THE UNIVERSITY *of* EDINBURGH

Notch signalling pathway in murine embryonic stem cell derived haematopoiesis

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Declaration

I declare that the work presented here is my own and this thesis was composed by myself, unless otherwise stated in the text.

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Abstract

Haematopoiesis is the process to produce haematopoietic stem cells (HSCs), haematopoietic progenitors (HPCs) and terminally differentiated cell types. In the adult, HSCs resided in bone marrow while in the embryo, haematopoiesis occurred sequentially in several niches including yolk sac, aorta-gonad-mesonephros (AGM) region, placenta and fetal liver. The AGM region is the first place where HSCs arise *in vivo* and therefore should provide important factors to induce haematopoiesis. The mouse embryonic stem cells (mESC) system is a powerful platform to mimic the development process *in vitro* and is widely utilized to study the underlying mechanisms because they are pluripotent and can be genetically manipulated. A novel co-culture system has been established by culturing differentiating mESCs with primary E10.5 AGM explants and a panel of clonal stromal cell lines derived from dorsal aorta and surrounding mesenchyme (AM) in AGM region. Results of these co-culture studies suggested that the AM-derived stromal cell lines could be a potent resource of signals to enhance haematopoiesis. Molecular mechanism involved in haematopoiesis is a key research direction for understanding the regulation network of haematopoiesis and for further clinical research. A series of studies have demonstrated involvement of the Notch signalling pathway in haematopoiesis during development but with controversial conclusions because of the difference of models concerning various time windows and manipulating populations.

This project aimed to investigate the role of Notch signalling pathway during haematopoiesis in the AGM environment. We analyzed the expression of Notch ligands in AGM-derived stromal cells with or without haematopoietic enhancing ability. No correlation was observed between ligand expression and haematopoietic enhancing ability in stromal cell lines or between Notch activity in EBs and haematopoietic enhancing ability. We demonstrated that inhibition of the Notch

signalling pathway using the γ-secretase inhibitor could abrogate Notch activity in both mES-derived cells and the haematopoietic enhancing AM stromal cell line. To better understand the involvement of the Notch signalling pathway in a more specific spatial-temporal environment, we established a co-culture system of haemangioblast like cells ($Flk1^+$) with one of AM region derived stromal cell lines with haematopoietic enhancing ability . We found that the AM stromal cell line could enhance $Flk1^+$ derived haematopoiesis as assessed by haematopoietic colony formation activity and production of $CD41^+cKit^+$ progenitor cells. Based on the issue that the inhibitor could potentially affect both the ES cells and stromal cells, we carried out genetic approaches to overexpress or knock down Notch signalling pathway in this $Flk1^+$/AM co-culture system. Interestingly, it was found that when Notch activity was enhanced in $Flk1^+$ cells, the production of haematopoietic progenitors was inhibited and the number of cells expressing the pan-haematopoietic marker CD45 was reduced. By using the inducible dominant negative MAML1 system to knock down Notch activity, it was found that the haematopoiesis in the $Flk1^+$/AM co-culture system was not affected, which could be accounted for the low Notch activity in this system. These results supported the hypothesis that the Notch signalling pathway plays a role in modulating $Flk1^+$ derived haematopoietic differentiation within the AGM microenvironment.

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Contents

List of Figures and Tables

Chapter 1:

List of Abbreviations

AGM	Aorta-gonad-mesonephros region
AM	Dorsal aorta and surrounding mesenchyme of AGM region
BFU-E	Erythroid burst forming unit
BL-CFC	Blast colony forming cell
BM	Bone marrow
BMP4	Bone morphogenic protein 4
Bry	Barchyury gene
CAFC	Cobblestone-area forming cells
CFC	Colony forming cell
CFU	Colony forming unit
CFU-A	Colony forming unit A
CFU-E	Erythroid colony forming unit
CFU-EryP	Primitive erythroid colony forming unit
CFU-M	Macrophage colony forming unit
CFU-GM	Granulocyte/macrophage colony forming unit
CFU-GEMM	Granulocyte, erythrocyte, macrophage and megakaryocyte colony forming units
EB	Embryoid body
EGFP	Enhanced green fluorescent protein
EPO	Erythropoietin
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast growth factor
H.d	Hanging drop
HPC	Haematopoietic progenitor cell
HPP-CFC	High proliferative potential colony forming cell
HSC	Haematopoietic stem cell
ICM	Inner cell mass
IL	Interleukin
LIF	Leukaemia inhibitory factor
LTR/LT	Long term repopulating
LPM	Lateral plate mesoderm
Mac/Ery	Macrophage and erythrocytes colony forming units
M-CSF	Macrophage colony stimulating factor
MEF	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
OP9	Stromal cell line derived from *op-/-* mice
P-Sp	Para-aortic splanchnopleura region
SCF	Stem cell factor

SLAM	Signaling lymphocyte activation molecule
SP	Side population
STR/ST	Short term repopulating
UGR/UG	Urogenital ridges of AGM region
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells

Chapter 1: Introduction

1.1 Introduction

Haematopoiesis is the process of producing haematopoietic stem cells (HSCs), haematopoietic progenitors (HPCs) as well as differentiation to terminal lineages to form the mature blood and immune system. *In vitro* haematopoietic differentiation using embryonic stem cells (ESCs) has been developed as a pivotal model to investigate the steps of haematopoietic differentiation and the underlying molecular mechanisms, to identify novel cytokines and/or markers for haematopoiesis, to optimize strategies for haematopoietic induction and expansion *in vitro*, and finally to provide alternative sources of haematopoietic stem cells (HSCs) for use in clinical applications.

A number of models utilizing the addition of cytokines cocktails or gene modification have produced an extraordinary induction and expansion of haematopoietic cells and based on this, molecular mechanisms have been analyzed, but these *in vitro* systems, although very powerful are possibly not reflecting the precise mechanisms that exist in the *in vivo* environment. Thus, this project developed a culture system to direct haematopoietic differentiation using ESC system that more closely mimics *in vivo* microenvironment without exogenous powerful induction factors and we carried out a series of investigation for understanding how the Notch signaling pathway- a widely published pathway related to haematopoietic differentiation was involved in this system. This introduction reviews the background of adult haematopoiesis, embryonic haematopoiesis, mouse embryonic stem (ES) cell derived haematopoietic differentiation and the published evidence to date for the role of Notch signaling in haematopoiesis *in vivo* and *in vitro*.

1.2 Ontogeny of haematopoiesis

During mammalian embryogenesis and adult development, haematopoiesis occurs at different niches. Here we introduce the ontogeny of haematopoiesis according to the spatiotemporal sequence during development (Figure 1.1).

1.2.1 Niches for embryonic haematopoiesis

During embryonic development, haematopoiesis is reported to take place at discrete anatomical niches including yolk sac (YS), aorta-gonad-mesonephros (AGM), umbilical and vitelline, placenta, fetal liver and bone marrow. So far based on the characteristics of haematopoietic cells produced, haematopoiesis is widely defined as being established in primitive and definitive waves. However during the long history of research on haematopoiesis, the definition of primitive and definitive are still ambiguous among different reports. Here, haematopoiesis was categorized according to the function of haematopoietic cells or the origin of haematopoietic tissues.

1.2.1.1 Primitive and definitive haematopoiesis

In mammals, the primitive wave is initiated in the yolk sac at E7 in the extra-embryonic region, which is marked by the formation of blood islands. This process produces nucleated primitive erythrocytes expressing a defined set of hemoglobins (ζ, β-H1 and ε) (Kingsley et al., 2004; McGrath et al., 2003; Silver and Palis, 1997). Other primitive cell lineages have been described in the early YS, including primitive megakaryocytes which could rapidly mature with an accelerated production of platelets as well as bi-potential progenitors for megakaryocytes and primitive erythrocytes at E7.25 (Tober et al., 2007; Xu et al., 2001). In addition, a unique type of macrophage which differentiates bypassing the monocyte stage during their maturation is also defined as a primitive lineage (Bertrand et al., 2005b; Naito et al., 1989).

Following primitive haematopoiesis, definitive haematopoiesis develops, which can be further divided into two separate waves. The formation of multi-potential

progenitors is defined as the first wave of definitive haematopoiesis. These multi-potential progenitors that originate from the yolk sac and P-Sp (earlier AGM region) have the potential to form erythroid, myeloid and lymphoid lineage in *in vitro*, *ex vivo* culture and in transplantation (Cumano et al., 1996; Godin et al., 1995; Palis et al., 1999; Yoder, 2001; Yoder et al., 1997a; Yoder et al., 1997b; Yoshimoto et al., 2011). These definitive haematopoietic progenitors can be further categorized as Pro (erythroid-myeloid progenitor), Meso (lymphoid-erythroid-myeloid progenitor or multipotent low level repopulating progenitor), and Meta (neonatal repopulating HSC or CFU-S) as summarized by Dzierzak and Speck before E10.5 (Dzierzak and Speck, 2008) (Figure 1.1).

Finally, the long-term adult repopulating HSCs are formed in the AGM at E10.5, which is defined as the permanent-definitive haematopoiesis (Medvinsky and Dzierzak, 1996; Medvinsky et al., 1996; Muller et al., 1994).

