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Effects of HOXB4 downstream targets on the haemopoietic differentiation of pluripotent stem cells

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Declaration

I declare that this thesis was composed entirely by myself and that all work presented is my own, unless otherwise stated in the text. No part of it has been submitted for any other degree or professional qualification.

Maria Kydonaki

August 2015

Abstract

Attempts for the *in vitro* differentiation of reconstituting human HSCs from ESCs have been unsuccessful as key factors of HSC specification remain unclear. Enforced HOXB4 expression can enhance haemopoietic differentiation of mouse and human ESCs and generate reconstituting HSCs from mouse ESCs. We have previously shown that HOXB4 can enhance haemopoietic differentiation of mouse ESCs in a paracrine manner. Microarray analysis identified a number of secreted factors upregulated by HOXB4 potentially mediating this paracrine effect. The aim of this study was to assess whether these factors alone and/or in combination can enhance the *in vitro* haemopoietic differentiation of mouse and human ESCs. We first developed a defined, serum and feeder-free protocol to test the effects of these secreted factors on haemopoietic differentiation. This defined protocol allowed us to compare the haemopoietic potential of mouse ESCs with the recently derived epiblast stem cells (EpiSCs), thought to be comparable to hESCs. Haemopoietic colony forming assay and flow cytometry analysis showed that serum-free conditions generated 8-10 fold more CD144+CD41+ and c-Kit+CD41+ haemopoietic progenitor cells (HPCs) compared to serum conditions from both ESCs and EpiSCs, with ESCs giving the most significant increase. We then validated the panel of HOXB4 target genes by QRT-PCR and selected those increased in expression by at least 2.5-fold when HOXB4 was activated. We used this defined, serum-free protocol to assess the effects of our panel of secreted factors, FGF17, RSPO3 and APLN, on the haemopoietic differentiation of mouse ESCs. We demonstrated that FGF17 can mediate HOXB4 haemopoietic activity by enhancing the generation of c-Kit+ HPCs. On the other hand increasing concentrations of RSPO3 inhibited haemopoietic development by reducing the numbers of CD41+ and CD41+CD45+ HPCs, while, APLN did not have any effects on the haemopoietic activity of the cells. We finally used the secreted factors in human ESC and iPSC differentiation cultures. We observed differences in the activity of the tested factors not only between species but also between human cell lines. These results suggest that HOXB4 haemopoietic activity is partly mediated by paracrine signalling but more complex cell interactions are probably required for it to fully exert its effects. More importantly, HOXB4 regulatory pathways differ between mouse and human cells stressing the need for careful translation of data between the two species and more detailed analysis of key human haemopoietic factors for the successful generation of reconstituting HSCs.

Lay Summary

Haemopoietic stem cells (HSCs), can replicate themselves and mature into all the blood cells found in our body. Currently, HSC transplantation is the only available treatment for blood disorders including certain types of cancer, such as leukaemia, myeloma, and lymphoma. Unfortunately, the only source of HSCs is the donation of bone marrow and blood, which provides a very limited supply. Therefore, scientists have turned to the use of pluripotent stem cells (PSCs) as an alternative source. PSCs can be obtained from very early stage embryos or from the genetic modification of adult somatic cells. They have the ability to replicate themselves indefinitely in culture, and when placed under the appropriate conditions, they can give rise to specialised cells. Although cells with similar properties to HSCs have been produced by PSCs in culture by replicating the conditions found inside our bodies, these cells are not fully functional and they cannot be used for clinical applications. The reason for this failure is that many key factors which determine HSC development have not been identified yet and used in culture protocols. Additionally, because animal products or animal model organisms are many times used in culture protocols to understand human physiology, discrepancies arise between studies and species.

An important regulator of HSC development is the transcription factor HOXB4. Transcription factors are proteins that act by regulating, in a well-orchestrated manner, the expression of other genes and consequently inducing the completion of biological processes. In this study, we investigated the effects that certain HOXB4 target genes have on the generation of HSCs in culture from PSCs and we compared these effects between mouse and human cells in well-defined conditions free of animal products.

Initially, we established a culture protocol for mouse PSCs, as the mouse is the most reliable and widely used experimental model that has provided great insight into human physiology. In our protocol we ensured that our culture medium and substrate contained no animal products or undefined factors, which might have influenced our results. We compared the differentiation potential of two cell populations, the mouse embryonic stem cells (ESCs) and mouse epiblast stem cells (EpiSCs), with the latter being more comparable to human ESCs. We found that our protocol could generate HSC-like cells with great efficiency and more stably for mouse ESCs. Therefore, we proceeded to testing the HOXB4 targets in mouse ESC cultures. We tested three HOXB4 targets alone or in combination: FGF17, RSPO3 and

APLN. We demonstrated that the factor FGF17 can enhance the generation of HSC-like cells, RSPO3 reduces their production and APLN does not have any effects. We then used a similar well-defined protocol to assess the effects of the factors in human cells. We tested two human cell populations, human embryonic stem cells (hESCs), which are derived from embryos, and human induced pluripotent stem cells (hiPSCs), which are derived from adult skin cells. We showed that none of the added factors had an effect on hiPSCs. On the other hand the generation of HSC-like cells was reduced in hESCs treated with FGF17 or RSPO3, while APLN did not confer any changes.

Conclusively, our study identified two new regulators of haemopoietic development, FGF17 and RSPO3. Interestingly, these two regulators have different effects not only between mouse and human cells, but even within different cell types of the same species, in our case the human. Our study therefore, stresses the necessity of conducting comparative studies, where multiple cell populations are being tested in order to draw definite conclusions regarding the factors that regulate haemopoietic development and how they can be used in the lab for the generation of therapeutic cells. Finally, we demonstrate the effectiveness of using well-defined, xenogeneic-free culture conditions to achieve the above objectives.

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AGM	Aorta-gonad-mesonephros
APLN	Apelin
bFGF	Basic fibroblast growth factor
BFU-E	Burst forming unit erythroid
BL-CFC	Blast colony forming cell
BM	Bone marrow
BMP4	Bone morphogenic protein 4
Bry	Brachyury
CFU-C	Colony forming unit cell
CFU-M	Colony forming unit macrophage
CFU-GM	Colony forming unit granulocyte/macrophage
EB	Embryoid body
EMP	Erythro-myeloid progenitor
EpiSC	Epiblast stem cell
EryP	Erythroid primitive
ESC	Embryonic stem cell
FCS	Fetal calf serum
FGF17	Fibroblast growth factor 17
GFP	Green fluorescent protein
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
HOXB4	Homeobox B4
HPC	Haemopoietic progenitor cell

HSC	Haemopoietic stem cell
iPSC	Induced pluripotent stem cell
LIF	Leukaemia inhibitory factor
mESC	Mouse embryonic stem cell
P-Sp	Para-aortic splanchnopleura
RSPO3	R-spondin 3
SCF	Stem cell factor
VEGF	Vascular endothelial growth factor
YS	Yolk sac

Chapter 1 : Introduction

1.1 Introduction

The haemopoietic system, which produces more than 10 different mature blood cell types with variable functions, and life spans of just a few days up to several years, is one of the most complex and yet well-regulated systems of the human body. The easy isolation of large numbers of blood cells and their high tolerance in *ex vivo* conditions have enabled the study of haemopoiesis over the last couple of centuries and established the haemopoietic system as a paradigm for the study of tissue development and regenerative medicine. It has been long demonstrated that remarkably, all blood cells derive from a single progenitor cell, the haemopoietic stem cell (HSC), which has the ability to self-renew, maintaining an adequate stem cell pool throughout the life of an organism, while continuously providing the organism with short lived mature cell types through differentiation (1, 2, 3). Haemopoiesis occurs through successive stages of HSC maturation and amplification during which potency gets restricted progressively leading to the segregation of different haemopoietic lineages (Figure 1.1).

The transplantation of adult HSCs from bone marrow, umbilical cord or peripheral blood, has become the most successful and widely used clinical cell therapy for hematologic disorders (4). However, this clinical treatment is often hampered by the limited donor supply and the difficulty in expanding HSCs *ex vivo*. Such limitations heighten the demand for an alternative source of HSCs, free from infections and with unlimited availability. The discovery of human embryonic stem cells (hESC) (5), as well as, the generation of human induced pluripotent stem cells (hiPSC) (6) brought up exciting opportunities for their use in regenerative medicine. Pluripotent stem cells have the ability to self-renew for extended periods of time in an undifferentiated state, and to generate all the mature cell types found in the adult body, under the appropriate culture conditions. These properties render them an incomparable model for the detailed study of haemopoiesis, as well as, an efficient tool for the generation of transplantable HSCs and mature blood cells. Laying the foundations for the clinical use of hESCs and hiPSCs, decades of previous studies using mouse ESCs as a model have provided a wealth of information regarding haemopoietic development and have led to the development of pioneering *in vitro* differentiation protocols which have been in many occasions directly translated to use in human cells.

However, while both *in vivo* and *in vitro* studies have proven that the haemopoietic differentiation process is highly conserved between mouse and human species, they have also shown that there are critical differences between mouse and human HSC development

(7). Although the murine model overcomes many technical and ethical restrains posed by the human model allowing a more detailed examination of developmental haemopoiesis, years of evolutionary pressure have left a translational gap between the two species. These species differences emphasize the importance of conducting parallel studies between the two organisms. Examining differences in the activity of key factors involved in haemopoiesis between mouse and human systems will help narrow the translational gap for the successful generation of transplantable human HSCs.

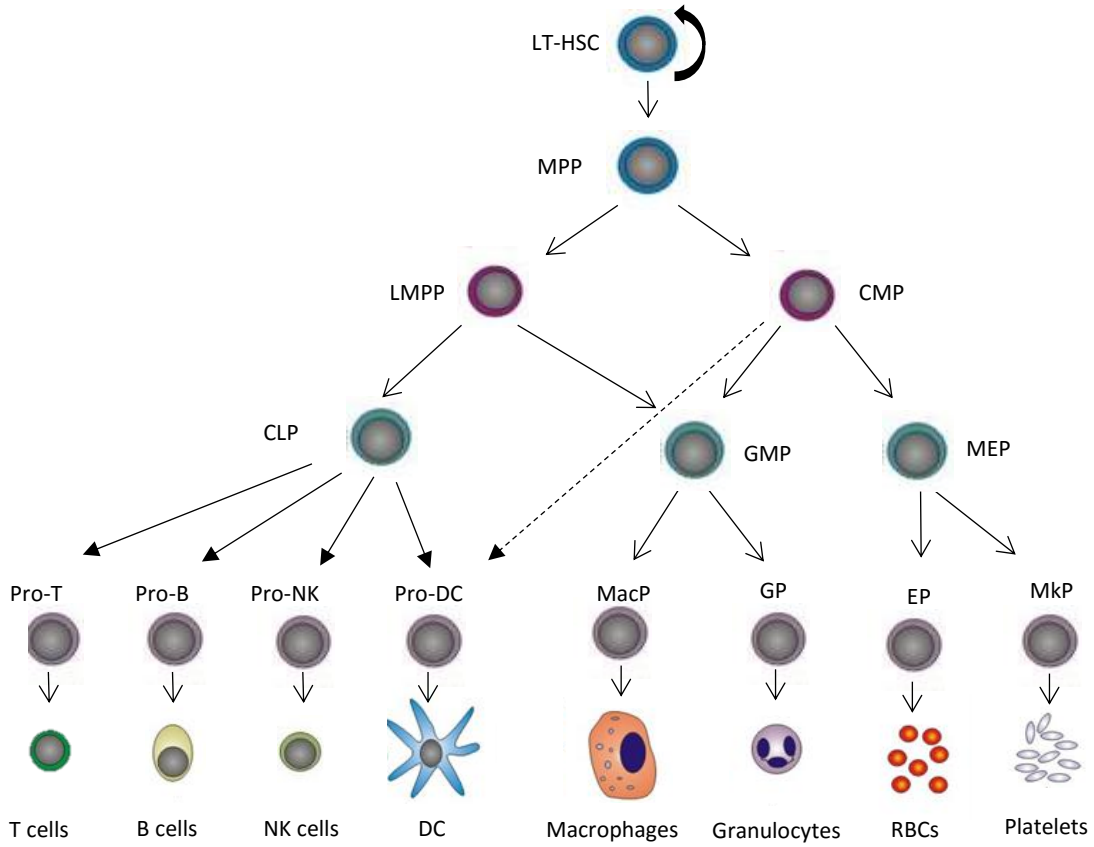

Figure 1.1: Haemopoietic hierarchy.
During differentiation HSCs lose their self-renewal capacity and progressively get lineage restricted to become mature functional cells. LMPP, lymphoid-primed multipotent progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; EP, erythrocyte progenitor; GMP, granulocyte/macrophage progenitor; GP, granulocyte progenitor; HSC, hematopoietic stem cell; MacP, macrophage progenitor; MEP, megakaryocyte/erythrocyte progenitor; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; NK, natural killer (8, 9).

1.2 Embryonic haemopoiesis

Although research on the murine model has dominated over other organisms, the origin of HSCs has been tracked down in a variety of vertebrates including frog, chick, zebrafish and with many recent advances human. It is now known that haemopoiesis is an evolutionary conserved process which occurs in distinct yet overlapping stages during embryogenesis, in multiple anatomic locations. Haemopoiesis had long been distinguished in two major types related to the location and type of blood cells it generated. The primitive one which occurred extra-embryonically in the yolk sac and gave rise only to short-lived blood cells of restricted lineage, and the definitive one which occurred intra-embryonically in the aorta-gonad-mesonephros (AGM) region and generated HSCs capable of long-term multilineage differentiation. However, due to increasing evidence indicating the contribution of the yolk sac, as well as other embryonic tissues to the long-term adult pool of HSCs it is currently considered more appropriate to define primitive and definitive haemopoiesis exclusively based on the type of cells it produces and not on the embryonic location it ensues.

Taking into consideration decades of studies on the time, location and cells produced, haemopoietic development can now be described as follows (Figure 1.2). The first wave of haemopoiesis, the primitive one, appears in the extra-embryonic yolk sac (YS) early in embryogenesis, around embryonic day E7.25 in the mouse (10) and during week 3 of human gestation (11). This wave gives rise to transient nucleated erythrocytes and primitive myeloid cells (10), which lack the ability to engraft when transplanted into irradiated recipients (12) and their purpose is to oxygenate tissues of the developing embryo. Following the onset of circulation, on E8.25 for the mouse and around day 21 for human, a second transient wave of definitive haemopoiesis follows, again in the yolk sac and later colonising the fetal liver (FL), which generates erythro-myeloid progenitors (EMP) with a high proliferative erythroid and myeloid lineage potential persisting through adulthood, however, without bone marrow homing ability (10, 13, 14, 15). The third wave of blood development, which gives rise to bona fide HSCs with multilineage potential and repopulating activity, occurs in multiple locations including the placenta, vitelline and umbilical arteries, yolk sac and predominately the aorta-gonad-mesonephros (AGM) region, on E10.5 (16) and week 5 (17) for mouse and human respectively. These definitive HSCs later colonise the fetal liver where they expand, before finally homing to the bone marrow (BM) and maintaining haemopoiesis for the whole adult life.

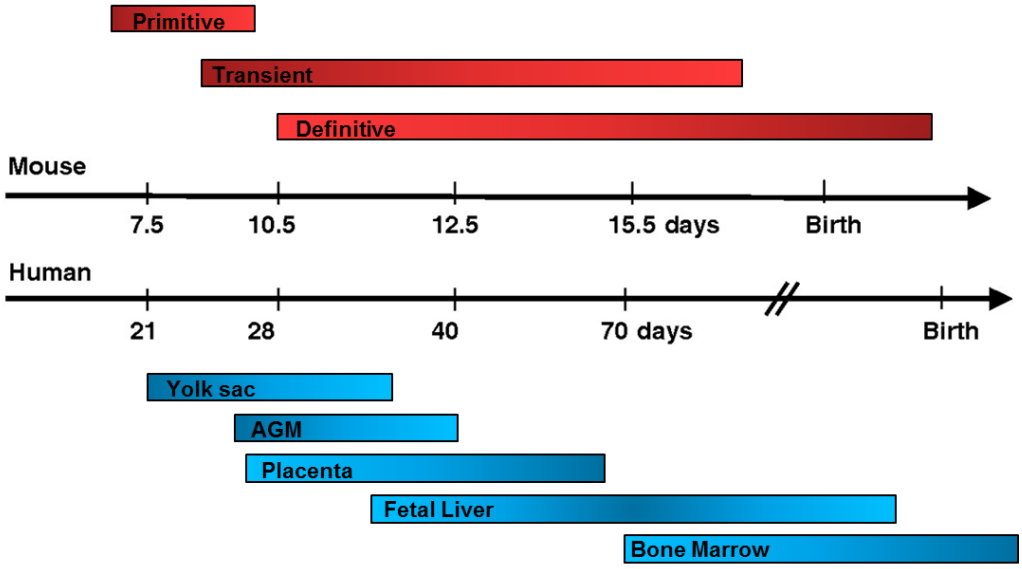

Figure 1.2: Embryonic haemopoiesis.
Haemopoietic cell development occurs in distinct yet overlapping waves. The first primitive wave in the yolk sac generates short lived primitive erythrocytes and macrophages. The second transient wave also occurs in the yolk sac and produces high proliferative, mixed lineage cells persisting until late gestation. The third wave gives rise to definitive HSCs in multiple anatomic locations that finally home the bone marrow through adult life.

1.2.1 Emergence of the first haemopoietic progenitors in the yolk sac

The emergence of the first blood cells in the yolk sac of the developing embryo, through the formation of cell clusters called blood islands, led for many years to the belief that the yolk sac is the source of definitive HSCs. The first experiments suggesting the yolk sac origin of HSCs were conducted in 1967 by Moore and Owen using the avian model (18). They demonstrated using markers between male and female animals that transplantation of 7 day yolk sac cells into 14 day old irradiated chicken embryos led to the colonisation of adult haemopoietic organs by yolk sac cells. Confirmation of their findings soon came from the murine system in 1970 by Moore and Metcalf (19). Transplantation of cultured yolk sac and fetal liver cells into irradiated adult recipients showed that the day 8 yolk sac could

reconstitute adult hosts, whereas, fetal liver could only reconstitute recipients after day 10 of gestation, with the establishment of circulation. Additional data from the culture of 7-day old embryos with intact yolk sacs and separated embryos or yolk sacs showed that cells capable of haemopoietic colony formation *in vitro* were found only in the yolk sac. The first challenge of the yolk sac as the ultimate source of HSCs came in 1975 by Dieterlen-Lievre (20). Using chimeras of quail embryos grafted on chick yolk sacs before the establishment of circulation, it was demonstrated that all blood cells colonising the spleen, thymus and bone marrow were of quail origin, thus suggesting an intra-embryonic source of HSCs. Ever since a debate on the role of yolk sac in definitive haemopoiesis began which remains unanswered until today.

In the early 1990s accumulating data in the murine system confirmed that the yolk sac can only generate transient cells and the source of definitive HSCs is found intra-embryonically. Medvinsky and Dzierzak demonstrated, using explant culture of rudiments from embryos as early as E9, that the intra-embryonic AGM region had a much higher capacity of forming colonies in the spleen of irradiated mice (CFU-S) than the yolk sac (21). Later work by Cumano and colleagues, who again isolated and cultured yolk sacs prior however to the onset of circulation on E8, showed that the yolk sac cells were unable to generate lymphoid progeny and had a reduced myeloid potential in *in vitro* colony forming assays, while *in vivo*, they could only provide short-term myeloid reconstitution when injected into *Rag2γc*$^{-/-}$ adult recipients (22, 23). Extensive work by Yoder questioned the above findings and complicated once again our understanding of yolk sac haemopoiesis. He demonstrated that even though E9.5 yolk sac cells lack the ability to home the bone marrow of adult recipients, they can exhibit long-term reconstitution activity when injected into the facial vein or liver of newborn mice and that bone marrow from these pups can reconstitute adult recipients (24, 25). This work suggests that precursor yolk sac cells are indeed capable of multilineage long-term engraftment, yet, they need to proceed through further maturation inside the embryo proper.

Work on *Ncx1*-null embryos, which fail to generate a heartbeat and die by E10.5, came to support the above hypothesis. It was shown that *Ncx1*-null embryos can produce normal numbers of early erythrocytes and CFU-Cs in their yolk sac but the embryo bodies were nearly devoid of any haemopoietic progenitors, confirming that intra-embryonic haemopoiesis is dependent on colonisation and further maturation of yolk sac cells (26). A recent study using an *in vitro* single-cell multipotency assay also showed that a well-defined cell population capable of multilineage differentiation is predominantly localized to the yolk

sac compared to the AGM and fetal liver between E9.5 to E10.5 which can yield far greater engraftment potential when cultured *in vitro* prior to transplantation (27). Further evidence supporting the long-term reconstituting ability of yolk sac HSCs come from studies focusing on *Runx1*. Lineage tracing experiments used a *Runx1* Cre-estrogen receptor (ER) reporter and showed that induction of the reporter on E7.5, when the yolk sac is the only tissue to express *Runx1*, resulted in the marking of fetal liver and adult haemopoietic cells (28). However, this study has been disputed by some as interpretation of the data is dependent on timing of *Runx1* expression which is considered not clearly defined. Additionally, inducible rescue of *Runx1* expression in *Runx1* knockout embryos demonstrated that definitive haemopoiesis could only be rescued at the developmental stages when *Runx1* expression was restricted to the YS (29).

Due to ethical limitations and the scarcity of human embryos, the role of yolk sac in human haemopoiesis has not been as extensively studied. Nonetheless, the majority of studies so far indicate that contrary to the mouse, the human yolk sac solely provides a primitive wave of blood cells. The first functional study of human yolk sac haemopoiesis in 1986 (15), examined the kinetics of haemopoietic progenitors in embryos from 4.5 to 10 weeks of gestation. It showed that 4.5 week yolk sac cells were able to give rise to multipotent progenitors, as well as, early or late erythroid and granulomacrophage progenitors, as monitored by *in vitro* colony assays. The clonogenic ability of the yolk sac dropped dramatically by week 6 with the liver becoming the major haemopoietic site. Later studies by Huyhn and co-workers confirmed the multipotent potential of yolk sac cells (30). When placed in colony assay, yolk sac cells gave rise to both erythroid and non-erythroid colonies since gestation day 25. Interestingly, cells from the embryo could also generate both types of colonies and a higher proportion of non-erythroid progenitors in later stages of development, indicating an intra-embryonic origin of blood progenitors. These studies, however, were performed after the onset of circulation not excluding the possibility of cells migrating from the yolk sac to the embryo proper. More recent work of Tavian and colleagues, using explant organ culture before the onset of circulation, proved that the human yolk sac is only capable of limited myeloid lineage while the intra-embryonic para-aortic splanchnopleura region can generate multilineage lympho-myeloid progenitors as assessed by *in vitro* differentiation (31). Finally, *in vivo* work by Ivanovs has demonstrated that yolk sac cells are capable of reconstituting adult recipients only after the generation of repopulating HSCs intra-embryonically, suggesting the migration of HSCs to the yolk sac from the embryo proper (17).

1.2.2 The AGM as the prominent site of intra-embryonic haemopoiesis

Since the first proof of an intra-embryonic source of HSCs (20), nearly a decade past before its location was characterised in the *Xenopus* model. Transplantation of tissues between two *Xenopus* subspecies demonstrated that haemopoietic progenitors arise in two different locations of the developing embryo, the ventral blood island (VBI) and the dorsal lateral plate mesoderm (DLP), which comprises the prospective mesonephros (32). It was later confirmed that the VBI produced progenitors of early larval haemopoiesis, mainly transient erythroid cells, whereas, the DLP generated precursors of late larval and adult haemopoiesis, including myeloid and lymphoid cells (33). Extensive work by the Dzierzak group identified as the equivalent of the DLP region in the mouse system the AGM region, which develops from lateral plate mesoderm through the initial formation of the para-aortic splanchnopleura (P-Sp). It was shown that AGM cells of E10 embryos, when directly transplanted into adult hosts, could restore their haemopoietic system, albeit in low frequency, while yolk sac and liver cells failed to do so. By day E11 all organs could repopulate the recipients, though the AGM with a much higher repopulation frequency (12), indicating the generation of definitive HSCs in the AGM region and their subsequent migration to other organs. Later work of the group, using an explant culture system of embryonic organs to prevent cell circulation amongst them, showed that the AGM is indeed able to autonomously generate definitive HSCs by embryonic day E10 and that the AGM can further expand *in vitro* the HSCs (16). Work by Cumano and Godin (22, 23) confirmed the AGM as an autonomous source of HSCs. Cultured explants of mouse E8 para-aortic splanchnopleura before the onset of circulation could give rise to multilineage progenitors *in vitro,* and to multipotent *in vivo* reconstituting cells in immunocompromised *Rag2γc*$^{-/-}$ recipients, whereas, mouse yolk sac tissue could not. The reconstitution efficiency was however, strikingly lower than that of the E11 AGM.

The specific location of HSC development within the AGM region has been investigated in more recent studies. The transplantation of E11.5 dorsal aorta and urogenital ridges (UGR) into adult hosts showed that the dorsal aorta and particularly its ventral domain could give long-term reconstitution (34). Explant culture of E10.5 dorsal and ventral parts of the aorta, stage at which high-level repopulating HSCs are absent, followed by transplantation into irradiated hosts, revealed the exclusive capacity of the ventral part to generate HSCs. Additionally, more recent imaging work showing the emergence of haemopoietic cells directly from the endothelium of the ventral part of the dorsal aorta, either in AGM sections

of the mouse embryo in culture (35) or in live zebrafish embryos (36, 37, 38), left no doubt of the ability of the region to autonomously generate HSCs.

In humans, Tavian and colleagues have contributed greatly to the understanding of intra-embryonic haemopoiesis. The first indication of haemopoietic progenitors arising inside the embryo came with the detection of $CD34^+$ haemopoietic clusters on the ventral side of the dorsal aorta (39), which appeared on day 27, expanded rapidly and disappeared on day 40 of development, shortly before liver colonisation (11). The co-culture of $CD34^+$ cells with MS-5 stromal cells resulted in much higher numbers of progenitor cells compared to the liver as monitored by methylcellulose colony assay (39). Further analysis before and after gestation day 21, which marks the onset of circulation, confirmed the AGM as the site of an independent origin of adult blood cells (31). Successively the splanchnopleura, para-aortic splanchnopleura and AGM region tissues, in parallel with matching yolks sacs, were dissected, cultured *in vitro* and assayed on their haemopoietic potential. It was demonstrated that as opposed to the yolk sac, even 19-day splanchnopleura had the ability to form long-term haemopoietic cell cultures. Looking into lineage potential, explanted yolk sac cells could only give rise to myeloid and NK cells *in vitro*, while P-Sp and AGM cells generated both myeloid and lymphoid cells. In more recent studies of Ivanovs and colleagues, functional *in vivo* analysis confirmed the AGM as the intra-embryonic source of adult HSCs (17). Transplantation of human AGM cells into NSG mice resulted in long-term multilineage reconstitution as early as gestation day 32 with a very high self-renewal potential, whereas, human yolk sac cells could repopulate the recipients at least 5 days later. These highly regenerative HSCs were particularly localised in the ventral part of the dorsal aorta, similarly to the mouse model, as proven by transplantation of bisected aortas and urogenital ridges into irradiated mice, which resulted into increasing levels of human haemopoietic engraftment exclusively from the ventral part of the aorta (17, 40).

1.2.3 Other sites of haemopoietic activity in the embryo

Besides the yolk sac and the AGM region, additional haemopoietic sites contributing to the HSC pool have been discovered in vertebrate embryos. Initial studies in avian embryos revealed that the allantois generates haemopoietic cells, as quail allantois grafted into chicken embryos resulted in partial colonisation of the bone marrow with quail cells (41). In mammals the allantois gives rise to the umbilical vessels which fuse with the chorionic plate to form the placenta. The umbilical vessels together with the vitelline vessels, which connect the embryo with the yolk sac, have been shown to harbour HSCs in the mouse model by de

Bruijn and colleagues. In E10.5 embryos, umbilical and vitelline HSCs are found in frequencies comparable to the AGM region, and increase through days E11 and E12. Mice injected with cells from umbilical and vitelline arteries show long-term multilineage repopulation as well as haemopoietic reconstitution of secondary recipients (42). Nonetheless, explant cultures of these tissues have been unsuccessful, thus their ability to autonomously generate definitive HSCs remains unclear.

The placenta has been identified as another major haemopoietic organ. Haemopoietic progenitors in the E9 mouse placenta were first identified by *in vitro* assays (43). Later *in vivo* repopulating assays demonstrated that the E10.5-11.5 placenta contains long-term reconstituting HSCs in numbers parallel to those of the AGM region (44, 45), which surprisingly, can expand rapidly between E11.5-12.5 up to 15-fold more than the AGM (44). In *Ncx1*-null mice, devoid of circulation, it has be shown that placenta explant cultures can still produce multilineage precursors *in vitro*, though in lower numbers compared to wild type animals (46). These data indicated that the placenta can independently generate HSCs, yet transplantation experiments were not performed to confirm the engraftment potential of the cells. In human development it has been shown that placentas from gestation week 6 and onwards, contain progenitors capable of multilineage differentiation *in vitro* and of homing the bone marrow of NSG mice (47). However, early human placentas of weeks 4-6 provide only long-term T cell repopulation potential of maternal origin in NSG mice (17) leaving unanswered whether the placenta initiates HSC development or it can only support the maturation of HSCs originating from other sites.

The fetal liver in mid-gestation becomes the main haemopoietic organ where HSCs expand rapidly and differentiate until they colonise the bone marrow before birth. Although little is known about the fetal liver HSC niches, it is believed that the liver cannot produce HSCs *de novo* but it is seeded by cells emerging in other haemopoietic locations (48, 49). Very recently additional haemopoietic sites in the embryo have been discovered. The head vasculature has been shown to generate *de novo* HSCs with multilineage engraftment potential (50), while the endocardium, even in the absence of circulation, can give rise to erythro-myeloid progenitors that persist until late gestation (51).

Evidently all these studies emphasise that developmental haemopoiesis is a particularly complex and dynamic process and that our understanding of it is far from complete. The variability of extra- and intra-embryonic tissues that contribute to the adult HSC pool complicates the analysis of when and where these cells are initially generated. Furthermore,

the stringent assay of transplanting embryonic cells into adult recipients to test their HSC properties may be hindering the results by not providing the required microenvironment for the full potential of the cells to unravel. What can be concluded with certainty is that multiple sites within the embryo have intrinsic HSC potential which is timely dictated by the *in vivo* microenvironment. However, in order to understand how HSCs first appear in these sites it is essential to take a step back and look into their molecular pathways and cellular sources.

1.3 Cellular origins of haemopoietic progenitors

The close spatial and temporal association of endothelial and haemopoietic cells during embryogenesis has long been observed by researchers and has led to various stipulations regarding the cellular origin of HSCs. The two prevailing theories are those of the hemangioblast, a common bipotent progenitor, and the one of hemogenic endothelium, specialised vascular endothelial cells with blood forming potential.

1.3.1 The hemangioblast

In the early 1900s Sabin and Murray observed in the chick yolk sac that early blood islands appear as tight clusters of cells surrounded by mesenchyme. These clusters soon differentiate with the inner cells becoming erythrocytes and the outer cells forming an endothelial lining. This morphological observation led to the hypothesis of a bipotent common progenitor, which gives rise to both haemopoietic and endothelial lineages, the hemangioblast (52, 53).

Gene targeting experiments firmly supported this common developmental pathway of endothelial and haemopoietic cells by identifying a number of genes indispensable for normal development of the two lineages. Extensive work by Shalaby and co-workers on the mouse system proved that embryos deficient for the vascular endothelial growth factor receptor 2 (*Vegfr2* or *Flk-1)* fail to develop blood islands in the yolk sac or any organised blood vessels in the embryo body and yolk sac resulting in embryonic lethality (54). They also demonstrated that *Flk-1*$^{-/-}$ ESCs fail to contribute to primitive and definitive haemopoiesis or to endothelial development in chimeras (55). In the zebrafish, the *cloche* mutation results in lack of endocardium and greatly reduced numbers of blood cells, while *Scl* knockdown causes loss of primitive and definitive haemopoiesis, and vasculogenic defects predominately in the dorsal aorta (56, 57). Moreover, deletion of the Etsrp/Er71 gene has been found to affect critically the development of endothelial and haemopoietic lineages

in the mouse and zebrafish (58, 59). Despite the valuable evidence in these studies linking the two lineages, they could not directly identify a common cell progenitor.

In vitro culture studies were the first to successfully identify and characterise cells with hemangioblast properties. In the avian embryo, mesodermal cells isolated from the early blastodisc expressing the *Flk-1* homologue could form either haemopoietic or endothelial colonies when cultured in the absence or presence of VEGF respectively (60). Using mouse and human ESC differentiation cultures the Keller group has provided the strongest evidence for the existence of hemangioblasts to date. The mouse hemangioblast was initially identified as a blast colony-forming cell (BL-CFC) that appeared transiently, expressed both endothelial and haemopoietic markers, including CD34, CD31 and Flk-1, and was capable of differentiating into both cell lineages in response to VEGF and SCF (61). Nearly a decade later the existence of the human hemangioblast, that expresses the VEGFR2 (also known as KDR) and has the ability to generate endothelial and blood cells, was similarly confirmed in ESC cultures (62). More importantly, the same group using GFP-Bry$^{+/-}$ mouse embryos was the first to detect cells with properties similar to the BL-CFC *in vivo* (63). They identified a population of Brachyury and Flk-1 positive cells appearing transiently in the posterior region of the primitive streak of E7-7.5 embryos which could give rise to endothelial and haemopoietic colonies when cultured *in vitro.* However, the ability of these cells to also generate smooth muscle cells, in addition to the fact that their differentiation potential was measured using *in vitro* assays, similarly to the previous studies, raised doubts as to whether there is indeed an exclusive bipotent precursor *in vivo* or whether the BL-CFC is just an *in vitro* artefact.

Lineage-tracing studies in a variety of species have not yet answered this question with certainty. Orthotopic transplantation of *LacZ* expressing E6.5-7.5 primitive streak cells into wild type mouse embryos showed that labelled cells could give rise to either endothelial or haemopoietic cells in the yolk sac blood islands, with slightly different timing, thus not supporting the presence of hemangioblasts (64). Work by Ueno and Weissman also did not favour the concept of the hemangioblast. They established multichimera mice by injecting three ESC lines marked with different flourogenic proteins into blastocysts and demonstrated that E7.5 yolk sac blood islands were of polyclonal origin, mainly with separate progenitors giving rise to endothelial or haemopoietic cells (65). Nevertheless, the existence of hemangioblasts could not be negated as they also found individual progenitors within an island contributing to both lineages, in lower but yet considerable frequency. Similar results were produced in single-cell labelling experiments in the zebrafish. Vogeli and colleagues

found that while most labelled cells gave rise to either Flk-1 expressing or Runx1 expressing cells, indicating endothelial or haemopoietic cells respectively, a substantial subset of labelled cells co-expressed the two markers, contributing to both lineages and confirming the presence of hemangioblasts *in vivo* (66). Very recent work by Padron-Barthe and colleagues presents evidence against the existence of hemangioblasts. Using a clonal labelling technique, where inducible *Cre* and reporter alleles were ubiquitously expressed, once again only a small percentage of bilineage clones was identified in the yolk sac blood islands. These clones however, appeared to derive from labelled cells in the early epiblast before specification of the blood- and endothelial-forming regions. To narrow down more on rare hemangioblasts they used an inducible *Tie2-Cre* model which resulted in significantly reduced bilineage cells labelling the yolk sac endothelium. The writers therefore concluded that the two lineages are specified independently in the epiblast, migrate sequentially in the yolk sac where finally haemopoietic cells arise from the yolk sac hemogenic endothelium (67).

The conflicting data have still neither proven nor rejected with certainty the theory of the hemangioblast. The rarity of bi-potent hemangioblasts *in vivo* constitutes the main argument refuting their existence. Nonetheless, the presence of bipotent cells in the developing embryo even in low numbers cannot be disregarded. As the majority of *in vivo* and *in vitro* reports stress the transient presence of cells with hemangioblast properties, it is quite possible they could have been missed in tracing experiments due to the short time window of their presence. Moreover, the possibility of studies focusing at a stage after the segregation of the endothelial and haemopoietic lineages cannot be excluded. Additional single-cell tracing and live imaging experiments during early stages of embryogenesis are required to clarify the existence of the hemangioblast and the lineage identity of its direct progeny.

1.3.2 Hemogenic endothelium

About a century ago, clusters of haemopoietic cells attached to the endothelial lining of the dorsal aorta were first observed, leading to the idea that blood cells are generated by specialised endothelial cells termed hemogenic endothelium (68). Evidence of the capacity of aortic endothelial cells to generate haemopoietic progenitors came many decades later. It was first observed in the chick embryo that aortic cells harboured multipotent progenitors, as demonstrated by their ability to give rise to all types of haemopoietic colonies *in vitro* (69). Soon after, the hemogenic potential of $CD34^+$ intra-aortic clusters was confirmed in human embryos (39). Work from the same group later identified $CD45^-$ $CD34^+$ or $CD31^+$

endothelial cells, capable of yielding myeloid and lymphoid progenitors in culture, not only in the aorta but also in the yolk sac, liver and bone marrow of human embryos (70). Additionally, in the mouse, both the yolk sac and embryo proper were found to contain VE-Cadherin, CD31, Flk-1 and CD34 expressing endothelial cells which could generate erythro-myeloid and lymphoid colonies *in vitro* (71). Further research revealed that other major arterial regions in the mouse embryo, such as the vitelline and umbilical arteries, could give rise to HSCs supporting even more the idea of an endothelial origin of blood cells (42).

A large number of lineage tracing and *in vivo* imaging studies have also provided direct evidence of an endothelial origin of blood cells *in vivo*. Lineage tracing experiments were first performed in the avian embryo (72). Jaffredo and co-workers labelled the aorta with AcLDL-Dil, which was specifically uptaken by Flk-1^+ endothelial cells, prior to the emergence of $CD45^+$ haemopoietic cells. It was found that newly formed haemopoietic clusters on the ventral side of the dorsal aorta contained cells expressing CD45 while retaining Dil labelling. Interestingly some $CD45^+$ Dil labelled cells were also located in the underlying mesenchyme implicating it in the early specification of HSCs. Similar Dil labelling experiments of $CD34^+$ $CD31^+$ endothelial cells in the mouse yolk sac and embryo that gave rise to definitive erythroid progenitors, confirmed the hemogenic potential of endothelium (73). Work by de Bruijn and colleagues, using *Sca-1 (Ly-6A)-GFP* mouse embryos, identified transgene expression in cells of the endothelial layer of the dorsal aorta, vitelline and umbilical arteries, but not in the surrounding mesenchyme. GFP^+ cells expressed additional haemopoietic and endothelial markers and were capable of long-term reconstitution of irradiated recipients (74). More recent work by Zovein and colleagues used an inducible Cre-lox system to track the lineage of VE-Cadherin expressing cells. They demonstrated that hemogenic endothelial cells are present in the aorta, yolk sac, placenta, vitelline and umbilical arteries of the developing mouse embryo and that the haemopoietic cells emerging from this endothelium are capable of long-term, multilineage differentiation. The underlying aortic mesenchyme was found unable to directly generate HSCs and could only do so through an endothelial intermediate, suggesting once again that within the subendothelial mesenchyme lie the precursors that give rise to hemogenic endothelium (48).

Inarguably, the strongest proof for the presence of hemogenic endothelium came from recent *in vivo* and *in vitro* time-lapse imaging of endothelial cells in the ventral wall of the dorsal aorta. Zebrafish embryos, which are transparent and therefore a unique tool for live imaging, were used by three different groups to monitor the generation of haemopoietic cells from the aortic wall. In all three cases lineage tracing of *Flk-1, cMyb, CD41*, or *Runx1* expressing

cells revealed that endothelial cells acquire their haemopoietic identity through progressive morphological and phenotypical changes. The endothelial cells contract and bend in the sub-aortic space, while upregulating haemopoietic markers, before finally rounding up and entering the blood circulation without further cell division to colonise the thymus and kidneys (36, 37, 38). In the mouse, live sections of embryos, which maintain free cell movement, were cultured under confocal microscope for time-lapse imaging. It was demonstrated that $CD31^+$ $Sca\text{-}1^+$ endothelial cells from the ventral endothelium budded into the lumen of the aorta and co-expressed the haemopoietic markers c-kit and CD41 upon their emergence (35).

As both conflicting concepts of the hemangioblast and hemogenic endothelium have received confirmation a definite answer regarding the exact cellular origins of HSCs has not been provided. Moreover, even though the existence of hemogenic endothelium has been proven beyond doubt, little do we know about the origin of these cells. Whether there is a lineage relationship between hemangioblasts and hemogenic endothelium also remains unclear. In an effort to address this question Lancrin and co-workers showed that haemopoietic cells develop from hemangioblasts through a hemogenic endothelium intermediate (75). ESC-derived mouse hemangioblasts defined by Bry and Flk-1 expression gave rise to blast colonies consisting of tight adherent $Tie2^+c\text{-}kit^+CD41^-$ endothelial cells. As differentiation progressed these cells acquired a round non-adherent morphology while downregulating Tie2 and c-kit to eventually become $CD41^+$ haemopoietic cells. These $Tie2^+c\text{-}kit^+CD41^-$ hemogenic endothelial cells were also identified in the embryo in developing blood islands and the AGM region, and were capable of forming haemopoietic colonies in culture. Although this study reconciles the two theories it still leaves a huge gap between the $Bry^+Flk\text{-}1^+$ hemangioblast cells in the primitive streak and the downstream $Tie2^+c\text{-}kit^+CD41^-$ hemogenic endothelium in the yolk sac and AGM. The identification of appropriate markers and the direct lineage tracing of hemogenic endothelium precursors *in vivo* will answer many questions and generate more information critical not only for our understanding of haemopoiesis but also for the generation of haemopoietic stem cells *in vitro* in the hope of developing successful regenerative medicine applications.

1.3.3 Phenotype of developing HSCs

As demonstrated above, cell surface markers have played a pivotal role in defining the lineage relationships between early mesodermal precursors and HSCs. Common hemogenic endothelial origins have been revealed for yolk sac, placenta and AGM-derived

haemopoietic cells, nonetheless, the cells behave differently in transplantation studies, depending on their site of origin. Functional differences between developing HSCs may be reflected on phenotypical ones, which in turn can enable the characterisation and isolation of fully functional HSCs from different anatomic locations. Indeed many studies have confirmed that the phenotype of HSCs changes throughout development, with different sets of markers being characteristic of early and late haemopoiesis.

Work by Ferkowicz and colleagues demonstrated that the first haemopoietic progenitors arising in the E7 mouse yolk sac are Flk-1^{+}CD41^{dim} and represent the primitive erythroid progenitors (76). Soon after on E8.25 a second CD41^{bright} population emerges which marks the onset of definitive haemopoiesis (76). As confirmed by Mikkola and colleagues, by E9.5 the yolk sac HSCs become CD41^{bright}CD34^{+}c-kit^{+} and are capable of generating multilineage haemopoietic colonies and repopulating irradiated newborn recipients (76, 77). CD45 expression is also initiated in E9.5 yolk sac cells, however, clonogenic progenitor activity is equally detected in the CD45^{+}CD41^{+}c-kit^{+} and CD45^{-}CD41^{+}c-kit^{+} fractions (77). With development progressing, CD41^{+} cells emerge in the vitelline and umbilical arteries as well as the AGM region (77). Detailed work by Rybtsov and colleagues has confirmed that specification of HSCs in the AGM region is initiated by CD41 expression and has shown that it progresses in a stepwise manner. On E9.5 a haemopoietic committed VE-Cad^{+}CD41^{+}CD43^{-}CD45^{-} population of pro-HSCs appears in the dorsal aorta. Pro-HSCs mature rapidly into type I pre-HSCs with the upregulation of CD43, followed by upregulation of CD45 in type II pre-HSCs by E11.5, before becoming fully competent HSCs (78, 79). All types of HSCs are also marked by c-kit expression, while, the gradual increase in CD43 and CD45 expression levels is accompanied by the gradual decrease in CD41 expression (79). Sca-1, a hallmark of adult HSCs, and CD41 co-expression also appears in the E11 AGM region (80). On E12.5 the placenta harbours CD34^{+}c-kit^{+} reconstituting HSCs which are heterogeneous for CD41 as its levels decrease (44, 81). Work from various groups has demonstrated that as haemopoiesis transitions to the liver, repopulating HSCs are predominately found in the CD45^{+}CD41^{-}CD34^{+}c-kit^{+} fraction of the E12.5 and E14.5 fetal liver (76, 77, 80). Expression of CD34 is finally downregulated and with the establishment of haemopoiesis in the bone marrow, repopulating potential is restricted in the CD41^{-}CD34^{-}c-kit^{+} cells (76). Further analysis of bone marrow cells has identified HSC activity particularly in the CD34^{-}Lin^{-}Sca-1^{+}c-kit^{+} population, while the use of SLAM antigens allows for higher enrichment of repopulating HSCs in the CD150^{+}CD48^{-}Lin^{-}Sca-1^{+}c-kit^{+} population (82, 83). Interestingly, SLAM markers also identify repopulating HSCs in the E14.5 fetal liver, but not in the earlier yolk sac, placenta or AGM region, where CD150 and CD48 are

absent (84, 85). Conclusively, in the mouse, c-kit marks all HSC populations throughout development. In the early yolk sac and AGM stages between E8.5 and E11.5 when HSCs first emerge, CD41 and CD34 identify repopulating HSCs. Downregulation of CD41 and later on of CD34, together with the increase in expression of CD45 after E11.5 in the AGM region marks the transition to liver haemopoiesis and further HSC expansion. With the establishment of the fetal liver as the major site of haemopoiesis around E14.5, SLAM antigens mark fully mature repopulating HSCs that finally home the bone marrow.

Although detailed examination of haemopoietic tissues for HSC markers throughout human gestation is more challenging than in the mouse, human haemopoietic cells have been successfully characterised in different anatomic locations. Early work by Huyhn and co-workers identified a population of $CD34^{+}CD38^{-}CD33^{-}$ cells capable of generating haemopoietic colonies *in vitro* as the first blood cells in the 35-40 gestation day human yolk sac and embryo proper (30). Subsequent analysis of haemopoietic clusters in the developing AGM region further characterised a population of CD34+CD31+CD45+CD38-Lin- cells capable of multilineage differentiation *in vitro* as early as gestation day 27 (31, 39). As later proven by Ivanovs and colleagues it is those AGM residing haemopoietic cells on gestation day 32 that are the first fully functional reconstituting HSCs in human development and are characterised by the following phenotype $CD34^{+}$VE-cadherin$^{+}CD45^{+}$C-KIT^{+}THY-1^{+}Endoglin^{+}RUNX1$^{+}CD38^{-/lo}CD45RA^{-}$ (17, 40). As embryogenesis progresses HSCs are identified in the 6 week placenta with their repopulating activity peaking on week 9 and persisting until term (47). HSC activity is in both $CD34^{+}$ and $CD34^{-}$ fractions, however, as time progresses it becomes restricted to the $CD34^{+}$ fraction. Immunostaining of placental villi stroma shows co-expression of CD34 and CD45 in cells budding from the vasculature. Highly proliferative and engraftable VE-$Cad^{+}CD34^{+}CD45^{+}$THY-$1^{+}CD38^{-}$ HSCs subsequently appear in the 7-8 week embryonic liver (86). Analysis of human cord blood and bone marrow by the Dick group has demonstrated that fully mature human HSCs are enriched in the $Lin^{-}CD34^{+}CD38^{-}CD90^{-/+}CD45RA^{-}CD49f^{+}Rho^{lo}$ population of hematopoietic cells (87, 88). CD34 is thus the marker identifying HSCs throughout human development, while the use of CD45 and CD38 can further specify HSC populations in different sites of haemopoietic activity.

