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Circulation-Independent Differentiation Pathway from Extraembryonic Mesoderm toward Hematopoietic Stem Cells via Hemogenic Angioblasts

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SUMMARY

A large gap exists in our understanding of the course of differentiation from mesoderm to definitive hematopoietic stem cells (HSCs). Previously, we reported that Runx1$^+$ cells in embryonic day 7.5 (E7.5) embryos contribute to the hemogenic endothelium in the E10.5 aorta-gonad-mesonephros (AGM) region and HSCs in the adult bone marrow. Here, we show that two Runx1$^+$ populations subdivided by Gata1 expression exist in E7.5 embryos. The hemogenic endothelium and the HSCs are derived only from the Runx1$^+$Gata1$^-$ population. A subset of this population moves from the extra- to intraembryonic region during E7.5–E8.0, where it contributes to the hemogenic endothelium of the dorsal aorta (DA). Migration occurs before the heartbeat is initiated, and it is independent of circulation. This suggests a developmental trajectory from Runx1$^+$ cells in the E7.5 extraembryonic region to definitive HSCs via the hemogenic endothelium.

INTRODUCTION

How definitive hematopoietic stem cells (HSCs) emerge is a major unresolved issue in hematology. For more than three decades, researchers have repeatedly addressed this question using different technologies. The consensus can be summarized in two points: (1) the earliest blood cell (BC) formation occurs in the extraembryonic region, and (2) the first definitive HSCs that contribute to adult hematopoiesis appearing in the embryo proper and placenta most likely differentiate directly from endothelial cells (ECs) (Cai et al., 2000; Chen et al., 2011; Medvinsky and Dzierzak, 1996; Medvinsky et al., 2011; Ottersbach and Dzierzak, 2005; Rhodes et al., 2008; Zovein et al., 2010). The major point of contention has been how these two events, which occur at different embryonic sites, are related. However, because of a lack of knowledge concerning the differentiation of hemogenic ECs from mesoderm, the answers proposed for this question so far have been ambiguous.

Null mutations in either the *Flk1* or *Etv2*/*ER71* gene result in a lack of both ECs and BCs during embryogenesis, and *Etv2* null embryos completely lack Flk1$^+$ mesoderm distributed over extraembryonic space, which is also Etv2$^+$ in normal embryos (Kataoka et al., 2011; Koyano-Nakagawa et al., 2012; Lee et al., 2008; Shalaby et al., 1995, 1997). Thus, the Flk1$^+$Etv2$^+$ mesoderm is the latest point from which the progenitors of all ECs and BCs are segregated from other lineages. Some of these precursors migrate into the intraembryonic region and form intraembryonic vasculature, including the dorsal aorta (DA), as angioblasts (Cleaver and Krieg, 1998; Shalaby et al., 1997; Drake and Fleming, 2000). Gata1 is a key regulator of erythroid gene transcription and a primitive erythrocyte progenitor marker, and is expressed in blood islands (BIs) at the onset of primitive erythropoiesis (Fujiwara et al., 1996; Onodera et al., 1997; Silver and Palis, 1997). Runx1 is an essential transcriptional factor of definitive hematopoiesis (Okuda et al., 1996; Wang et al., 1996) and plays an important role in the generation of HSCs from hemogenic endothelium (North et al., 1999). We previously showed that embryonic day 7.5 (E7.5) Runx1$^+$ cells contribute to HSCs and this Runx1 expression is required for the later emergence of HSCs (Samokhvalov et al., 2007; Tanaka et al., 2012). In this study, we used lineage-tracing analysis and time-lapse live imaging to examine how E7.5

Runx1$^+$ cells contribute to HSCs. Our findings suggest a possible origin of HSCs.

RESULTS

Runx1$^+$ Cells Are Derived from Flk1$^+$Etv2$^+$ Cells in E7.5 Embryos

To further understand the contribution of E7.5 Runx1$^+$ cells to definitive hematopoiesis, we first had to determine which Runx1$^+$ cells contribute to HSC formation. Runx1 expression is detected in the hematopoietic and vascular components of BIs, chorionic mesoderm, allantois, and yolk sac (YS) visceral endoderm at E7.5 (Daane and Downs, 2011; Iacovino et al., 2011; Lacaud et al., 2002; North et al., 1999; Zeigler et al., 2006) (Figure S1A). At E7.5, almost all Flk1$^+$ cells express Etv2 (Figure S1B). Given that all BCs and ECs come from Flk1$^+$Etv2$^+$ cells at this time point (Kataoka et al., 2011; Koyano-Nakagawa et al., 2012; Lee et al., 2008), E7.5 Runx1$^+$ cells that contribute to definitive hematopoiesis should be derived from Flk1$^+$ cells in the embryo. In *Runx1$^{IRESGFP/+}$* embryos, most GFP$^+$ cells (Runx1$^+$ cells) at the midstreak stage were Flk1$^+$ but started to lose Flk1 expression by the late-streak stage, suggesting that Runx1$^+$ cells appear from the Flk1$^+$ population during development (Figure 1A). We also confirmed that Runx1$^+$ cells were detected within the Etv2$^+$ cell population in *Etv2$^{Venus/+}$* embryos at both the neural-plate (NP) and head-fold (HF) stages (Figures 1B and S1C). Furthermore, no Runx1 expression was detected in *Etv2* null embryos at E7.5 and E8.5 (Figure S1D). Thus, the E7.5 Runx1$^+$ cells that contribute to HSCs are derived from Flk1$^+$Etv2$^+$ mesoderm.

