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Chapter 4

Human Embryonic Stem Cell-Derived Primitive and Definitive Hematopoiesis

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Additional information is available at the end of the chapter

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1. Introduction

It is well believed that human embryonic stem cells (hESCs [1]) and induced pluripotent stem cells (hiPSCs [2]) are of great potential use for tissue substitutes (for example, blood cells) and to cure various congenital disorders. In mammals, hematopoiesis has already been precisely described in murine system but not yet in human. Early development of hematopoietic system can be well defined by a series of waves from primitive hematopoiesis (early embryogenesis) to definitive ones (late fetal stages). In vitro induction of undifferentiated hESC to functionally mature blood cells may mimic the early hematopoietic development during human embryonic and fetal stages. It also provides an ideal model to uncover molecular and cellular mechanisms controlling early development of human hematopoiesis. On the other hand, functionally matured blood cells derived from hESC/hiPSCs are expected to be widely used for clinical cellular therapies. Although almost all kinds of the mature blood cells can be generated from hESCs, there still lacks solid evidence for the generation of reconstituting hematopoietic stem cells (HSCs) from hESC or hiPSC. So far until now, in vitro hESC-derived blood cells possess phenotypical maturity and partial functions while still more or less share embryonic/fetal characteristics, differing greatly from their adult counterparts. This indicated that in vitro culture systems are not perfect enough to exert full mature activities. Lack of knowledge about the molecular and cellular regulations in human early hematopoiesis has handicapped the development of research on hESC/hiPSC-derived hematopoiesis.

Having been focusing on basic and clinical research on hESC/hiPSC-derived functionally mature blood cells for long, our group has established an efficient method to induce large-scale production of multipotential hematopoietic progenitor cells by coculturing hESC/hiPSCs with murine hematopoietic niche-derived stromal cells [3-6]. By this method, large quantity of

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matured erythrocytes and other functional blood cells could be harvested. In this chapter we will discuss the latest progress in this research field along with our recent discoveries. We will emphasize on the origin, evolution and the development of both primitive and definitive hematopoietic waves, especially those derived from hESCs in vitro systems. The critical problems need to be solved and the research prospects of this field will also be addressed at the end of the chapter.

2. The primitive and definitive waves of hematopoiesis in mammals

2.1. Anatomical sites of hematopoiesis at different developmental stages

Hematopoiesis takes place in some discrete anatomical niches that change temporally and spatially in mammals. Its maturation along with developing ontogeny is a successive event initiated from yolk sac (YS) and then to intra-embryonic sites. The classical opinion believes that para-aortic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM) ought to be the sole location for the emergence of intra-embryonic hematopoiesis, where the earliest HSCs exist. During midgestation, these HSCs move to fetal liver (FL), a predominant hematopoietic place until birth [7, 8]. Hematopoietic precursors seed the bone marrow (BM) in late gestation, where maintaining the principal site of HSC activity lifelong. However, a recent discovery provided solid proof that before HSCs enter the circulation, the embryonic day (E) 10.5–11.5 mouse head is an unappreciated site for HSC emergence within the developing embryo independent to the AGM region [9].

Yolk Sac. The first wave of blood cell generation begins at embryonic day E7.0 at YS and is termed primitive hematopoiesis, producing large erythroblasts that express embryonic hemoglobins [10, 11]. The second wave, termed definitive hematopoiesis, produces smaller erythroblasts that express adult hemoglobins and various other blood cells [12]. Long-term repopulating HSCs (LTR-HSC) appear only in the second wave [13].

The murine YS is a bilayer organ composed of extra-embryonic mesoderm cells apposing to visceral endoderm cells. Its mesoderm layer produces the first blood cells within blood islands [14, 15]. Between E8.0 and E9.0, the outer layer's cells of the blood island in YS differentiate into endothelial cells and form a spindle shape while the vast majority of the inner cells gradually lose their intercellular attachments along with their differentiation into primitive erythroblasts [16].

The research of Yoder's group support that the YS not only acts as the sole site of primitive erythropoiesis but also possibly serves as the first source of definitive hematopoietic progenitors during embryonic development [15,17]. The result of BFU-E assay indicated that following the early wave of primitive erythropoiesis, definitive erythroid progenitors appear at 1-7 somite pairs (E8.25) and solely exist within the YS. After that the definitive erythroid (according to the CFU-E assay), mast cell and bipotential granulocyte/macrophage progenitors develop in the YS [18]. Another proof comes from Ncx1-/- embryo, which could not initiate a heartbeat on embryonic day E8.25 while development continued through E10. There is similar amount

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of primitive erythroid progenitors and definitive HPCs in YS of Ncx1-/- and WT mice through E9.5, while the P-Sp region in Ncx1-/- mice lacks primitive erythroblasts and definitive hematopoietic progenitor cells (HPCs) from E8.25 to E9.5 [17]. So it is reasonable to believe that primitive erythroblasts and nearly all definitive HPCs seeded the fetal liver after E9.5 are generated from the YS between E7.0-E9.5 and are re-distributed into the embryo proper via the systemic circulation [17]. The definitive hematopoiesis may originate from primitive hematopoiesis during embryonic development and migrating HSCs come from the murine YS, which seed the liver and initiate hematopoiesis on 10.0 day postcoitus (dpc). But whether YS cells isolated before day 11.0 dpc possess any long-term repopulating HSC activity remains controversy. Yoder et al proved that donor day 9.0 dpc YS cells could establish long-term hematopoietic system in conditioned newborns, but not in adult recipients [19]. When these early YS cells were co-cultured for 4 days with AGM-S3, an AGM region-derived stromal cell line, they obtained such capacity of reconstitution [20]. All the evidence so far agree that YS itself couldn't provide long-term repopulating HSC activity while the hematopoietic cells originate from YS could own this activity through proper "education" by definitive hematopoiesis niches or the stromal cell line derived from them [20, 21].

