Chapter 3

Embryonic Stem Cell Differentiation – A Model System to Study Embryonic Haematopoiesis

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Additional information is available at the end of the chapter

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1. Introduction

Haematopoiesis, the process of generation of blood cells, is one of the most extensively studied developmental systems. The whole spectrum of blood cells produced in mammalian organisms includes primitive erythrocytes and definitive haematopoietic cells such as myeloid, lymphoid, definitive erythroid and haematopoietic stem cells (HSCs).

Haematopoiesis takes place in several locations during ontogeny and in adult life. The embryonic origin of blood cells has been studied for more than a century. However, studies on haematopoiesis *in vivo* are challenging as embryos, and in particular mammalian embryos, are extremely small and difficult to access at these very early stages of development. Moreover, the number of cells per embryo is limited and all the successive developmental events take place very fast. Therefore different approaches have been developed to facilitate these studies *in vitro* and one of them involves the use of embryonic stem (ES) cell *in vitro* differentiation. In this chapter, we will highlight some recent results on studies of the development of the haematopoietic system obtained in particular using the *in vitro* differentiation of murine ES cells. We will also present the methods we routinely use in our laboratory to work with wild type or genetically modified murine ES cells.

2. Early haematopoietic development

2.1. How can embryonic stem cells be used to study early embryonic haematopoiesis?

Studying haematopoiesis in the mouse embryo *in vivo* remains challenging, in particular at the very early stages of development when the embryo is small, difficult to access and the number of cells is limited. One of the alternative approaches is the *in vitro* differentiation of ES cells which are defined as pluripotent cells, able to give rise to three primary germ cell layers (endoderm, mesoderm and ectoderm) [1]. ES cells are isolated from the inner cell mass of the blastocyst and under appropriate conditions can be maintained undifferentiated in culture [2] or alternatively allowed to differentiate. By scaling up cultures of differentiated ES cells, it is relatively easy to access large number of cells that would be unattainable *in vivo*. ES cells represent a unique tool to study the molecular and cellular mechanisms of normal haematopoietic development, or the perturbations of these mechanisms leading to pathogenesis. In addition, with the advent of human ES cells, and induced human pluripotent stem (iPS) cells, the differentiation of these stem cells toward haematopoiesis could represent an exciting approach to generate cell populations to treat haematological disorders.

2.2. Sites of haematopoietic development

In 1920, the embryologist Florence Sabin observed that endothelial and haematopoietic cells were closely located in the yolk sac of avian embryo [3]. These structures, later called "blood islands", were thought to be derived from mesodermal cells undergoing differentiation towards endothelial and haematopoietic lineages [4]. In the mouse embryo, the first blood cells were shown to emerge around day E7.5 in the extra-embryonic yolk sac, within the blood islands [5]. These first haematopoietic cells are primitive erythrocytes that transport large amounts of oxygen required to support the rapidly growing embryo. In the final days of gestation, their number decreases rapidly as other haematopoietic cells overtake their function.

For a long time, the yolk sac was thought to generate only primitive erythrocytes. However, detailed studies indicated that other cell lineages such as definitive erythroid progenitors, mast cells and bipotential granulocyte/macrophage progenitors are also generated in the murine yolk sac before circulation [6]. By day E8.5, circulation in the mouse embryo is established and the newly formed blood vessels connect the extra-embryonic yolk sac to intra-embryonic tissues. From that time onward, other haematopoietic tissues within the embryo proper become actively involved in haematopoiesis. In 1994, a seminal study by Muller and co-workers demonstrated that at day 10 p.c. (post coitus), the aorta-gonad-mesonephros (AGM) region contains long term repopulating haematopoietic stem cells (HSCs)-the foundation of the blood system in adult organisms [7]. The AGM region is an intra-embryonic site that will later develop into major internal organs. Following the discovery of these first HSCs in the AGM region it was important to distinguish whether these cells were generated in this region or emigrated from other embryonic locations through the blood circulation. The work of Medvinsky and Dzierzak established that definitive HSCs, capable of long term multilineage haematopoietic reconstitution emerge but also expand within the AGM region [8]. More recently, the placenta, both in mouse and human was reported to contain HSCs [9, 10],

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although whether these HSCs are *de novo* generated within the placenta remains unknown. During adult life, the main site of haematopoiesis is the bone marrow, where HSCs are found. The capacity of HSCs present in the bone marrow to rebuild the whole haematopoietic hierarchy in recipient organism is routinely used by clinicians to treat many blood-related diseases through bone marrow transplantations.

2.3. Haemangioblast and haemogenic endothelium – Is there a connection?

The search for the cellular origin of blood cells started nearly 100 years ago, when Sabin noticed that endothelial and haematopoietic lineages are located in close proximity within the blood islands, suggesting the existence of a common precursor called a haemangioblast [3]. Few years later, in 1924 Alexander Maximow observed that the blood islands represent mesodermal masses that differentiated towards endothelial and haematopoietic cells [4]. *In vitro* experiments based on embryonic stem (ES) cell differentiation were the first experiments providing substantial data supporting the existence of the haemangioblast [11-13]. First, Choi and co-workers identified a precursor called blast colony forming cell (BL-CFC), expressing FLK1 – the VEGR receptor 2, that upon culture gave rise to blast colonies containing precursors for both endothelial and haematopoietic cells [11]. These BL-CFCs were further shown to express the *Brachyury* (*T*) gene as well as the *Scl* (Stem Cell Leukaemia) transcription factor [12, 13]. Later, studies on mouse embryos demonstrated the existence of the hemangioblast *in vivo* and indicated that it is found prominently in the posterior primitive streak [14]. It probably migrates from there to the yolk sac where the generation of blood, endothelial and vascular smooth muscle cells take place [14]. The existence of haemangioblast was also more recently documented in human with human ES cells [15] and *in vivo* in zebrafish [16].

Another concept of development proposes that a mature endothelial cell with haematopoietic potential, a haemogenic endothelium, give rise to blood cells. Several *in vitro* studies demonstrated that endothelial cells have the potential to generate blood cell lineages [17, 18]. In these studies, the authors isolated cells expressing both FLK1 and the endothelial marker VE-Cadherin and observed that these cells were able of *de novo* production of blood cells, marked by the expression of CD45. The generation of blood cells from endothelial progenitors was also demonstrated *in vivo* by Jaffredo and collaborators [19]. These authors specifically labelled endothelial cells in the avian embryo and observed that haematopoietic cells are later generated from these fluorescent endothelial progenitors.

