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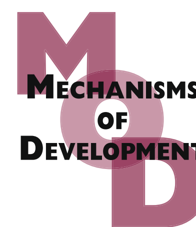

In vivo fate mapping with SCL regulatory elements identifies progenitors for primitive and definitive hematopoiesis in mice

Ernesto Bockamp [a,*,1], Cecilia Antunes [a,1], Stefan Liebner [b], Steffen Schmitt [c], Nina Cabezas-Wallscheid [a], Rosario Heck [a], Svetlana Ohnngemach [a], Barbara Oesch-Bartlomowicz [a], Christof Rickert [d], Maria José Sanchez [e], Jan Hengstler [f], Bernd Kaina [a], Anne Wilson [g], Andreas Trumpp [h], Leonid Eshkind [a]

[a] Medical Centre of the Johannes Gutenberg-University Mainz, Institute for Toxicology, Obere Zahlbacher Str. 67, 55131 Mainz, Germany
[b] Institute of Neurology (Edinger-Institute), Medical School, Johann Wolfgang Goethe-University Frankfurt, Deutschordenstr. 46, 60528 Frankfurt/Main, Germany
[c] Flow Cytometry Core Facility, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[d] Institute of Genetics, Johannes Gutenberg-University Mainz, Saarstrasse 21, 55122 Mainz, Germany
[e] Center for Developmental Biology (CABD), CSIC/Universidad Pablo de Olavide, Carretera de Utrera 1, 41013 Seville, Spain
[f] Leibniz Research Centre for Working Environment and Human Factors, Ardeystr. 67, 44139 Dortmund, Germany
[g] Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland
[h] Division of Stem Cells and Cancer, Deutsches Krebsforschungszentrum, DKFZ-ZMBH Alliance and Heidelberg Institute for Stem Cell Technologies and Experimental Medicine (HI-STEM), Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

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ABSTRACT

One of the principal issues facing biomedical research is to elucidate developmental pathways and to establish the fate of stem and progenitor cells *in vivo*. Hematopoiesis, the process of blood cell formation, provides a powerful experimental system for investigating this process. Here, we employ transcriptional regulatory elements from the stem cell leukemia (SCL) gene to selectively label primitive and definitive hematopoiesis. We report that SCL-labelled cells arising in the mid to late streak embryo give rise to primitive red blood cells but fail to contribute to the vascular system of the developing embryo. Restricting SCL-marking to different stages of foetal development, we identify a second population of multilineage progenitors, proficient in contributing to adult erythroid, myeloid and lymphoid cells. The distinct lineage-restricted potential of SCL-labelled early progenitors demonstrates that primitive erythroid cell fate specification is initiated during mid gastrulation. Our data also suggest that the transition from a hemangioblastic precursors with endothelial and blood forming potential to a committed hematopoietic progenitor must have occurred prior to SCL-marking of definitive multilineage blood precursors.

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* Corresponding author. Tel.: +49 6131 3933361; fax: +49 6131 230506.
E-mail address: bockamp@uni-mainz.de (E. Bockamp).
1 These authors contributed equally to the work.

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1. Introduction

Hematopoiesis, the process of blood cell formation, provides a powerful system for studying lineage specification and cellular commitment. Although the ontogeny of blood cells has been extensively investigated, the initial emergence of blood cell progenitors and hematopoietic stem cells (HSCs) is still controversial (Cumano and Godin, 2007; Lux et al., 2008; Rhodes et al., 2008). It is also not well understood when vascular and hematopoietic lineages diverge during ontogeny and when hematopoietic specification is first established (Flamme et al., 1997; Haar and Ackerman, 1971; Huber et al., 2004; Kinder et al., 1999; Lugus et al., 2005; Ueno and Weissman, 2006). In mice, evidence for hematopoietic activity occurs around day 7.2 (E7.2) of gestation, which is characterized by primitive erythroid progenitors (Ery^P) that will give rise to large, nucleated erythrocytes (Palis, 2008). This first wave of primitive blood cell formation is followed by the emergence of definitive hematopoietic progenitors, proficient to generate the complete repertoire of adult blood (Godin and Cumano, 2002; Mikkola and Orkin, 2006).

Several lines of evidence suggest that the basic helix-loop-helix transcription factor SCL (also known as TAL1 or TCL5) is a key regulator for primitive and definitive hematopoiesis (Begley and Green, 1999; Lecuyer and Hoang, 2004). Genetic studies indicated that SCL function is required for the specification of primitive erythroblasts deriving from a mesodermal precursor (Ery^P) at the origin of blood and endothelium (D'Souza et al., 2005; Shivdasani et al., 1995). In addition, SCL is necessary for the establishment of definitive HSCs in the embryo proper (Dooley et al., 2005; Patterson et al., 2005; Porcher et al., 1996; Robb et al., 1995). Though these studies indicated a functional requirement for SCL in hematopoietic development, they did not elucidate the origin of primitive and definitive SCL^+ blood progenitors. The use of SCL-reporter mice is also not appropriate for correlating marker gene expression and lineage fate because SCL activity is down-regulated within some cells of the hematopoietic system and maintained in others (Delabesse et al., 2005; Elefanty et al., 1999; Gottgens et al., 2000, 2004; Sanchez et al., 1999, 2001; Sinclair et al., 1999). To be able to trace the origin of primitive and definitive hematopoietic cells and to establish the progenitor–progeny relationship between SCL^+ HSCs and more differentiated blood cells, it is necessary to use a lineage-tracking system in which cells that express SCL, or are the progeny of SCL-expressing cells, will be permanently marked.

Here, we have used the previously established SCL-tTA-2S knock-in mouse model (Bockamp et al., 2006) for conditional *in vivo* tagging and lineage tracing. We report that the first SCL-labelled cells arise in the mid to late streak embryo and find that these progenitors give rise to primitive erythropoiesis. Restricting *in vivo* marking to different stages of foetal development revealed a second population of progenitors, which have the potential to contribute to adult blood. Most strikingly, permanent SCL-labelling failed to mark endothelial cells of the developing conceptus. Our findings provide a developmental map for the initial specification of hematopoiesis in the mouse foetus.