AGM. Following the onset of circulation at E8.5, hematopoietic progenitors rapidly move within the embryo. Determining the anatomical origins of definitive hematopoiesis is a complicated and controversial topic. It is now widely recognized that main source of definitive hematopoiesis originates from the AGM region [8]. Using irradiated adult mice as the recipients, Müller et al reported that LTR-HSC first appeared in the AGM region at 10 dpc and expanded in 11 dpc AGM region [13]. Medvinsky et al then demonstrated that, at day 10 in gestation, hematopoietic stem cells initiate autonomously and exclusively within the AGM region under in vitro organ culture condition [8]. All these findings suggested that the AGM region at 10 to 11 dpc provides a microenvironment suitable for generation of LTR-HSC. Xu et al obtained a stromal cell line derived from the AGM region of 10.5 dpc mouse embryo (AGM-S3) that could support the growth and proliferation of hematopoietic progenitor/stem cells from adult mouse bone marrow and human cord blood without additional cytokines [21]. The same research group found that no definitive hematopoiesis-derived colony-forming cells were generated from YS and P-Sp cells at 8.5 dpc before co-cultured with AGM-S3 cells. However after 4-day co-culture of 8.5 dpc YS and P-Sp cells with AGM-S3, spleen colony-forming cells and HSCs capable of reconstituting definitive hematopoiesis in adult mice simultaneously appeared [20]. It is proposed that precursors that had the potential to generate definitive HSCs appear in both extra-embryonic (YS) and intra-embryonic (P-Sp) region, the latter providing microenvironment to support the definitive hematopoiesis from both precursors.

Fetal liver and bone marrow. At 9 dpc, the liver rudiment begins to form an evagination of gut into the septum transversum. The liver does not generate hematopoietic cells de novo but is instead colonized at late E9 by hematopoietic cells generated in other tissues [22; 23]. The first erythroblasts are visible in the liver at 9 dpc. From 10 dpc onwards, the erythroid lineage begins to develop definitive characteristics. Myeloid CFU-Cs appears in the fetal liver at 9 dpc and macrophages and B cells are present at 10-11 dpc [24]. Although most of these differenti-

ated cell types are found early in liver development, the more immature cell types, such as the CFU-S progenitor and the LTR-HSC [7, 25], can be detected only beginning at 11 dpc. Since the liver rudiment is colonized by exogenous blood cells [26]HSCs must arise elsewhere.

Previous study on mouse embryo supported that HSCs able to engraft adult mice were present in the liver beginning at E11–E12 [27-29]. These fetal liver–derived HSCs expressed CD34, c-kit, AA4.1, and Sca-1 surface markers, and were thought to migrate to the fetal BM after E15 [30], the latter providing a continuous supply of mature blood cells for the lifespan (Figure 1).

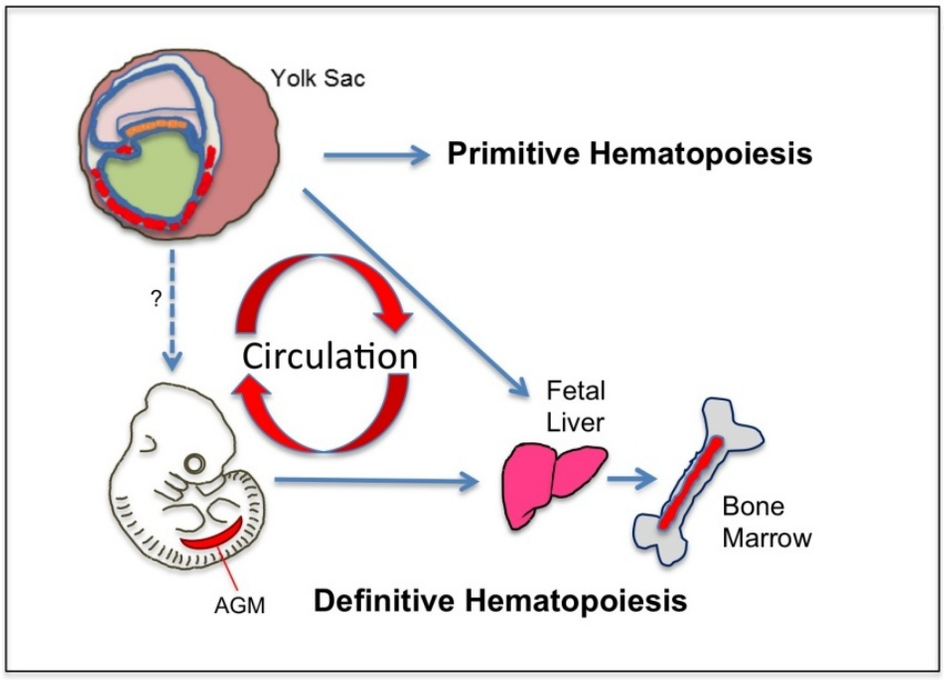


Figure 1. Primitive and definitive hematopoiesis

2.2. Characterization of the progenies derived from primitive and definitive hematopoiesis

The embryonic and fetal erythrocytes only come from primitive hematopoiesis wave while definitive HSCs and T lymphocytes only come from definitive hematopoiesis wave [31]. We will summarize the cellular and molecular characteristics of these cells in primitive and/or definitive hematopoiesis.

2.2.1. Erythrocytes

The erythroid cells within the blood islands of YS are known as primitive or embryonic erythrocytes, specially named as EryP. Differing from the ones found in the FL and adult BM, these YS primitive erythrocytes are typically large in size, nucleated and express embryonic pattren of hemoglobins [32, 33]. The development of primitive erythroid lineage is so transient that its progenitors reach top number in the developing YS at E8.25 and soon become undetectable at E9.0. They would never occur in the late stage of embryonic development [18, 33].

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Following the early wave of primitive erythropoiesis and before the generation of adult-repopulating HSCs, a transient erythromyeloid wave of definitive hematopoietic progenitors (erythroid/myeloid progenitors [EMPs]) emerges in the YS. This YS-derived definitive wave begins E8.25 and colonizes the liver by E10.5. All maturation stage of erythroid precursors and the first definitive erythrocytes could be observed in the liver and circulation at E11.5 [34]. Initially, the development and differentiation of both primitive and definitive erythroid precursor cells depend on growth factor signaling [35],while the later committed erythroid progenitor is different [18, 36].

