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Review article

Embryonic hematopoiesis under microscopic observation

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ABSTRACT

Hematopoietic stem cells (HSCs) are at the origin of adult hematopoiesis, providing an organism with all blood cell types needed throughout life. During embryonic development a first wave of hematopoiesis (independent of HSCs) allows the survival and growth of the embryo until birth. A second wave of hematopoiesis that will last into adulthood depends on the production of HSCs that begins at mid-gestation in large arteries such as the aorta. HSC production occurs through a hemogenic endothelial to hematopoietic transition (EHT) process and the formation of hematopoietic clusters in most vertebrate species. Advances in understanding EHT, cluster formation and HSC production were triggered by combined progresses made in the development of *in vivo* assays, microscopy, imaging and fluorescence tools. Here, we review the current knowledge on developmental hematopoiesis with a focus on the first step of HSC production in the aorta and how microscopic approaches have contributed to a better understanding of the vital process of blood cell formation.

1. Introduction

The initial discovery of blood producing cells concurs with the progresses made in the field of microscopy in the early 20th century. Modern microscopes allowed direct visualization of individual cells and tissue structure leading to important scientific dogma. On the basis of his observations, the German pathologist Franz E.C. Neumann identified in the mid 19th century the bone marrow as the site of adult hematopoiesis and proposed the concept of a unique cell at the foundation of the entire blood system. The Russian scientist Alexander A. Maximow coined the term "hematopoietic stem cell" (HSC) for this very particular cell type (Maximow, 1909). At the same time, another partisan of the stem cell concept, Vera Dantschakoff, observed clusters of "hemoblasts" attached to the endothelial layer of the aorta of a chicken embryo (Dantschakoff, 1909). Those clusters rapidly appeared as a common feature of early vertebrate development since they were observed in the aorta of most animal species, including human (Dieterlen-Lievre et al., 2006; Jordan, 1917). The close proximity of clusters to the endothelium soon led to the hypothesis that cluster cells might be originating from or descendants of the endothelium. Finding the origin of clusters, the process of their formation and the connection between clusters and HSCs has been the focus of intense research for decades. In this review, we will report the current knowledge on HSC-independent and dependent hematopoiesis as it occurs during embryonic development and how microscopic (besides cellular and molecular) approaches have been crucial to better understand blood formation.

2. How microscopy development and fluorescence discovery have revolutionized developmental research

The development of microscopes and the discovery of fluorescence have been crucial to pave the way to our current research discoveries and techniques, especially in the field of developmental biology. Formally, the history of microscopy begins in the first century when glass was invented and the Romans discovered that primitive glass lenses could magnify objects (Fig. 1). In the 13th century, Salvino D'Armate made the first eye glass. The Dutch lens grinders Zacharias Jansen and his father built the first compound microscope featured with several lenses connected to a hollow cylinder (originally magnifying 3×–9×) in 1590–1595. However, it was in the late 17th century that the Dutch tradesman and scientist Antoni van Leeuwenhoek first made and used a microscope with an impressive (for his time) magnifying power of 270X. While the early microscopes were mainly used for merriments, van Leeuwenhoek investigated unicellular (such as bacteria and yeast) and multicellular organisms. Among his many important discoveries was the blood corpuscules that he could observe circulating in the tail capillaries of a living fish, emphasizing the close relation between the discoveries made in the hematopoietic field and microscopy. Van Leeuwenhoek's work was confirmed and further advanced by the English scientist Robert Hooke who published the first work of microscopic studies in 1665 (Title: "Micrographia: or some physiological descriptions of minute bodies made by magnifying glasses with observations and inquiries thereupon"). Over the two next

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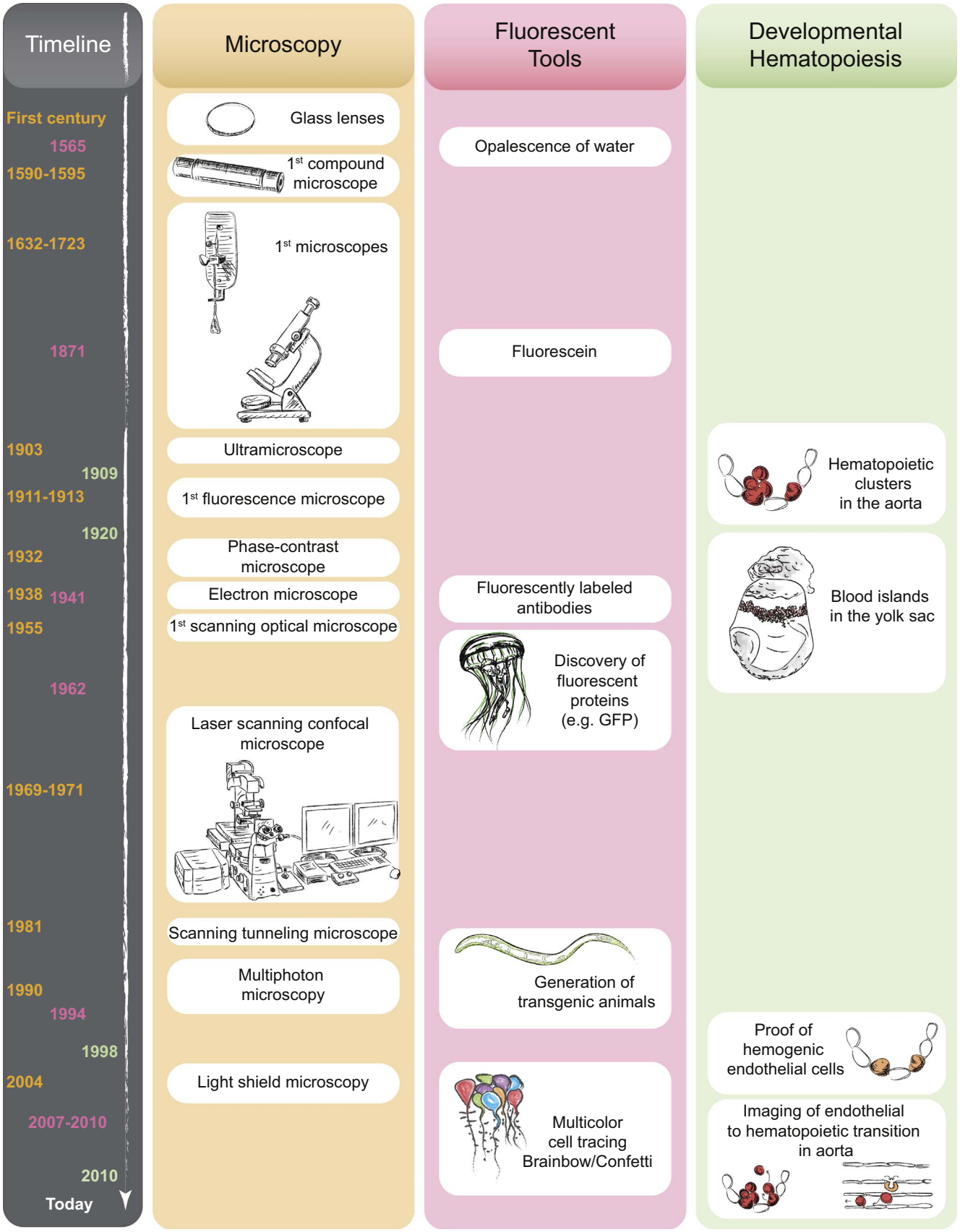