2. Results

2.1. *SCL-tagged cells first emerge in the extra-embryonic mesoderm and are subsequently localized to the yolk sac and the developing vasculature*

To mark putative hematopoietic progenitors *in vivo*, we generated triple transgenic mice containing the SCL-tTA-2S transgene (Bockamp et al., 2006) together with an inducible Cre-recombinase (Saam and Gordon, 1999) and the ROSA26 flox-STOP-flox *lacZ* Cre-reporter gene (R26R *lacZ* (Soriano, 1999)) that allows visualizing recombined cells and their progeny (Fig. 1). In accordance with the described onset of SCL activity, tTA-2S mRNA expression was present in E7.2 SCL-tTA-2S embryos (data not shown). Furthermore, analysis of triple transgenic foetuses between E7 and E7.75 demonstrated the emergence of β-galactosidase$^+$ (β-gal$^+$) cells at embryonic day E7.2 (S1). In these mid to late streak foetuses, SCL-tagged β-gal$^+$ cells were localized to the extra-embryonic mesoderm (Fig. 2A). As development proceeded to the neural plate and headfold stage, SCL-tagged cells were confined to the mesoderm of the visceral yolk sac (Fig. 2B). Interestingly, SCL-tagging did not target intra-embryonic sites in presomitic and early somitic embryos (<5 somites). As shown in Fig. 2C, in 5–7 somite embryos β-gal$^+$ cells started to emerge in the dorsal aorta of the embryo proper and localized to the yolk sac blood islands and the allantois. At embryonic day E9.5 intense *lacZ* staining highlighted the small and large vitelline vessels of the yolk sac (S2) and the vascular network of the embryo

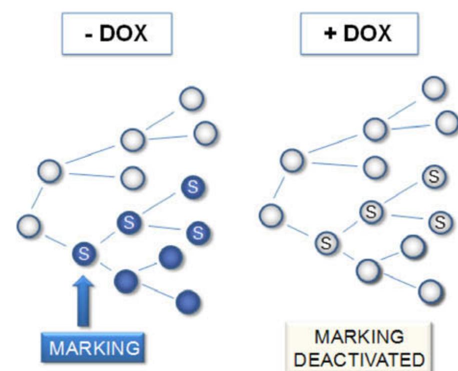

Fig. 1 – Schematic representation of DOX-dependent cell marking and lineage tracking. Induction of *lacZ* reporter gene expression is restricted to cells (indicated by S) which express the tTA-2S activator from the SCL knock-in locus. In the absence of doxycycline (−DOX) the tTA-2S transactivator induces the expression of Cre-recombinase which in turn initiates *lacZ* reporter gene expression (marking) from the ROSA 26 locus (on the left). Note that *lacZ* reporter gene activity is preserved in cells without active tTA-2S expression but which are originally derived from a tagged ancestor (blue cells that are not marked by an S). By contrast, cell marking is extinguished by application of DOX (+DOX) thus preventing the tagging process (on the right). White dots represent unmarked cells and blue dots represent irreversibly marked cells.

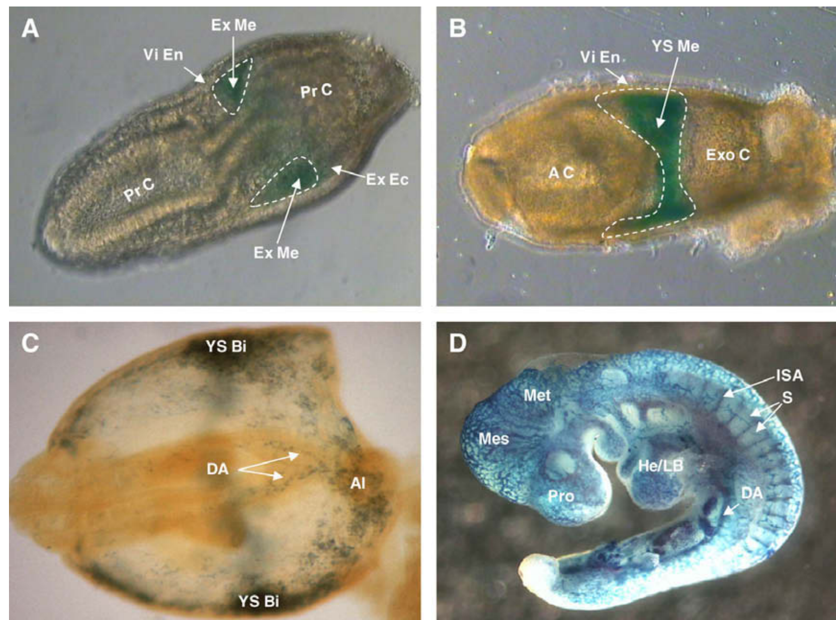

Fig. 2 – Expression of β-galactosidase reveals the localization of SCL-tagged cells at different stages of embryonic development. (A) E7.2 wholemount view of the mid to late streak SCL-tagged embryo with β-gal$^+$ cells appearing in the extra-embryonic mesoderm (Ex Me). (B) In the E7.8 pre-somite stage embryo all β-gal$^+$ cells are confined to the extra-embryonic mesoderm of the yolk sac (YS Me). (C) First appearance of β-gal$^+$ cells in the dorsal aorta (DA) of the embryo proper. At this developmental time point (somites > 5) SCL-tagged cells are also present in the yolk sac blood islands (YS Bi) and the allantois (Al). (D) Wholemount E9.5 embryo with β-gal$^+$ cells localized to the newly formed vascular network. Vi En, visceral endoderm; Ex Ec, extra-embryonic ectoderm; PrC, proamniotic cavity; AC, amniotic cavity; Exo C, exocoelomic cavity; Pro; prosencephalon; Mes, mesencephalon; Met, metencephalon; He/LB, heart and liver bulge; ISA, intersomitic artery; S, somites.

proper (Fig. 2D). These findings suggested that SCL-tagging targets early hematopoietic and/or vascular development.

2.2. *SCL-tagging targets primitive erythropoiesis but does not contribute to the newly formed vasculature of the conceptus*

Primitive erythroblasts are the first hematopoietic cell type to form during mammalian embryogenesis. To investigate whether SCL-tagged cells contributed to primitive erythropoiesis, we used the RAGE flox-STOP-flox *EGFP* mouse line (RAGE *EGFP* (Constien et al., 2001)) as a Cre-reporter for SCL-marked cells. Analogous to our previous findings (Fig. 2), in this mouse model SCL-tagged EGFP$^+$ cells were completely confined to the extra-embryonic mesoderm of early somite foetuses (S3 and S4). As shown in Fig. 3A, flow cytometric analysis of E8.5 dissected extra-embryonic yolk sacs indicated that about one third of the analyzed cells expressed EGFP together with the Ter119 pre-foetal liver erythroid-specific marker. The expression of Ter119 on SCL-marked cells therefore demonstrated that these cells were primitive erythrocytes.