After HSC expansion in the fetal liver between E12.5 and E16.5, the definitive erythroid progenitors are exponentially increased, which produce massive number of definitive erythroid precursors exclusively expressing adult globins [37, 38]. Thus, HSCs that colonize the fetal liver are commonly described as the origin of "definitive" hematopoiesis in the mammalian embryo.

2.2.2. Macrophage

Another mature hematopoietic cells present in the early YS are macrophages. Progenitor cells of functional macrophages were first found in YS of the mouse between day 7 and 8 of gestation. Pro-monocytes and macrophages were firstly identified in the 10-day YS and 11-day FL, indicating that the primitive macrophage progenitors are "proximal" to the promonocyte on the pathway of sequential macrophage maturation [39]. The observation that they mature rapidly, bypassing the monocyte stage of development, and express lower level of certain genes than later stage macrophages suggests that they could represent a unique population [40, 41].

However, the distinction of primitive macrophages is not as clear as the primitive erythroid lineage, since it is unknown if the molecular events leading to their development differ significantly from other types of macrophages. Because that is a close relationship between microphages and the maturation of erythroid cells, the special developmental trait of microphages probably is the adaptation for the primitive erythrogenesis process [12, 18]

2.2.3. Mast cells

In fetal stages, a large number of MC precursors could be found in murine YS and fetal blood, indicating a strong wave of MC development taking place in early embryo [42, 43]. But little is known about human MC development during the embryonic and fetal stages. The human and non-human primates MCs could be generated from ES cells when co-cultured with mouse AGM or OP9 (a fetal bone cell line from M-CSF knockout mouse) cells [4, 44], providing important information about human MC development in early embryonic stage.

In humans, two types of MCs have been identified based on their neutral protease compositions [45]. Connective tissue-type MCs (CT-MCs)mainly located in skin and sub-mucosal area and express tryptase, chymase, MC carboxypeptidase, and cathepsin G in their secretory granules. Mucosal-type MCs (M-MCs) that locates in alveolar wall and small intestinal mucosa express only tryptase. The non-human primate ESC-derived MCs are similar to CT-MC in

phenotype and functionally identical to human skin counterparts, indicating that a different pathway may occur in early development for this two type of MCs. [4].

2.2.4. Lymphocytes

It is ambiguous to obtain common opinion about the origin site and time of lymphocytes during embryonic development. Some researchers persist that lymphoid precursors could be detected in the YS (extraembryo) prior to the embryo, which is as early as Day 8 of gestation [46, 47]. The opposite opinion believe that lymphoid precursors appear in the embryo proper before the YS [48]. Other researchers believe that they appear in both sites at the same developmental stage [49, 50]. The clonal assays of Godin et al. suggested that this controversy might be due to difference in the experimental condition among these groups to favor lymphoid potential but not lymphoid commitment [51]. If the latter as a standard, the early fetal liver was the first place to produce committed lymphoid precursors [52]. Different to myelopoiesis, lymphogenesis has no primitive wave and is specifically derived from definitive hematopoiesis.

2.2.5. LTR-HSC

The definitive hematopoiesis is defined by LT-HSCs that could reconstitute the hematopoietic system in irradiated adult mouse. The primitive hematopoiesis could not support such a reconstitution in adult mouse but only in fetal ones [8]. The primitive hematopoiesis has too simple and incomplete hematopoietic hierarchy compared with definitive hematopoiesis and could not be detected of the complete activity of LT-HSC [24]. The mechanism controlling developmental difference between two waves of hematopoiesis has been obviously observed, but whether "primitive" HSC exists is still a controversial topic [53]. Some researches support the opinion that LT-HSC also originates from YS. Only after the "education" on AGM, these YS LT-HSC progenitors become the functional LT-HSCs, for the hematopoietic cell isolated from E9.5 YS could reconstitute hematopoietic system in irradiated adult mouse after 4 days co-cultured with mAGM-S3 cells [20]. Immediately after primitive hematopoiesis at YS, the definitive hematopoietic progenitors could be detected by generating CFU-GM and BFU-E, indicating that HSCs probably originate from YS and finally mature at AGM region [18]. But other groups persist that LT-HSC has no relation to YS and was produced de novo from AGM [54].

2.3. Molecular mechanisms controlling the transition from primitive to definitive hematopoiesis

Recently, findings from different gene targeting experiments have demonstrated that the primitive and definitive hematopoietic lineages develop from a common precursor by distinct molecular programs, and that the respective cell populations are regulated by different growth factors. Though the controversy still exists about their origins, the YS-derived cell population that only have primitive hematopoiesis potential could obtain definitive hematopoiesis potential by the "education" on mAGM cells in vitro [20]. The YS cell grafted to fetal liver or fetal marrow could also obtain LT-HSC activity [19, 55]. This provides strong evidences to

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support that the primitive hematopoietic progenitor cells could transfer to definitive ones in the microenvironment of fetus definitive hematopoiesis origin, such as mouse AGM, fetal liver and fetal marrow. It is obvious that molecular signals released by definitive hematopoiesis niche cells play a key role in such "education".

Because the component of hemoglobin provides clear trace to describe the developmental stages of red blood or its precursor that distinguish the primitive from definitive hematopoiesis, the erythropoiesis serves as an ideal model to research both waves of hematopoiesis. It also provides clue to dissect their molecular switch mechanism, which is also the main object in our discussion about the molecular regulation of both primitive and definitive hematopoiesis.

In situ studies of the early embryo have demonstrated that genes known to play a role in the onset of hematopoietic development (e. g., GATA-2,scl/tal-1, rbtn2) are expressed prior to the appearance of the blood islands [56]. This suggests that the molecular program that leads to hematopoietic commitment begins shortly after gastrulation at approximate Day 7.0 of gestation. Under the control of tal-1, rbtn2, GATA-2, and GATA-1 that was expressed orderly, primitive mammalian erythropoiesis takes place in a subpopulation of extra-embryonic mesoderm cells during gastrulation. Though these transcriptional factors were also expressed in other region of embryo in the same stage (tal-1 and rbtn2 also in posterior embryonic mesoderm and GATA-1 and GATA-2 expression also in extra-embryonic tissues of ectodermal origin), their expression pattern in extra-embryonic mesoderm cells still play a key role in understanding the molecular mechanism of hematopoietic commitments [56].

