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Citation for published version:
Li, Z, Vink, CS, Mariani, SA & Dzierzak, E 2016, 'Subregional localization and characterization of Ly6aGFP-expressing hematopoietic cells in the mouse embryonic head', Developmental Biology.
https://doi.org/10.1016/j.ydbio.2016.05.031

Digital Object Identifier (DOI):
10.1016/j.ydbio.2016.05.031

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Developmental Biology

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Short Communication

Subregional localization and characterization of Ly6aGFP-expressing hematopoietic cells in the mouse embryonic head

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1. Introduction

The generation of the hematopoietic cells by vascular endothelial cells in the mouse embryo has been demonstrated by lineage tracing (Chen et al., 2009; Zovein et al., 2008) and 3-dimensional static/live imaging studies of the embryonic aorta (Bertrand et al., 2010; Boisset et al., 2010; Kiss and Herbomel, 2010). Clusters of hematopoietic cells along the midgestation dorsal aorta and vitelline and umbilical arteries have been phenotypically characterized, imaged in situ and show a transition from the expression of endothelial markers such as VE-cadherin, Flk1 and Tie2 in the cells underlying the clusters, to the expression of hematopoietic markers CD41, ckit, CD45 and others in cluster cells (Robin et al., 2011; Rybtsou et al., 2011; Yokomizo and Dzierzak, 2010; Yokomizo et al., 2011). All cluster cells along these arteries express ckit and quantitative analyses show that the number of clusters peaks to about 650 at E10.5, when HSCs are first detected (Yokomizo and Dzierzak, 2010). Importantly, the Ly6aGFP marker defines all HSCs in the mouse midgestation AGM, aorta/vitelline/umbilical arteries and placenta, some cluster cells and underlying ventral aortic endothelial cells (de Bruijn et al., 2002; Ottersbach and Dzierzak, 2005) and time lapse imaging of the embryonic aorta shows that the Ly6aGFP expressing endothelial cells undergo endothelial-to-hematopoietic transition (EHT) (Solaimani Kartalaei et al., 2015). Other highly vascular tissues such as the yolk sac, placenta and embryonic head also generate hematopoietic cells (Li et al., 2012; Rhodes et al., 2008; Lux et al., 2008). Recently it has been shown that EHT occurs in the yolk sac to give rise to hematopoietic progenitor cells (Frame et al., 2016). Here we examine the head and the head vasculature of Ly6aGFP embryos for hematopoietic cells, HPC and HSC function and show that Ly6aGFP expression marks some vascular endothelial and hematopoietic cells and all HSCs, but find little evidence of multicellular hematopoietic cluster formation or characteristic of EHT.

2. Methods and materials

2.1. Mouse and embryo production

Ly5.1 female (6–8 week) mice and C57BL/6 mice were obtained (Charles River, Harlan). Ly6aGFP mice were maintained as hemizygotes on a C57BL/6 background, and transgenic embryos were phenotyped by tail GFP fluorescence. Day of plugging was
considered as embryonic day (E) 0. E10.5 corresponds to embryos with 34–40 somite pairs (sp); E11.5 with > 40 sp; E12.5 by eye pigmentation and limb webbing. Dissections and cell preparation were done as previously described (Medvinsky et al., 2008). The cell numbers at E10.5 for whole head were 7.8 ± 3.4 × 10⁶, for forebrain (FB) 2.3 ± 0.6 × 10⁵, for midbrain (MB) 1.0 ± 0.5 × 10⁵, for hindbrain and branchial arches (HBA) 3.2 ± 1.3 × 10⁵ and at E11.5 for whole head 4.8 ± 9.1 × 10⁶, for FB 1.5 ± 7.3 × 10⁵, for MB 4.4 ± 2.9 × 10⁵, for HBA 1.9 ± 1.0 × 10⁵. At E12.5 whole head contained 9.9 ± 1.3 × 10⁵ cells. All animal procedures were approved under UK Home Office regulations and performed in compliance with Standards for Care and Use of Laboratory Animals.

