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Imaging the founder of adult hematopoiesis in the mouse embryo aorta

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Hematopoietic stem cells (HSCs) are multipotent and capable of self-renewal. Therefore, HSCs can produce and replenish all blood cell types during the entire life, with no HSC pool depletion. HSCs are the key element for long-term clinical therapies in blood-related genetic diseases and leukemia. But HSC clinical use and fundamental research are impeded due to the limiting number of HSCs available in tissues. Moreover, HSCs are very difficult to maintain or expand ex vivo without loss of stem potential, and so far impossible to generate in vitro. Thus it is essential to identify the mechanisms controlling HSC commitment and fate to develop new strategies for in vitro HSC generation/expansion and fate manipulation.

Adult HSCs are mainly located in the bone marrow (BM) where they are in close contact to osteoblasts (endosteal niche) and vascular/perivascular cells (vascular niche). Such niches create a protective microenvironment against apoptotic and differentiation stimuli, and maintain the pool of HSCs quiescent. Adult HSCs are not generated in the BM but during embryonic development. There, HSCs will transit via several hematopoietic sites that offer a suitable microenvironment for HSC generation, survival and expansion. In mouse embryo, adult-type HSCs are first detected, starting at precise embryonic day (E)10.5, in the dorsal aorta of the Aorta-Gonad-Mesonephros (AGM) region (as shown by transplantation into irradiated wild-type adult recipients). Slightly later, HSCs are also found in the yolk sac, as well as in the placenta and fetal liver, two important HSC reservoirs where the cells massively expand before to colonize the BM before birth.[1] The precise anatomical and cellular origin of HSCs has been questioned over the past decades. Interestingly, clusters of cells are found attached to the aortic endothelium of many different embryo species (including human). These cells co-express endothelial and hematopoietic markers, strongly suggesting a developmental relationship between both lineages. Moreover, mouse embryos lacking transcription factors known to be crucial for HSC production (e.g., *Runx1* knock-out) are devoid of intra-aortic hematopoietic clusters. All these observations have raised the hypothesis that HSCs are first generated in the aorta and reside in the clusters. First demonstrated in chicken embryos,[2] recent studies performed in vitro and in vivo in mouse have also shown that specialized endothelial cells, so-called hemogenic, are most likely the direct precursors of adult-type HSCs.[3-6] However, one question remained: does the hemogenic endothelium-to-HSC transition actually occur in the aorta?

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Embryo opacity and in utero development has impaired so far the direct in vivo observation of the deeply located aorta. To bypass these obstacles, we developed cutting procedures of non fixed embryos to access and image the aorta by using a confocal microscope.[7] We either removed the somites to image the aorta (located just behind) from the dorsal perspective (**Fig. 1A-a**) or we cut the embryo transversally into thick slices to image the aorta from an anterior/posterior perspective (**Fig. 1A-b**). The aortic endothelium was stained, prior confocal imaging, with antibodies (directly conjugated with fluorochrome) against CD31. For imaging, *Ly-6A*(Sca1)*-GFP* transgenic and *CD41-YFP* knock-in reporter mouse lines were used to visualize HSC generation (as both markers are expressed by HSCs).[8,9] Embryonic tissues were imaged directly under a confocal microscope for periods of up to 15 h. We observed Ly-6A-GFP$^+$ cells budding directly from the ventral aortic endothelial floor into the lumen side (**Fig. 1B**). All newly generated cells were stained for CD31, proof of their endothelial origin (knowing that the aortic endothelium was stained only ones and prior imaging). In addition, we stained the sections at the end of imaging with antibodies against c-kit, a reliable marker of embryonic HSCs. It appeared that the newly generated cells co-expressed Ly-6A-GFP, CD31 as well as c-kit. Another interesting marker is CD41 (αIIb integrin) because it is most likely the first marker of hematopoietic commitment during development. Using *CD41-YFP* embryos, we observed the emergence of cells that started to express YFP (indicative of CD41 expression) as they were budding off the aortic endothelium (YFP$^-$). The newly formed CD41-YFP$^+$ cells also co-expressed CD31 and c-kit. Although the frequency of emergence event is extremely low (<2 per embryo), it correlates with the number of HSCs per aorta as estimated by transplantation experiments. Importantly, no cell emergence was ever observed in embryos younger than E10.5 (<33 somite pairs) or in aortas from *Runx1* knockout embryos, where no adult-type HSC activity is detected. Moreover, cells were generated on the ventral aspect of the aorta where HSC activity is restricted. Altogether, our data imply that adult-type HSCs are