1.4 Molecular regulation of haemopoiesis

Equally important to tracing HSC precursors *in vivo* and determining their phenotype, is unravelling the intrinsic determinants of their development. It is only by understanding HSC regulators and using them *in vitro* that cells for clinical transplantation can be generated. Studies focusing on this task have revealed a complex genetic program consisting of a large number of transcription factors and signalling pathways. These molecular regulators have a highly dynamic expression pattern during development, and it is their interaction at different stages of haemopoiesis that eventually determines cell fate. The major molecular determinants of haemopoiesis are highlighted below.

1.4.1 Transcription factors involved in haemopoietic development

Two transcription factors that act early in development and largely regulate primitive haematopoiesis, are Gata1 and Pu.1. Gata1 is a central mediator of erythroid and megakaryocytic development, as *Gata1* null mice die between E10.5 and E11.5 from severe anaemia with cells arrested at the pro-erythroblast stage (89). On the other hand Pu.1 is a master regulator of myeloid development, with disruption of the gene leading to defects in macrophage and granulocyte generation, and death at late gestation (90). The two proteins have been found to interact physically and antagonize each other's activity. In the zebrafish, *Gata1* knockdowns switch blood progenitors to the myeloid fate, whereas, inhibition of *Pu.1* promotes erythroid development at the expense of the myeloid fate (91, 92).

The basic-loop-helix-loop (bHLH) factor Scl/Tal1, and the Lim-domain containing Lmo2 are two more transcription factors that act in parallel and have drown a lot of scientific interest. They are involved in both primitive and definitive haemopoiesis, and play a critical role in the transition to hemogenic endothelium. The two factors are expressed in vascular endothelial and haemopoietic tissues during development (93, 94). It has been shown, using *Scl* or *Lmo2* null mice, that both factors are essential for primitive haemopoiesis as mutant embryos die *in utero* around E9.5-E10.5 due to failure of yolk sac erythropoiesis (95, 96). Because of the early embryonic lethality, the effects of Scl and Lmo2 in definitive haemopoiesis were studied using chimeric mice made by injecting blastocysts with embryonic stem cells deficient in the factors. The stem cells failed to contribute to any of the haemopoietic lineages in adult mice, while reintroduction of gene expression could rescue the ability of the cells to contribute to blood cell development, confirming the necessity of the genes in definitive haemopoiesis (97, 98, 99). Lmo2 directly interacts with Scl to form a

complex which regulates haemopoietic lineage specification, and evidence have suggested this complex functions within the hemangioblast to specify a haemopoietic rather than a vascular fate (100, 101). Interestingly, it has been shown that although Scl is crucial for the transition of the hemangioblast to the hemogenic endothelium stage, it is then dispensable for the emergence of HSCs from it (102, 103).

Another member of the GATA family of transcription factors, Gata2, has been identified as an essential regulator of definitive haemopoiesis. Initial work by Tsai and co-workers demonstrated that Gata2 is essential for definitive haemopoiesis. *Gata2*$^{-/-}$ mice die by E11.5 due to severe anaemia, and *Gata2*$^{-/-}$ embryonic stem cells cannot contribute to definitive haemopoietic cells in mouse chimeras (104). Later studies showed that *Gata2* is expressed in the endothelium of haemopoietic sites, including the AGM region and the fetal liver, and its presence is required for HSC emergence from the endothelium (105, 106). More recently, work by the Dzierzak group revealed a dual role for Gata2. It is not only required for HSC generation in the AGM region, but also for HSC survival and proliferation at later stages of development (107). Transcription factor *Runx1* has also been identified as an essential factor for the endothelial-to-haemopoietic transition in the AGM region. *Runx1* deletion in mouse embryos results in severe haemorrhaging and eventual embryonic death around E12.5. Although the embryos are capable of undergoing primitive haemopoiesis, they fail to proceed to definitive HSC development (108, 109). North and colleagues showed that *Runx1* is expressed in intra-aortic clusters of the dorsal aorta in the AGM region, as well as, the endothelium of the yolk sac, the vitelline and umbilical arteries. However, in *Runx1* deficient embryos, haemopoietic clusters in the dorsal aorta are absent and sequentially HSCs do not form (110). As opposed to Gata2, once HSC emergence is completed, Ruxn1 function is no longer required (111).

1.4.2 Signalling pathways regulating haemopoiesis

Notch signalling plays a key role in vascular development and HSC specification. In mammals, four transmembrane receptors (Notch 1-4) interacting with five ligands located in adjacent cells (Jagged1, Jagged3, Delta1, Delta3, Delta4) have been identified (112). This interaction leads to two proteolytic cleavages of the receptor, allowing its intracellular domain to translocate to the nucleus and regulate gene transcription. It has been proven that a number of Notch signalling pathway proteins are critical for definitive HSC specification. In mouse and zebrafish embryos, targeted disruption of *Cls* or *Mib*, both of which are required for Notch signalling, does not impair primitive haemopoiesis but diminishes HSC

development (106, 113, 114). As shown by Krebs and co-workers *Notch1*$^{-/-}$ mouse embryos display severe vascular and aortic defects (115). Interestingly, later work by Kumano and colleagues demonstrated that *Notch1*$^{-/-}$ embryos do not have a decrease in the population of hemogenic endothelial cells, suggesting that Notch1 is dispensable for hemogenic endothelium specification. Nonetheless, the embryos have severely impaired HSC development indicating that Notch1 activity is essential for the endothelial-to-haemopoietic transition (116). Additionally, the fact that *Notch1*$^{-/-}$ embryonic stem cells can give rise to short-term haemopoietic progenitors in chimeric mice but show no contribution to the adult haemopoietic system, indicates that Notch1 has to act in a cell-autonomous manner in order to switch to definitive haemopoiesis (117). More recently, it has been shown that Notch3 within the somites exhibits a non-cell-autonomous activity and can specify HSCs in the neighbouring precursor cells (118). The Notch ligand, Jagged1, has also been found to contribute to HSC specification. Jagged1 knockout mouse embryos have normal arterial development but decreased numbers of Gata2 and Runx1 expressing HSCs on E10.5, suggesting multiple Notch regulators are required for HSC specification (119).

The Wnt pathway is another important regulator of many biological processes, with HSC specification among them. The pathway comprises of 19 secreted glycoproteins that function by associating with the G protein-coupled Frizzled receptors (Fzd) and the LDL-receptor related proteins (LRP) co-receptors (120). Wnt signalling is either β-catenin dependent (known as canonical pathway) or β-catenin independent (known as non-canonical pathway). Upon binding of Wnt proteins to the receptors, the canonical pathway inhibits the degradation of β-catenin in the cytoplasm and results in its translocation to the nucleus and the activation of target genes. On the contrary, the non-canonical Wnt pathway leads to β-catenin degradation and is rather mediated by intracellular calcium ion or JNK. Similar to the Notch pathway, various Wnt proteins are required for HSC regulation. As shown by Luis and colleagues *Wnt3a* deficient mice have decreased numbers of fetal liver HSCs and impaired reconstituting capacity upon serial transplantation (121). Work by the same author, using a negative modulator of the canonical Wnt pathway, showed that the haemopoietic system is highly sensitive to Wnt signalling dosage. Low Wnt levels enhanced HSC self-renewal, whereas, high levels skewed lineage distribution and impaired HSC function (122). A dose- and time-dependent β-catenin requirement has also been demonstrated in the mouse AGM endothelium for the successful production of HSCs. Deletion of β-catenin in VE-Cadherin^{+} endothelial cells results in loss of haemopoietic potential without affecting endothelium development. However, β-catenin deletion in committed haemopoietic precursors has no effect on HSC production, indicating that Wnt signalling is crucial for the

initial haemopoietic specification of the endothelium (123). Finally, in the zebrafish both canonical β-catenin and non-canonical Wnt16 signalling are required for HSC specification (124, 125).

Bone morphogenic proteins (BMPs) are part of the transforming growth factor-β (TGFβ) superfamily, an important group of morphogens involved in many cellular processes and fate decisions during early embryonic development. BMPs bind to type I and type II receptors and trigger their heterodimerisation. These complexes will in turn phosphorylate Smad proteins which regulate the expression of multiple genes (126). BMP4 is required for mesoderm development (127) and also has a key role later in HSC emergence. In the E11 mouse AGM region BMP4 is expressed in the mesenchyme underlying HSC aortic clusters. When BMP signalling is inhibited HSC numbers are reduced, whereas, when AGM explants are cultured in the presence of BMP4 HSCs display enhanced repopulating potential (128). Similarly, in the zebrafish embryo BMP4 is expressed in the mesenchyme surrounding the developing dorsal aorta and conditional knockdown results in a loss of HSCs in the ventral wall of the dorsal aorta (129). Inhibition of the downstream effectors of BMP signalling, Smad1 and Smad5, in the zebrafish embryo represses the specification of hemogenic endothelium and ultimately the generation of definitive HSCs (130, 131). Interestingly, however, knockout of *Smad1* and *Smad5* in specified fetal liver and bone marrow HSCs does not affect their self-renewal or their reconstituting capacity (132). Collectively, these data suggest that BMP signalling is essential during HSC specification from the endothelium but dispensable after HSC commitment.

Additional pathways have been implicated in the specification of hemogenic endothelium and HSCs. As shown by Peeters and colleagues, components of the Hedgehog signalling are expressed in the murine E10 AGM region, mainly in the mesenchyme underlying the dorsal aorta (133). When early E10 AGM explants are cultured with low doses of Hedgehog proteins HSCs display enhanced repopulating activity, while, when anti-Hedgehog antibodies are added HSC function is reduced. These data suggest a time- and dose-dependent requirement of Hedgehog signalling in HSC specification. Studies on the developing zebrafish embryo demonstrated that Hedgehog proteins are expressed in the notochord and floor plate, and are required for the formation of the dorsal aorta and the maintenance of its arterial identity. Additionally, targeted deletion or inhibition of Hedgehog signalling components leads to loss of definitive haemopoiesis (134, 135). More importantly, these studies have shown that Hedgehog acts upstream of the vascular endothelial growth factor (Vegf) signalling, another major regulator of endothelial and haemopoietic

development, which in turn regulates the Notch pathway. Particularly, Hedgehog induces the expression of the Vegfa ligand in the somites which binds to the Vegf receptor expressed throughout the vasculature. Subsequently, Vegf signalling in endothelial cells activates the expression of Notch receptors, therefore, allowing the cells to receive the required signals for HSC specification (134, 135).

In conclusion, a lot of progress has been made in defining the molecular signals that determine HSC emergence. Although further work is required to accurately synthesise their interaction network, many transcription factors and signalling pathways have been characterised and successfully used in replicating, to a certain degree, haematopoietic induction *in vitro*. The development of the appropriate culture systems is equally important and has attracted a lot of interest in HSC research.

1.5 *In vitro* haemopoietic differentiation from pluripotent stem cells

Despite the detailed study of *in vivo* haemopoiesis and the wealth of information it has provided regarding the development and characteristics of adult HSCs, attempts to maintain and expand these cells *ex vivo* have been unsuccessful (reviewed in 136). With HSC transplantation remaining the most established cellular therapy and the only curative treatment for a variety of life-threatening haematologic disorders including anaemias, blood cancers, autoimmune and immune deficiency syndromes, the demand for engraftable HSCs is soaring. The only current sources of HSCs are bone marrow and cord or peripheral blood, which pose severe limitations due to the scarcity of donors. In addition the efficiency of the transplantations and the patient survival rate are significantly reduced by the transmission of infectious diseases and immunocompatibility problems. Therefore, many studies have focused on the generation of transplantable HSCs from alternative sources, with pluripotent stem cells (PSCs) either ESCs or iPSCs, being the most promising, if not the only alternative resource. As mentioned earlier, in many occasions PSCs have been successfully used in developmental studies of early haemopoiesis as they can recapitulate development *in vitro* (61-63, 75). With the same rational, a large number of protocols have focused on the directed differentiation of PSCs into fully mature HSCs that like their *in vivo* counterparts will have long-term multilineage engraftment potential.

1.5.1 Pluripotent stem cells

Pluripotent stem cells (PSCs) can either be derived from blastocyst-stage embryos, embryonic stem cells (ESCs), or from adult somatic cells, induced pluripotent stem cells (iPSCs). Regardless of their origin PSCs are distinguished by two important features that make them an unparalleled *in vitro* tool for biomedical applications. Firstly, they have the ability to self-renew robustly in an undifferentiated state in culture, thus providing an unlimited renewable source of cells. Secondly, under the appropriate conditions they can differentiate into any mature cell type of the adult body, creating enormous potential for the treatment of a large number of diseases. Moreover, PSCs can readily be genetically manipulated which allows the generation of cells with desirable genetic features. Finally, the ability to culture and differentiate PSCs under sterile conditions provides a source of safe, infection-free transplantable cells.

1.5.1.1 Embryonic stem cells

The study of PSCs began with the derivation of the first stable mouse embryonic stem cell lines (mESCs) from the inner cell mass of blastocyst-stage embryos in 1981 (137, 138). Mouse ESCs can differentiate into all three germ layers *in vitro* and can form chimeras or teratomas when reinjected into developing embryos or adult mice respectively. The first cell lines were maintained in an undifferentiated state by co-culture with mouse embryonic fibroblasts (MEFs) supplemented with fetal calf serum (FCS) and/or conditioned media from teratocarcinoma stem cell cultures. The use of leukaemia inhibitory factor (LIF) allowed for the maintenance of mESCs in feeder-free cultures on gelatinised surfaces supplemented with FCS (139). To further minimise the animal-derived factors and the variability they present a number of serum- and feeder-free culture conditions have been established to date. Ying and co-workers initially used a combination of LIF and bone morphogenic protein 4 (BMP4) to efficiently derive and maintain mESCs, while more recently they showed that use of GSK and MEK inhibitors, 3i medium, can successfully maintain undifferentiated mESC self-renewal (140, 141).

The first successful generation of human ESCs occurred in 1998 by Thompson and colleagues (5). Human ESCs were derived from donated embryos produced by *in vitro* fertilisation and like mESCs, they could retain their pluripotency when co-cultured with mouse MEFs and generate teratomas when injected into immunocompromised mice. As the use of animal-derived culture products can prevent the use of hESCs in clinical therapies due to transfer of pathogens or increased risk of immune reactions, the development of serum-

and feeder-free culture systems has also been a priority in hESCs. Extracellular matrices such as matrigel and fibronectin have been widely used to substitute for feeder cells. Nonetheless, MEF conditioned media supplemented with basic fibroblast growth factor (bFGF) was initially required for the maintenance of hESCs in feeder-free conditions (142, 143). It was later demonstrated that high concentrations of bFGF together with ActivinA or the BMP inhibitor noggin can sustain the feeder and serum-free undifferentiated proliferation of hESCs. (144, 145). Commercially available xeno-free culture media supplemented with human recombinant proteins has also been used, however, with limited efficiency (146).

1.5.1.2 Induced pluripotent stem cells

Despite the exciting clinical potential of hESCs, the destruction of human embryos for the derivation of hESC lines has raised major ethical and political concerns. Additionally, while undifferentiated ESCs express low levels of MHC antigens, their expression significantly increases during differentiation therefore also increasing the risk of immune reactions and rejection upon transplantation in mismatched hosts. The groundbreaking generation of induced pluripotent stem cells (iPSCs) from somatic cells can overcome ethical limitations and more importantly allow for patient-specificity.

The first mouse iPSCs were initially generated by the Yamanaka group through retroviral transduction of the pluripotency-associated transcription factors Oct3/4, Sox2, Klf4, c-Myc to embryonic and adult fibroblasts (147). Similarly to mESCs they could form multilineage teratomas upon transplantation into immunodeficient hosts and contribute to the development of chimeric mice following injection into blastocysts. A year later human iPSCs were generated using the same methodology by both the Yamanaka and Thompson groups (6, 148). Although the efficiency of human iPSCs generation was significantly lower than the murine, human iPSC lines could again contribute to all three germ layers in teratomas or *in vitro* conditions. Even though iPSCs resolve the issue of immune rejection, they bring up the risk of malignant transformation due to the use of viral vectors and proto – oncogenes. Recent studies have tried to overcome this problem with the viral-free transient gene expression or the direct delivery of reprogramming proteins (149, 150, 151). Although questions regarding the similarities and differentiation potential of iPSCs compared to ESCs remain, this technology is evolving rapidly and holding great promises for the generation of patient-specific HSCs.

1.5.1.3 Mouse epiblast stem cells

Even though there are currently a number of established human ESC lines, mouse cells still serve as an efficient tool to further decipher haemopoietic development and translate the acquired knowledge to the human system. However, fundamental differences between mouse and human ESC maintenance and differentiation strategies do not allow for the direct application of knowledge produced from one species to the other. Particularly, LIF which is essential for the maintenance of mESCs does not have an effect on hESCs, while BMP4, required for mESC self-renewal, induces differentiation of hESCs. In addition, bFGF an essential factor for hESC maintenance induces neural differentiation of mESCs. Another critical factor for hESC maintenance, ActivinA, fails to maintain mESCs. Differences in the regulatory pathways of mouse and human ESCs may reflect not only differences between species but also between the developmental stage and origin of the cells.

Mouse epiblast stem cells (EpiSCs), derived from post-implantation blastocyst embryos, share similar expression and signalling patterns with hESCs (152, 153). EpiSCs cannot be derived in the presence of LIF and/or BMP4, the two critical factors for mESC maintenance and self-renewal. Conversely their pluripotency depends on the Activin/Nodal pathway similar to hESCs. EpiSCs maintain the expression of pluripotency genes such as Oct4, Sox2 and Nanog, however, they are characterized by the downregulation of inner cell mass markers (Klf4, Stella) and the upregulation of early germ layer genes including Foxa2, Fgf5 and T (Bry). The similarities between human ESCs and mouse EpiSCs make EpiSCs a promising new model for the understanding of human development. More important, the primed state of EpiSCs towards the mesoderm lineage, marked by the expression of T, may confer to them a developmental advantage over mESCs for the study of haematoendothelial specification.

1.5.2 Approaches for the directed haemopoietic differentiation from PSCs

A variety of protocols for the directed differentiation of PSCs into the haemopoietic lineage have been developed to date using different technical approaches that include co-culture with feeder cells that mimic the embryonic haemopoietic environment, embryoid body (EB) formation that spontaneously differentiate into the three germ layers mimicking the embryonic development, and growth in extracellular matrix-coated dishes that promote proliferation and provide an attachment scaffold (Figure 1.3). The addition of serum or haemopoietic cytokines is used in these protocols to increase their efficiency and indeed the successful production of hematopoietic progenitor cells (HPCs) and mature blood cell types

has been well documented. However, the generation of HSCs capable of long-term engraftment in adult recipients and multilineage reconstitution has been more challenging and current protocols are constantly being improved.

1.5.2.1 Differentiation of PSCs in feeder cell co-culture

The 2-dimensional co-culture of PSCs together with a layer of feeder cells has proven quite successful in inducing and supporting haemopoiesis. Feeder cells provide the necessary cellular interactions and signalling molecules for the maturation of HSCs, similar to those found in haemopoietic niches *in vivo*. Even though future clinical applications will require feeder-free generated HSCs, feeder cell lines have been derived from a variety of anatomical sites including the bone marrow, the AGM region, the yolk sac and the fetal liver, and have been widely used alone or in combination with serum or haemopoietic factors both in mouse and human PSC cultures.

One of the most widely used feeder cell lines derived from mouse bone marrow, the OP9, was first used by Nakano and colleagues in mouse ESC cultures and was capable of supporting their erythroid, myeloid and lymphoid differentiation as shown by haemopoietic colony assay and cell surface marker expression (154). OP9 feeder cells were later successfully used for the generation of $CD34^{+}$c-$kit^{+}Sca\text{-}1^{+}$ haemopoietic cells capable of multilineage differentiation *in vitro* through a Flk-1-expressing mesodermal progenitor from mouse iPSCs (155). Similar to the mouse, human ESCs and iPSCs have been co-cultured with the OP9 line, generating high levels of $CD34^{+}CD43^{+}$ multipotent yet not engraftable haemopoietic progenitors (156, 157). In addition to the OP9 line, work by Kauffman and co-workers showed that the mouse bone marrow cell line S17 and the mouse yolk sac endothelial cell line C166 were capable of inducing the haemopoietic differentiation of human ESCs (158). Following the co-culture, hESCs gave rise to $CD34^{+}$ progenitors that expressed the haemopoietic transcription factors TAL-1, LMO-2 and GATA-2, and were capable of generating myeloid, erythroid and megakaryocyte colonies *in vitro* similar to those produced by human adult bone marrow cells.

In addition to bone marrow derived lines, feeder cells from the AGM region, mainly from the aorta-mesonephros subregion, have been extensively used in co-culture strategies and have proven quite successful in enhancing haemopoietic progenitor production from mouse and human PSCs. Weisel and colleagues generated a large panel of AGM-derived cell lines and evaluated their ability to support the haemopoiesis of mESCs. They demonstrated for the first time that several AGM lines were able to induce the generation of $CD45^{+}$ haemopoietic

cell to levels comparable with the OP9 line (159). Similar findings were provided by Krassowska and co-workers. Co-culture of mESCs with primary AGM region resulted in significantly enhanced haemopoiesis compared to mESCs in feeder-free culture as assayed by *in vitro* colony formation. Moreover, co-culture with the AM20-1B4 feeder cell line derived from the aorta and surrounding mesenchyme subregion yielded high numbers of haemopoietic colonies and c-kit, Sca-1 or CD45 expressing progenitors, while the haemopoietic potential of the urogenital ridge-derived UG26-1B6 and the fetal liver-derived EL08.1D2 lines was limited (160). The effects of primary feeder cells from the murine AGM region and fetal liver, as well as the established AM20-1B4, UG26-1B6 and EL08-1D2 cell lines on hESCs differentiation were subsequently tested. In all cases CD34 expressing cells were increased compared to non-feeder controls and most importantly some of these progenitors could reconstitute primary and secondary recipients, albeit in low numbers (161). Feeder cell lines derived from human fetal liver or bone marrow have also been reported to induce the generation of CD34+ progenitors in hESC differentiation, however, their use has not been widely applied suggesting low reproducibility (162, 163).

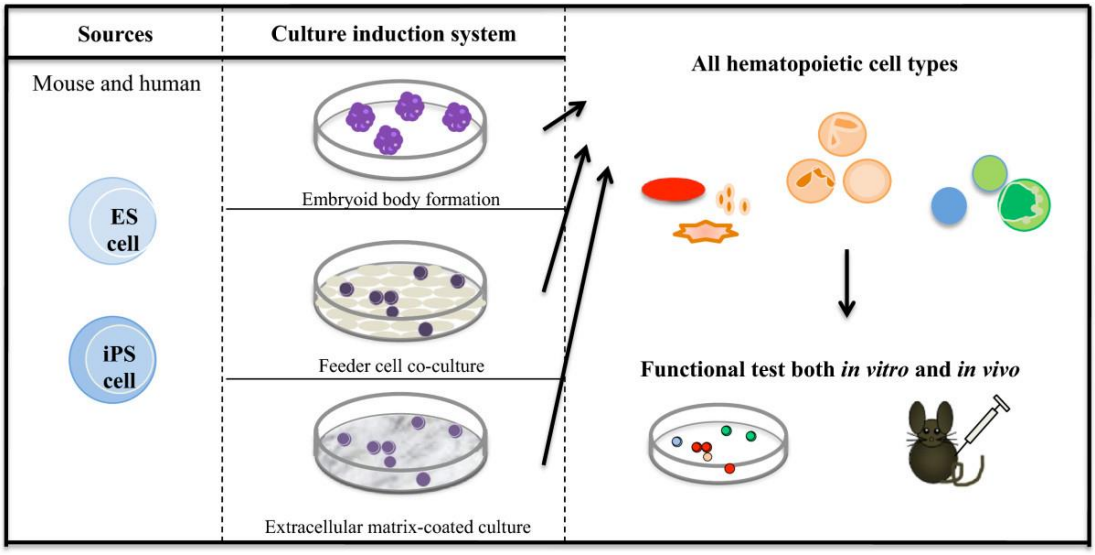

Figure 1.3: *In vitro* haemopoietic differentiation of pluripotent stem cells.
Embryoid body formation, feeder cell co-culture and extracellular matrix-coated dishes can be used for the directed haemopoiesis of mouse and human ESCs and iPSCs. Progenitors and mature blood cells are tested for their properties both *in vitro* and *in vivo.* Image adapted from Lim et al., 2013 (164).

1.5.2.2 Differentiation of PSCs in EB cultures

When removed from culture conditions that maintain self-renewal, mouse ESCs spontaneously form 3-dimensional spherical cell aggregates that closely mimic the early stages of embryonic development as they exhibit cell movement, form all three germ layers and differentiate into committed cell types (165). EBs are formed through a number of methods including suspension culture, hanging drops, spin EBs and are subsequently cultured in defined media with various combinations of cytokines and growth factors. EB mediated differentiation can overcome problems imposed by feeder cells such as passage limitation, feeder cell senescence, variability due to undefined factors and presence of animal-derived components, thus allowing highly reproducible and efficient yields.

The spontaneous generation of blood cells in EBs grown in the presence of serum has been long recorded in the mouse ESC system by Doetschman and colleagues (166). A number of cytokines, IL-3, IL-1, M-CSF, GM-CSF, were later identified that in combination with FCS enhanced haemopoietic differentiation of mouse ESCs (167). Interestingly, Burt and co-workers reported the long-term repopulating ability of c-kit+CD45+ cells generated from EBs treated with SCF, IL-3 and IL-6 in the presence of serum (168). However, their protocol has not been reproduced suggesting that FCS variability may have inhibitory effects and stressing the need for well-defined serum-free conditions. Early work by Nakayama and co-workers showed that serum-free differentiation of mouse EBs, particularly in the presence of knockout serum replacement (KOSR), BMP4 and VEGF resulted in increased numbers of $CD34^+$, $CD31^+$ and $CD45^+$ progenitors with multilineage *in vitro* potential (169). Park and colleagues confirmed the above findings by demonstrating that BMP4 was required for the generation of $FLK1^+$ and SCL^+ cells, while VEGF for the expansion of SCL^+ haemopoietic progenitors. Activin A was also found to augment the BMP4 and VEGF synergistic effect (170). Nevertheless, KOSR still contains unknown components that may affect some steps of the differentiation. Therefore, more robust and well-defined serum-free EB differentiation protocols were subsequently developed which did not contain KOSR and mainly relied again on the activity of BMP4 and VEGF. In one case the development of $CD34^+CD41^+$ colony-forming haemopoietic progenitors was greatly enhanced by the combined effects of BPM4, VEGF and TPO, while in another similar results were obtained by the step-wise addition of BMP4, Activin A, bFGF and VEGF (171, 172). However, none of these studies have demonstrated *in vivo* repopulating ability of the EB-derived haemopoietic progenitors.

Similar to mouse, human ESCs can differentiate into all three embryonic germ layers upon EB formation (173). Initial work by Chadwick and colleagues showed that EB culture in the

presence of BMP4 in combination with other cytokines can significantly enhance the generation of $CD34^+CD45^+$ progenitors of multiple lineages, however the particular approach included the use of serum in the first steps of the protocol (174). Extensive work by the Elefanty group has led to the development of a highly efficient serum-free EB differentiation protocol. To generate EBs of uniform size a defined number of hESCs were spun down to enforce aggregation and cultured in low-attachment plates. The spin EBs resulted in significant increase in the number of CD34 and RUNX1 expressing haemopoietic progenitors compared to protocols published by others, and were capable of generating colonies of the erythroid and myeloid lineages (175). Further work by the group proved that the culture of spin EBs in the presence of BMP4, VEGF, SCF and bFGF maximised the yield of haemopoietic progenitors as shown by the high numbers of colonies and the expression of transcriptional factors, SCL, GATA2, RUNX1, and cell-surface haemopoietic markers, CD34, CD45, CD117 (176). This serum- and feeder-free spin EB protocol has also been applied in human iPSCs successfully generating CD34, CD43 and CD45 expressing haemopoietic progenitors (177).

1.5.2.3 Differentiation of PSCs on extracellular matrix-coated dishes

While the 3-dimensional environment, provided by EBs, mimics quite faithfully the early stages of embryogenesis it may not be ideal for modelling later events. The limited cellular movements and lack of anatomical structures can inhibit the diffusion of critical molecules and important cellular interactions for optimal haemopoietic differentiation. To overcome these limitations novel 2-dimensional serum- and feeder-free differentiation systems have been developed that allow the development of PSCs as a monolayer on extracellular protein matrices.

Chiang and Wong developed a defined, serum-free differentiation protocol, where mESCs were plated in low density onto laminin-, Matrigel or Cell-Tak-coated plates and differentiated in the presence of BMP4, bFGF, Activin A and VEGF (178). In this system $CD41^+Runx1^+$ haemopoietic precursors, capable of haemopoietic colony formation *in vitro,* arose with high efficiency budding off from adherent endothelial cells. More importantly, this differentiation system allowed the direct time-lapse imaging of the differentiation process from single progenitors. In the same year, work by two different groups demonstrated the serum- and feeder-free monolayer haemopoietic differentiation of human ESCs and iPSCs. Human PSCs plated onto human fibronectin or mouse or human collagen IV, gave rise to floating $CD34^+CD43^+$ haemopoietic progenitors that under the appropriate

cytokine cocktails could further mature into several blood lineages (179). Interestingly, hypoxic conditions resembling the *in vivo* environment of the developing embryo increased the efficiency of the protocol. In a similar approach hPSCs were plated onto growth factor-reduced Matrigel, and under the influence of a haemopoietic cytokine cocktail generated $CD34^{+}CD45^{+}$ HPCs capable of multilineage differentiation *in vitro* (180). More recently, a combination of the EB and monolayer culture systems has been developed by Park and co-workers that takes advantage of the benefits the two systems provide. Human PSCs were allowed to initially form suspension EBs in defined serum-free conditions giving rise to hemato-endothelial precursors. The EBs were then dissociated and plated as single-cells onto human fibronectin-coated plates. The floating progenitors arising from the monolayers were characterised by CD34 and CD45 expression and high CFC activity (181). The ability of the progenitors generated in these studies to repopulate adult recipients has not been demonstrated yet.

1.5.3 Enhancing haemopoietic engraftment by genetic manipulation

While the differentiation protocols described above have been quite successful in generating haemopoietic progenitors that express haemopoietic markers and form multilineage colonies *in vitro*, studies reporting the engraftment of PSC-generated haemopoietic cells show either very low engraftment efficiency or limited reproducibility. In an effort to increase the repopulating potential of these *in vitro* progenitors, researchers have focused on the upstream molecular regulators of haemopoietic development and have identified a number of key transcription factors directing HSC specification. The enforced expression of these genes has been used in combination with the above protocols and in many occasions have resulted in enhanced *in vivo* engraftment.

In the mouse system *Stat5* overexpressing ESCs co-cultured with OP9 feeders have repeatedly been reported to not only increase the number of colony-forming haemopoietic cells but most importantly enhance their repopulating ability in primary and secondary recipients (182, 183). The LIM-homeobox transcription factor, *Lhx2*, was initially demonstrated to generate multipotent haemopoietic precursors from mouse ESCs capable of remaining in an undifferentiated state in culture for prolonged periods of time (184). More recent work has shown that its enforced expression in mouse ESCs and iPSCs can produce $Lin^{-}c\text{-}kit^{+}Sca\text{-}1^{+}$ cells capable of *in vivo* long-term engraftment (185). In human ESCs and iPSCs expression of *RUNX1a*, a *RUNX1* isoform, during EB differentiation produces progenitors expressing haemopoietic-related factors that demonstrate multilineage

haemopoietic reconstitution *in vivo* (186). An important transcription factor of definitive haemopoiesis that has attracted a lot of attention due to its ability to enhance the production of PSC-derived haemopoietic progenitors in both the mouse and the human system, as reported in a large number of studies, is HOXB4. However, while HOXB4 can confer long-term repopulating activity in precursors produced in mouse ESC cultures, it has not to date led to the maturation of repopulating HSCs in the human system (187, 188). Therefore, a huge amount of research has been invested in understanding the regulatory network behind HOXB4 activity and identifying critical factors that mediate its activity in an effort to advance the generation and expansion of PSC- derived HSCs.

1.6 HOXB4: an effector of definitive haemopoiesis

Overexpression of the transcription factor HOXB4 is currently the most efficient strategy for expanding adult HSCs, inducing repopulating activity in embryonic yolk sac HSCs and increasing the haemopoietic differentiation potential of PSCs. This remarkable ability, has subjected HOXB4 to extensive analysis of its mechanism of action and resulted in the development of a variety of protocols for HSC expansion and differentiation *in vitro*. Nonetheless, the critical factors triggered by HOXB4 necessary for the generation of human functional HSC remain elusive and warrant further investigation.

1.6.1 HOXB4 in development and haemopoiesis

HOXB4 is a member of the highly conserved family of the homeotic, *HOX* genes that encode DNA-binding transcription factors. In mammals *HOX* genes are organised into four major genomic clusters, A, B, C and D, and play a crucial role in directing the position of tissue development along the anterior-posterior axis during embryogenesis. Interestingly, the order of the *HOX* genes on their chromosome mirrors the order in which *HOX* genes are expressed along the head-to-tail axis in the developing embryo, a characteristic known as spatial colinearity (189). Genes involved in head formation are located in the 3' end of the chromosome, while, genes linked to the posterior are found on the 5' end. In vertebrates *HOX* genes also display temporal colinearity. Genes become progressively activated during development in a temporal sequence that corresponds to their position on the chromosome, with 3' genes being expressed before 5' genes (190). As described by Deschamps and colleagues the earliest 3' *HOX* gene expression is initiated during the late streak stage in the posterior primitive streak, in a cohort of cells that contributes to extraembryonic mesoderm.

Expression of more 5' genes also initiates in the posterior primitive streak at progressively later stages and in different cells due to cell movement during gastrulation. *Hox* expression then expands rostrally along the streak past the node reaching the definitive anteriormost boundaries, earlier for 3' genes and later in more posterior positions for 5' genes, in neurectoderm, mesoderm and endoderm structures (191. 192). Retinoic acid gradients, Fgf, and Wnt signalling are among the factors modulating early *Hox* gene expression, possibly through the jointed action of the *Cdx* transcription factors. Finally, once the anteriormost boundaries of *Hox* expression are established, the *Polycomb* and the *Trithorax* group genes start operating and maintaining them throughout embryonic development (191,192).

The majority of *HOX* genes, mainly clusters A, B and C have also been implicated in patterning haemopoietic mesoderm into definitive HSCs. They are highly expressed in stem and progenitor populations and downregulated upon lineage commitment (193, 194, 195). Using a YFP reporter, Hills and co-workers were able to track HOXB4 expression in the developing haemopoietic niches in the mouse embryo (196). They showed that HOXB4 is expressed in emerging haemopoietic cells in the AGM region, the yolk sac and the placenta. Although HOXB4 expression appears reduced in the fetal liver it increases again upon homing of the bone marrow with HOXB4-expresssing HSCs displaying long-term repopulating ability. Consistently, HOXB4-deficient mice exhibit significantly decreased cellularity of haemopoietic organs including bone marrow and spleen, and reduced numbers of haemopoietic progenitors (197). Thus HOXB4 is required for normal haemopoietic development, yet its loss can be compensated by other *HOX* genes.

Overexpression of several *HOX* genes followed by transplantation has been used to enhance the proliferation and expansion of HSCs. However, in many cases it has resulted in myeloproliferative disorders, leukaemias and defects in the differentiation of various lineages (198, 199, 200). Surprisingly, HOXB4 is the only factor that can significantly enhance HSC self-renewal in the murine system without leading to malignant transformation of the cells. As demonstrated by the Humphries group, transplantation of HOXB4-transduced bone marrow cells into lethally irradiated mice promotes competitive reconstitution of all haemopoietic lineages (201). Additionally, HOXB4-transduced HSCs display significantly increased *in vitro* expansion levels, without exceeding normal repopulation levels upon their transplantation (202). Retrovirally mediated HOXB4 expression in adult human bone marrow and cord blood HSCs has also been proven to enhance their repopulating ability without impairing differentiation or causing disease (203). Direct delivery of HOXB4 protein, which minimises genetic intervention, has also been

successful in expanding mouse and human HSCs and increasing their engraftment potential (204, 205). The initial excitement of the potent effects of HOXB4 on the expansion and engraftment in non-human primate $CD34^+$ HSCs demonstrated by Zang and colleagues, was hampered by his later work showing that HOXB4 overexpression can increase the risk of leukaemogenesis in larger animal models (206, 207). Additionally, high level of HOXB4 expression in $CD34^+$ human cells have been shown to result in impaired lymphoid and myeloerythroid differentiation, thus indicating the concentration dependent nature of HOXB4 activity and the need for extreme caution in its clinical applications (208).

1.6.2 Improving *in vitro* HSC differentiation by HOXB4 induction

The enhancing effects of HOXB4 on the *ex vivo* expansion of adult HSCs sparked a lot of interest in its use for improving the *in vitro* haemopoietic differentiation of PSCs. The first breakthrough came by Kyba and colleagues over a decade ago (187). Using a tetracycline-inducible *HoxB4* transgene, they induced *HoxB4* expression in EB-derived mouse cells followed by OP9 co-culture, which resulted in the generation of haemopoietic cells capable of reconstituting the myeloid and lymphoid pools of primary and secondary recipients. Interestingly, in the same study *HoxB4* also conferred repopulating activity on early embryonic yolk sac cells. Since then a variety of HOXB4 overexpression studies have been produced that despite variations in the induction strategy, they have resulted in enhanced HSC differentiation and in many cases long-term engraftment while providing insight on HOXB4 action. Work by Pilat and co-workers examined the effects that different ectopic HOXB4 levels have on the lineage decisions of EB-derived reconstituting cells and demonstrated that increased amounts of HOXB4 expression enforced myeloid development while supressing lymphoid and erythroid development (209). Accordingly, later work showed that transient rather than constitutive HOXB4 expression can significantly increase the generation of CD41, CD45 or Sca-1 expressing cells derived from mouse ESCs and iPSCs (210). Wang and colleagues showed that combined ectopic expression of *HoxB4* and another homeobox transcription factor *Cdx4*, further increased multilineage engraftment and improved the generation of lymphoid cells than *HoxB4* alone in mouse haemopoietic cells derived from EBs followed by OP9 co-culture (211). All the above approaches, however, included the use of serum or feeders that can obscure HOXB4 activity, the reproducibility of results and their clinical translation. Work by Lesinski and colleagues has successfully overcome these problems. They managed to develop a serum- and feeder-free protocol where ectopic HOXB4 expression together with hypoxic conditions is sufficient to produce

ESC-derived haemopoietic cells, phenotypically similar to definitive bone marrow HSCs and capable of long-term multilineage engraftment (212).

Generation of mouse PSC-derived definitive HSCs using HOXB4 raised the expectations that the same results can be achieved in human PSCs. However, while many studies have reported the enhanced generation of haemopoietic progenitors *in vitro*, the robust production of transplantable human HSCs based on HOXB4 expression has not been achieved to date. Bowles and co-workers reported that stable HOXB4-expressing hESCs generated significantly higher numbers of $CD45^+$ progenitors with upregulated levels of GATA1 and SCL/Tal1, yet *in vivo* reconstitution was not examined (213). Work by Wang and colleagues also demonstrated that lentiviral-based ectopic HOXB4 expression increased the total cell expansion of ESC-derived haemopoietic cells in culture, however, it failed to enhance blood colony formation *in vitro* or *in vivo* engraftment (188). Similar results were obtained by Lu and co-workers, who in an effort to avoid the potential risks associated with genetic manipulation of ESCs in clinical applications, they added a recombinant tPTD-HOXB4 protein in their differentiation protocol. Their approach resulted in increased numbers of multilineage colony forming progenitors but not in repopulating capacity (214). As in the murine system, expression levels of HOXB4 have been shown to affect the differentiation potential of human ESCs, and it can therefore be possible that modulation of HOXB4 levels may be the key for improving the efficient differentiation of repopulating human HSCs (215).

Despite the variability between reports it does appear that HOXB4 is the most powerful *in vitro* tool for adult HSC expansion and PSC-derived haemopoiesis. In order to eventually achieve the production of reconstituting human HSCs it is essential to understand the molecular mechanisms behind HOXB4 activity. Therefore, a number of gene expression studies have been performed on adult and ESC-differentiating haemopoietic cells that have uncovered a wealth of information regarding the molecular pathways controlled by HOXB4 and opened up the door for novel approaches in the use of HOXB4.

1.6.3 Characterising molecular pathways underlying HOXB4 activity

In an effort to identify HOXB4 target genes, genome-wide expression profiling was performed by Scheindlmeier and co-workers in murine adult bone marrow HSCs/HPCs and ESC-derived haemopoietic cells that expressed inducible forms of *HoxB4* (216). They identified 133 gene targets of HOXB4 in adult HSCs/HPCs the majority of which were

confirmed by qRT-PCR assays. The targets included genes shown to be crucial for self-renewal, survival and maintenance of adult HSCs such as *Cdkn1b, Mad, Foxo3a, Ptgs2,* and *Zfx.* A much larger number of HOXB4 targets, >700, was identified in differentiating ESCs, as they included non-haemopoiesis related genes of the three germ layers formed in developing EBs. Between the two datasets they found 52 overlapping genes which were characterized as 'universal targets' of HOXB4. Genes in this list are involved in pathways important for HSC self-renewal, maintenance and differentiation such as Wnt/β-catenin, Notch, TGF-β/Activin/BMP and FGF, as well as genes associated with cell cycle, apoptosis and response to stress. A later study by Oshima and colleagues combined ChIP-on-Chip with microarray analysis on ESC-derived HPCs overexpressing *HoxB4,* to provide further insight on direct and indirect HOXB4 targets (217). The ChIP-chip analysis revealed that HOXB4 regulates genes involved in a variety of functions, including cell proliferation, cell cycle and chromosomal organization and biogenesis. Most importantly they identified transcription factors essential for the maturation of developing HSCs into repopulating cells such as *Gata2, Scl/Tal* and *Runx1* as direct targets of HOXB4. Unexpectedly, none of the top 20 ranked direct HOXB4 targets have been characterized before as related to haemopoiesis. Additionally, their set of differentially expressed HOXB4 targets contained 13 genes overlapping with the universal HOXB4 targets identified by Scheindlmeier. More recently, ChIP-Seq coupled with gene expression analysis at 4 sequential time points of HOXB4-mediated ESC differentiation by Fan and colleagues, demonstrated that the HOXB4 regulatory network is highly dynamic as it expands during the differentiation process and different pathways are activated at distinct developmental stages (218). HOXB4 targets in the early stages of differentiation included genes related to early embryonic development and patterning such as *Dll1, Sox2, Nodal, Tbx3* and *Gli2.* At later time points HOXB4 induced genes already implicated in early hematopoiesis including *Meis1, Gata2, Runx1, Cited2, Id2* and *Stat5,* as well as genes of later lineage specific stages such as myeloid and lymphoid proliferation. Interestingly, it was shown that HOXB4 regulates haemopoiesis by targeting several chromatin modification enzymes.

Despite the insight that the above studies provided into HOXB4 activity, they only focused on its cell autonomous effect in partially or fully mature HSCs. Our group, based on the observation that HOXB4 is expressed in the primitive streak of the gastrulating embryo prior to the onset of hematopoiesis, looked into its potential hematopoietic inductive effect on the developing mesoderm of differentiating ESCs (219). Using an inducible HOXB4 expression system it was demonstrated that activation of HOXB4, before the emergence of hematopoietic progenitors, can increase hematopoietic differentiation. Expression profiling

at this stage of ESC differentiation, identified for the first time as targets of HOXB4 genes associated with paraxial mesoderm (Tbx6, Frzb, Dll1, Dll3, Foxc1, Fst, and Noggin), from which the hematopoietic niche is derived. This suggested a role for HOXB4 in the generation and/or modulation of the niche in differentiating ESCs. Target genes identified in this analysis also included a large number of secreted factors such as VEGFA and SCF that act as ligands for cell surface receptors of HPCs. Cell mixing experiments of HOXB4 overexpressing cells with constitutively expressing eGFP cells, resulted in increased hematopoietic differentiation of GFP+ cells indicating a paracrine mechanism of HOXB4 activity. Collectively these data suggest that HOXB4 might induce hematopoietic differentiation by regulating the formation of the hematopoietic microenvironment which further enhances hematopoiesis through a paracrine effect. Results of earlier work conducted in our group further support this hypothesis. When HOXB4 overexpressing ESCs were co-cultured on AGM-derived feeder cell lines, the HOXB4-mediated and feeder-mediated induction of hematopoiesis, were not additive, suggesting that this could be due to shared mechanisms (220).

A wide range of novel candidate regulators for HSC specification and development targeted by HOXB4 have been identified. These target genes allow HOXB4 to act not only in a cell autonomous but also in a paracrine mechanism. It remains to be tested whether the manipulation of these targets can improve the *in vitro* generation of engraftable HSCs. Additionally, as the above studies have been conducted exclusively on the murine system, it is interesting to see if the identified HOXB4 regulatory mechanisms also apply in humans and if they can bring us closer to the production of HSCs for clinical use.