More recently, a study by Lancrin and colleagues merged the haemangioblast and haemogenic endothelium theories into one linear model of development, in which the haemogenic endothelium is an intermediate stage during the generation of blood progenitors from the haemangioblast [20]. The presence of a haemogenic endothelium cell population was established both *in vitro* during ES cell differentiation as well as *in vivo*, in E7.5 mouse embryos [20-22]. In 2010, the generation of blood cells by haemogenic endothelium was directly visualised in embryos. This endothelial to haematopoietic transition (EHT) was observed both during murine [23] and zebrafish embryogenesis [24-26]. A schematic representation of the successive stages of haematopoietic commitment is presented in Figure 1.

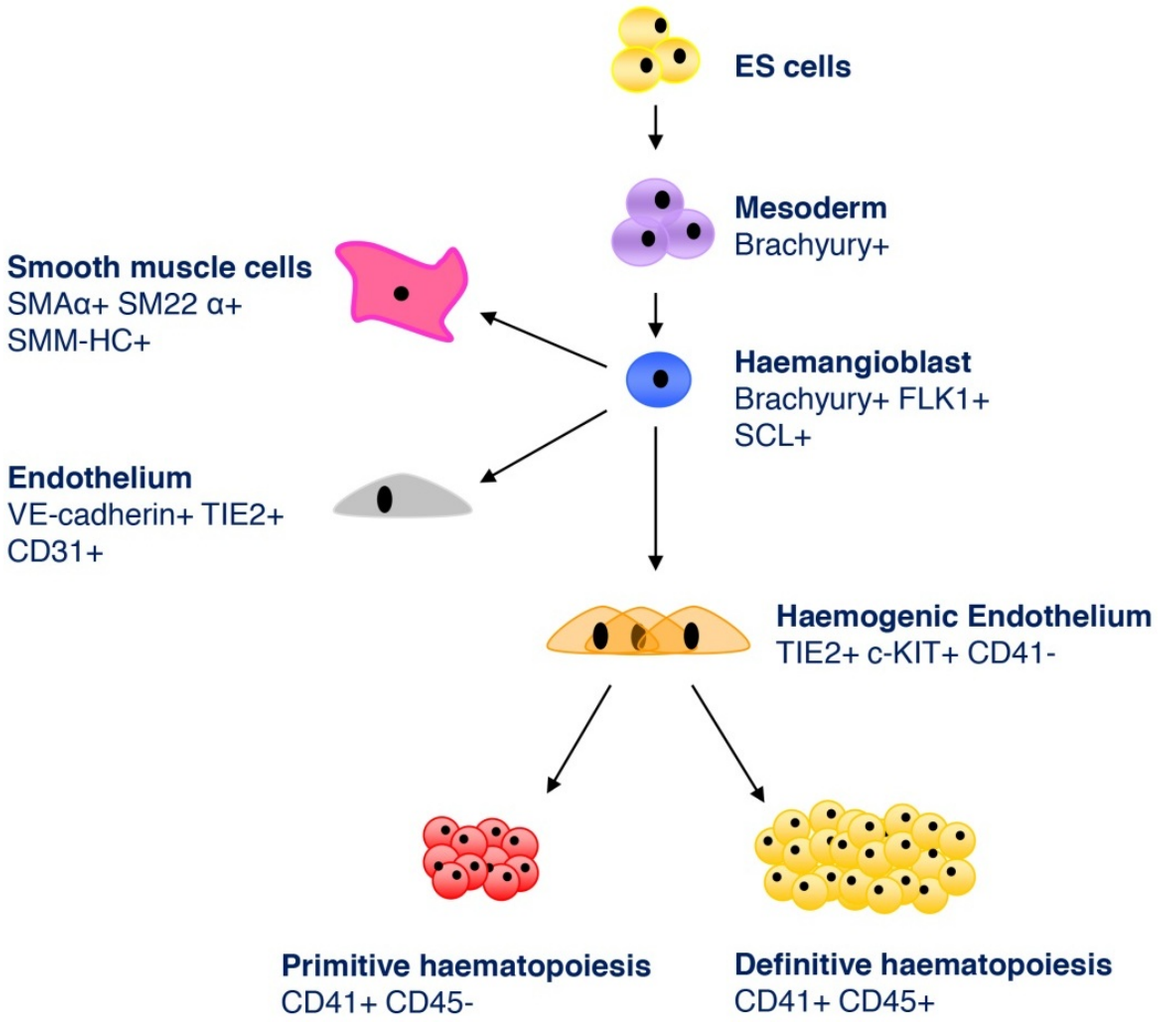

Figure 1. The process of generation of blood cells from the haemangioblast through a transient haemogenic endothelium cell population. Specific markers for each cell population are depicted. This figure has been adapted from [27].

2.4. Primitive erythrocytes – First blood cells in the embryo

Primitive erythrocytes, the first blood cells that emerge during embryogenesis in mammals, are large and nucleated. Their main function is to transport large quantities of oxygen to support the rapid growth of the embryo. It has been shown that these cells firstly appear *in vivo* in the yolk sac's blood islands around day E7.5 [28]. Although it was initially thought that these cells were nucleated, more recent studies have indicated that their nuclei are lost during the final days of mammalian gestation [29]. Studying primitive erythropoiesis remains challenging for several reasons; not only is the murine embryo around day 7 of gestation

extremely small and difficult to access, but also there are no specific cell surface markers to specifically label this cell population. Therefore, transgenic mouse models were developed to directly address this later limitation.

Two mouse models used the expression of the same haemoglobin – ε-globin, as a reporter to track primitive erythropoiesis during ontogeny. In the first model [30], the authors coupled the ε-globin promoter to the KGFP (jellyfish-derived) fluorescent protein. With this new tool, they were able to isolate circulating primitive erythrocytes at different stages of murine embryogenesis and also to define the cell surface markers expressed by these cells between day E9.5 and E12.5 such as TER119, CD71, CD24, CD55 or CD147 [30]. In a second model, the fluorescent reporter used was a H2B-EGFP fusion protein [31]. This model allowed study of the complex process of maturation of primitive erythrocytes within the foetal liver [31]. More recently, the same group monitored the emergence of primitive erythrocytes at the very early stages of development *in vivo*, starting from day E6.75 and defined key pathways governing the emergence of this cellular lineage [32]. Other studies have more directly examined the cell signalling pathways supporting the emergence of primitive erythrocytes using both *in vivo* mouse models, as well as *in vitro* using the ES cell differentiation approach. In 2008, the importance of Wnt signalling for the emergence of primitive erythrocytes from FLK1 positive mesoderm was demonstrated [33]. Later, Cheng and co-workers expanded those findings and showed that not only the activation of Wnt pathway is crucial, but also the inhibition of Notch signalling is important for the emergence of primitive erythrocytes from FLK1 positive cells [34].