1.2.1.2 Niche for embryonic haematopoiesis

a) Yolk sac

In the 1970s, Moore and Metcalf suggested that the yolk sac was the source of the adult haematopoietic system by demonstrating the presence of primitive erythrocytes, erythro-myeloid progenitors, colony-forming unit-spleen (CFU-S) and HSCs in the yolk sac at E7, E8, E8.5 and E11 respectively (Moore and Metcalf, 1970). Primitive erythrocytes which mark primitive haematopoiesis emerged at E7 in the yolk sac exclusively and declined sharply at E9. Following the first wave of primitive erythropoiesis and before the circulation is established at E8.5, definitive haematopoiesis is established when erythro-myeloid progenitors are detected in the yolk sac as detected *in vitro* colony formation assay and explant culture, which then enter the embryo proper via the circulation without lymphoid potential (Cumano et al., 1996; Palis, 2001). Cumano and colleagues demonstrated that yolk sac cells between E7.5 and E8.5 could only provide myeloid short-term reconstitution in *Rag2γc*$^{-/-}$ models lacking NK cells before circulation happened (Cumano et al., 2001). Recent two knock out models, Cdh5$^{-/-}$ and Ncx1$^{-/-}$ that abrogate circulation

revealed that erythro-myeloid progenitors are generated autonomously in the yolk sac within the *in vivo* system (Lux et al., 2008; Rampon and Huber, 2003). Furthermore, two recent reports using this model revealed the lymphoid potential of yolk sac cells independent of the intra-embryonic sites before HSC formation in AGM (Yoshimoto et al., 2011; Yoshimoto et al., 2012). Thus, these recent data provides evidence that the yolk sac is a haematopoietic site for both primitive and definitive haematopoiesis including lymphopoiesis.

After circulation, potent myeloid progenitors capable of forming colonies in the spleen in irradiated mice (CFU-S) are identified in both the yolk sac and AGM region from E9 (Medvinsky et al., 1993). Furthermore, a $cKit^{+}CD34^{+}$ population from yolk sac was identified at E9 with the potential to long-term reconstitute newborn mice with erythro-myeloid and lymphoid potential, which was different from E10.5 AGM derived HSCs with adult reconstitution ability (Yoder et al., 1997a; Yoder et al., 1997b). However, these progenitors could originate from cells migrating from intra-embryonic haematopoietic tissues because circulation has been established by E8.5. The inability to reconstitute adult mice indicates that these multi-potential progenitors need to circulate within the embryo proper for further maturation which indicates that they might be preHSCs or that these progenitors are less efficient compared to E10.5 AGM derived HSCs in homing or responding in the adult haematopoietic microenvironment. Although recently, contribution of yolk sac to produce long-term adult reconstituting HSCs was proposed by Samokhvalov and colleagues using Runx1 (a marker for definitive haematopoiesis) lineage tracing model based on Cre/loxP system (Samokhvalov et al., 2007). However, the interpretation of this data is highly dependent on the variable timing of expression of Runx1. At E11.5, long term adult reconstitutive HSCs could be detected in yolk sac, while which could also be based on migration from other origins (Kumaravelu et al., 2002). Thus, yolk sac cells relative contribution to adult haematopoiesis remains unclear.

b) Aorta-Gonad-Mesonephros (AGM)

In the 1970s, the yolk sac was thought to be the origin of haematopoiesis in the embryo. However this notion was challenged by quail-chick engraftment experiment

(quail embryos on to chick yolk-sacs) by Dieterlen-Lievre in 1975 demonstrating an intra-embryonic origin of haematopoiesis (Dieterlen-Lievre, 1975). A series of studies have since been carried out to identify the intra-embryonic sites critical for the ontogeny of haematopoiesis (Godin et al., 1995). It is widely accepted that definitive haematopoiesis in the mouse initiates from intra-embryo tissue para-aortic splanchnopleura (P-Sp) (E8.5 to E10), which first originates from lateral plate mesoderm (LPM) and later develops into the aorta-gonad-mesonephros (AGM) (E10 to E11.5). A progenitor with lymphoid-myeloid potential is also found in the P-Sp region of the embryo following explant culture as early as E7.5 prior to circulation (Cumano et al., 1996). These progenitors from explant culture could further achieve low level but multilineage repopulation including lymphoid lineages in adult immunodeficient *Rag2γc*$^{-/-}$ mice, in which yolk sac cells could only achieve short-term myeloid repopulation (Cumano et al., 2001).

After circulation, the cKit^{+}CD34^{+} population with capacity to repopulation newborn mice but not adult mice was also identified in E9 P-Sp region, while with a lower repopulation ability compared to yolk sac (Yoder et al., 1997a). After identification of multi-potential progenitors in the AGM region at E9 with CFU-S formation ability higher than yolk sac (Medvinsky et al., 1993), Definitive HSCs with long-term multilineage repopulation ability in adult recipient were first identified in E10 AGM region as shown by explant culture for 2 to 3 days or direct cell transplantation (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Therefore, E10.5 AGM region is believed to be the earliest site within the embryo proper for generating definitive long-term HSCs autonomously. Slightly thereafter, HSCs are found in other tissues; the placenta, arteries, yolk sac, and liver.

c) Placenta, circulation in arteries and fetal liver

In addition to the AGM and yolk sac, multi-potent progenitors and HSCs are also found to harbor other embryonic sites. During mammalian development, two main circulatory routes, vitelline and umbilical, develop to connect the fetal haematopoietic organs during midgestation. The yolk sac is connected to the upper dorsal aorta and the fetal liver via the vitelline artery. The placenta is connected to

the caudal dorsal aorta and the fetal liver via theumbilical vein. Pools of definitive HSCs have been identified in these arteries (de Bruijn et al., 2000). Onset of erythro-myeloid progenitors within the placenta were identified at E9 and HSCs at E10.5- E11 with an expansion until E12.5- E13.5 indicating that the placenta has an important role in the establishment of HSCs (Alvarez-Silva et al., 2003; Gekas et al., 2005; Ottersbach and Dzierzak, 2005). A recent study using the $Ncx^{-/-}$ model to abrogate circulation suggested that HSCs could also initiate independently in placenta (Rhodes et al., 2008).

At E9.5-10.5, the fetal liver is first seeded by erythro-myeloid progenitors which probably derive from yolk sac through circulation as the yolk sac microenvironment did not support terminal differentiation into definitive blood cell lineages,. Furthermore, fetal liver is the primary fetal haematopoietic organ where HSCs expand and differentiate without HSCs initiation but could be colonized by HSCs circulated from AGM, yolk sac and placenta from E11.5 (Kumaravelu et al., 2002). Thereafter, HSCs are generally stated to exhibit significant expansion from E12.5 then colonize the spleen, thymus and bone marow before birth.

1.2.2 Development of HPCs and HSCs in haematopoiesis

As introduced above, during embryogenesis, haematopoiesis occur in consecutive waves to form erythro-myeloid progenitors, lymphoid-erythro-myeloid progenitors, CFU-S, neonatal repopulating HSCs with long-term repopulating HSCs relatively late in this process. To better understand how these progenitors or HSCs originate, studies have focused on the cell origin during embryogenesis. So far, only blood island in yolk sac consisting of erythrocytes and endothelial cells and the ventral aorta in P-Sp/AGM have been identified unambiguously as the in situ origin of haematopoiesis. Thus, we review the studies on how haematopoietic cells emerge *in vivo* in these two sites.

1.2.2.1 Formation of mesoderm

In mice both embryonic haematopoiesis in yolk sac and intra-embryonic sites start after gastrulation which initiates at E6.5. During gastrulation, mesoderm cells emerge

from the posterior part of primitive streak, a posterior midline structure of the epiblast, and then migrate posteriorly into extra-embryonic yolk sac to differentiate into blood islands. During mid-streak stage and late-streak stage in the intra-embryonic site, nascent mesoderm cells within the primitive streak migrate anteriorly and laterally to further form lateral and paraxial mesoderm (Kinder et al., 2001; Mikawa et al., 2004; Tam and Behringer, 1997). The lateral plate mesoderm later evolves into AGM region. However, how mesoderm cells differentiate to HPCs or HSCs is still full of controversies. So far, two major notions are proposed: haemangioblast and haemogenic endothelium.

1.2.2.2 Haemangioblast

Co-localization of haematopoietic cells and endothelial cells in blood islands in yolk sac, emergence of haematopoietic cluster in the endothelium layer in dorsal aorta, and a series of observation in embryonic development *in vivo* revealed a close developmental relationship between the haematopoietic and endothelial lineages. In light of this direct ontogenic link, a bi-potent precursor for these two lineages, the "haemanbioblast", was could be traced back early in 1924.

Failure to form blood islands and no haematopoietic cells in fetal liver was observed in the vascular endothelial growth factor receptor 2 (VEGFR2, Flk1) deficient mice model, indicating the existence of haemangioblast related to both primitive and definitive haematopoiesis *in vivo* as well as the involvement of Flk1 (Shalaby et al., 1997; Shalaby et al., 1995). A series of *in vitro* studies by Keller's group from 1997 first identified blast colony-forming cells (BL-CFCs) with bi-potential forming haematopoietic and endothelial cells using ES cells system *in vitro* co-expressing Barchyury and Flk1 (Choi et al., 1998; Fehling et al., 2003; Kennedy et al., 1997). Later, Huber and colleagues (2004) first detected an equivalent population *in vivo* with haemangioblast potential in the posterior primitive streak at mid-streak stage from E7 to E7.5, with co-expression of Barchyury and Flk1 (Huber et al., 2004). Yao et al also supported the notion of the haemangioblast by demonstrating a bi-potential cell located in the P-Sp region at E8.5 till AGM at E10.5-E12.5, but not in yolk sac, which was able to differentiate to endothelial and haematopoietic cells (Yao et al.,

2007). However, these two studies both measured the potential using the *in vitro* colony assay after removal from the *in vivo* microenvironment, which did not provide direct evidence to demonstrate the existence of the haemangioblast *in vivo*. Cell tracking techniques have also been applied to solve this problem. So far, a bi-potential progenitor for haematopoietic and endothelial lineages have been identified in developing zebrafish using a $Flk1^+$ single-cell tracking strategy supporting the existence of the haemangioblast (Vogeli et al., 2006). Plus, by permanently marking $Flk1^+$ cells and their progenies using Cre/loxP system in a mouse model, it was noted that both primitive and definitive blood cells originated from $Flk1^+$ cell (Lugus et al., 2009). These studies together suggest that Flk1 is a critical marker for haemangioblast cells.