By gene-targeting studies on transcription factors essential for development of all hematopoietic lineages, the key control genes for primitive and/or definitive hematopoiesis (such as Gata1, Gata2, AML1, C-myb, EKLF, rbtn2) were well analyzed.

GATA gene family. GATA1 expression is highly restricted in erythroid cells, megakaryocytic cells, eosinophils, dendritic cells, and MCs of hematopoietic cell [57]. It is essential for red blood cell (RBC) development because GATA-1-/- mice will die between E10.5–E11.5 in mid-gestation by anemia [58]. In such mutated embryo, only primitive erythroid cells could be found in the peripheral blood, which are arrested at a proerythroblast stage and express βH1, α and ζ-globin transcripts, then die by apoptosis [59]. Matured definitive erythroid cells were completely absent in GATA-1-/- mice [60-62]. GATA-2, another important member of GATA family, was expressed in many other multi-lineage progenitors and HSCs [63] and also plays important role in proliferation, survival and differentiation of early hematopoietic cells, though the result of its functional mutation is relatively mild compared with GATA-1 [59].

At the late stage of erythrocyte development the "GATA switch" is the key molecular mechanism for erythroid differentiation companied by down-regulating GATA2 and up-regulating GATA1 expression. This is the process that GATA-1 occupies the GATA binding site on the upstream element of GATA2 gene and represses the expression of the latter [64]. In general, after terminal erythroid differentiation start, GATA1 directly opens the expression of erythroid lineage-affiliated genes such as β-globin, Alas2, and Gata1 itself while at the same time

represses Gata2, c-Kit, c-Myb, and c-Myc, responding for the proliferation of progenitors in earlier stages of hematopoiesis [65].

AML1/Runx1. Homozygous mutations of AML1/Runx1 did not interfere normal morphogenesis and YS-derived erythropoiesis, but completely inhibit FL hematopoiesis, leading to the death of embryo around E12.5. The same mutation in ES cells do not influence their differentiation potential into primitive erythroid cells in vitro while stop occurence of any definitive myeloid or erythroid progenitors in both the YS and FL after injected to blastocysts to produce chimeric animals. Above proofs support the key role of AML1 and AML1-regulated target genes to all lineages in definitive hematopoiesis [10, 66]

c-Myb. c-Myb is highly expressed in immature hematopoietic cells and its expression is down-regulated as they become more differentiated [67, 68]. c-Myb controls self-renewal and differentiation of adult HSCs and its disruption seriously depletes the HSC pool and inhibits the definitive hematopoiesis [69].

EKLF. EKLF is zero or very weakly expressed in hematopoietic stem cells and multipotential myeloid progenitors, while arise since more matured stages and play a role all the time later for RBC differentiation. Its expression is up-regulated when myoloid and erythriod progenitors were committed to the erythroid lineage while down-regulated when differentiate toward megakaryopoiesis [70]. During the global expansion of erythroid gene expression in primitive and definitive lineages, EKLF also plays a direct role in globin switching. EKLF is weakly expressed during embryonic and fetal development, which led to a low expression of adult β-globin, Bcl11a and a high one of γ-globin. While in adults, EKLF is highly expressed in definitive RBCs that results in high levels of adult β-globin and Bcl11a expression, and represses γ-globin expression [71]. Finally, EKLF stop the cell cycle of RBCs at the terminal maturation [72]. EKLF/KLF1 mutations will change the RBC phenotypes or even lead to disease. [73, 74]

Rbtn2. Rbtn2 is a nuclear protein expressed in erythroid lineage in vivo, which is essential for erythroid development in mice. The homozygous mutation of rbtn2 inhibits YS erythropoiesis and leads to embryonic lethality around E10.5. YS tissue from homozygous mutant mice and double-mutant ES cells could not process erythroid development in in vitro differentiation system, showing a key role for Rbtn2 in erythroid differentiation, which is high related to GATA-1 [75]

EPO/EPOR. Erythropoietin is a glycoprotein produced primarily by kidney and is the principal factor to regulate RBC production, mainly functioning on erythroid progenitors within the FL and adult BM [76]. The erythroid progenitors before BFU-E stage and RBC after late basophilic erythroblast stage are not responsive to EPO. Proliferation in CFU-E stage could highly responsive to EPO and this response is very transient. The affinity between erythropoietin and its receptor (EPO-R) and their concentration decide strength of such response during the erythropoiesis. EPO-R signaling pathway is necessary for both primitive and definitive erythropoiesis [77, 78].

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3. hESC-derived primitive and definitive hematopoietic cells

The first hESC line was established by Thomson's group in 1998 [1] and then the first hiPSC line by Yamanaka's group in 2007 [2]. Both cells provide possibility to uncover various normal or diseased mechanisms in early human development. By in vitro differentiation system, factors controlling the primitive and definitive hematopoiesis could be investigated in detail using the method of embryoid body (EB) forming [79], or co-culture with hematopoietic niche-derived stromal cell lines [3-6, 80, 81]. Among them, method of EBs could obtain large quantity of blood cells that were not well matured, mimicking the primitive hematopoietic cells [82, 83]. Then, co-culture with OP9 cells was applied to promote the differentiation based on EB method and large-scale production of mature erythrocytes with some definitive properties could be obtained [84, 85]. Although OP9 co-culture system could obtain robust growth of matured blood cells, it is clearly not a natural process and could not be used as proper model to elucidate the natural mechanism controlling early human hematopoiesis. The stromal cells isolated from early hematopoietic niches, such as AGM region, FL and late-stage fetal BM, should be more reasonable candidates to support the in vitro differentiation of hESC/hiPSC-derived hematopoietic stem/progenitor cells. The Lako's group compared the differences among several in vitro differentiation systems based on stromal cell co-culture and optimized their culture conditions. Their result suggested that AGM-derived cell line was most proper for the hematopoiesis differentiation of ESC. [86]

Based on in vitro differentiation systems, researchers tried to make clear the details of early human hematopoiesis using hESCs as a model. Keller's group found two distinct types of hemangioblasts during hESCs differentiation culture, one could give rise to primitive erythroid, macrophages and endothelial cells, while the other one generated only primitive erythroid and endothelial cells [87]. Their work provided the first evidence to prove the existence of hemangioblasts derived from hESCs in vitro. The follow-up work showed that under the control of growth factors and hematopoietic cytokines the hematopoiesis and myelopoiesis will happen during the later stage; and common bi-potent progenitor capable of generating erythroid and megakaryocytic cells could be observed, mimicking the process in vivo [88, 89]. Through the analysis of hemoglobin components, an erythroid maturation could be observed by the ratio of β-globin expression during hESC differentiation culture, which seems a maturation switch but not lineage switch [90]. Above researches indicated that the in vitro hESC differentiation system could reflect the in vivo hematopoietic process during the early human embryonic development.

