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**Of hemangioblast, hemogenic endothelium and primitive versus definitive
hematopoiesis**

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The types of progenitors generated during the successive stages of embryonic blood development are now fairly well characterized. The terminology used to describe these waves, however, can still be confusing. What is truly primitive? What is uniquely definitive? These questions become even more challenging to answer when blood progenitors are derived *in vitro* upon the differentiation of embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Similarly, the cellular origin of these blood progenitors can be controversial. Are all blood cells, including the primitive wave, derived from hemogenic endothelium? Is the hemangioblast an *in vitro* artefact or is this mesoderm entity also present in the developing embryo? Here we discuss the latest findings and propose some consensus relating to these controversial issues.

Embryonic hematopoiesis

During embryogenesis, the hematopoietic system is established in successive waves that are each temporally and spatially restricted and that each gives rise to specific blood progenitors (Figure 1) ¹. At E7.25, shortly following gastrulation, the onset of blood emergence takes place in the yolk sac where extra-embryonic mesoderm differentiates to form blood islands ². The first blood precursors generated at this stage of development give rise to primitive erythroid precursors, erythroid cells with unique characteristics, only found during early embryogenesis and which role is to rapidly deliver oxygen to the fast expanding embryo ^{3,4}. Macrophage and megakaryocyte progenitors are also generated during this earliest wave of blood emergence ^{3,5}. The next wave of blood specification starts a day later around E8.25 with the emergence of erythro-myeloid progenitors (EMPs) within the yolk sac ⁶. These EMPs produces definitive erythrocytes and most myeloid lineages. By E9.0-9.5, B and T lymphoid progenitors are generated in the yolk sac and in the intra-embryonic para-aortic splanchnopleura region ^{7,8}. All types of fetal and adult T cells are produced by these early progenitors, including $\alpha\beta$ and $\gamma\delta$ subsets ⁸. In the case of B lymphocytes, the potential of this first wave of progenitors is restricted to the production of innate-type B1 and marginal zone B cells subsets ^{7,9}. Recent studies have also established that tissue resident macrophages of the brain, lung and liver are generated during these earliest waves of hematopoietic development ¹⁰,

macrophage populations persisting in the adult organism throughout life. It is only by E10.5 that the first long-term multilineage adult-engrafting hematopoietic stem cells (HSCs) are generated¹¹. HSCs emerge within the major arteries of the developing embryo including the dorsal aorta, the vitelline and umbilical arteries¹². By E11.5, HSCs are also found in the yolk sac and placenta^{13,14}; whether this occurs through de novo generation or through the circulation of HSCs generated elsewhere is still unknown. Newly generated HSCs migrate to the fetal liver where they undergo massive expansion; by E16.5 they start to colonize the bone marrow where they will reside throughout adult life, self-renewing and producing a continuous supply of all blood lineages.

Primitive versus definitive hematopoiesis

In the developing mouse embryo, primitive hematopoiesis is most often defined as the initial wave of blood cell production taking place around E7.25 in the blood islands within the yolk sac^{2,5,15}. As stated above, this first wave gives rise to primitive erythrocytes, macrophages and megakaryocytes (Figure 2). All subsequent waves of blood emergence in the embryo, from E8.25 onward, are defined as definitive hematopoiesis. This includes EMPs produced in the yolk sac which give rise to definitive erythrocytes, macrophages, megakaryocytes and other myeloid lineages, early T and B progenitors produced in the yolk sac and para-aortic splanchnopleura and HSCs produced in the dorsal aorta, vitelline and umbilical arteries (Figure 2). Alternate definitions for primitive hematopoiesis can be found in the literature: e.g. primitive hematopoiesis representing all pre-circulation hematopoiesis¹⁶, primitive hematopoiesis encompassing all blood cells produced prior to HSC emergence or primitive hematopoiesis representing all blood lineages except for definitive erythrocytes, T cells and HSCs¹⁷. But overall these alternate definitions of primitive hematopoiesis are not widely used and are not based on strong scientific grounds.

Primitive erythrocytes are easily distinguishable from definitive erythrocytes by their cellular and molecular characteristics. They are much larger than definitive erythrocytes and they predominantly express embryonic forms of globin^{18,19}. However, like definitive erythroid cells, primitive erythroid cells ultimately enucleate

at late stage of maturation ²⁰. In contrast, macrophages and megakaryocytes generated during the primitive wave of hematopoiesis are hardly distinguishable from their definitive counterparts ⁵. Defining and characterizing primitive hematopoiesis in the embryo is straightforward since this wave is restricted in time and space. However, defining this primitive wave during the *in vitro* differentiation of ESCs or iPSCs is more challenging and one can only identify with certainty primitive erythroid precursors as being part of this primitive wave. Macrophages and megakaryocytes can be equally generated from primitive or definitive hematopoiesis.