Interestingly, Ueno and Weissman demonstrated that yolk sac blood islands did not have a clonal origin by co-injecting three ES cell lines marked with different colours into blastocysts and analyzed individual blood islands from yolk sac at E7.5. It was noted that each blood island was contributed by more than one ES cell line, indicating the polyclonal nature of the blood islands (Ueno and Weissman, 2006). Correlated to studies by Huber claiming the existence of the haemangioblast in the posterior primitive streak from E7 to E7.5 with another potential to vascular smooth muscle cells (VSMCs), it was revealed that haemangioblast could have already undergone differentiation into endothelial and haematopoietic progenitors before seeding the yolk sac (Huber et al., 2004). Studies using a Cre/loxP strategy to track $Flk1^+$ cells progeny in mice also provided the evidence that $Flk1^+$ cells are progenitors for muscles (Alvarez-Silva et al., 2003). Therefore, these evidences indicate that the "haemangioblast" as defined by Flk1 or Brachyury and Flk1 co-expression could be a multi-potent progenitors that gives rise to more than haematopoietic and endothelial lineages.

Lineage tracing carried out by Jaffredo et al in chick embryos at later stages during definitive haematopoiesis by cardiac injection of LacZ-expressing retroviral vector demonstrated that haematopoietic progenitors are derived from the aortic endothelium, suggesting a more specific definition of strict bi-potent precursor for

haematopoietic and endothelial cells—"haemogenic endothelium" (Jaffredo et al., 2000). Thus, "haemangioblast" could possible endeavor more potentials than "haemogenic endothelium" which suggesting an early role in the development of haemangioblast. Alternatively, based on the fact that studies of the haemangioblast are so far mostly based on the extra-embryonic microenvironment or ES cell system, it is possible that the origin of extra-embryonic and intra-embryonic haematopoiesis could be different.

1.2.2.3 Haemogenic endothelium

It was first identified in mouse E9.5 embryos that the VE-Cadherin^{+} endothelial cells derived from yolk sac and intra-embryo co-expressed PECAM1, Flk1, and CD34 and could further differentiate into lympho-erythromyeloid progenitors expressing CD45 *in vitro* (Nishikawa et al., 1998b).

A series of *in vivo* or *de novo* studies investigating the emergence of HSCs in the AGM region have revealed the close association of HSCs or haematopoietic cells with endothelium (Jaffredo et al., 1998; Yokomizo and Dzierzak, 2010). When AGM was sub-dissected into dorsal aorta and urogenital ridge segments for transplantation into irradiated adult recipients, it was noted that HSCs first appeared in the dorsal aorta and the surrounding mesenchyme subregion (AM) but not urogenital ridge (UGR, UG) (de Bruijn et al., 2000) (Appendix Figure S1.1). Further cell tracing using transgenic mice carrying GFP under regulation of Sca1 promoter, a well-known HSCs marker, showing that the first definitive HSCs were raised in the endothelial layer or dorsal aorta, though Sca1 could not solely define the adult repopulating HSCs (de Bruijn et al., 2002). Taoudi et al have identified a PECAM1^{high} CD45^{+} VE-Cadheren^{+} population containing definitive HSCs which predominantly localized to the intra-aortic clusters attached to the endothelial layer of the ventral domain of dorsal aorta (Taoudi et al., 2008; Taoudi and Medvinsky, 2007; Taoudi et al., 2005). Indeed, reviewed by Dzierzak and Speck, HPCs and HSCs shared many markers in common with some or all endothelial cells in the ventral domain of the dorsal aorta in the AGM region at E10 to E11, including Sca1, cKit, CD34, Runx1, SCL and Gata2. VE-Cadherin, the marker for endothelial cells,

was also expressed in intra-aortic cluster in AGM (Dzierzak and Speck, 2008). Therefore, these studies revealed the close association of haematopoietic cells and endothelial cells at locations *in vivo*.

In light of this, a cell tracing strategy was widely applied to investigate the origin of haematopoietic cells from endothelium. Zovein et al tracked the progeny of VE-cadherin^{+} cells, which was supposed to be endothelial-specific, in AGM region before the onset of definitive haematopoiesis using inducible Cre/loxP system. It was noted that these VE-cadherin^{+} population could give rise to adult haematopoietic cells (Zovein et al., 2008). As what has been carried out in avian model by Jaffredo revealing origin of haematopoietic from endothelium layer, in mice model, the same strategy was applied to confirm this. AclDL-Dil was applied to label endothelial cells coexpressing PECAM1 and CD34 with cardiac injection. These endothelial cells could then give rise to an adult type of erythroid cells in clonal culture and *in vivo* after longer development suggesting the origin of haematopoietic cells from endothelium (Sugiyama et al., 2003). A *de novo* slice culture of mouse AGM region with live imaging at the dynamic emergence of HSCs population defined by PECAM1^{+}Sca1^{+}CD41^{+} directly from ventral aortic haemogenic endothelial cells supported this theory (Boisset et al., 2010). Correlately, two zebrafish models of cell tracking at Flk1 or cMyb strategy also suggested that haematopoietic cells emerged from haemogenic endothelium (Bertrand et al., 2010a; Kissa and Herbomel, 2010).

Hirschi's group also carried out a series of studies to find the haemogenic endothelium like cells in the yolk sac. They succeeded in defining a haemogenic endothelial population within the yolk sac as well as embryo proper which were Flk1^{+}cKit^{+}CD45^{-} and with dye-efflux properties (so-called "side population", SP cells) exhibiting haematopoietic potential at clonal level (Goldie et al., 2008; Nadin et al., 2003). In agreement with this, Tie2^{+} Flk1^{dim} CD41^{-} population was identified in E8.25 yolk sac with potential to haematopoietic lineages expressing CD41. Tie2 was widely used as a late differentiation marker for vascular morphogenesis (Li et al., 2005; Suri et al., 1996). Together with studies by Ema et al demonstrating that during gastrulation, an endothelial like population co-expressing Flk1, PECAM1,

VE-cadherin, CD34, endoglin, and Tie2 within the extra-embryonic mesoderm layer of the yolk sac could give rise to primitive erythroid colonies *in vitro*, these data supporting the notion of haemogenic endothelium in the extra-embryonic site (Ema et al., 2006).

1.2.2.4 Progenitors in mesenchymal area

Several studies also raised progenitors in mesenchyaml area as another plausible origin for this HPCs and HSCs, or the mesenchymal area could provide the microenvironment for differentiation of haemangioblast or haemogenic endothelium. Some studies have suggested that HSCs existed within the mesenchymal area underneath the endothelium of the ventral domain of dorsal aorta as detected by Runx1 (a definitive haematopoietic transcription factor) expression in mesenchymal area, or the discrete patches ventral-lateral to the dorsal aorta (sub-aortic patches) according to the observation that repopulation ability into immune-deficient adult recipients of HSCs defined by $CD45^{-}cKit^{+}AA4.1^{+}$, though at low engrafting efficiency (Bertrand et al., 2005a; North et al., 2002). Interestingly, a recent study suggested that, the VE-$Cadherin^{+}CD45^{+}$ pre-HSCs in the intra-aortic clusters enriched HSCs could be derived from an earlier population defined by VE-$Cadherin^{+}$CD45-$CD41^{+}$ (Taoudi et al., 2008; Rybtsov et al., 2011). More importantly, they noted that this earlier population was located in luminal endothelial lining and intra-aortic clusters, however also in the subluminal compartment of the dorsal aorta (Rybtsov et al., 2011). These data suggested that mesenchymal area could be a potential environment for production of HSCs. Thus, it is also possible that the so called “haemogenic endothelium” represents the transient state that pre-HSCs progress through from the underlying mesenchyme to form haematopoietic clusters. More importantly, this suggests that microenvironment provided by the mesenchyme could be critical for formation or maturation of HSCs, perphaps from mesoderm, or haemangioblast stage.

1.2.3 Lineage commitments of HPCs and HSCs

As introduced above, after HSCs emerge from embryonic niches, they colonized the

bone marrow. These HSCs are a rare population residing in the adult bone marrow and are mostly quiescent with the ability to repopulate the whole haematopoietic system when transplanted into irradiated adult recipient. When necessary, LT-HSCs are able to differentiate into terminal lineages to form blood and immune system, through a series of steps losing multi-potential finally restricting to unipotent lineages (Orkin and Zon, 2008). Although LT-HSCs sit at the top of this hierarchy, it should be the same for embryonic derived pre-HSCs (ie, erythro-myeolid progenitors, lymphoid-erythromyeolid progenitors) to follow the same diagram becoming more limited in potential as differentiation proceeds (Figure 1.2).