Slukvin's group applied PO9 co-culture system to explore the detail pathway from hESCs to definitive hematopoiesis. They firstly characterized a population of differentiating hematopoietic cells defined by the expression of CD43, which is distinct to endothelial and mesenchymal cells. Then they defined the erythro-megakaryocytic progenitors (CD34+CD43+CD235a+CD41a+/-CD45-) and multi-potent lymphohematopoietic progenitors (CD34+CD43+CD235a-CD41a-CD45-) in later stage, which replicated the beginning of definitive hematopoiesis in some degree [91, 92]. The human myelomonocytic cells could also be generated from expansion and differentiation of pluripotent stem cell-derived lin-

CD34+CD43+CD45+ progenitors. All the defined population above could be detected during the in vitro differentiation for different hESCs or hiPSCs lines [92].

By different culture conditions, nearly all of the blood lineages could be obtained from hESCs according to the experience from murine ESC protocols, which pave the way to the clinic application [93]. Jame's group also identified a wave of hemogenic endothelial development during the transition from endothelial to hematopoietic cell [94], reflecting the classic property of definitive hematopoiesis. Their result showed that the definitive hematopoiesis could be researched in vitro differentiation system and most progenitor population could be obtained in such a system.

Although research on the early development of human hematopoiesis using human embryo is rigorously restricted by ethics, so far accumulated data have clearly demonstrated that in vitro hESC-derived hematopoiesis is more or less similar to the events happening in murine fetal development (Figure 2). Thus, research on hESC-hematopoiesis should contribute greatly to understand the developmental controls of human early hematopoiesis.

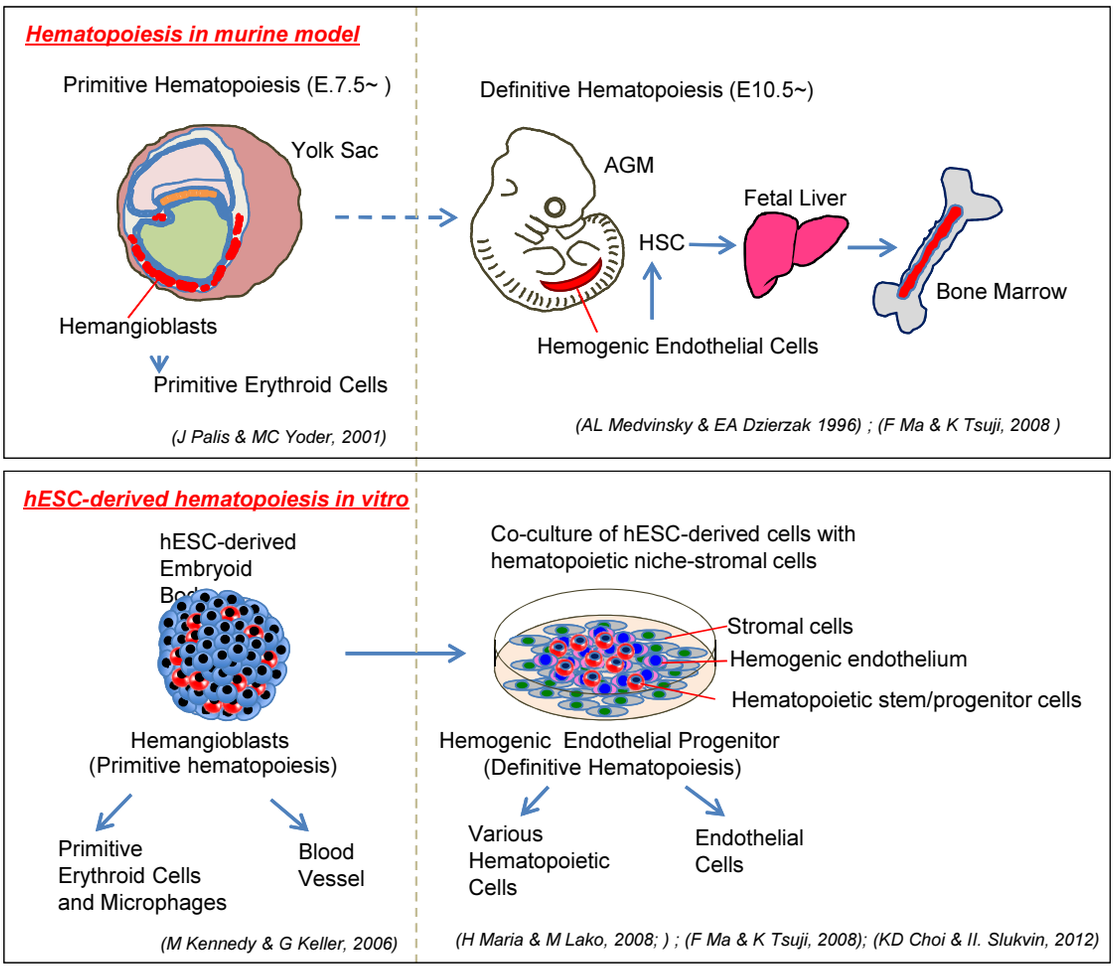


Figure 2. Comparison of mouse model and in vitro hESC/hiPSC-derived hematopoiesis