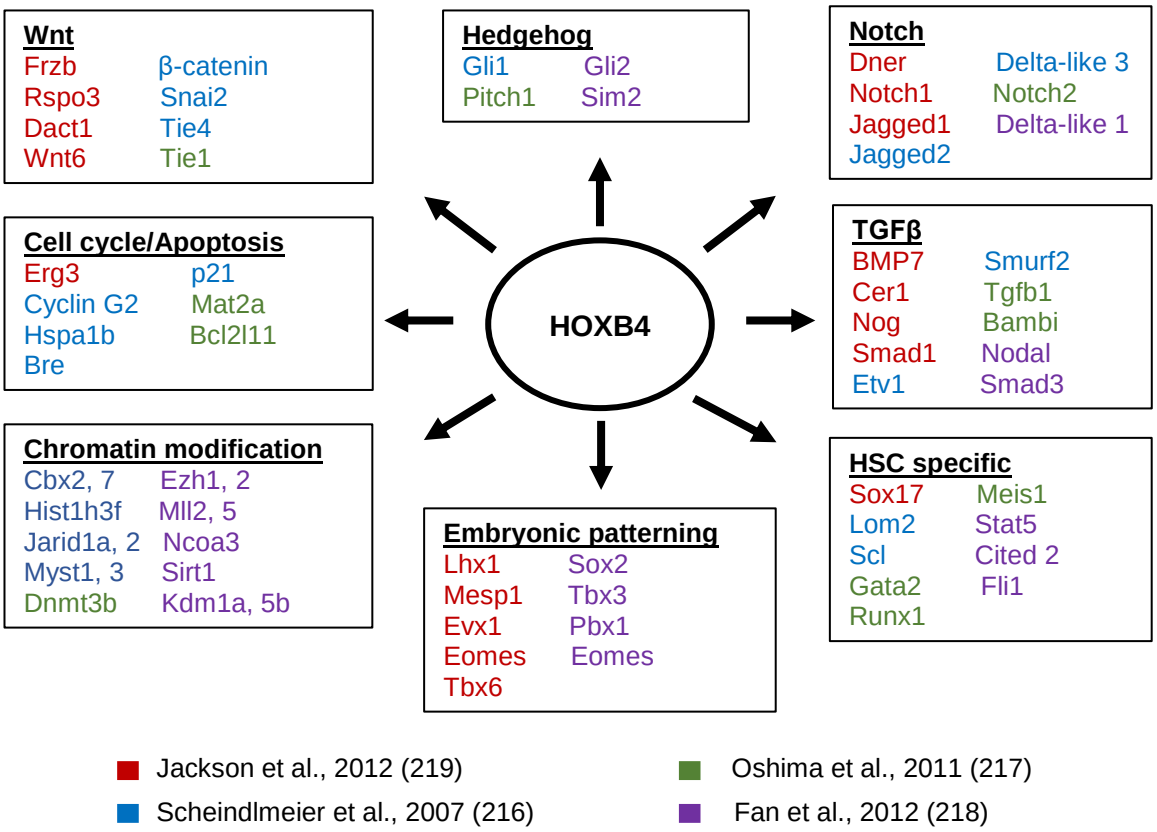

Figure 1.4: Selected important genes regulated by HOXB4.
HOXB4 regulates the expression of genes involved in different stages of development and different signalling pathways important for embryonic patterning and HSC specification.

1.7 Thesis aims

The overall aim of the thesis is to further investigate the paracrine mechanism of HOXB4 action on HSC specification suggested by our group. Identifying secreted factors that mediate this paracrine haemopoietic effect will not only further clarify HOXB4 regulatory network, but also lead to a differentiation method based on simply adding these factors in the cell culture. This method will have the great advantage of avoiding the genetic manipulation of ESCs, and therefore, providing not only a more practical but also safer source of definitive HSCs.

1.7.1 Hypothesis

HOXB4 expression in pre-hematopoietic mesoderm promotes paraxial mesoderm differentiation and could therefore result in the formation of the hematopoietic niche. Downstream HOXB4 targets secreted by the niche can promote the formation, maintenance and survival of engraftable HSCs. (Figure 1.4).

1.7.2 Experimental strategy

In order to test the above hypothesis the specific aims are:

1. Develop a feeder-free, serum-free mouse haemopoietic differentiation system to provide a well-defined, reproducible background in which to test HOXB4 induced secreted factors. Mouse ESCs as well as EpiSCs, were cultured in feeder- and serum-free conditions and assessed on their differentiation potential by colony-forming assay and surface phenotype.

2. Identify secreted factors induced by HOXB4 critical for haemopoietic differentiation. HOXB4 target genes identified in our previous microarray analysis were validated by qRT-PCR analysis and were used in the novel culture system to monitor their effects on haemopoietic differentiation.

3. Translate the above findings into the human system. Using a serum-free and feeder-free human haemopoietic differentiation system established in our group the secreted factors tested in the mouse system were used in human ESC and iPSC cultures to investigate differences not only between species but also cell lines.

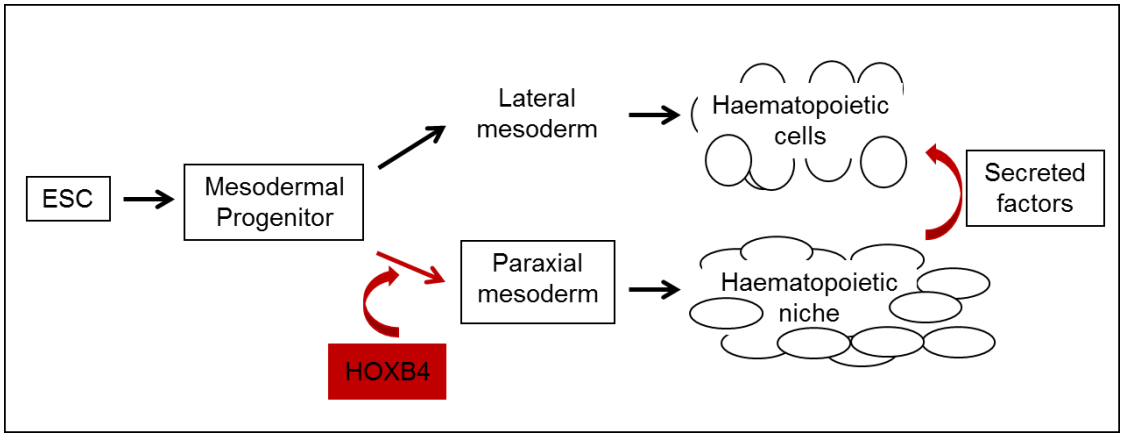

Figure 1.5: Proposed mechanism of HOXB4 activity.
Overexpression of HOXB4 modulates mesoderm differentiation by inducing genes associated with paraxial mesoderm that gives rise to the hematopoietic niche. Secreted factors by the niche are also upregulated in response to HOXB4 and enhance the differentiation of hematopoietic progenitors.

Chapter 2 : Materials and Methods

2.1 Tissue Culture

All cell culture procedures were carried out under aseptic conditions in a specialised tissue culture facility fitted with laminar flow hoods. Cells lines were tested mycoplasma free before use in experiments by Mrs Helen Henderson and Mrs Marilyn Thompson. Serum batch testing, LIF production and some of the preparation of tissue culture stock, were carried out by Mrs Helen Taylor, Mrs Helen Henderson and Mrs Marilyn Thompson. Human cells were maintained and passaged by Mrs Helen Taylor. All cells were incubated at 37^{o}C in a humidified 5% CO_2 atmosphere in the absence of antibiotics unless otherwise stated.

2.1.1 Maintenance and differentiation of mouse embryonic stem cells (mESCs)

2.1.1.1 Maintenance of mESCs

A subclone of CGR8 ESCs, known as CGR8.5, was routinely maintained onto 0.1% gelatin (Sigma) coated 25cm^2 tissue culture flasks, in 1x Glasgow Minimal Essential Medium (GMEM) (Gibco) supplemented with 10% fetal calf serum (FCS) (Lonza), 1% non-essential amino acids (Gibco), 0.1mM L-glutamine (Gibco), 2mM sodium pyruvate (Gibco) and 0.1mM β -Mercaptoethanol (Sigma). The medium was supplemented with 100U/ml Leukaemia Inhibitory Factor (LIF) as required.

Cells were passaged every 2 or 3 days when they reached 80-90% confluency. Prior to passaging, a new 25cm^2 flask was coated with 5ml of 0.1% gelatine in PBS for 10 minutes. Old medium was aspirated and cells washed with 5ml PBS to remove residual medium. Cells were subsequently incubated at 37^{o}C for 3min with 2ml trypsin solution (0.025% trypsin (Sigma), 1% chick serum (Gibco) and 1.3mM EDTA in PBS). The flask was tapped sharply to lift the cells, which were then transferred in 8ml of medium to deactivate the trypsin in the presence of FCS, and finally centrifuged at 120g for 5 minutes. The supernatant was aspirated and cells resuspended in single cell suspension with 10ml of fresh medium. Cell number was determined using a Neubauer haemocytometer. 1x10^6 cells were seeded onto the new gelatinised flask and supplemented with medium to 10ml and LIF at 100U/ml before placed in the incubator.

2.1.1.2 Thawing and cryopreservation of mESCs

Cells were thawed rapidly by placing the cryovials at 37^{o}C. Cell suspension from one cryovial was then transferred into 8ml of medium and centrifuged at 120g for 3 minutes.

After aspirating the supernatant, the cell pellet was resuspended in 10ml of fresh medium and transferred onto a gelatinised 25cm^2 flask with 100U/ml LIF. Medium was replaced after 5 hours or the following day.

To freeze mESCs, cells from an 80-90% confluent 25cm^2 flask were trypsinised and pelleted down as for passaging. After centrifugation the cell pellet was resuspended in 1ml of freezing medium, which consisted of maintenance medium with 10% dimethyl sulphoxide (DMSO) (Sigma). The cell suspension was then divided into 2 labelled cryovials and placed on dry ice for 10 minutes. The vials were then kept at -80^{o}C overnight and transferred at -150^{o}C for long-term storage.

2.1.1.3 Serum-based differentiation of mESCs

In this method, also known as the hanging drop method, mESCs were differentiated for 6 days under the influence of FCS without the addition of any other cytokines.

Day -2

Cells were treated as for passaging and counted. 6x10^5 cells were added in 20ml final volume of maintenance medium with 100U/ml LIF. Using a multi-channel pipette 10µl droplets (300 cells per droplet) were pipetted onto the upturned lid of a square petri dish. The lid was then turned over and placed back onto the petri dish so that the droplets were hanging down. This allowed the cells in suspension to congregate at the bottom of the droplet and form an embryoid body (EB). 10ml of tissue culture grade water was added at the bottom of the petri dish to keep the EBs hydrated and then placed in the incubator.

Day 0

After 48 hours, EBs were harvested by tapping the corner of the dish on the bottom of the hood, to allow for droplets to run down the edge of the dish. Using a plastic pipette the suspension was transferred into a centrifuge tube and spun down at 80g for 3 minutes. Supernatant was aspirated and the EBs were gently resuspended in 20ml of fresh medium without LIF to initiate differentiation. The EB suspension was placed into a 90mm bacterial grade petri dish which would prevent the EBs from attaching on the bottom of the dish and 1:100 dilution of Penicillin/Streptomycin (2000 units of Penicillin and 2mg of Streptomycin, Sigma) was added to prevent contamination.

Day 1 to day 6

After 24 hours new flasks were gelatinised, typically two 75cm^2 flasks per 20ml of EB suspension. EBs were collected from the petri dish and transferred into a 20ml tube. EBs were gently pipetted a couple of times to disperse them and the suspension was then split into the two flasks. Medium was topped up to 40ml and flasks were placed in the incubator. On day 4 of differentiation a complete medium change was performed by aspirating the old medium and adding 40ml of fresh. On day 6 of differentiation, EBs were collected and dissociated into single cells for further analysis following the typical trypsinisation process as for passaging.

In experiments done with the HOXB4-ER^{T2} clone, 4-OHT at 200nM final concentration was added in the flasks on day 1 and cells were harvested on day 3 of differentiation.

2.1.1.4 Serum-free differentiation of mESCs

In the serum-free protocol, cells were differentiated for 6 days in the absence of serum under the influence of cytokines in N2B27 medium. The N2B27 medium consisted of 1:1 ratio of Neurobasal : DMEM/F12 without L-Glutamine (Invitrogen), supplemented with 0.5% N2 (Invitrogen), 2% B27 (Invitrogen), 1% non-essential amino acids (Gibco), 0.1mM L-glutamine (Gibco) and 0.1mM β -Mercaptoethanol (Sigma).

Day -2

2 days prior to differentiation mESCs, typically maintained in FCS supplemented GMEM medium, were plated onto a 25cm^2 gelatinised flask in N2B27 medium supplemented with 100U/ml LIF and 10ng/ml BMP4 to remove any FCS residues and allow the cells to adjust to serum-free conditions.

Day 0

On Day 0 of differentiation the N2B27 medium was aspirated and the cells washed with 5ml PBS. 1ml of Accutase solution (Sigma) was added in the flask and incubated at 37^{o}C for 2-3 minutes. The flask was then tapped to lift the cells, the cells were quenched in 9ml of N2B27 medium and spun at 120g for 3 minutes. The supernatant was aspirated, cells resuspended in single cell suspension in fresh N2B27 and counted. 3x10^5 cells were put in a clean tube and N2B27 was added to a final volume of 10ml supplemented with the cytokines outlined in

Table 2.1. The cell suspension was then placed in a 60mm bacterial grade petri dish or split in 3 wells an ultra-low attachment 6-well plate (Stemcell Technologies), depending on the experiment plan. Embryoid bodies were formed in suspension by the next day.

Day 2 to day 6

On day 2 of differentiation 2ml of 0.1% gelatine were placed per well of a 6-well tissue culture plate and allowed to incubate for 10 minutes. The EB suspension was transferred in a centrifuge tube and spun at 80g for 3 minutes. The media was aspirated and the EBs were gently dispersed in 30ml N2B27 media supplemented with the appropriate cytokines (Table 2.1). 5ml of the EB suspension were placed in each well and incubated for 4 more days. By day 4 of differentiation the EBs had fully attached to the bottom of the plate and were fed with 0.5ml N2B27 with fresh cytokines.

To harvest the cells on day 6, the floating fraction, with any cells present in it, was transferred into a centrifuge tube. The EBs were gently washed with 2ml of PBS and this was added to the tube with the floating fraction. 500μl of Accutase were added per well and incubated at 37^{o}C for 5min. EBs were quenched with 3ml fresh media, pipetted a few times to dissociate and added to the centrifuge tube with the rest of the floating fraction. The suspension was spun down for 3 minutes at 120g. Cells were resuspended in the appropriate volume of N2B27 medium and counted before further analysis.

To examine the effects of HOXB4 targets, the recombinant proteins of interest were added from day 0 to day 6 in the following concentrations: mRSPO3 (RnD Systems) at 10, 30, 100 and 300ng/ml, mFGF17 (RnD Systems) at 10, 30, 100 and 300ng/ml, APELIN13 and APELIN36 (AnaSpec) at 30nM, 100nM and 300nM.

Day of differentiation	Cytokines
Day 0 to 2	ActivinA 5ng/ml, bFGF 10ng/ml, BMP4 5ng/ml
Day 2 to 6	bFGF 10ng/ml, BMP4 5ng/ml, VEGF 15ng/ml

Table 2.1: Cytokines used in serum-free haemopoietic differentiation of mESCs

2.1.2 Generation, maintenance and differentiation of mouse epiblast stem cells (mEpiSCs)

2.1.2.1 Generation and maintenance of mEpiSCs

Mouse epiblast stem cells were generated *in vitro* by culturing mouse embryonic stem cells in the appropriate conditions. Particularly, mESCs were prepared in single cell suspension as for passaging and plated at a density of 8 x 10^4 cells per well of a 6-well tissue culture plate pre-coated for 30 minutes with 8μg/ml of human Fibronectin (Sigma) in PBS. For the first 24 hours the cells were grown in conventional mESC maintenance medium, thereafter, the cells were kept in N2B27 medium supplemented with 20ng/ml ActivinA (Peprotech) and 10ng/ml bFGF (RnD Systems).

The cells were passaged every 2 or 3 days when they reached 70-80% confluency. At the beginning cells were passaged at high density, 1/3 or 1/4, and once established after a minimum of 5 passages at lower density, 1/6 to 1/8 depending on confluency. To passage the mEpiSCs, the cells were washed gently with 2ml of PBS and then incubated in 300μl Accutase for 5 minutes at 37^{o}C. Following this period 3ml of N2B27 medium were used to collect the cells into a centrifuge tube and they were spun down for 3 minutes at 120g. The pellet was then resuspended in N2B27 supplemented with ActivinA and bFGF by gently pipetting up and down and the appropriate amount of cells was plated onto a new Fibronectin pre-coated 6-well plate. Culture medium was replaced daily.

2.1.2.2 Thawing and cryopreservation of mEpiSCs

Cryovials were placed at 37°C to allow for rapid thawing of the cells. The cells from one cryovial were transferred into 9ml of N2B27 medium and spun down for 5 minutes at 130g. The supernatant was aspirated and cells resuspended in 3ml of N2B27 medium supplemented with ActivinA and bFGF before being plated onto a Fibronectin pre-coated well of a 6-well tissue culture plate. The medium was replaced 5 hours later or the following day after the cells had adhered to the plastic.

Mouse EpiSCs were frozen down using knock-out serum replacement (KOSR, Invitrogen) with 10% DMSO. One confluent well was washed with PBS and the cells lifted with a 3 minute Accutase incubation at 37°C. The cells were quenched with fresh N2B27 medium and pelleted down for 3 minutes at 120g. The supernatant was aspirated and the pellet resuspended with 1ml of freezing medium. The cell suspension was placed into one cryovial and quickly frozen on dry ice for 10 minutes. The vial was then kept at -80°C overnight and transferred at -150°C for long-term storage.

2.1.2.3 Serum-free differentiation of mEpiSCs

Mouse EpiSCs were differentiated using the same protocol described for the serum-free differentiation of mouse ESCs (Table 2.1). One confluent well of mEpiSCs was washed with PBS and incubated with 300µl of Accutase to lift the cells as for passaging. Following the centrifugation, the pellet was resuspended in 10ml of N2B27 supplemented with day 0 cytokines. The cell suspension was divided into 3 wells of an ultra-low attachment 6-well plate. Following the protocol detailed above, on day 2 of differentiation the EBs were collected and plated onto a Fibronectin pre-coated 6-well tissue culture plate where they were further matured up until day 6.

2.1.3 Maintenance and differentiation of human embryonic and induced pluripotent stem cells (hESCs and hiPSCs)

2.1.3.1 Maintenance of hESCs and hiPSCs

hESCs and hiPSCs were maintained on CELLstart (Invitrogen) coated 6-well tissue culture plates (Costar) in StemPro medium comprising of DMEM/F12 with Glutamax (Invitrogen), 1.8% BSA (Invitrogen), StemPro supplement (Invitrogen), 0.1mM β-Mercaptoethanol (Invitrogen) and 20ng/ml human basic FGF (Invitrogen). Maintenance medium was replaced

daily. Cells were passaged mechanically when they reached about 80% confluency using EZ passage tool (Invitrogen). Prior to passaging, cells were fed with fresh medium and new tissue culture plates were coated with 750µl CELLstart in DPBS containing Mg^{2+} and Ca^{2+} at 1:50 dilution for an hour minimum at 37^{o}C. Cells were then cut into small pieces with EZ passage tool, gently suspended by pipetting with a plastic Pasteur pipette and finally transferred into the new coated plate with fresh medium at appropriate ratios.

Cell lines used in the study were the hESC line H1 (5) and the hiPSC line SFCi55. The SFCi55 cell line was generated by Roslin Cells (http://roslincells.com/) from skin fibroblasts using episomal expression of Yamanaka factors. SNP analysis performed by Edinburgh Genomics (https://genomics.ed.ac.uk/) to detect deletions, duplications and other abnormalities across the genome detected no abnormalities.

2.1.3.2 Thawing and cryopreservation of hESCs and hiPSCs

To thaw the cells, the cryovials were warmed up quickly in the operator's palms. Cells from one cryovial were immediately transferred into 1ml of pre-warmed media and centrifuged for 5 minutes at 200g. After aspirating the supernatant, the cells were resuspended in fresh maintenance medium and transferred into a CELLstart coated 6-well plate. Medium was replaced 24 hours later that the cells had adhered to the plastic.

To freeze cells, cells were fed with fresh medium and Mr Frosty (Nalgene) was cooled down at 4^{o}C for a minimum of 30 minutes prior to freezing. The EZ passage tool was used to cut the cells of an 80-90% confluent well. Cells were spun down for 5 minutes at 200g, supernatant was aspirated and cells were resuspended in 1.5ml of cold Cryostor CS 10 reagent (Biolife Solutions). 0.5ml of the cell suspension was finally transferred in labelled cryovials. The vials were then placed in Mr Frosty and into 4^{o}C for 10 minutes. Mr Frosty was subsequently transferred at -80^{o}C overnight, after which time, vials were placed at -150^{o}C for long-term storage.

2.1.3.3 Differentiation of hESCs and hiPSCs

hESCs and hiPSCs were differentiated under serum-free conditions for a total of 10 days as outlined in Table 2.2. Cells were collected and analysed on days 7 and 10. One confluent well of cells in a 6-well plate was typically split into 2 wells for differentiation.

Day 0 to day 2

On day 0, the maintenance medium was aspirated and replaced by 1ml of Stemline II haemopoietic cell expansion medium supplemented with cytokines as described in Table 2.2. Cells were cut with EZ passage tool, suspended by gentle pipetting and split into 2 wells of an ultra-low attachment 6-well plate (Stemcell Technologies) which allows for formation of suspension EBs. Cells were topped up with 2ml of Stemline II with the appropriate cytokines before being placed in the incubator. On Day 2 cells were checked for the formation of EBs and each well was topped up with 0.5ml of Stemline II media supplemented with the required cytokines.

Day 3 to day 7

On day 3 EBs were harvested into centrifuge tubes and spun down for 3 minutes at 200g. The supernatant was aspirated and 0.5ml Accutase (Life Technologies) was added to the cells and allowed to incubate at 37^{o}C for 3 minutes. EBs were dissociated to single cell suspension by pipetting a few times and quenched with 2ml of medium. They were subsequently spun down for 3 minutes at 200g and resuspended in 1ml of fresh medium. Cells were counted using a Neubauer haemocytometer and 2x10^{5} cells were plated per well of a 6-well tissue culture plate. Wells were topped up with 3ml of Stemline II with day 3 cytokines. On day 5 cytokines were renewed by adding 0.5ml of cytokine supplemented medium.

Day 7 to day 10

On day 7, cells to be analysed were collected into centrifuge tubes by repeatedly pipetting a few times to detach them from the culture plastic and disperse any clumps. They were then spun down for 3 minutes at 200g and resuspended in 1ml of medium before being counted to use in flow cytometry and colony forming assays. For the cells to be propagated to day 10 a complete media change was performed on day 7. The medium was gently aspirated from the wells, to disturb the cells the least possible, and transferred into a centrifuge tube. 1ml of fresh medium with day 7 cytokines was added to the wells to prevent cells from drying while the old medium with any cells present in it was centrifuged for 3 minutes at 200g. Following the centrifugation, the supernatant was discarded and the cell pellet was resuspended in 2ml of medium with cytokines and finally added to the wells. On day 9 cells were fed with 0.5ml medium with fresh cytokines. On day 10 cells were collected and their number was determined for further analysis as described for day 7.

For the testing of HOXB4 targets, the recombinant proteins were added from day 0 to day 10 in the following concentrations: hRSPO3 (RnD Systems) at 30 and 100ng/ml, hFGF17 (RnD Systems) at 30 and 100ng/ml, APELIN13 and APELIN36 (AnaSpec) at 100nM.

Day of differentiation	Cytokines
Day 0 to 2	BMP4 10ng/ml, VEGF 10ng/ml, Wnt3a 10ng/ml
Day 2 to 3	BMP4 20ng/ml, VEFG 20ng/ml, Wnt3a 10ng/ml
Day 3 to 7	BMP4 20ng/ml, VEGF 30ng/ml, FGFa 10ng/ml, SCF 30ng/ml, IGF2 10ng/ml, TPO 10ng/ml, Heparin 5µg/ml
Day 7 to 10	BMP4 20ng/ml, VEGF 30ng/ml, FGFa 10ng/ml, SCF 30ng/ml, IGF2 10ng/ml, TPO 10ng/ml, Heparin 2.5µg/ml

Table 2.2: Cytokines used for the haemopoietic differentiation of hESCs and hiPSCs

2.1.4 Methylcellulose-based haemopoietic progenitor colony assay

Methylcellulose-based haemopoietic progenitor assay was routinely used to enumerate haemopoietic progenitors at single cell level for both mouse (MethoCult™ GF M3434, Stemcell Technologies) and human (MethoCult™ H4434, Stemcell Technologies) cells. Cells were collected from culture and prepared into single cell suspension. 1.5ml of methylcellulose medium were added into a 35mm low attachment dish. For each cell treatment 2 dishes were set up in parallel at densities $1x10^5$ and $5x10^4$ for mouse cells and at $1x10^4$ and $5x10^3$ for human cells unless otherwise stated. The dishes were placed in big

round dish containing a 60mm petri dish with 10ml of tissue culture grade water to prevent the methylcellulose from drying out. The dishes were incubated at 37°C for 12-14 days. The colonies were classified based on the morphology using light microscopy and scored according to the manual.

2.1.5 Proliferation assay

$1x10^6$ mouse cells, were seeded onto $25cm^2$ tissue culture flasks in the appropriate medium and cultured for 48 hrs. The cells were then dissociated into single cell suspension and counted using a Neubauer haemocytometer. Proliferation rates, denoted as cell doubling times (DT), were calculated using the following formula: DT = (t−t0) log2/(logN−logN0) where t, t0 indicate time points at counting and initial plating, respectively, and N, N0 indicate number of cells at respective time points. Results are presented as mean doubling times ± standard deviation of 5 consecutive passages.

2.2 Molecular biology techniques

2.2.1 RNA extraction and cDNA synthesis

Total RNA from fresh or frozen cell pellets was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. On-column DNaseI digest was performed with the RNase-Free DNase Set (Qiagen) to remove residual genomic DNA. RNA concentration was measured on Nanodrop and the samples used directly for cDNA synthesis or stored at -80°C.

For cDNA synthesis, the SuperScript® VILO™ Master Mix Kit (Invitrogen) was used. The reaction was set up with 400ng of RNA per 10µl reaction. Samples were incubated at 25°C for 10 minutes for primer annealing, then at 42°C for 60 minutes for cDNA synthesis and finally at 85oC for 5 minutes to inactivate the reaction. cDNA was normally stored at -20°C.

2.2.2 Quantitative RT-PCR analysis

qRT-PCR reactions were set up in triplicate in 96-well plates and performed on a ABI 7500 FAST qPCR machine (Applied Biosystems). The program used with TaqMan based analysis

was: 95^{o}C for 3 seconds, followed by 40 cycles of at 95^{o}C for 20 seconds to denature the cDNA and 60^{o}C for 30 seconds to allow annealing and extension. For SYBR Green based analysis, a final dissociation step was added to check primer specificity. Two types of primer-probe systems were used, the TaqMan and the UPL, which were designed by the author. SYBR Green primer sequences were given by Dr Anestis Tsakiridis. The housekeeping genes Hprt or Tbp were used as endogenous controls to normalise cDNA quantity loaded. Data analysis was performed using the SDS v1.4 software (Applied Biosystems). Gene expression was calculated using the ΔΔCT method, where each gene of interest was first normalized to the endogenous control and the data was then shown as fold change to a calibrator chosen according to each experiment.

Primer and probe sequences are listed in Appendix Table S1.

2.3 Flow cytometry analysis

At different time points during differentiation cells were harvested for flow cytometric analysis of surface marker expression. A minimum of 2x10^{5} to a maximum of 1x10^{6} cells were added in each FACS tube. Antibodies were then added according to predetermined optimal concentrations and incubated for 20 minutes at 4^{o}C. Cells were washed in 2ml of DPBS containing 1% BSA and centrifuged at 400g for 5 minutes. Pellets were resuspended in 200μl of DBPS with 1% BSA and stained with 7-AAD viability dye (eBioscience).

Samples were analysed on a 5 laser BD LSRFortessa™ flow cytometer (Becton Dickinson) at the Centre for Inflammation Research, Queen's Medical Research Institute (QMRI), with the help of Mrs Shonna Johnston and Dr Will Ramsay, or on a BD Accuri™ C6 cytometer (Becton Dickinson) at the MRC Centre for Regenerative Medicine, with the help of Mrs Fiona Rossi and Dr Claire Cryer. Data were analysed using FlowJo or the BD Accuri™ C6 cytometer analysis software.

Gating strategy and controls used are outlined in Supplementary Figures S1 and S2 for mouse and human cell respectively. Antibodies used for the analysis of mouse and human cells are listed in Appendix Table S2.

2.4 Protein analysis

2.4.1 Immunofluorescence

Cells were grown on appropriately pre-coated 24-well tissue culture plates to 90% confluency. Cells were washed twice with PBS and fixed with 4% PFA for 10 minutes at room temperature. Following a quick wash with PBS, the cells were incubated in PBST (0.1% Triton-X 100 in PBS) for 10 minutes at room temperature to permeabilise and then the fixation was stopped by adding 0.5M Glycine for 15 minutes at room temperature. Cells were then washed 2 x 5 minutes with PBST and blocked with 3% donkey serum in PBST for 2 hours at room temperature. Cells were subsequently incubated with primary antibodies, Oct4 (Santa Cruz) and Brachyury (RnD Systems), at 1:200 dilution in blocking buffer overnight at 4^{o}C. 3 x 5 minutes followed by 4 x 25 minutes washes with PBST at room temperature on shaking platform at the lowest speed were applied the next day. Secondary antibodies, Alexa fluor 488 and 568 at 1:1000 dilution in blocking buffer were then added for 2 hours at room temperature in the dark. Unbound antibodies were removed by final washes of 3 x 5 minutes followed by 4 x 25 minutes with PBST. DAPI at 1:7000 dilution was added for 10 minutes before the final wash. PBST was added in the wells and cells were observed under an Olympus IX51 inverted fluorescence microscope using the Volocity imaging software.

2.4.2 Protein extraction

To extract proteins, ice cold RIPA buffer (Thermo Scientific) with 1% protease inhibitor (Sigma) was added to cell pellets and incubated for 30 minutes on ice. The solution was vortexed for 10 seconds every 10 minutes during this incubation and finally centrifuged at 13000 rpm for 20 minutes at 4^{o}C. Supernatants were transferred to clean eppendorf tubes and used directly for Western blots or stored at -20oC.

2.4.3 Gel electrophoresis and Western blot

Protein electrophoresis was performed using the NuPAGE® SDS-PAGE Gel System (Invitrogen) with 4-12% Bis Tris Gels. The protein extracts were warmed up for 10 minutes at 80^{o}C with 1/10 volume of β-Mercaptoethanol and loaded onto the gel. Gels were run at 200V for about 35 minutes in MES buffer according to manufacturer's instructions. Semidry transfer onto nitrocellulose membranes was performed using the Biorad transfer system with

cold transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 15V for 1 hour. Membranes were subsequently blocked with 5% semi skimmed milk in TBST (25mM Tris-HCl, pH 8.0, 125mM sodium chloride, 0.1% Tween-20) over night at room temperature. Primary antibodies diluted in blocking buffer were incubated for 1 hour, followed by TBST washes of 3 x 15 minutes. Horseradish (HRP)-conjugated secondary antibodies, were subsequently added at 1:1000 dilution in blocking buffer for an hour at room temperature and finally washed 3 x 15 minutes with TBST. Equal volumes of ECL reagent A (0.1M Tris-HCl pH 8.6, 25mM luminal, 0.4mM coumaric acid) and reagent B (0.1M Tris-HCl pH 8.6 and 0.02% hydrogen peroxide) were mixed and added to the membrane for 1 minute before exposure to film to visualise the proteins. Primary antibodies used were: rat anti-HOXB4 (DSHB) diluted at 1:500 in blocking buffer and goat anti-mouse GAPDH (Santa Cruz), serving as loading control, at 1:1000 dilution.

2.5 Data analysis

Data presented in this thesis were analysed and plotted with Microsoft Office Excel 2013 and GraphPad Prism software (version 6.0). Data are summarised as average $\pm$ standard deviation. Statistical significance was evaluated using two-tailed Student's *t*-test. Image analysis of immunofluorescence was performed using ImageJ software (NIH).

Chapter 3 : Development of an efficient mouse haemopoietic differentiation protocol: comparing the differentiation potential of ESCs to EpiSCs in feeder- and serum-free conditions

3.1 Introduction

Since their discovery mouse ESCs have been the gold standard for the investigation of haemopoiesis *in vitro*, generating a significant amount of knowledge regarding the required lineage induction events. As detailed in Chapter 1, numerous differentiation methods have been developed leading to the successful generation of haemopoietic progenitors, mainly relying in the use of serum and/or supportive feeder cells. Nevertheless, the presence of serum or feeders in the differentiation medium increases variability and introduces many undefined molecules that can affect positively or negatively the haemopoietic process and interfere with the analysis of signalling factors. Therefore, many research groups have turned to the use of defined culture conditions that increase reproducibility and allow for better understanding of the molecular mechanisms of cell fate specification. Similarly, our group until today, has used either serum or feeder cell lines for the investigation of haemopoietic development, which despite their efficiency and the valuable data they have provided, they still require the tedious work of serum batch testing to ensure reproducibility and involve the presence of unknown regulating factors. Since the overall aim of this study is to investigate the effects that newly identified HOXB4 targets have in the differentiation output, it was deemed necessary to establish a feeder-free and serum-free differentiation protocol in the lab that would allow testing individual factors in a well-defined and controlled background.

Another major issue to be tackled in the mouse ESC differentiation system is the translational gap with the human system. Even though major developmental pathways are highly conserved, critical interspecies differences in the activity of regulatory factors exist that would have an impact on the clinical translation. Such is the case of HOXB4, which unlike in the mouse system, its *in vitro* use has not yet generated engraftable human ESC-derived HSCs. Mouse EpiSCs are considered more comparable to human ESCs not only due to similarities in the signalling pathways controlling their maintenance and self-renewal but also due to their shared mechanisms regulating early cell fate decisions and linage commitment (221, 222). The ultimate purpose of this study is to assess the data produced in the mouse system regarding HOXB4 activity, in our human ESC differentiation system. Therefore, the haemopoietic potential of mouse EpiSCs was also assessed in the novel differentiation protocol. Additionally, the primed state of EpiSCs towards the mesoderm lineage as indicated by their *T* (*Bry*) expression (152), may offer them a developmental advantage over conventional mouse ESCs.

3.2 Aims

1. Develop a defined, feeder-free and serum-free mouse haemopoietic differentiation protocol to use in the study of HOXB4 targets.

2. Assess whether mouse EpiSCs under defined conditions provide an improved system for haemopoietic differentiation compared to ESCs.

3.3 Experimental strategy

1. To test whether the new feeder-free and serum-free protocol is capable of supporting haemopoietic differentiation, mouse ESCs were cultured in parallel in the new and our standard serum-based protocols. The differentiation efficiency was assessed by flow cytometry analysis of surface haemopoietic markers and haemopoietic colony formation as a functional readout.

2. To ensure maximum comparability between ESC and EpiSC populations, EpiSCs were derived from the ESC cell line used in the study. The two populations were then differentiated in the new serum-free protocol in parallel and assessed for HPC production at different time points to closely monitor their developmental progression.

3.4 Results

3.4.1 Development of a well-defined mouse ESC haemopoietic differentiation system

Mouse ESCs were cultured in feeder- and serum-free conditions, from now referred to as serum-free/SF, using EB formation in the presence of four growth factors (BMP4, Activin A, bFGF and VEGF) that have been implicated in the differentiation of both mouse and human ESCs to haemopoietic lineages (127, 223, 224). Cells were passaged in serum-free conditions two days prior to the onset of differentiation to ensure serum traces that might affect the outcome are eliminated and allow for the cells to adapt to the new culture medium. As a control cells were cultured in parallel in our routinely used EB differentiation protocol which relies on the use of FCS to induce haemopoietic development, and will be referred to as serum-based/SR. The two protocols are outlined in detail in Figure 3.1.

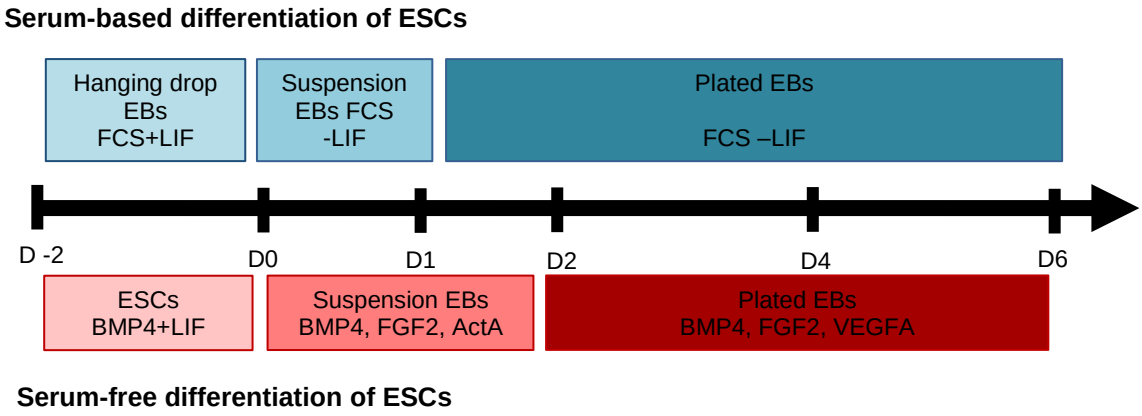

Figure 3.1: Mouse ESC differentiation protocols.
In serum-based conditions EBs were formed by the hanging drop method and induced to differentiate in suspension upon LIF withdrawal after two days. The EBs were then plated on gelatin and allowed to fully differentiate to day six. In serum-free conditions ESCs were passaged once in media supplemented with LIF and BMP4, and subsequently placed in suspension to form EBs with the specified cytokines. After two days the EBs were plated on gelatin in the appropriate medium and cultured up to six days. Cells were collected for analysis at different time points as specified in the text.

3.4.1.1 Serum-free conditions are capable of maintaining ESCs in an undifferentiated state

The serum-free ESC culture protocol established by Ying and colleagues (141) was used for cell maintenance before initiation of differentiation. A critical point to test before proceeding with the study was whether the particular culture system could support expansion of our cell line without inducing differentiation and therefore affecting the results. To this end ESCs were passaged in serum-free N2B27 medium supplemented with LIF and BMP4 for 5 consecutive times and compared on their proliferation rates and expression profile to cells maintained in our conventional FCS and LIF supplemented medium for the equivalent time period. Morphologically the two culture media showed no obvious morphological differences. Colonies of cells in serum-free medium maintained a dense, rounded, dome-like morphology characteristic of ESCs even after 5 passages similar to the cells maintained in FCS with no signs of spontaneous differentiation and maintaining their adherent properties (Figure 3.2 A, B). The proliferation rates of each cell culture system were assessed based on the cell doubling times for 5 sequential passages. Interestingly, serum-free maintenance conditions enhanced the proliferation rate, by significantly reducing the doubling time to 13 hours, from 15 hours observed in the serum-based culture (Figure 3.2 C).

Finally, after 5 passages the cells were collected and analysed on the expression of genes specific for undifferentiated ESCs, as well as, genes characteristic of early germ layer specification. As expected, the pluripotency related markers *Oct4*, *Sox2* and *Nanog*, exhibited similar expression levels without any significant differences in the two culture systems (Figure 3.2 F). Likewise expression levels of inner cell mass related genes, *Klf4* and *Rex1*, remained equivalent in the two cell populations without any distinct differences (Figure 3.2 E). Comparing the expression levels of *Fgf5* and *Bry*, which can be indicative of spontaneous cell differentiation, it was observed that cells in serum-free conditions had a 2-fold and 10-fold reduction in expression levels respectively, compared to serum grown cells (Figure 3.3 D). Even though the experiments were repeated only twice our data strongly demonstrate that serum-free conditions have no detrimental effects on the maintenance of mESCs. On the contrary, the enhanced proliferation rates together with the reduced levels of germ layer genes suggest that serum-free culture conditions are capable of maintaining ESCs in an undifferentiated state even more efficiently than serum supplemented culture.

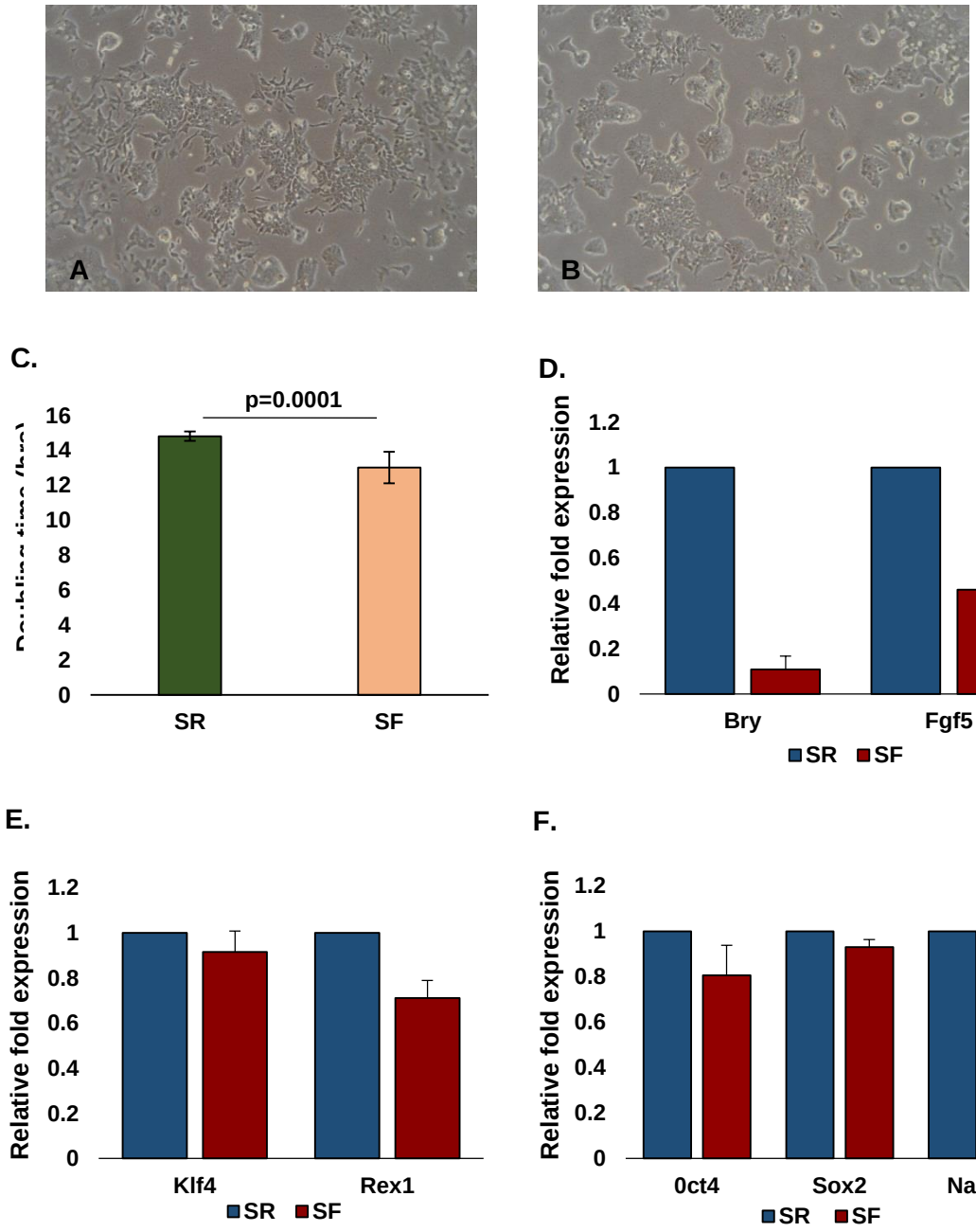

Figure 3.2: Characterisation of mouse ESCs cultured in serum-free medium.
Phase-contrast photographs of ESC colonies grown in serum **A** or serum-free **B** conditions (20x). Cells were passaged for 5 consecutive times and assessed on their doubling time **C**, results are means $\pm$ sd for 5 passages (n=2). Quantitative RT-PCR at passage 5 for ESC associated and early germ layer genes, **D-F**. Tbp expression was used for normalisation and ESCs grown in serum as a calibrator. Cells were analysed in triplicate from 2 independent experiments and represent means + sd. SR: serum-based conditions, SF: serum-free conditions.

3.4.1.2 Serum-free conditions enhance the haemopoietic differentiation of mouse ESCs

Mouse ESCs were differentiated with the two protocols detailed in Figure 3.1 and cells were collected for analysis on day 6 of the protocols. On microscopic examination of day 6 EBs the two differentiation systems exhibited profound morphological differences. EBs grown in the presence of serum retained an intact core with very little visible differentiation in the periphery (Figure 3.3 A, B), whereas, EBs grown in serum-free conditions under the influence of cytokines displayed extensive differentiation and had separated into two fractions, an adherent stroma-like one, which covered the entire area of the culture plastic, and a non-adherent, floating fraction of various sized cells (Figure 3.3 C-F). When cells were harvested it became obvious that serum-free conditions had generated mature blood cells, which were mainly found in the floating population when the fractions were separated, as indicated by the red colour of the pellet (Figure 3.3 H, I), something that has never been observed in the serum-based system (Figure 3.3 G). Cells were then examined in detail on their haemopoietic activity and phenotype.

Dissociated cells were plated in methylcellulose medium to assess the presence of HPCs. Even though haemopoietic colonies were generated by cells grown in serum-based conditions, their numbers were notably low. Additionally, secondary EBs with no signs of haemopoietic differentiation formed upon replating were mainly present in the methylcellulose medium (Figure 3.4 E-G). In contrast, serum-free conditions yielded very high numbers of colonies of various types (Figure 3.4 H-M). Particularly, serum-free conditions generated 6-fold higher numbers of CFU-M and CFU-GM than serum conditions (Figure 3.4 A, B). Additionally, CFU-Mix colonies, which are evident of multipotent progenitors, showed a 10-fold increase in the serum-free protocol (Figure 3.4 C). Finally, erythroid colonies, BFU-E, were only formed in serum-free conditions (Figure 3.4 D).

Flow cytometry was also used to determine the phenotype of cells generated in the protocols. A variety of haemopoietic markers were used which are characteristic of mouse HSCs at different stages of development. Cells differentiated in serum-free conditions showed a dramatic, more than 4-fold increase in CD41 expression compared to serum conditions, marking about 45% of the population (Figure 3.5). CD41 was also expressed on more than half of the population of VE-Cadherin or CD144 expressing cells, which appeared as a very distinct population in the case of serum-free conditions (Figure 3.5 B). CD45 expression remained low in both culture conditions between 3 to 6%, however, it formed a well-defined $CD45^{+}CD41^{+}$ population of cells more apparent again in serum-free conditions. Expression

of c-Kit was high in both protocols, however as it is not an exclusively haemopoietic marker, its co-expression with other markers was assessed. The serum-free protocol generated nearly ten times more $CD41^{+}c\text{-}Kit^{+}$ cells compared to the serum-based protocol, reaching 20% of the population. When c-Kit, Sca-1 co-expression was assessed, double positive populations, even though at low levels, were only found in the case of cells grown in serum-free conditions.