2.5. *In vivo* studies of the first haematopoietic stem cells (HSCs) – the foundation of the adult blood system

There are several definitive haematopoietic lineages generated during embryogenesis such as myeloid, lymphoid, definitive erythroid and haematopoietic stem cells. HSCs are the foundation of the blood system in the adult organism as these cells can differentiate towards all definitive haematopoietic cells.

HSCs arise first in the AGM, they possess the ability to self renew and, upon transplantation, they provide multilineage haematopoietic reconstitution [35]. Various studies, spanning several decades, aimed to characterise HSCs. In 1993, Huang and Auerbach reported that at E9.0 the murine yolk sac contains HSCs [36]. These cells, however, were unable to provide long-term multilineage haematopoietic reconstitution. A few months later, Muller and co-workers demonstrated that at day E10.5 the AGM region of the mouse contains fully functional HSCs – able to provide long-term haematopoietic reconstitution [7]. These findings were then expanded and HSCs were shown to emerge and expand within the anterior part of the AGM region [8]. It was also observed that definitive HSCs are present in the placenta, both in mouse [9, 37, 38] and human [10]. Interestingly, more recently mouse embryonic head tissues were shown to contain HSCs [39].

Several research groups investigated the cellular origin of haematopoietic stem cells *in vivo*. Zovein and co-workers (2008) demonstrated that HSCs emerge from the endothelium by performing lineage tracing experiment to specifically label either the endothelium or mesen-

chyme [21]. Furthermore, the emergence of putative haematopoietic cells from haemogenic endothelium has been visualised in the mouse embryonic aorta [23]. Similar results were obtained in zebrafish [24-26]. However whether these blood cells display any long-term repopulation activity remains to be directly assessed.

2.6. Molecular regulation of early embryonic haematopoiesis

Specific transcription factors regulate the developmental potential of different cells and progenitors during blood formation. In this section, we will discuss the role and function of a restricted set of these players.

One of the first genes implicated in mesoderm leading to blood development is the *Brachyury* gene. This transcription factor belongs to the T-box gene family [40] and is expressed by all nascent mesodermal cells [41]. Its expression is detected in murine embryo as early as E6.5 [42] and its deletion results in serious developmental defects and lethality by midgestation [42, 43]. In 2003, Fehling and co-workers generated a transgenic ES cell line in which GFP was targeted to the *Brachyury* locus. Further differentiation of this ES cell line allowed them to isolate mesodermal cells. The authors demonstrated that when combined with FLK1 (*Fetal Liver Kinase 1*) it was possible to separate three distinct populations corresponding to pre-mesoderm (negative for both FLK1 and *Brachyury*), mesodermal (positive for *Brachyury* only) and finally haemangioblastic, positive for both FLK1 and *Brachyury,* populations [13].

Flk1 encodes the receptor 2 for vascular endothelial growth factor (VEGF-R2) [44]. This gene is expressed by intra-and extra-embryonic mesoderm and later by endothelial cells in the vasculature [45, 46]. In 1995, Shalaby and colleagues demonstrated that in *Flk1* deficient embryos blood vessels and blood islands are not formed and that hardly any haematopoietic progenitors are present in these embryos [47]. As a result, *Flk1* deletion is embryonic lethal and embryos die between day E8.5 and E9.5 [47]. A few years later, they also determined, by evaluating the contribution of *Flk1* deficient ES cells to chimaeric mice, that the expression of *Flk1* is crucial for the migration of mesodermal progenitors from the intra-embryonic locations to the yolk sac [44]. This finding was later confirmed using *in vitro* differentiation of ES cells. In 1998 Choi and co-workers demonstrated that during ES cell differentiation, *Flk1* expression marks the blast colony-forming cell (BL-CFC) [11]. Later, it was shown that although Flk1 deficient ES cells are able to give rise to endothelial and haematopoietic lineages upon *in vitro* differentiation, they generate reduced number of blast colonies [48]. To date FLK1 remains, with *Brachyury*, the best marker of haemangioblast.

Etv2, a transcription factor of the *Ets* family, is another important regulator of haematopoietic specification. Murine embryos deficient for the expression of *Etv2* were shown to die at around E10.5 and to lack blood cells and vessels [49]. More recently, it was shown that *Etv2* is not required for the specification of primitive mesoderm, but is indispensable in the commitment of FLK1-positive mesoderm towards haematopoietic and endothelial programmes [50]. Using a transgenic *Etv2* ES cell line and mouse line it was shown that the expression of *Etv2* marks the endothelium and in particular haemogenic endothelial cell population [51]. In the absence of this transcription factor, both *in vivo* and *in vitro*, no haemogenic endothelium was observed [51]. Furthermore, it was demonstrated using a Cre-mediated deletion of *Etv2*, that this

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transcription factor is acting at the *Flk1* stage and that re-introduction of the *Scl* transcription factor in Etv2-deficient ES cells can fully rescue the haematopoietic potential of these cells [52].

The transcription factor *Scl* (Stem Cell Leukaemia Factor) was originally identified due to its involvement in chromosomal translocation in T-cell leukaemias [53] and was later demonstrated by both *in vitro* and *in vivo* studies to play a significant role during embryonic blood development. Murine embryos lacking this transcription factor do not develop neither primitive nor definitive haematopoietic cells and die by E9.5 [54, 55]. In addition, *Scl*-/-ES cells do not generate blast colonies (Robertson et al., 2000) or any haematopoietic cells [56]. Blast colony forming cells (BL-CFCs) were shown to express Scl during *in vitro* ES cell differentiation [12]. More recently it was demonstrated that *Scl* is critical for the generation of the haemogenic endothelium [20].

SOX7, with SOX17 and SOX18, form the F-subgroup of SRY-related (HMG-box) family of transcription factors [57]. During embryonic development SOX7 transcripts are detected in various tissues such as brain, heart, lung, kidney and spleen [58]. SOX7 and SOX18 knockdowns performed in zebrafish and *Xenopus* embryos revealed critical roles of these transcription factors in cardiogenesis and vasculogenesis [59-61]. Recently, Wat and colleagues developed a mouse model lacking the expression of Sox7 that is embryonic lethal at E10.5 due to cardiovascular abnormalities [62]. SOX7 was recently shown to be also implicated in early stages of blood development. SOX7 expression is upregulated at the haemangioblast stage and transiently expressed in the first CD41 – positive blood progenitors emerging from the FLK1-positive haemangioblasts [63]. Its enforced expression in haematopoietic progenitors, marked by the expression of CD41, results in the arrest of haematopoietic differentiation of these cells, a property shared by Sox18 but not Sox17 [64]. Recently, it was also reported that SOX7 is expressed at the haemogenic endothelium stage, where it regulates the expression of the endothelial marker VE-Cadherin [65].