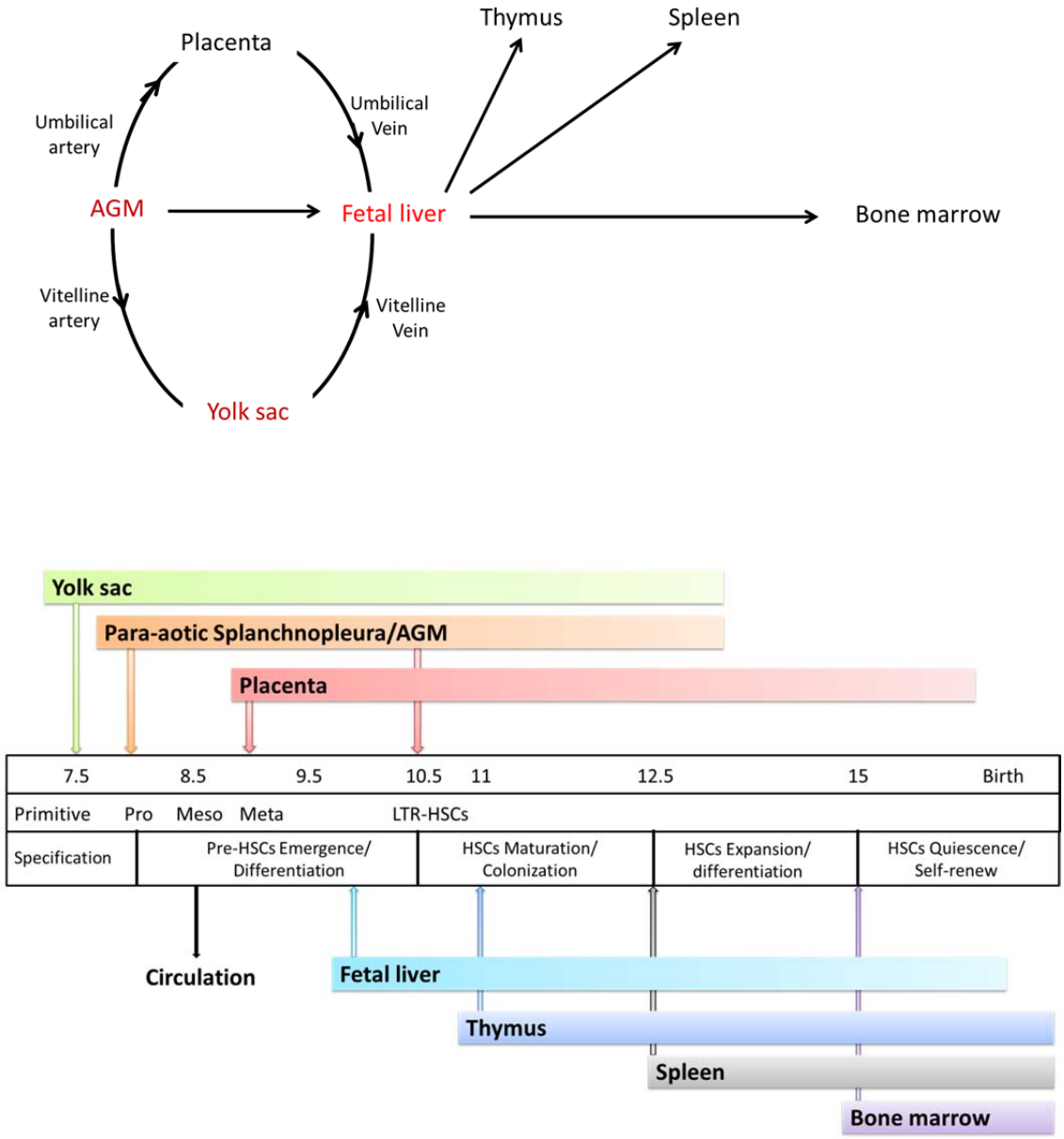

Figure 1.1 Sites of embryonic to adult haematopoiesis and possible migration and colonization.

Upper figure represented the connection of niches for embryonic haematopoiesis before birth; Lower figure represented the timing and sites of specification, emergence, maturation and migration of HSCs from primitive streak. Abbreviations: Pro, erythroid-myeloid progenitor; meso, lymphoid-erythroid-myeloid progenitor or multipotent low level repopulating progenitor; meta, neonatal repopulating HSC or CFU-S; HSCs, haematopoietic stem cells; LTR-HSCs, long-term repopulating HSCs. These diagrams were adapter from figures in the following review articles: Medvinsky et al., 2011; Dzierzak and Speck, 2008.

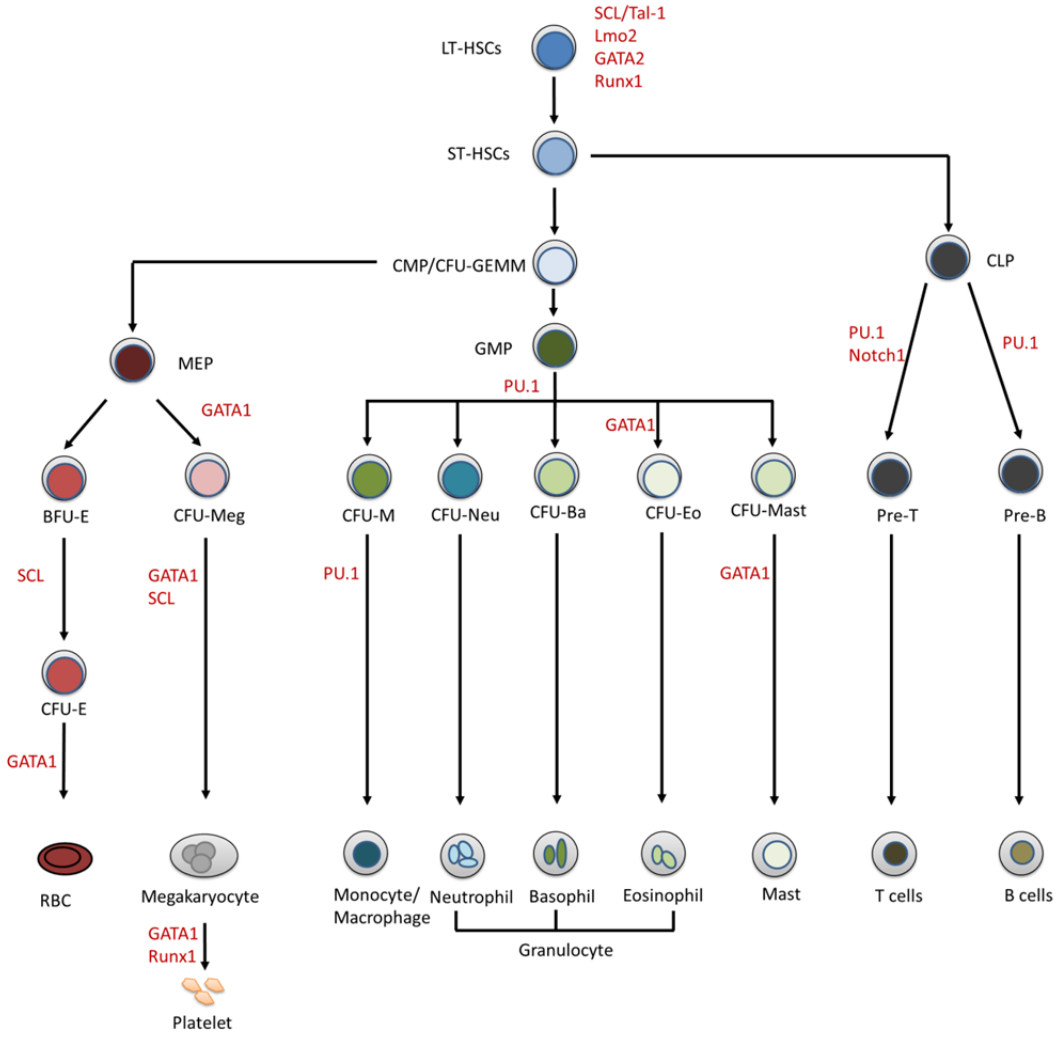

Figure 1.2 Hierarchy of haematopoietic differentiation from LT-HSCs.
This is a classical model and there is some evidence for slight differences. This diagram was adapted from figure in reiews by Orkin and Zon (2008).

1.3 Detection of embryonic derived HPCs and HSCs

As introduced above about the niches and origins of haematopoiesis, a series of strategies are required to detect and assess the function of HPCs and HSCs. Here we briefly summarised the mainly used methods for determining HPCs and HSCs.

1.3.1 *In vivo* repopulation

1.3.1.1 Adult repopulating assay

In vivo repopulating assays can be applied to assess the presence of long-term HSCs (LTR-HSCs) with full and long term reconstitution ability into all haematopoietic lineages in irradiated or immunodeficiency adult recipients, for more than 6 months after transplantation. Based on this, limiting dilution and competitive long-term repopulation assays have been developed to determine the number of LTR-HSCs (Orlic and Bodine, 1994; Szilvassy et al., 1990; Szilvassy et al., 1989).

1.3.1.2 Neonatal repopulating assay (Newborn repopulating assay)

This assay has been widely applied to repopulate neonatal (new born) mice treated with busulfan to enhance engraftment efficiency with HSCs or HPCs. The cells able to repopulate neonatal mice are long lived multilineage progenitors but are not necessarily able to repoplulate adult irradiated recipients because they could lack homing receptors that are required to migrate to adult bone marrow (Yoder and Hiatt, 1997).