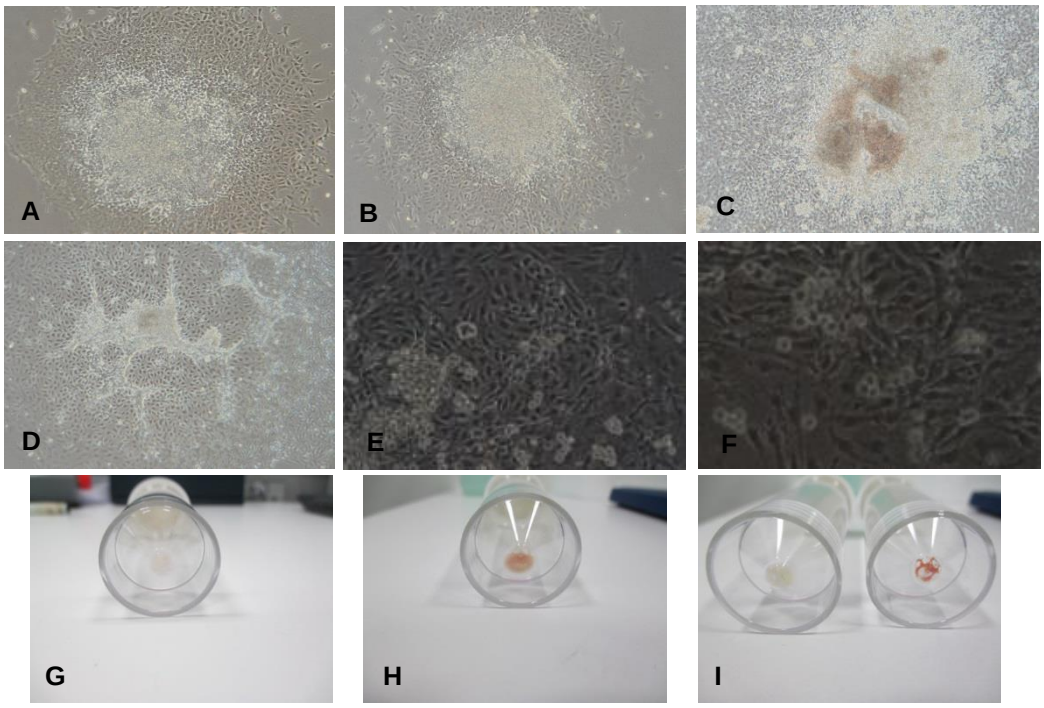

Figure 3.3: Differentiation of day 6 EBs in serum and serum-free conditions.
EBs grown in the presence of serum show minimum differentiation while they maintain their compact morphology **A, B** (20x)**.** EBs cultured in serum-free conditions separate into two fractions of differentiated cells, an adherent stromal fraction and non-adherent of floating single cells **C**, **D** (20x) **E**, **F** (40x). There are no visible signs of mature blood cells in serum grown EBs **G**, whereas, mature blood cells are evident by the red colour of EBs **C** or cell pellets upon harvesting **H** in serum-free conditions. When the two serum-free fractions are centrifuged separately the majority of mature cells is found in the non-adherent fraction (right tube) as opposed to the adherent fraction (left tube) **I**.

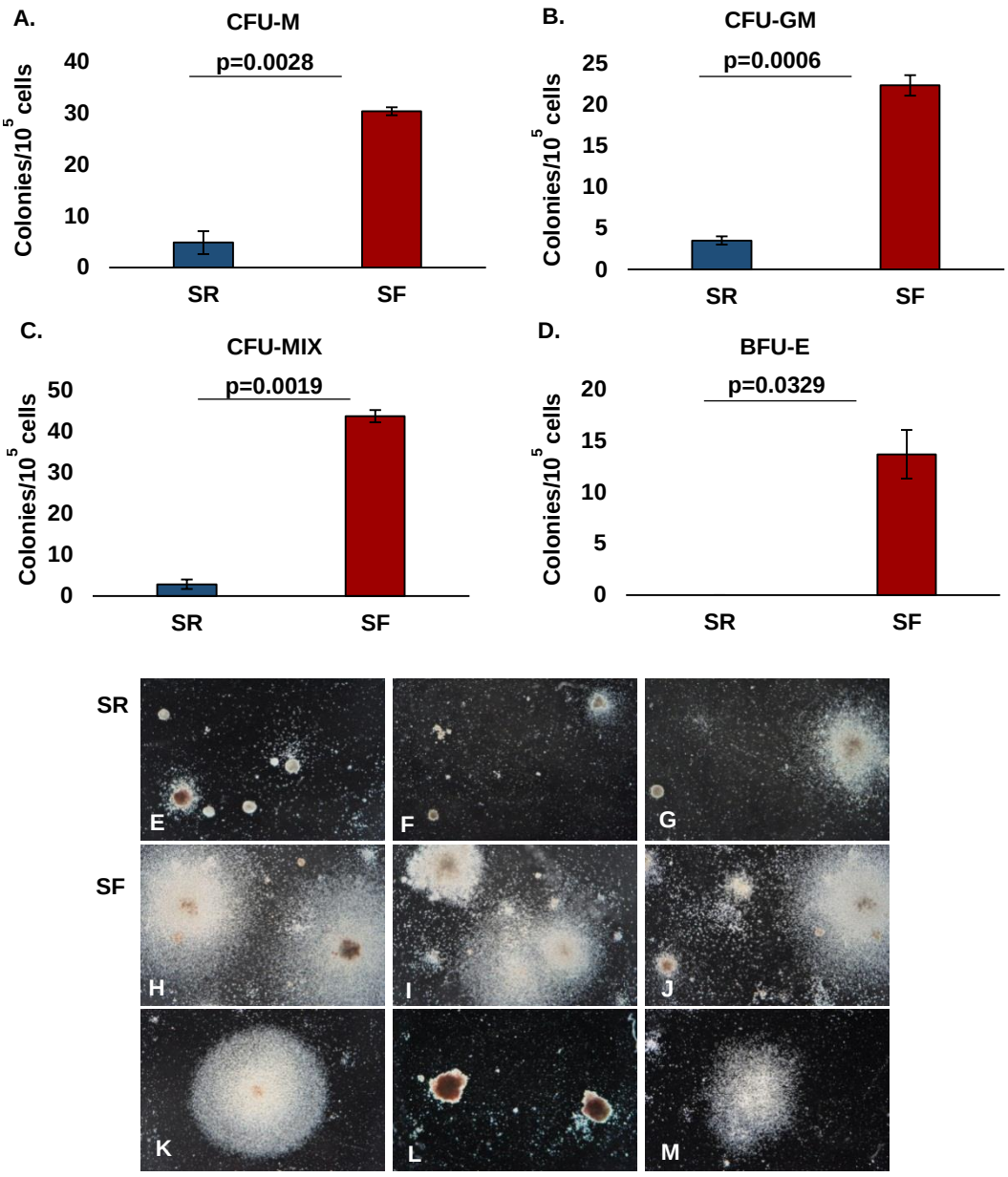

Figure 3.4: Haemopoietic colonies formed by day 6 EBs in serum and serum-free conditions.

Number of colony types produced by day 6 EBs **A-D**. Data represents 3 independent experiments ± sd. P values calculated with Student's t test. Representative pictures of haemopoietic colonies in methylcellulose assay (10x). Serum conditions yielded mainly undifferentiated EBs **E-G.** Serum-free conditions generated large numbers of various colonies **H-M.**

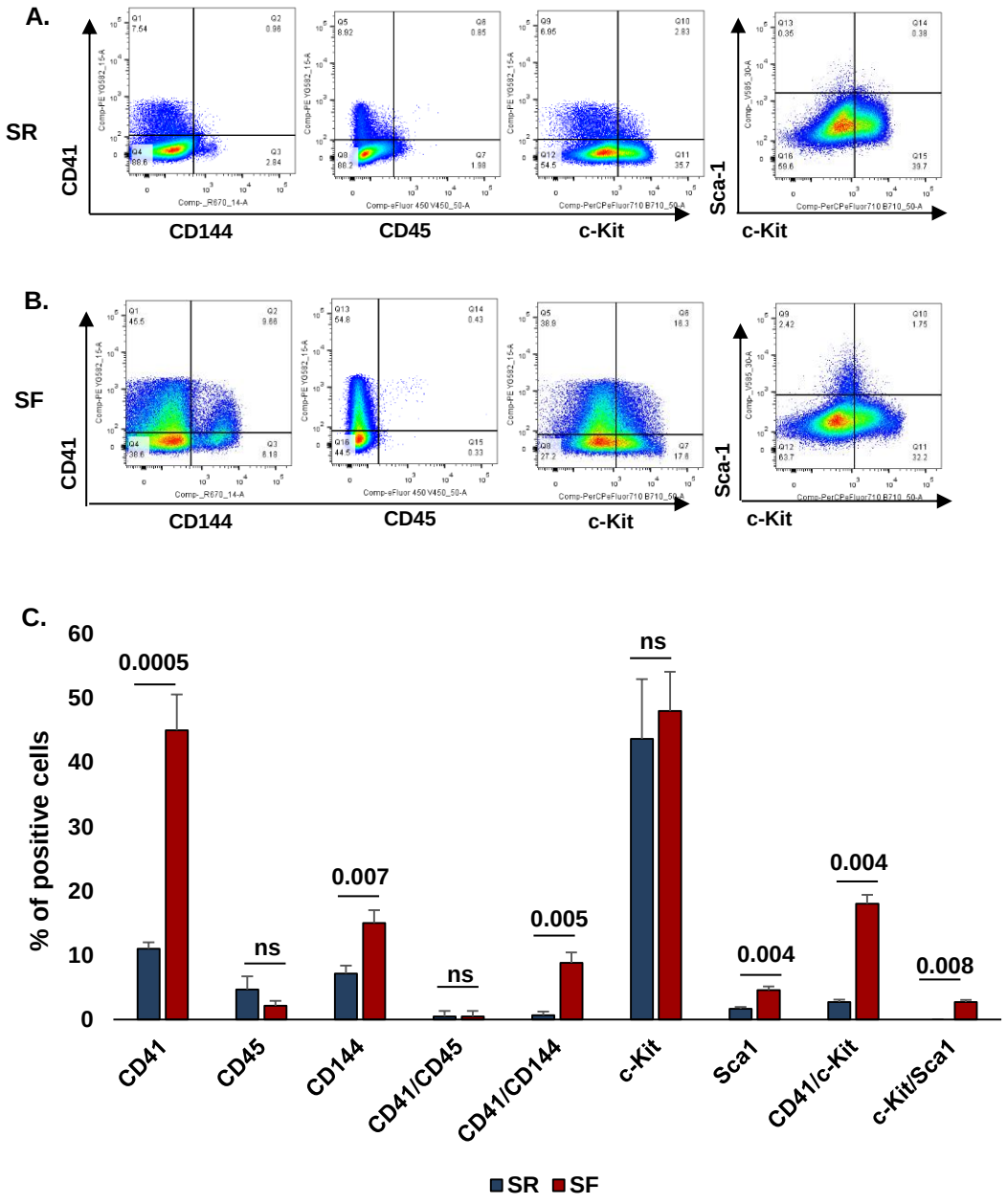

Figure 3.5: Flow cytometry analysis of day 6 EBs.
Representative scatter plots of cells differentiated in serum **A** and serum-free conditions **B**. Expression levels of haemopoietic markers on day 6, **C**. Data are representative of 3 independent experiments, error bars represent standard deviation. P values calculated with Student's t test. ns: not significant.

3.4.1.3 Localisation of HPCs in serum-free conditions

The distinct cell populations observed in the serum-free protocol prompted us to investigate whether haemopoietic progenitors were exclusively localised in one of the two fractions. It has been well documented *in vivo* that haemopoietic cells emerge through an epithelial-to-mesenchymal transition and it could be possible that this was the case in our system with progenitors budding off from the adherent layer into the supernatant (33-36). Cells were differentiated to day 6 of the protocol and the two resulting fractions were separated for analysis. Particularly, media containing the non-adherent population was collected and the remaining cells were washed with PBS to ensure all non-adherent cells were collected. The adherent population was then lifted off the culture plastic and the two fractions were analysed in parallel.

The functional potential of the two cell populations were assessed by haemopoietic colony formation. Interestingly, the total number of haemopoietic colonies from the adherent fraction were slightly yet significantly increased compared to the non-adherent one (Figure 3.6 A). When the various types of colonies were analysed in detail this increase was not found significant for any of the colonies types, thus suggesting that there is no pronounced distinction of HPC presence in the two populations (Figure 3.6 B). However, flow cytometric analysis of surface marker expression, showed there are distinct differences between the two fractions (Figure 3.7). The non-adherent population consisted almost exclusively of CD41 expressing cells, which were nearly 3 times more than in the adherent fraction, marking more than 50% of the population (Figure 3.7 B, C). On the other hand, adherent cells exhibited higher expression levels of all other surface markers compared to the non-adherent cells (Figure 3.7 A, C). Adherent cells were mainly characterised by c-Kit expression, present in 50% of the population. CD41 was expressed in 22% of the adherent population, half of which co-expressed c-Kit reaching 10% of the total population, while $CD41^{+}CD144^{+}$ cells were found in 5% of the total population. $CD41^{+}CD45^{+}$ and $c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells, though in low numbers, were predominately found in the adherent fraction, more clearly demonstrated in representative scatter plots (Figure 3.7 A, B). Yet the presence of minimal numbers of $CD41^{+}CD45^{+}$, $CD41^{+}CD144^{+}$ and $CD41^{+}c\text{-}Kit^{+}$ cells in the floating population is sufficient to account for the observed colonies in the CFU-C assay. Therefore, in order to ensure that the maximum number of HPCs is collected for analysis both fractions were pooled and analysed together in subsequent experiments.

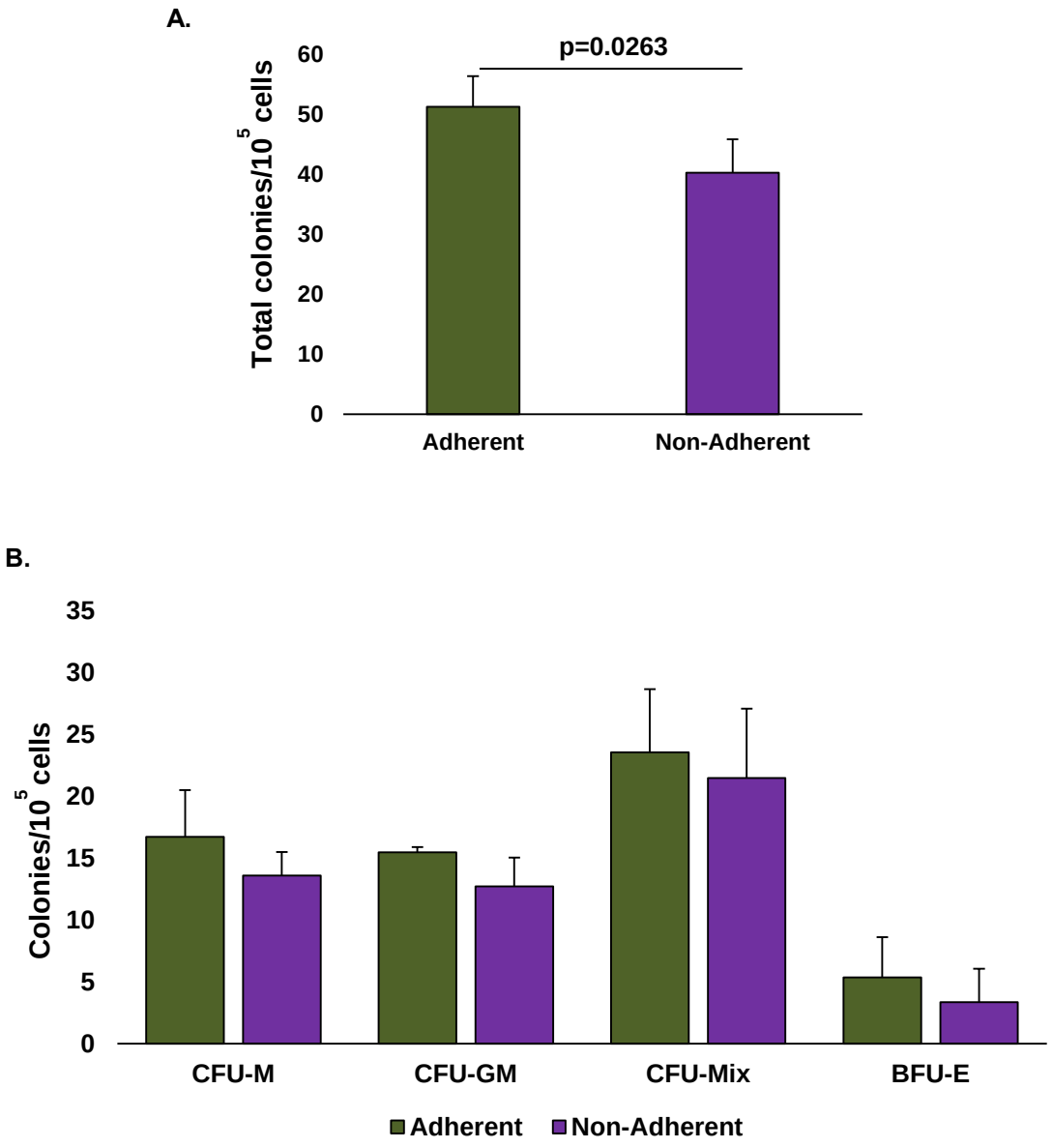

Figure 3.6: Assessment of haemopoietic colony formation by day 6 adherent and non-adherent population in serum-free conditions.

ESCs were differentiated in the serum-free protocol and the resulting fractions were separated and placed in CFU-C assay. **A.** Total colony numbers. **B.** Detailed analysis of numbers of different colony types. Data are representative of 4 independent experiments, error bars represent the standard deviation.

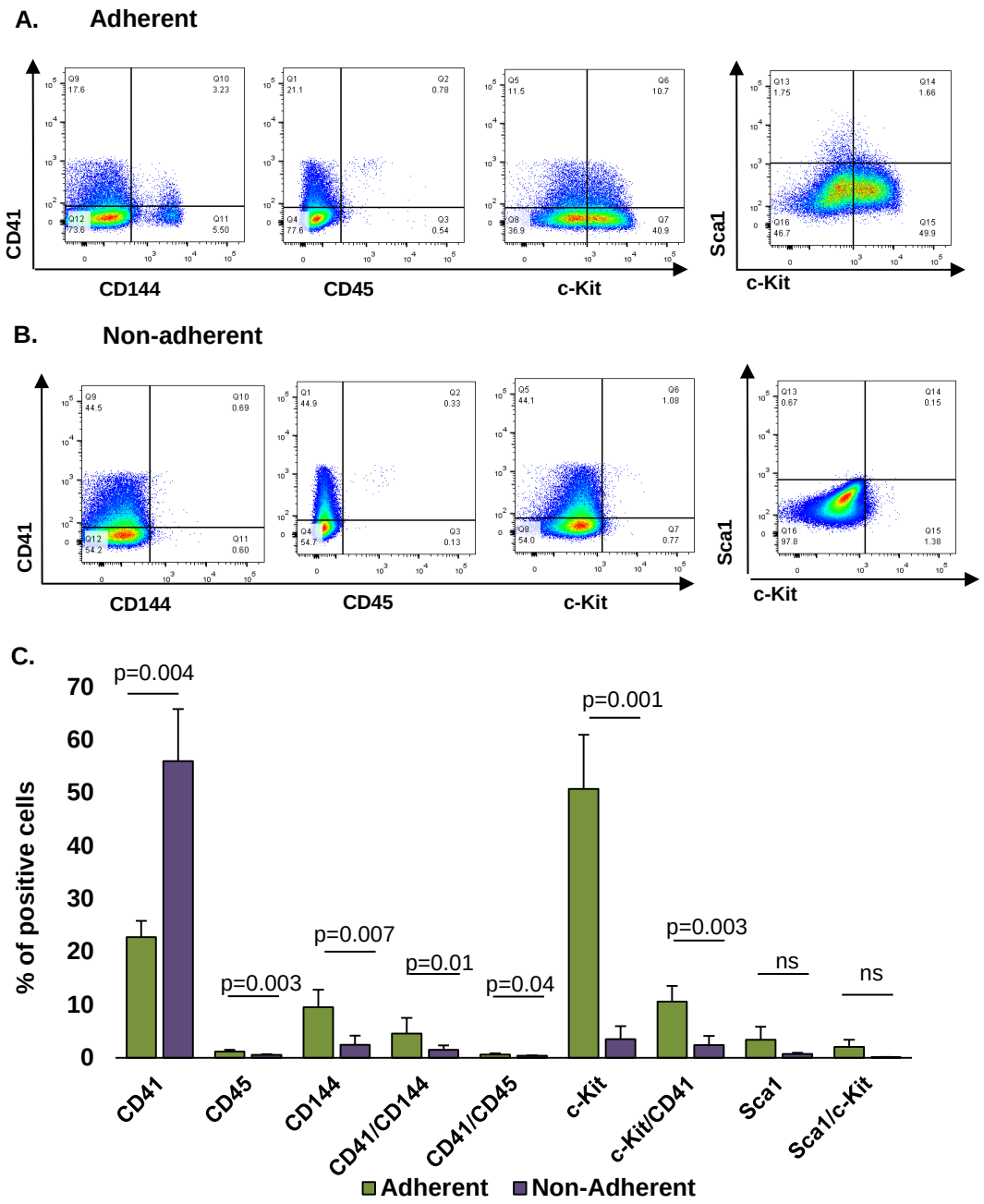

Figure 3.7: Flow cytometry analysis of the two resulting populations in serum-free differentiation conditions.

Representative scatter plots of day 6 adherent **A** and non-adherent fractions **B**. Quantification of haemopoietic marker expression in the two fractions **C**. Data representative of 4 independent experiments. Error bars represent standard deviation. P values calculated by Student's t test. ns: not significant.

3.4.2 Assessment of EpiSCs as a model of haemopoietic differentiation

Mouse EpiSCs were discovered several years ago, nonetheless, their *in vitro* differentiation potential has not been fully explored. It is now considered that pluripotency exists in two distinct states, the naïve and the primed one, with ESCs and EpiSCs being the *in vitro* representatives of each state respectively (226). These two classes of pluripotency are marked by epigenetic and gene expression differences, which may favour or restrict lineage choices. With *Bry* expression marking the primed state of EpiSCs we sought to investigate whether they may exhibit a developmental advantage over ESCs in haemopoietic differentiation.

3.4.2.1 *In vitro* derivation of EpiSCs from ESCs

Mouse EpiSCs can be derived in two different ways, either *in vivo* from the post-implantation embryo or *in vitro* by culturing ESCs under the appropriate conditions (227). In order to allow for maximum comparability between ESC and EpiSC haemopoietic potential, EpiSCs were derived *in vitro* from the ESC line used for the establishment of the serum-free differentiation protocol. ESCs were plated in standard culture media for one day before changing to EpiSC culture media consisting of N2B27 plus ActivinA and bFGF. After an initial wave of cell death in the first 2 or 3 passages, the cells recovered quickly and started forming typical EpiSC colonies by passage 5. The colonies consisted of cells displaying a high nuclear to cytoplasmic ratio with big nucleoli typical of stem cells, however, they appeared more flat as opposed to the more domed colonies of ESCs (Figure 3.8 A, B). The produced EpiSCs were assessed on their gene expression profile compared not only to the ESC line they were derived from but also to embryo-derived EpiSCs. Quantitative RT-PCR analysis showed that they expressed the core determinants of pluripotency, *Oct4*, *Sox2* and *Nanog*, while they displayed a dramatic downregulation of inner cell mass genes such as *Rex1* and *Klf4*, which are expressed at high levels in mouse inner cell mass but silenced in early epiblast cells after implantation (Figure 3.8 C, D). In contrast, the expression of *Fgf5* and *Bry* was significantly higher in EpiSCs compared to ESCs that had barely detectable levels of expression (Figure 3.8 E). Similar expression pattern was observed in the embryo-derived EpiSCs. Immune staining was also performed to confirm that Bry was co-expressed with Oct4 in stem populations and not in spontaneously differentiating cells. Indeed Bry and Oct4 were co-expressed in EpiSCs while Bry was not detected in ESC cultures (Figure 3.8 F, G).

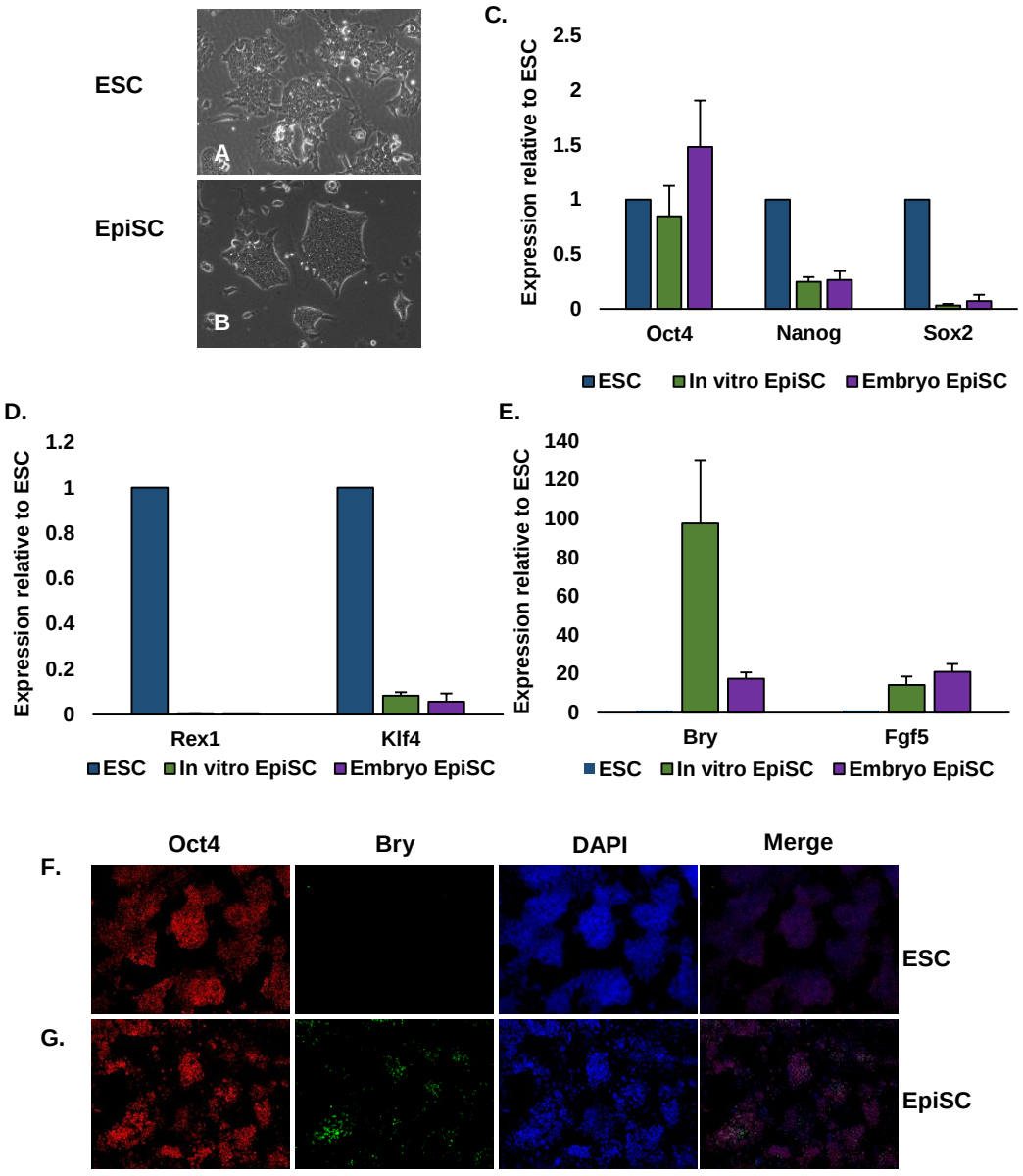

Figure 3.8: Generation of EpiSCs.
Mouse ESCs were cultured in N2B27 medium supplemented with Activin A and bFGF for a minimum of 5 passages to convert to EpiSCs. Typical ESC **A** and EpiSC colonies **B** (10x). Quantitative RT-PCR analysis to confirm the conversion of ESC to EpiSC assessing expression of pluripotency genes **C**, inner cell mass genes **D** and epiblast related genes **E**. Tbp expression was used for normalisation and ESCs grown in serum as a calibrator. Cells were analysed in triplicate from 3 independent experiments and represent means plus standard deviation, sd. Representative photos of Oct4 and Bry expression in ESC **F** and EpiSC **G** cultures analysed by immunofluorescence. Dapi was used as nuclear staining.

3.4.2.2 EpiSCs display a developmental advantage over ESCs

As a first attempt to test whether the primed state of EpiSCs can result in differences in their haemopoietic potential compared to ESCs, cells were put directly in colony forming assay without any of the prior steps of EB formation and subsequent culture. When placed in the methylcellulose both cell types formed secondary EBs which did not show any signs of differentiation in the first 7 days of incubation. However, by the end of the second week EpiSCs had differentiated at very high rates giving rise to various types of colonies (Figure 3.9 D-F), whereas, ESCs remained as undifferentiated EBs (Figure 3.9 A-C). In order to verify that the increase in colony number is due to the different developmental stage and not an *in vitro* effect of the cytokines used for EpiSC maintenance, embryo-derived EpiSCs were also placed in methylcellulose assay without any prior differentiation steps. Similarly to ESC-derived EpiSCs, the embryo-derived EpiSCs differentiated into haemopoietic colonies (Figure 3.9 G-I).

It should be noted however, that the majority of colonies observed were EB associated colonies both in the case of embryo-derived and ESC-derived EpiSCs (Figure 3.9 J). These colonies do not represent mature haemopoietic progenitors. They develop from undifferentiated EBs that form in the methylcellulose medium following cell plating and differentiate under the influence of haemopoietic cytokines present in the medium. They have a compact EB-like core with few erythroid and/or myeloid cells developing in the core or the periphery (Figure 3.9 D, E, G, H). More mature haemopoietic colonies were also present (Figure 3.9 F, I), though in much lower numbers (Figure 3.9 K). As no prior *in vitro* differentiation occurred, their development can possibly be due to more primed state EpiSCs that again managed to differentiate under the influence of methylcellulose cytokines. Additionally, even though EpiSCs never failed to give rise to mature colonies they did have a quite high variability rate between experimental repeats suggesting they are a more dynamic system than ESCs. Nonetheless, it was certain that EpiSCs display a developmental advantage over ESCs which needed further clarification.

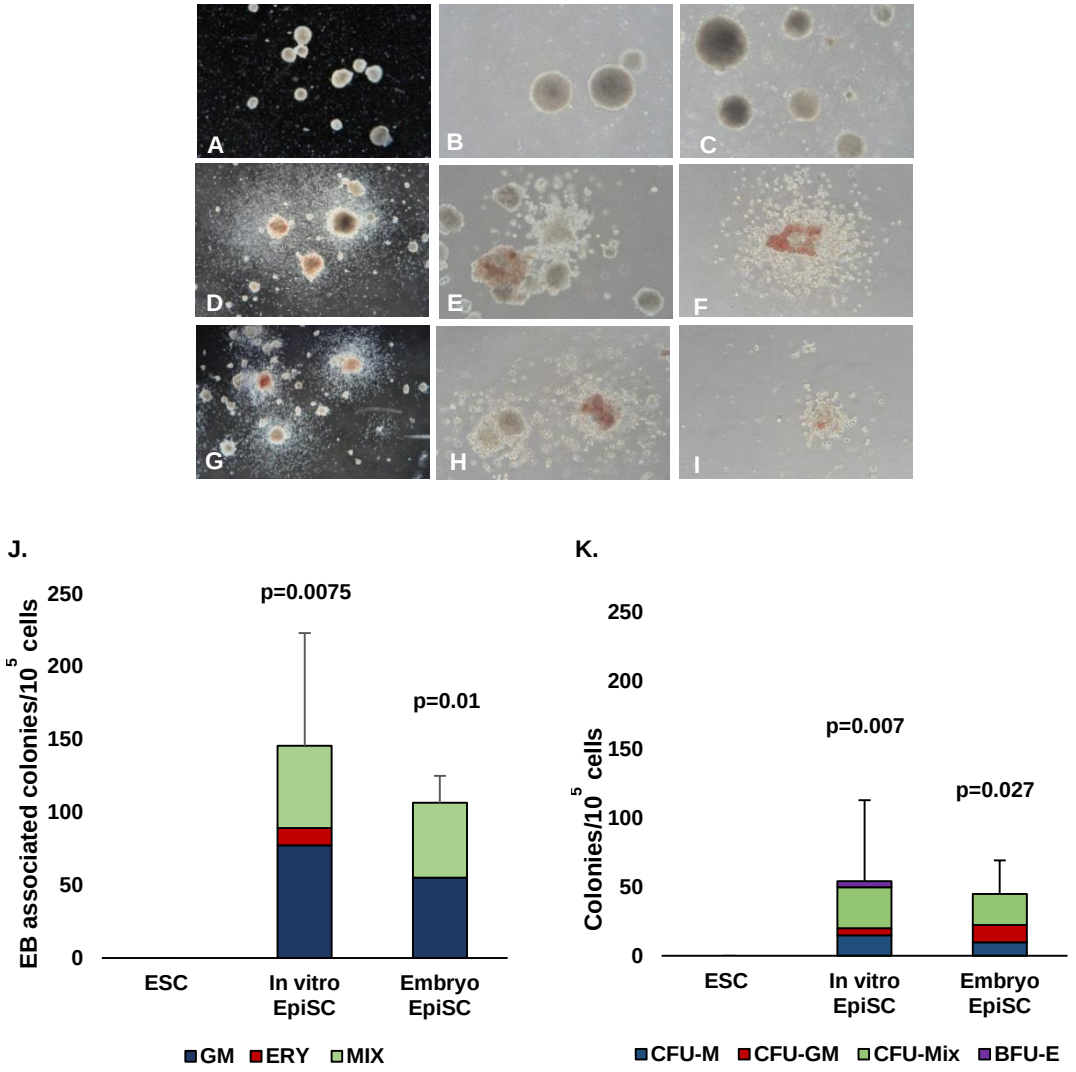

Figure 3.9: Initial assessment of EpiSC haemopoietic potential.
ESCs **A-C**, ESC-derived EpiSCs **D-F** and embryo-derived EpiSCs **G-I** plated in methylcellulose assay without any prior differentiation. Numbers of EB associated haemopoietic colonies scored in the assay **J**. Mature haemopoietic colony numbers **K**. Data represent means of 5 independent experiments, error bars represent standard deviation, p values were calculated with Student's t test.

3.4.2.3 Detailed characterisation of differences in the haemopoietic differentiation of ESCs and EpiSCs

In order to further clarify the differences in developmental progression between ESCs and EpiSCs the novel serum-free protocol was implemented. The two cell populations were cultured in parallel and cells were analysed on day 0, day 2, day 4, day 5 and day 6 of differentiation by colony forming assay and flow cytometry. ESCs were also cultured in the serum-based protocol to serve as a baseline of differentiation.

ESCs grown in serum-based conditions formed very low numbers of colonies, as expected, which only appeared on days 5 and 6 of differentiation (Figure 3.10 A). ESCs in serum-free conditions followed a similar trend where colony numbers increased with time, yet in much higher numbers as previously shown. The first colonies appeared on day 4 and by the end of the differentiation protocol their total number had increased more than 4-fold (Figure 3.10 B). EpiSCs displayed a different progression pattern to the two other culture systems (Figure 3.10 C). As observed in previous colony assays, Day 0 EpiSCs had a high degree of colony formation. Surprisingly though, day 2 cells failed to generate any haemopoietic colonies. By day 4, however, cells rebounded generating many colonies. A slight increasing trend was observed the following time points of analysis however the change was not significant. Day 6 colony numbers were analysed more thoroughly to compare in more detail differences between the culture systems (Figure 3.11). Numbers of CFU-M, CFU-GM and CFU-Mix were very comparable between ESCs and EpiSCs grown in serum-free conditions. Both cell populations had a more than 5-fold increase in each colony type compared to ESCs grown in the presence of serum. BFU-E numbers were more than double in serum-free ESCs compared to EpiSCs and again ESCs in the serum-based protocol failed to generate any of this type of colonies. Strikingly, although EpiSCs generated at times much higher numbers of haemopoietic colonies compared to ESCs in serum-free conditions, they exhibited large variation between experimental repeats, thus the difference between the two cell types was not found significant.

Time course expression of haemopoietic surface markers was conducted in parallel to provide more insight on the HPC populations that developed in the different culture systems. Similarly to the CFU-C assay, ESCs differentiated in serum-free conditions had overall higher marker expression levels compared to both EpiSCs and serum grown ESCs. Particularly, CD144 expression remained low for serum grown ESCs throughout differentiation (Figure 3.12 A). In serum-free conditions for both ESCs and EpiSCs CD144

reached a peak of expression on day 4, at around 20% of the populations, and then steadily declined, with ESCs expressing higher levels than EpiSCs by day 6. Representative scatter plots in Supplementary Figure S 1 show the distinct CD144 population that appears on day 4 serum-free ESCs and EpiSCs and gradually disappears in the case of EpiSCs. CD45 levels remained low and comparable between all three culture systems peaking on day 6 (Figure 3.12 B). CD41 showed a steady increase in ESCs both in serum and serum-free conditions with serum-free cells expressing significantly higher levels (Figure 3.12 C). EpiSCs, however, had a sharp CD41 increase on day 4, followed by a sharp decrease by day 5 reaching similar expression levels with serum grown ESCs and remaining low on day 6. $CD144^{+}CD41^{+}$ cells dramatically increased on day 4 in serum-free conditions, coinciding with the appearance of haemopoietic colonies, with ESCs maintaining significantly high expression levels and EpiSCs decreasing by day 6 (Figure 3.11 D). Co-expression of CD41 and CD45 remained low for all cell populations (Figure 3.11 E), however, double positive cells formed a well-defined population clearly visible in serum-free ESCs and EpiSCs scatter plots (Supplementary Figure S.2). Expression of c-Kit remained at similar levels for all culture systems throughout the differentiation process (Figure 3.11 F), with the population showing a slight positive shift with time progression (Supplementary Figure S.3). $CD41^{+}$c-Kit^{+} cells appeared on day 4 and continued to increase until day 6 in all populations, with serum-free ESCs generating significantly more $CD41^{+}$c-Kit^{+} cells (Figure 3.11 G). Sca-1 levels appeared comparable in serum-free differentiated cells and increased compared to serum grown ESCs (Figure 3.11 H). C-$Kit^{+}Sca\text{-}1^{+}$ cells were detected only in serum-free ESCs and EpiSCs, with ESCs expressing stably higher levels compared to serum ESCs (Figure 3.11 I).

The above data clearly demonstrate that despite the initial developmental advantage EpiSCs showed over ESCs, they fail to further enhance haemopoietic differentiation. It should be noted that even though EpiSCs always generated significantly more HPCs than ESCs in serum-based conditions, at times they performed poorer compared to serum-free ESCs. This variability makes them a more unpredictable model even under controlled differentiation conditions. It was therefore decided to use mouse ESCs is serum-free conditions for the subsequent experiments in this thesis.

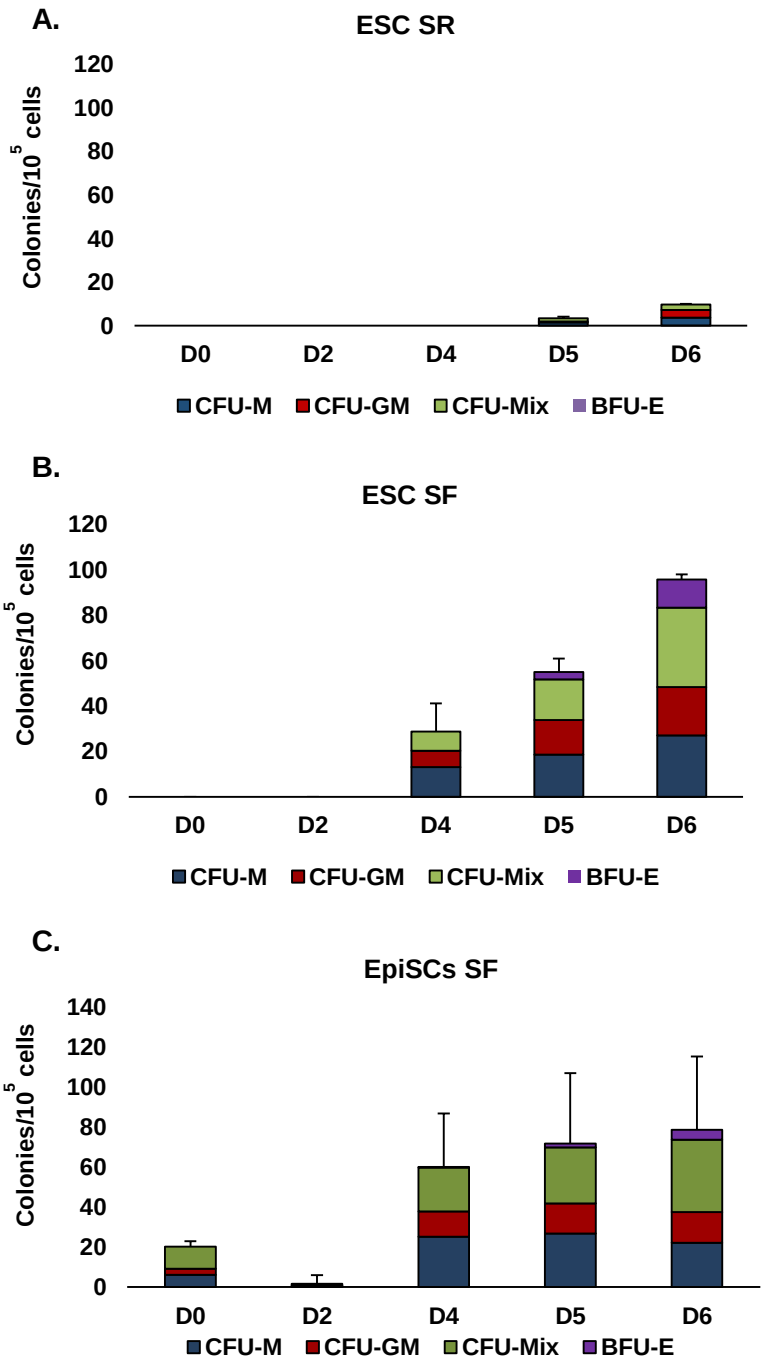

Figure 3.10: Development of haemopoietic colonies during haemopoietic differentiation.

Cells were cultured under serum or serum-free conditions and collected for CFU-C assay at different time points. ESCs were differentiated in serum conditions as a baseline A. The serum-free protocol was used for the differentiation of ESCs B and EpiSCs C. Plots represent means of 4 independent experiments. Error bars represent standard deviation.

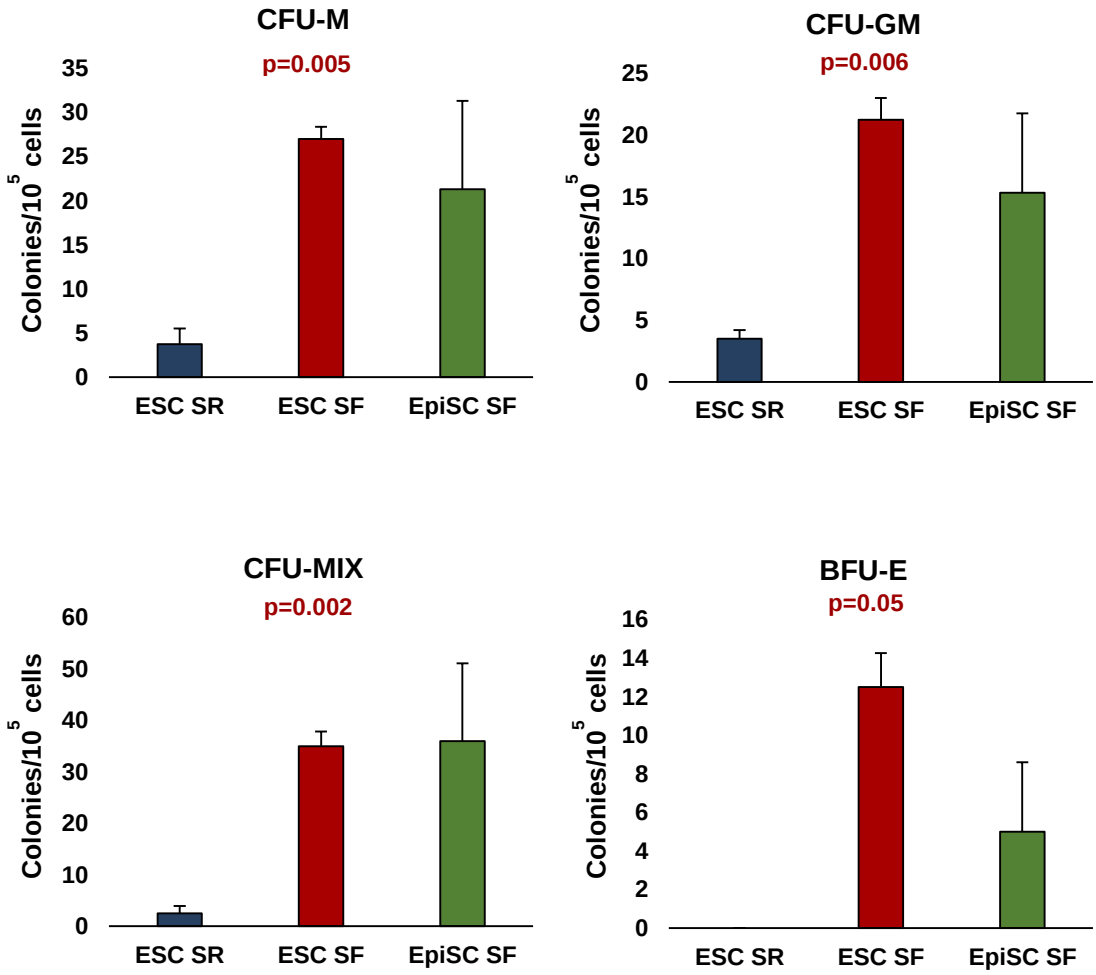

Figure 3.11: Detailed analysis of day 6 haemopoietic colonies.
Serum-free conditions were used for the differentiation of ESCs and EpiSCs, while serum ESC differentiation was used as a control. Despite similar numbers of colonies generated by both cell populations in serum-free conditions, only ESCs were found statistically significant. Date represents 4 experiment plus standard deviation.