The transcription factor RUNX1, encoded by the *Runx1/AML1* gene is considered a master regulator of definitive haematopoiesis. Indeed *Runx1* deficient embryos completely lack definitive haematopoietic cells [66]. The deletion of *Runx1* gene is embryonic lethal by E11.5 and E12.5 of gestation, and these embryos present multiple haemorrhages [67]. *Runx1* was also shown to be critical *in vitro*. *Runx1*-/-ES cells generate only a few blast colonies and these are restricted to primitive haematopoietic programme [68]. Furthermore the kinetic of the development of the haematopoietic system has been shown to be dependent of a gene dosage effect of *Runx1* [69]. Finally, *Runx1* is essential for the formation of haematopoietic progenitors from the haemogenic endothelium [20, 70, 71]. This critical role of RUNX1 in the endothelial to haematopoietic transition has spurred efforts to identify and characterize its direct transcriptional targets [72-74].

Although, numerous molecular regulators of haematopoietic specification have been identified, it is likely that many others remain to be discovered. In addition, the events they regulate and how they interact to orchestrate blood development remain largely unknown. The specific requirement for several of these regulators is depicted in Figure 2.

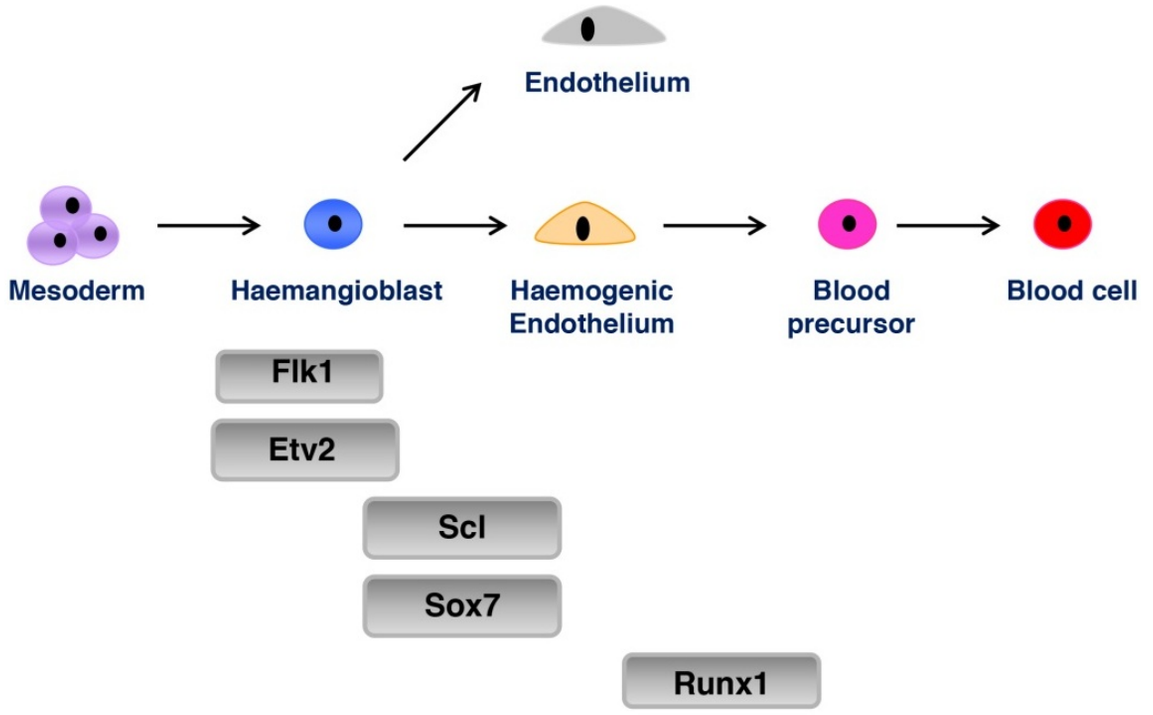

Figure 2. Molecular regulation of early embryonic haematopoiesis. The stages of blood development where the function of the different genes is critical are indicated.

3. Methods and procols to study haematopoitic development during es cell differentiation

ES cell differentiation provides a relatively easy and accessible system to study early embryonic haematopoiesis. Using well-defined protocols, it is possible to effectively study the events happening *in vivo* using this *in vitro* approach. This experimental system was shown to recapitulate the early *in vivo* events of development of the haematopoietic system.

3.1. Mouse embryonic fibroblasts (MEFs)

There are several methods to keep ES cells undifferentiated. One of them consists of growing ES cells on mouse embryonic fibroblasts (MEFs). Before working with ES cells, it is recommended to prepare a good stock of MEFs to be used as feeder cell layer. For that, wild type ICR or DR4 (resistant to four drugs) [75] MEFs are harvested from E14.5 embryos and cultured in Iscove's modified Dulbecco medium (IMDM, Lonza) supplemented with 50 μg/ml penicil-

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lin-streptomicin (Gibco), 2mM L-Glutamine, 10% of FCS (PAA Laboratories) and $1,5x10^{-4}$ monothioglycerol (MTG, Sigma) under low oxygen conditions. When amplified, MEFs are harvested (TrypLE, Invitrogen) and irradiated at 30Gy to stop the cells proliferation. The cells should be frozen at around 1 million cells per ml of IMDM supplemented with 50% FCS and 10% of dimethyl sulfoxide (DMSO). Cells should be stored at-80 ^{0}C. Thawed MEFs should be replated in one six-well plate previously coated with gelatine and let to adhere to the plastic wells overnight. Upon microscopic examination, MEFs should cover the entire surface of the cell-culture dish and be ready to be seeded with ES cells.

3.2. ES cell culture

ES cells are cultured on irradiated MEFs in a media constituted of Dulbeco's modified Eagle Medium (DMEM, Gibco) supplemented with 50 μg/ml penicillin-streptomycin, 2mM L-Glutamine, 15% FCS (PAA Laboratories), 2% Leukaemia Inhibitory Factor (LIF) (conditioned medium from LIF-generating cell line, see [76]) or 50 units of recombinant ESGRO LIF/ml (Millipore) and $1,5x10^4$ MTG (Sigma). Leukaemia inhibitory factor (LIF) – is a cytokine inhibiting differentiation. ES cells, when cultured on MEFs feeder cell layer in the presence of LIF remain undifferentiated. Upon microscopic observation they form tightly associated clusters of cells that are bright and shiny in appearance (Fig. 3A).