Fig. 1. Historic timeline. Chronological list of particularly important or significant inventions and discoveries in microscopy that allowed crucial observations to advance the field of developmental hematopoiesis.

centuries the optics and handling of microscopes improved, especially by the work of the German engineer Carl Zeiss, making microscopes more popular among the scientific world. Most of cell biological findings were only permitted through the development of modern compound microscopes. Among them were the ultramicroscope (with a resolution below the wavelength of light, invented in 1903 by Richard Zsigmondy), the phase-contrast microscope (allowing imaging of colorless or transparent biological samples, in 1932 by Frits Zernike), the electron microscope (with a higher resolving power than light microscopes allowing to investigate the ultrastructure of a wide range of specimens, in 1938 by Ernst Ruska) and the scanning tunneling microscope (allowing 3D imaging down to the atomic level, in 1981 by Gerd Binnig and Heinrich Rohrer) (Fig. 1).

Fluorescence microscopes were initially developed by Otto Heimstaedt and Heinrich Lehmann in 1911–1913 as a development of the UV microscope. They were mainly used to observe the auto-fluorescence of bacteria, plant and animal tissues. In 1955, Marvin Minsky built the first working scanning optical microscope (Minsky, 1988), a predecessor of the present widely used confocal laser scanning microscope. Unfortunately, it was not sensitive enough for fluorescence and the image resolution in biological specimens was very poor (due to the movement of the stage, creating vibration artifacts). It was only in the 1970s that fluorescence found a wide variety of applications in research with the first commercially available laser scanning confocal microscope (Amos and White, 2003; Davidovits and Egger, 1969). White and colleagues provided the first detailed and convincing confocal imaging of various biological structures such as microtubules, chromosomal structures and neuronal dendrites, with a tremendous improvement compared to conventional imaging (White et al., 1987). Confocal microscopy offers many advantages such as the elimination of out of focus light by placing a pinhole in front of the light source and a better resolution. Confocal microscopy also permits to collect serial optical sections from thick specimens, either fixed or living cells and tissues labeled with one or more fluorescent probes, for later 3D reconstructions.

Major advancements in living cell and organism imaging have been made by improving the photon efficiency and thereby by decreasing phototoxicity. These progresses led to a new field of research, the live cell confocal imaging that is acquired not only in 3D, but also over time in a time-lapse manner (multidimensional imaging) (Mohler and White, 1998; Paddock, 2001). Another important technical advancement was the multiphoton microscopy in which fluorophores are excited with two or more photons with lower energy to induce an excited state that was commonly reached by exciting with one photon of higher energy (Denk et al., 1990; Svoboda and Yasuda, 2006; Zipfel et al., 2003). The pinhole is no longer required for the confocal effect as the laser only excites at the point of focus. By decreasing phototoxic effects, the cell viability is improved. Also, the penetration efficiency in thick samples is 2–3 times deeper than with single photon imaging (Helmchen and Denk, 2005). More recently, a technique was developed that allows the imaging of living samples from different angles at a subcellular resolution, the scanned light-sheet microscopy (Huisken et al., 2004; Keller et al., 2010). Optical sectioning is hereby achieved with a thin sheet of laser light with the objective and detector perpendicular to it. The advantage of light-sheet microscopy is low phototoxicity with a high acquisition speed. In 2008, all nuclei of a developing zebrafish embryo were imaged during the first 24 h of development (Keller et al., 2008). More recently, a precise cellular map of the vascular system was obtained in the brain of a zebrafish embryo (Park et al., 2015). The light-sheet fluorescence microscopy now provides the toolbox to visualize the dynamic process of morphogenesis without perturbing the development of the biological sample, opening new exciting areas to *in toto* image embryogenesis (at least in transparent specimens).

Concomitant with the development of microscopes was the discovery and understanding of the fluorescence phenomena. The first "fluorescent" observation was most likely made by the Spanish physician and botanist Nicolás Monardes who described a bluish opalescence of water after infusion of a small Mexican tree wood in 1565 (Fig. 1). Such infused water became also popular for its medicinal virtues. The first fluorescent compound identified by the German chemist Adolph von Baeyer was fluorescein in 1871. However, it is only in 1941 that the field of immunofluorescence started with Albert Coons who first labeled antibodies with FITC (Fluorescein isothiocyanate). Shimomura, Johnson and Saiga discovered the Green Fluorescent Protein (GFP) in the Aequorea victoria jellyfish in 1962, which became the most widely used protein in biochemistry and cell biology (Shimomura et al., 1962; Tsien, 1998). GFP was first used in C. elegans as a genetically stable fluorescent marker that could be followed and imaged by confocal microscopy (Chalfie et al., 1994). Since then, a large variety of engineered GFP derivatives (Heim and Tsien, 1996), spectral variants of GFP as blue, yellow and cyan fluorescent proteins and others such as DsRed have been generated and extensively used as markers of gene expression and protein targeting in intact cells and organisms. Fluorescent proteins continue to be optimized, resulting in a multitude of fluorescent colors now commercially available and the possibility to combine fluorescent light microscopy with electron microscopy (Bos et al., 2015; Giepmans et al., 2006). A great example of these advancements was the spectacular multicolor imaging of living neurons obtained in a single sample with up to 90 different colors by using the *Brainbow* technique with a laser scanning confocal microscope equipped with a spectral detector (Livet et al., 2007). Multicolor labeling in living animals has become an important practical tool for developmental research.