3.1. hESC-derived erythrocytes

Research on hESC-derived erythrocytes is one of the hot points because of the fact that development of erythrocytes shows distinct trait between primitive and definitive waves. More important is that the matured RBCs have no nucleus, which could avoid the exogenous gene interference when clinically applied to treat patients. Much labor has been donated to optimize the method of in vitro differentiation of RBCs from hESCs. For example, Lu's group firstly use EBs formation and then co-culture with OP9 to obtain a high yield of erythrocytes with some efficiency of enucleation. However, these erythrocytes were still immature because only 16% of these hESC-derived erythrocytes expressed β-globin [84]. Similar work has been done by several other groups using OP9 system [95, 96]

In our laboratory, we have recently established efficient blood cell-yeilding systems by co-culture of hESC/hiPSCs with murine AGM and FL stromal cells (Figure 3). In the co-culture, undifferentiated hESC/hiPSC colonies grow up and differentiate firstly to a mesoderm-like structure, then to hematopoietic progenitor cells on days 10 to 14. In the second suspension culture, these hESC/hiPSC-derived hematopoietic progenitors are further induced to some specific blood cell lineages, such as erythrocytes, mast cells, and eosinophils, etc. [3]. By a clone-tracing method, we gained concrete results that hESC-derived erythrocytes kept continuously progressing toward maturation over a time course. The expression of β-globin vs ε-globin showed a typical switching pattern, mimicking the normal development of human erythrocytes. On day 12 of the co-culture with murine FL-derived stromal cells, hESC-derived erythrocytes (BFU-E) express β-globin at about 60%, but up-regulated to almost 100% with additional 6 days of culture. These matured hESC-derived erythrocytes can undergo enucleation and release oxygen [5].

Our results showed that the in vitro differentiation from hESCs to matured hematopoietic cells is a progressive process if proper co-culture condition provided. Functionally matured blood cells similar to those from the definitive hematopoiesis could be obtained in large-scale production by this method.

3.2. hESC-derived HSCs

The most challenging task for research on hESC/hiPSC-derived hematopoiesis is to obtain the real HSC from in vitro differentiation system [97]. Although hESC/hiPSC-derived hematopoietic cells that could be engrafted in immune compromised mice have been reported [86, 98-102], effort to obtain real HSCs from hESCs has largely been done in vain during past years. In most experiments, the engraftment rate was very low and mostly restricted to the myeloid lineage, and it was ambiguous if these engrafed cells were derived from real HSCs. The co-culture with S17 could induce hESCs to HSC-like properties with low capacity of RBC potential [103]. If a modified cell line generated from mAGM (AM20.1B4) was used the RBC activity of HSC will be increased much [86]. But these hESC-derived HSC-like cells could not repopulate in NOD-SCID mice, representing they were not true definitive HSCs that satisfy the functional definition.

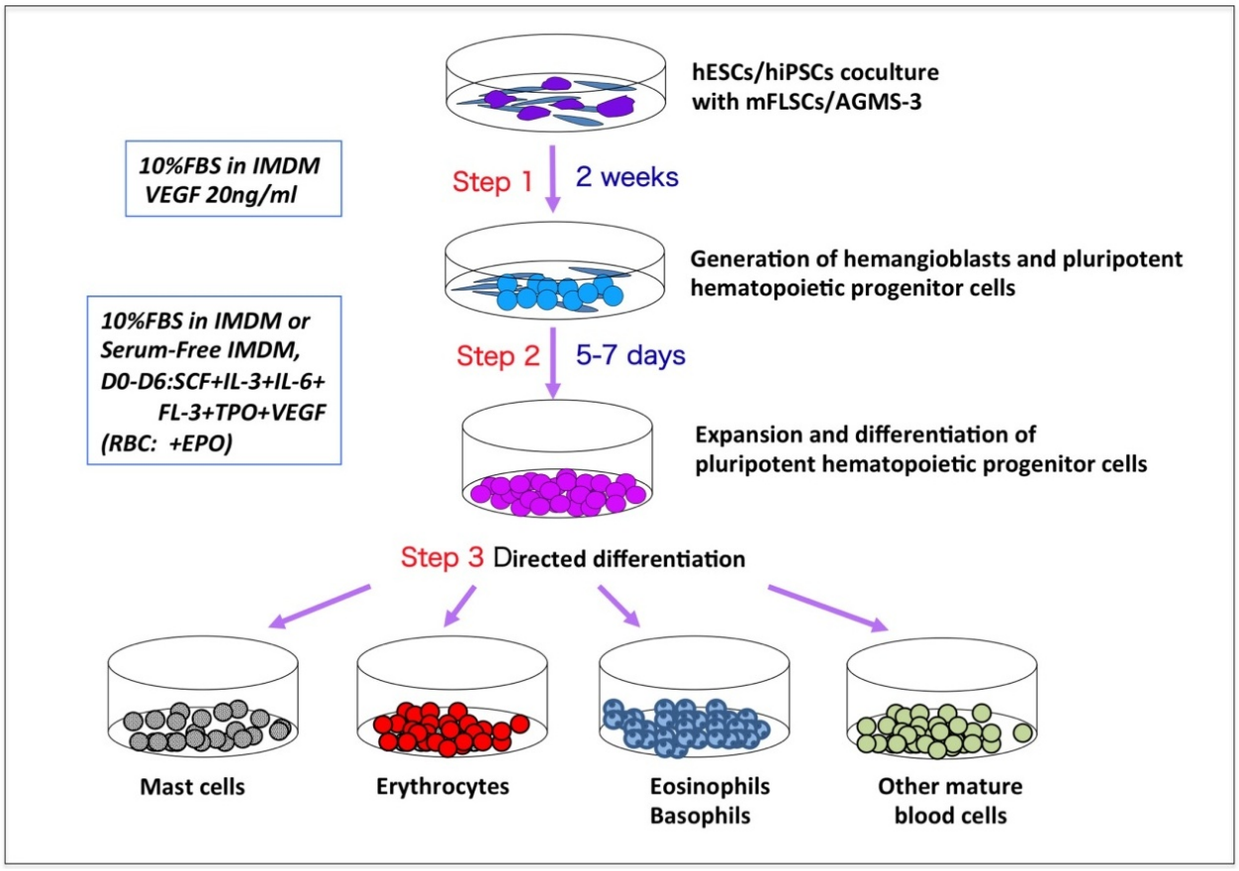


Figure 3. Co-culture of hESC/hiPSCs with murine AGM or FL stromal cells

3.3. hESC-derived lymphocytes

A recent report showed that when co-cultured with OP9-DL1 cells, the hESC-derived hematopoietic progenitor cells that formed endothelium-lined cell clumps could be induced into T-lineage cells [104]. These hESC-derived T cells expressed sequential surface markers from T-lymphoid progenitors (CD34+CD7+) to matured T cells (double positive CD4+CD8+and finally maturedCD3+CD1-CD27+). The T-lineage cell production provided concrete evidence that hESC-derived hematopoiesis endowed with definitive property. The functional matured hESC-derived NK cells could also be generated by sequential co-culture on different feeder cells [105, 106]. But the detail of HMC molecular and globulin class-switching during this process has still not been elucidated.