One might ask why it is so important to have a clear understanding and definition of primitive versus definitive or successive waves of blood emergence during the *in vitro* differentiation process. A long-standing quest in the field of ESC differentiation to blood has been the *in vitro* generation of HSCs ^{21,22}. In keeping with embryonic development, it has been proposed that *in vitro* hematopoietic differentiation also occurred in sequential steps with a primitive wave followed by a definitive wave and the production of HSCs ²³⁻²⁵. Indication of definitive hematopoiesis emergence has been monitored by following the generation of T lymphocyte progenitors ^{17,26}. While indeed T cell clearly represents a definitive lineage, it is however not indicative of HSC emergence since T cell progenitors are generated at E9.5 in the yolk sac and embryo proper one full day prior to the emergence of the first HSCs ⁸. It is therefore clear that T cell potential cannot be used to track HSC emergence. In fact, only one single hematopoietic lineage appears to be specifically generated from adult-repopulating HSCs and it is the B2 adaptive B cell lineage. The emergence of B cell progenitors prior to HSC generation is restricted to the formation of innate-specific B1 and marginal zone B cells ^{7,9}. Therefore, one might speculate that to monitor HSC emergence *in vitro* it may be best to monitor the emergence of B2 lymphoid cells. Interestingly, there are very few if any reports of HSC generation *in vitro*, and there are equally very few reports of B cells generation *in vitro* ²⁷⁻²⁹. Furthermore, whether B1 or B2 lymphoid subsets were obtained in these studies was not determined. In our recent study using mouse ESCs ³⁰, we did report the *in vitro* generation of multilineage engrafting blood progenitors.

Strikingly, B2 lymphocytes were generated from these engrafting progenitors, suggesting that they might be true HSCs though with limited self-renewal capacity.

***In vitro* and *in vivo* evidences or lack thereof for the presence of hemangioblast**

The term hemangioblast was initially coined by Murray in 1932³¹ and referred to a mass of cells derived from the primitive streak mesoderm that contains both endothelium and blood cells. This was meant to complement and contrast the term angioblast, previously discussed by Sabin³² which only referred to vessels or endothelium. The hemangioblast, as originally described by Murray, was not a clonal mesoderm precursor giving rise to both blood and endothelium. The concept of the hemangioblast as a clonal precursor gained traction in the late 90's when it was shown that single mesodermal cells isolated from *in vitro* differentiating mouse ESCs could give rise to both blood cells and endothelium^{33,34}. Hemangioblast were subsequently identified in the mouse embryo³⁵, in Zebrafish³⁶, in *Drosophila*³⁷ and in *in vitro* differentiating human ESCs³⁸. To date, however, there are still no conclusive evidences demonstrating that in higher vertebrates a hemangioblast does indeed give rise to both endothelium and blood cells *in vivo*. Experiments performed using mouse embryos only showed that upon *ex vivo* culture hemangioblast gave rise of both blood and endothelium. This has led to the proposition that hemangioblast is a state of competency which is never fulfilled *in vivo* due to the restriction and constraint imposed by the microenvironment³⁹.

In vivo lineage tracing in mouse embryo have so far failed to demonstrate the existence of hemangioblast. Ueno and colleagues⁴⁰, using multicolour chimera embryos and FLK1-cre lineage tracing, concluded that blood islands were derived from multiple precursors and that most blood cells did not derive from FLK1-expressing precursors. However, given that hemangioblast are mostly localized in the posterior primitive streak³⁵, by the time these mesoderm progenitors reach the yolk sac to form the blood islands, they have already divided and their daughter cells have already initiated their fate specification toward endothelium or blood. Given the highly migratory behaviours of cells in the gastrulating embryo^{41,42}, it is quite unlikely that all daughter cells of one hemangioblast will migrate to the same location in the rapidly expanding yolk sac to generate a clonal blood island.

Nevertheless, one of the main conclusions in this study was that most blood cells were not derived from FLK1-expressing precursors. This suggests potential problems with the experimental approach and/or the interpretation of the data. As indeed, it has been unequivocally established since that all blood cells do derive from FLK1-expressing mesoderm ⁴³. The second study by Padron-Barthe and collaborators ⁴⁴ has also unfortunately flaws. For example, *Tie2-cre* was used in this study to track hemangioblast; however TIE2 is not expressed in mesoderm of the primitive streak where the hemangioblast has been detected ³⁵. The expression of *Tie2* is only switched on upon commitment of mesoderm to angioblast and hemogenic endothelium ^{45,46}. While both studies concluded that hemangioblast does not exist *in vivo*, they are both technically flawed and therefore their negative conclusion on the presence of hemangioblast *in vivo* remains questionable. To demonstrate whether hemangioblast is indeed an *in vivo* mesodermal precursor with both blood and endothelium or only a state of competency will require either novel experimental approaches or the identification of a marker specific to this mesodermal subset.