3.3. Generation of embryoid bodies

Embryoid bodies (EBs) are three-dimensional structures spontaneously generated by ES cells during differentiation. They contain precursors for the three primary germ layers ectoderm, endoderm and mesoderm. Two passages on gelatine are performed to remove the MEFs that would hamper ES cells differentiation. The first passage is performed in DMEM-ES media (described above), whereas for the second passage DMEM is replaced with IMDM. The ES cells are then harvested by trypsinisation and seeded into liquid cultures in non-tissue culture Petri dishes (Sterilin) in differentiation medium containing: IMDM supplemented with 15% FCS serum selected for differentiation (PAA Laboratories), 2mM L-Glutamine, 180 μg/ml transferring (Roche), 25 μg/ml Ascorbic Acid (AA, Sigma) and $4,6x10^{-4}$ MTG. The density of cell seeding should be adjusted in function of the day at which the cultures will be harvested, varying from $1,5x10^4$ cells/ml (for day 4-6) up to $3,0 \times 10^4$ cells/ml (for days 2.5-3.5). 10-20ml of "Differentiation medium" should be used per one Petri dish.

By performing two passages on gelatine and removing feeder cell layer and LIF, ES cells become primed for differentiation and formation of three-dimensional embryoid bodies in liquid culture (Figure 3B). Early EBs contain precursors for the three primary germ layers. By day 7, hemoglobinisation can be observed as red areas present within the EBs (Figure 3C). This system is versatile and allows to access and study in details several subsequent stages of blood development such as the emergence of haemangioblast, production of blast colonies and the development of primitive and definitive blood precursors (Figure 4).

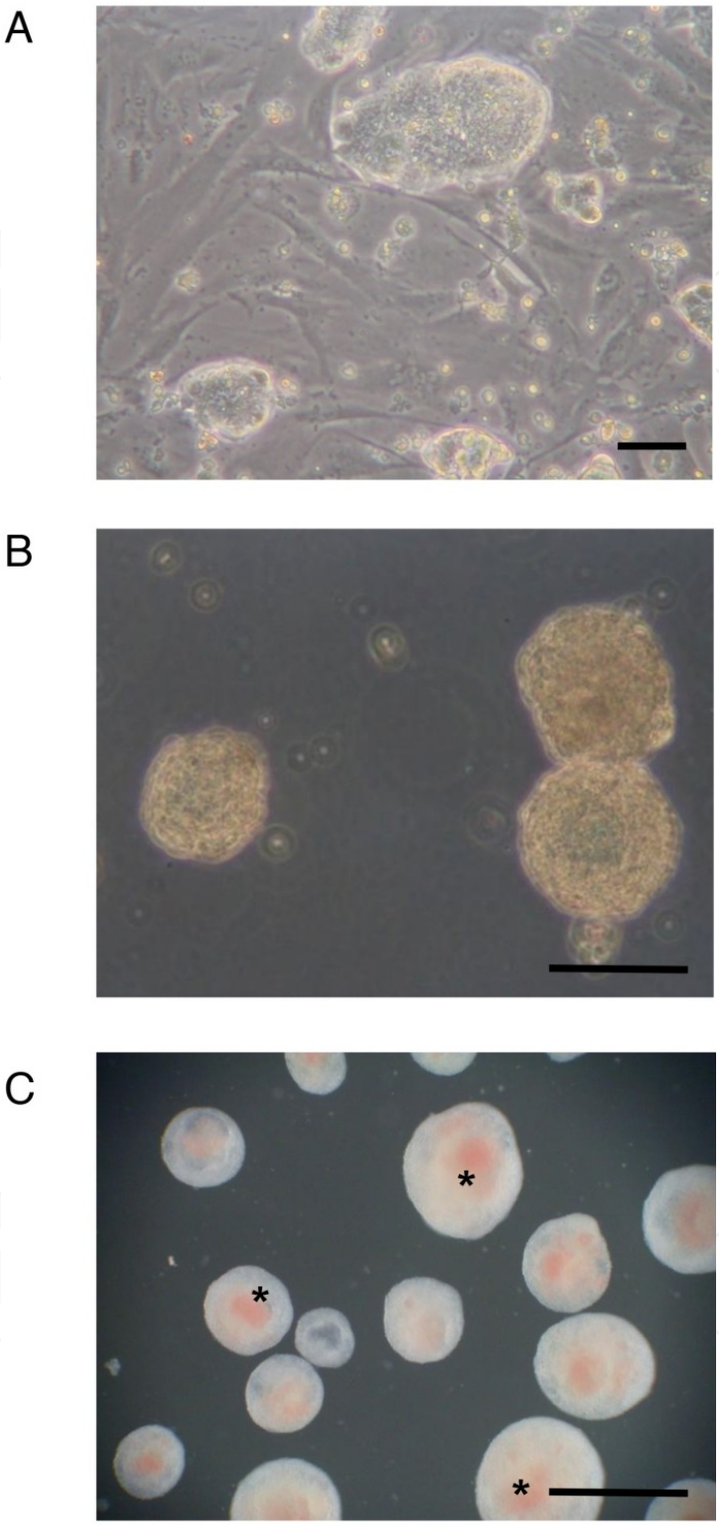

Figure 3. Morphology of ES cells and embryoid bodies (EBs). A) Typical appearance of ES cell in culture. Cells form bright, shiny adherent colonies and are cultured on a layer of MEFs feeder cell layer. B) Typical appearance of EBs in culture. These three-dimensional structures are formed during ES cell differentiation and contain precursors for three primary germ cell layers. C) Embryoid bodies at day 7 of differentiation containing haemoglobin (indicated with asterisks). Scale bar 300µm.