3. Unraveling embryonic hematopoiesis, a success story of microscopic advances

Advances in the field of hematopoiesis and HSC research have often been triggered by important microscopic observations. For example, a close look at human and rabbit bone marrow saps has led Ernst E.C. Neumann to think that the bone marrow was the seedbed of red blood cell (Neumann, 1868) and leukocyte formation. He also suggested the existence of a common stem cell for all blood cells. It was in complete opposition with the view of dualists such as Ehrlich at the time, who favored the existence of some lymphocytes with separate cell lineages. Decades of subsequent research proved the presence of HSCs and their central role to replenish the entire blood system upon transplantation. Indeed, HSCs are endowed with a multilineage potential that allows single HSCs to produce all cells from both myeloid and lymphoid lineages. They are also able to self-renew to maintain a constant pool of HSCs throughout the entire existence of an organism. Long thought to be responsible for blood homeostasis, it was recently shown that a large number of long-lived progenitors would in fact be responsible for the steady-state blood production throughout life (Sun et al., 2014). However, it remains debated to present (Biasco et al., 2016; Notta et al., 2016), indicating that the HSC research field is more than ever in the spotlight. As Neumann postulated, most adult HSCs reside in the bone marrow in mammals. It is also the case in avian. However, HSCs reside in the kidney (referred to as kidney marrow) in fish. Yet, HSCs are initially generated during embryonic development where they transit through different anatomical sites before to reach the bone marrow. Embryonic hematopoiesis has been extensively studied in various animal species (fish, amphibian, avian, rodent and other mammals). In the following paragraphs, we will focus on the advances made in this exciting field by the continuous improvement of imaging techniques, with a particular focus on the chicken, zebrafish and mouse models.

3.1. The yolk sac is a site of HSC-independent hematopoietic waves

The mesodermal layer of the extra-embryonic yolk sac is the site where the first waves of hematopoiesis occur in mouse and chicken embryos. Careful microscopic observation of the chicken yolk sac first revealed a progressively flattening cell population, the endothelial cells, lining red corpuscles forming the so-called blood islands (Sabin, 1920). The close association of endothelial and erythroid cells in the blood islands led early embryologists to hypothesize that they were derived from bipotent progenitors. At the beginning of the 21st century, these cells were described first as angioblasts and later as hemangioblasts with the property to generate both vessels and hematopoietic cells in the mouse and chicken models (Murray, 1932; Sabin, 1920). However at the early-streak stage, erythropoietic precursors of the yolk sac emerge earlier than the bulk of the vitelline endothelium therefore suggesting that these lineages may arise independently (Kinder et al., 1999). Fate-mapping (Ueno and Weissman, 2006) and cell labeling (Padron-Barthe et al., 2014) studies indeed revealed that most blood islands in the mouse yolk sac are not clonal and only very few truly derive from hemangioblasts.

The first wave, also referred to as primitive erythropoiesis, leads to the production of primitive erythroblasts in the blood islands, starting at embryonic day (E)2 of chicken and E7.25 of mouse development (Haar and Ackerman, 1971; Jaffredo and Yvernogeau, 2014; Maximow, 1909; Moore and Owen, 1965) (Fig. 1). The mouse primitive erythroblasts, which express embryonic globins, are released in the circulation when the heart starts beating and begin to enucleate at E12.5 to produce erythrocytes that continue to transiently circulate in pups after birth (Fraser et al., 2007; Kingsley et al., 2004; Wong et al., 1986). The early production of primitive erythrocytes coincides with the need of a proper oxygenation of the growing embryo. Primitive megakaryocyte and macrophage progenitors are also produced at E7.25 in the mouse yolk sac (Moore and Metcalf, 1970; Xu et al., 2001). Megakaryocytes and platelets are found soon after, playing a role in the development of blood and lymphatic vasculatures during embryonic development (Bertozzi et al., 2010). Primitive hematopoiesis transiently produces cells that do not last in adult except for primitive macrophages that were recently shown to produce microglia in the brain of adult mice (Ginhoux et al., 2010), as it is also the case in chicken (Cuadros et al., 1992). A second wave, named definitive, produces erythroid progenitors (burst-forming unit erythroid or BFU-E) in E8.25 mouse yolk sac (McGrath et al., 2011). After colonization of the fetal liver, BFU-E will produce the first definitive erythrocytes. Different types of myeloid progenitors (*e.g.* granulocyte-macrophage progenitors, mast cell progenitors, high proliferative potential colony-forming cells) also appear in the yolk sac at E8.25 before colonizing the fetal liver where they will produce mature cells after E10.5 (Palis et al., 1999). Erythro-myeloid progenitors (EMPs) start to emerge in the yolk sac at E8 and will be responsible for the production of definitive erythrocytes, granulocytes and macrophages in the fetal liver (Bertrand et al., 2005). Altogether the yolk sac primitive and definitive hematopoietic waves allow the survival and growth of the embryo to term without the need of HSCs (Palis, 2016).

Hematopoiesis begins only 11 hours post fertilization (hpf) in the intra-embryonic lateral plate mesoderm in the zebrafish larvae. It is specified in a posterior region, giving rise to erythroid cells, and an anterior region generating macrophages (Detrich et al., 1995). In concordance with other vertebrates, this initial blood cell production is followed by a wave of EMP hematopoiesis as shown by confocal imaging of reporter fish for various hematopoietic markers such as GATA1, LMO2, MPX and CD41 (Bertrand et al., 2007). Primitive macrophages were recently shown to be involved in extracellular matrix degradation by means of metalloproteases in the aorta of zebrafish embryos (Travnickova et al., 2015). This process facilitates the migration of the newly emerged hematopoietic stem and progenitor cells (HSPCs) through the sub-aortic mesenchyme and the extravasation in the axial vein circulation.