1.3.1.3 CFU-S

First proposed by Till and McCulloch, the single multipotent haematopoietic progenitors can be identifed by injecting donor bone marrow cells into lethally irradiated recipient mice and the number of colonies formed in the spleen calculated 8-12 days later. Each colony (often composed of granulocyte/megakaryocyte and erythroid precursors) represents one single progenitor with multi-potential and is thus defined as the colony forming unit in spleen (CFU-S). This assay first provided the evidence of clongenity of haematopoietic progenitors (Becker et al., 1963; Siminovitch et al., 1963; Till and Mc, 1961). The progenitors forming colonies slightly later after transplantation ($CFU\text{-}S_{11\text{-}14}$) represent a less committed state than

the CFU-S_8 (Magli et al., 1982). Some but not all of primary colonies in spleen could further reconstitute secondary irradiated recipient indicated that CFU-S likely represent a short-term repopulating HSCs, not the LTR-HSCs (Jones et al., 1989; Jones et al., 1990; Siminovitch et al., 1963).

1.3.2 *In vitro* colony assay

During the evolvement of strategies to measure HSCs and HPCs *in vitro*, several *in vitro* colony assays have been developed.

1.3.2.1 Cobblestone-area forming cells (CAFC)

Co-culture of tested cells on stromal layers (ie OP9) has been used for long-term culture *in vitro*. HSCs or HPCs co-cultured on stromal layers form cobblestrone-area (CA) through differentiating and migration. These CAs have been analysed to determining their potency and at different time points. The longer it takes for a CA to appear, the less commited and more potent the originating cell is likely to be (Dexter et al., 1984; Dexter and Testa, 1976; Ploemacher et al., 1989).

1.3.2.2 Methylcellulose-based colony assay

More recent studies have used colony assay of semi-solid medium supplemented with a combination of cytokines as the the major assay to determine haematopoietic progenitors in which progenitors could differentiate and proliferate to form colonies. Each colony is the product of a single progenitor and is known as a colony forming unit/cell (CFU/CFC). Colony forming unit-A (CFU-A) and HPP-CFC (high proliferative potential colony forming cell) assays have been applied in the past to measure haematopoietic progenitors. However CFU-A are only able to detect more mature cells and HPP-CFC only distinguish HPCs of various committed states.

Methylcellulose-based colony assays have been widely used to detect haematopoietic progenitors at the single cell level in semi-solid medium methylcellulose supplemented with combination of cytokines including IL-3 (Interleukin-3), IL6 (Interleukin-6), stem cell factor (SCF) and erythropoietin (Epo) at specific concentrations. In these assays, individual progenitors called colony-forming

units/cells (CFU/CFC) form cell clusters or colonies consisting of one or more types of mature haematopoietic lineages: BFU-E/CFU-E (erythrocytes), CFU-GM (granulocyte and macrophage), CFU-M (macrophage), as well as CFU-GEMM (granulocyte, erythrocytes, macrophages, megakaryocytes). This assay gives information about the types of progenitors with different potentials, however, do not well distinguish primitive or definitive by morphology.

1.3.3 Surface phenotyping by flow cytometry

So far, flow cytometric analysis for surface phenotypes has been applied as a pivotal tool to define haematopoiesis along the roadmap from mesoderm to hematopoietic fate. A brief summary was shown to summarize the studies on surface phenotypes of embryonic-derived HSCs and HPCs, which are mostly based on *in vivo* observation (Figure 1.3).

Flk1 (VEGF receptor 2) is expressed in the yolk sac at E7 later than gastrulation and is strictly required for the establishment of haematopoietic and endothelial cells in both extra- and intra-embryonic compartment: the migration induction ability responding to the VEGF (Vascular endothelial growth factor) being expressed by the underlying endoderm (Schuh et al., 1999; Shalaby et al., 1997; Shalaby et al., 1995). Based on this, Brachyury and Flk1 have been applied together to define the haemangioblast or the *in vitro*-equivalent, BL-CFC (Fehling et al., 2003; Huber et al., 2004).

Haemogenic endothelium, as an alternative origin of haematopoietic cells or an intermediate stage between the haemangioblast and haematopoietic cells, has been defined by $Tie2^{high}$c-$Kit^{+}CD41^{-}$ as observed in yolk sac (Lancrin et al., 2009). This population was also confirmed in extra-embryonic $Flk1^{+}$ population as having an endothelial phenotype co-expressing a series of endothelial markers including PECAM1, Flk1, MECA32, CD34, VE-Cadherin and endoglin and also able to give rise to primitive haematopoietic cells (Ema et al., 2006).

HSCs are derived from pre-HSCs, which emerge and mature from haemangioblast, haemogenic endothelium or mesenchyme under the dorsal aorta endothelium. As described above, haematopoietic and endothelial lineages share a series of markers in common. CD41 is originally identified as a cell adhesion molecule expressed in the megakaryocytic lineage. However CD41 is later characterized as the first and most specific surface marker distinguishing cells committed to the haematopoietic lineage from the endothelial lineage and is widely expressed in haematopoietic progenitors and embryonic HSCs (Corbel and Salaun, 2002; Corbel et al., 2005; Ferkowicz et al., 2003; Mikkola et al., 2003; Mitjavila-Garcia et al., 2002). Corbel et al found that a $CD41^{+}cKit^{+}$ population from the embryo has erythroid, myeloid, and lymphoid potential but has very low repopulating ability when purified from bone marrow (Corbel and Salaun, 2002; Corbel et al., 2005). In support of this, Mikkola et al demonstrated that CD41 was co-expressed with embryonic HSC markers cKit and endothelial/haematopoietic marker CD34 in yolk sac as well as embryoid bodies (EBs) formed from ES cells. Using *in vitro* colony forming assay, they also confirmed that definitive haematopoietic progenitors enriched in CD41 and cKit co-expressing population from E9.5 yolk sac and day6 EBs, though repopulating assays were not carried out to confirm whether they represented long term repopulating HSCs. Furthermore, this work also investigated CD45, a pan-haematopoietic cells marker, claiming that HPCs with multi-potential were enriched in the $cKit^{+}CD41^{+}CD45^{-}$ compartment in E9.5 yolk sac and ES cell derived EBs. Interestingly, in E14.5 fetal liver HPCs were found to be enriched in the $CD45^{+}CD41^{-}$ population which suggests down regulation of CD41, but also indicates potential differences in cell surface marker expression between the HPCs that appear before and after LT-HSCs have emerged. In agreement, Ferkowicz et al also suggested that competitive repopulating HSCs were enriched in $CD41^{lo/-}$ cells from bone marrow and fetal liver cells (Ferkowicz et al., 2003). $CD34^{+}cKit^{+}$ cells from E9 yolk sac have been defined as multi-potent progenitors able to repopulate newborn recipients but not adults (Yoder and Hiatt, 1997; Yoder et al., 1997a; Yoder et al., 1997b).

Medvinsky's group also carried out a series of studies to investigate the surface phenotype definition of pre-HSCs in the intra-embryonic region. They demonstrated that in the E11.5 AGM region the VE-cadherin^{+}CD45^{+}PECAM1^{high} fraction in the intra-aortic cluster were enriched for HSCs that did not have endothelial potential. VE-cadherin expression was lost during maturation to adult BM type HSCs (Taoudi et al., 2008; Taoudi and Medvinsky, 2007; Taoudi et al., 2005). Based on this, Medvinksy's group further identified a novel earlier pre-HSCs VE-cadherin^{+}CD45^{-}CD41^{+} located in mesenchyme area which could further develop into VE-cadherin^{+}CD45^{+} CD41^{-} pre-HSCs (Rybtsov et al., 2011). Similarly to this, Bertrand and colleagues suggested another potential origin of HSCs from mesenchyme area around aorta, they identified a pre-HSCs which was CD41^{+}cKit^{+}CD45^{-}PECAM1^{+}AA4.1^{+} with limited repopulation ability (Bertrand et al., 2005a).

Sca1 (Ly-6A), a critical marker for adult HSCs together with cKit, was also defined as a marker for embryonic derived HSCs. This was demonstrated using a transgene mouse model in which only Sca1-GFP population from AGM region were able to repopulate irradiated recipient post explant culture (de Bruijn et al., 2002). In addition, the SLAM family of receptors (CD150^{+}CD244^{-}CD48^{-}) has been widely used to define HSCs from fetal liver to adult haematopoiesis (Kiel et al., 2005). Combining the SLAM family phenotype together with the CD41, CD45, cKit and CD34 it was reported that with cKit expressed consistently, CD41 and CD34 decreased while CD45 and CD150 increased during HSCs development (McKinney-Freeman et al., 2009).

For lineage commitment from HPCs and HSCs, Ter119 has been used to monitor mature erythroid differentiation. Mac1 (CD11b) and GR1 (Ly-6G) have been used to monitor the myeloid lineage. CD45, as a pan-haematopoietic cells marker, was expressed by haematopoietic cells except mature erythrocytes. B220, CD4 or CD8 expressed by lymphoid lineages. These markers are not expressed by the LT-HSCs which should be cKit^{+}Thy1.1^{low}Lin^{-}Sca1^{+} (Okada et al., 1991).