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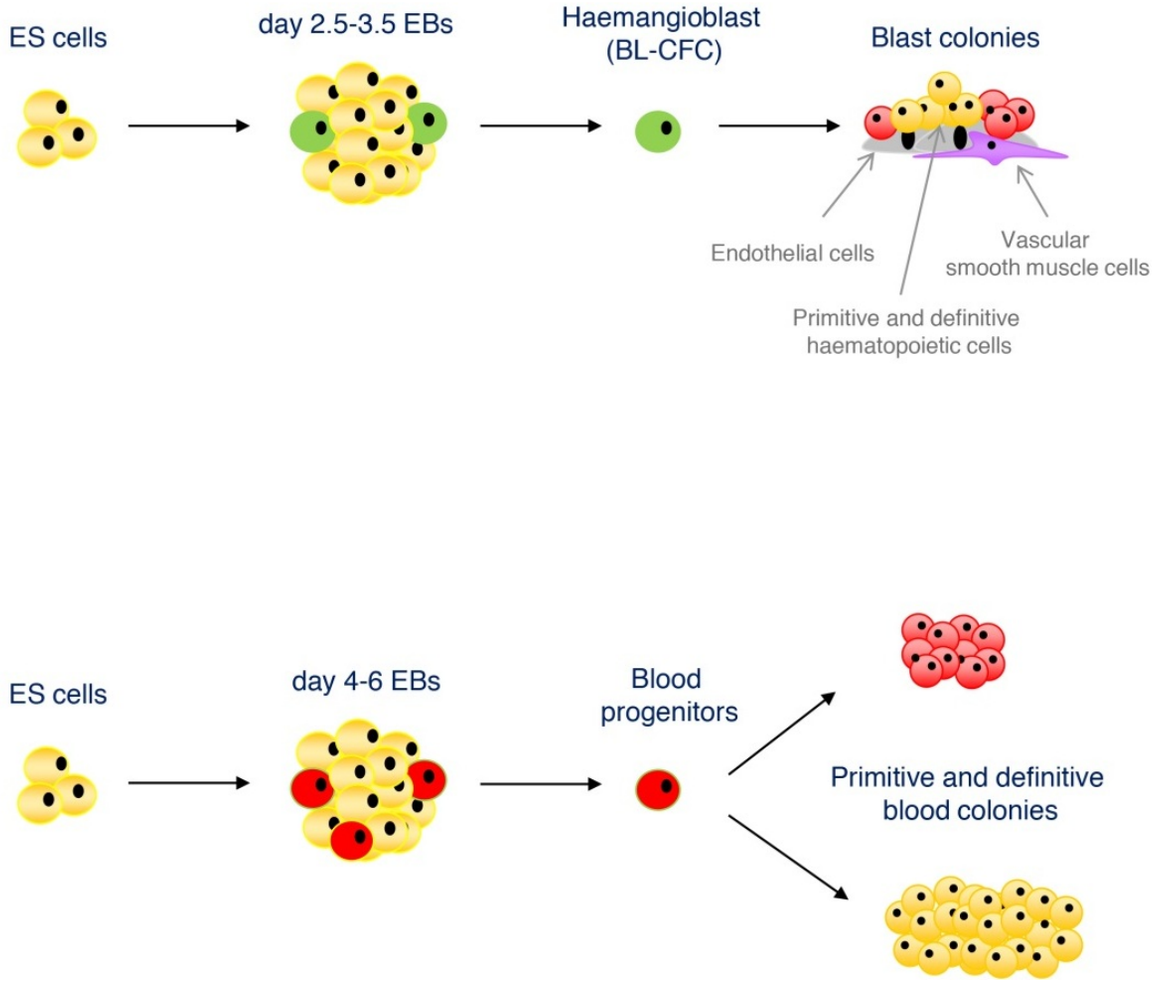

Figure 4. The ES/EB differentiation system. Upon differentiation of ES cells, three-dimensional structures, called embryoid bodies (EBs) are formed. Sorting EBs at day 2.5-3.5 for the expression of FLK1 enrich for BL-CFC, the *in vitro* equivalent of the haemangioblast. Upon culture, the BL-CFCs generate blast colonies that contain precursors for haematopoietic, endothelial and vascular smooth muscle cells. Day 4-6 EBs contain haematopoietic progenitors that can give rise to various primitive and definitive haematopoietic colonies.

3.4. Blast colonies

There are two alternative approaches to study the development of blast colonies; liquid culture on gelatine or semi-solid culture in methylcellulose. Liquid culture on gelatine facilitates harvesting of the cells for flow cytometry analysis or time-lapse imaging techniques. Alternatively, the semi-solid culture is a clonogenic assay allowing the growth of of individual blast colonies and their quantification. Individual blast colonies can then further be isolated and their haematopoietic potential assessed. To perform blast colony culture, EBs are harvested between day 2.5 and 3.5 and the cells are enriched for haemangioblast by sorting for the expression of FLK1, the receptor 2 for VEGF. Isolated FLK1 positive cells are then replated on gelatinised cell culture plates at the density of 8,5 x 10^4 cell/10cm^2 in a medium containing IMDM supplemented with 10% FCS, 2mM L-Glutamine mM, 180 μg/ml transferrin, 25 μg/ml ascorbic acid, 4.6 x 10-4 M MTG, 15% endothelial cell line-D4T conditioned medium [11], 10

ng/ml interleukin 6 (IL-6) (Peprotech) and 5 ng/ml vascular endothelial growth factor (VEGF, Peprotech). For semi-solid cultures, FLK1 positive cells are seeded in 35 mm x 10 mm (BD Falcon) dishes at a density of 1.5 x 10^4 cells/ml in IMDM medium supplemented with 10% FCS, 2mM L-Glutamine mM, 180 μg/ml transferrin, 25 μg/ml ascorbic acid, 4.6 x 10-4 M MTG, 15% endothelial cell line-D4T conditioned medium [11], 10 ng/ml interleukin 6 (IL-6) (Peprotech) and 5 ng/ml vascular endothelial growth factor (VEGF, Peprotech). The medium is additionally supplemented with 10 g/L methylcellulose (dissolved in IMDM, Alfa-Aesar). Blast colonies are scored 3-4 days after replating. Plating 3.0 x 10^4 cells per 1 ml of semi-solid medium should result in the formation of around 300-400 of blast colonies.

Sorting EBs between day 2.5 and 3.5 for the expression of FLK1 enrich for blast colony forming cell (BL-CFCs) [11, 13]. During the formation of blast colonies, several distinct morphological stages can be observed. First, there is the formation of tight clusters of adherent cells and later, single round cells emerging from these tight clusters. These cells then proliferate (Figure 5A). Alongside these morphological changes, there is first an upregulation of endothelial markers, such as TIE2 and VE-Cadherin during the formation of the tight clusters. Later the expression of these endothelial markers is gradually downregulated whereas the expression of haematopoietic markers (such as CD41 and then CD45) is upregulated (Figure 5B). This correlates with the emergence of round floating–haematopoietic-cells.