2.2. Hematopoietic progenitor and stem cell assays

Clonogenic analysis was performed on sorted cells plated in methylcellulose (M3434; StemCell Technologies). Hematopoietic colonies were counted at day 6 and 12. HSC activity of sorted or unsorted Ly5.2 head cells (various cell doses) was analysed by in vivo transplantation. Cells were intravenously coinjected with 2 × 10⁵ ly5.1 spleen cells into irradiated (9 Gy split-dose, γ irradiation) Ly5.1 recipients. After 16 weeks, donor chimerism (CD45.2) was analysed by flow cytometric analysis on blood after erythrocyte lysis (Beckman Coulter) and antibody staining (7-amino-actinomycin D or Hoechst staining for viability). Multi-lineage donor chimerism was analysed in recipient blood, bone marrow, spleen, lymph node and thymus with antibodies specific for macrophages (CD11b), granulocytes (Gr1), B (CD19) and T (CD3, CD4, CD8) lymphocytes and erythroid cells (Ter119). For secondary transplantsations, BM cells (3 × 10⁶) cells from primary recipients were injected into irradiated Ly5.1 recipients.

2.3. Immunostaining

Immunostaining was performed as previously described (Ling et al., 2004). E10.5, E11.5 and E12.5 Ly6aGFP embryos were fixed (2% paraformaldehyde/PBS, 4 °C, 1 h for E10 head, 2 h for E11.5 head and 2.5 h for E12 head). Embryonic heads were equilibrated in 20% sucrose/PBS at 4 °C overnight and then embed in the Tissue Tek before freezing. 10-μm cryosections were prepared. En- dogenous biotin activity was blocked by Avidin/Biotin blocking kit. The fixed head sections were incubated with primary antibodies (ckit (2B8), GFP, Runx1 (EP3099)) or secondary antibodies (Anti-Rabbit Alexa Fluor® 488 IgG (H+L), anti-rat Alexa Fluor 555 IgG (H+L), Anti-Rabbit Alexa Fluor® 647 IgG (H+L:1–2) into PBS-block (PBS containing 0.05% tween and 1% BSA) overnight and washed three times in PBS-T (PBS with 0.05% tween). Samples were stained with DAPI for 10 min, room temperature and then mounted with mounting buffer. Images were acquired with an inverted confocal microscope (Leica SP5) and processed using Leica AF Lite.

3. Results and discussion

3.1. Embryonic head contains Ly6aGFP expressing hematopoietic cells

Since the head is composed of large numbers of non-hematopoietic cells, we attempted to localize head hematopoietic cells by subdissection (Fig. 1A) according to developmentally defined regions – forebrain (FB), midbrain (MB) and hindbrain (with attached brachial arches: HBA). In addition, we used the Ly6aGFP transgenic mouse model (de Bruijn et al., 2002; Ma et al., 2002) as a localization and potential enrichment marker for head hemogonic endothelium and HSCs. Analysis of the whole E11.5 head and the three head subregions showed large numbers of CD45+ and F4/80+ cells. The percentages of these cells was similar between the 3 subregions (Fig. 1B and C) and are likely to be yolk sac tissue resident macrophages that are dispersed throughout the brain as others have reported (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015). No Gr1+ cells were detected.

To more specifically examine the hematopoietic and endothelial compartments, Ly6aGFP transgenic embryos were examined. Whole head confocal imaging (Fig. 1D) showed the strongest GFP expression along some areas of the major head vasculature, such as the rostral extension of the dorsal aorta (carotid artery (arrow)) and single GFP+ cells scattered throughout the head. At E10.5, E11.5 and E12.5 flow cytometric analyzes detected 0.20 ± 0.04%, 0.39 ± 0.03% and 0.97 ± 0.16% GFP+ cells respectively (n = 3) in whole head cell suspensions (Fig. 1E). Other markers such as ckit and/or CD31 that characterize aortic hematopoietic cluster cells and endothelial cells were found by flow cytometric analysis to be expressed by some cells of the embryonic head. At E10.5 1.26 ± 0.42% and E11.5 0.52 ± 0.07% of head cells were ckit+, and 1.89 ± 0.81% of E10 and 1.03 ± 0.13% of E11 head cells were CD31+ (n = 3). Interestingly, the frequency of CD31+ ckit+ GFP+ cells (phenotypic HSCs) increased between E10.5 and E11.5 (Fig. 1F and G). These frequencies were not different between the subregions indicating that the phenotypic hematopoietic cells were distributed throughout the embryonic head.

3.2. HPCs in the embryonic head are predominantly Ly6aGFP negative

Ly6aGFP is a distinguishing marker of the most immature hematopoietic cells as they are generated in the mouse embryo. It is expressed by all HSCs (de Bruijn et al., 2002; Ma et al., 2002), about 30% of HPCs (Solaimani Kartalaei et al., 2015) and progenitors with lymphoid potential in the E10.5/E11.5 AGM (Li et al., 2014). To examine whether head HPCs express this marker, E10.5 and E11.5 whole head and subregions were isolated from Ly6aGFP embryos and sorted into GFP+ and GFP− fractions. Of the 271 CFU-C obtained from the E10.5 whole head (Fig. 2A), majority (78%) were in the GFP− fraction. Although few CFU-C were found in the GFP+ fraction, all colony types were found in both the GFP+ (except CFU-E) and GFP− fractions. CFU-C localized to all three subregions and were in equal distribution when the total number of cells in each region was considered (see legend to Fig. 1A).