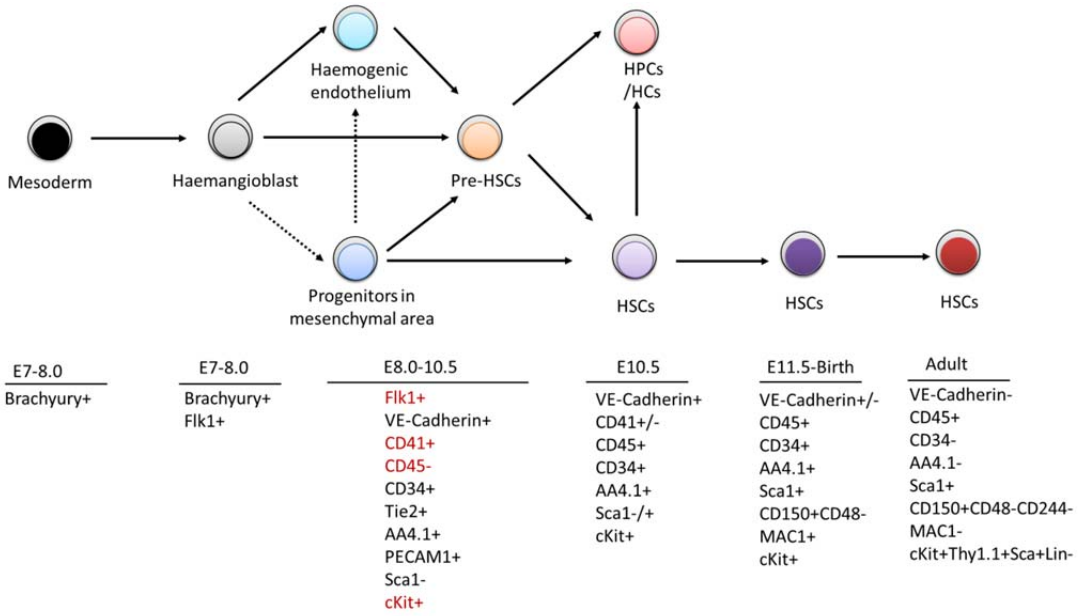

Figure 1.3 Development of HSCs from mesoderm stage and surface phenotypes of each differentiation stages as assessed by vivo studies.

Red highlighted markers represent widely applied surface markers in this project. Dashed arrows represent the process not confirmed by experiments solidly.

1.4 In vitro haematopoiesis with embryonic stem (ES) cells

Embryonic stem (ES) cells system provides a platform to understand the development of haematopoietic system and haematopoietic disorders as well as make it easier to apply a genetic modification or treatment to overcome certain specific disorders.

1.4.1 Mouse Embryonic Stem cells (mES cells)

Mouse embryonic stem cells (mES cells) are derived from the inner cell mass (ICM) of the day3.5 embryo known as the blastocyst, which are rigorously defined by their ability to self-renew *in vitro* and to differentiate into a variety of cell lineages and tissues derived from all three germ-layers of the embryo once injected into host blastocysts or induced under proper factors *in vitro*. (Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981). ES cells can be maintained as undifferentiated cells with pluripotent potential *in vitro* by co-culturing on murine embryonic fibroblasts (MEF) or in the presence of exogenous leukaemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). Later, a combination of LIF and bone morphogenic protein 4 was confirmed able to maintain mouse ES cells *in vitro* bypassing the use of serum or a feeder layer (Ying et al., 2003). Upon withdrawal of LIF, mouse ES cells are able to differentiate spontaneously into a variety of cell types of ectodermal, mesodermal and endodermal origin, including the haematopoietic, vascular and cardiac lineage.

For therapeutic purposes and to investigate the ontogeny of haematopoiesis, *in vitro* differentiation systems using mES cells have been developed to mimic the *in vivo* pattern of haematopoietic differentiation as described above (Section 1.2, 1.3). Here we briefly summarize the current studies in the mES cells system on haematopoietic ontogeny and differentiation.

1.4.2 mES cells derived haematopoietic progenitors (HPCs)

To date, haematopoietic differentiation of mES cells has been mostly carried out in

two microenvironments, a 3-dimensional structure of formation of embryoid bodies (EBs) or a 2-dimensional co-culture of ES cells on stromal cells from haematopoietic niches. Based on these two systems, addition of defined cytokines, targeting of transcription factors or combination of EBs with stromal cell lines were further applied for increasing efficiency, investigating molecular mechanisms and discovering novel factors related to haematopoiesis.

1.4.2.1 HPCs induced in embryoid bodies by mES cells

Upon withdrawal of LIF or feeder cells, EBs are formed by ES cells in suspension aggregating spontaneously or using a hanging drop strategy to obtain cell aggregates of uniform size. Initiated by Doetschman and colleagues in 1985, they observed that blood precursors formed in EBs and could further differentiate into "blood islands" analogous to those found in the embryonic yolk sac indicating the autonomous emergence of haematopoietic differentiation in EBs. Progenitors for myeloid and erythroid lineages were also present in EBs (Burkert et al., 1991; Doetschman et al., 1985; Hole et al., 1996; Keller et al., 1993; Kennedy et al., 1997; Wiles and Keller, 1991). This haematopoietic differentiation was further confirmed by assaying surface phenotypes or transcription marker expression as well as function assays (ie methylcellulose assay). It was suggested that the EBs formed in the presence of serum could synthesize cytokines autonomously to initiate haematopoiesis at an early stage because exogenous addition of cytokines like interleukin-3 (IL-3), IL-1, IL-6, IL-11, erythropoietin, and Kit ligand did not affect haematopoietic initiation in EBs before day10 (Keller et al., 1993). However addition of cytokines has been helpful to increase the efficiency of terminal lineages production. It was noted that addition of erythropoietin (Epo) and IL-3 could increase erythropoietic activity as well as myeloid mature cells production within EBs (Wiles and Keller, 1991).

In light of the *in vivo* ontogenesis from mesoderm formation, increasing studies focused on haematopoietic differentiation in EBs in the absence of serum with addition of exogenous cytokines. In EBs cultured in serum-free condition, it was found that bone morphogenetic protein 4 (BMP4) was required for the production of haematopoietic progenitors (erythro-myeloid progenitors and lymphoid progenitors)

formation synergized by vascular endothelial growth factor (VEGF) (Nakayama et al., 2000). In support of this, Park and colleagues demonstrated that BMP4 was critical for $Flk1^+$ mesoderm and SCL^+ progenitor formation while VEGF functions in the expansion of these progenitors (Park et al., 2004). Recently, Pearson and colleagues have reported a step-wise addition of BMP4, Activin A, bFGF and VEGF (Pearson et al., 2008). Bmp4 promotes the formation of mesoderm with an induction to haemangioblast by bFGF and activin A. VEGF mediated maturation of haemangioblast cells into committed haematopoietic progenitors as confirmed by surface phenotypes and colony assays. Thus, these serum-free systems provided a clean and stable platform for research into the molecular mechanism involved in haematopoiesis, though no reconstitution assay being further investigated to measure production of HSCs in this system (Pearson et al., 2008).

1.4.2.2 HPCs induced by co-culture of stroma or stromal cell line with mES cells

In addition to EBs cultured in serum with autonomous haematopoiesis or serum-free condition with cytokines induction, co-culture of ES cells with stroma, stromal cells or extracellular matrices have also been used to provide a microenvironment for haematopoietic induction. A co-culture system was first described by Nakano's group in 1994 in which formation of EBs or exogenous cytokines was replaced by co-culture on the OP9 stromal cell line. This cell line was derived from calvaria of newborn osteopetrotic *op/op* mice, which do not express functional macrophage colony-stimulating factor (M-CSF) to minimize macrophage differentiation and allow differentiation of mES cells into other haematopoietic cell types (Kodama, et al., 1994). It was demonstrated that co-culturing OP9 stromal cell lines with mES cells could induce mES cells differentiation into erythroid, myeloid, B cell lineages. Furthermore, application of stromal cell lines could enable identification of novel factors for haematopoiesis. For example, Ueno and colleagues reported a membrane protein mKirre expressed by OP9 which could contribute haematopoietic supporting ability of OP9 (Nakano et al., 1994; Ueno et al., 2003).

With regards to the theory that E10.5 days AGM region is a putative region to give rise to the first long term repopulation HSCs, it is highly possible that this niche can

provide essentially inductive signals for haematopoiesis. Based on this, several groups have set up co-culture system with AGM-derived stromal cell lines to investigate their influences on embryonic and adult haematopoiesis. Oostendorp and colleagues derived a panel of stromal clones from E10.5 and E11.5 AGM region (AM and UGR region separately) (Appendix Figure S1.1), yolk sac and fetal liver (Oostendorp et al., 2005; Oostendorp et al., 2002a; Oostendorp et al., 2002b). Based on this, our group reported a co-culture system of AGM explants with EBs showing enhancing effects on haematopoiesis (Krassowska et al., 2006). Furthermore, haematopoietic differentiation of ES cells was significantly enhanced when EBs were co-cultured with a stromal cell line AM20.1B4 derived from dorsal aorta and the surrounding mesenchyme subregion (AM). This was confirmed by *in vitro* colony assay and surface marker expression (Gordon-Keylock et al., 2010). Furthermore, it was found that this haematopoietic enhancement could be mediated post mesoderm. In light of this, brachyury^{+} and brachyury^{-} fractions by a GFP-Bry reporter ES cell line were co-cultured on another AM-derived stromal cell line AM14.1C4 and of note, the enhancing effect of AM14.1C4 applied on brachyury^{+} fraction according to colony assay (Fehling et al., 2003; Gordon-Keylock et al., 2010). In agreement with this, another E10.5 AGM-derived stromal cell line AGM-S62 was also published as a potent inducer of haematopoietic differentiation of mES cells to induce haematopoietic differentiation (Weisel et al., 2006)

1.4.3 mES cells derived haematopoietic stem cells (HSCs)

So far, formation of EBs and co-culture of stromal cells as well as addition of cytokines have been applied to induce HPCs formation as described above or haematopoietic lineages as summarized by Olsen et al (Olsen et al., 2006). However, efficient ES cell systems to induce transplantable HSCs are still limited. Initiated by Muller in 1993, it has been reported that transplantation of EB-derived cells into irradiated recipient is only able to obtain a limited level of reconstitution, existing for a short time *in vivo*, and/or require purification and direct intra-femoral cavity injection (Burt et al., 2004; Hole et al., 1996; Muller and Dzierzak, 1993). This could be possibly be accounted for by the lack of a suitable microenvironment for induction, maturation of HSCs to enable them to home to bone marrow as suggested

for preHSCs from yolk sac. Alternatively there could be a limited frequency of transplantable HSCs due to the lack of suitable culture conditions required for expansion at the HSCs stage.