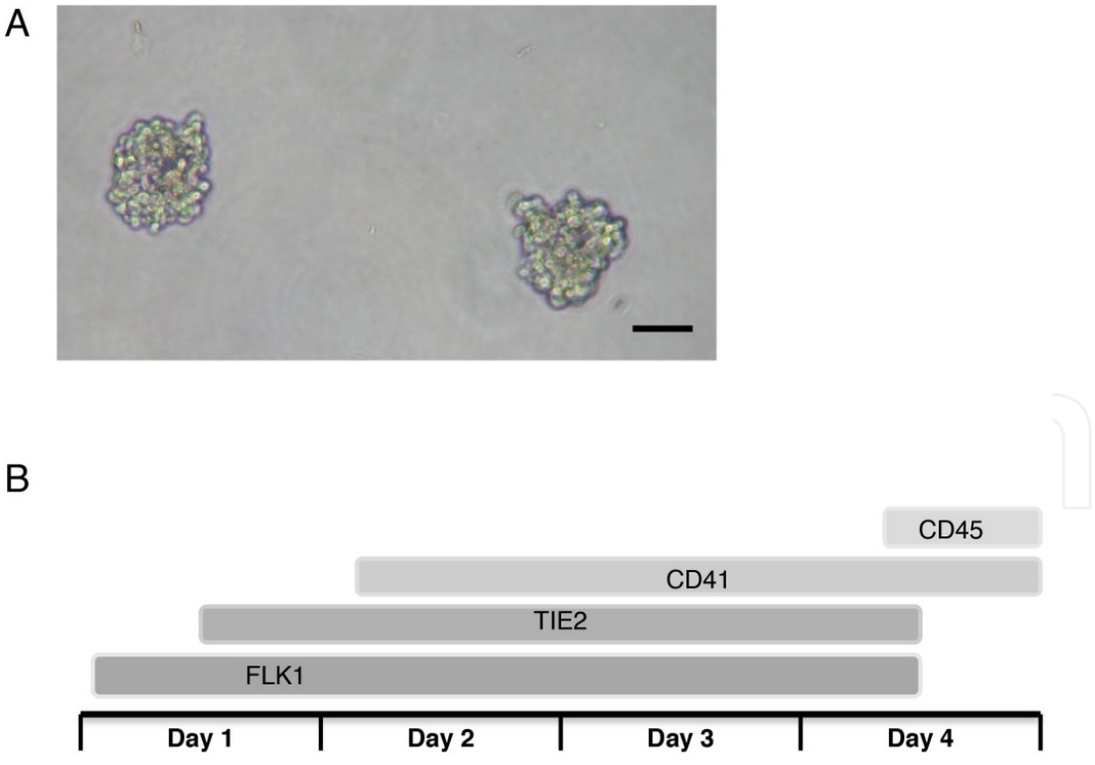

Figure 5. The development of the blast colony during four days of differentiation. A) Microscopic image showing representative blast colonies. Scale bar 100μm. B) Schematic representation of the different cell surface markers expressed on cells during blast colony differentiation.

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It was recently shown that the haemangioblast progenitor can also generate vascular smooth muscle cells in addition to endothelial and haematopoietic cells [14, 77, 78]. Accordingly, Yamashita and co-workers also established that FLK1 positive cells can differentiate towards endothelial and mural cells [78]. Smooth muscle cells appear, under the microscope as large flat adherent cells. These cells express smooth muscle specific genes such as smooth muscle actin α, transgelin, calponin, and smooth muscle myosin heavy chain [79]. A typical immunostaining of α-smooth muscle actin is presented in figure 6A. We have recently analysed in more detail the generation of smooth muscle cells and showed that these cells are largely generated independently from the haemogenic endothelium [80].

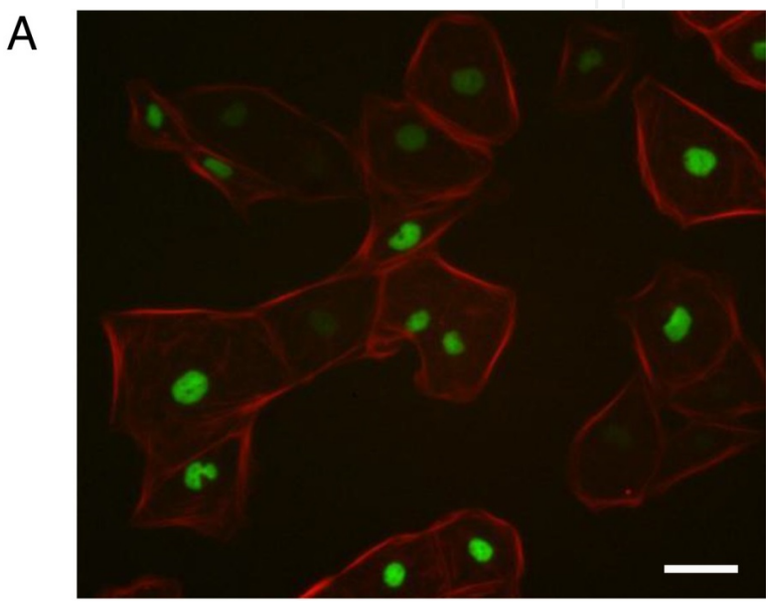

Figure 6. The differentiation of ES cells towards smooth muscle cells. Representative image of smooth muscle cells stained with SMAα-Cy3 antibody. These cells also carry a transgenic BAC containing H2B-VENUS cDNA under the control of the α-smooth muscle actin transcriptional regulatory elements. Scale bar 100μm.

3.5. Culture of haemogenic endothelium

During blast colony development *in vitro*, tight clusters of adherent cells that represent haemogenic endothelium are observed after around two days. This population of cells corresponds to an intermediate stage in the formation of blood cells from the haemangioblast. It was recently shown that these cells express TIE2 and VE-cadherin (markers of endothelial cells), c-KIT (expressed by both haematopoietic and endothelial cells) and are negative for CD41 (first haematopoietic marker) expression [20]. To isolate haemogenic endothelium, cells in day 2 liquid blast cultures are harvested and FACS sorted for the expression of these markers [20]. It is also possible to isolate a more advanced haemogenic endothelium cell population, positive for the expression of CD41 [70]. The cells are next plated onto gelatin-coated plates at a density of 1.2 x 10^5 cells/cm^2 in IMDM medium supplemented with 10% FCS, 2 mM L-Glutamine, 180 μg/ml transferrin, 25 μg/ml ascorbic acid, 4.6 x 10-4 M MTG, 10 ng/ml Oncostatin M (R&D Systems) and 1 ng/ml bFGF (Peprotech). Cells are grown in standard culture

conditions. Upon culture, at least 1-2% of these cells generate primitive and definitive haematopoietic cells [20]. During this process, the haemogenic endothelial cell population (TIE2+,c-KIT+,CD41-) cells gradually acquire the expression of CD41. The cells then progress further and lose their expression of endothelial markers.