To further determine the relative percentages of distinct primitive red blood cell subtypes prior to the appearance of the first definitive erythroblasts, EGFP$^-$ and SCL-tagged EGFP$^+$ cells from whole E9 conceptuses were analyzed by flow cytometry. As shown in Fig. 3B, only about 0.18% of all viable cells were EGFP-tagged. Based on their expression for CD71 and Ter119 five subpopulations could be established (quadrants I to V in Fig. 3B). Compared to the EGFP$^-$ fraction, E9 EGFP$^+$ cells were much less abundant in the progenitor and early proerythroblasts population (Fig. 3B, upper plot, quadrant I). Since CD71 is not exclusively expressed on erythrocytes but also on highly proliferating cells (Trowbridge and Omary, 1981) the reduced percentage of CD71$^+$/Ter119$^-$ EGFP-expressing cells in quadrant I most likely reflects selective SCL-marking of early proerythroblasts and the lack of marking of non-hematopoietic cells. However, SCL-tagged proerythroblasts, early and late basophilic erythroblasts, chromatophilic and orthochromatophilic erythroblasts were present in the E9 conceptus (Fig. 3B, upper plot, quadrants II, III and IV). By contrast, only very few EGFP-expressing cells were detected in the late orthochromatophilic erythroblast and reticulocytes fraction (quadrant V), the most part of which contains enucleated cells that have lost their ability to express the EGFP reporter gene. Our analysis thus revealed that EGFP-tagged cells expressed CD71 and Ter119 in a manner which recapitulated the stepwise developmental maturation pattern for primitive erythroblasts (Fraser et al., 2007).

Next, we wanted to clarify whether SCL-tagged cells also contribute to the three-dimensional network of tubules that constitutes the primary vascular plexus, which later undergoes reorganization, sprouting and remodelling to form the vitelline vessels. Given the shared expression of known endothelial markers on both primitive blood and blood vessel cells (Ema et al., 2006; Redmond et al., 2008) it is not possible to unambiguously discriminate between these two lineages by flow cytometry. To circumvent this problem, SCL-induced *lacZ* reporter gene activation was analyzed by confocal microscopy.

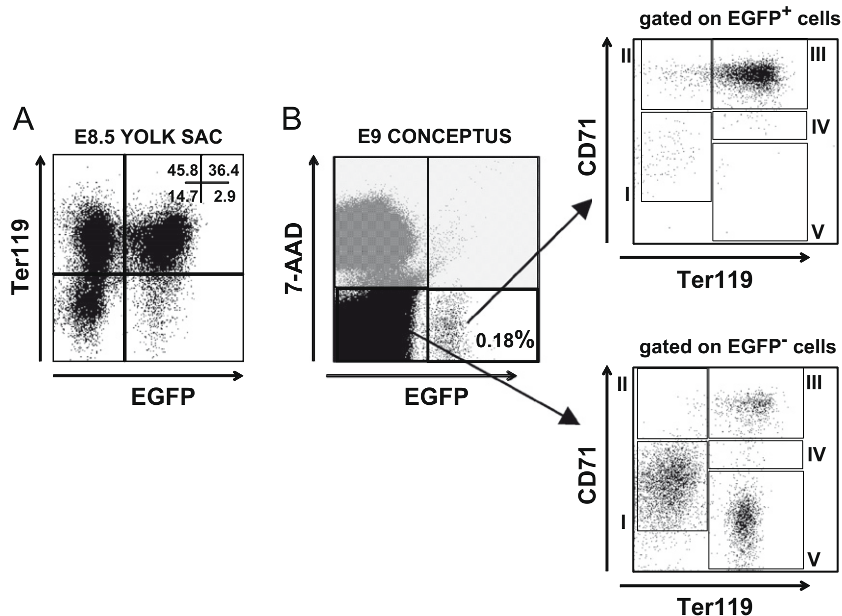

Fig. 3 – SCL-tagged cells contribute to primitive erythropoiesis. (A) Flow cytometric analysis of extra-embryonic tissues pooled from E8.5 triple transgenic embryos for the erythroid marker Ter119. The presence of EGFP/Ter119 double positive cells demonstrates tagging of primitive red blood cells. (B) Flow cytometric analysis of EGFP$^+$ SCL-marked cells pooled from E9 triple transgenic whole conceptuses. Viable SCL-tagged EGFP$^+$ and EGFP$^-$ cells excluding the dye 7-AAD were gated and assessed for expression of Ter119 and CD71. Roman numerals indicate: I, primitive progenitor cells and proerythroblasts; II, proerythroblasts and early basophilic erythroblasts; III, early and late basophilic erythroblasts; IV, chromatophilic and orthochromatophilic erythroblasts; V, late orthochromatophilic erythroblasts and reticulocytes.

Strikingly, in E9 SCL-marked embryos, no co-localization of SCL-tagged cells (depicted in blue) with the endothelial cell-containing tubes of the yolk sac capillary network (in green) was detected (Fig. 4A–F). In line with the previously observed tagging of primitive erythrocytes, we found expression of β-gal in large round cells. Equally, the microscopic analysis of intra-embryonic structures revealed no contribution of SCL-marked cells to the endothelial networks of the E9 embryo proper. Fig. 4G–L shows representative images of this analysis. Again the presence of large round β-gal$^+$ cells within β-gal$^-$ vessels was apparent. Furthermore, no co-staining of SCL-tagged cells with endothelial structures was found in E10.5 and E12.5 embryos (S5 and data not shown). Taken together these results demonstrate that the SCL-based system did not target endothelial structures of the developing embryo till E12.5.

2.3. *SCL-labelling targets progenitors for adult blood*

With the tTA-2S mouse model it is possible to conditionally deactivate cell marking at any given time ((Bockamp et al., 2006; Wilson et al., 2008) and Fig. 1). To determine the time required for shutting down SCL-marking in the developing embryo, pregnant mothers were exposed to doxycycline (DOX) for different time periods before the initial appearance of SCL-labelled β-gal$^+$ cells. Pregnant mothers receiving DOX for 42 h prior to the onset of SCL-mediated *lacZ* reporter gene activation failed to give rise to SCL-marked foetuses (S6). This finding indicated that DOX application for 42 h is sufficient for completely extinguishing SCL-mediated cell tagging in the developing foetus. This kinetic not only takes into account the time DOX needs to pass the placenta and to prevent irreversible Cre-recombination, but also accommodates for the known asynchronous embryonic development during gestation (Downs and Davies, 1993).