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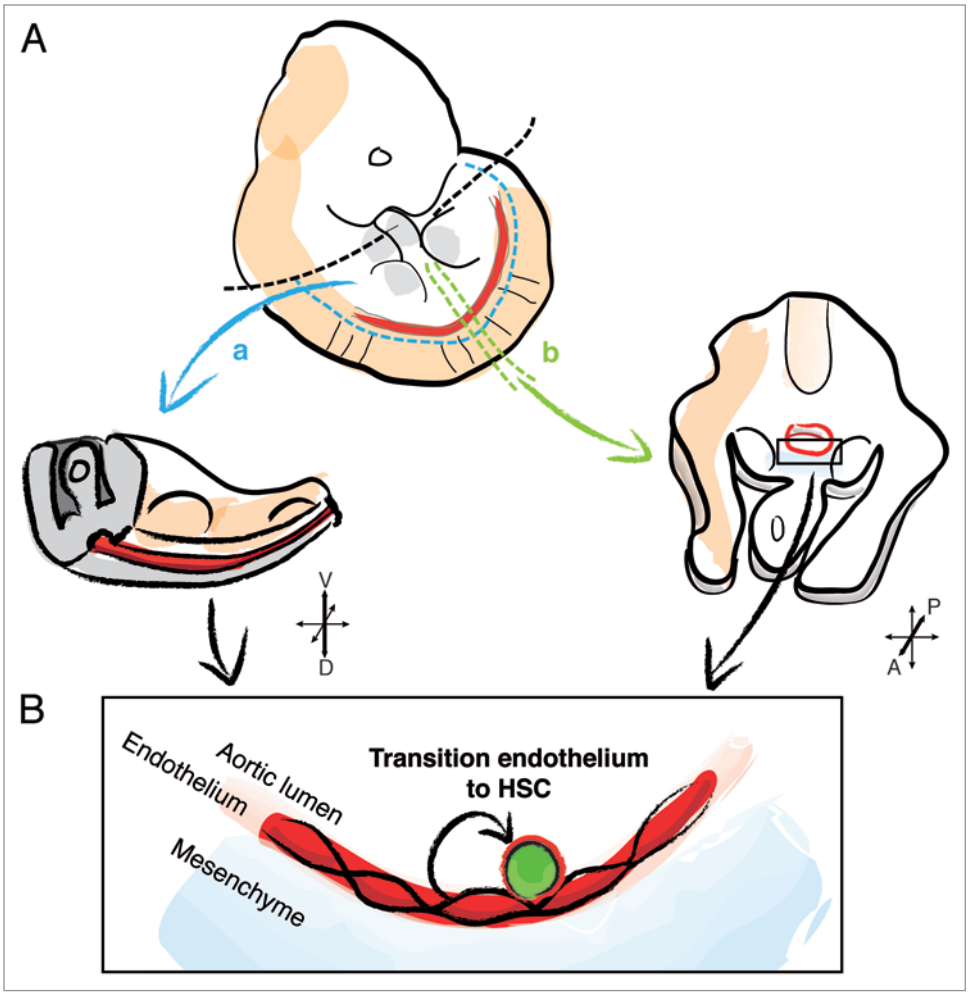

Figure 1. Protocol to image the transition hemogenic endothelium into hematopoietic stem cells in live mouse embryo aorta. (A) Embryo head and tail is first removed (black dashed line). Then the aorta of embryos (E10.5) is accessible for imaging after cutting away the somites (a, blue dashed line) or by slicing the embryos into transversal thick slices (b, green dashed line). (B) Schematic representation of the transition hemogenic endothelium into hematopoietic stem cells in the ventral part of the aorta. Arrows represent the axis of imaging according to the embryo/embryo slice orientation (V: ventral, D: dorsal, A: anterior, P: posterior).

directly generated from hemogenic endothelial precursors located in the ventral part of the mouse embryo aorta.[7]

Two other studies performed in live zebrafish embryos corroborate our conclusion.[10,11] Both groups showed, using time-lapse in vivo imaging, the emergence of HSCs from the ventral endothelial floor of the aorta. These cells (as defined by Lmo2, cmyb, CD41 and/or kdrl expression) were at the origin of the blood system in adults. Hematopoietic cells appeared to emerge in the space between the aorta and the caudal vein: the zebrafish AGM equivalent, before to reach the vein blood flow. Interestingly, the endothelial-HSC transition in zebrafish does not involve asymmetrical division of hemogenic endothelial cells but important cell bending and remodelling.[11] The process behind HSC generation remains unclear in the mouse aorta. Another interesting question is whether extra-embryonic sites (e.g., yolk-sac or placenta) are also capable of HSC production via an endothelial transition (similar to the aorta). Now that the dynamic emergence of HSCs can be observed directly in vivo, it will assist in understanding the complex mechanisms involved in HSC birth.

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