Hemogenic endothelium versus hemogenic angioblast

It has long been recognized that blood and endothelium are two closely related lineages that emerge in close proximity during embryonic development ^{31,32,47,48}. The formal demonstration that blood progenitors are generated from an endothelium cell population was achieved through lineage tracing ^{49,50} and time lapse imaging ^{45,51-55}. This specialized endothelial population, termed hemogenic endothelium, is thought to give rise to blood cells through an endothelial to hematopoietic transition rather than through an asymmetric division ⁵¹. By definition, a hemogenic endothelium is an endothelial cell with the potential to become a blood cell and is characterized by an endothelial-specific gene expression signature, endothelial-specific cell morphology and is localized within the endothelial layer of a blood vessel.

Hemogenic endothelium as a cell population giving rise to blood cells has now been described in most species studied to date. In the mouse embryo, E8.25 yolk sac EMPs ⁵⁶, E9.5 T and B progenitors ^{7,8} and E10.5 intra-embryonic progenitors and HSCs ⁵³ have all been shown to emerge from hemogenic endothelium (Figure 2).

In contrast, the cellular origin of the E7.25 wave of primitive hematopoiesis is still disputed. It seems unclear whether primitive hematopoiesis emerges directly from mesoderm, from hemogenic endothelium or from another type of precursor. This first wave of blood development occurs prior to the formation of the vasculature and cannot therefore as such emerge from a bona fide hemogenic endothelium. There are however clear evidences in the literature demonstrating that primitive hematopoiesis do arise from precursors expressing endothelial markers including TIE2, VE-cadherin and CD31^{45,48,57,58}. This endothelial precursor is not localized within the lining of a blood vessel but rather within a mass of endothelial-expressing cells^{45,59}. In line with Sabin's original observation³² and as suggested by Tanaka and colleagues⁴¹, we propose that this precursor should be termed hemogenic angioblast as it is clearly no longer a mesoderm precursor but also not a hemogenic endothelium (Figure 2).

Together, it is now clear that all blood cells emerge from hemogenic endothelial-expressing cells through an endothelial to hematopoietic transition. Whether these endothelial-expressing cells are termed angioblast or endothelium depends on their localization within the developing vasculature. What will be important to determine in the future is how each hemogenic subset is fated to give rise to specific blood progenitors. Is this a cell-intrinsic pre-determined property or is this determined by cues provided by the local micro-environment?

Conclusion

Understanding how the hematopoietic system develops during embryogenesis will provide critical knowledge to translate to the *in vitro* derivation of blood progenitors for use in the clinic. However, while there are many similarities between *in vitro* and *in vivo* blood cell emergence, there are also clear differences. An intriguing observation in our recent study on *in vitro* blood emergence was that all progenitors including primitive and definitive erythrocytes, myeloid, T cells, B cells and engrafting cells were generated at the same time from the mesoderm suggesting that there are no sequential waves of blood specification *in vitro*³⁰. This suggests that *in vitro* populations of hemogenic endothelium with the ability to give rise to all lineages are all generated at once. However, given that within the

embryoid bodies, blood lineages do emerge in sequential order ⁶⁰, this suggests that the timing and further differentiation of hemogenic endothelium subsets might be intrinsically controlled. Could this inform us on the emergence of hemogenic endothelium during embryonic development? What is the cellular origin of hemogenic endothelium population giving rise to EMPs in the yolk sac or HSCs in the dorsal aorta? Recent studies have suggested that endothelial progenitors generated around E7.0-7.5 in the extra-embryonic yolk sac migrate to intra-embryonic sites and contribute to the formation of the dorsal aorta ^{41,42}. Furthermore, we showed that in these migrating endothelium progenitors, blood specification was impaired through the active silencing of *Runx1* via a BMI1-dependent mechanism ⁴². In line with these findings, RUNX1-expressing cells at E7.5, labelled by tamoxifen, have been shown to contribute to the hemogenic endothelium in the dorsal aorta at E10.5 and to some extent to adult HSCs ^{61,62}. Together, these data support the notion that all hemogenic endothelial cells might have an early extraembryonic origin.

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Figure legends

Figure 1: The sequential waves of embryonic hematopoiesis. Schematic representation of hematopoietic development during embryogenesis with the embryonic day (E) of emergence, the type of progenitors generated and the localization of each wave.

Figure 2: Cellular origin of primitive and definitive hematopoiesis. Schematic representation depicting hemogenic angioblast or endothelium emergence and lineages contribution of primitive and definitive hematopoiesis.