In the E11.5 whole head the total number of CFU-C increased by 9–12 fold (3287) as compared to E10.5 (Fig. 2B). The GFP− fraction still contained most (77.2%) of the CFU-C (2538). However, the number of CFU-C in the GFP+ fraction increased by a factor of 10. Over 80% of the CFU-C, both GFP+ and GFP−, are localized to the FB and HBA regions. The GFP+ fractions (whole head and sub-regions) contained about 78.5% of the CFU-Mix, whereas the GFP− fraction contained all head CFU-E, and most BFU-E and CFU-M+GM (Fig. 2C). The fact that such a large percentage of CFU-C are GFP− is agreement with the data of others supporting the notion that yolk sac derived primitive progenitors and EMPs colonize the brain, and that these cells are Sca1 negative (McGrath et al., 2015). These head results are similar to AGM data showing that Ly6aGFP expression correlates to cells with more immature hematopoietic potential and suggest that changes in the hematopoietic cell composition could be related to in situ hematopoietic cell generation.

3.3. HSCs localize to HBA and FB, and are exclusively Ly6aGFP positive

Previously, we demonstrated by the in vivo transplantation...
Fig. 1. Localization of phenotypic hematopoietic and Ly6aGFP expressing cells in the embryonic head. (A) Subregional dissection of an E11.5 mouse head into the forebrain (FB), midbrain (MB) and hindbrain with brachial arches (HBA). Number of cells x 1000 per E10.5 and E11.5 whole head = 78.2 ± 34.7 and 484 ± 91.7; FB = 23.9 ± 6.3 and 154.0 ± 73.4; MB = 10.0 ± 4.8 and 43.79 ± 29.0; HBA = 32.2 ± 13.4 and 190.7 ± 101.2 respectively. (B) Representative flow cytometric cell density plots for E10.5 whole head single cell suspensions. Viable CD45 positive cells (against forward scatter (FSC)) are gated and analysed for F4/80 and Gr1 expression. (C) Bar graph showing percentages of CD45+ cells are shown for whole head (WH, blue), forebrain (FB, red), midbrain (MB, green) and hindbrain with arches (HBA, purple). n = 3. Mean ± SD. nd = not detected. (D) Confocal image of whole mount E11.5 Ly6aGFP head (right halfsection) showing GFP expression (green fluorescence) in the carotid artery (CA, arrowhead). An outline around the boundaries of the head is drawn to facilitate orientation. (E) Representative flow cytometric cell density plots for non-transgenic and Ly6aGFP E10.5, E11.5 and E12.5 whole head single cell suspensions. Percentages of GFP+ cells are shown. n = 3. (F) Representative flow cytometric density plots showing percentages of ckit+CD31+ cells and GFP+ cells in the gate indicated. (G) Bar graph showing the percentage of ckit+CD31+GFP+ cells in E10.5 and E11.5 whole head (blue) and head subregions FB (red), MB (green) and HBA (purple). n = 3 Mean ± SD.
 assay that HSCs are present in the E10.5 head at limiting numbers, and that head HSC numbers increase at E11.5 and E12.5 (Li et al., 2012). To examine whether HSCs are localized to any of the head subregions, we transplanted E11.5 FB, MB and HBA cells and measured donor cell hematopoietic engraftment at 16 weeks post-injection (Fig. 3A). The most robust and frequent engraftment was found with HBA cells, with 33% of recipients showing multilineage chimerism (36.5 ± 32.8%). HSCs were found also in the FB, but at a lower frequency (25%) and yielded lower chimerism levels (13.6 ± 11.6%). Only rare, very low engraftment (0.1%, 3.3%) was found from MB cells. Secondary transplantations performed with HBA-repopulated primary recipient BM (71.5% and 45.4% donor-derived), showed an average of 8.7 ± 2.5% (n = 3) and 14.9 ± 4.6% (n = 3) long-term donor-derived engraftment, thus demonstrating that HBA HSC are self-renewing.