3.4. Hemangioblasts

The blood islands region in YS consists of clusters of primitive erythroblasts surrounded by mature endothelial cells. The close development relationship between hematopoietic and endothelial cells in such a region indicated that these lineages ought to share a common progenitor named as the hemangioblast [16, 33, 107]. This concept was provided nearly one century ago [108,109] while it has been circumstantially supported by the latest molecular genetic and embryological proofs [110-114]. More direct evidence came from the in vitro

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differentiation system based on ESCs. Some differentiation models identified a progenitor with properties of the hemangioblast [115, 116], indicating that the primitive hematopoiesis derived from ESCs should pass through a hemangioblast stage. Further studies showed that these hemangioblasts might have more potential than hematopoietic and endothelial cells, such as smooth muscle [117, 118].

hESC-derived hemangioblasts have also been identified in differentiation cultures [87]. These hESC-derived hemangioblasts were defined by the expression of KDR and could generate clonal cells sharing both hematopoietic and vascular potential. There were two distinct types of hESC-derived hemangioblasts: one gave rise to primitive erythroid cells, macrophages, and endothelial cells and the other generated only the primitive erythroid population and endothelial cells. This finding provided evidence that hESC-derived hematopoiesis mimicked the normal development pattern of human earliest stage of hematopoietic commitment.

3.5. Hemogenic endothelium

Different to hemangioblasts from YS, the concept of "hemogenic endothelial" come from the research of AGM, which is in the ventral wall of the aorta and buds off HSCs [13]. The molecular control of hemogenic endothelium is different to that of hemangioblasts. For example, Runx1 was indispensable for the hematopoiesis originated from hemogenic endothelia, but not hemangioblasts [119, 120]. And the hemogenic endothelium did not originate from hemangioblasts but presumptive mesoangioblasts, which could express endothelial-specific genes and ultimately express HSC-associated markers [121]. Since hemogenic endothelium is closely related to definitive hematopoiesis and has been regarded as the necessary stage for generation of HSCs, the details of its potential and developmental process to produce HSCs should be finely elucidated [122-125].

In some in vitro differentiation systems, hESC-derived CD34+ hemogenic progenitor cells could also be detected with the endothelium potential [3, 126]. In an OP-9 co-culture system, hESC-derived hemogenic endothelium progenitors (HEPs) were identified pinpoint by VE-cadherin+CD73-CD235a/CD43- intermediate phenotype, which arise at the post primitive streak stage of differentiation directly from a hematovascular mesodermal precursor (KDR +APLNR+PDGFRa$^{low/-}$). These HEPs differ from non-HEPs (VE-cadherin+CD73+) and early hematopoietic cells (VEcadherin+CD235a+CD41a−) [124]. This subtle finding may provide clue to facilitate generation of HSCs from hESCs (Figure 4).

3.6. Switching mechanisms controlling primitive hematopoiesis to definitive one

The origin of two waves of hematopoiesis was distinct in the sight of embryogenesis by the research for Xenopus [127], mice [128] and human [54]. However, the reciprocal transplant test in Xenopus embryo proved that the hematopoietic progenitor cell in VBI(corresponding to YS) and DLP(corresponding to AGM) could change their potential according to microenvironment of the graft site [129]. Similarly, blast-like cells derived from murine ESCs could differentiate to both primitive and definitive lineages [130]. In our study by a clonal tracing method, hESC-derived erythrocytes showed the primitive properties at the early stage and progressively