In order to determine the ability of SCL-marked progenitors to contribute to adult blood, SCL-tagging was deactivated at separate time points during embryogenesis and the fate of labelled progenitors was traced into adulthood. As shown in Fig. 5 and S7, in triple transgenic adult animals permanently exposed to DOX (+DOX), we detected no SCL-marked cells. By contrast, we found up to 10% of EGFP$^+$ cells in triple transgenic mice, which never received DOX (−DOX). Therefore, cell marking was completely dependent on DOX with no illegitimate reporter gene activation taking place. When tagging was allowed until E7.75 (application of DOX at E6), SCL-marked cells failed to contribute to adult hematopoiesis (Fig. 5 and S7). By contrast, when SCL-labelling was allowed until E8.75 (DOX E7.0), a small proportion of EGFP$^+$ cells appeared in the bone marrow and the spleen of adult mice (Fig. 5 and S7). Marking levels further increased when labelling was extended until E9 and E9.25 (DOX application at E7.25 and E.7.5). These findings indicated that conditional SCL-mediated *in vivo* tagging targeted two independent populations, namely Ery^{P}s, giving rise to primitive erythroid development and definitive progenitors which contribute to adult blood.

2.4. *SCL-tagged progenitors have multilineage potential*

The long-term function of HSCs that permits them to support hematopoiesis throughout life is thought to depend on

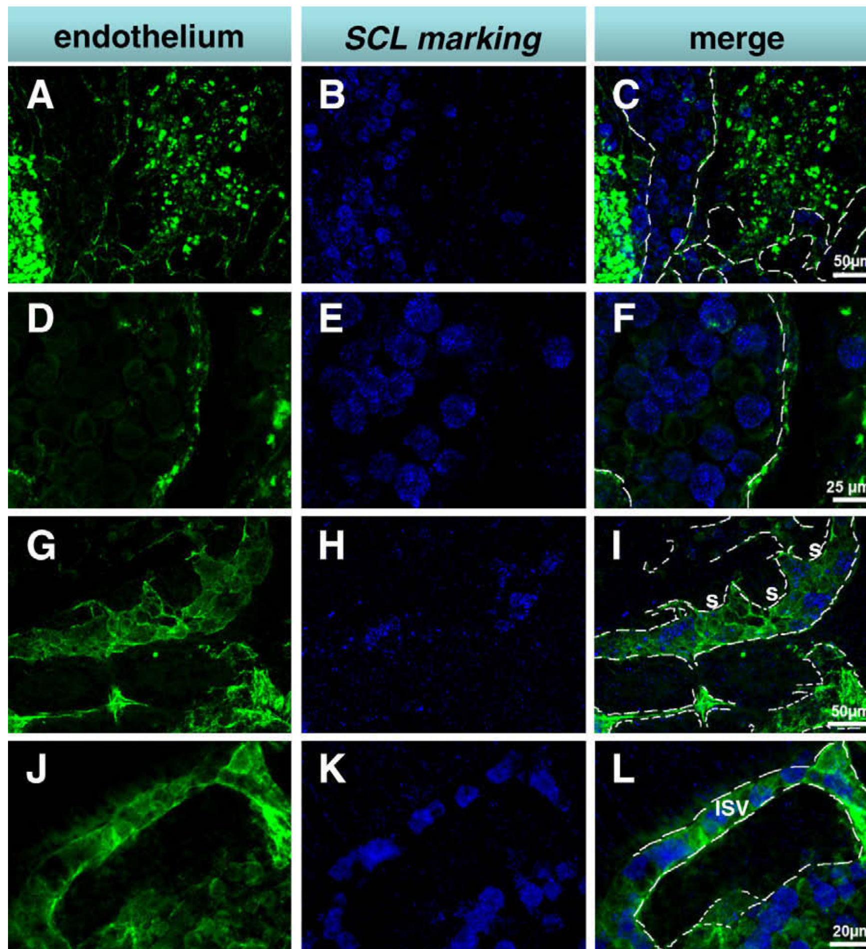

Fig. 4 – *In vivo* SCL-tagging does not contribute to the nascent vascular system of the yolk sac and the embryo proper. (A–F) Confocal micrographs showing a section of the yolk sac. The vascular architecture of the yolk sac is highlighted by CD31 staining in green (endothelium) and SCL-marked β-gal$^+$ cells are shown in blue (SCL marking). Panels D–F show a higher magnification. (G–L) Confocal micrographs showing a section of the embryo proper. The newly forming endothelial network of the embryo is highlighted by CD31 staining in green and SCL-tagged β-gal$^+$ cells are shown in blue. Panels J–L show a higher magnification. White lines have been introduced in the merged image to indicate endothelial boundaries. S, somites; IVS, inter somatic vessel.

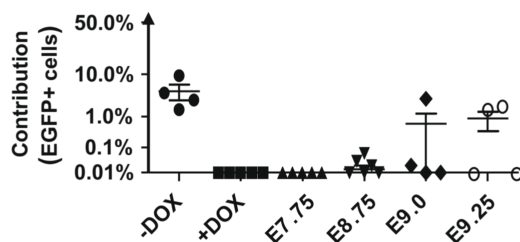

Fig. 5 – Temporal restriction of SCL-tagging identifies a population of progenitors surviving to adulthood. The presence of EGFP-positive cells was determined by FACS analysis from bone marrow of five month old mice. The marking process was stopped at embryonic day E7.75, E8.75, E9.0 and E9.25. Control mice were kept without DOX (−DOX) or permanently exposed to DOX (+DOX). Symbols represent individual mice. The mean and the standard deviation are indicated for each group.

definitive hematopoietic progenitors which arise during embryonic development (Godin et al., 1995; Gothert et al., 2005; Medvinsky and Dzierzak, 1996; Rhodes et al., 2008; Samokhvalov et al., 2007). Our above results indicated that cells genetically tagged between E8.75 and E9.25 were proficient to contribute to adult hematopoietic tissues. To test whether these cells were able to give rise to all mature hematopoietic lineages, potentially EGFP-expressing cells were isolated from five month old mice and analyzed by flow cytometry. As expected, no EGFP$^+$ cells were present in triple transgenic animals continuously exposed to DOX (Fig. 6A). By contrast, in mice never exposed to DOX, CD3$^+$ T- and CD19$^+$ B-lymphoid cells, CD11b$^+$ and Gr1$^+$ myeloid cells, CD41$^+$ megakaryocytes, Ter119$^+$ erythrocytes, DX5$^+$ natural killer cells, CD11c$^+$ dendritic cells and CD23$^+$ mast cells were EGFP-labelled. Furthermore, EGFP$^+$ cells were also present in the lineage negative (lin$^-$) and c-kit-expressing (c-kit$^+$) population of cells, that contains HSCs and progenitors (Fig. 6B). Therefore, perma-