3.2. The aorta is the cradle of the first HSCs

Until the 1970s, the yolk sac blood islands were assumed to be at the origin of definitive hematopoiesis and therefore the site of HSC production (Moore and Metcalf, 1970). However, this dogma was challenged after grafts were performed in the avian and amphibian models, allowing the first tracing of hematopoietic cells in developing embryos. The creation of chicken/quail chimeras and the possibility to distinguish quail embryonic cells (dark nuclei) (Le Douarin, 1969) from chicken yolk sac cells demonstrated the intra-embryonic origin of adult blood (Dieterlen-Lievre, 1975). Indeed, the early extra-embryonic yolk sac progenitors (chicken origin) were short-lived and became gradually replaced by long-lived blood cells of intra-embryonic quail origin. It was confirmed in the amphibian model (*Xenopus*) by performing grafting experiments (Ciau-Uitz et al., 2000; Turpen et al., 1981). Indeed, the dorsal lateral plate (mesodermal region containing the dorsal aorta) mainly contributed to adult blood production while the ventral blood island region did not. These findings, supporting an intra-embryonic region comprising the aorta as the cradle of HSCs, contradicted the dogma of a yolk sac HSC origin in mammals (Moore and Metcalf, 1970). The grafting strategies, applicable for avian and amphibians due to the external development of the embryo, are unfortunately impossible to apply to mammals. The use of organotypic *in vitro* cultures revealed the presence of multipotent precursors in the mouse intraembryonic splanchnopleura region (before blood circulation) while it was not the case in the yolk sac (Cumano et al., 1996). It therefore demonstrated that the yolk sac does not contribute to definitive hemopoiesis in the mouse. In mammals, the HSC potential is tested in an *in vivo* transplantation assay, where cells are injected intravenously in primary and secondary wild-type irradiated adult recipients to test at long-term their multilineage and self-renewal capacities (Robin and Dzierzak, 2005). Using this assay, the first HSCs were detected in the aorta of the aorta-gonad-mesonephros (AGM) region (which develops from the para-aortic splanchnopleura), the vitelline and umbilical arteries and the head at E10.5 (de Bruijn et al., 2000; Li et al., 2012; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Starting at E11-E11.5, HSCs are also found in the yolk sac, placenta and fetal liver (Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). The placenta and the fetal liver become important HSC reservoirs at mid-gestation before HSCs start to colonize the bone marrow at E17 (Christensen et al., 2004).

3.3. The relationship between hemogenic endothelium, hematopoietic clusters and HSCs

In the mouse embryo, clusters of round cells have been found tightly associated with the endothelial layer of large arteries such as the aorta (referred to as intra-aortic hematopoietic clusters or IAHCs) (Fig. 1; Fig. 2**A–F**), and the vitelline and umbilical arteries (Yokomizo and Dzierzak, 2010). Similar clusters are also found in the vascular labyrinth of the placenta (Rhodes et al., 2008), more precisely in the chorioallantoic vasculature and the fetal vessels near the chorionic plate (Azevedo Portilho et al., 2016). Immunostaining on whole embryo or sections have shown that IAHC cells express numerous hematopoietic markers (*e.g.* c-kit, CD41, CD45) also expressed by embryonic HSCs (Boisset et al., 2010; Brachtendorf et al., 2001; Corbel et al., 2005; Garcia-Porrero et al., 1998; Taoudi et al., 2008). While the morphology and phenotype of IAHC cells strongly suggested a hematopoietic identity, the link between IAHC cells and HSCs was established after close observation of hematopoietic mutants lacking im-

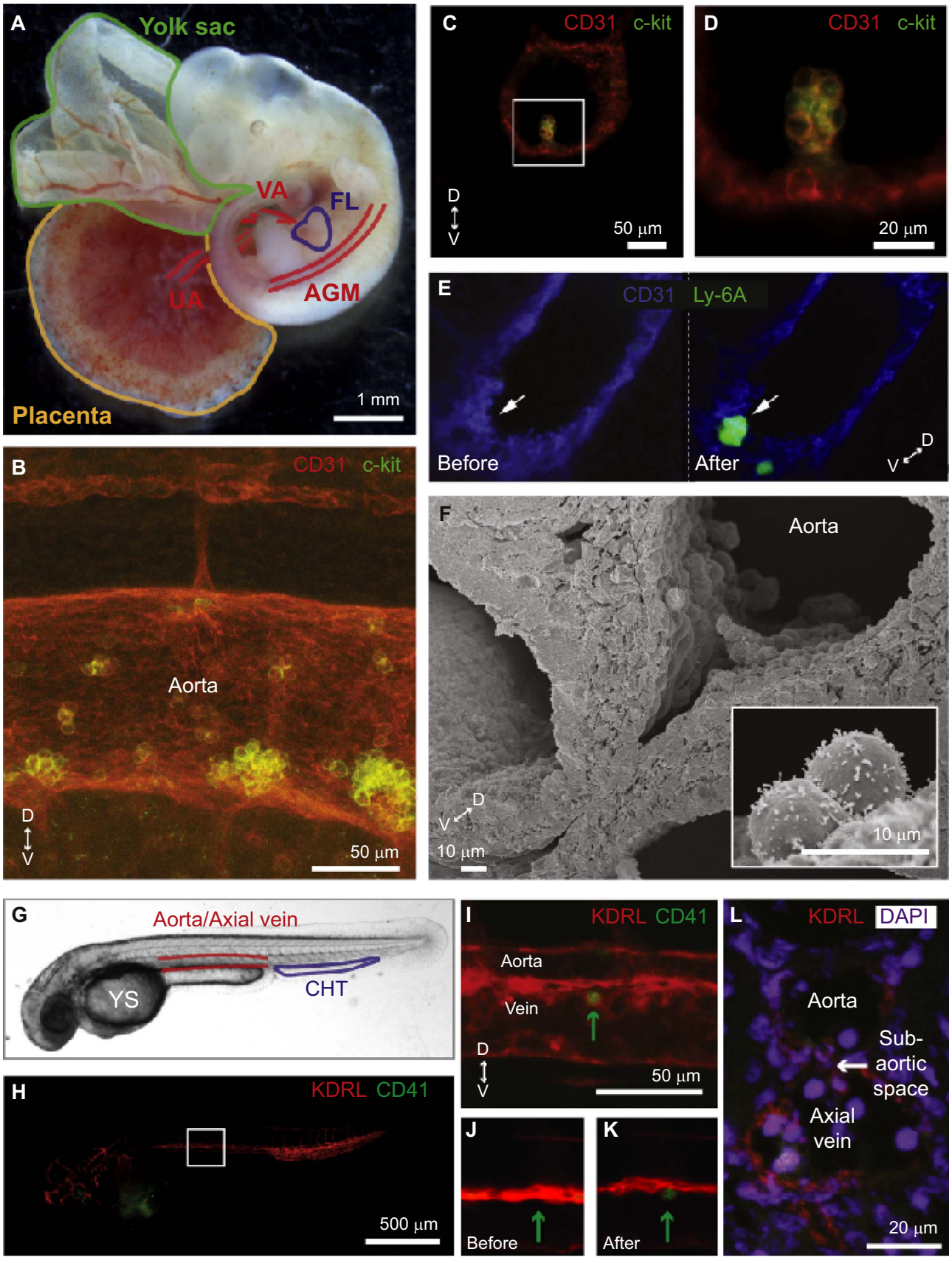
A
Yolk sac
VA
FL
UA
AGM
Placenta
1 mm
B
CD31 c-kit
Aorta
D
V
50 μm
C
CD31 c-kit
D
V
50 μm
D
CD31 c-kit
20 μm
E
CD31 Ly-6A
Before
After
D
V
F
Aorta
D
V
10 μm
10 μm
G
Aorta/Axial vein
YS
CHT
H
KDRL CD41
500 μm
I
KDRL CD41
Aorta
Vein
D
V
50 μm
J
Before
K
After
L
KDRL DAPI
Aorta
Sub-
aortic
space
Axial
vein
20 μm