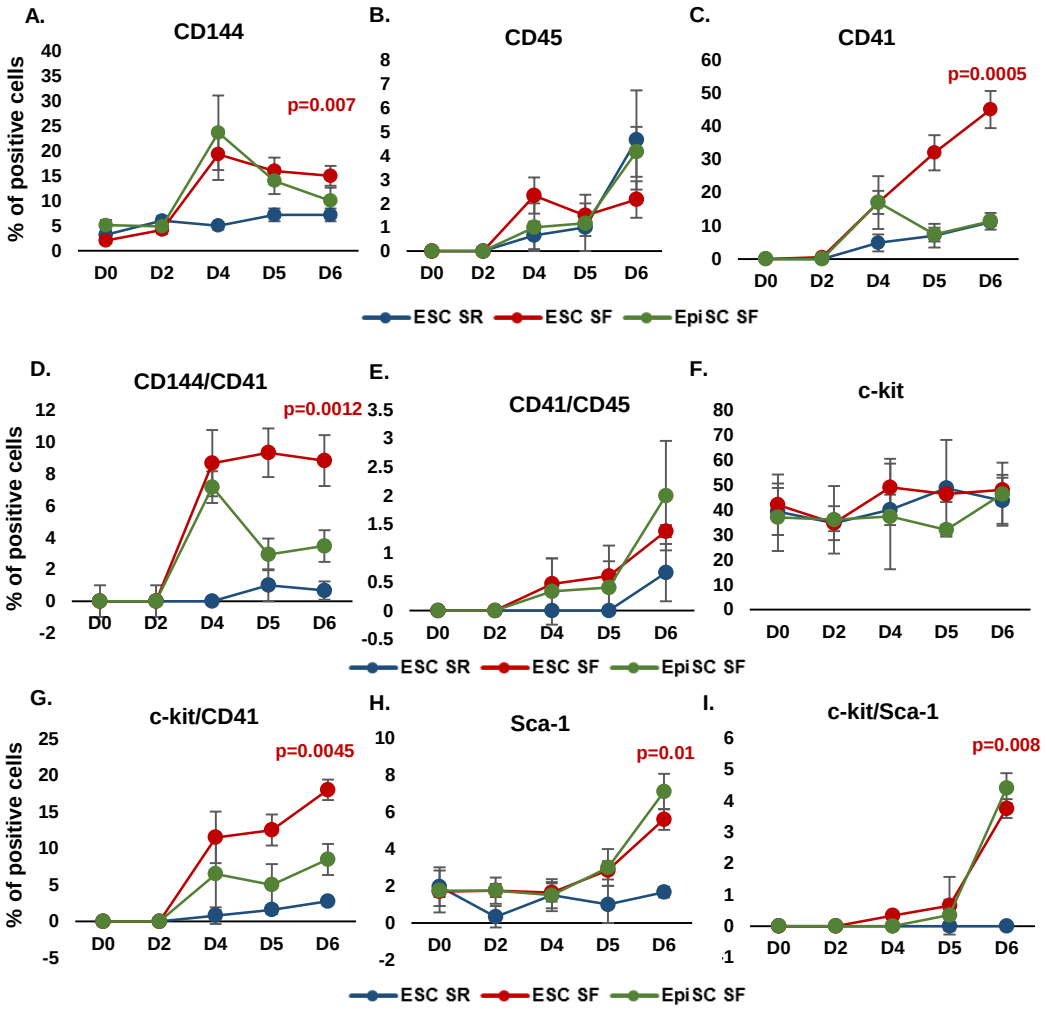

Figure 3.12: Monitoring haemopoietic marker expression during differentiation.
Expression levels of surface molecules marking emerging haemopoietic progenitors were measured at different time points of serum and serum-free differentiation of ESCs and EpiSCs. Data represent means of 3 experiments ±sd. Representative flow cytometry charts are shown in Supplementary Figures S1-4.

3.5 Conclusion

A well-controlled system of *in vitro* haemopoietic development of mouse ESCs was developed. The protocol was used to compare the haemopoietic potential of mouse ESCs and the previously uncharacterised EpiSCs. The data generated indicate that:

- The use of just four factors in minimal concentrations was sufficient to direct the differentiation of ESCs to mature haemopoietic cells (Figure 3.3).
- The serum-free conditions could dramatically enhance the generation of HPCs capable of multilineage differentiation *in vitro* compared to serum conditions (Figure 3.4).
- The serum-free protocol increased the expression of haemopoietic markers that characterise HSC populations at different developmental stages *in vivo*, such as $CD144^{+}CD41^{+}$, c-$Kit^{+}CD41^{+}$ and c-$Kit^{+}Sca\text{-}1^{+}$ cells (Figure 3.5).
- EpiSCs are characterised by a primed state which was reflected not only in their expression profile but also in their ability to proceed to haemopoietic commitment easier compared to ESCs (Figure 3.8, 3.9).
- Despite the developmental edge EpiSC display, they were not capable of exceeding the haemopoietic potential of ESCs when cultured under the same conditions (Figure 3.11, 3.12).

3.6 Discussion

3.6.1 Haemopoietic progenitor development under the influence of cytokines

In this chapter we sought to create a defined haemopoietic culture environment that would allow us to investigate HOXB4 activity. Haemopoiesis is controlled by the coordinated action of many signalling pathways, and various mediators of these pathways have been widely used in PSC differentiation protocols (169-177). To allow for the most subtle changes that HOXB4 targets to be tested might have in the differentiation output, we used a limited number of cytokines and in low concentrations, involved in pathways deemed critical for the

directed differentiation of ESCs to the haemopoietic lineage (127, 223-225). We demonstrated that the use of Activin A, bFGF and BMP4 for the first 2 days of differentiation, followed by substitution of Activin A by VEGF for the remaining 4 day culture is sufficient to rapidly generate mature blood cells (Figure 3.3). We were able to produce strikingly increased numbers of HPCs compared to the serum control, which could develop into multiple blood lineages *in vitro* (Figure 3.4). More importantly, extensive analysis of these HPCs revealed that they phenotypically resemble HSCs emerging during embryogenesis in various anatomic sites (Figure 3.5).

Similar to our findings, work by Park and colleagues had previously shown that the combined action of Activin A, BMP4 and VEGF could successfully substitute for the use of serum in ESC differentiation (170). Yet their serum-free protocol failed to generate higher numbers of HPCs compared to their serum control, as opposed to our system, where the difference between serum and serum-free conditions was dramatic. This difference could be due to the use of bFGF in our system that appears to be important for the survival and proliferation of haemopoietic cells. A recent report demonstrated that treatment with bFGF *in vivo* expanded mouse haemopoietic stem and progenitor cells by inducing proliferation of supportive stromal cells and upregulating c-Kit expression in mouse bone marrow and spleen (228). It is possible that bFGF acts in a similar mechanism in our system as suggested by the development of the adherent stromal population in serum-free conditions and the high expression levels of c-Kit. Very recently Pearson and co-workers using the same cytokines as we did, though in slightly different timing and combinations, reported the generation of c-Kit expressing repopulating HSCs. Although they did not proceed to any detailed phenotypic characterisation of the repopulating cells, the numbers of c-Kit$^+$ CD41$^+$ and CD45$^+$ cells were significantly lower than the ones generated in our system (229). It would be interesting to see if the cells generated in our system are also engraftable, and whether the higher expression levels of c-kit, CD41 and CD45 reflect higher engraftment rates.

An interesting point of our protocol was the separation of differentiating EBs into two distinct fractions, an adherent and a non-adherent fraction. We addressed the question of whether the haemopoietic progenitors are located in one of the two cell populations. Colony forming assays showed no striking differences in the haemopoietic profile of the two fractions, which generated quite comparable numbers and types of colonies (Figure 3.6). We then proceeded with surface marker expression analysis, which initially indicated that HPCs were located in the floating fraction as CD41 expression marked more than half of the cell population (Figure 3.7). The emergence of CD41$^+$ HSCs from the aortic endothelium of the

AGM region into circulation has been monitored *in vivo* in the mouse and the zebrafish, suggesting that our culture system acted in a similar mechanism (35, 36). However, when we examined CD41 co-expression with other markers of emerging HSCs, such as CD45 or c-kit, contrary to what we expected the majority of HPCs was located in the adherent fraction. This can be explained by the fact that the compact 3-dimensional form of the EBs does not provide all the *in vivo* structures and environmental cues present in the AGM region. Cellular movements and signalling can therefore be restricted, preventing the release of the majority of HPCs into the supernatant. One study using a monolayer differentiation protocol monitored the transition of adherent CD144 endothelial cells to floating CD41 haemopoietic cells (178). Although direct cell tracking was not possible in our system, the presence of clearly defined $CD144^+$ and $CD144^+CD41^+$ cells exclusively in the adherent population followed by the accumulation of $CD41^+$ floating cells indicates a similar cell lineage progression. However, in the above study the $CD41^+$ floating cells could generate predominately erythroid colonies suggesting it replicated primitive erythropoiesis as opposed to our protocol where mixed colonies constituted the majority of the colonies formed. The predominant presence of CD41 in the floating population can be explained by taking into consideration that it is also a marker of terminally differentiated cells. CD41 was originally recognised as a megakaryocyte/platelet marker (230). It is therefore probable that the $CD41^+$ cells found in the floating fraction mainly consist of more differentiated blood cells instead of early progenitors. This is further supported by the presence of red blood cells in the supernatant (Figure 3.3 I). In summary, differentiating EBs in our system form a stromal layer which supports haemopoietic maturation. The core cell population rapidly differentiates to the haemopoietic lineage with multipotent HPCs remaining attached to the stromal layer, while upon full maturation terminally differentiated cells are released into the supernatant.

It can be deduced from the above that the use of the appropriate cell surface markers is critical for monitoring the *in vitro* generation of haemopoietic cells and how they correspond to their *in vivo* counterparts. As detailed in Chapter 1 the phenotype of HSCs changes during development making their identification more challenging. In an effort to closely monitor haemopoietic development in our protocol we used various markers widely used to detect HSCs *in vivo*. The most prevalent population of haemopoietic progenitors in our serum-free culture system were $CD41^+$c-Kit^+ cells which constituted 20% of the day 6 population. Studies by different groups have shown that $CD41^+$c-Kit^+ cells are found in the embryonic yolk sac, AGM and liver *in vivo*, and can not only generate multilineage colonies but also confer long-term multilineage reconstitution upon transplantation into irradiated recipients

(76, 77). Furthermore, CD144 and CD41 co-expression detected in our serum-free protocol has been shown to mark AGM type I pre-HSCs (78). With upregulation of CD45 these cells transit to CD144+CD41+CD45+ type II pre-HSCs before fully maturing to reconstituting CD41+CD45+ HSCs (78). Although we failed to detect intermediate CD144+CD41+CD45+ type II pre-HSCs at any time point following $CD144^{+}CD41^{+}$ emergence (Supplementary Figure S5) our system did produce fully mature $CD41^{+}CD45^{+}$ cells. It is possible that type II pre-HSCs appear so transiently in our protocol that their detection was not possible. Day 6 serum-free cells also co-expressed low yet detectable levels of the more mature HSC markers c-Kit and Sca-1. It would be interesting to see if day 6 $c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells co-express SLAM antigens or whether prolonged culture can result in further maturation of the generated cells into adult-type HSCs. Conclusively, our protocol can generate HPCs with multilineage potential, phenotypically similar to embryonic rather than adult-type bone marrow HSCs.

3.6.2 Differentiation potential of EpiSCs

When initially discovered, mouse EpiSCs attracted a lot of scientific interest partly due to their similarities to human ESCs and partly due to their potential predisposition to differentiate into particular germ-layer derivatives based on the expression of early lineage markers in self-renewing conditions. Yet, in the years that followed their discovery, limited progress was made towards investigating their differentiation potential. The expression of *Bry*, a known mesodermal and more importantly a pre-haemopoietic marker, also attracted our attention. Aiming to establish an efficient mouse differentiation system for our study we investigated the haemopoietic potential of EpiSCs compared to conventionally used ESCs.

In order to minimise genetic variability between cell lines that could affect our results we cultured ESCs under the appropriate conditions and successfully generated EpiSCs with the morphology and expression profile observed in their embryo derived counterparts (Figure 3.8). Confirming our expectations initial analysis of their differentiation potential suggested that their primed state offers them an advantage over ESCs. Ground state ESCs when placed directly in haemopoietic colony assay never managed to form colonies, however, EpiSCs could very effectively differentiate and give rise to various types of colonies (Figure 3.9). We then examined in more detail differences between the two cell populations by analysing the cells at different time points of the serum-free protocol we developed. We demonstrated that EpiSCs generally reached the maximum of their differentiation potential by day 4 of the protocol without significant increase for the rest of the culture period as shown by colony

assays and analysis of surface markers (Figure 3.10, 3.12). On the other hand ESCs exhibited a stable increase of haemopoietic profile which peak later on day 6, confirming that the primed state of EpiSCs allows them to proceed faster to haemopoietic maturation. Interestingly, on day 2 of the protocol we observed the inability of EpiSCs to generate haemopoietic colonies. This can be simply due to the stress caused to the cells by successive changes in culture conditions. Single cell dissociation has been repeatedly reported to induce wide-spread cell death to EpiSCs and in our protocol the cells were subjected to it twice within a very short time frame (152, 153). Cultured EpiSCs were dissociated into single cells, cultured in suspension for 2 days and then again dissociated to be used in the CFU-C assay. Therefore, the difference in colony numbers between day 2 and day 4 may represent a lag time for cell recovery.

An interesting point regarding EpiSC differentiation was also the more pronounced variability they exhibited between experimental repeats. Studies focusing on embryo-derived EpiSC lines have shown that there is a great variability not only between independent cell lines but even within an individual cell line. Bernemann and colleagues characterized a panel of EpiSC cell lines derived in several laboratories and found that although the lines have some conserved pluripotent stem cell characteristics, some of them have differences in the expression of mesendodermal markers, such as *Bry*. This expression signature reflects slightly different developmental stages, which do not seem to correlate with the embryonic day on which cells have been derived, and may have implications for functional readouts such as directed differentiation to a particular lineage (231). Additionally, work conducted by Han and co-workers demonstrated that EpiSC lines comprise of distinct subpopulations, Oct4-GFP^{+} and Oct4-GFP^{-}, with different expression profiles and the ability to continuously interchange between states (232). These subpopulations represent again distinct developmental stages resulting in discrepancies in the pluripotential capacity of EpiSCs. Although no insight has been provided for the *in vitro* derived EpiSCs we did ourselves observe a variability in *Bry* expression between EpiSC colonies in immunofluorescence analysis of the cells (Supplementary Figure S6). It can be assumed that similarly to the embryonic EpiSCs a shift of subpopulations might occur any time during culture resulting in a difference of developmental potential, in our case in colony output and marker expression. Therefore, despite the developmental advantage EpiSCs may have over ESCs in terms of timing, their haemopoietic efficiency remains comparable, while the instability they exhibit within the cell population makes them a less reliable model system.

Chapter 4 : Investigating the paracrine action of HOXB4: effects of HOXB4-induced secreted factors in mESC haemopoiesis

4.1 Introduction

The unique ability of HOXB4 to generate repopulating mouse HSCs from ESCs *in vitro* has attracted a lot of interest in the mechanisms though which it exerts its haemopoiesis promoting effects. Many studies have focused on understanding HOXB4 activity in fully committed haemopoietic progenitors, yet our group has tried to shed light on the role it plays in the earlier stages of haemopoietic commitment. Work conducted in our group, using a tamoxifen-inducible expression system, revealed a key role of HOXB4 in the specification of pre-haemopoietic mesoderm in differentiating ESCs. HOXB4 was activated prior to the emergence of haemopoietic progenitors and microarray analysis was performed. We identified as HOXB4 targets a large set of paraxial mesoderm genes, a population that contributes to the formation of the haemopoietic niche. Additionally, cell mixing experiments demonstrated the ability of HOXB4-expressing cells to enhance the haemopoietic activity of neighbouring cells. Collectively, these data supported the idea that HOXB4 enhances haemopoietic activity by promoting the formation of the haemopoietic niche (219).

The concept of HOXB4 providing a supportive niche environment has been increasingly gaining popularity. Liu and Hematti have reported the generation of mesenchymal/stromal stem cells (MSCs) from HOXB4-expressing hESCs. These HOXB4-expressing MSCs, similar to bone marrow stromal cells, presented the typical MSC phenotype and were able to differentiate into adipocytes and osteocytes (233). However, they did not provide any data regarding the ability of these MSCs to support the differentiation or proliferation of HPCs. A recent study by Chen and co-workers demonstrated that co-transplantation of HOXB4-expressing MSCs with human bone marrow $CD45^+$ cells or umbilical cord blood $CD34^+$ cells into irradiated mice markedly increased their engraftment potential and the haemopoietic recovery rates, indicating the contribution of HOXB4 in the regulation of the haemopoietic niche and the proliferation of HSCs (234).

Despite all the proof of this novel role of HOXB4 there is little, if any evidence, regarding the exact mechanisms that mediate its activity. Interestingly, among the HOXB4 targets identified in our expression analysis we observed a large number of secreted molecules some of which have already been implicated with haemopoiesis *in vivo* and *in vitro*. We thus hypothesised that HOXB4 acts through a paracrine mechanism, where HOXB4-expressing cells secrete the necessary molecules for the development and proliferation of neighbouring HPCs. The possibility of using these secreted molecules purified and directly in

differentiation cultures opens up the prospects for an efficient, reproducible and clinically safe method of HSC generation. We therefore, focused on examining whether these targets individually or in combination could mediate HOXB4 activity and generate the same enhancing effects in our culture system as the ones observed with HOXB4 overexpression.

4.2 Aims

1. Validate as HOXB4 targets the genes encoding secreted factors detected in the microarray analysis of our inducible overexpression system.

2. Assess the effects that secreted proteins encoded by HOXB4 target genes have on the haemopoietic differentiation of mESCs.

4.3 Experimental strategy

1. To validate HOXB4 target genes, wild type and HOXB4-ER^{T2} expressing cells were differentiated under the same conditions as in the microarray analysis study. Cells were collected and analysed by qRT-PCR for the expression levels of the genes of interest.

2. To assess the effects of specific HOXB4 targets wild type cells were cultured in the serum-free protocol developed in this study. HOXB4 targets were added in the differentiation system in various concentrations, alone or in combination and cells were analysed by colony forming assay and surface marker expression for changes in their haemopoietic activity.

4.4 Results

4.4.1 Validation of HOXB4 targets

The HOXB4 targets identified in the microarray analysis included genes encoding secreted factors, transcription factors, signalling receptors and adhesion molecules (219). In this study we focused on the secreted factors as potential mediators of the paracrine effect of HOXB4 and selected targets with a minimum expression increase of 1.5-fold in HOXB4-ER^{T2} cells compared to control cells after 4-OHT addition for further analysis. This initial screening revealed a number of secreted proteins. Some of these proteins, such as KITL and VEGFA, have already been widely used in protocols for mouse and human ESC hematopoietic differentiation, while others like NTN1, RSPO3, FST have not been studied previously in this context.

To narrow down the number of secreted factors to be tested for possible enhancing effects on haemopoietic differentiation, the microarray targets were validated as HOXB4 targets by quantitative real time RT-PCR. Initially primers were designed and validated for the genes of interest. Subsequently, HOXB4 induction experiments were repeated under the same conditions as for the microarray study to collect material for analysis. Briefly, wild-type and HOXB4-ER^{T2} expressing mESCs were differentiated using our serum-based protocol. Embryoid bodies (EBs) formed in hanging drops for two days in the presence of LIF and serum and then placed in suspension culture for one day in the absence of LIF to initiate differentiation. Day 1 EBs were then plated onto gelatinized flasks in the presence or absence of 200nM tamoxifen (4-OHT) and harvested two days later (day 3) for RNA extraction and subsequent analysis. In order to exclude any leakiness of the system or possible effects of tamoxifen on gene expression, comparison was made between HOXB4-ER^{T2} ESCs plus 4-OHT and control ESCs plus 4-OHT.

All genes analysed by qPCR were upregulated in response to HOXB4 induction and even in much higher levels compared to the microarray data, indicating that they are indeed activated by HOXB4 (Figure 4.1 A). More detailed statistical analysis showed that 5 out of the 8 analysed genes demonstrated a statistically significant increase in expression ($p<0.05$ t test) (Figure 4.1 B) with the level of induction of the three genes that were excluded (*Cer1, Ntn1,* and *Sema3f*) being high yet very variable.

A.

Gene	Gene Symbol	Fold increase in array	Fold increase in qPCR
R-spondin 3	Rspo3	4.3	7.4
kit ligand	Kitl	3	4
netrin 1	Ntn1	3	13.5
cerberus 1	Cer1	2.7	29
follistatin	Fst	2	3.5
apelin	Apln	1.8	8.8
semaphorin-3f	Sema3f	1.5	3.6
fibroblast growth factor 17	Fgf17	1.5	2.7

B.

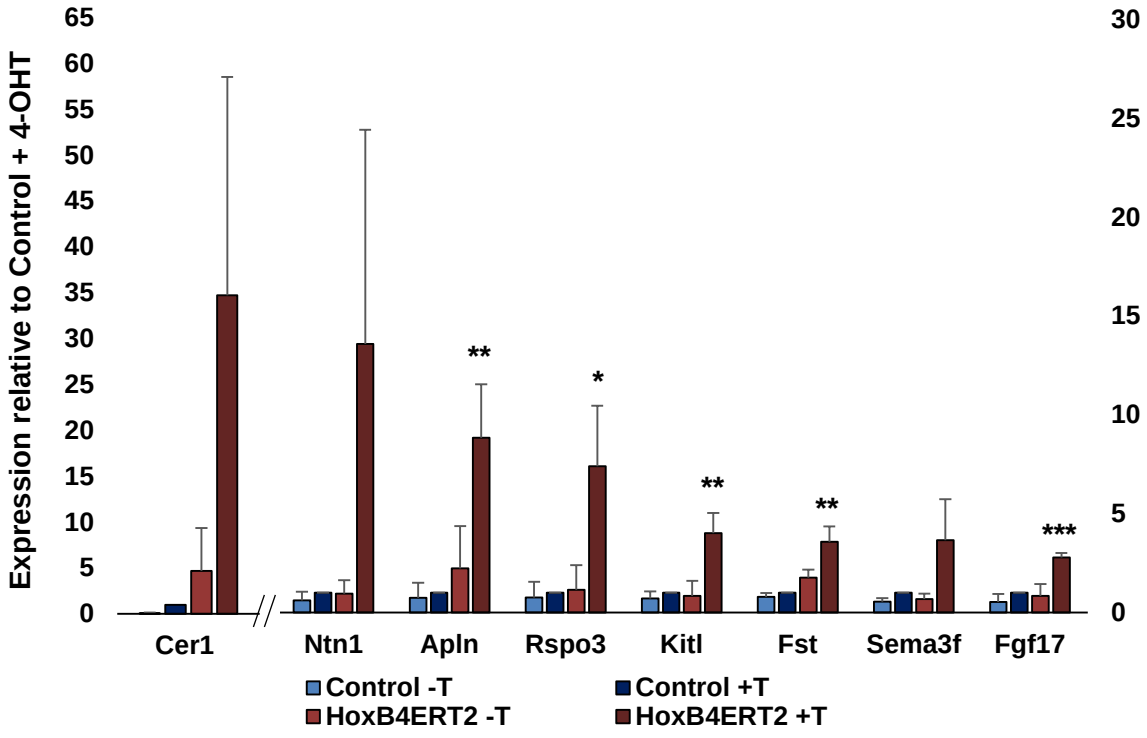

Figure 4.1: Validation of genes identified as HOXB4 targets by microarray analysis.
A. Mean fold increase in expression levels after HOXB4 activation identified by microarray and qPCR. **B**. Quantitative real-time RT-PCR performed on RNA isolated from day 3 EBs derived from HOXB4-ER^{T2} ESCs and control wild type ESCs in the presence (+T) or absence (-T) of 4-OHT. 5 of the genes showed a significant increase (*≤0.05, **≤0.01, ***≤0.001 t test) in HOXB4-ER^{T2} ESCs in the presence of 4-OHT compared to control ESCs. Data represent 3 independent experiments, error bars represent standard deviation.

4.4.2 Testing the HOXB4-ER^{T2} induction system in serum-free conditions

Even though the serum-free ESC differentiation protocol developed in this study provides a stable and clear background in which to test HOXB4 targets, it was necessary before proceeding with its use to ensure that the haemopoiesis promoting effects of our HOXB4-ER^{T2} inducible system are reproducible in serum-free conditions. If the HOXB4-ER^{T2} system failed to behave in a similar manner in the serum-free conditions, then the testing of HOXB4 targets in these conditions would not provide any useful information. HOXB4 target analysis in serum-based conditions was conducted during a short time window on day 3 before the appearance of the first HPCs. Given the significantly faster progression of ESCs grown in serum-free conditions to the haemopoietic lineage, as detailed in Chapter 3, it was not possible to identify a time point in serum-free conditions corresponding to day 3 of the serum protocol and to proceed with a detailed expression analysis. We therefore focused on the CFU-C activity of the cells.

Wild type and HOXB4-ER^{T2} expressing cells were differentiated in serum-based and serum-free conditions, in the presence or absence of tamoxifen, and collected for analysis on day 6 of the protocols. In agreement with our previous data (219), induction of HOXB4 in serum-based conditions resulted in a dramatic nearly 10-fold increase of haemopoietic colony numbers in HOXB4-ER^{T2} cells compared to the wild type control in the presence of tamoxifen (Figure 4.2 A). HOXB4 overexpressing ESCs differentiated in serum-free conditions also resulted in significantly increased colony numbers compared to wild type cells after tamoxifen addition (Figure 4.2 B). It was thus evident that our HOXB4 induction system even under different culture conditions could still increase the haemopoietic differentiation of mESCs. We then continued with the assessment of HOXB4 targets in order to determine whether the use of the validated secreted factors is sufficient to produce the same degree of haemopoietic increase observed in serum-free conditions by HOXB4 induction.

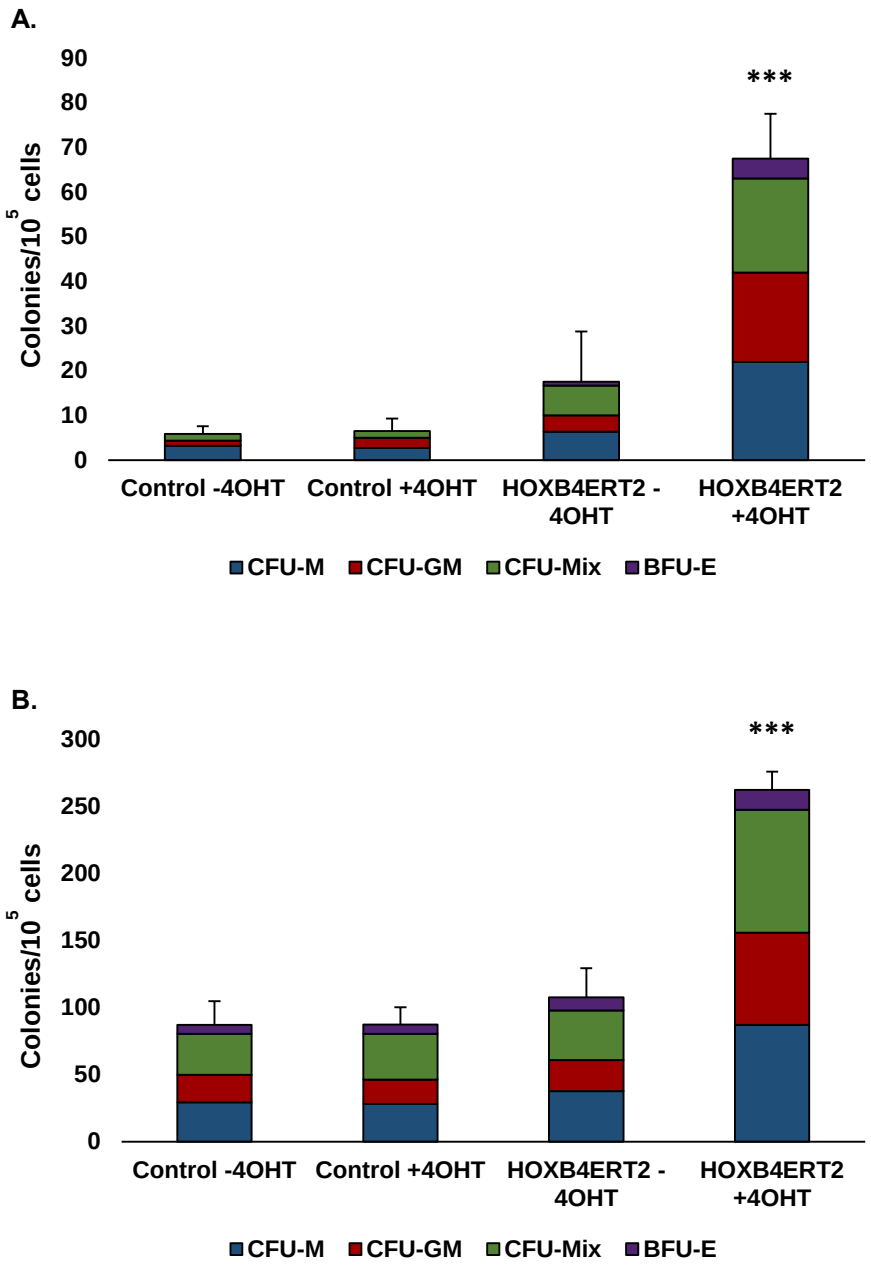

Figure 4.2: Testing of HOXB4 induction in serum-free conditions.
Wild type and HOXB4-ER^{T2} cells were differentiated in serum **A**, and serum-free **B** conditions in the presence or absence of 200nM of tamoxifen (4-OHT). Cells were harvested on day 6 of differentiation and assayed on their haemopoietic activity by CFU-C assay. Graphs represent 3 independent experiments, error bar represent standard deviation. Comparison was made between wild type and HOXB4-ER^{T2} cells in the presence of tamoxifen, ***$\leq$ 0.001.

4.4.3 Assessment of HOXB4 targets in haemopoietic differentiation

Following the validation of HOXB4 target genes and of the ability of our serum-free protocol to respond to HOXB4 activation we asked whether the proteins encoded by the targeted genes can mediate HOXB4 activity in culture. To this end wild type ESCs were initially cultured in the presence of proteins encoded by individual HOXB4 target genes in different concentrations to assess specific effects they may have in the haemopoietic output. Subsequently, the proteins were added together in various combinations to look into synergistic effects they may have on haemopoietic differentiation.

4.4.3.1 FGF17 enhances the haemopoietic differentiation of mESC

Fgf17 was the gene that even though had the lowest expression increase in response to HOXB4 of all genes analysed in the qRT-PCR analysis (2.7-fold), it was the one with the highest statistical significance. Members of the FGF superfamily have been shown to regulate haemopoiesis both through autocrine and paracrine mechanisms by acting directly on haemopoietic cells or the supporting stromal and endothelial cells (235). FGF17 is a member of the FGF8 subfamilly and has been shown to play an important role during embryonic development predominately in the patterning of embryonic brain (236, 237). FGF17 expression in the mouse embryo has been detected in major wall arteries, including the E12.5 descending aorta, mesenchymal cells and osteoblast precursors of the long bones implicating it in the development of these tissues (236). Both the aorta and the long bones are sites of haemopoiesis in the mouse thus suggesting that FGF17 might have an active role in regulating haemopoiesis.

Recombinant FGF17 was added in differentiation cultures at 10, 30, 100 and 300ng/ml and day 6 cells were collected for analysis. CFU-C assays demonstrated that FGF17 at 100 and 300ng/ml could enhance the generation of HPCs resulting in increased numbers of all types of haemopoietic colonies compared to untreated cells, with 100ng/ml giving the most significant increase (Figure 4.3). Flow cytometry analysis of HPC markers showed that there was not marked change for the majority of markers examined (Table 4.1). Upon closer inspection, we detected a significant increase in the expression levels of c-Kit, probably accounting for the increase in colony numbers, which was more visible as a defined population on scatter plots at 100 and 300ng/ml (Figure 4.4 A, B). Analysis of c-Kit co-expression with CD41 or Sca-1 also showed an increase in double positive cells, again more pronounced at 100ng/ml which was not found however statistically significant (Figure 4.4C).

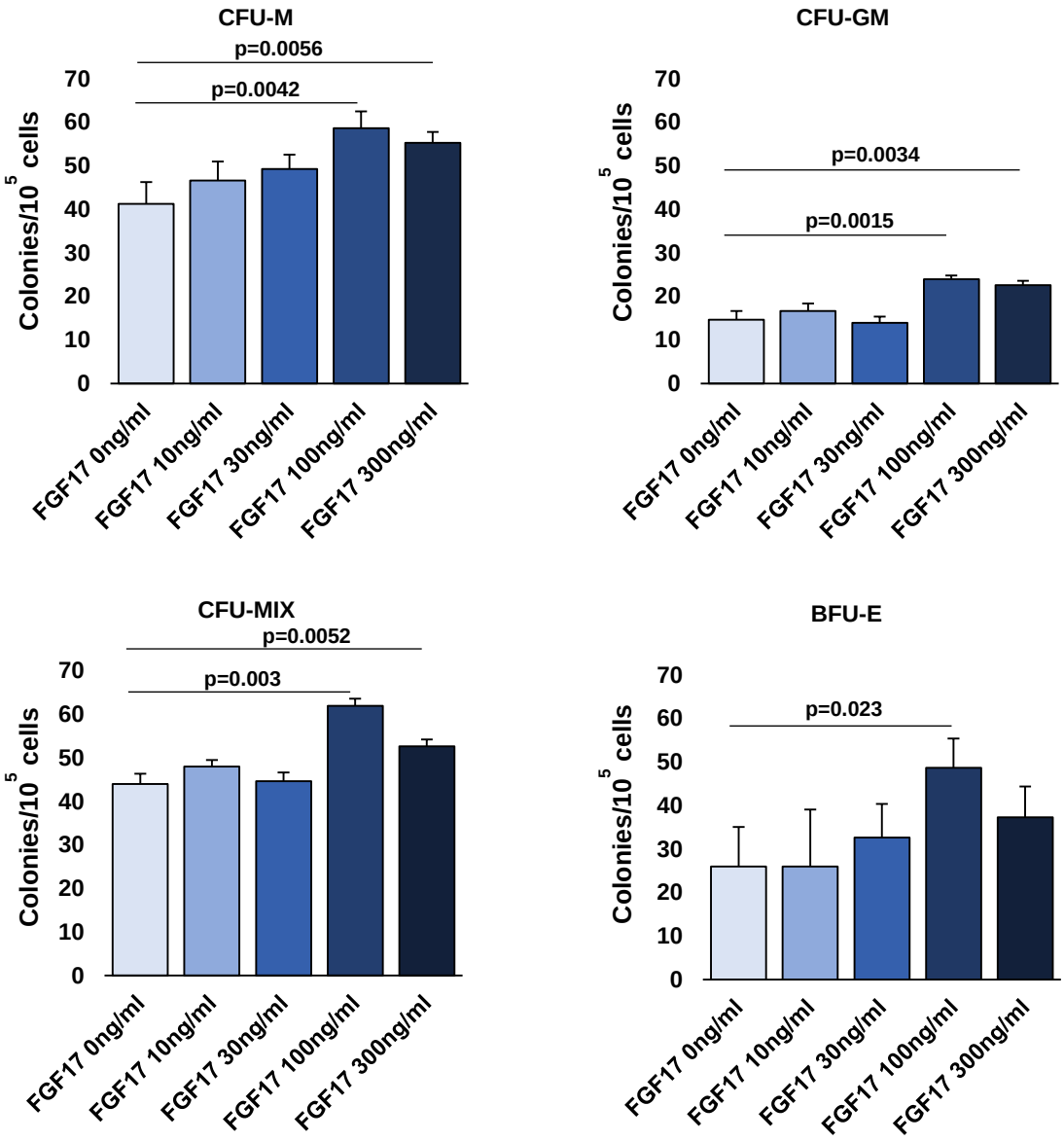

Figure 4.3: CFU-C analysis of FGF17 treated cells.
Wild type mESCs were differentiated in serum-free conditions in the presence of 0, 10, 30, 100 and 300ng/ml FGF17. Cells were analysed for their haemopoietic activity on day 6. Data represent 3 independent experiments +standard deviation. P values calculated with Student t test.

	FGF17 0ng/ml	FGF17 10ng/ml	FGF17 30ng/ml	FGF17 100ng/ml	FGF17 300ng/ml
CD144	10.7 ± 0.3	10.8 ± 0.5	11 ± 1.4	10.8 ± 1	11 ± 1.2
CD41	46 ± 2.8	45 ± 8.5	45.5 ± 2.1	54 ± 7	48 ± 8
CD45	2.4 ± 0.5	2.2 ± 0.2	2.6 ± 0.1	2.4 ± 0.9	2.5 ± 0.3
CD41/CD144	7.3 ± 0.3	6.8 ± 0.4	7 ± 1	8.6 ± 0.5	7.3 ± 0.5
CD41/CD45	1.1 ± 0.5	0.8 ± 0.1	0.7 ± 0.2	1 ± 0.4	0.5 ± 0.3
c-Kit	43 ± 5	40 ± 7	49 ± 5	**55 ± 5.6***	**57 ± 6***
c-Kit/CD41	8 ± 0.6	9.3 ± 0.8	9 ±0.9	11.6 ± 0.6	9.8 ± 2.6
Sca-1	3.4 ± 0.1	3.8 ± 0.5	3.8 ± 0.9	4.1 ± 0.1	3 ± 0.9
c-Kit/Sca-1	1.9 ± 0.2	2.2 ± 0.2	2.2 ± 0.5	2.6 ± 0.1	2.3 ± 0.6

Table 4.1: Flow cytometry data of day 5 cells treated with FGF17.
Cells were differentiated in serum-free conditions in the presence of different concentrations of FGF17 and analysed for expression levels of surface markers. Data represent 3 independent experiments ± standard deviation.

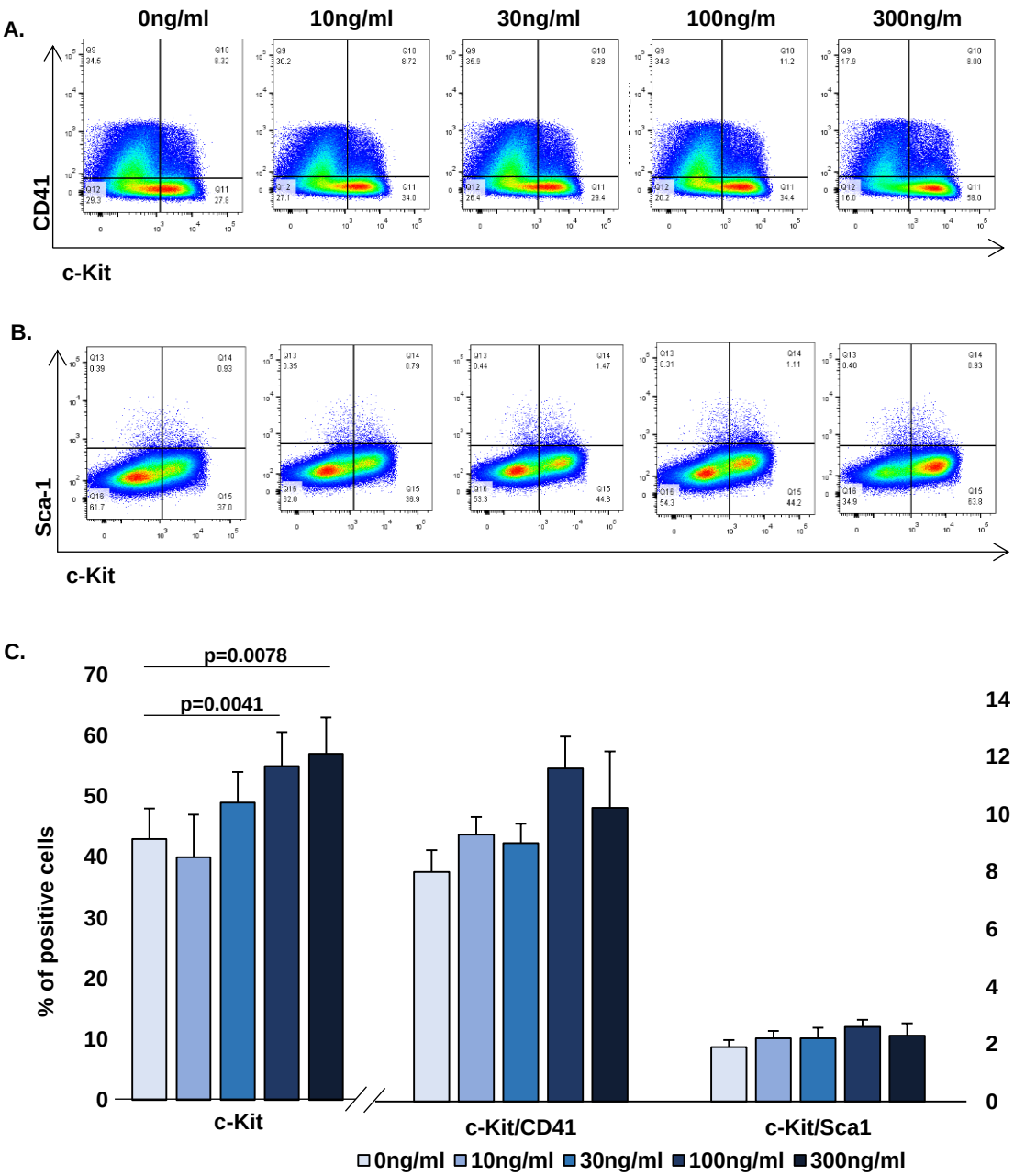

Figure 4.4: Analysis of c-Kit expression of FGF17 treated cells.
A. Representative plots of CD41/c-Kit co-expression. **B**. Representative plots of Sca-1/c-Kit co-expression. **C**. Statistical analysis of c-Kit, CD41, Sca-1 expression levels. Data represent 3 independent experiments, error bars represent standard deviation.

4.4.3.2 Increasing RSPO3 concentrations reduce the generation of HPCs

The second validated HOXB4 target to be tested was RSPO3, the expression of which was increased 7.4-fold by activation of HOXB4 as assessed by qRT-PCR. R-spondin family members (*Rspo1-4*) are known mediators of Wnt/b-catenin and Wnt/PCP signalling pathway (238). RSPO3 has been studied in the developing mouse and *Xenopus* embryo and found to be expressed in the posterior primitive streak and allantois in the mouse, and the dorsal lateral plate and ventral blood islands in *Xenopus*, which are sources of hematopoietic cells. Moreover, RSPO3 is essential in the regulation of endothelial cells and it is expressed in sites of vasculogenesis and angiogenesis, as well as, the placenta and umbilical cord (239, 240). Targeted disruption of *Rspo3* in mice leads to embryonic lethality due to vascular defects in the placenta, yolk sac and embryo (239). The expression pattern of RSPO3, relating it to the vascular niche, and the close developmental proximity of hematopoietic and endothelial cells, made it an interesting candidate.

RSPO3 was added in differentiation cultures at a wide concentration range and day 6 cells were analysed on their haemopoietic activity. CFU-C assay showed that RSPO3, contrary to what we expected, had an inhibitory effect in HPC generation. Increasing RSPO3 concentrations were accompanied by a decreasing trend in haemopoietic colony numbers. Particularly RSPO3 at 100 and 300 ng/ml significantly reduced the numbers of CFU-M, CFU-Mix and BFU-E colonies, while CFU-GM colony numbers, even though not significantly, also decreased (Figure 4.5). We then proceeded to detailed surface marker expression analysis to look into the population of cells affected by the addition of RSPO3 in culture. As outlined in Table 4.2, the reduction in colony numbers was due to a small yet statistically significant decrease in the $CD41^+$ cells (Table 4.2). Even though CD41 expression was significantly downregulated at 30, 100 and 300ng/ml, the reduction in colony numbers was observed only for the two higher concentrations. This was probably due to a decreasing trend in $CD41^+CD45^+$ cells which was observed at 100 and 300ng/ml. These data suggest that RSPO3 acts through different pathways compared to FGF17, which affected different cell populations, showing the complexity of HOXB4 regulatory network. Although the decrease in CD41 and CD45 expression could be easily missed as it was not clearly noticeable on flow cytometry plots (Figure 4.6) it was enough to alter the haemopoietic output, suggesting that even the slightest changes can affect the haemopoietic balance and confirming that our serum-free system is a very reliable tool for the *in vitro* study of haemopoiesis.

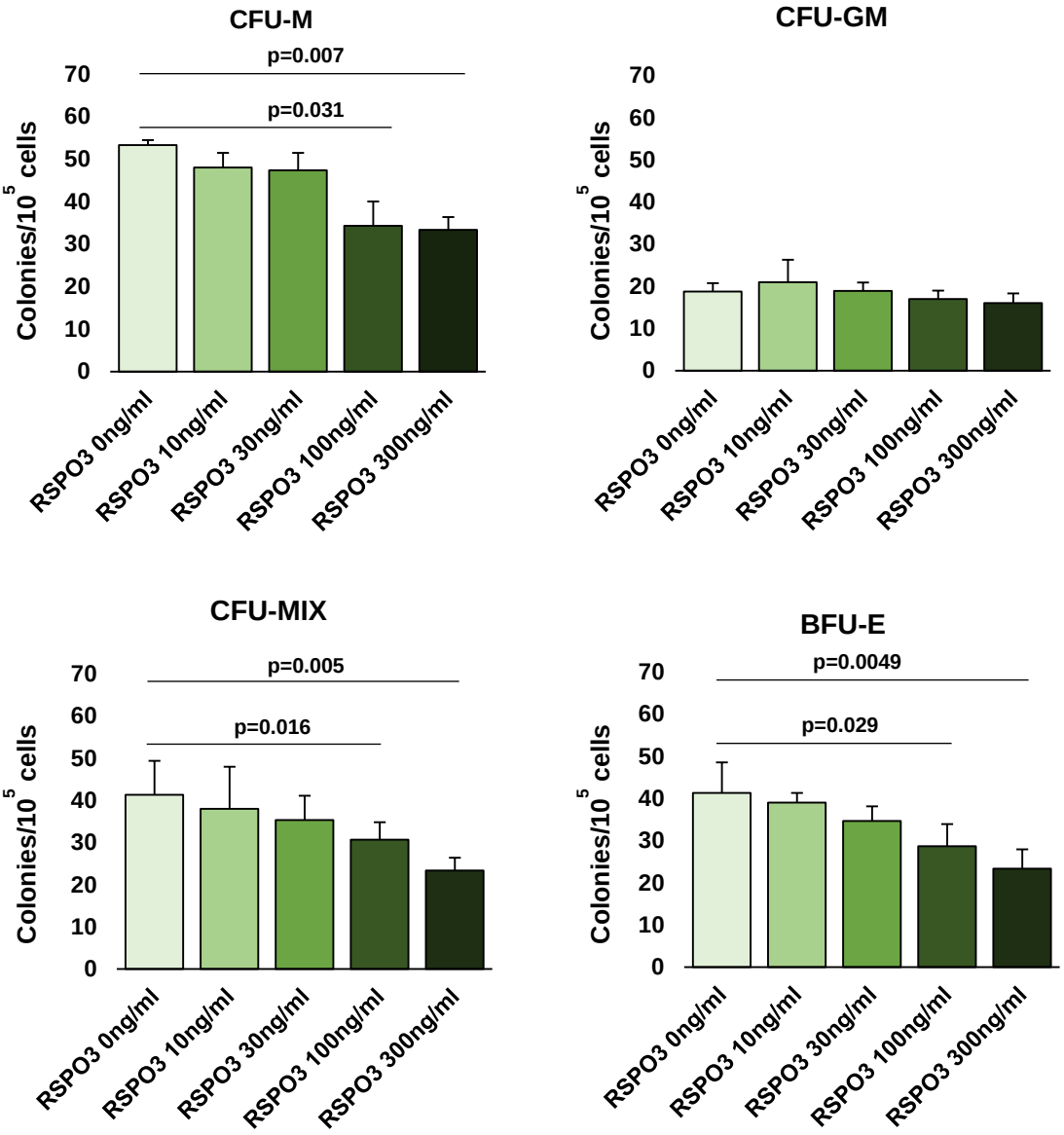

Figure 4.5: CFU-C assay of mESCs differentiated in the presence of RSPO3.
Cells were differentiated in serum-free conditions in the presence of increasing RSPO3 concentrations and assayed on day 6 of differentiation. Data represent 3 independent experiments +standard deviation. P values calculated by Student's t-test.