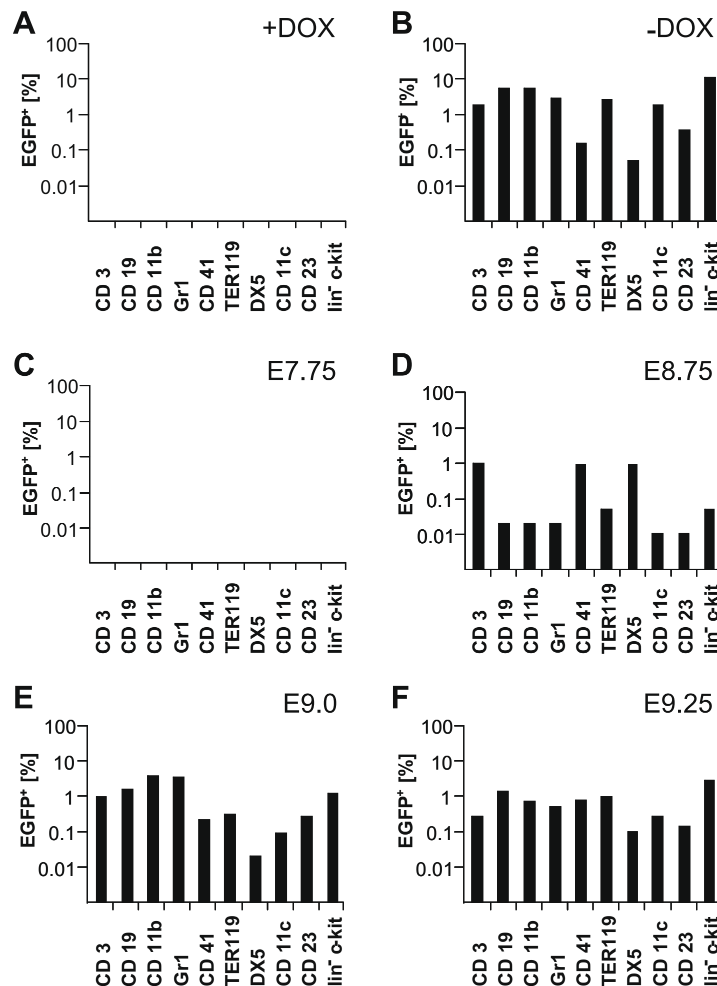

Fig. 6 – SCL-tagging between E8.75 and E9.25 targets a population of progenitors giving rise to erythroid, myeloid and lymphoid development. CD3⁺ T- and CD19⁺ B-lymphocytes, CD11b⁺ and Gr1⁺ myeloid cells (macrophages and granulocytes respectively), CD41⁺ megakaryocytes, Ter119⁺ erythrocytes, DX5⁺ natural killer cells, CD11c⁺ dendritic cells, CD23⁺ mast cells and lin⁻/c-kit⁺ HSCs/progenitors were analyzed for EGFP-expression using FACS. The bar diagrams represent the analysis of a single mouse showing the overall contribution of SCL-tagged cells to each hematopoietic lineage. Contribution of SCL-tagged cells to adult blood lineages in each cohort of mice was verified in at least three additional experiments using different mice. Note that progenitors which were EGFP-marked until day E8.75 contributed to all different hematopoietic lineages.

nent SCL-marking tagged all adult hematopoietic lineages and lin⁻/c-kit⁺ HSCs and progenitors. Consistent with our previous analysis, no lineage contribution was detected when SCL-tagging was stopped at E7.75 (Fig. 6C). To assay the lineage potential of reporter-labelled cells marked between E8.75 and E9.25, cells from this cohort were also subjected to lineage analysis. As shown in Fig. 6D–F, analysis of adult hematopoietic cells derived from embryonic progenitors, labelled between E8.75 and E9.25, demonstrated the presence of a small proportion of EGFP⁺ cells in all adult blood lineages and also in the population of lin⁻/c-kit⁺ HSCs and progenitors. Our findings thus indicate that SCL-tagging targets a population of definitive hematopoietic progenitors in the developing mouse embryo, proficient to contribute to adult erythroid, myeloid and lymphoid development. We conclude that SCL-tagging targets progenitors that support adult hematopoiesis and that these progenitors can be detected at E8.75, when the transition from the early somite stage to functional foetal circulation is initiated.

3. Discussion

The goal of the present paper was to investigate the origin of primitive and definitive hematopoiesis during embryonic ontogeny. Using lineage marking we demonstrate primitive erythroid lineage specification *in vivo*. Furthermore, time-restricted labelling revealed a population of definitive blood cell progenitors capable of contributing to adult hematopoiesis.

In the past a number of studies have investigated the origin of primitive hematopoiesis in the early mouse conceptus (Ema et al., 2006; Furuta et al., 2006; Huber et al., 2004; Kinder et al., 1999; Lancrin et al., 2009; Ueno and Weissman, 2006). While these studies provided direct proof for the existence of a common hemangioblastic progenitor for blood and endo-

thelial cells and for the subsequent specification of blood and endothelial lineages, it is still unclear when primitive blood cell development is first initiated. Our mouse model now extends these studies and uses *in vivo* tagging to trace the developmental origin of hematopoiesis. This approach has distinct advantages over prior experimental strategies since it avoids the removal of cells from their native developmental niches and also provides the opportunity to stop marking at any given time. Congruent with the expected origin of foetal hematopoiesis, we found the first SCL-tagged cells in the extra-embryonic mesoderm of mid to late streak gastrulating embryos (Fig. 2A). Subsequently, marked cells assembled in the yolk sac blood islands and finally localized to the vascular network of the developing embryo and the vitelline vessels of the yolk sac (Fig. 2B–D and S2). Flow cytometry and confocal microscopy verified that SCL-tagging targeted primitive erythropoiesis but failed to mark the newly forming vascular network of the yolk sac and the embryo proper (Figs. 3 and 4 and S5). The observed lack of endothelial contribution demonstrates that initial SCL-marking must have started downstream of a common precursor with endothelial and hematopoietic potential and strongly supports the previously proposed concept that primitive hematopoietic and endothelial lineage commitment diverges prior to the establishment of the visceral yolk sac (Kinder et al., 1999; Ueno and Weissman, 2006). Importantly, in the present paper we demonstrate for the first time the emergence of genetically labelled progenitors, proficient in giving rise to primitive erythrocytes *in vivo*. Our findings thus position these progenitors at the origin of primitive embryonic hematopoiesis.