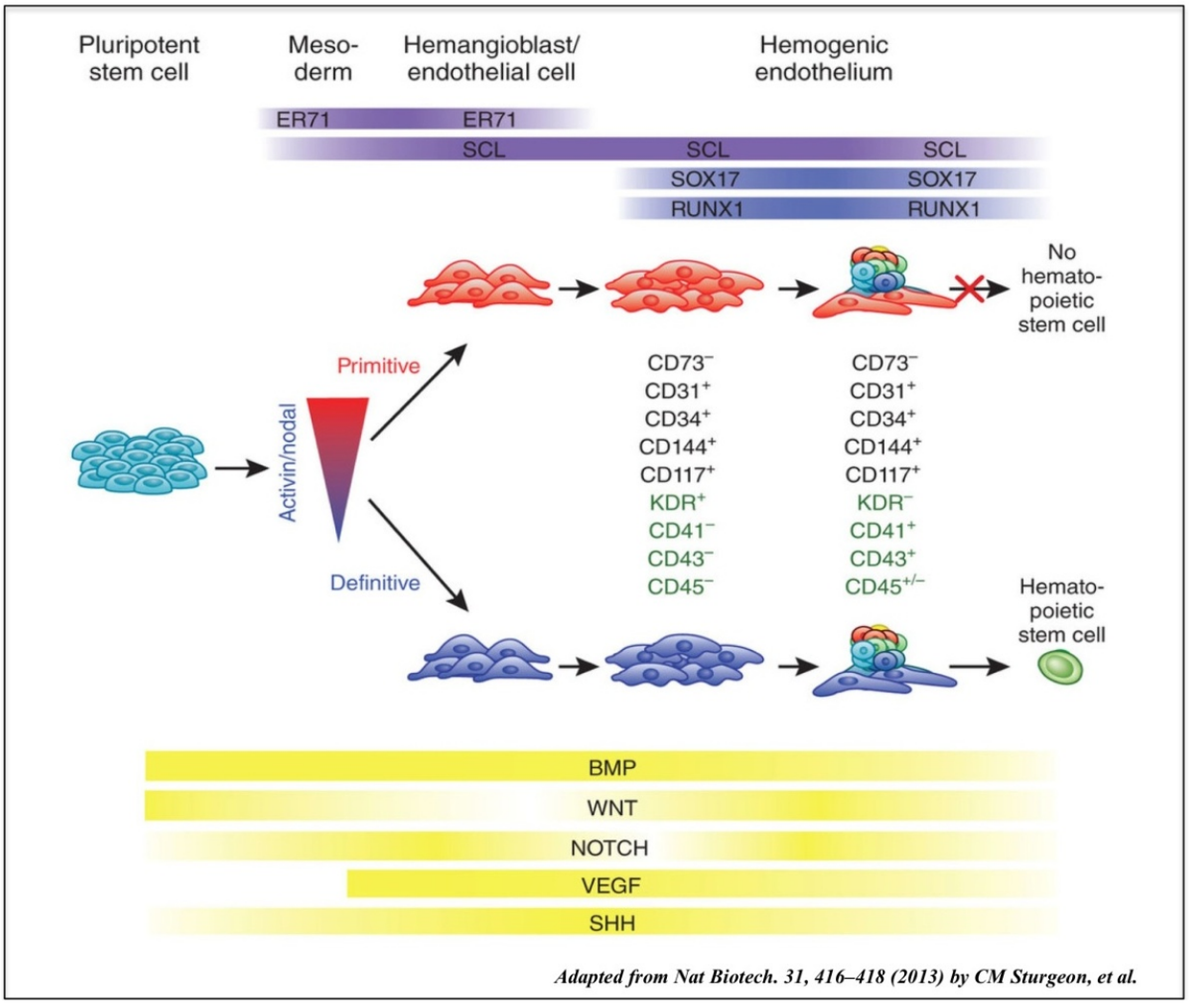


Figure 4. Development of hemangioblasts and hemogenic endothelia from pluripotent stem cells

turned to be definitive ones along with co-culture on FL cells [5], indicating that there exists a possible switching mechanism controlling the conversion.

However, the molecular mechanism controlling such a switch remains unclear so far. The switch observed in process of hESC-derived hematopoiesis probably is caused by the influence or induction of the definitive hematopoietic niche-derived stromal cells (AGM, FL). The cellular and molecular signals, including the growth factors and adhesion proteins expressed by feeder cells and growth factors added in medium ought to play a key role to activate the switch controlling the definitive hematopoiesis (such as Hedgehog, Notch1 and BMP signal pathways). Among them, HoxB4 had been identified as the key factor to promote such a switch [131]. Many key factors expressed in primitive and/or definitive erythrogenesis could also be tracked in ESC-derived hematopoiesis [94, 132]. Since the hemoglobin switch of erythrocyte could be used as the indicator of premitive and definitive hematopoiesis, the erythrogenesis-related factors, such as GATA1, GATA2, AML1, SCL, EKLF, ought to be changed their

expression profile during such a switch. A thorough transcriptome analysis by deep sequencing or gene chip should be useful tools to detect hESC-derived hematopoietic switch.

4. Concluding remarks and future perspectives

The in vitro production of functionally matured blood cells from hESC/hiPSCs has provided an excellent model for both basic research and clinical applications. hESC/hiPSC-derived RBCs are highly fancied because of their unlimited use as the substitute for blood transfusion, not to mention the the utilization of hESC/hiPSC-derived HSCs in transplantation. On the other hand, hiPSCs can provide patient-tailored models for analyzing the pathogenesis of malignant blood diseases and thus develop individual treatment by molecular corrections. Innate immune-related cells differentiated from hESC/hiPSCs will also help us to reveal the initial establishment of human immune system, and to explore drug screening system and cell therapy model for deficiencies of innate immunity.

However, before the successful application of hESC-derived cellular therapy, there are still many problems needed to be solved. The molecular mechanisms controlling both primitive and definitive hematopoiesis should be clarified at first. The HSC-like cells and functional matured blood cells derived from hESC / hiPSC in vitro must also be proven their activities in vivo. Besides, more efficient culture system free from xenobiotics must be optimized.

In order to realize above aims, the following efforts need to be done to promote the research in this field:

1. The latest molecular biological technology and other new technique tool should be applied in the future research.

Currently, Tetracycline (tc) and Tet repressor (TetR) system (tet-on and off) is one of the most matured and widely applied regulatory systems [133] in organisms ranging from bacteria to mammals. The technique by Transcription activator-like effector nucleases (TALENs [135, 136]) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems [136, 137] have also been developed. These methods let us be able to do molecular manipulation in genomic scale(named "genome edit") by unlimited times against single cell line without obvious influence to the cell characteristic, thus provide an ideal model to investigate the development events in hESC-derived hematopoiesis.

2. A highly efficient animal-source-free 3D culture system should be developed to search for the generation of hESC-derived HSCs.

A big problem that hampers the progress in research on hESC-derived hematopoiesis is a lack of a proper culture system that fully mimics the microenvironment of early development of human hematopoiesis. An ideal culture model should be completely free of any animal source substitutes and to great extent imitate a live structure as done in vivo circumstances. Since HSCs develop in a complicated niche composed of various cell types (endothelial cells, osteoblasts, mesenchymal cells, etc.), a 3D culture model mixed by several hematopoietic niche cells should benefit the efficiency of generating true HSCs from hESCs.

3. The new concept and theory should be introduced to conduct the research of hESC-derived hematopoiesis.

According to the recent researches by Deng and other group [138, 139], the pluripotency of stem cells is only the balance between several transcription factor groups controlling layer lineage differentiation. Their finding denied the existence of special pluripotent factors and showed that maintenance of ESC/iPSCs may be only because that all the way to any differentiation direction has been blocked by mutually antagonistic lineage specifiers [140]. Such concept may also help us to uncover the controlling balance by some specifiers in early hematopoiesis, especially for those controlling primitive and definitive ones. Since we always try to obtain more definitive blood cells for clinic application, to discover the key factor leading to definitive hematopoiesis is a challenging task to do. Moreover, how to define and describe the essence of HSCs at molecular level is also an issue need to be re-addressed.

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