	RSPO3 0ng/ml	RSPO3 10ng/ml	RSPO3 30ng/ml	RSPO3 100ng/ml	RSPO3 300ng/ml
CD144	12.3 ± 1	11 ± 1.4	12.7 ± 1.7	13 ± 2.8	12.3 ± 0.4
CD41	36 ± 5	31.5 ± 6.3	**29.5 ± 6 ***	**29 ± 1.4 ***	**25.5 ± 2 ***
CD45	3 ± 0.5	2.8 ± 0.3	2.5 ± 0.5	2.6 ± 0.2	2.5 ± 0.2
CD41/CD144	9 ± 0.5	9.6 ± 0.6	8.8 ± 0.4	8.4 ± 0.6	9.3 ± 0.1
CD41/CD45	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1 ± 0.1	1 ± 0.06
c-Kit	38.5 ± 3.5	31.5 ± 6	35 ± 10	34 ± 4	38 ± 6
c-Kit/CD41	12 ± 0.4	13 ± 0.5	12 ± 1	14 ± 1.7	13 ± 1.6
Sca-1	4 ± 0.7	3.5 ± 0.5	3 ± 0.9	3.5 ± 0.7	4 ± 0.5
c-Kit/Sca-1	2 ± 0.3	2.5 ± 0.1	2.3 ± 0.6	2.3 ± 0.4	2.2 ± 0.4

Table 4.2: Flow cytometry analysis of RSPO3 treated mESCS.
Cells were differentiated in serum-free conditions in the presence of increasing RSPO3 concentrations and analysed on day 6 of differentiation for expression levels of surface markers. Data represent 3 independent experiments ± standard deviation.

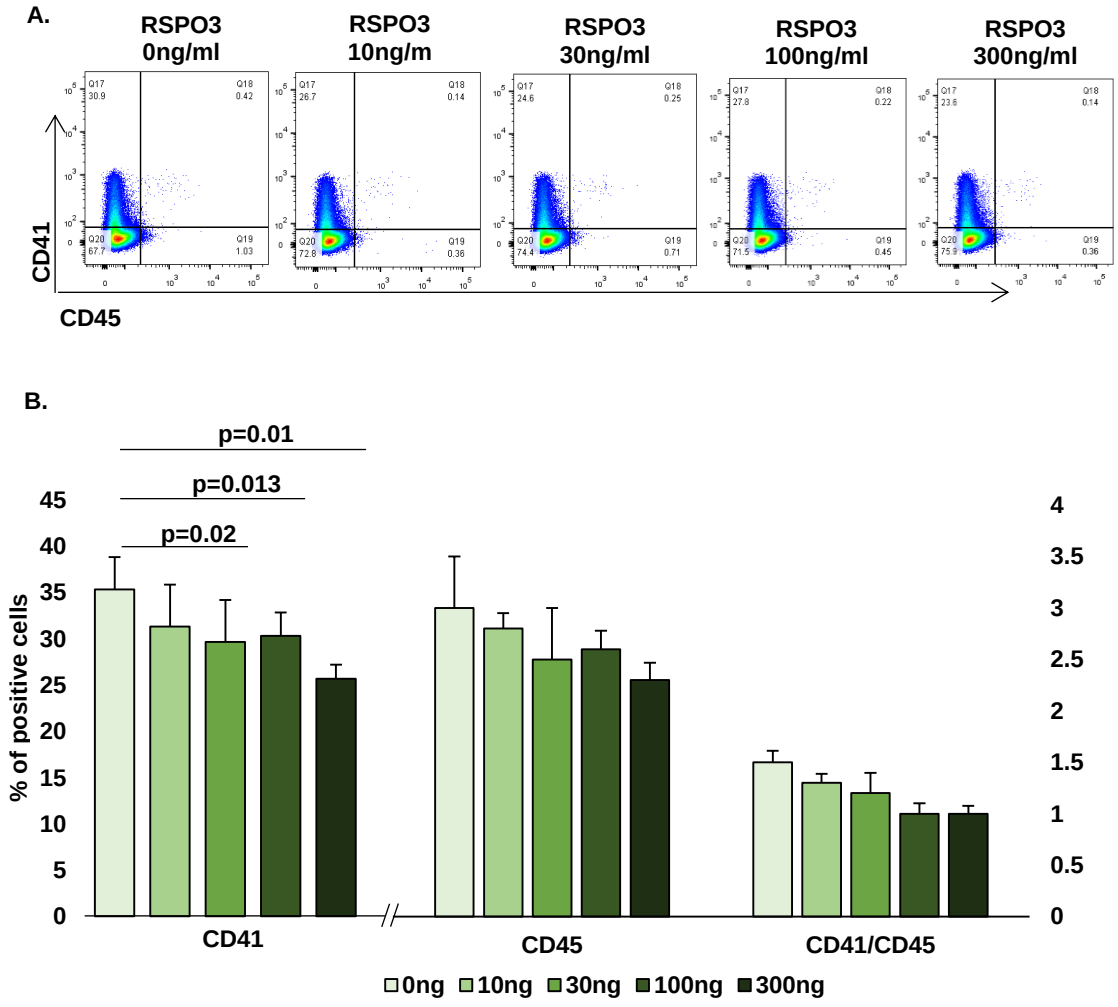

Figure 4.6: Detailed analysis of CD41 expression in RSPO3 treated cells.
A. Representative scatter plots of CD41/CD45 co-expression. **B**. Statistical analysis of CD41 and CD45 expression compared to untreated cells. Data represent 3 independent experiments, error bars represent standard deviation. P values calculated with Student t test.

4.4.3.3 Apelin peptides do not alter the haemopoietic activity of mESCs

The last candidate soluble factor to be examined in culture was Apelin. Apelin had the highest significant increase in expression by qPCR analysis (8.8-fold), and the gene encoding its receptor, Agtrl1 or Apj was the top upregulated gene by HOXB4 in the microarray with a 13.5-fold increase and further validated increase of 6.6-fold by qPCR analysis (219). The Apelin gene encodes a 77-amino acid pre-protein, which after translocation to the endoplasmic reticulum, can be cleaved to several active peptides whose number has been reported to reach up to 46 isoforms, including Apelin 36 corresponding to the amino acid sequence 42-77 and Apelin 13 corresponding to the sequence 65-77 (241). The Apelin-APJ pathway has been reported widely to play a critical role in the development and function of both the cardiac and vascular system (242, 243) that share common mesodermal origins with the hematopoietic system. Additionally, it has been reported, using a human ESC differentiation system, that the Apelin receptor marks a common mesoderm derived precursor of mesenchymal and endothelial stem cells (244). This finding is consistent with our hypothesis that HOXB4 can act to induce the formation of a stromal/mesenchymal population.

The two most common isoforms of Apelin (Apelin 13 and Apelin 36) were used separately in differentiation experiments at a concentration of 0, 30, 100 and 300nM. Colony forming assays, following the addition of Apelin 13 in culture, showed a slight but not significant increase of CFU-M, CFU-GM and CFU-Mix colonies at 300nM (Figure 4.7). Flow cytometry analysis, however, showed that there was no change in the expression of cell surface markers for any of the Apelin 13 concentrations tested (Table 4.3). Looking at the Apelin 36 isoform, a similar pattern of increased but statistically insignificant colony numbers was observed at 300nM (Figure 4.8). As outlined in Table 4.4, surface marker expression again did not differ markedly for any of the tested concentrations. The two Apelin isoforms were also added together in culture at 300nM concentration, to look into any synergistic effects, yet again there was no marked increase of haemopoietic activity as observed in both CFU-C assay and flow cytometry (Figure 4.9). The above experiment was conducted only once, as combined with the data of Apelin isoforms used alone in culture, it was conclusive to the fact that Apelin does not confer any marked changes on haemopoietic activity. Since any changes, even though minimal, were observed at 300nM it is possible that Apelin isoforms are required in even higher concentrations than the once used here for any effects to be more clearly evident.

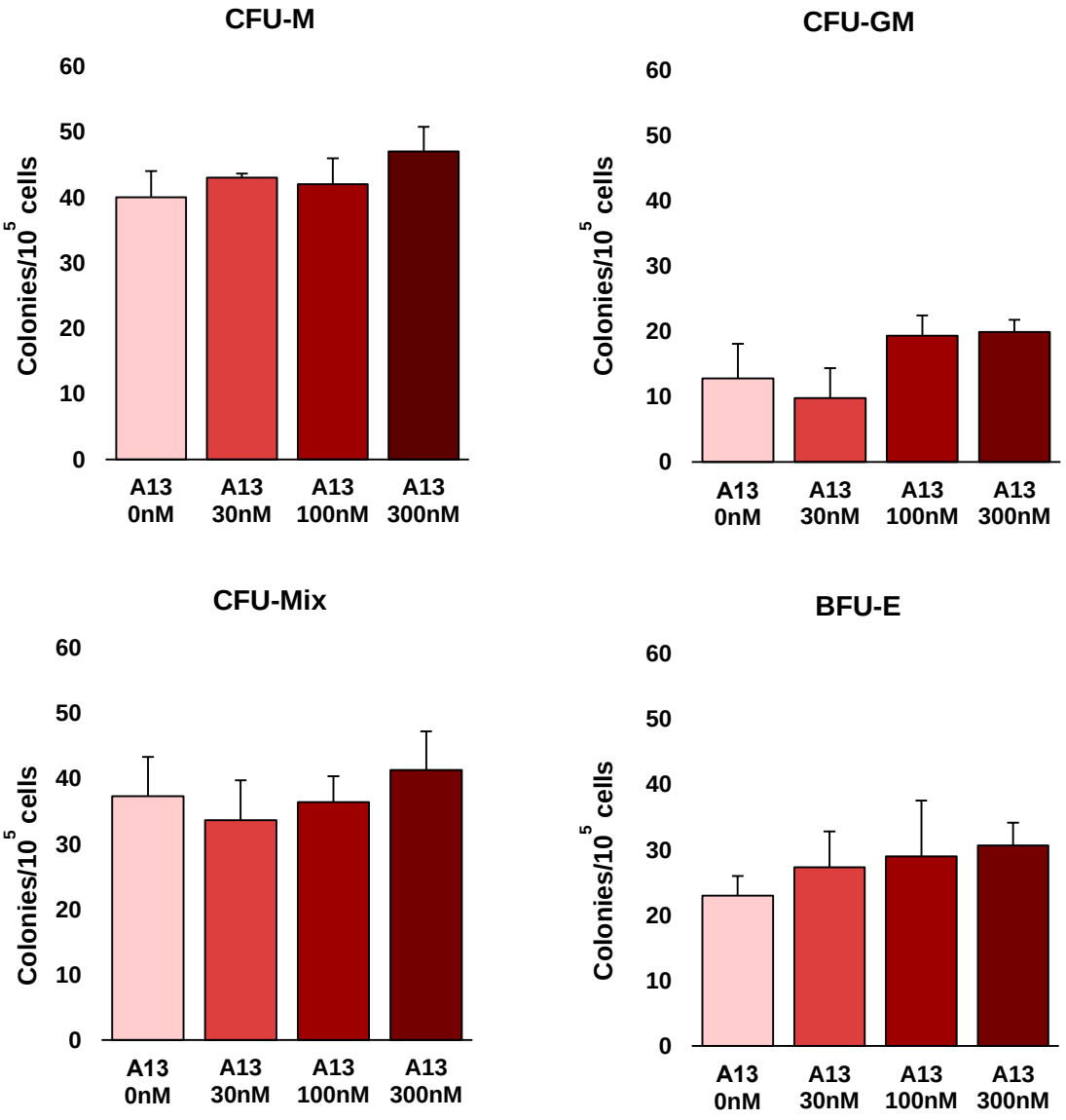

Figure 4.7: Haemopoietic colony forming activity of mESCs treated with Apelin 13. Cells were differentiated in serum-free conditions supplemented with different Apelin 13 (A13) concentrations. Cells were collected and plated in colony forming assay on day 6. Data represent 3 independent experiments +sd.

	A13 0nM	A13 30nM	A13 100nM	A13 300nM
CD144	10 ± 1.4	11 ± 1.7	10 ± 2	12 ± 1.4
CD41	49 ± 9.9	47 ± 8.4	45 ± 11	42 ± 13
CD45	1.9 ± 0.6	2.2 ± 0.5	1.8 ± 0.7	2 ± 0.2
CD41/CD144	7 ± 1.2	6 ± 0.9	6.8 ± 0.5	7.3 ± 1
CD41/CD45	0.8 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.9 ± 0.1
c-Kit	38.5 +2.1	43 ± 8.4	39 ± 9.7	37 ± 9
c-Kit/CD41	7 ± 1.4	8 ± 1.5	6.2 ± 2	8.4 ± 1.4
Sca-1	2.7 ± 0.4	2.6 ± 0.1	2 ± 0.6	1.8 ± 0.7
c-Kit/Sca-1	1.3 ± 0.5	1.5 ± 0.2	1.7 ± 0.4	1.1 ± 0.3

Table 4.3: Flow cytometry analysis of Apelin 13 treated mESCs.
Cells were differentiated in serum-free conditions in the presence of different concentrations of Apelin 13 and analysed on day 6 of differentiation for expression levels of surface markers. Data represent 3 independent experiments ± standard deviation.

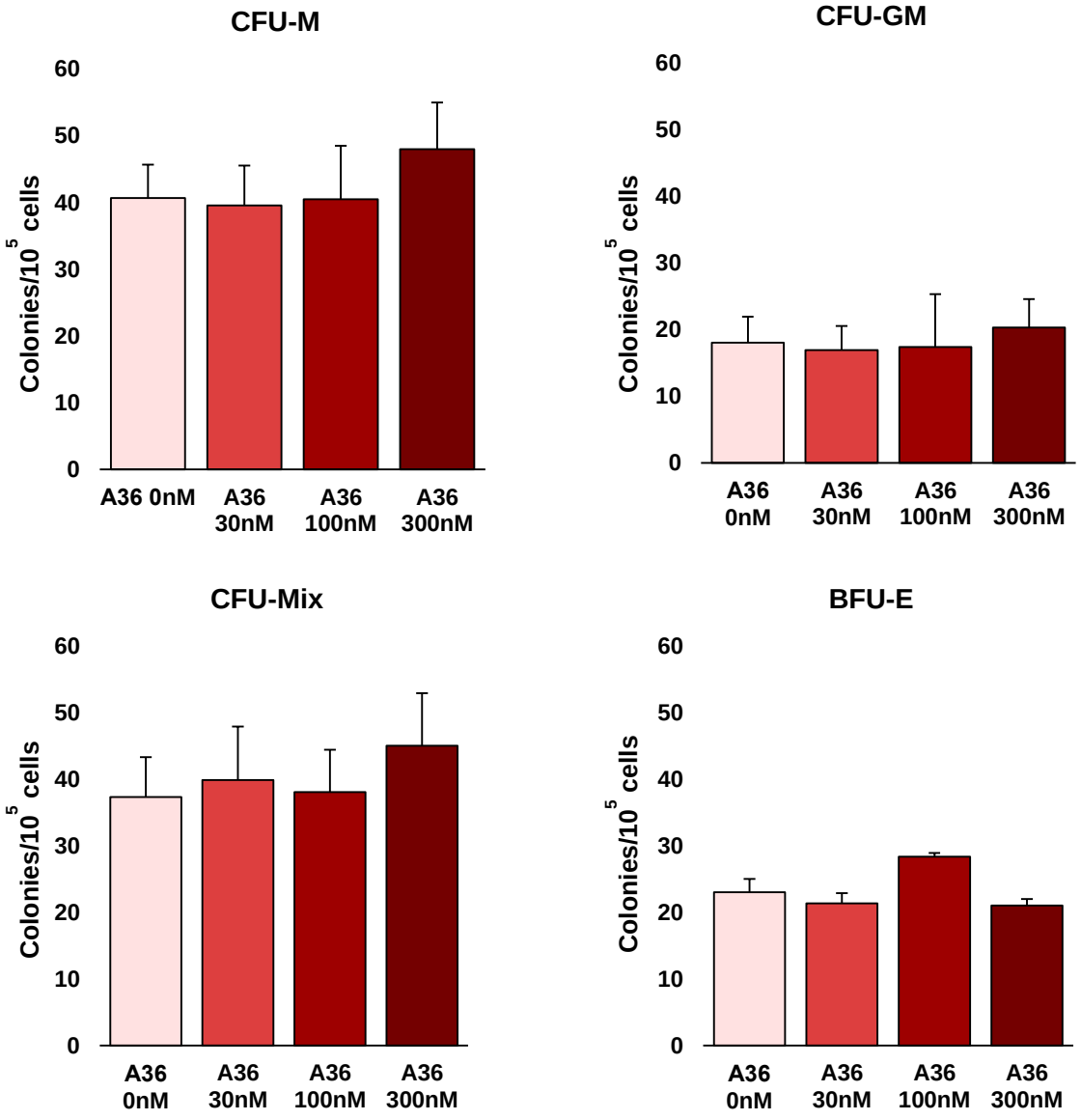

Figure 4.8: Haemopoietic colony forming activity of mESCs treated with Apelin 36.
Cells were differentiated in serum-free conditions supplemented with various Apelin 36 (A36) concentrations. Cells were collected and plated in colony forming assay on day 6. Data represent 3 independent experiments +sd.

	A36 0nM	A36 30nM	A36 100nM	A36 300nM
CD144	10 ± 1.4	10 ± 0.6	11 ± 1	9 ± 1.7
CD41	49 ± 9.9	43 ± 8	37 ± 11	35 ± 13
CD45	1.9 ± 0.6	1.5 ± 0.2	1 ± 0.7	1.8 ± 0.4
CD41/CD144	7 ± 1.2	5 ± 1.5	6 ± 0.5	7 ± 1
CD41/CD45	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.5 ± 0.3
c-Kit	38.5 +2.1	39.07 ± 6.8	34 ± 9.7	30 ± 7
c-Kit/CD41	7 ± 1.4	5 ± 1.2	7 ± 0.7	8 ± 0.5
Sca-1	2.7 ± 0.4	2.5 ± 0.1	2.5 ± 0.3	2.4 ± 0.1
c-Kit/Sca-1	1.3 ± 0.5	1.2 ± 0.2	1 ± 0.7	1.6 ± 0.2

Table 4.4: Flow cytometry analysis of Apelin 36 treated mESCs.
Cells were differentiated in serum-free conditions in the presence of various Apelin 36 concentrations and analysed on day 6 of differentiation for expression levels of surface markers. Data represent 3 independent experiments ± standard deviation.

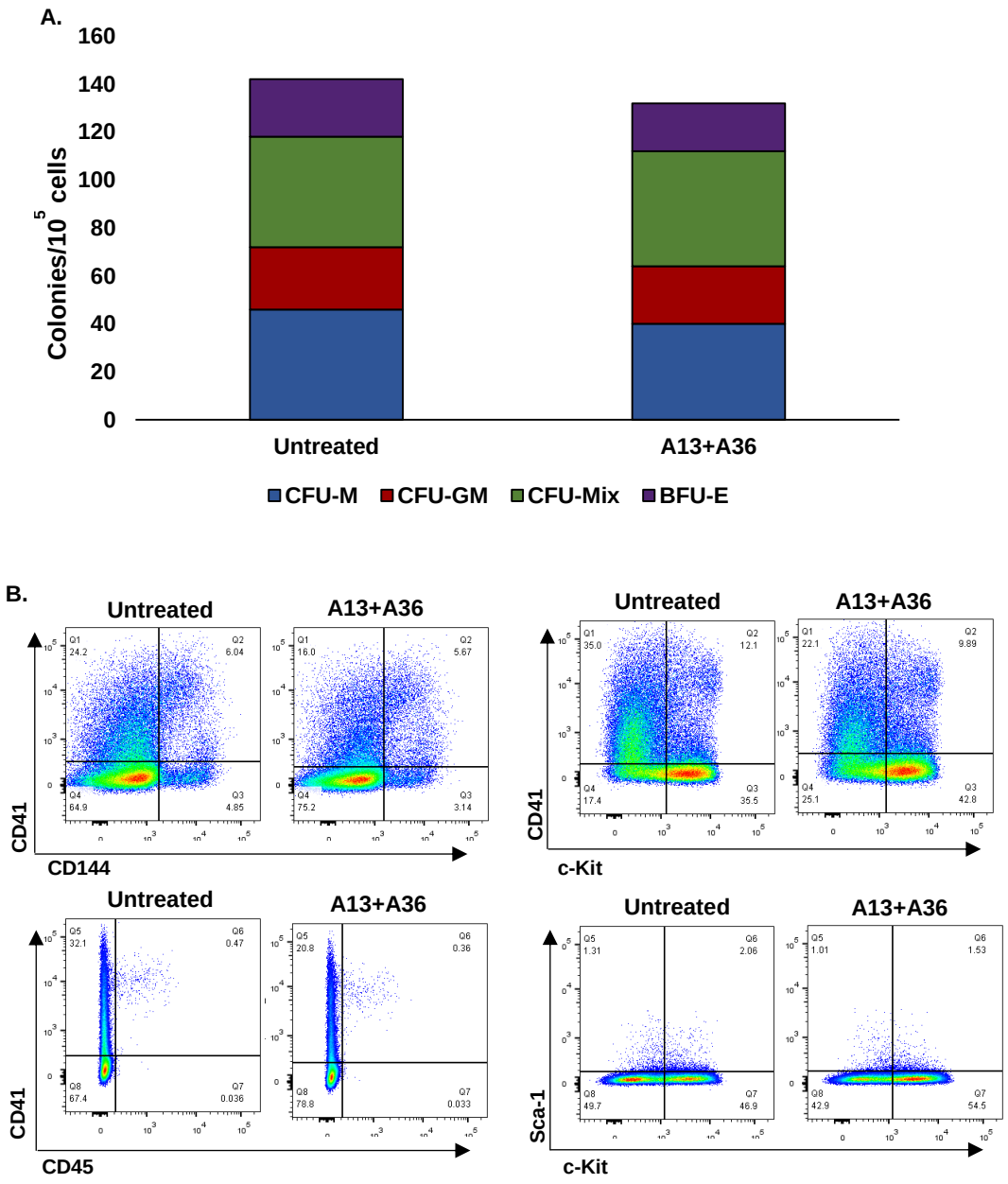

Figure 4.9: Analysis of combined Apelin isoforms effects on mESC haemopoietic differentiation.

Mouse ESCs differentiated in serum-free conditions in the presence of 300nM Apelin 13 (A13) and Apelin 36 (A36). **A.** Cells plated in methylcellulose assay on day 6. **B.** Flow cytometry charts of surface marker expression. N=1.

4.4.3.4 Combined action of HOXB4 targets

Given the large number of genes induced by HOXB4 it was consider unlikely that the use of a single factor is not enough to generate the same response as HOXB4 overexpression. We therefore decided to use the factors we individually assessed above, in combination to look into any additive effects. The factors were used in various combinations and at concentrations that seemed to favour an enhancing effect. Particularly, FGF17 was used at 100ng/ml as it was the concentration with the most significant increase in HPCs, and Apelin peptides were used at 300nM. As RSPO3 at high concentrations had an inhibitory effect, it was used at 30ng/ml which was considered high enough to have an effect but not as much as inhibiting the differentiation process.

When cells were analysed on day 6 of the serum-free protocol, we did not observe any striking difference in haemopoietic activity between untreated cells and any of the factor combinations used. Hematopoietic colonies were formed at comparable numbers for all cell treatments with no notable differences in the type of colonies either (Figure 4.10) In the cases where FGF17 was added in the differentiation culture an increase in c-Kit cells was observed again but not in significantly higher levels compared to its use alone suggesting there was no interaction between FGF17 and the other factors (Table 4.5). The experiments were repeated twice as it was evident that the factors did not increase the haemopoietic potential of the cells to a degree that could be suggesting that they can indeed mediate HOXB4 activity.

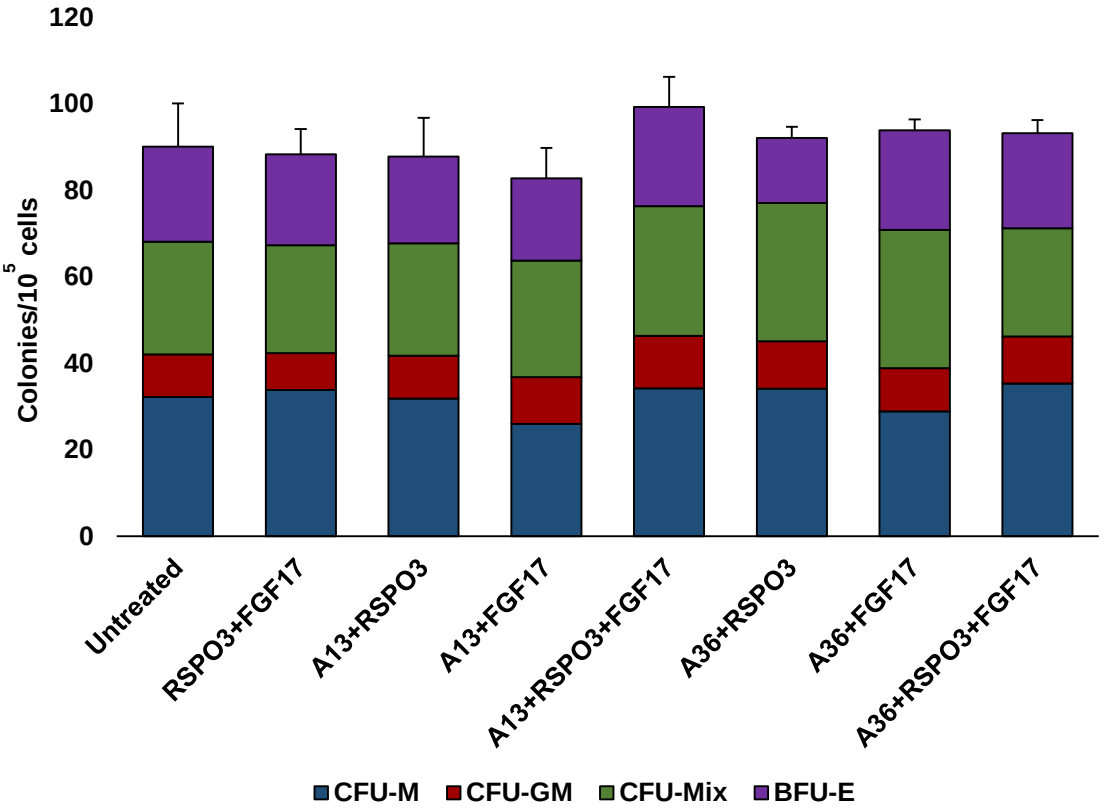

Figure 4.10: Colony forming assay on day 6 mESCs differentiated in different combinations of HOXB4-induced factors.
FGF17 was used at 100ng/ml, RSPO3 at 30ng/ml and Apelin peptides A13 and A36 at 300nM. Data represent 2 independent experiments.

	Untreated	RSPO3+FGF17	A13+RSPO3	A13+RSPO3+FGF17	A13+FGF17	A36+RSPO3	A36+RSPO3+FGF17	A36+FGF17
CD144	7 ± 1.4	6.5 ± 0.7	6 ± 1.4	7.1 ± 0.1	6.2 ± 0.2	7 ± 1.2	6.5 ± 1.3	7.2 ± 0.3
CD41	48.5 ± 9	42 ± 2	46 ± 2	38.5 ± 6	37 ± 4	42.5 ± 4	37.5 ± 6	36 ± 3
CD45	2.2 ± 0.2	2.1 ± 0.8	1.4 ± 0.1	1.8 ± 0.5	2.9 ± 1.6	2.2 ± 1	2.2 ± 1	2.75 ± 1.7
CD41/CD144	4.7 ± 0.3	4.3 ± 0.4	3.8 ± 0.6	4.4 ± 0.5	4.7 ± 0.2	5 ± 0.8	4 ± 0.7	5.5 ± 0.5
CD41/CD45	0.9 ± 0.1	0.9 ± 0.3	0.7 ± 0.1	0.7 ± 0.4	1 ± 0.4	1 ± 0.2	0.8 ± 0.6	0.9 ± 0.5
c-Kit	47 ± 1.4	53 ± 4.2	45 ± 4.2	52.5 ± 1.7	54.5 ± 2	46 ± 7	54 ± 4	57.5 ± 2.7
c-Kit/CD41	11 ± 0.9	14 ± 2.8	13 ± 1.6	13 ± 2.3	10 ± 2	13 ± 0.3	12 ± 1.4	10 ± 0.5
Sca-1	2 ± 0.2	2.7 ± 0.1	2.7 ± 0.5	2.9 ± 0.6	2.7 ± 0.1	2.5 ± 0.1	2.5 ± 0.2	3.2 ± 0.8
c-Kit/Sca-1	1.4 ± 0.1	1.9 ± 0.1	2 ± 0.3	2 ± 0.7	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.2	2.3 ± 0.7

Table 4.5: Analysis of flow cytometry data of mESCs differentiated in the presence of combined HOXB4 targets.
FGF17 was used at 100ng/ml, RSPO3 at 30ng/ml and Apelin peptides A13 and A36 at 300nM. Data represent two independent experiments ± standard deviation.

4.5 Conclusion

The activity of a novel set of HOXB4 targets was assessed in a well-controlled environment. It was shown that even though induced by the same transcription factor the tested proteins generate different results in haemopoietic differentiation demonstrating the complexity of HOXB4 regulatory network. In particular:

- We identified as HOXB4 targets a number of secreted factors, whose direct role in haemopoietic differentiation has not been previously investigated (Figure 4.1).
- Addition of FGF17 in the differentiation cultures promoted the haemopoietic potential of mESCs by increasing c-Kit expression (Figure 4.3, 4.4).
- RSPO3 inhibited the haemopoietic activity of mESCs by reducing the number of $CD41^+$ and $CD41^+CD45^+$ cells (Figure 4.5, 4.6).
- Apelin peptides did not affect the differentiation potential of mESCs even when the different isoforms were used together (Figure 4.7, 4.8, 4.9).
- The combined action of these tested factors in culture was not sufficient to generate the same enhancing effects as HOXB4 overexpression in haemopoietic differentiation (Figure 10).

4.6 Discussion

4.6.1 Reproducibility of HOXB4-ER^{T2} induction system in serum-free conditions

An important aim of this chapter was to essentially translate the data obtained by HOXB4 induction in our serum-based culture system, to the newly developed serum-free protocol. Therefore, a major question we came across was whether serum-free conditions can allow for HOXB4, and ultimately the factors induced by it, to generate the same enhancing effects on haemopoiesis. Since the first study reporting that HOXB4 overexpression can improve the definitive haemopoiesis of mESCs a large number of overexpression systems have been

developed for mouse and human cells. Even though these overexpression systems have generally improved the haemopoietic outcome, variations in the levels of HOXB4 and timing of expression among them have many times generated conflicting results. To date there is little evidence regarding the reproducibility of these HOXB4 induction systems, let alone the regulatory pathways of their action. A retroviral HOXB4 expression system developed by Klump and co-workers (245), has been used to compare the growth and differentiation of ESC-derived and bone marrow HSCs (209). The authors demonstrated that HOXB4 transduced bone marrow and ES cells behaved almost indistinguishably both in culture, where they exhibited a competitive growth advantage over wild type cells, and *in vivo* after transplantation, showing the same repopulation efficiency and lineage bias. Although they did not compare the expression profiles of the two cell populations the data they presented were enough to suggest that HOXB4 mediates the same cell fates in the two populations, resulting in identical cell behaviour.

As already mentioned the fast progression to the haemopoietic fate observed in our serum-free protocol made it difficult to pin point the corresponding time to the day 3 of expression analysis in serum-based conditions. We did consider however, following the same reasoning with the above study, that if the HOXB4 targets we identified in serum grown cells are crucial factors that mediate its activity they should be induced even under different culture conditions, enforce the same cell fate decisions and increase the haemopoietic outcome of the differentiation process. Hence we focused on analysing the haemopoietic output of HOXB4 activation. We showed that our inducible system could indeed generate significantly increased numbers of HPCs as shown by colony forming assays (Figure 4.2) in both culture conditions confirming its reproducibility and suggesting conserved regulatory factors. We then aimed at determining whether the secreted factors we validated were the ones accounting for the observed increase in HPCs.

4.6.2 Assessment of FGF17 effects on mESC haemopoietic differentiation

FGF17 was one of HOXB4 targets that as we expected increased the haemopoietic activity of mESCs. We used a wide range of FGF17 concentrations to detect the appropriate amount for it to exert its effects, as well as, any dose-dependent variations. We demonstrated that 100 and 300ng/ml of FGF17 could markedly increase the number of HPCs capable of differentiating into multiple lineages *in vitro* (Figure 4.3). Flow cytometry analysis suggested that the increase in haemopoietic activity was due to the upregulation of c-Kit expression which in turn led to an increase in the numbers of c-Kit+CD41+ and c-Kit+Sca-1+ cells

(Table 4.1, Figure 4.4). Interestingly, the increase in colony forming and c-Kit$^+$ cells was more prevalent at 100ng/ml suggesting there is not only a minimum but also a maximum threshold for optimal FGF17 activity.

Similar results have been described *in vivo* for another member of the FGF superfamily. Treatment with bFGF in mice resulted in expansion of bone marrow stromal cells and an increase in c-Kit$^+$ cell proliferation leading to improved repopulation and CFU formation rates (228). It is possible that similarly, HOXB4-induced FGF17 promotes the development of a haemopoiesis supportive population while also enhancing the proliferation of c-Kit$^+$ HPCs per se. Although our study did not include detailed analysis of the stromal population generated in our serum-free protocol for possible changes induced by FGF17, the increase in c-Kit expressing cells clearly points to the proliferative activity of FGF17. A study led by Nezu and colleagues, where FGF17 expression was largely detected in human haemopoietic tumour cells lines and in patients with acute leukaemia, also confirmed the proliferation activity of FGF17 (246). When human haemopoietic tumour cell lines were cultured in the presence of FGF17, their growth rates increased in a dose-dependent manner. Even though in our study the overall increase in HPCs induced by FGF17 treatment was not equivalent to the one generated by HOXB4 overexpression we showed that FGF17 is in part mediating HOXB4 activity. It would be interesting to see if this increase in HPC numbers with FGF17 treatment can also result in improvement of the repopulating ability of the cells.

4.6.3 Assessment of RSPO3 effects on mESC haemopoietic differentiation

We and others have identified as targets of HOXB4 genes involved in the regulation of the Wnt pathway, which in turn is an important regulator in the development and proliferation of HSCs (216, 217, 247, 248). RSPO3, which was in the top ten genes identified as HOXB4 targets by our group, is a potent activator of the Wnt pathway (238). This led us to hypothesise that it might be a major mediator of its enhancing haemopoietic activity. To our great surprise, our results indicated a negative role of RSPO3 in haemopoietic differentiation. We found that increasing RSPO3 concentrations resulted in decreasing numbers of HPCs. We observed significantly reduced numbers of haemopoietic colonies at 100 and 300ng/ml which were accompanied by a decrease in the populations of CD41$^+$ and CD41$^+$CD45$^+$ cells (Figure 4.5, 4.6).

In agreement with our data, Scheller and colleagues have shown that continuous activation of Wnt signalling in the haemopoietic system impairs the function of HSCs. Cells are forced

into the cell cycle, thus exhausting the stem cell pool and ultimately blocking their ability to repopulate irradiated hosts and to differentiate into all haemopoietic lineages (249). Another study monitoring Wnt signalling pathway during mESC haemopoietic differentiation has shown that while members of the Wnt signalling are expressed in ESCs and early stages of haemopoietic commitment, they are significantly downregulated as differentiation progresses (250). It becomes evident from the above studies that well-regulated Wnt stimulation is crucial for efficient haemopoiesis to occur. The continuous presence of high RSPO3 concentrations in our serum-free differentiation protocol might have possibly tipped the fine balance required for optimal Wnt signalling activity, therefore leading either to impairment of HPC generation or to increased cell death which can account for the reduction in colony numbers and CD41, CD45 expressing cells. It is important to keep in mind that in our expression analysis RSPO3 induction by HOXB4 was detected on day 3 of differentiation prior to the emergence of HPCs. It is possible that RPSO3 expression is subsequently turned-off to allow for HPCs to develop. A more detailed study where cells are treated with RSPO3 only for a short period at the early stages of differentiation, or with reducing doses throughout the protocol, might provide more evidence regarding its activity.

4.5.4 Assessment of Apelin in the haemopoietic differentiation of mESCs

Apelin and its receptor, APJ, were both identified as HOXB4 targets by our group thus clearly pointing to their involvement in mediating HOXB4 activity. Combined with our data, the expression of APJ in the AGM vasculature of the mouse embryo, as well as, in a mesenchymal and endothelial progenitor with enriched hemangioblast colony-forming ability in OP9-cultured hESCs, suggested a role for Apelin and its receptor in haemopoiesis (251, 244). We looked into the effects of the two more common Apelin isoforms, Apelin 13 and 36, into haemopoietic mESC differentiation. We did not detect any change in the numbers or populations of haemopoietic progenitors developed in our system neither when the two isoforms were tested separately nor when they were used in combination.

Contrary to our data, work by Yu and co-workers, showed that Apelin 13 in hESC haemopoietic differentiation cultures increased the number of hemangioblast colony-forming cells and the expression of haemopoietic markers (252). The inconsistency between our data and the above study might be due to interspecies differences. Nonetheless, it would not explain why while it is clearly shown that the mouse Apelin-APJ system is activated by HOXB4, addition of Apelin in culture fails to produce any effect. An important issue to consider, which could account for our negative data, is the unstable nature of Apelin

peptides. The use of Apelin has been repeatedly restricted due to the fast degradation of the peptides which results in very short half-life of less than 10 minutes (253, 254). The fast degradation of Apelin in our culture system would not allow for any effects to be seen even in very high concentrations. Very recently, improved delivery methods that increase peptide stability have been developed that will enable the more reliable use of Apelin for research and clinical purposes (255).

4.6.5 Secreted factors and HOXB4 activity

Taking into consideration that a single secreted factor is unlikely to fully account for the haemopoietic promoting activity of HOXB4 we used the factors in combination. Nevertheless, the haemopoietic output of the cells did not increase for any of the tested combinations (Figure 4.10, Table 4.5). It is possible that a better fine-tuning of the amount and timing of factors tested is required for their combined activity to be effective. It is also important to consider that the haemopoiesis supporting environment promoted by HOXB4 similar to *in vivo* niches may have a more complicated mechanism of action. Haemopoietic niches maintain and generate HSCs through a fine balance of secreted and membrane-bound factors, cell adhesion molecules and matrix proteins. The expression profiling in our group apart from the secreted factors, identified as HOXB4 targets a number of receptors and cell adhesion molecules such as APJ, Plexin A, Cdh11, NCAM. It is likely that in addition to the paracrine activity of the soluble factors, direct cell-to-cell contact is necessary for the full potential of HOXB4 activity to unravel.

In agreement with this concept a number of studies have demonstrated the importance of direct cell contact for development and expansion of HSCs. Previous work in our group has shown that cell-to-cell interaction between haemopoiesis supportive feeder cell lines and differentiating mESCs is necessary for the feeder lines to enhance the generation of HPCs (220). In the same study the combined use of feeder cell lines and HOXB4 overexpression did not have an additive effect in the haemopoietic activity of the cells, suggesting common molecular mechanisms such as the requirement for direct cell contact. The requirement for cell-to-cell contact has also been demonstrated between embryonic HSCs and the feeder cell lines, to maintain HSC activity in culture (256). Furthermore, some studies have reported the synergistic effects of using recombinant growth factors and supportive feeder cells, in the expansion of HSCs (257, 258). An efficient way to define whether paracrine signalling is sufficient for mediating HOXB4 activity would be the use of supernatant from HOXB4-expressing cell cultures in the differentiation protocol. Additionally, the generation of a

HOXB4 expressing feeder cell line would clarify if direct cell interaction is an essential part for HOXB4 activity and which soluble factors and adhesion molecules are critical in regulating it.

Chapter 5 : Translating HOXB4 paracrine activity to the human system: testing HOXB4-induced secreted factors in the haemopoietic differentiation of human PSCs

5.1 Introduction

Mouse and human haemopoietic development bear striking similarities in the molecular and cellular events leading to the generation of HSCs. This has allowed scientists to differentiate human haemopoietic cells from PSCs *in vitro,* by directly translating information gained from the study of the mouse system. Supportive feeder cell lines derived from mouse haemopoietic niches have been successfully applied for the differentiation of human HPCs, while, cytokines able to induce the haemopoietic lineage in mouse ESCs have also been used for human (161, 174). HOXB4 overexpression, which has been effective in generating mouse cells capable of long-term multilineage engraftment, can increase as well the generation of human HPCs (213). However, HOXB4 has not been as effective in rendering human cells engraftable (188). The observed difference has been attributed to variability in the dosage or the temporal window of exogenous HOXB4 which might require further optimising (215). Nonetheless, key differences between species, regarding the role of HOXB4 or the regulatory mechanisms underlying its activity, cannot be excluded. So far, studies looking into the regulatory network of HOXB4 have been conducted exclusively in mouse adult and ESC-derived HSCs (216-219). Therefore, the way HOXB4 modulates human haemopoietic specification and how it compares to the mouse one remains an unexplored area. In this chapter we aimed to gain some insight into HOXB4 interspecies differences by testing, in human ESC haemopoietic differentiation cultures, the HOXB4-induced secreted factors that we had identified in the mouse ESC differentiation system.

Taking into consideration the fast progress in hiPSCs research and the immense future clinical opportunities they offer for patient-specific therapy we decided to also investigate the haemopoietic activity of hiPSCs compared to hESCs in our differentiation system and their response to treatment with HOXB4-induced factors. Human ESCs and iPSCs are remarkably similar not only in their self-renewal but also in their multilineage potential (6, 148). However, gene expression and DNA methylation analysis of hESC and hiPSC cell lines has revealed marked differences between the two cell types which could affect the differentiation propensity of the cells (259). Studies focusing on the haemopoietic differentiation ability of hiPSCs have shown that despite variations in their efficiency, their phenotype and haemopoietic activity is very similar to hESCs (157, 260). Nevertheless, even the slightest differences between hESCs and hiPSCs may be critical and lead to variable results in identifying factors that can confer repopulating ability to the cells, with important implications in clinical cell therapy.

5.2 Aims

1. Monitor the emergence of HPCs in the serum-free and feeder-free haemopoietic differentiation of hESCs and hiPSCs.

2. Assess how HOXB4-induced secreted factors affect the generation of HPCs in hESC and hiPSC haemopoietic differentiation cultures.

5.3 Experimental strategy

1. Human ESCs and iPSCs were differentiated in serum-free and feeder-free conditions to set a baseline for their haemopoietic activity, as well as, for any differences between them. Cells were collected at different time points and monitored for the generation of HPCs through colony forming assay and flow cytometry analysis of surface markers.

2. To study the effects of secreted factors induced by HOXB4, hESCs and hiPSCs were cultured in the presence of the factors in different concentrations and changes in the haemopoietic activity of the cells were measured through colony assays, cell expansion rates and expression of haemopoietic markers.

5.4 Results

5.4.1 Human ESC and iPSC lines follow similar stages of haemopoietic differentiation

A robust serum- and feeder-free differentiation protocol has been established in our lab for the generation of erythroid cells. Although the ultimate aim of the protocol is to produce mature red blood cells, the generation of HPCs is a part of the differentiation process and we have observed that HPCs are produced very efficiently over a wide time window. In this study we used a modified version of the protocol, where again only the most crucial factors required for haemopoiesis were used, to allow for even the slightest changes caused by HOXB4 targets to be detected. We differentiated hESCs and hiPSCs and analysed their phenotype and lineage potential to identify the time point during the protocol when production of multipotential HPCs is optimal. The human differentiation protocol as outlined in Figure 5.1 followed similar steps to the mouse protocol. Suspension EBs were formed for the first 3 days. EBs were then dissociated and single cells were plated down and allowed to mature for a further week. Cells were collected on day 7, when the first colony forming progenitors are detected, and on day 10 before their transfer to conditions that favour erythroid differentiation.

Colony forming assay analysis showed that hESCs and hiPSCs follow a similar development pattern. A wave of primitive erythroid progenitors is generated on day 7 which is followed by more mature multipotent HPCs on day 10. Particularly on day 7 the vast majority of haemopoietic colonies for both cell populations consisted of small primitive erythroid colonies, EryP (Figure 5.2 A-D). Although myeloid and multilineage colonies were also present on day 7 they were of small size and quite low numbers. By day 10 however, there was a significant increase in the numbers and size of CFU-M, CFU-GM and CFU-Mix (Figure 5.2 E-I). The primitive erythroid colonies had disappeared and mature BFU-E colonies had developed. Interestingly, by day 10 a clear lineage bias difference between the two cell lines was observed. There were significantly more myeloid progenitors developed by hiPSCs compared to hESCs, while, hESCs had a significantly higher yield of multilineage progenitors (Figure 5.2 I).

We conducted flow cytometry analysis to look into the phenotype of the developing HSCs. We examined the expression of CD34 which marks haemopoietic stem and progenitors cells throughout development in embryonic and adult human haemopoietic tissues (40, 86, 87).

We observed that CD34 expression was in comparable levels, 55-60% of cells, between the two cell populations on day 7, and was subsequently reduced on day 10 with hiPSCs expressing significantly lower levels than hESCs (Figure 5.3). We included in our analysis the surface markers CD235a and CD43 which have been shown to mark *in vitro* primitive erythroid and multipotent progenitor cells respectively (157). We found that $CD34^{+}CD235a^{+}$ cells constituted nearly half of the population of day 7 cells, while by day 10 they were expressed in less than 10% of the population suggesting that CD235a marks the cells generating the primitive erythroid colonies in our culture system. $CD34^{+}CD43^{+}$ cells were present in high numbers on both days of analysis with no significant change for the two cell populations. However, we did observe that $CD34^{+}CD43^{+}$ cells were significantly more in hESC differentiation cultures than in hiPSC, suggesting that different PSC lines display variable haemopoietic activity in our system.

It was concluded from the above data that the optimal time of appearance of mature HPCs capable of multilineage differentiation was at day 10 of our human differentiation protocol. HPCs were characterised by CD34 and CD43 expression and their levels were comparable in the two pluripotent cell lines tested (one hESC and one hiPSC line). Cell sorting experiments conducted by Dr Rui Ma in our lab have confirmed that the haemopoietic potential of day 10 cells is predominately found in the CD34+CD43+ fraction. Therefore, analysis in subsequent experiments was performed on day 10 cells, and surface marker analysis examined CD34 and CD43 expression.