In addition to primitive blood cell development, SCL-tagging targeted a second population of cells, capable of contributing to adult blood. The sites of origin and the initial appearance of definitive hematopoietic progenitors has been a longstanding and controversial issue of intense investigation. Now there is evidence that progenitors with definitive hematopoietic potential do not arise from a single embryonic origin but instead are autonomously generated in different sites which include the yolk sac, the paraaortic splanchnopleura/AGM-region and the placenta (Godin et al., 1995; Medvinsky and Dzierzak, 1996; Rhodes et al., 2008; Samokhvalov et al., 2007; Zovein et al., 2008). These studies also suggested that the spatio-temporal appearance of definitive hematopoietic progenitors takes place in two consecutive waves, namely in the yolk sac of the E7.5 embryo (Samokhvalov et al., 2007) and subsequently in the AGM-region and the placenta (Godin et al., 1995; Medvinsky and Dzierzak, 1996; Rhodes et al., 2008). In addition, a recent study by Zovein and colleagues demonstrated that definitive hematopoietic progenitors arising in the AGM-region and possibly also in the placenta originate from an endothelial precursor population (Zovein et al., 2008). Using time-restricted *in vivo* labelling, we found in the present study that SCL-tagged cells arising during gastrulation completely failed to give rise to adult blood (Fig. 5 and S7, E7.75). However, when marking was extended until E8.75 and further, we were able to target a second population of cells capable of reaching adulthood (Fig. 5 and S7). The fact that E8.75 genetically SCL-marked cells have progeny which can be detected postnatally as late as one year (data not shown) strongly suggests that these progenitors are multilineage hematopoietic stem cells. By contrast, no contribution of SCL-labelled cells arising during gastrulation was observed in aged mice. The lack of multilineage and delayed hematopoietic potential within the initially SCL-labelled progenitor population is in contrast to the finding that Runx1-tagged yolk sac progenitors give rise to adult blood cell formation (Samokhvalov et al., 2007). This different spatio-temporal *in vivo* labelling pattern is most likely to be explained by the use of dissimilar transcriptional elements (SCL and Runx1) and tagging systems (tetO-Cre/RAGE and MER-Cre-MER/ROSA26-YFP). In addition, SCL-mediated marking activity of definitive progenitors was less efficient than tagging of primitive blood cells. Therefore the amount of DOX needed for saturating free tTA-2S molecules and thus for extinguishing genetic marking of definitive blood cell progenitors might have been lower than for primitive blood cells. For this reason we cannot completely rule out the possibility that SCL-marked definitive progenitors might have emerged prior to E 8.75. However, since DOX application of 42 h was sufficient for preventing marking of primitive blood cells, it is safe to conclude that definitive SCL-marked progenitors were present at E 8.75.

One intriguing result of our *in vivo* lineage experiments is the fact that only a fraction of definitive blood cells underwent Cre-recombination even when the system was continuously activated (Fig. 5, −DOX). By contrast, when the same SCL-tTA-2S effector mouse was used in combination with the tetO-H2B-GFP system, 80% of hematopoietic stem cells were labelled (Wilson et al., 2008). This discrepancy is most likely due to the use of distinct DOX-responsive elements (tetO-Cre/RAGE-EGFP or tetO-H2B-GFP), which might differently respond to the SCL-tTA-2S stimulus. Although the number of recombined adult blood cells derived from E8.75 tagged cells was low, it was sufficient to determine the adult fate of these progenitors by flow cytometry. As evidenced by these experiments, E8.75 SCL-tagged cells contributed to erythroid, myeloid and lymphoid lineages and to $lin^-/c\text{-}kit^+$ HSCs and progenitors (Fig. 6D). Since the initial wave of SCL-tagged cells did not contribute to adult hematopoiesis and since labelling of definitive hematopoietic precursors took place at the same time when the first SCL-marked cells emerge in the dorsal aorta (Fig. 2C), it is tempting to speculate that the second wave of SCL-marking targeted AGM-derived hematopoietic progenitors. However, in our experiments definitive progenitors were first labelled at a developmental time point when embryonic circulation is already initiated, and therefore the precise anatomical origin of these cells remains to be determined.

Using continuous SCL-marking, we did not identify any contribution of SCL-tagged cells to the vasculature of the developing embryo (Fig. 4, S5 and data not shown). Given that low level SCL activity is detectable in endothelial tissues of the early embryo (Drake et al., 1997; Drake and Fleming, 2000; Kallianpur et al., 1994), it is evident that the expression levels of the tTA-2S effector did not reach the threshold required to switch on the downstream tetO-Cre and R26R *lacZ* allele in endothelial cells. Since all descendants of SCL-tagged ancestor cells preserve a genetically activated reporter gene, it is safe to conclude that SCL-tagged cells did not have any potential to contribute to the endothelial compartment and thus represent a population which is already committed to the

hematopoietic fate. Developmental restriction of embryonic progenitor cells to the hematopoietic fate as seen in our experiments and also previously reported by other researchers (Samokhvalov et al., 2007; Stadtfeld and Graf, 2005) together with the recent finding that AGM and possibly also placenta derived endothelial cells have multilineage hematopoietic potential (Zovein et al., 2008) strongly suggest a polyclonal origin of progenitor cells, able to contribute to definitive hematopoiesis.

In summary, our data provide direct *in vivo* evidence for the establishment of primitive hematopoiesis in the mid to late streak embryo and demonstrate that a population of multilineage hematopoietic progenitors contributing to erythroid, myeloid and lymphoid development is present at E8.75. Our results emphasize the importance of studying developmental pathways in their native environment and underscore the experimental potential of this novel approach. The ability to specifically mark blood progenitors and to identify discrete hematopoietic pathways *in vivo* will now provide the opportunity to explore further the regulatory mechanisms and developmental niches required for hematopoietic specification and lineage establishment.

4. Experimental procedures

4.1. *Animals*

The SCL-tTA-2S knock-in mouse (Bockamp et al., 2006), the tetO-Cre responder mouse line (Saam and Gordon, 1999), the R26R flox-STOP-flox *lacZ* (Soriano, 1999) and RAGE-EGFP (Constien et al., 2001) Cre-reporter lines have been previously described.