To examine whether head HSCs are Ly6aGFP expressing, E11.5 and E12.5 GFP⁺ and GFP⁻ head cells were injected into irradiated adult recipients. At 16 weeks post-transplantation only GFP⁺ cells provided high level, multilineage chimerism (22.6 ± 10.8%; Fig. 3C). These HSCs were self-renewing as determined by secondary transplantations of BM from primary (30.5% and 10.3% donor-derived), showed an average of 8.7 ± 2.5% (n = 3) and 14.9 ± 4.6% (n = 3) long-term donor-derived engraftment, thus demonstrating that HBA HSC are self-renewing.

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To localize HP/SCs, Ly6aGFP head sections were immunostained with antibodies specific for GFP, Runx1 and ckit, and confocal imaging was performed. We focused on the HBA because the carotid arteries were GFPhigh expressing (Fig. 1D) and HSC activity was found in this subregion. E10.5 (Fig. 4A), E11.5 (Fig. 4B) and E12.5 (Fig. 4C) HBA sections showed Ly6AGFPhigh expressing cells along the wall of the carotid arteries (CA). These vascular endothelial cells did not express ckit or Runx1, and is in agreement with the findings of others (Iizuka et al., 2016). GFPlowRunx1⁺ cells were found scattered through the branchial arches (BA) of E10.5 heads (Fig. 4A). In some areas (Fig. 4Ai) the majority of these cells were ckit⁻ (enlarged inset, orange arrowheads). In another section from the same embryo (more posterior), the majority of the GFPlowRunx1⁺ cells were cKit⁺ (Fig. 4Aii). Interestingly, some of the GFPlowRunx1⁺ ckit⁺ cells are clustered (enlarged inset, white arrowheads), and they appear to be outside the vessels (Fig. 4Aii). GFPlowRunx1⁺ ckit⁺ cells were also found surrounding the neuroepithelium (NE) and the roof of the hindbrain (Fig. 4A, low magnification panels).

By E11.5, the head is much larger. GFPlowRunx1⁺ ckit⁺ cells were observed as single cells near and within the vein lateral to the CA (Fig. 4Bi, white arrowheads). Some GFP⁺ Runx1⁺ ckit⁻ cells are also found in this area (Fig. 4Bii, orange arrowheads). Interestingly,
Fig. 3. Head HSCs are GFP⁺ and localized to the HBA and FB. (A) Percentage donor cell chimerism in the peripheral blood of adult Ly5.1 recipients injected with Ly5.2 cells (4–7 embryo equivalents (ee)/recipient) from E11.5 forebrain (FB, red), midbrain (MB, green) and hindbrain with brachial arches (HBA, purple) at 16 weeks post-transplantation. n=3. Chimerism was analysed in peripheral blood by flow cytometry for CD45.2. (B) Hematopoietic repopulation by E11.5 (blue squares) and E12.5 (green dots) Ly6GFP head cells. GFP⁺ and GFP⁻ sorted head cells were injected into irradiated adult Ly5.1 recipients and percentage donor chimerism (Ly5.2) determined by flow cytometry at 16 weeks post-transplantation. 0.5–1.8 ee of E11.5 (n=5) and 0.5–2.5 ee of E12.5 (n=3) were injected per recipient. E11.5 AGM GFP⁺ cells were injected as a control (1.1–2.15 ee/recipient, n=5). Horizontal lines indicate mean chimerism of engrafted mice (only those mice with 5% or greater donor chimerism were considered engrafted. (C) Representative multilineage repopulation analysis of a head GFP⁺ recipient mouse (30.5% engrafted) in panel B. Flow cytometric analysis for percentage of donor-derived (CD45.2⁺) hematopoietic cells in peripheral blood, BM, thymus, lymph node and spleen is shown. Gated CD45.2⁺ cells were analysed for myeloid lineage cells (granulocytes, Gr1⁺ and macrophages, CD11b⁺), B cells (CD19⁺), T cells (CD3⁺) and erythroblasts (Ter119⁺). FSC=forward scatter.