Fig. 2. Hematopoietic production in mouse and zebrafish embryos. (A-F) Main sites of hematopoietic production during mouse embryonic development. (A) E11 mouse embryo with its yolk sac connected by the vitelline vessels and the placenta connected by the umbilical vessels. Hematopoietic stem cells (HSCs) are first found in the aorta-gonad-mesonephros (AGM) region, vitelline artery (VA), umbilical artery (UA) and head. Later on, HSCs are also found in the yolk sac, placenta and fetal liver (FL). (B) Three-dimensional reconstitution of the aorta of an E10.5 mouse embryo showing c-kit$^+$CD31$^+$ clusters attached to the ventral aortic floor. Red, CD31; green, c-kit. (C) Thick E11 Ly-6A-GFP embryo slice showing a ventral intra-aortic hematopoietic cluster (IAHC). The aortic endothelium and IAHC are stained with anti-CD31 antibodies (injected directly inside the aorta before embryo slicing). Boxed area is shown enlarged in (D). Red, CD31; green, c-kit. (E) Time-lapse imaging of a E10 Ly-6A-GFP embryo slice stained with anti-CD31 antibodies. Left panel is a picture of the aorta at the beginning of imaging and the right panel is a picture of the aorta after 16 h of imaging, revealing the emergence of a Ly-6A-GFP$^+$ IAHC. Blue, CD31; green, Ly-6A-GFP. (F) Scanning electron microscopy of E10 thick embryo slice. Close-up view where IAHCs are visible inside the aorta. The close-up panel shows two IAHC cells with surface microvilli. (G-L) Main sites of hematopoietic production during zebrafish embryonic development. (G) The location of the yolk sac, aorta, axial vein and caudal hematopoietic tissue is shown on a zebrafish embryo (36 hpf). (H) Global view of a (Tg(*kdrl: mCherry-CAAX)/-6.0itga2b: eGFP)*) double transgenic embryo at 36 hpf. mCherry fluorescence highlights the whole vasculature. Red, KDRL; green: CD41(or Itga2b). The boxed area is shown in (I) and reveals the presence of a newly emerged hematopoietic stem and progenitor cell (HSPC) from the aortic floor (green arrow). (J, K) Time-lapse imaging of a (Tg(*kdrl: mCherry-CAAX)/-6.0itga2b: eGFP)*) double transgenic embryo (~42 hpf). Pictures of the aorta at the beginning of imaging (J) and after 16 h of imaging (K), revealing the emergence of a CD41-eGFP$^+$ HSPC from the aortic floor towards the sub-aortic space (green arrow). (L) Transversal section of a KDRL–mCherry-CAAX zebrafish embryo (36 hpf) showing the aorta, sub-aortic space and axial vein. Red, KDRL; blue, DAPI.

portant transcription factors such as Runx1. The fact that Runx1$^{-/-}$ mutant embryos, devoid of HSCs, also lack IAHCs formally linked HSCs and IAHCs (North et al., 1999; Okuda et al., 1996; Wang et al., 1996). Surprisingly, hematopoietic clusters were also recently observed by using 3D confocal imaging of whole mouse embryos in non-hematopoietic sites such as the head, heart and somites (Yzaguirre and Speck, 2016b). Similar clusters of cells have been microscopically identified in extravascular sites where they bud from the vitelline and umbilical arteries by vascular remodeling or extrusion (Yzaguirre and Speck, 2016a; Zovein et al., 2010). They are most likely involved in the vascular bed remodeling and possibly contribute to a certain extent to hematopoiesis. In the yolk sac, EMP-associated clusters are found in the arterial and venous vasculature (Frame et al., 2016). In contrast to yolk sac, clusters have only been reported to be associated to arteries and not veins in the embryo.

