker CD41 or erythroid cell marker Ter119 (x-axis) and EYFP (y-axis) to assess the origin of these lineages within the E9.5 yolk sac. C) Summary data of multiple experiments (n = 10) analyzing the yolk sacs of *Tie2-Cre;Rosa26R-EYFP* mice. Data are presented as the percentage of Ter119^{+} and CD41^{+} that are also EYFP^{+} ± S.D. of the mean percentage of each lineage.

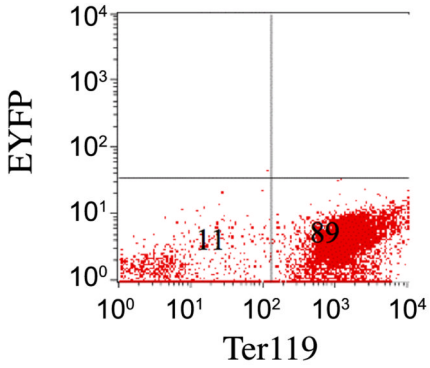

Figure 3. The Tie2-Cre transgene is not active in circulating embryonic blood cells

Peripheral blood cells were collected from *Tie2-CreER;Rosa26R-EYFP* mice at E12.5 and induced *in vitro* with 4OTH for 24 hours in suspension. Cells were then analyzed for EYFP expression within the $Ter119^+$ population. No significant expression of EYFP was observed.