Several transcription factors critical for HSCs self-renewal have been introduced into ES cell system in attempt to improve haematopoietic reconstitution. For example, HoxB4 was a transcription factor shown to be pivotal for mouse HSCs expansion (Antonchuk et al., 2001; Antonchuk et al., 2002; Bjornsson et al., 2003). A co-culture system of EBs with OP9 co-culture and overexpression of HoxB4 in the presence of SCF, VEGF, TPO and Flt-3 ligand has been developed and shown to produce HSCs able to repopulate irradiated primary and secondary recipients, however, with low levels of lymphoid reconstitution ability. Based on this, a combination HoxB4 and Cdx4, a modulator of Hox genes, was further applied and derived HSCs successfully repopulating primary and secondary recipient and expressed SLAM surface markers (Kyba et al., 2002; McKinney-Freeman et al., 2009; Wang et al., 2005).

1.4.4 Haematopoietic ontogeny in mES cell system

The mES cell system could provide an alternative source for HSCs, HPCs, and haematopoietic cells. Establishment and optimization of these systems *in vitro* were basically parallel to, or even supplement *in vivo* observation. Herein, we reviewed a series of *in vitro* studies on ontogeny of haematopoiesis in mES cell system.

Analysis of the temporal production of haematopoietic precursor and lineages in differentiating EBs showed a similar development pattern to yolk sac and early fetal liver haematopoiesis (Keller et al., 1993). More detailed analysis of early stage EBs (day2.5 to day 4) identified a progenitor known as the blast colony-forming cell (BL-CFC) co-expressing Flk1 and Bry which could form colonies consisted of haematopoietic (primitive and definitive)and endothelial precursors in response to VEGF and SCF (Choi et al., 1998; Fehling et al., 2003; Kennedy et al., 1997). Later a study by Ema suggested a smooth muscle potential of BL-CFC population, which could be abrogated by SCL (Ema et al., 2003). This progenitor was assumed to

represent the early stage haematopoeisis and comparable to the haemangioblast *in vivo*.

As the controversies *in vivo* about haemangioblast and haemogenic endothelium, *in vitro* ES cell system, an alternative theory was proposed by Nishikawa's group. They applied a 2-dimensional mESC differentiation system on collagen IV to induce $Flk1^{+}VE\text{-}cadherin^{-}$ and $Flk1^{+}VE\text{-}cadherin^{+}$ population. Further co-culture of these two populations on OP9 with addition of Epo, SCF, IL-3 and G-CSF revealed their bi-potential of haematopoietic and endothelial cells. $Flk1^{+}VE\text{-}cadherin^{+}$ represented a diverging point of haematopoietic and endothelial lineages (Nishikawa et al., 1998a). Eilken and colleagues also demonstrated that nascent blood progenitors could derive from endothelial cells by detatching from an endothelial colony. Plus, not all the colonies transformed into haematopoietic cells but also contributed to endothelial cells. This further supported the existence of endothelial cells with haematopoietic potential (Eilken et al., 2009). Thereafter, the concepts of the haemangioblast and the haematogenic endothelium were first connected by Lancrin et al in 2009 that haemangioblast ($Bry^{+}Flk1^{+}$) first generated the haematogenic endothelium ($Tie2^{high}cKit^{+}CD41^{-}$) then further produced haematopoietic cells (Lancrin et al., 2009).

Of note, the $Flk1^{+}$ population displayed a better progeny to haematopoietic direction than Bry according to a series of studies in EB differentiation. Kouskoff and colleagues demonstrated that Bry^{+} cells had differential Flk1 expression and that BL-CFC activity was enriched in $Bry^{+}Flk1^{+}$ from day 3.25 EBs while $Bry^{+}Flk1^{-}$ had a preference for cardiac differentiation. Later studies revealed a re-specification by Notch4 on $Bry^{+}Flk1^{+}$ to cardiac direction (Chen et al., 2008; Kouskoff et al., 2005). Purified $Flk1^{+}$ population from EBs could re-aggregate in serum-free media supplemented with VEGF to undergo primitive and definitive haematopoiesis (Cheng et al., 2008). Interestingly, Keller's group further reported that $Flk1^{+}$ cells derived from EBs at day3.25 and day5.25 displayed characteristics of haematopoiesis in yolk sac and P-Sp region in the early embryo (Irion et al., 2010).

As mentioned in section 1.3.3 Mikkola and colleagues, also screened surface phenotypes during haematopoiesis not only in yolk sac but also differentiating EBs. Using the ES cell system, they confirmed that CD41 was expressed in EBs from 4.25 to 4.75 till day7. CD45 was expressed in EBs from day6.75 but with very low level at around 2%. Definitive haematopoietic progenitors were enriched in $CD41^+$ fraction from EBs co-expressing CD34 and cKit. Of note, colony assays revealed that haematopoietic activity was more highly enriched in the $CD41^+CD45^-$ $cKit^+$ fraction compared to $CD41^+CD45^+cKit^+$ fraction from day6 EBs, suggesting CD41 as an earlier marker than CD45 in EB system (Mikkola et al., 2003). Thus, $CD41^+cKit^+$ can be used as a convincing surface phenotype to define definitive haematopoietic progenitors in the EB system. CD45 can be applied to measure a later stage of haematopoietic differentiation in this system.

1.5 Molecular mechanism involved in regulation of haematopoiesis

The induction, maturation and lineage specification of HPCs and HSCs in embryonic and adult haematopoiesis are essentially regulated by a molecular network consisting of transcription factors, regulators and signaling pathways. Herein, we briefly summarized studies on effects of these key regulators on haematopoietic ontogeny and differentiation during the development based on *in vivo* and *in vitro* studies (Figure 1.4, 1.5).

1.5.1 Transcription factors and regulators of haematopoiesis

1.5.1.1 Runx1 and Gata2

According to *in vivo* studies, the transcription factor Runx1 is an essential regulator in definitive haematopoiesis, while being dispensable for primitive haematopoiesis. Deletion of Runx1 in the mouse embryo results in embryonic lethality with internal bleeding at around E12.5. These embryos have the ability to produce primitive erythrocytes but they have a complete lack of haematopoiesis in fetal liver or definitive HPCs formation in E10 yolk sac indicated its role in definitive haematopoiesis (Okuda et al., 1996; Wang et al., 1996). Runx1 reporter mice were developed and demonstrated Runx1 expression in intra-aortic clusters, the ventral endothelial layer of the dorsal aorta and underlying mesenchyme in AGM region as well as in the endothelial cells of the yolk sac, the vitelline and umbilical arteries. Thus loss of Runx1 abrogated formation of intra-aortic clusters and sequentially inhibited HSCs formation (Cai et al., 2000; North et al., 1999; North et al., 2002). In support of this, it was noted that embryoid bodies generated from $Runx1^{-/-}$ ES cells failed to commit to definitive haematopoiesis but not primitive one (Lacaud, 2002). Further studies *in vitro* or *in vivo* using conditional knock out or rescue models of Runx1 suggested that Runx1 was required for haematopoietic differentiation only from haemogenic endothelium marked by Tie2 or VE-cadherin (Chen et al., 2009; Li et al., 2006; Liakhovitskaia et al., 2009). In addition, Runx1 was also widely expressed in the adult haematopoietic cells except erythroid lienages (de Bruijn and Speck, 2004; North et al., 2004).

Gata2 was found to essential for early stage of haematopoiesis in embryo and expansion of multi-potential hematopoietic progenitors (Tsai et al., 1994; Tsai and Orkin, 1997). It was reported to be involved into erythroid and megakaryocytes differentiation later from HSCs (Dore and Crispino, 2011). Interestingly, Robert-Moreno and colleagues demonstrated that Gata2 was crucial for the onset of definitive haematopoiesis in AGM region which functioned upstream of Runx1 and was regulated by the Notch signaling pathway (Robert-Moreno et al., 2005). Gata2 being downstream of Notch signaling was also confirmed in 32D myeloid progenitors (Kumano, 2001; Robert-Moreno et al., 2005). Conditional induction of Gata2 in the ES cells system indicated that Gata2 could promote haemangioblast generation, precocious commitment to erythroid lineages and increased endothelial cell generation (Lugus et al., 2007).

1.5.1.2 SCL and Lmo2

SCL (T-cell leukaemia oncoprotein, Tal-1) was first identified through its involvement in a chromosomal translocation in human leukaemia. During embryogenesis, SCL is widely expressed in the vascular endothelium and in primitive and definitive haematopoietic cells (Elefanty et al., 1999; Kallianpur et al., 1994). Deficiency of SCL causes the failure of yolk sac haematopoiesis and SCL null animals die at E8-10.5 due to the lack of primitive haematopoiesis resulting in severe anaemia (Robb et al., 1995; Shivdasani et al., 1995). Analysis of chimeric mice generated by injecting $SCL^{-/-}$ ES cells into a wild type blastocyst demonstrated the contribution of $SCL^{-/-}$ only to non-haematopoietic lineages revealing a crucial role for the SCL in definitive hematopoiesis (Porcher et al., 1996; Robb et al., 1996). Thus SCL was invovled in both primitve and definitive haematopoiesis. Of note, in murine and ES cells models, it has been confirmed that SCL is required for the transition of the haemangioblast into haemogenic endothelium, but not later haematopoietic commitment from haemogenic endothelium (D'Souza et al., 2005; Gering et al., 1998; Lancrin et al., 2009; Schlaeger et al., 2005).