Microscopic observations of the close spatial connection between IAHCs and the vessel wall led to the pioneer idea that the endothelium or specialized endothelial cells might give rise to IAHCs (Dantschakoff, 1909). The endothelium capable of generating hematopoietic cells was named hemogenic endothelium. Immunostaining of whole embryos or sections have shown that IAHC cells, besides hematopoietic markers, also express endothelial markers (*e.g.* VE-Cadherin, endomucin, CD31, CD34, endoglin, Tie2) (Boisset et al., 2010; Brachtendorf et al., 2001; Garcia-Porrero et al., 1998; Taoudi et al., 2008; Yokomizo and Dzierzak, 2010). It therefore supported a developmental relationship between endothelial and hematopoietic lineages, but experimental proof was missing. Due to the *in ovo* development of the embryo, the avian model is an attractive system for cell manipulation and cell tracing experiments *in vivo*. Therefore, it is not surprising that the first functional proof of an endothelial origin of IAHC cells was obtained in chicken embryos (Jaffredo et al., 1998) (Fig. 1). The aortic endothelium was labeled by injecting acetylated-low density lipoproteins (Ac-LDL) into the heart of a chicken embryo before IAHC emergence. Twenty-four hours later, all IAHC cells were labeled with this traceable dye, formally proving their endothelial origin. Such findings were confirmed by labeling the endothelium of the chicken embryo with retroviral vectors (Jaffredo et al., 2000). Further experiments performed in the avian system have permitted a better comprehension of the organization and origin of the hemogenic endothelium in the aorta (Fig. 3**, left panel**). Grafting experiments of mesodermal fragments between chicken and quail embryos suggested that the aortic endothelium derived from two distinct endothelial cell lineages. While the ventro-lateral portion of the aorta is derived from the splanchnopleural lateral mesoderm, the roof is derived from the paraxial mesoderm (Pardanaud et al., 1996). It appeared that only the splanchnopleural derived ventral endothelium is hemogenic, starting at E2.5 by thickening and expressing the pan-hematopoietic marker CD45 and progressively transforming into IAHCs (Fig. 3**, left panel**). Non-hemogenic endothelial cells of somitic origin gradually replace the disappearing hemogenic endothelium (Pouget et al., 2006). The precise location of the hemogenic endothelium and its progressive replacement regulate both in time and space IAHC production in the aorta. While part of IAHC cells is released in the lumen of the aorta, many cells also ingress into the underlying mesenchyme forming para-aortic foci of CD45$^+$ hematopoietic cells (Dieterlen-Lievre and Martin, 1981; Jaffredo et al., 2000). These foci are considered as a mammalian fetal liver equivalent (Guedes et al., 2014; Kingsbury et al., 1956), providing a transient and maybe proliferative niche before the cells colonize definitive hematopoietic sites (spleen, thymus, bone marrow, bursa of Fabricius) (Dunon et al., 1998, 1999; Lassila et al., 1980). The use of mouse embryonic stem cell cultures confirmed the need of an endothelial step for the production of hematopoietic cells from hemangioblasts (Eilken et al., 2009; Lancrin et al., 2009). Indeed, the continuous long-term single-cell imaging and tracking of mouse mesodermal cells allowed to directly observe hemogenic endothelial cells giving rise to hematopoietic cells *in vitro* (Eilken et al., 2009).

Various experimental approaches have been employed to prove the endothelial origin of IAHCs and HSCs *in vivo*. Cre recombinase was expressed under the control of the endothelial cell specific promoter VE-Cadherin and induced at a specific time-point of early development (Oberlin et al., 2010; Zovein et al., 2008). The stable genetic labeling of endothelial cells was detectable thereafter in the hematopoietic embryonic and adult progeny, clearly demonstrating the endothelial origin of HSCs. The conditional deletion of Runx1 in VE-Cadherin$^+$ endothelial cells leading to the absence of IAHC and HSC formation in mice confirmed the endothelial origin of both (Chen et al., 2009). These first experiments provided crucial findings. However, they were experimentally limited to the imaging of fixed tissues collected at various time points of development and did not permit the direct and dynamic observation of IAHC and HSC formation inside the aorta. The development of confocal microscopes and the discovery of stable fluorescent proteins (*e.g.* GFP) were crucial to pave the way for generating fluorescent transgenic animals, which allow to visualize and trace cells *in vivo*. Transgenic zebrafish embryos represent a powerful tool for live imaging since they are translucent, develop *ex utero* and establish a functional blood circulatory system within 24 hpf (Fig. 2**G**). Despite different anatomical niches, the zebrafish shares the regulatory machinery governing hematopoiesis and lineage specification into HSCs with other vertebrates. Using endothelial KDR-GFP transgenic reporter fish, HSPCs were shown to emerge from the ventral aortic endothelium between 33 hpf and 54 hpf, with a peak at 48 hpf (Bertrand et al., 2010; Kissa and Herbomel, 2010; Lam et al., 2010). HSPC emergence coincides with the expression of HSC markers, such as CD41 (Fig. 2**H-K**), RUNX1, LMO2 and c-MYB. The excellent confocal microscopic resolution of zebrafish allowed a precise description of the so-called endothelial into hematopoietic transition (EHT) necessary for HSPC production (Kissa and Herbomel, 2010). Hemogenic endothelial cells start to bend away from the aortic endothelium towards the sub-aortic space (Fig. 3**, middle panel;** Fig. 2**L**). Neighboring endothelial cells are thereby brought in proximity before HSPCs bud into the sub-aortic mesenchyme, where they either divide or directly enter the circulation *via* the axial vein (Kissa and Herbomel, 2010).