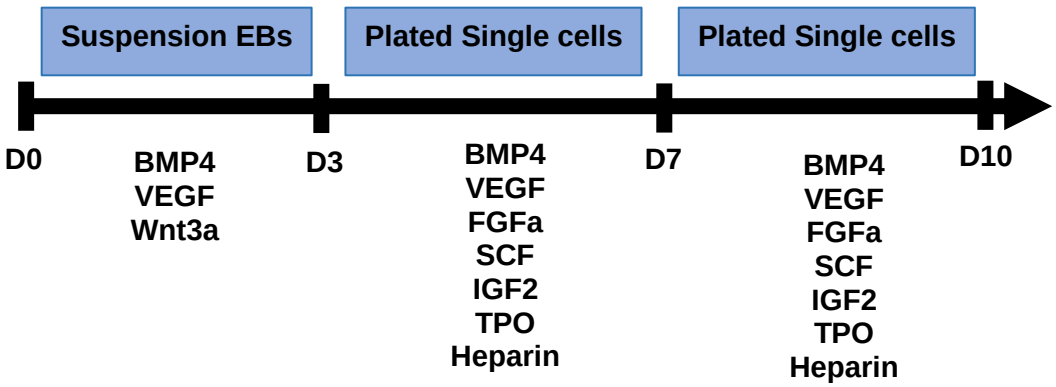

Figure 5.1: Human PSC haemopoietic differentiation protocol.
Human ESCs and iPSCs were cultured in serum- and feeder-free conditions. Cells were scraped from maintenance cultures and placed in suspension in the presence of cytokines to initiate differentiation. On day 3 EBs were dissociated and single cells were plated down and differentiated for 7 more days in the cytokines outlined above. Cells were collected for analysis on days 7 and 10.

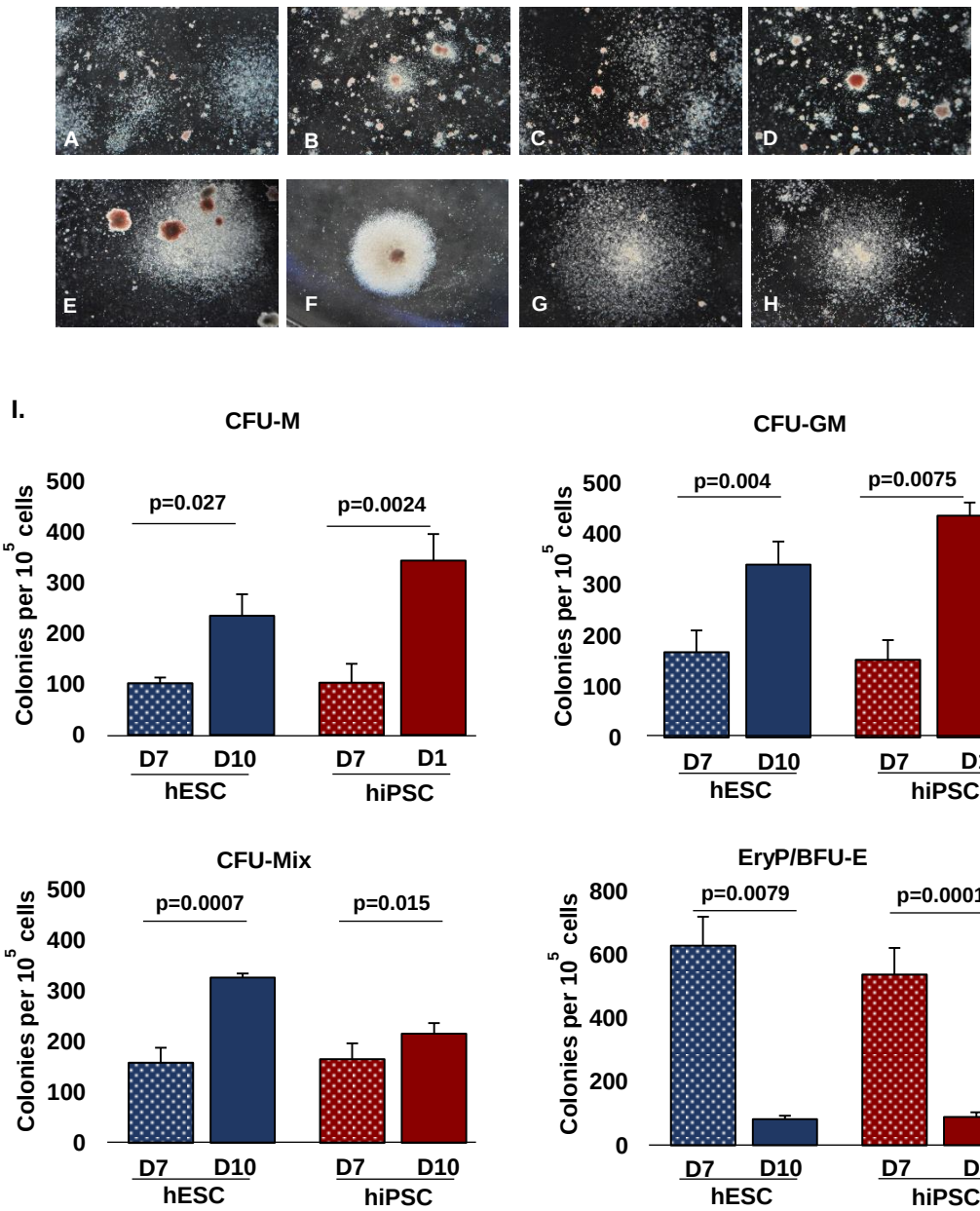

Figure 5.2: Colony forming assay on day 7 and day 10 hESCs and hiPSCs
Cells formed mainly primitive erythroid colonies on day 7 (**A-D)**, while day 10 cells generated mature colonies of multiple lineages (**E-H)**. Detailed analysis of colony numbers on days 7 and 10 (**I**). Graphs represent 4 and 5 independent experiments for hESCs and hiPSCs respectively. Error bars represent standard deviation.

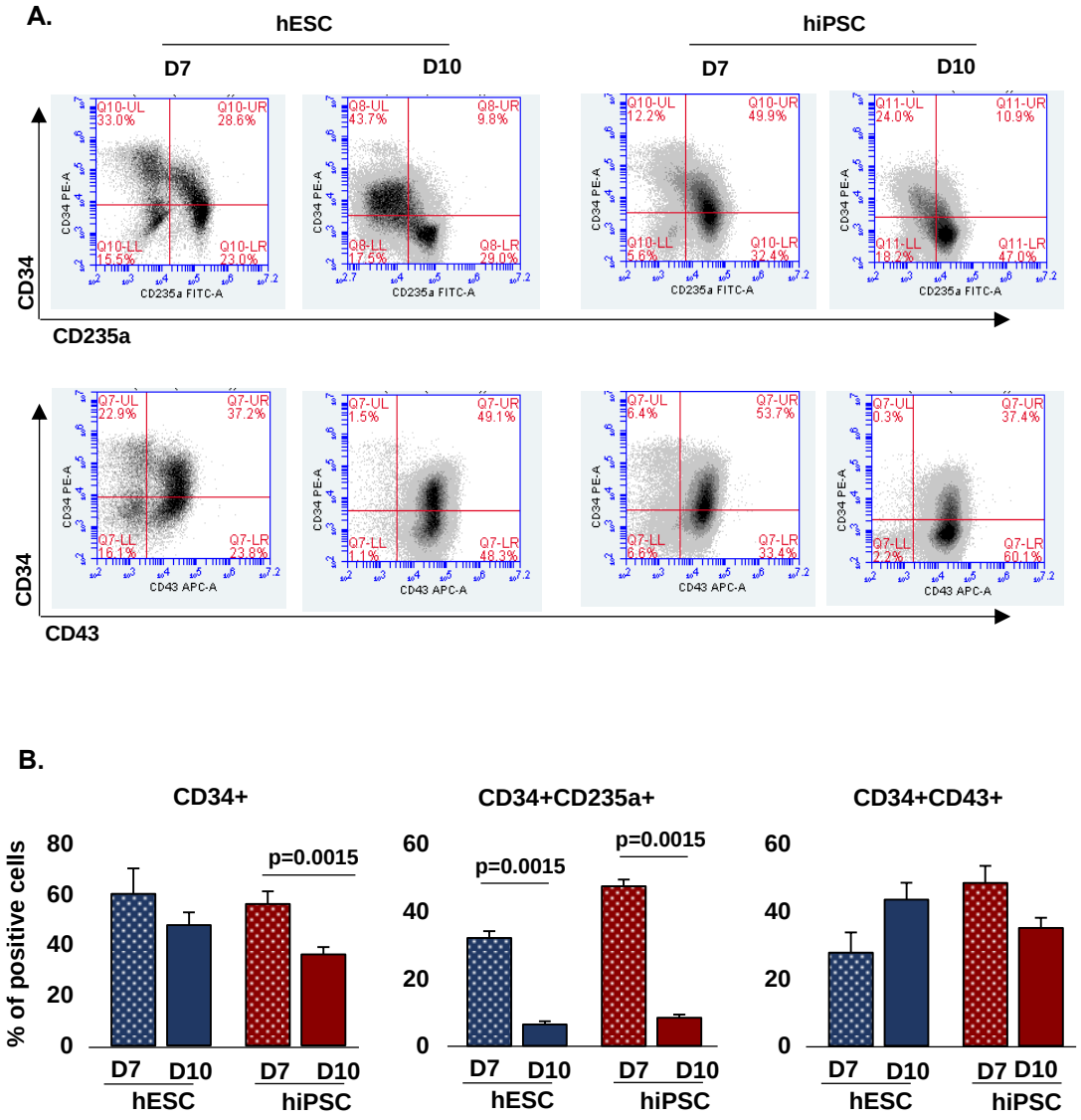

Figure 5.3: Flow cytometry analysis of day 7 and day 10 hESCs and hiPSCs.
Cells were differentiated in serum- and feeder-free conditions and analysed for the expression of haemopoietic markers. A. Representative flow cytometry plots. B. Percentage of cells expressing haemopoietic markers CD34, CD43 and CD235a. Graphs represent 4 and 5 independent experiments for hESCs and hiPSCs respectively. Error bars represent standard deviation. P values calculated by Student's t test.

5.4.2 Effects of HOXB4-induced secreted factors on human HPC differentiation

Similar to the experiments performed on our mouse differentiation system, we used the secreted proteins encoded by HOXB4 target genes in different concentrations to monitor any specific effects on the haemopoietic activity of human PSCs.

5.4.2.1 Addition of FGF17 reduces $CD34^+CD43^+$ HPC generation by hESCs but not hiPSCs

FGF17 was used at two different concentrations: a low concentration of 30ng/ml and a higher concentration of 100ng/ml, which had the most significant enhancing effect on the mouse *in vitro* haemopoiesis (Chapter 4). Haemopoietic colony assays initially showed that FGF17 did not have any effects on the number of HPCs generated by hESCs or hiPSCs (Figure 5.4). There was no significant difference observed between control and treated cells neither in the total numbers of colonies nor in the numbers of individual types of colonies. Interestingly, however, flow cytometry analysis revealed that hESCs and hiPSCs respond differently to FGF17. While there was no marked change on the percentage of cells expressing CD34 or CD43 in the hiPSC line, 30ng/ml FGF17 decreased the portion of $CD34^+$ cells and consequently of $CD34^+CD43^+$ HPCs that were generated from ESCs (Figure 5.5). The decrease in $CD34^+$ and $CD34^+CD43^+$ cells was small (approximately 6%) but significant. There are a number of reasons for this apparent discrepancy. It is possible that this small change detected by flow cytometry would not be detected in CFU-C assays because these assays are inherently more variable. Alternatively it is possible that not all CFU-C activity is contained in the $CD34^+$ or $CD34^+CD43^+$ cells, and is therefore not reduced by the small decrease of these populations.

It has been demonstrated that FGF17 can regulate the growth rates of human haemopoietic tumour cell lines (246). In that study FGF17 had an enhancing proliferative effect. We wondered whether the reduction in $CD34^+$ and $CD34^+CD43^+$ cells was also due to the action of FGF17 on cell growth, which in our experiments could be promoting the growth of non-colony forming cells. In such case CFU numbers would remain stable, while, the percentages of $CD34^+$ and $CD34^+CD43^+$ cells in the overall population would be affected. To assess this potential explanation we examined the cell expansion rates for the two cell lines following FGF17 treatment. As described above, on day 3 of the human differentiation protocol the EBs are dissociated and single cells are plated down for further maturation. This allows us to monitor cell expansion for the remaining time of the protocol. Cells were seeded at 2×10^5 cells per well of a 6-well plate and cell numbers were counted on day 7 and day 10 of

differentiation. However, we observed that treatment with FGF17 did not confer any positive or negative changes on the proliferation of the two cell lines we examined (Figure 5.6).

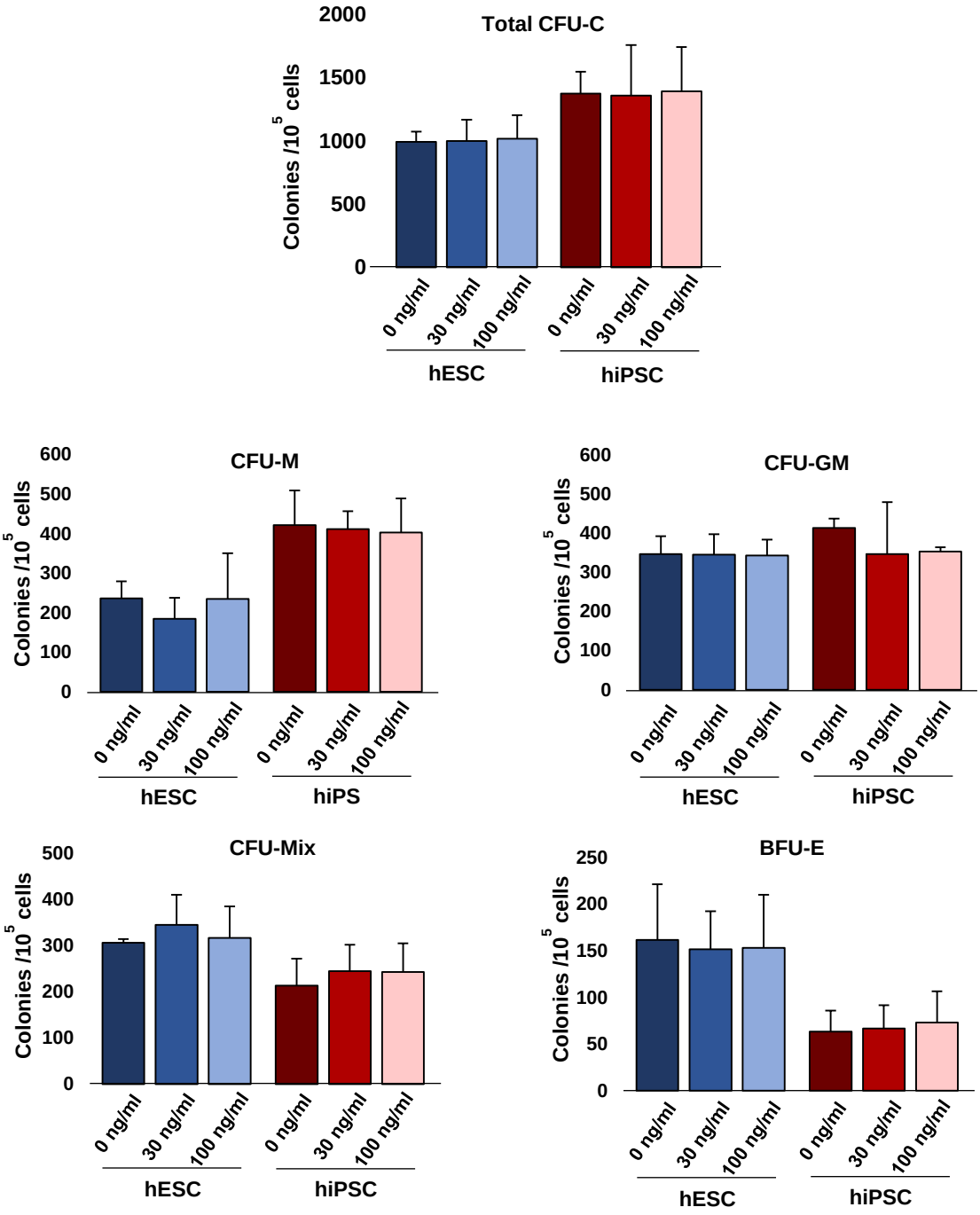

Figure 5.4: Haemopoietic colony assay of hESCs and hiPSCs treated with FGF17.
Cells were differentiated in the presence of 0, 30 and 100 ng/ml of FGF17 and analysed on day 10 of differentiation. Data represent 3 independent experiments. Error bars represent standard deviation.

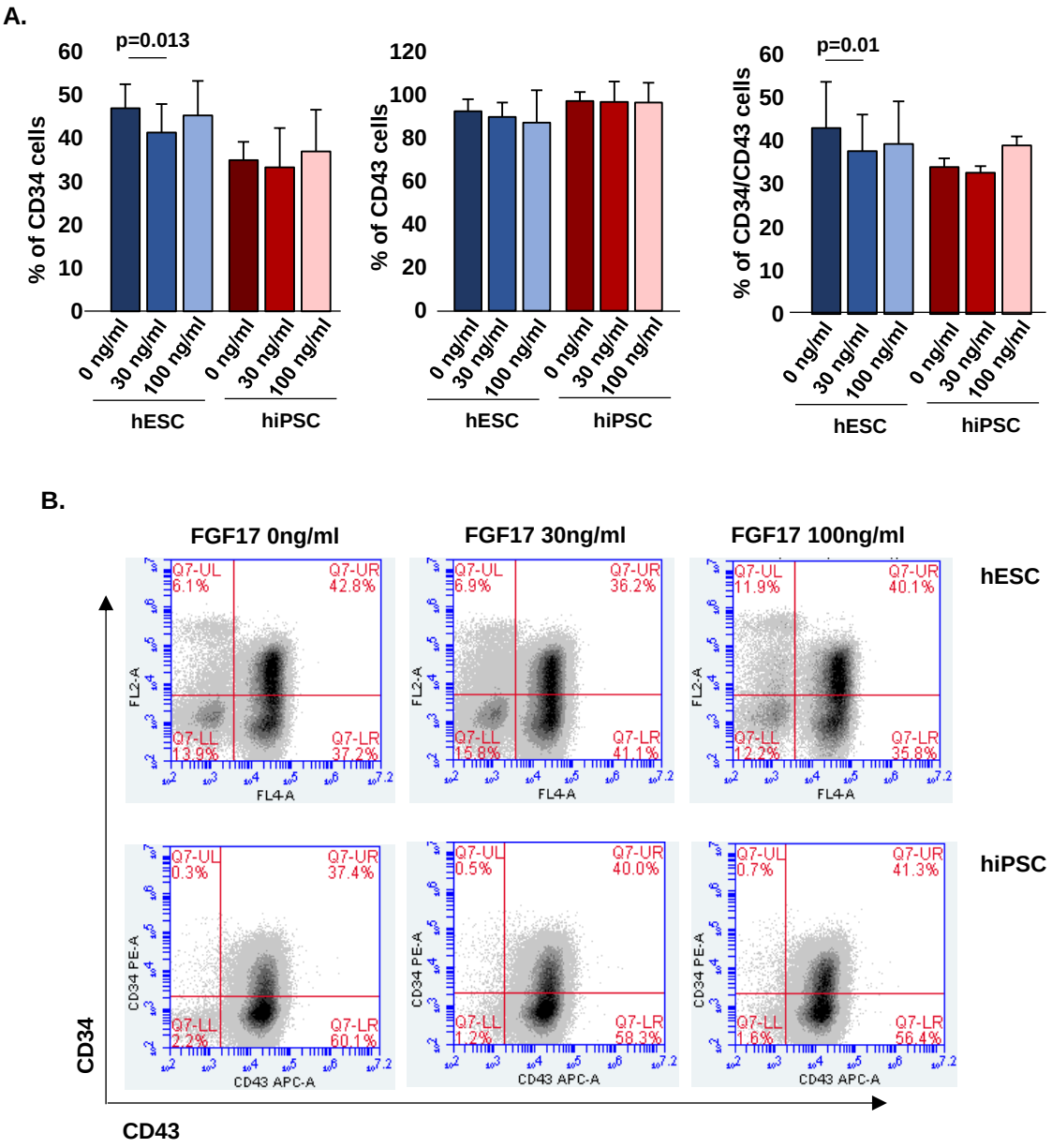

Figure 5.5: Flow cytometry analysis of day 10 hESCs and hiPSCs.
Human cells were differentiated in serum-free conditions in the presence of FGF17 and analysed for surface marker expression. **A.** Quantification of haemopoietic marker expression. Data represent 3 independent experiments. Error bars represent standard deviation. P values calculated with Student's t test. **B.** Representative scatter plots.

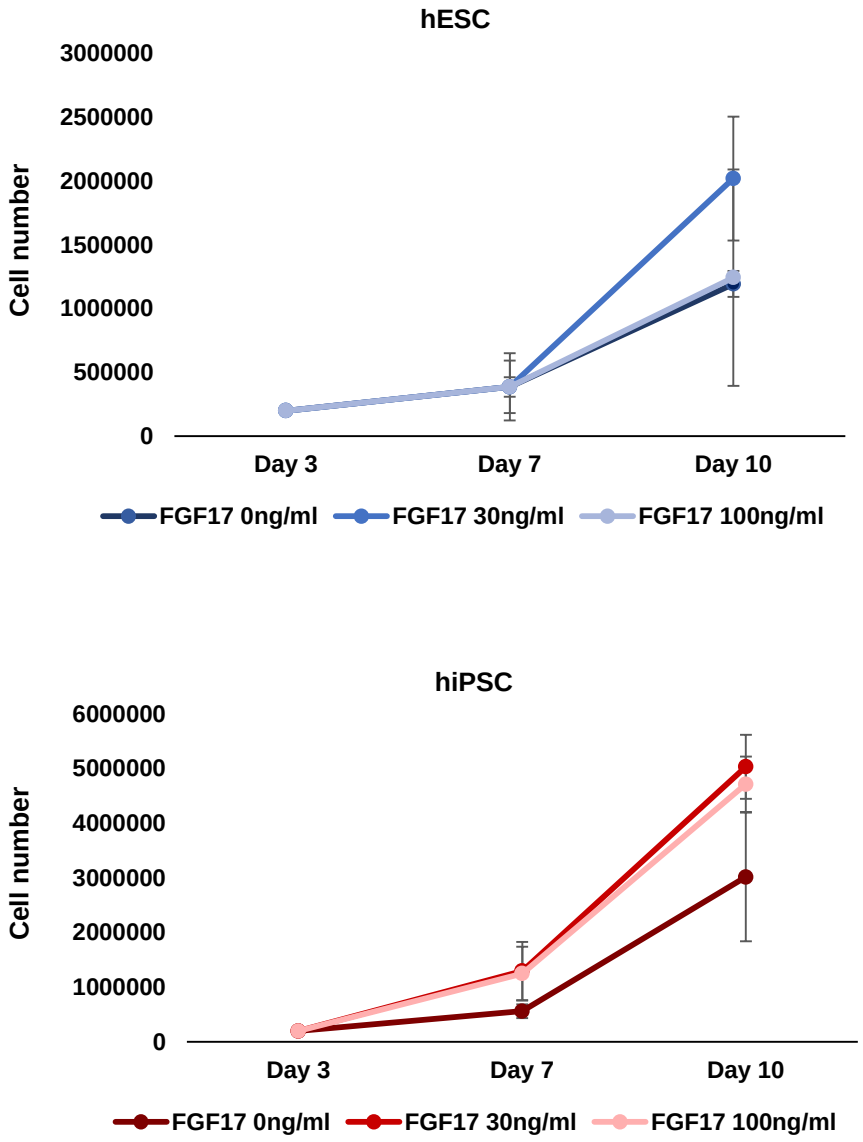

Figure 5.6: Cell growth of FGF17 treated hESCs and hiPSCs.
Cells were seeded at 2 x 10^5 density on day 3 of differentiation and counted on day 7 and day 10. Data represent 3 independent experiments $\pm$ sd.

5.4.2.2 RPSO3 decreases the haemopoietic differentiation of hESCs but not of hiPSCs

Cells were treated with 30 and 100ng/ml of RSPO3. Once again as in the case of FGF17 treatment, we observed that the two cell lines responded differently to RSPO3. In the case of hESCs, CFU-C assay showed that the differentiation was negatively affected by the increased concentration of RPSO3. Total colony numbers were significantly reduced at 100ng/ml, with the difference being more profound for CFU-M and CFU-Mix colonies (Figure 5.7). These data are in agreement with our results for RPSO3 effects on mESCs. In contrast, addition of RSPO3 in hiPSCs cultures had no effect on the differentiation of HPCs as all colony types gave comparable numbers between treated and untreated cells (Figure 5.7). Flow cytometry analysis demonstrated that in hESCs 100ng/ml of RSPO3 significantly reduced the expression of both CD34 and CD43 markers (Figure 5.8). Interestingly, we observed a significant decrease in the numbers of $CD34^{+}CD43^{+}$ progenitors not only at 100ng/ml but also at 30ng/ml of RSPO3. In agreement with the haemopoietic colony assay, addition of RSPO3 in hiPSCs did not result in any change in the expression of surface markers. We also monitored the cell expansion rates of the cells to look into any effects of RSPO3 on cell proliferation. No significant effect on cell proliferation was observed in either hESCs or iPSCs (Figure 5.9).

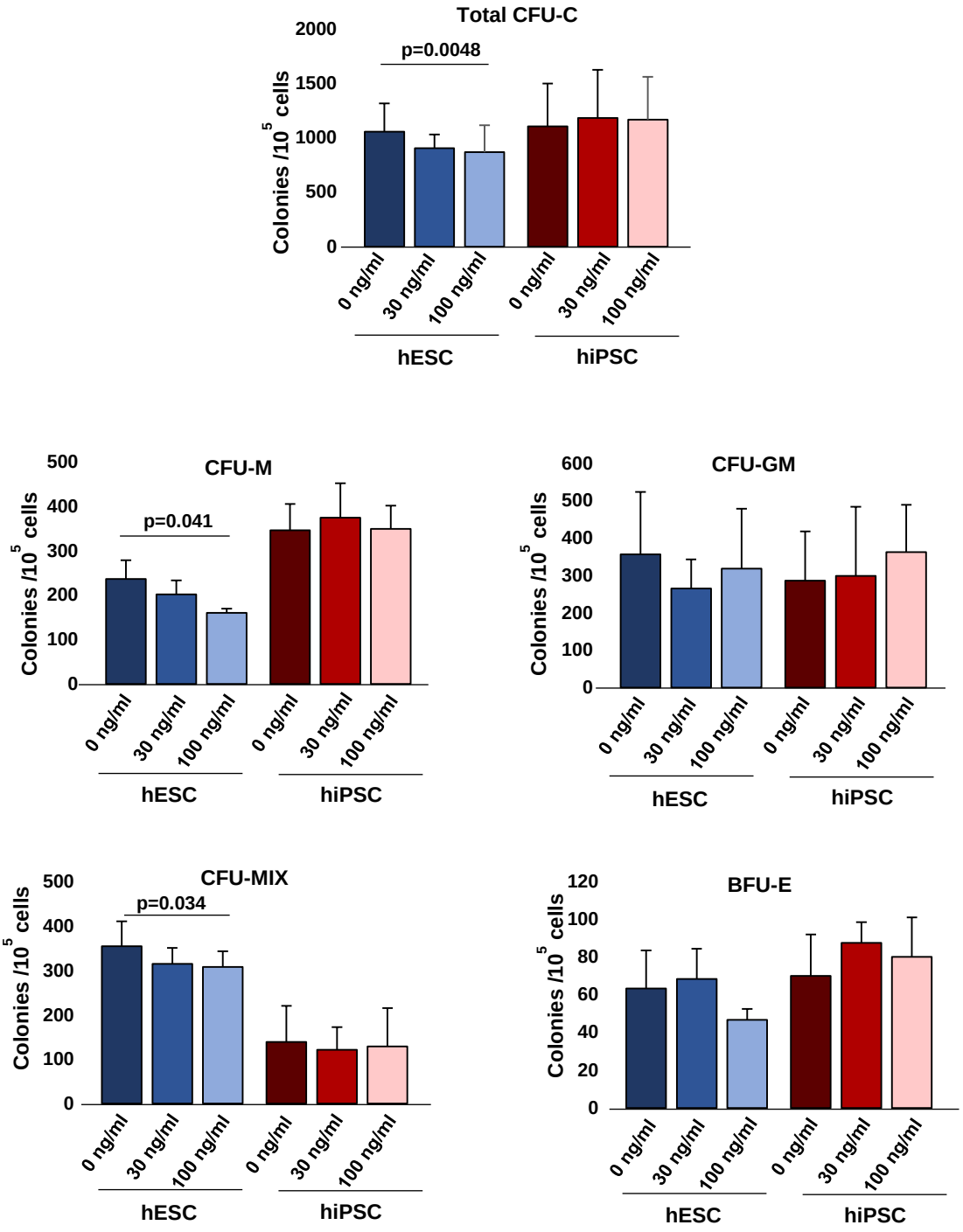

Figure 5.7: Number of haemopoietic colonies of RSPO3 treated hESCs and hiPSCs.
Cells were differentiated in serum-free conditions in the presence of 0, 30 and 100ng/ml RSPO3. Cells were collected and analysed on day 10. Data represent 3 and 4 independent experiments, error bars represent standard deviation.

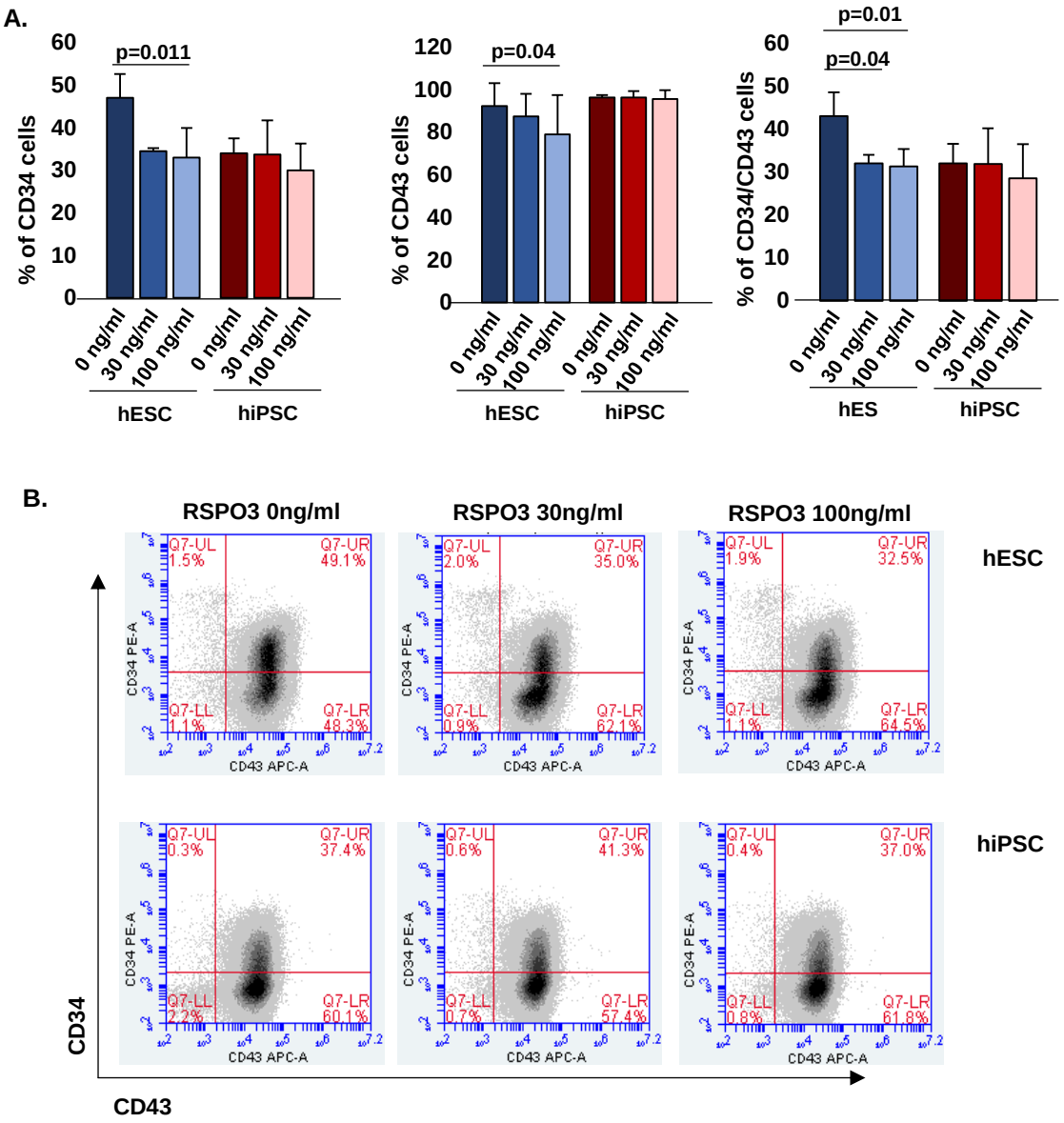

Figure 5.8: Flow cytometry analysis of surface markers in hESC and hiPSC lines treated with RSPO3.

A. Analysis of day 10 cells treated with 0, 30, 100ng/ml of RSPO3. Data represent 3 and 4 independent experiments +sd. P values calculated with Student's t test. **B.** Representative flow cytometry plots.

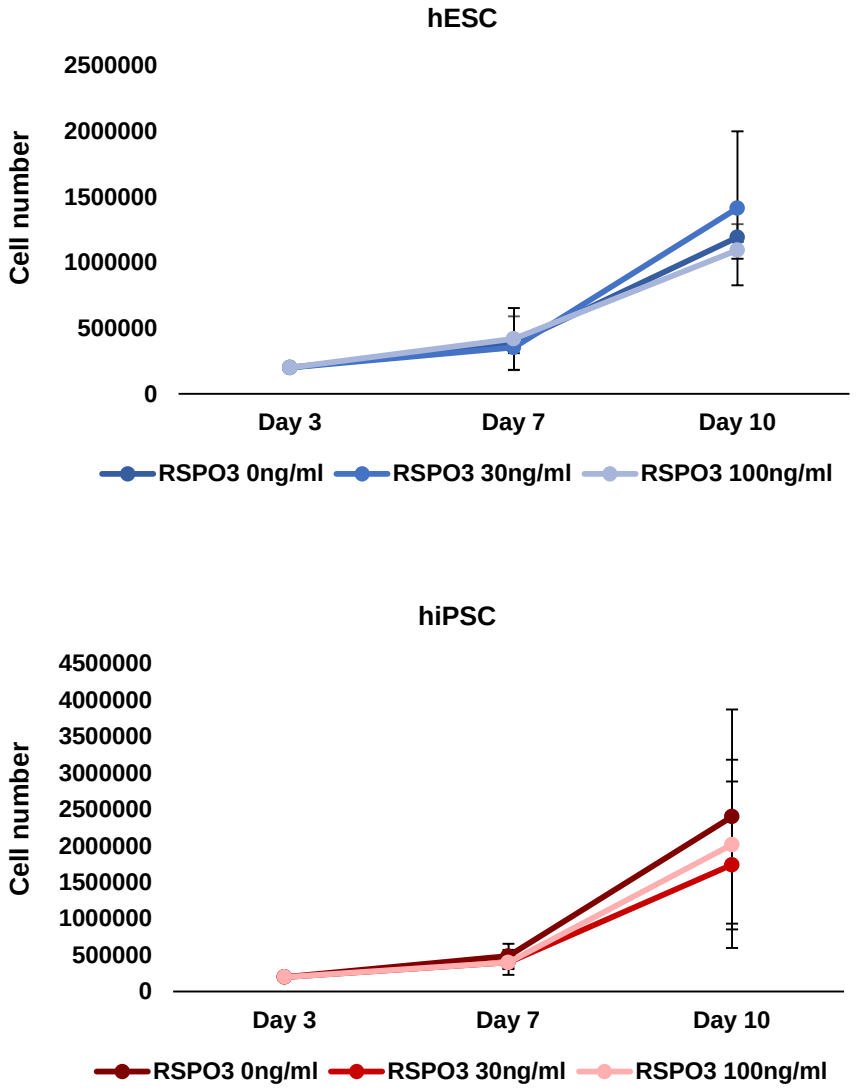

Figure 5.9: Cell expansion of hESCs and hiPSCs differentiated in the presence of RSPO3.

Cells were differentiated in serum-free conditions in the presence of RSPO3. On day 3 cells were seed at 2 x10^5 density and counted on day 7 and day 10. Data represent 3 and 4 independent experiments $\pm$ sd.

5.4.2.3 Apelin does not affect the haemopoietic differentiation of hESCs and hiPSCs

The last HOXB4 target we tested in our human differentiation system was Apelin. We tested the two Apelin isoforms, Apelin 13 and Apelin 36 at 100nM. Experiments on hESCs were performed by Dr Melany Jackson and Mrs Helen Taylor. As shown on Figure 5.10, haemopoietic colony forming assay did not detect any changes in the numbers or types of haemopoietic progenitors generated by either cell line in response to the two Apelin peptides. Although a decreasing trend in total colony numbers of hESCs was observed for both Apelin isoforms, the decrease was not statistically significant. In agreement with the CFU-C data, flow cytometry analysis showed that neither Apelin 13 nor Apelin 36 affected the cell differentiation. Surface markers were expressed in equal percentages for all treatments in the two cell populations (Figure 5.11). Nonetheless, the possibility that the Apelin peptides degraded too fast when added in culture before exerting their effects cannot be excluded.

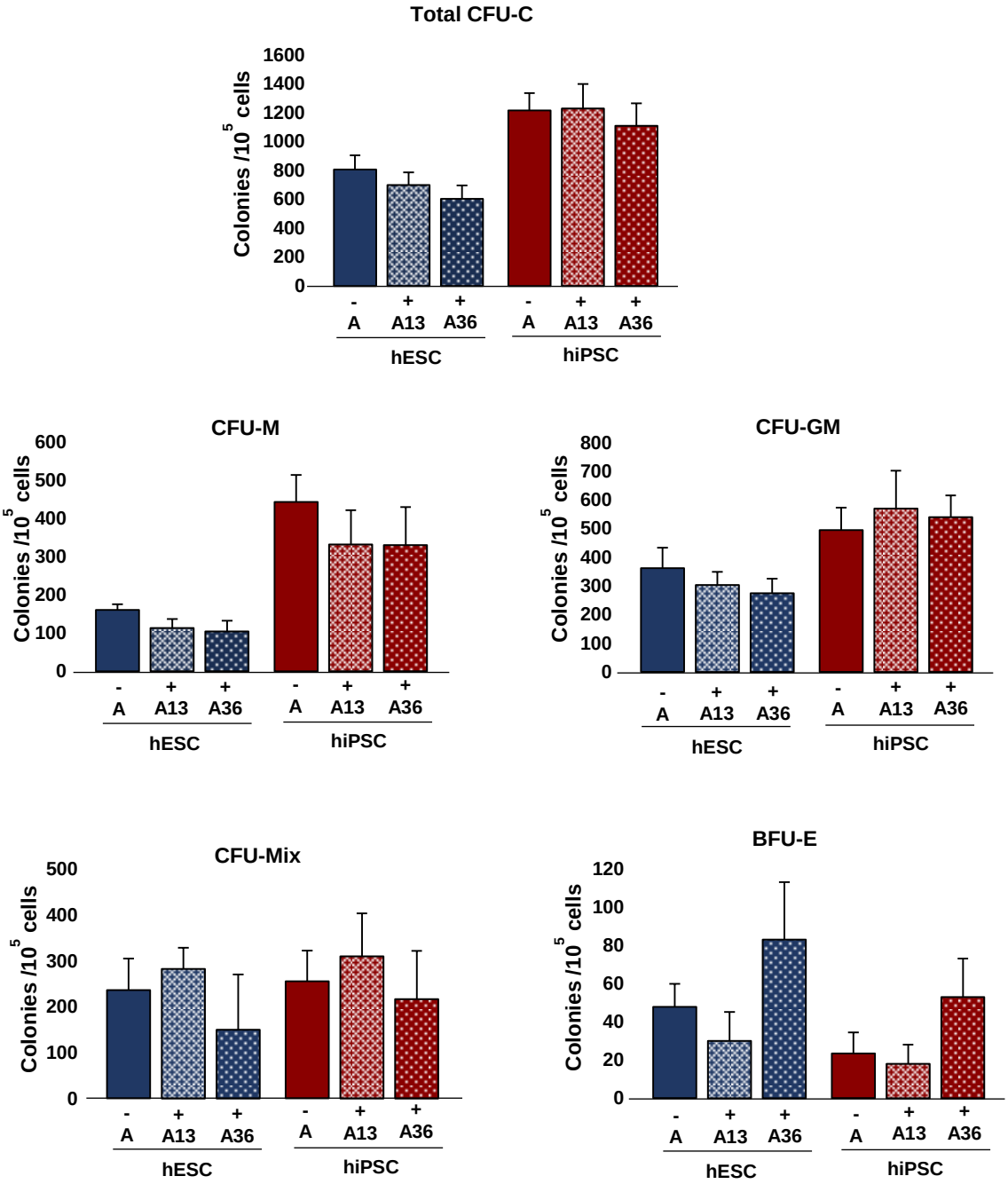

Figure 5.10: Number of haemopoietic colonies of Apelin treated hESCs and hiPSCs. Cells were differentiated in serum-free conditions in the presence of 100nM A13 or A36 and analysed on day 10. Data represent 5 and 3 independent experiments, error bars represent standard deviation.

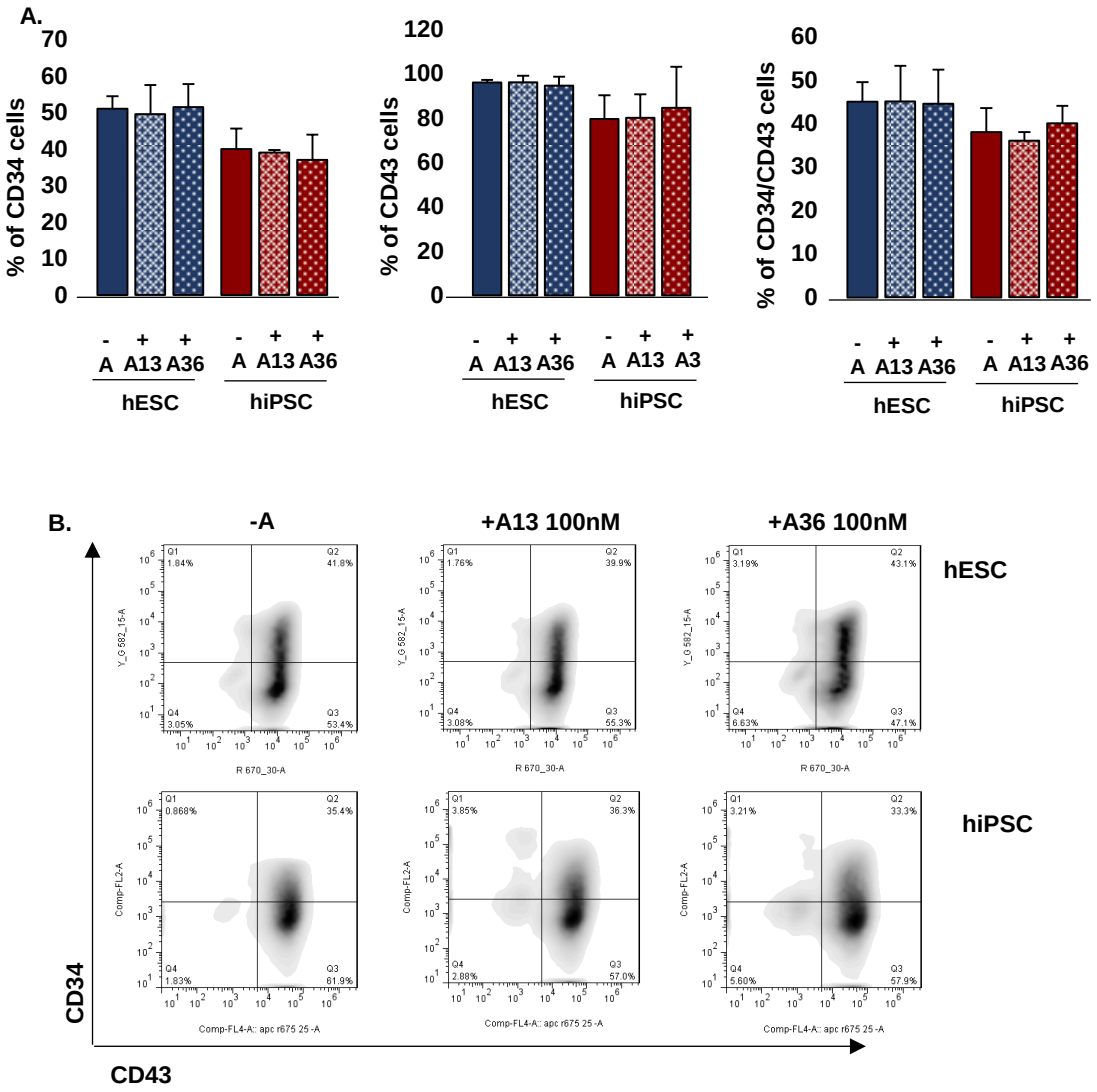

Figure 5.11: Flow cytometry analysis of surface markers in hESC and hiPSC lines treated with Apelin peptides.

A. Analysis of day 10 cells treated with 100nM of Apelin 13 (A13) or Apelin 36 (A36). Data represent 5 and 3 independent experiments +sd. P values calculated with Student's t test. **B.** Representative flow cytometry plots.

5.5 Conclusion

Human ESCs and iPSCs were differentiated to the haemopoietic lineage in well-defined, serum-free conditions. The two cell populations exhibited slight variations in their haemopoietic potential, nonetheless they followed a similar developmental pattern. When treated with secreted factors induced by HOXB4, the two cell populations responded differently not only compared to mouse ESCs but also to one another. Our data suggest:

- When cultured in serum-free conditions hESCs and hiPSCs follow a differentiation pattern similar to *in vivo* haemopoiesis. A transient wave of erythroid progenitors arises on day 7 and is then followed by a second wave of more definitive-type multipotent progenitors on day 10 (Figure 5.2, 5.3).

- Human PSC lines can exhibit lineage bias and variability in their haemopoietic efficiency even when differentiated under the same well-defined conditions (Figure 5.2, 5.3).

- Low concentration of FGF17 inhibits the haemopoietic differentiation of hESCs by decreasing the number of $CD34^+$ and $CD34^+CD43^+$ HPCs, while it does not affect the haemopoietic potential of hiPSCs (Figure 5.5).