Please cite this article as: Li, Z., et al., Subregional localization and characterization of Ly6aGFP-expressing hematopoietic cells in the mouse embryonic head. Dev. Biol. (2016), http://dx.doi.org/10.1016/j.ydbio.2016.05.031
Fig. 4. Phenotypic hematopoietic cells are not in clusters. (A) Schematic drawing of an E11.5 head with diagonal lines indicating the plane of transverse sections in the confocal fluorescent images. Confocal images of immunostained Ly6aGFP E10.5 (A, 33 somite pairs (sp)), E11.5 (B, 46 sp) and E12.5 (C) hind brain/branchial arch (HBA) head sections. Ly6aGFP (green), Runx1 (magenta), ckit (red), and DAPI (blue) fluorescent images are shown individually and/or merged. Enlarged images of the boxed areas (Ai, Aii, Bi, Bii, Biii and Ci) are shown for GFPlowRunx1⁺ ckit⁺ cells (white arrowheads) and GFPlowRunx1⁺ ckit⁻ cells (orange arrowheads) cells. Bar = 50 μm. E10.5 (n = 2 embryos; 33 and 34 sp); E11.5 (n = 3 embryos; 43, 45, 46 sp); E12.5 (n = 2 embryos). CA = carotid artery, BA = branchial arches, NE = neuroepithelium, V = ventricle, CV = cardinal vein.
the ventricle of the hindbrain contains both single GFP⁺ Runx1⁺ ckit⁺ hematopoietic cells (Fig. 4Bii, white arrowheads) and cluster-like GFP⁺ Runx1⁺ ckit⁺ cells (Fig. 4Biii, asterisks), which are along the ventricle face of the neuroepithelial layer. In the E12.5 HBA, GFP⁺ Runx1⁺ ckit⁺ cells are found in the ventricle cavity and tongue primordia, and single GFP⁺ Runx1⁺ ckit⁺ and GFP⁺ Runx1⁺ ckit⁻ cells are scattered throughout the branchial arches (data not shown). The region lateral to the GFP⁺ cardinal vein (CV) contains single GFP⁺ Runx1⁺ ckit⁻ cells (Fig. 4Ci).

The variety of cell types in the embryonic head confounds the identification and localization of the emerging cells of the hematopoietic system. No GFP⁺Runx1⁺ ckit⁻ cells were found in the lumen or closely associated with the wall of the carotid artery. Despite the lack of arterial clusters, it is possible that the single phenotypic HP/SCs (expressing Ly6aGFP, Runx1 and ckit) scattered throughout the HBA sections are in capillary beds/sinusoidal areas or veins which are not visible in these images. Interestingly, we found hematopoietic cells clustered in an ablumenal location near the cardinal vein (Fig. 4Aii).

Although SP-A Cre directed RosaSTOPLacZ recombination showed LacZ expression in vascular cells (CD31⁻) in the E12.5 embryonic head (Li et al., 2012), it is as yet uncertain how these HSCs are generated. Similar to the aorta (de Bruijn et al., 2002), we found here that Ly6aGFP is strongly expressed in the carotid arteries within the HBA and marks functionally potent HPC and HSC. The fact that phenotypic HP/SCs stand alone, isolated and without a special association with vasculature suggests that HP/SC generation in the head occurs differently. EHT may occur infrequently in the head and cells may immediately home to other niches. The zebrafish embryo models such an alternative mode of HP/SC generation and EHT. In the production of hematopoietic cells, aortic endothelial cells bulge in an ablumenal direction (Kiss and Herbold, 2010) into the subaortic space, rather than the luminal direction as in the mouse aorta (Boisset et al., 2010). No hematopoietic clusters form and these cells move immediately to the circulation in the zebrafish axial vein (Kiss and Herbold, 2010).

Others have shown that the early head-fold and neuroectodermal cells possess hematogenic potential if stimulated by morphogens (Baron, 2001; Dyer et al., 2001; Kanatsu and Nishikawa, 1996) and hence our result showing GFP⁺ Runx1⁺ ckit⁺ cells along the neuroepithelium is intriguing. Hence, the process of head HP/SC generation is likely to be different, or alternatively, EHT may be too infrequent/rapid to be observed. In this regard, it is of interest to further understand the microenvironment (morphogens, shear stress/pressure, metabolism, and oxygen levels) of the head and how it promotes hematopoietic cell development. Knowledge of what factors and the balance of the factors necessary for the efficient production of HPC and HSC from the head as compared to the aorta and other hemogenic tissues such as the yolk sac and placenta, should inform current approaches attempting HSC production ex vivo.

Authorship contributions

ZL performed research. CSV and SAM performed/analysed flow cytometric data and CSV provided mouse support. ZL and ED designed experiments, analysed and interpreted data. ZL and ED wrote the manuscript.

Acknowledgements

We thank members of the lab for critical comments on this work, S. Johnston and W. Ramsay for flow cytometric support. This research was supported NIH (RO37 DK54077), ZonMW TOP (91211068) and ERC Advanced Grant (341096). The authors declare no conflicts of interest.

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