4.2. *In vivo lineage tracing using timed mated mice*

To ensure accurately-timed mating periods SCL-tTA-2S male mice were mated with bi-transgenic tetO-Cre/RAGE-EGFP or tetO-Cre/R26R *lacZ* female mice for 2 h in the morning. The marking process was terminated by administering the mice a solution of 3 mg DOX (Sigma)/ml water containing 1% sucrose. To ensure the immediate uptake of DOX, pregnant dams were deprived of liquid for 1 day prior to administering the DOX-containing drinking water.

4.3. *X-gal staining and intra-embryonic visualization of lacZ⁺ cells*

Wholemount X-gal staining was performed as described (Bockamp et al., 2006). To be able to detect *lacZ*$^+$ cells localized within the embryo, X-gal stained wholemount animals were dehydrated using graded steps into 100% methanol and subsequently transferred into equal parts of benzyl benzoate:benzyl alcohol (1:1).

4.4. *FACS analysis*

Lineage contribution of EGFP-marked blood cells was analyzed with a FACSCalibur (Becton Dickinson, BD) by co-staining with PE-conjugated antibodies against CD11b, CD19, Gr1, Ter119, CD71 (BD), CD3, CD11c, DX5 (Caltag), CD23 (Southern) or with purified antibodies against CD41 (BD) detected with anti-rat-PE (Caltag). The stem cell fraction was defined by lin-PE$^-$ and c-kit-APC$^+$ (BD Biosciences) staining. For determining Ter119 and CD71 expression of EGFP-marked primitive blood cells, pooled E8.5 yolk sacs ($n = 30$) or E9 conceptuses ($n = 20$) were suspended in PBS (phosphate buffered saline pH 7.4) containing 5% foetal calf serum using repeated pipetting. Prior to staining with the first antibody, cells were blocked with PBS containing 20% rat serum. Specific staining was confirmed using appropriate isotype controls.

4.5. *Confocal microscopy and immunostaining*

Wholemount staining with rat monoclonal anti-CD31 antibody (MEC13.3 provided by Elisabetta Dejana) has been previously described (Liebner et al., 2004). Briefly, embryos were fixed with 2% (w/v) paraformaldehyde in PBS, permeabilized with 0.02% (v/v) NP-40 in PBS, and stained for β-gal activity. For subsequent immunofluorescent staining whole embryos were blocked with 1% (v/v) BSA, 0.5% TritonX-100 in PBS overnight at 4 °C. Incubation with primary antibodies was carried out overnight at 4 °C. After washing in PBS fluorescently labelled streptavidin was added for 2 h at room temperature. After additional washing in PBS, specimens were mounted using PolyMount (Polysciences Europe) and digitally documented using a spectral imaging confocal microscope (C1*si*, Nikon). The non-fluorescent blue *lacZ* staining was documented in reflection mode using a 488 nm laser. Images were processed using the NIH ImageJ software and Adobe Photoshop CS. Optical sections through the E8 conceptus were obtained with a Leica TCS SP2 confocal microscope, using Leica LSC image acquisition software. Movies were produced with Marcomedia Director.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.07.005.

REFERENCES

Begley, C.G., Green, A.R., 1999. The SCL gene: from case report to critical hematopoietic regulator. Blood 93, 2760–2770.

Bockamp, E., Antunes, C., Maringer, M., Heck, R., Presser, K., Beilke, S., Ohngemach, S., Alt, R., Cross, M., Sprengel, R., Hartwig, U., Kaina, B., Schmitt, S., Eshkind, L., 2006. Tetracycline-controlled transgenic targeting from the SCL locus directs conditional expression to erythrocytes,

megakaryocytes, granulocytes, and c-kit-expressing lineage-negative hematopoietic cells. Blood 108, 1533–1541.

Constien, R., Forde, A., Liliensiek, B., Grone, H.J., Nawroth, P., Hammerling, G., Arnold, B., 2001. Characterization of a novel EGFP reporter mouse to monitor Cre recombination as demonstrated by a Tie2 Cre mouse line. Genesis 30, 36–44.

Cumano, A., Godin, I., 2007. Ontogeny of the hematopoietic system. Annu. Rev. Immunol. 25, 745–785.

Delabesse, E., Ogilvy, S., Chapman, M.A., Piltz, S.G., Gottgens, B., Green, A.R., 2005. Transcriptional regulation of the SCL locus: identification of an enhancer that targets the primitive erythroid lineage in vivo. Mol. Cell. Biol. 25, 5215–5225.

Dooley, K.A., Davidson, A.J., Zon, L.I., 2005. Zebrafish scl functions independently in hematopoietic and endothelial development. Dev. Biol. 277, 522–536.

Downs, K.M., Davies, T., 1993. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development 118, 1255–1266.

Drake, C.J., Fleming, P.A., 2000. Vasculogenesis in the day 6.5 to 9.5 mouse embryo. Blood 95, 1671–1679.

Drake, C.J., Brandt, S.J., Trusk, T.C., Little, C.D., 1997. TAL1/SCL is expressed in endothelial progenitor cells/angioblasts and defines a dorsal-to-ventral gradient of vasculogenesis. Dev. Biol. 192, 17–30.

D'Souza, S.L., Elefanty, A.G., Keller, G., 2005. SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development. Blood 105, 3862–3870.

Elefanty, A.G., Begley, C.G., Hartley, L., Papaevangeliou, B., Robb, L., 1999. SCL expression in the mouse embryo detected with a targeted *lacZ* reporter gene demonstrates its localization to hematopoietic, vascular, and neural tissues. Blood 94, 3754–3763.

Ema, M., Yokomizo, T., Wakamatsu, A., Terunuma, T., Yamamoto, M., Takahashi, S., 2006. Primitive erythropoiesis from mesodermal precursors expressing VE-cadherin, PECAM-1, Tie2, endoglin, and CD34 in the mouse embryo. Blood 108, 4018–4024.

Flamme, I., Frolich, T., Risau, W., 1997. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. J. Cell. Physiol. 173, 206–210.

Fraser, S.T., Isern, J., Baron, M.H., 2007. Maturation and enucleation of primitive erythroblasts during mouse embryogenesis is accompanied by changes in cell-surface antigen expression. Blood 109, 343–352.

Furuta, C., Ema, H., Takayanagi, S., Ogaeri, T., Okamura, D., Matsui, Y., Nakauchi, H., 2006. Discordant developmental waves of angioblasts and hemangioblasts in the early gastrulating mouse embryo. Development 133, 2771–2779.