- Increasing concentrations of RSPO3 reduce the CD34 and CD43 expressing HPCs capable of multilineage differentiation, derived from hESCs but not from hiPSCs (Figure 5.7, 5.8).

- Apelin peptides have no effect on the haemopoietic potential of neither hESCs nor hiPSCs (Figure 5.10, 5.11).

5.6 Discussion

5.6.1 Efficient differentiation of human ESC and iPSC lines to haemopoietic progenitors

In this chapter we aimed to assess the effects that specific secreted molecules have in the haemopoietic activity of human PSCs. We focused our experiments on factors that we had

shown to be regulated by HOXB4 and that we had tested in our mouse ESC differentiation system. To this end, we had to ensure that human HPCs could be efficiently generated in a well-defined culture system, and that this could provide a clear background to assess and control the presence of additional factors. By taking advantage of a robust serum-free and feeder-free erythroid differentiation protocol established in our lab we managed to track the development of human HPCs *in vitro.* We demonstrated that our differentiation protocol successfully recapitulates early embryonic haemopoiesis and that it is reproducible between cell lines.

Within the first 7 days of differentiation, our protocol generated a striking number of 1000 CFU-Cs per 10^5 cells plated in haemopoietic colony forming assay (Figure 5.2). More than 60% of the colonies were small bright red primitive erythroid colonies, termed EryP. Flow cytometry analysis demonstrated that 50-70% of day 7 cells expressed the erythroid marker CD235a (261), which also marked nearly the entire $CD34^+$ population (Figure 5.3). Therefore, our data clearly indicated that day 7 progenitors closely resemble primitive yolk sac erythropoiesis. On day 10 of analysis a second wave of definitive-type multipotent progenitors was observed. These progenitors could generate significantly increased numbers of CFU-M, CFU-GM and CFU-Mix colonies. Additionally, erythroid colonies had dramatically reduced in numbers, were of more mature type, BFU-E, large and with a darker red colour. CD235a marked a minor cell population, whereas, CD34 and CD43 were co-expressed in more than 35% of the HPC population.

In agreement with our data, extensive work by the Slukvin group, using co-culture with OP9 feeder cells, has demonstrated that hESC-derived multipotent lymphohaemopoietic progenitors reside within the $CD34^+CD43^+CD235a^-$ population of cells (157, 262, 263). Transplantation experiments, conducted by Dr Jennifer Easterbrook in our group, have shown that day 10 HPCs cannot reconstitute irradiated recipients. It is therefore possible that the day 10 HPCs generated in our system more closely resemble definitive erythro-myeloid progenitors (EMPs) rather than definitive HSCs. EMPs have broad erythroid and myeloid potential, distinct phenotype from primitive progenitors but lack lymphoid and long-term engraftment potential (264). Lymphoid *in vitro* differentiation of day 10 HPCs will clarify whether our cells represent EMPs or truly multipotent progenitors that still lack the ability to engraft. Nonetheless, considering that long-term engraftment has not been achieved yet, and that haemopoietic differentiation efficiency is measured mainly through colony formation and surface marker expression, our protocol has been found significantly more efficient in

generating $CD34^+$ CFU-Cs compared to most published human differentiation protocols using either feeder cell lines or haemopoietic cytokines (158, 161, 163, 174, 176, 180, 262).

The differentiation protocol was also tested in hiPSCs, which can potentially serve as an embryo-free, patient-specific alternative source for cell therapy of haematologic disorders. Consistent with previous published reports, we found that hiPSCs behaved similar to hESCs, followed the same maturation stages and could efficiently generate multipotent HPCs (157, 179, 180, 181, 260). Nonetheless, we did observe differences in the haemopoietic activity of the two cell lines. The hiPSCs gave rise to significantly less $CD34^+CD43^+$ cells which was reflected in reduced CFU-Mix colony numbers. Similar to our findings, a number of studies have shown that even though hiPSCs are capable of generating haemopoietic cells, they exhibit large variability. Feng and co-workers have demonstrated that haemato-endothelial progeny of hiPSC lines have increased apoptosis, and dramatically reduced expansion and colony forming activity compared to hESCs (265). Additionally, work by Choi and colleagues proved that hiPSC lines present intrinsic variability. While some hiPSC lines could generate more than 1500 CFUs per 10^5 cells, colony forming ability could get severely limited to less than 400 CFUs per 10^5 cells in some others (157). Cell line variability has also been a major issue in hESC lines, hindering their clinical potential (266, 267). Variability may be due to passage number, genetic background, quality of the embryos at derivation in the case of hESCs, or tissue of origin and epigenetic memory for hiPSCs. It therefore becomes evident that large-scale comparative studies are necessary for hPSCs to be reliably used in clinical applications. These studies will help identify and carefully select cell lines that stably reproduce and enhance the yield of the desired lineage. Currently more hiPSC lines are being tested in our lab to further investigate the reproducibility of our protocol and variations between cell lines.

5.6.2 Intraspecies and interspecies differences in the activity of HOXB4

It is only reasonable to assume that the cell line variability described above may have functional consequences in the testing of exogenous factors. Indeed we observed that hiPSCs and hESCs responded quite differently to the addition of secreted proteins encoded by HOXB4 target genes. Depending on the secreted protein and the concentration that was used, the haemopoietic differentiation of hESCs was affected differently. In contrast, none of the tested proteins had any effects on the differentiation output of hiPSCs. There have been numerous reports suggesting that there are marked genomic differences between hESCs and hiPSCs, which could explain the observed variability. A comprehensive genome-wide

analysis conducted by Chin and colleagues, which included gene expression, RNA and histone modification profiling, investigated differences between early and late-passage hESC and hiPSC lines. They concluded that hiPSCs are more similar to each other than to hESCs and that they should be considered a unique subtype of PSCs (268). Interestingly, among the differentially expressed genes identified in early-passage cells, was *HOXB4* together with a large number of genes from the *HOXA* and *HOXB* clusters. Expression of genes from the *HOXB* cluster was also found significantly different between late-passage hESCs and hiPSCs. Additionally, work by Doi and colleagues demonstrated that hESCs and hiPSCs could be distinguished by differential methylation of tissue-specific CpG island shores (269). More recently Bock and co-workers showed that there are no clear-cut differences in DNA methylation and gene expression among hESC and hiPSC lines. Nonetheless, they cannot be considered as one cell population because variation in hiPSC lines is more prevalent compared to hESCs (259). Notably epigenetic and transcriptional variation was observed in this study as well, for genes of the *HOX* family. It is thus probable that differences between our cell lines in the expression of endogenous HOXB4 and/or genes related to its function affect their *in vitro* behaviour and response to added factors.

Human iPSCs remained unaffected by all HOXB4 targets tested in this study, clearly indicating that their regulatory network is completely different to mouse ESCs. On the other hand human ESCs showed not only differences but also similarities to their mouse counterparts suggesting some conserved mechanisms between species. FGF17 had an enhancing effect on the haemopoietic differentiation of mouse ESCs, but in contrast, human ESCs were negatively affected by its presence in culture, where we observed a reduction in the portion of $CD34^+$ and $CD34^+CD43^+$ cells (Figure 5.5). Our cell expansion data indicated that the observed difference was not a secondary effect of FGF17 proliferative activity (Figure 5.6) as we would expect, based on the growth effects it has on human haemopoietic tumour cell lines (246). Addition of RSPO3 generated the same response in both species. HPCs reduced in numbers as RSPO3 concentration increased (Figure 5.7, 5.8). As already mentioned RSPO3 is an activator of Wnt signalling (238), and numerous reports have demonstrated the important role of Wnt signalling in hESC haemopoietic development (270, 271). Nonetheless, as described by Gertow and colleagues, correct timing of Wnt signalling induction is crucial for the differentiation outcome. In their study, use of WNT3A in the early steps of differentiation promoted mesoderm development and generation of haemopoietic colonies. However, addition of WNT3A in later stages inhibited haemopoietic development, and instead resulted in the formation of mesenchymal colonies (272). It is possible that the prolonged use of RSPO3 in our protocol led to the development of non-

haemopoietic cells, which could account for the observed reduction in haemopoietic activity. In the case of Apelin peptides, the haemopoietic activity of neither mouse nor human ESCs was altered (Figure 5.10, 5.11). However, Yu and colleagues have reported the enhanced growth of hESC-derived haemopoietic and endothelial cells upon addition of Apelin in the media (252). It is quite possible that fast degradation of Apelin peptides accounts for our negative results, and repetition of our experiments using an effective peptide delivery method can clarify the role of Apelin in haemopoiesis.

It can be concluded from our ESC data that despite being partly conserved, the activity of proteins induced by HOXB4 varies between species. Therefore, directly extrapolating data will not always be effective. To date species-specific differences in HOXB4 activity have only been assessed based on the functional readout of developing HPCs. Our data clearly indicate that in order to clarify HOXB4 role and mechanism of action in human HSC *in vitro* differentiation, we need to focus on the subcellular level. An inducible HOXB4-ER^{T2} system in hESCs has been developed in our group. It would be interesting to see whether the genes found upregulated in response to HOXB4 in our mouse expression analysis also respond to HOXB4 induction during human haemopoietic differentiation. Additionally, the possibility that different *HOX* genes are required for the generation of human repopulating HSCs cannot be excluded. Molecular profiling by Salvagiotto and co-workers has shown that *HOXA* cluster genes are significantly reduced, whereas, *HOXB* cluster genes are highly expressed in hESC-derived HPCs compared to fetal liver HSCs (273). A similar study by Shojaei and Menendez comparing hESC-derived HPCs and fetal blood HSCs supported the above idea. They found that *HOXB4* was not differentially expressed between the two cell populations, confirming their earlier data that ectopic *HOXB4* expression fails to enhance the *in vitro* clonogenic and *in vivo* engraftment activity of hESC-derived HPCs (274, 188). Interestingly, they also found the expression of other members of the *HOX* family, and most significantly *HOXA13*, reduced in the hESC-derived cells suggesting their involvement in HSC specification. In conclusion further large-scale comparative studies are required for the identification of novel factors driving the generation of definitive HSCs from hESCs *in vitro.*

Chapter 6 : Summary and Perspectives

6.1 Summary

Human pluripotent stem cells, hESCs and hiPSCs, are promising sources for the *in vitro* generation of haemopoietic stem cells required in clinical applications. Even though progenitors capable of differentiating into multiple lineages *in vitro* have been successfully generated, they still lack the ability to repopulate recipients *in vivo*. In the mouse, the transcription factor HOXB4 has been shown to efficiently confer repopulating ability to embryonic and ESC-derived HSCs. Previous work in our group has suggested that this is achieved through a paracrine mechanism, where a supportive niche environment induced by HOXB4 secretes the necessary factors to promote HSC development. Even though the mouse and human haemopoietic systems are highly conserved, HOXB4 has not been efficient in conferring repopulating activity in human PSC-derived haemopoietic cells and studies looking into the underlying regulators are limited. In this study we investigated the suggested HOXB4 paracrine activity and how it differs between species. Particularly we assessed the effects that various secreted proteins, which we identified as HOXB4 targets, have in the haemopoietic differentiation of mouse and human PSCs.

We developed a serum-free and feeder-free mouse differentiation protocol that provided a clear background in which to monitor any effects induced by the tested proteins. We observed that this new protocol could significantly enhance the generation of haemopoietic cells compared to our standard serum-based protocol. We noted a 10-fold increase in the numbers of colony forming cells which were mainly characterised by the co-expression of CD41/CD144 or CD41/c-Kit. We used this protocol to characterise the more recently derived mouse EpiSCs. EpiSCs exhibit a more primed developmental state compared to mouse ESCs and more similarities to human ESCs, which can possibly render them an improved model system. Time-course analysis of the differentiation process revealed that EpiSCs generated colony forming cells at earlier time point thus proving they can progress faster to the haemopoietic lineage compared to mESCs. Nonetheless, mESC haemopoietic output was not exceeded. Surface marker analysis confirmed there were no significant qualitative differences between the two cell types as EpiSCs also gave rise mainly to $CD41^{+}CD144^{+}$ and $CD41^{+}$c-Kit^{+} cells. We concluded that our serum-free protocol is reproducible between cell lines, and it can efficiently generate HPCs which phenotypically resemble embryonic HSCs. Additionally, we demonstrated that although EpiSCs exhibit a developmental advantage over mESCs due to their primed state, they generate HPCs with the same efficiency.

Mouse ESCs were used in the serum-free protocol to test a number of secreted factors encoded by HOXB4 target genes, whose role in haemopoiesis had not been studied before. We observed that each tested factor had a different effect on the differentiation of the cells suggesting a complex regulatory network of HOXB4 activity. High concentrations of FGF17 increased the portion of cells expressing the surface marker c-Kit, leading to increased colony forming activity. In contrast, increasing concentrations of RSPO3 reduced the generation of $CD41^+$ cells, significantly decreasing the numbers of CFU-Cs. Addition of Apelin peptides did not affect either positively or negatively the differentiation of mESCs. Finally, the combined use of the secreted proteins did not increase the haemopoietic activity of the cells, as did the overexpression of HOXB4. Our data demonstrated that our well-defined protocol is highly efficient in detecting even slight changes caused by added factors. Regarding the paracrine activity of HOXB4 it is suggested that either a larger number of secreted factors are needed, or that a better fine-tuning of their timing and concentration is required. Given the complexity of *in vivo* niches it is also possible that direct cell contact with the supportive stromal population in combination with the soluble proteins is necessary for the full effects of HOXB4 to be seen.

We followed a similar strategy to assess the effects of the above factors in the haemopoietic activity of human PSCs. We established a serum-free and feeder-free protocol for the differentiation of human cells, which we used to monitor the differentiation of a hESC and a hiPSC line, in order to also examine variation between cell lines. When differentiated the two cell lines followed a similar development pattern. An initial wave of primitive $CD34^+CD235a^+$ erythroid progenitors was followed by definitive-type $CD34^+CD43^+$ multipotent HPCs. However, addition of secreted proteins encoded by HOXB4 revealed that there are marked differences between the two cell lines. In the case of hiPSCs none of the tested factors had any effect on the differentiation outcome. In contrast hESCs were affected variably depending on the added protein. FGF17 resulted in reduced percentage of $CD34^+$ cells. A reduction in the numbers of $CD34^+CD43^+$ colony forming cells was noted upon RSPO3 addition. We did not observe any changes with the use of Apelin peptides. We concluded that mouse and human paracrine HOXB4 activity is only partly conserved, therefore attention is needed when extrapolating data. More importantly, even within a species cell line variation may lead to different results stressing even more the need for comparative studies.

6.2 Perspectives

The data presented in this thesis not only answer some of our questions regarding HOXB4 activity, but also raise some new ones worthy of further investigation. Additionally, some important conclusions are drawn relating to the current methodologies followed in human PSC research and how they need to be adapted in the future.

6.2.1 Xenogeneic-free generation of human HSCs

One of the major hurdles to overcome before applying human PSC-based therapy in the clinic is the safety of culture conditions (275). With the increasingly tight regulatory guidelines on PSC-based therapy, the need for xenogeneic-free culture conditions, which avoid the risk of infections and immune reactions by animal products, is heightened. We established serum-free and feeder-free conditions, and managed to efficiently generate mouse and human haemopoietic cells. More importantly, the use of these well-controlled protocols allowed us to reliably reproduce our data between cell lines from both species, and examine the effects of additional factors. Therefore, the application of xeno-free conditions can provide a reliable and controllable model system for scientific research which will allow the generation of clinical grade HSCs in the future.

As indicated by our data in order to generate human HSCs with long-term multilineage repopulating ability addition of soluble factors in the culture media may not be sufficient. It will probably be necessary to develop culture systems that closely resemble the human *in vivo* haemopoietic niche environment. This is achievable through the use of supportive feeder cell lines. Currently, however, the established feeder cell lines used for human differentiation are of mouse origin, introducing animal products in culture. Although some efforts have been made to derive human feeder lines, there are no established cell lines shown to reproducibly support human haemopoiesis (162, 163). It is therefore essential to focus future research on the generation of supportive lines derived from human haemopoietic tissues. The use of human feeders in combination with serum-free media will provide the necessary cues for specification of clinical grade human HSCs, which will be easily collected through cell sorting assays.

6.2.2 Combined mouse and human studies

For decades the mouse has been an indispensable model system for studying *in vivo* and *in vitro* haemopoiesis. Using mouse ESCs, the cellular and molecular developmental principles that control HSC specification have been identified. Due to the high conservation of the haemopoietic system between mammals, the information gained in the mouse has helped in recognising critical regulatory pathways in human. Nonetheless, species-specific differences have also led to conflicting results, which impact clinical translation. We evaluated the activity of HOXB4 mediators in ESC haemopoietic differentiation *in vitro*, and identified such interspecies differences.

HOXB4 is only one example of the many differences in the molecular regulation, lineage commitment, phenotype and function between mouse and human haemopoietic cells (276, 277). Apart from the evolutionary divergence of the two species, mouse strains used in research are inbred. Therefore the results gained in these genetically homogeneous strains may not apply in precisely the same way in the heterogeneous human population. Continuous advances in the field of human PSCs have many times allowed scientists to complement the knowledge gained in the mouse. Undoubtedly the mouse system will continue being absolutely essential in deciphering haemopoiesis. Nonetheless, in our view, parallel studies between the two organisms are absolutely critical in understanding how this information can be applied in humans. Research studies need to carefully interpret data and to clearly state findings not applicable in the human system. In the future more combined mouse and human studies will lead to more efficient therapeutic applications.

6.2.3 HOXB4 use in HSC differentiation cultures

We aimed to identify soluble mediators that can substitute HOXB4 overexpression in haemopoietic differentiation cultures. We found that even though some of the tested proteins could affect the haemopoietic output, they could not enhance HPC generation to the same level as HOXB4 overexpression. It is possible that more proteins, and improved fine-tuning of the quantity and time they are added in culture is needed. Nonetheless, this approach can be proven too complicated and uneconomic. Additionally, it is possible that, if indeed HOXB4 promotes the formation of a haemopoiesis-supportive population, direct cell interactions are required. The generation of a HOXB4-overexpressing stromal cell line will ultimately answer our questions. Since HOXB4 expression levels have been shown to be critical for the differentiation outcome (215, 278, 279), it is possible that multiple cell lines with different HOXB4 expression levels will need to be generated to identify the optimal

level for HSC development. The fact that human HOXB4-expressing mesenchymal cell lines can be derived *in vitro* (240), is of critical importance as they can ensure clinically safe culture conditions free of animal products. Some may argue against the safety of using genetically modified cells in clinical applications. An alternative to genetic manipulation would be the addition of recombinant HOXB4 proteins in culture. Initial efforts have been made towards this direction with promising results (214). Further studies addressing the stability and the amount of the administered protein could enhance the generation of functional HSCs.

6.2.4 *In vivo* functional evaluation of HPCs

Secreted proteins encoded by HOXB4 target genes were use in differentiation cultures to assess whether they can mediate HOXB4 activity. We measured the effects that the tested proteins had in HPC generation using haemopoietic colony forming assay and flow cytometry analysis. Although valuable assays in measuring haemopoietic potential, they are not sufficient to measure the most important aspect of HSC activity: long-term multilineage repopulation. As scientific interest has focused on HOXB4 due to the repopulating ability it confers to mouse cells, *in vivo* repopulating assays are important in clarifying its activity in human cells. It is possible that the secreted factors we added in culture may have a qualitative rather than quantitative effect on differentiating cells. Therefore, even if there are no changes in the numbers of generated HPCs, the tested factors may change their engraftment potential. It would be interesting to address this possibility by transplanting our HPCs to irradiated mice and measuring their long-term engraftment potential.

6.2.5 Involvement of other factors in human HSC development

Although the ability of HOXB4 to confer repopulating activity to mouse ESC-derived haemopoietic cells has been repeatedly reported, HOXB4 success in human cells remains limited. This discrepancy may be due to the fact that the molecular regulators and signalling pathways of definitive haemopoiesis differ between mouse and human. Our results support this concept as none of the tested factors had enhancing effects in hESC or hiPSC differentiation. It is possible that even though HOXB4 plays a central role in human haemopoietic development, factors other than HOXB4 are needed for the full maturation of human PSCs into definitive HSCs.

Gene expression profiling studies comparing ESC-derived and *in vivo* derived haemopoietic progenitors point to that direction. In one study HOXB4 was found overexpressed in ESC-derived cells compared to HSCs from *in vivo* sources (274). Additionally, other members of the *HOXA* and *HOXB* clusters have been found underexpressed in ESC-derived HPCs suggesting their involvement in the maturation of HSCs (273, 274). Indeed many members of the *HOX* gene family are expressed in adult HSCs and have been shown to have redundant functions. Therefore, the combined activity of *HOX* genes may be required for fully functional human HSCs to develop. Additionally, in the above studies *in vivo* HSCs overexpressed a number of genes associated with HSC self-renewal and survival, such as *EZH2, MEIS1, MLL, ARHGAP1, ETV6*, and *HLF*, some of which are known upstream HOX regulators. In conclusion, while the involvement of HOXB4 in human haemopoiesis needs further clarification, future studies will need to address the role of other genes in establishing hPSC-derived cells with *in vivo* reconstituting activity.

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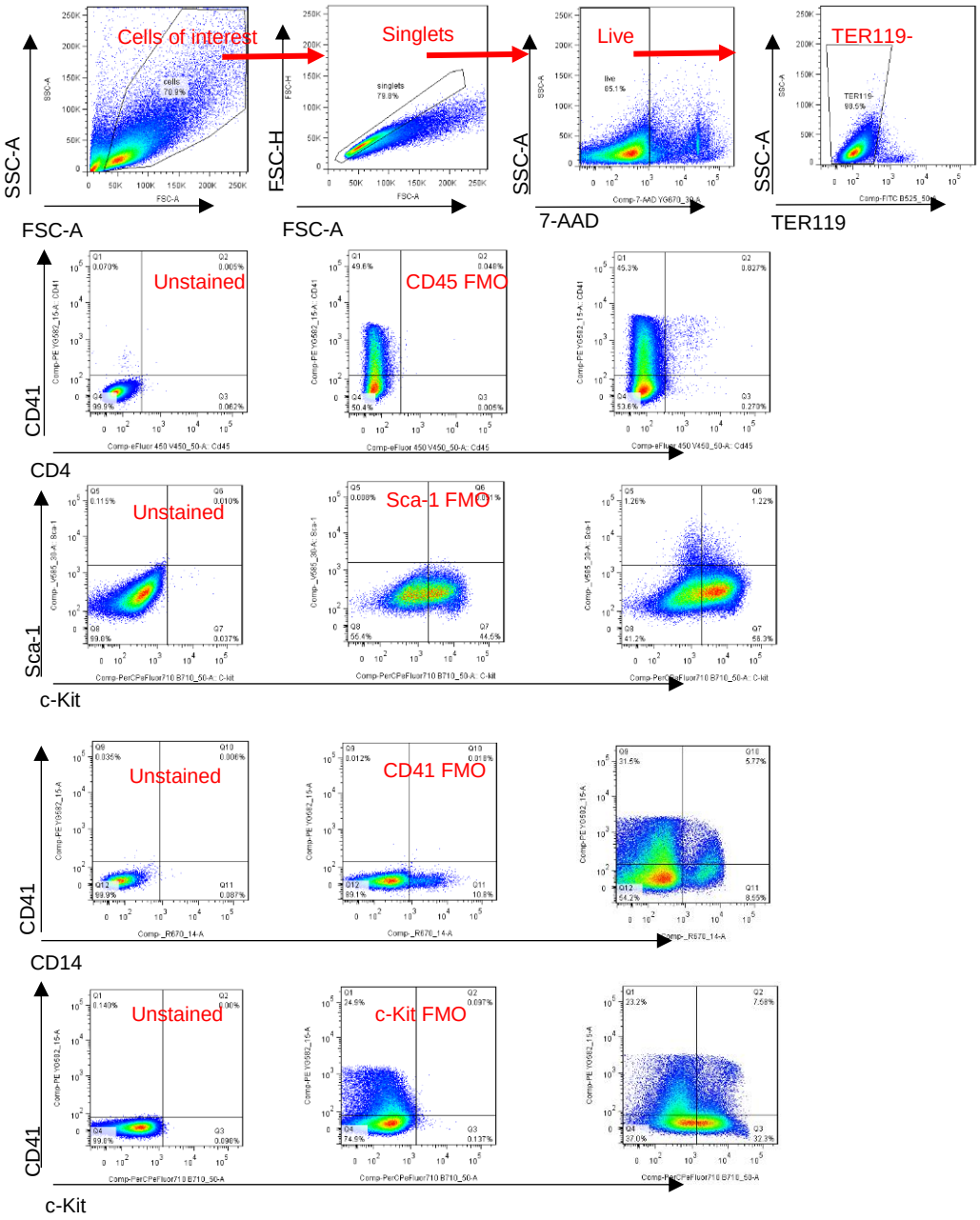
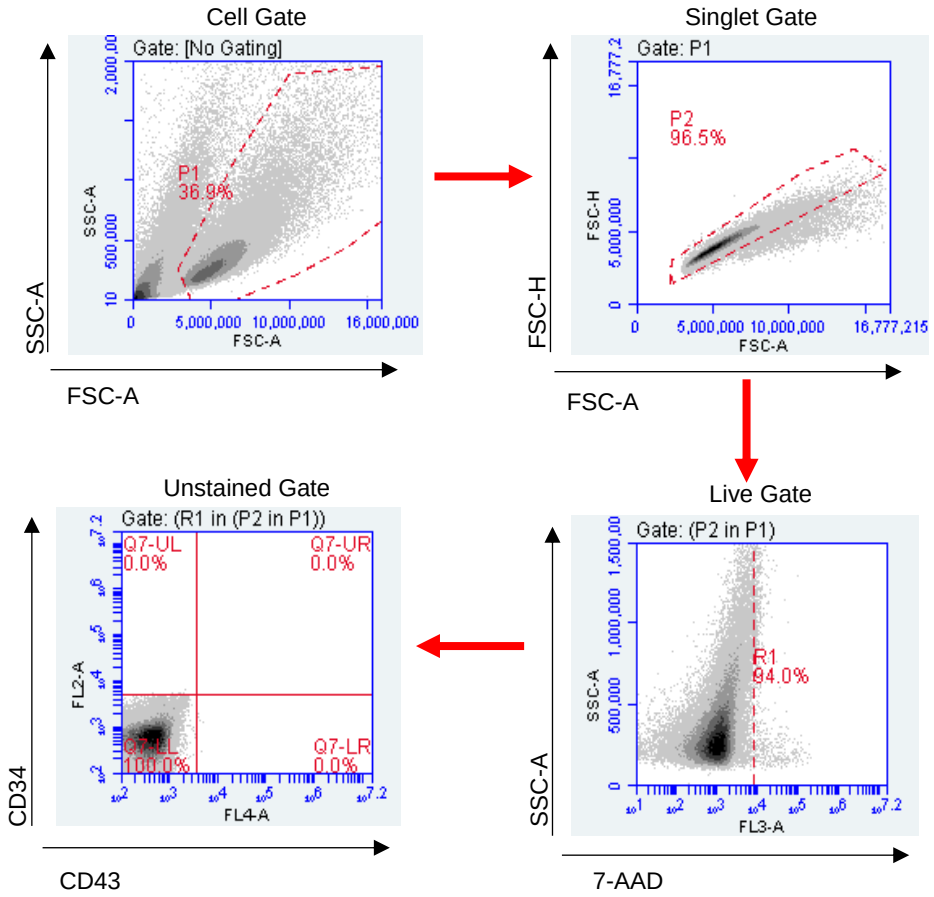
Appendix

Gene	Forward primer	Probe	Reverse primer
Tbp	GGGGAGCTGTGATGTGAAGT	SYBR Green	CCAGGAAATAATTCTGGCTCA
Oct4	GGCGTTCTCTTTGGAAAGGTGTTC	SYBR Green	CTCGAACCACATCCTTCTCT
Sox2	GGCGGCAACCAGAAGAACAG	SYBR Green	GCTTGGCCTCGTCGATGAAC
Nanog	CCTCCAGCAGATGCAAGAA	SYBR Green	GCTTGCACTTCATCCTTTGG
Rex1	ACGAGTGGCAGTTTCTTCTTGGGA	SYBR Green	TATGACTCACTTCCAGGGGGCACT
Klf4	GGCGAGAAACCTTACCACTGT	SYBR Green	TACTGAACTCTCTCTCCTGGCA
Fgf5	TTGCGACCCAGGAGCTTAAT	SYBR Green	CTACGCCTCTTTATTGCAGC
Bry	CCAAGGACAGAGAGACGGCT	SYBR Green	AGTAGGCATGTTCCAAGGGC
HoxB4	CCTGGATGCGCAAAGTTCA	CCAGCAGGTCCTGGAG	GTCAGGTAGCGATTGTAGTGAAACTC
Rspo3	AGGCGCCAGCGAAGAAT	ATCCTAATGTCAGTCAAGG	CACGTTGCACAGCCTCCTT
Apln	CCGAGTTGCAGCATGAATC	UPL 106	GCAACATCAGTGGCACTCC
Cer1	GACTGTGCCCTTCAACCAG	UPL 105	AGCAGTGGGAGCAGAAGC
Ntn1	GCAAGCTGAAGATGAACATGA	UPL 56	CTTTGTCGGCCTTCAGGAT
Fst	AAGCATTCTGGATCTTGCAACT	UPL 63	GATAGGAAAGCTGTAGTCCTGGTC
Sema3f	GCCTGCTACCCCTATCCAG	UPL 5	CCGGTGGCCTTAAGTTCTTT
Fgf17	TATGAACAAGAGGGGCAAGC	UPL 103	CTCGGTGAACACGCAGTCT
Kitl	AGCGCTGCCTTTCCTTATG	UPL 68	CCTTGGTTTTGACAAGAGGATT
Hprt	GCTCGAGATGTCATGAAGGAGA	AAAGAACTTATAGCCCCCCTTGA	CCATCACATTGTGGCCCTCTGTGTG

Table S 1: List of qRT-PCR primers and probes used in this project



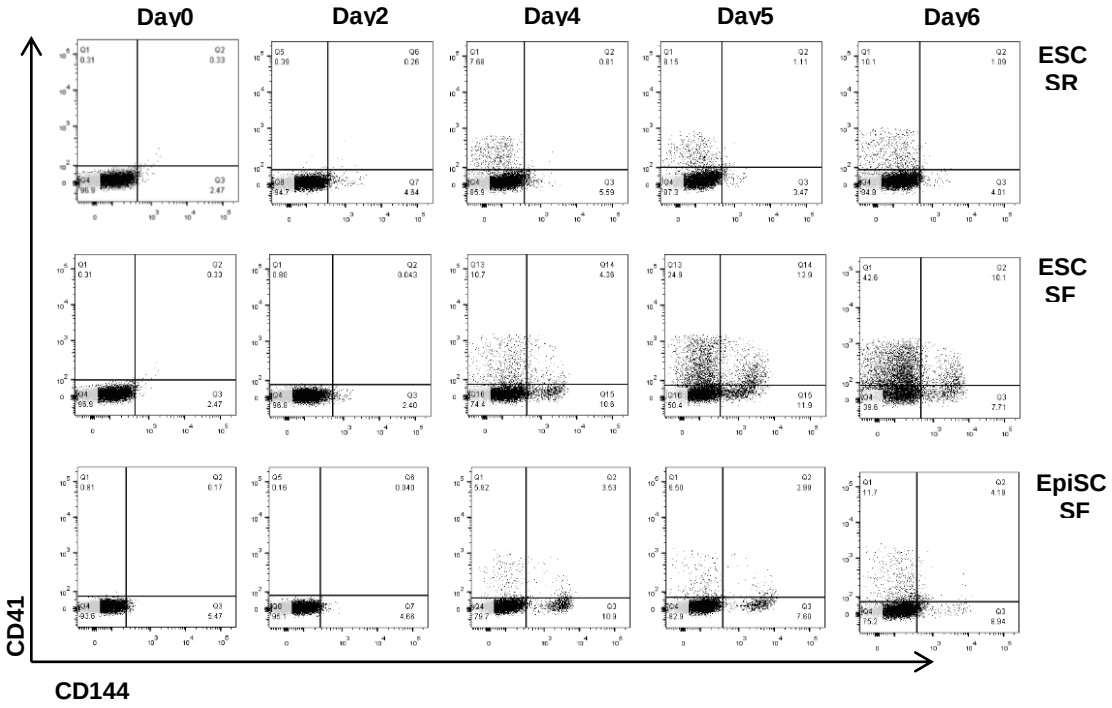
Supplementary Figure S 1: Gating strategy for flow cytometry analysis of mouse cells. The population of interest (SSC-A vs FSC-A) was gated for singlets (FSC-H vs FSC-A) and live cells with 7-AAD staining (SSC-A vs 7-AAD). The population was further gated on the TER119 negative cells (SSC-A vs TER119) to exclude autofluorescence from red cells. Unstained controls combined with FMOs were then used to gate on marker expression.



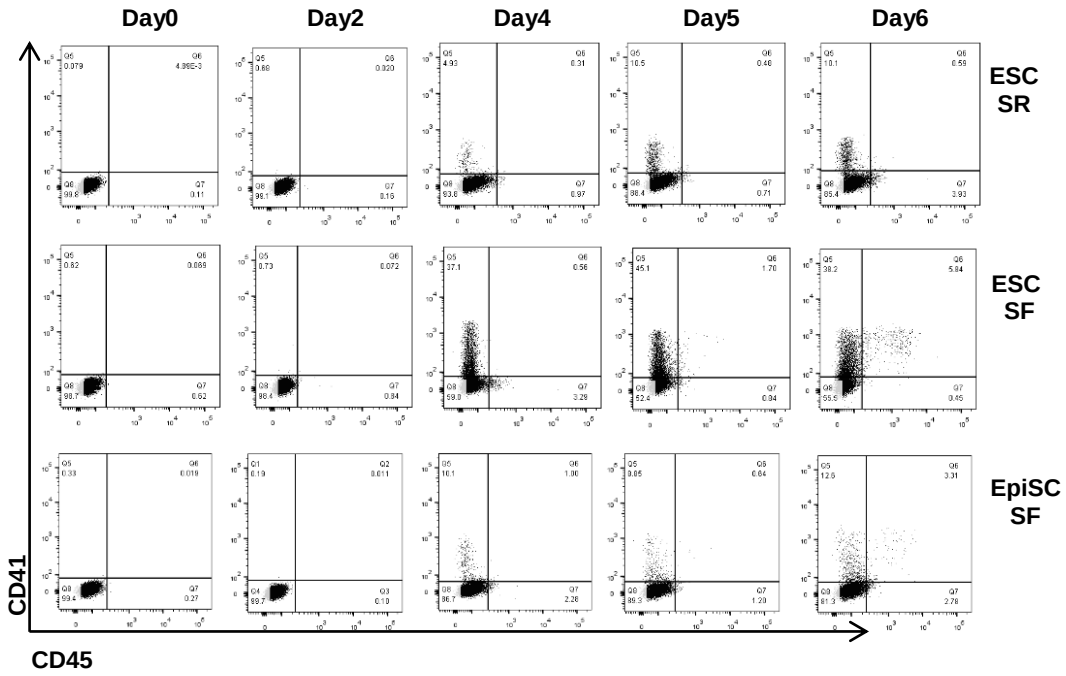
Supplementary Figure S 2: Gating strategy for flow cytometry analysis of human PSCs.
Cells were first gated for the population of interest (SSC-A vs FSC-A) and singlets (FSC-H vs FSC-A). The population of interest was further analysed for their uptake of 7-AAD to determine live versus dead cells and an unstained control was used to further gate the CD34 and CD43 population.

Antibody	Fluorochrome	Supplier
Anti-mouse CD144	eFluor450	eBiosciences
Anti-mouse CD144	Ef660	eBiosciences
Anti-mouse CD41	PE	eBiosciences
Anti-mouse CD45	V450	BD Biosciences
Anti-mouse CD45	FITC	eBiosciences
Anti-mouse Sca-1	V500	BD Biosciences
Anti-mouse CD117	APC	eBiosciences
Anti-mouse CD117	PerCP-eFlour710	eBiosciences
Anti-mouse TER-119	FITC	eBiosciences
Anti-human CD43	APC	eBiosciences
Anti-human CD34	PE	eBiosciences
Anti-human CD235a	FITC	BD Biosciences

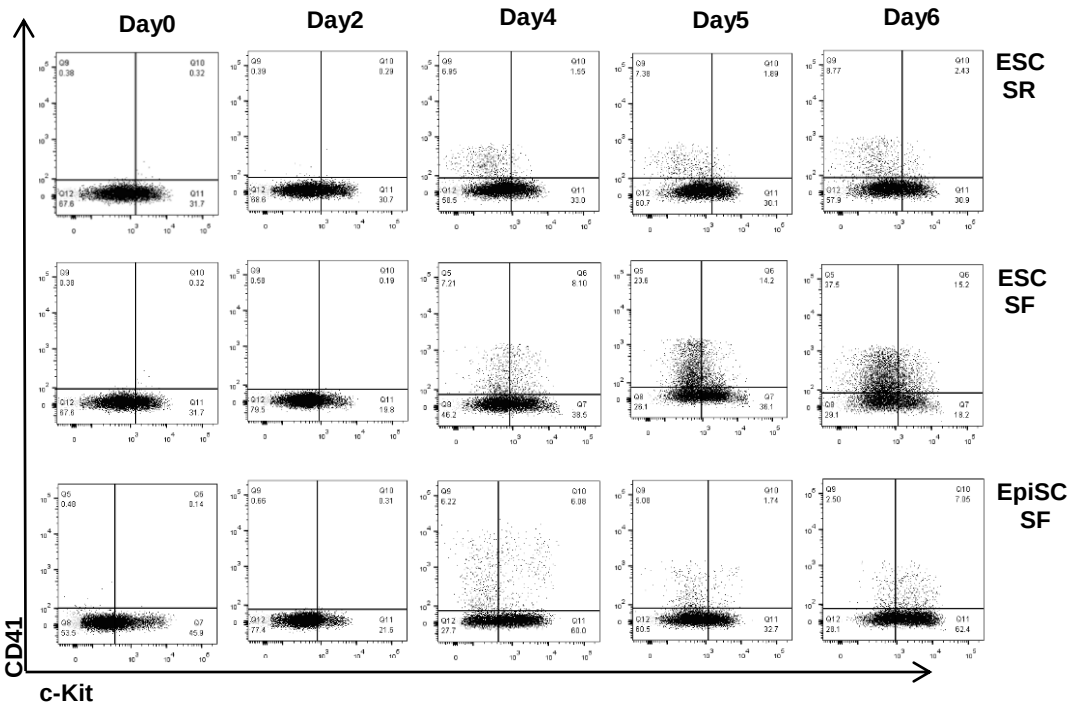
Table S 2: List of flow cytometry antibodies used in this project.



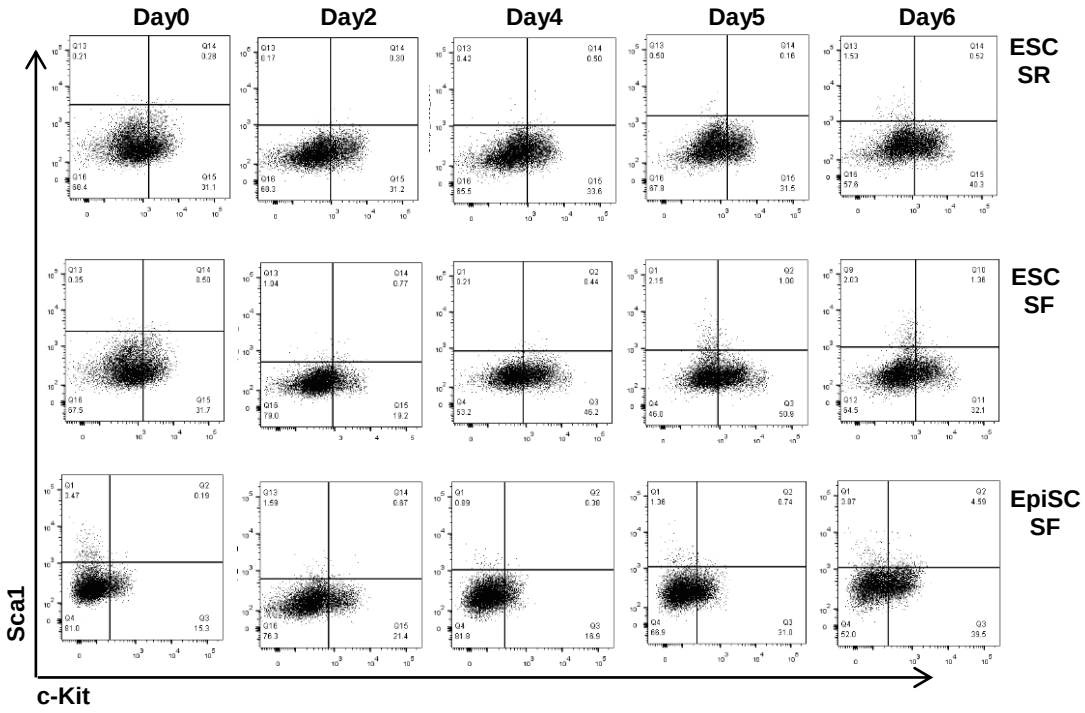
Supplementary Figure S 3: Expression of CD41/CD144 during mouse haemopoietic differentiation.
Representative flow cytometry scatter plots of haemopoietic differentiation between day 0 to day 6. SR: serum-based conditions, SF: serum-free conditions.



Supplementary Figure S 4: Expression of CD41/CD45 during haemopoietic differentiation.
Representative flow cytometry scatter plots of haemopoietic differentiation between day 0 to day 6. SR: serum-based conditions, SF: serum-free conditions.

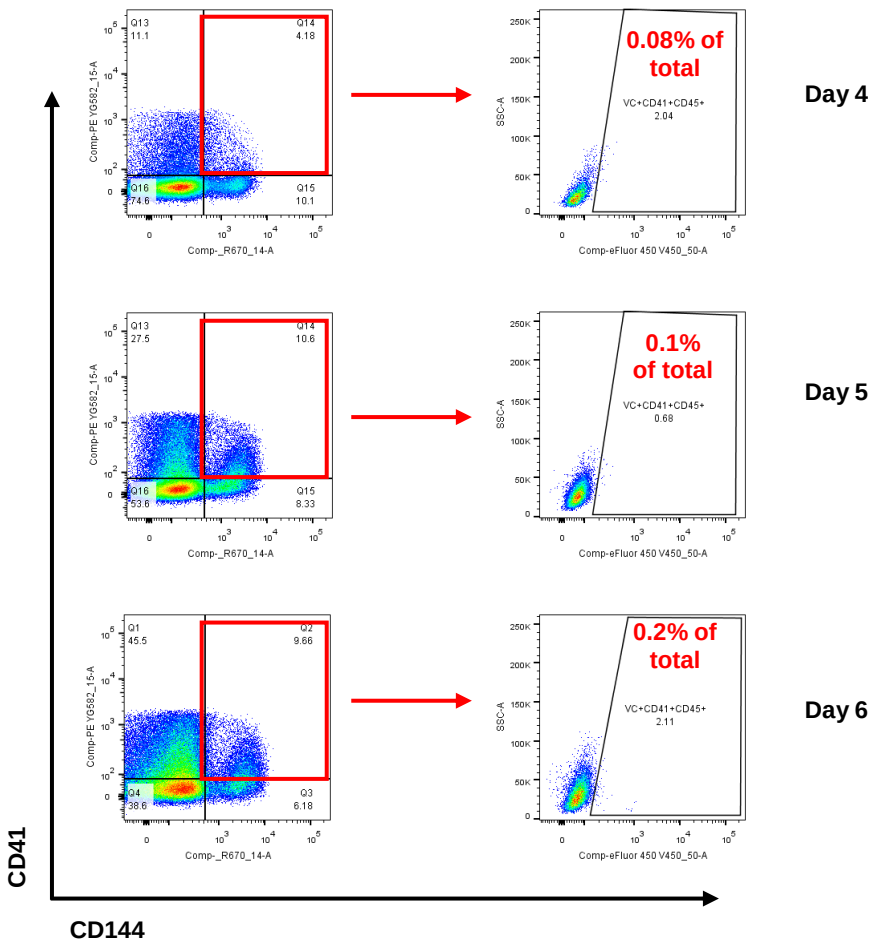


Supplementary Figure S 5: Expression of CD41/c-Kit during mouse haemopoietic differentiation.
Representative flow cytometry scatter plots of haemopoietic differentiation between day 0 to day 6. SR: serum-based conditions, SF: serum-free conditions.



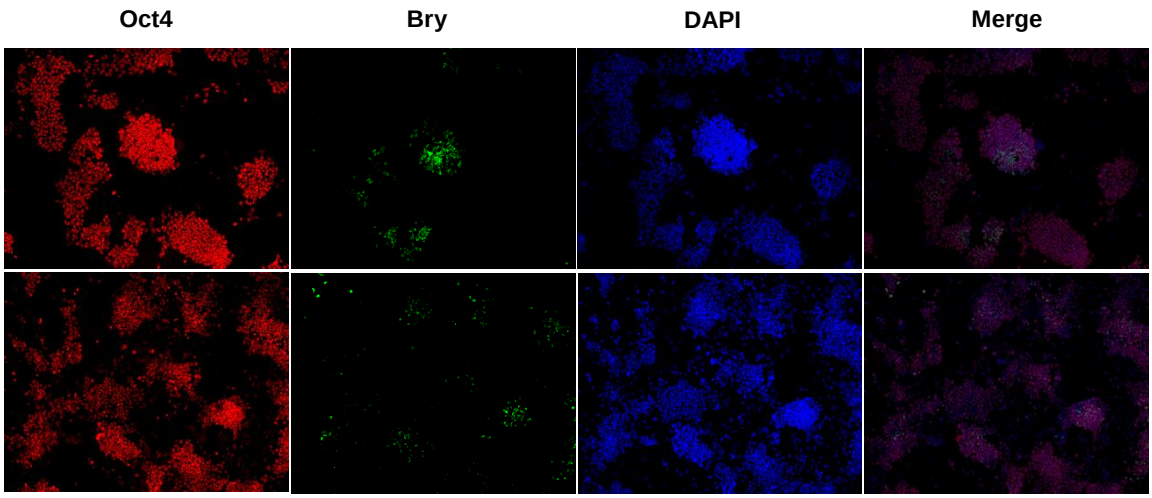
Supplementary Figure S 6: Expression of CD41/c-Kit during mouse haemopoietic differentiation.

Representative flow cytometry scatter plots of haemopoietic differentiation between day 0 to day 6. SR: serum-based conditions, SF: serum-free conditions.



Supplementary Figure S 7: Expression of CD144/CD41/CD45 during mouse haemopoietic differentiation.

Representative flow cytometry scatter plots of type II pre-HSC markers between day 4, when CD144/CD41 cells first appear, to day 6 of haemopoietic differentiation.



Supplementary Figure S 8: Immunohistochemistry of ESC-derived EpiSCs.
Representative pictures showing the variation in *Bry* expression between cell colonies of the culture. DAPI used as nuclear staining.