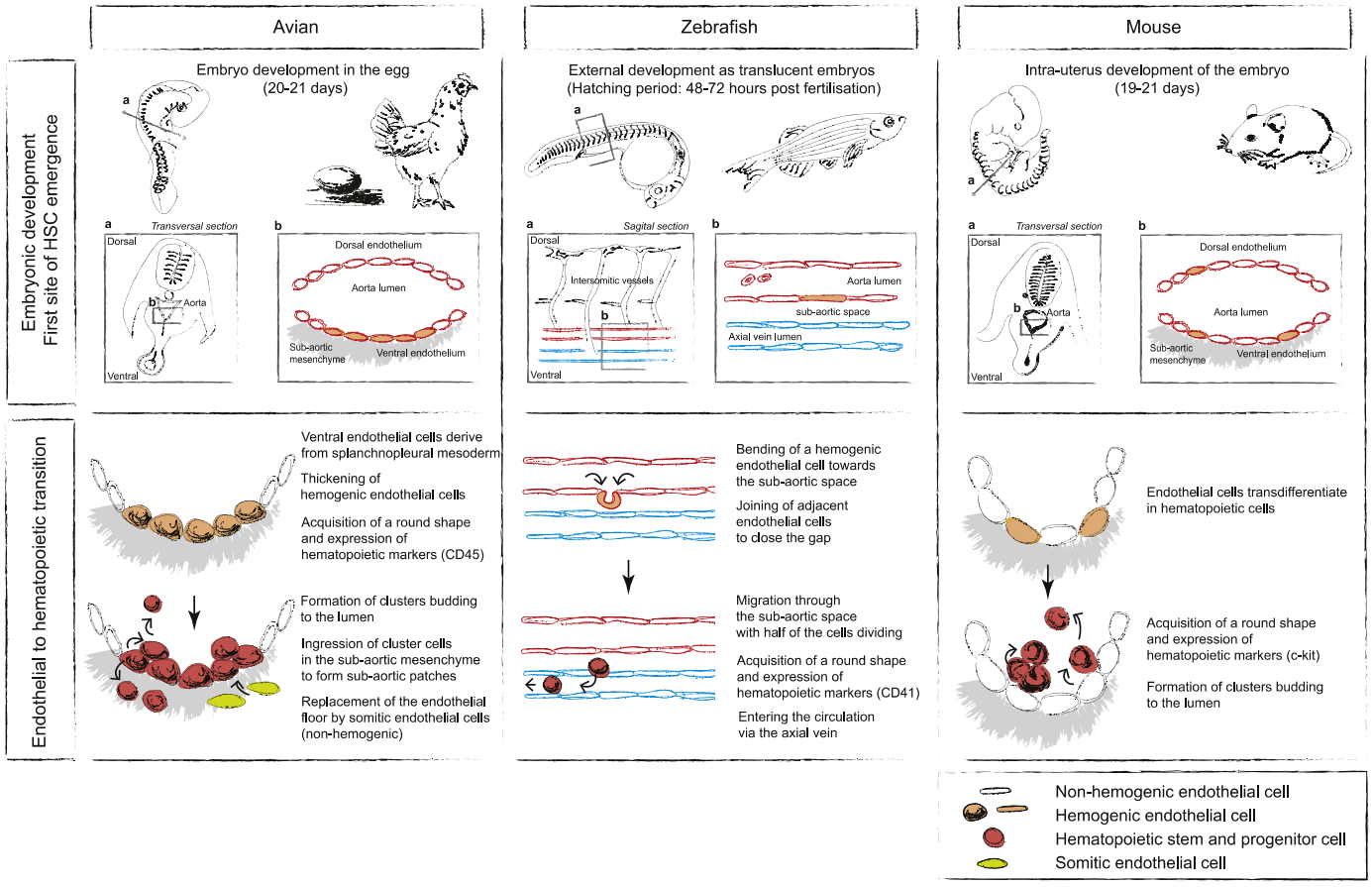

Fig. 3. Schematic representation of the embryonic development, first site of hematopoietic stem cell emergence and process of endothelial to hematopoietic transition in the avian, zebrafish and mouse embryo models. Hematopoietic stem cells (and intra-aortic hematopoietic clusters in the case of avian and mice) are generated from hemogenic endothelial cells located in the aorta. The endothelial to hematopoietic transition (EHT) is restricted to the ventral aspect of the aorta in the avian and zebrafish while it also occurs in the dorsal aspect of the aorta in mouse embryos. It is important to note that the EHT process differs between avian, zebrafish and mouse models.

Interestingly, HSPCs do not seem to bud into the aortic lumen as it is the case in other vertebrate species. Based on time-lapse imaging an estimate of 3 HSPCs enter the circulation per hour (Kissa et al., 2008), some of them contributing to definitive hematopoiesis and therefore being true HSCs (Bertrand et al., 2010; Ma et al., 2011). Once HSPCs enter the blood stream, they migrate to the caudal hematopoietic tissue (CHT, the equivalent for the mammalian fetal liver) (Fig. 2**G**). There they will induce the remodeling of endothelial and mesenchymal cells that form a proliferative stem cell niche (Tamplin et al., 2015), before colonizing the thymus and kidney marrow.

A landmark of single cell labeling and tracing was certainly the development of multicolor transgenic labeling strategies. In 2007, a mouse model (*Brainbow*) was developed expressing different fluorophores separated by loxP sites (Livet et al., 2007). Cre induction allows the stochastic recombination of either loxP sites, resulting in expression of one of the fluorophores. Initially, this single cell tracing approach was used in the context of neuronal cell fates. In the first generation of *Brainbow* mice, one of the fluorescent proteins was expressed by default. This limitation was overcome in the confetti mice, which was pivotal in the identification of intestinal stem cells (Snippert et al., 2010). The *Brainbow* targeting strategy was also applied to other species, including zebrafish (*Zebrabow*) (Pan et al., 2013)). Recently, HSPCs generated from the hemogenic endothelium of the aorta were traced during zebrafish embryonic development (Henninger et al., 2017). The *Zebrabow* was crossed to a *drl: cre*ER^{T2} line, expressing Cre in the lateral plate mesoderm and developing hemogenic endothelium after tamoxifen induction. The stochastic activation of the transgene resulted in a unique color code for each HSC and its cell progeny that was detectable in the adult fish by fluorescence-activated cell sorting. At the peak of HSPC emergence, about 30 different HSC clones contribute to definitive hematopoiesis. *CreER^{T2}* activation in *CD41*:eGFP fish supported these numbers, finally providing an estimate for embryonic HSCs at the foundation of adult hematopoiesis in the zebrafish. These numbers seem to be in line with mouse development, where ~2 HSCs and at least 12 transplantable pre-HSCs (Boisset et al., 2015) are present in the aorta that contribute to the 66 HSCs found at E12 in the fetal liver (Kumaravelu et al., 2002).

The *in utero* development and tissue opacity of mammalian embryos make live cell imaging and tracing very difficult in the developing embryo and particularly in the deeply located aorta. In order to optically access the aorta, we developed a technique that consists in slicing unfixed E10.5 mouse embryos into thick transversal slices (Boisset et al., 2010). After removing the head and tail of the embryo, fluorescently labeled CD31 antibodies were injected directly into the aorta to stain both the aortic endothelium and IAHC cells. Embryos from transgenic reporters for the (hemogenic) endothelium and HSCs (Ly-6A (Sca-1)-GFP) (de Bruijn et al., 2002; Ma et al., 2002) or first hematopoietic commitment (CD41-YFP (Ferkowicz et al., 2003; Mikkola et al., 2003; Zhang et al., 2007)) were used to stably label the cells of interest. Ly-6A is expressed by some hemogenic endothelial cells and IAHC cells, but not by the surrounding mesenchyme at E10.5 (de Bruijn et al., 2002). CD41 expression on the other hand is restricted to the newly formed IAHC cells (Boisset et al., 2010; Robin et al., 2011). Beside being an important IAHC cell marker, CD41 is also a marker of HSCs and has an important function in the maintenance of HSC activity in the aorta (Boisset et al., 2013). Overnight time-lapse

imaging of embryo slices showed CD31$^+$ endothelial cells undergoing cell shape rearrangement by budding into the lumen of the aorta (Boisset et al., 2010) (Fig. 1**;** Fig. 3**, right panel**). During budding, the cells started to express Ly-6A or CD41, as well as the HSC marker c-kit (as shown by immunostaining of the embryo slices after overnight imaging) (Fig. 2**E**). All in all, this new technique allowed the first imaging of the endothelial to hematopoietic (stem) cell transition as it most likely occurs *in vivo* in the aorta of the mid-gestation mouse embryo.