Godin, I., Cumano, A., 2002. The hare and the tortoise: an embryonic haematopoietic race. Nat. Rev. Immunol. 2, 593–604.

Godin, I., Dieterlen-Lievre, F., Cumano, A., 1995. Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. Proc. Natl. Acad. Sci. USA 92, 773–777.

Gothert, J.R., Gustin, S.E., Hall, M.A., Green, A.R., Gottgens, B., Izon, D.J., Begley, C.G., 2005. In vivo fate-tracing studies using the Scl stem cell enhancer: embryonic hematopoietic stem cells significantly contribute to adult hematopoiesis. Blood 105, 2724–2732.

Gottgens, B., Barton, L.M., Gilbert, J.G., Bench, A.J., Sanchez, M.J., Bahn, S., Mistry, S., Grafham, D., McMurray, A., Vaudin, M., Amaya, E., Bentley, D.R., Green, A.R., Sinclair, A.M., 2000. Analysis of vertebrate SCL loci identifies conserved enhancers. Nat. Biotechnol. 18, 181–186.

Gottgens, B., Broccardo, C., Sanchez, M.J., Deveaux, S., Murphy, G., Gothert, J.R., Kotsopoulou, E., Kinston, S., Delaney, L., Piltz, S., Barton, L.M., Knezevic, K., Erber, W.N., Begley, C.G., Frampton, J., Green, A.R., 2004. The scl+ 18/19 stem cell enhancer is not required for hematopoiesis: identification of a 5′ bifunctional hematopoietic-endothelial enhancer bound by Fli-1 and Elf-1. Mol. Cell. Biol. 24, 1870–1883.

Haar, J.L., Ackerman, G.A., 1971. A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. Anat. Rec. 170, 199–223.

Huber, T.L., Kouskoff, V., Fehling, H.J., Palis, J., Keller, G., 2004. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature 432, 625–630.

Kallianpur, A.R., Jordan, J.E., Brandt, S.J., 1994. The SCL/TAL-1 gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. Blood 83, 1200–1208.

Kinder, S.J., Tsang, T.E., Quinlan, G.A., Hadjantonakis, A.K., Nagy, A., Tam, P.P., 1999. The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. Development 126, 4691–4701.

Lancrin, C., Sroczynska, P., Stephenson, C., Allen, T., Kouskoff, V., Lacaud, G., 2009. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature 457, 892–895.

Lecuyer, E., Hoang, T., 2004. SCL: from the origin of hematopoiesis to stem cells and leukemia. Exp. Hematol. 32, 11–24.

Liebner, S., Cattelino, A., Gallini, R., Rudini, N., Iurlaro, M., Piccolo, S., Dejana, E., 2004. Beta-catenin is required for endothelial-mesenchymal transformation during heart cushion development in the mouse. J. Cell Biol. 166, 359–367.

Lugus, J.J., Park, C., Choi, K., 2005. Developmental relationship between hematopoietic and endothelial cells. Immunol. Res. 32, 57–74.

Lux, C.T., Yoshimoto, M., McGrath, K., Conway, S.J., Palis, J., Yoder, M.C., 2008. All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. Blood 111, 3435–3438.

Medvinsky, A., Dzierzak, E., 1996. Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897–906.

Mikkola, H.K., Orkin, S.H., 2006. The journey of developing hematopoietic stem cells. Development 133, 3733–3744.

Palis, J., 2008. Ontogeny of erythropoiesis. Curr. Opin. Hematol. 15, 155–161.

Patterson, L.J., Gering, M., Patient, R., 2005. Scl is required for dorsal aorta as well as blood formation in zebrafish embryos. Blood 105, 3502–3511.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F.W., Orkin, S.H., 1996. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell 86, 47–57.

Redmond, L.C., Dumur, C.I., Archer, K.J., Haar, J.L., Lloyd, J.A., 2008. Identification of erythroid-enriched gene expression in the mouse embryonic yolk sac using microdissected cells. Dev. Dyn. 237, 436–446.

Rhodes, K.E., Gekas, C., Wang, Y., Lux, C.T., Francis, C.S., Chan, D.N., Conway, S., Orkin, S.H., Yoder, M.C., Mikkola, H.K., 2008. The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. Cell Stem Cell 2, 252–263.

Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R.P., Metcalf, D., Begley, C.G., 1995. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proc. Natl. Acad. Sci. USA 92, 7075–7079.

Saam, J.R., Gordon, J.I., 1999. Inducible gene knockouts in the small intestinal and colonic epithelium. J. Biol. Chem. 274, 38071–38082.

Samokhvalov, I.M., Samokhvalova, N.I., Nishikawa, S.I., 2007. Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. Nature 446, 1056–1061.

Sanchez, M., Gottgens, B., Sinclair, A.M., Stanley, M., Begley, C.G., Hunter, S., Green, A.R., 1999. An SCL 3′ enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors. Development 126, 3891–3904.

Sanchez, M.J., Bockamp, E.O., Miller, J., Gambardella, L., Green, A.R., 2001. Selective rescue of early haematopoietic progenitors in Scl(−/−) mice by expressing Scl under the control of a stem cell enhancer. Development 128, 4815–4827.

Shivdasani, R.A., Mayer, E.L., Orkin, S.H., 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 373, 432–434.

Sinclair, A.M., Gottgens, B., Barton, L.M., Stanley, M.L., Pardanaud, L., Klaine, M., Gering, M., Bahn, S., Sanchez, M., Bench, A.J., Fordham, J.L., Bockamp, E., Green, A.R., 1999. Distinct 5′ SCL enhancers direct transcription to developing brain, spinal cord, and endothelium: neural expression is mediated by GATA factor binding sites. Dev. Biol. 209, 128–142.

Soriano, P., 1999. Generalized *lacZ* expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.

Stadtfeld, M., Graf, T., 2005. Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. Development 132, 203–213.

Trowbridge, I.S., Omary, M.B., 1981. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc. Natl. Acad. Sci. USA 78, 3039–3043.

Ueno, H., Weissman, I.L., 2006. Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. Dev. Cell 11, 519–533.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., Lio, P., Macdonald, H.R., Trumpp, A., 2008. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118–1129.

Zovein, A.C., Hofmann, J.J., Lynch, M., French, W.J., Turlo, K.A., Yang, Y., Becker, M.S., Zanetta, L., Dejana, E., Gasson, J.C., Tallquist, M.D., Iruela-Arispe, M.L., 2008. Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell Stem Cell 3, 625–636.