Runx1$^+$Gata1$^-$ Cells in E7.5 Embryos Are Angioblast-Like Cells

Most Runx1$^+$ cells in the BIs expressed Gata1, but a few Runx1$^+$Gata1$^-$ cells were detected in the periphery of the BIs at E7.5 (Figures 1B). Cryosections showed that Runx1$^+$Gata1$^+$ cells were detected only in the BIs, whereas a few Runx1$^+$ Gata1$^-$ cells were detected in the base of the allantois, amniotic mesoderm, and chorionic mesoderm (Figures S1A and S1C). In order to examine the expression pattern of Runx1 and Gata1 at the single-cell level, we generated a mouse strain that carried two fluorescent markers: *GFP* in a *Runx1* allele and *mCherry* in a *Gata1* allele (Figure S1E). Flow cytometric analysis showed that nearly all of the Gata1$^+$ (mCherry$^+$) cells coexpressed Runx1 (GFP). Although the majority of Runx1$^+$ cells expressed Gata1, a significant number of Runx1$^+$Gata1$^-$ cells were present (16% and 5.7% of Runx1$^+$ cells were Gata1$^-$ at the NP and HF stages, respectively; Figure 1C). Within the Flk1$^+$ population, 75.5% and 85.2% of Runx1$^+$ cells were Gata1$^-$ at the NP and HF stages, respectively, suggesting that the majority maintained endothelial identity. Indeed, Runx1$^+$Gata1$^-$ cells were distinct from the Gata1$^+$Runx1$^+$ population, displaying higher expression of endothelial markers such as Flk1, Pecam1, Tie2, VE-cadherin, and Endoglin (Figure 1D and S1F). Furthermore, hematopoietic colony-forming potential was detected only in the Runx1$^+$Flk1$^+$ cells at E7.5 (Figure S1G). These data suggested that Runx1$^+$ Gata1$^-$Flk1$^+$ cells contribute to definitive hematopoiesis. Given their location outside of the BIs, this cell population should be a subset of angioblasts.

Fate Analysis of Runx1$^+$ and Gata1$^+$ Populations in E7.5 Embryos

To determine whether Runx1$^+$Gata1$^-$ cells contributed to HSCs, we generated a mouse strain that carried *Mer-Cre-Mer* in a *Gata1* allele (Figures S2A and S2B). We compared the contribution of E7.5 Gata1$^+$ cells to HSCs and major hemogenic sites with that of E7.5 Runx1$^+$ cells. The progeny of E7.5 Gata1$^+$ cells contributed to BCs in the BIs and circulation at E8.5 and E9.5, respectively (Figure S2C). The labeled cells were only in the YS at E8.5 and were CD41$^+$VE-cadherin$^-$ (Figure S2D). These are likely to be primitive erythrocyte progenitors, not erythroid/myeloid progenitors (EMPs), because EMPs emerge from the YS hemogenic endothelium and should express VE-cadherin at E8.25 (Chen et al., 2011; McGrath et al., 2011). In the E13.5 fetal liver (FL), the labeled cells expressed TER119, but not CD45. However, the majority of TER119$^+$ cells were eYFP$^-$, suggesting that the labeled cells did not contribute to definitive erythropoiesis (Figure S2E). The labeled cells were almost completely lost by E16.5 in the FL and could hardly be detected in peripheral blood from 4-week-old mice (Figure S2F). Whole-mount immunostaining for eYFP and CD31 showed that no marked cells were found in any endothelium at major hemogenic sites such as the aorta-gonad-mesonephros (AGM), YS umbilical artery (UA), and vitellin artery (VA) at E10.5 (Figures 2A and 2B). Taken together, these results led us to conclude that E7.5 Gata1$^+$ cells do not contribute to HSCs or ECs.

By contrast, E7.5 Runx1$^+$ cells contributed to definitive hematopoiesis by E16.5 (Figure S2G). In addition to BCs, labeled ECs were found not only on the ventral side but also on the dorsal side of the DA at E10.5 (Figure 2A). Whole-mount immunostaining for CD31 and eYFP clearly showed that eYFP$^+$ cells contributed to endothelial cells in the DA (Figure 2C). The eYFP$^+$ ECs were also detected in the YS, UA, VA, and placenta (Figure 2D). Labeling of E7.5 Runx1$^+$ cells also marked VE-cadherin$^+$ cells of the YS at E8.5, suggesting that E7.5 Runx1$^+$ cells contribute to the EMPs (Figure S2G). Surprisingly, a few eYFP$^+$VE-cadherin$^+$ cells were detected in E8.25 caudal halves (CHs). Because labeling of Gata1$^+$ cells failed to mark either ECs or HSCs, we conclude that ECs and HSCs marked by the labeling of E7.5 Runx1$^+$ cells are derived from the E7.5 Runx1$^+$Gata1$^-$ population.

The Progeny of E7.5 Runx1$^+$Gata1$^-$ Cells within the Intraembryonic Region Originate from the Extraembryonic Region prior to Circulation

We next examined how E7.5 Runx1$^+$Gata1$^-$ cells contribute to HSCs. We have previously suggested that E7.5 Runx1$^+$ cells may stay in the YS until circulation starts and then move to intraembryonic HSC emergence sites by the circulation (Samokhvalov et al., 2007; Tanaka et al., 2012). An alternative possibility is that E7.5 Runx1$^+$Gata1$^-$ extraembryonic cells migrate directly to the intraembryonic sites in the same manner as angioblasts (Drake and Fleming, 2000; Shalaby et al., 1997). To investigate this further, we studied the distribution of the progeny of E7.5 Runx1$^+$Gata1$^-$ cells in the E8.25 embryo, the time point at which aortic primordia and lateral vascular networks start to form and the first intraembryonic lymphohematopoietic potential is detected (Cumano et al., 1996). Whole-mount X-gal staining of E8.25 *Runx1$^{SACre/+}$::R26R$^{LacZ/+}$* embryos labeled at E7.5