3.6. Haematopoietic colonies assays

To evaluate the presence of haematopoietic colonies, EBs should be harvested at day 4,5 or 6 and trypsinised. Cells from the EBs can be then directly used or alternatively sorted for the expression of a marker of haematopoietic progenitors, such as for example CD41. Approximately 3.0 x 10^4 unsorted cells should be plated in 35 mm x 10 mm (BD Falcon) dishes in 1 ml of semisolid medium containing IMDM, 15% plasma derived serum (PDS) (Antech), 10% protein free hybridoma medium (PFHM, Gibco), 2mM L-Glutamine, 180 μg/ml transferrin, 25 μg/ml ascorbic acid, 4.6 x 10-4 M MTG and cytokines such as: 1% c-KIT ligand supernatant, 1% interleukin 3 supernatant (IL-3) (see [76]), 1μg/ml GM-CSF, 1% thrombopoietin conditioned media, 10 ng/ml IL-6 (Peprotech), 10 ng/ml macrophage colony stimulating factor (M-CSF), 5 ng/ml IL-11 (R&D Systems) and 4 U/ml of Erythropoietin (Ortho-Biotech) and 10 g/L methylcellulose (dissolved in IMDM, Alfa-Aesar). Haematopoietic colonies are assessed and scored based on their morphology. Primitive erythroid colonies are scored at day 5, whereas definitive haematopoietic colonies are usually enumerated 8 days after replating. Morphologic landmarks are used to distinguish the different types of haematopoietic colonies. Haematopoietic progenitors can also be cultured in liquid conditions to allow easier access of the cells for subsequent flow cytometry analysis or cytospin assays. For that cells should be seeded at a density of 2.0 x 10^6 /ml in ultra low-adherence tissue culture plates (Costar) in the haematopoietic medium described above with methylcellulose being replaced with IDMD medium.

The onset of emergence of primitive erythroid cells is observed within EBs by day 4 of differentiation [81]. Definitive erythroid and macrophage precursors appear shortly after and are followed by mast cells and multilineage precursors [81]. Primitive colonies appear around day 4 of culture. These colonies are round, compact and bright red in colour. By day 6-7 of culture, morphologically distinguishable definitive haematopoietic colonies are detected (Figure 7).

4. Conclusions and future directions

In this chapter, we have reviewed recent progress in our understanding of the development of the haematopoietic system. We have emphasized the critical role that the use of ES cells, in particular murine ES cells, has played in these recent advances. ES cells have been instrumental to identify and characterise the elusive haemangioblast. More recently, this model system allowed the merging of the two conflicting theories of the origin of blood cells (haemangioblast and haemogenic endothelium) in a single linear model of development. In addition the precise roles and requirements of many critical regulators of this process have been to a large extent elucidated using this approach.

http://dx.doi.org/10.5772/57607

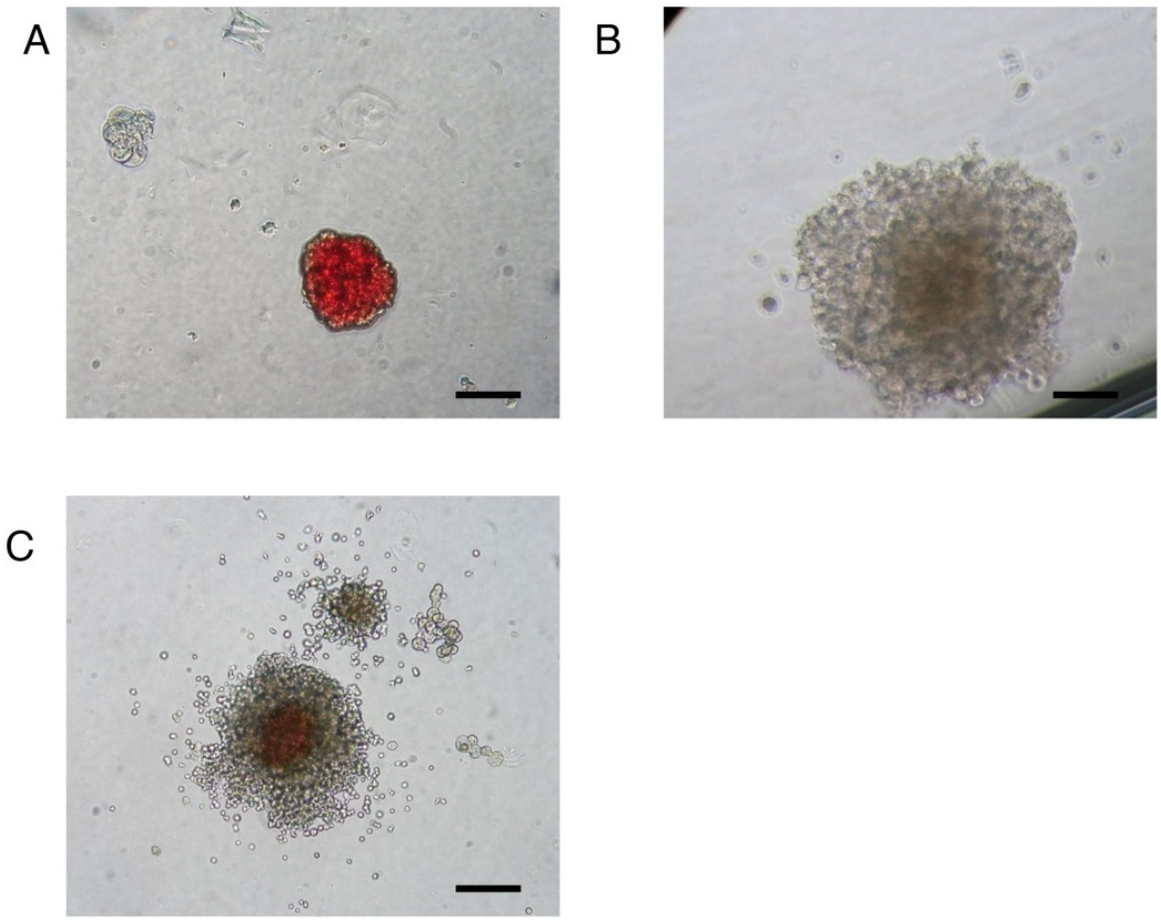

Figure 7. Examples of haematopoietic colonies obtained during ES cell differentiation A) Primitive erythroid colony – red in colour, compact and relatively small. B) Definitive myeloid colony – bigger in size, white and looser cells. C) Mixed haematopoietic colony. Scale bar 50µm.

Although only very succinctly discussed in this chapter, this system is also very amenable to examination of the cell signalling pathways that support the development of normal haematopoiesis [33, 34, 82-84]. Interestingly these pathways are also implicated in leukaemogenesis [85, 86]. Finally with the advent of novel human ES cells or human iPS cells [87], that recapitulate better the ground state and are easier to work with, and development of new methods facilitating genome editing [88-92], this experimental system is very likely to be instrumental for delivering new advances in our understanding of human haematopoietic development, that is otherwise very difficult to study *in vivo*.

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