3.4. Cell composition and role of intra-aortic hematopoietic clusters

During mouse embryonic development, IAHCs first appear at E9 in the vitelline and umbilical artery and shortly after in the aorta at E9.5. The meaning of such spatial and temporal emergence is still unknown. The number of IAHC cells (CD31$^+$c-kit$^+$), counted after whole embryo staining, steadily increases until E10.5 (~700 IAHC cells/aorta) and remains numerous until E11.5 (~500 IAHC cells), concomitant with HSC emergence in the aorta (Muller et al., 1994; Yokomizo and Dzierzak, 2010). Thereafter, their number gradually declines until E14.5, which coincides with the seeding of the fetal liver with HSCs and progenitors. Only few HSCs (1-3 HSCs at E10.5-E11.5 (Gekas et al., 2005; Kumaravelu et al., 2002; Robin et al., 2006)) and committed progenitors (an average of 22 progenitors/aorta clusters (Boisset et al., 2015)) are part of IAHCs that are composed of at least 25 times more cells at E10.5-E11.5 (Yokomizo and Dzierzak, 2010). IAHC cells are phenotypically heterogeneous, all cells expressing c-kit but not all expressing CD45 or Ly-6A at E10.5 (Boisset et al., 2015, 2010). It therefore suggests that an immature intermediate cell population between the hemogenic endothelium and HSCs might represent the majority of IAHC cells. Several lines of research concur with this hypothesis. A different type of *in vivo* transplantation assay has been established to reveal the potential of very immature cells (referred to as HSC precursors or pre-HSCs), that would be incapable to engraft adult recipients but could exert their HSC potential when transplanted in a more permissive environment (*e.g.* as the bone marrow of immunodeficient mice or the liver of newborns) (Cumano et al., 2001; Yoder and Hiatt, 1997). Using such an assay, the existence of pre-HSCs in the aorta has been demonstrated *in vivo* although there are very few (~12 pre-HSCs/aorta) (Boisset et al., 2015). The development of an *in vitro* culture system where AGMs are disaggregated and cells reaggregated in the presence of OP-9 cells and cultured for several days with cytokines has permitted the identification of different pre-HSC populations (Taoudi et al., 2008). Pro-HSCs (VE-cad$^+$CD45$^-$CD41^{low}CD43$^-$) are detected at E9.5 while Type I (VE-cad$^+$CD45$^-$CD41^{low}) and Type II (VE-cad$^+$CD45$^+$) pre-HSCs are present in E10.5–E11.5 AGM (Rybtsov et al., 2014, 2011). All these cells are able to mature into functional HSCs upon various periods of culture in reaggregates. The number of pre-HSCs present at E11.5 matches the number of definitive HSCs detected at E12.5 in the fetal liver, suggesting that the majority of IAHC pre-HSCs mature into HSCs after migration to the fetal liver to generate the adult HSC pool that will colonize the bone marrow before birth (Rybtsov et al., 2016). It is noteworthy that similar intermediate cell states have not yet been identified in other species.

4. Summary and outlook

After over a century of developmental hematopoietic research, the embryonic aorta appears as a central provider of pre-HSCs and HSCs. Various imaging approaches allowed to witness the hemogenic endothelial to hematopoietic transition in the aorta, revealing the similarities and differences of this conserved process between species. However, visualizing EHT without perturbing the development of the embryo remains a challenge. Embryonic hematopoiesis is highly susceptible to disturbance by environmental cues, such as hypoxia (Imanirad et al., 2014), blood flow (North et al., 2009) and shear stress (Adamo et al., 2009). Therefore, it remains possible that what has been observed so far, although very informative, might not completely reflect the dynamics of the *in vivo* situation. Microscopic techniques need to involve the imaging of complete embryos at a high resolution without affecting their development. A step further in that direction is the light-sheet microscopy technology, which now allows *in toto* imaging of embryogenesis at subcellular resolution. Even though the penetrance into the tissue is enhanced compared to single photon confocal microscopy, this technique is so far restricted to translucent organisms that develop *ex utero,* such as zebrafish embryos. Attempts in imaging mid-gestation mouse embryos for a long period of time *ex utero* have been proven unsuccessful so far. Whole-embryo culture of mid-gestation embryos is feasible but it remains restricted to up to 40 h in a rolling incubator system, which is incompatible with continuous live imaging (Kalaskar and Lauderdale, 2014). Moreover, the opacity of the mouse embryonic tissue remains an optical challenge. Until then, it will remain impossible to study mammalian HSCs in their immediate environment and to trace them and their progeny live and *in toto* in their successive hematopoietic niches during embryonic development. A promising line of research involves optogenetics that use targeted illumination of photosensitive proteins to control functions in cells. Photoactivatable Cre systems might for example enhance the specificity of Cre induction. These and other transgenic genetic engineering strategies promise to finally answer remaining fundamental questions in embryonic hematopoiesis. More and more light is shed on the biomechanics behind EHT and the factors controlling this process. Electron scanning microscopy of the surface of mouse IAHC cells shows a great number of cellular protrusions, possibly mediating cell-cell interactions and/or communication (Boisset et al., 2015) (Fig. 2**F**). During EHT, a flat endothelial cell rounds up or bends, thereby undergoing extensive cell shape rearrangements. Up to date, it is not clear how the cell-cell interactions, cell anchorage and cytoskeleton remodeling are modified during EHT and the acquisition of a hematopoietic fate. Most of these subcellular events can thus far not be followed in living cells and will remain an exciting field of developmental research at the cutting edge of microscopic imaging.

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