Lmo2 knockout mice die at E10.5 due to anaemia with failure of primitive erythropoiesis in yolk sac. Chimeric analysis also revealed its contribution to definitive haematopoiesis. It has been suggested that this regulator could interact with SCL to form a complex regulating haematopoietic lineage specification (Lecuyer et al., 2007; Warren et al., 1994; Yamada et al., 1998).

1.5.1.3 Gata1 and PU.1

A number of studies on thaematopoietic fate decisions have focused on the action of Gata1 and PU.1. It was found that Gata1 could promote erythroid/megakaryocytic differentiation while PU.1 promoted myeloid differentiation. These two proteins could physically interact and antagonize each other in fate decisions of HPCs. In the zebrafish model, inhibition of Gata1 expression by morpholinos resulted in the conversion of HPCs to a myeloid fate while inhibition of PU.1 converted HPCs to an erythroid fate (Galloway et al., 2005; Orkin, 2000; Rhodes et al., 2005).

1.5.2 Regulation of haematopoiesis by Notch signaling pathway

In addition to the transcription factors and regulators involved in haematopoiesis, signaling pathways such as Notch, Wnt and BMP signaling have been reported to regulate haematopoiesis at differentiation stages and to interact with each other as well as other regulators. Here we summarized the studies on Notch signaling pathway and its role in haematopoiesis.

1.5.2.1 Transduction of Notch signaling pathway

Notch is a transmembrane protein that acts as a signal receptor. The Notch signaling pathway is supposed to be an evolutionarily conserved mechanism that is widely used by invertebrates and vertebrates to control cell fate decisions, including proliferation, differentiation and apoptosis (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Lai, 2004; Lewis, 1998). So far, a series of specific trans-membrane ligands including Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2 have been identified to activate the Notch signaling pathway (Lai, 2004). Four Notch receptors (Notch1, Notch2, Notch3, Notch4) have been found in mammalian cells with different expression patterns and roles which are tissue and

cytokine dependent (Bigas et al., 1998; Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1992). Generation of function Notch ligands depend on the E3 ligase, Mib1. The Notch receptors consist of an extracellular domain, which functions in ligand binding and an intracellular domain (NotchIC) to interact with a number of cytoplasmic and nuclear proteins to permit signal transduction. Upon ligand binding, proteolytic cleavage occurs by a membrane-associated protease complex (γ–secretase) containing presenilin at the cell membrane (Karlstrom et al., 2002; Schroeter et al., 1998). After that the intracellular domain is released and interacts with a series of cytoplasmic and nuclear proteins. In the prevailing models for Notch signal transduction, the majority of studies have mainly focused on nuclear transduction where the transcription factor RBP-Jκ family protein (also known as CSL or CBF1/ Su(H)/ Lag-1) is involved. After translocation into the nucleus, NotchIC bind to and turns the RBP-Jκ co-repressor complex into a RBP-Jκ co-activator complex that then modulates downstream gene expression and cell fate decisions (Figure 1.4) (reviewed by Lai, 2004).

The main Notch downstream target genes activated by the interaction of NotchIC and RBP-Jκ co-activator complex belong to basic helix-loop-helix (bHLH) transcription factor family, such as the Hes (Enhancer of Split) and Hey (Hes related repressor) families which are supposed to mainly act as transcriptional repressors of lineage commitment genes (Iso et al., 2003). In addition to these basic components, a number of other Notch-associated proteins interact at various stages. For example, mastermind-like protein (MAML), p300 and SKIP are confirmed to positively affect Notch signaling, while Numb, Numb-like and Sel-10 (cdc4) act as negative regulators (Hansson et al., 2004; Kadesch, 2004). It has been reported that dominant-negative Mastermind-like1 (DNMAML), a truncated MAML, is a potent inhibitor of Notch signal pathway (Weng et al., 2003). DNMAML only encodes amino acids 13 to 74, providing the binding site to NotchIC but cannot recruit other co-activators such as p300 to form the RBP-Jk activator complex. Several studies have used DNMAML to inhibit Notch signaling pathway in haematopoietic differentiation (Maillard et al., 2008; Mercher et al., 2008; Yu et al., 2008).

1.5.2.2 Role of Notch signalling pathway in Haematopoiesis

A series of *in vitro* and *in vivo* models have been established to investigate the role of the Notch signaling pathway in the ontogeny of primitive and definitive haematopoiesis as well as haematopoietic differentiation from haematopoietic progenitors. As introduced in section 1.2, primitive haematopoiesis arises in the yolk sac from E7.5 to E8.5 followed by a definitive process to produce definitive HPCs able to form colonies in CFU assays and short-term HSCs in both yolk sac and P-Sp/AGM region. Finally, LTR-HSCs derived from the AGM region at E10.5 is defined as the later stage of definitive haematopoiesis.

a) Notch signaling in embryonic haematopoietic ontogeny

It was reported that Notch1, Notch4, Jagged1, Jagged2 and Delta-like4 are expressed in the ventral endothelium of the P-Sp/AGM aorta through E 9.5 to E10.5 in the mouse embryo before the appearance of LTR-HSCs (Robert-Moreno et al., 2005). Furthermore, Notch receptors and ligands are expressed in fetal liver from E12 to E17 indicating a role of Notch in definitive haematopoiesis (Walker et al., 2001). To investigate the role of Notch signaling, several knockout mouse models have been established including RBP-Jκ, Notch1, Jagged1, Dll1, Dll4, Hey1/Hey2 or Mib 1. Lethality at around E10.5 was observed caused by vascular defects indicating a critical involvement of Notch signaling in vascular development but abrogating a direct analysis of embryonic haematopoiesis (Duarte et al., 2004; Fischer et al., 2004; Hrabe de Angelis et al., 1997; Koo et al., 2005; Krebs et al., 2004; Krebs et al., 2000; Xue et al., 1999). Thus, ES cell system, explants culture, colony forming assay, chimaeras, heterozygous as well as conditional knock out/knock down have been used overcome early lethality and address the role of Notch in early haematopoiesis.

For primitive haematopoiesis: It was noted that Notch signaling did not affect yolk sac derived primitive haematopoiesis as assessed using colony assays from cells derived from Notch1$^{-/-}$ and Mib1$^{-/-}$ embryos. Although yolk sac from both models were taken from E9.5 and E8 to E8.5, respectively, when definitive HPCs had already been generated, these two studies confirmed primitive haematopoiesis was not dependent on Notch by determining expression of β-H1 (Kumano et al., 2003;

Yoon et al., 2008). CFU-EryP, the primitive erythroid colony formation units from whole embryo E7.5 to E8.5 were also measured and compared in Notch1$^{-/-}$ with wild type or heterozygous showing no difference, which further supported Notch signaling was dispensable for primitive haematopoiesis (Hadland, 2004). Other primitive lineages like primitive macrophages were also not affected in RBP-Jκ$^{-/-}$ yolk sacs at E9.5, however with an increase of both yolk sac derived primitive erythroid differentiation and adult erythrocytes formation due to reduced apoptosis. Erythrocytes increase observed in RBP-Jκ$^{-/-}$ model could be that it is a stronger model to block Notch signaling compared to Notch1 deficient model, which could be compensated by other receptors (Robert-Moreno et al., 2007). Therefore, Notch signaling did not affect or inhibit primitive haematopoiesis in yolk sac. This was also confirmed in the ES cell system, in which Flk1^{+} derived primitive haematopoiesis was inhibited by ectopic Notch1 but definitive haematopoiesis not affected (Cheng et al., 2008).

For definitive haematopoiesis: As introduced above in Figure 1.1, definitive haematopoiesis could be further devided into several steps, including formation of erythro-myeloid progenitor (pro), lymphoid-erythro-meyloid progenitors (meso), preHSCs for newborn mice repopulation (meta) and long-term definitive HSCs. It has been reported by different groups that haematopoietic colony forming units (CFUs) from RBP-Jκ$^{-/-}$ P-Sp or Mib1$^{-/-}$ P-Sp at E9.5 were reduced which indicated that Notch is crucial for the production of definitive multi-potential progenitors in P-Sp/AGM region (Robert-Moreno et al., 2005; Yoon et al., 2008). When explant culture of E9.5 P-Sp region on OP9 or cytokines were used to obtain later definitive progenitors, a severe reduction of CFUs was observed in these deficient models (Kumano et al., 2003; Robert-Moreno et al., 2005; Yoon et al., 2008). Compared to wild type mice, cells from both E9.5 yolk sac and P-Sp in Notch1$^{-/-}$ mice lost the ability to repopulation newborn animals (Kumano et al., 2003). Interestingly, a later study by Robert-Moreno using a Jag1$^{\Delta/\Delta}$/Sca1–GFP model showed a reduction of Sca1^{+} cells and CFUs in E10.5 AGM, however with normal arterial development indicating a non-cell autonomously regulation by Notch signaling (Robert-Moreno et al., 2008). Therefore, Notch signaling pathway was critical for definitive

haematopoiesis of P-Sp/AGM (CFUs and newborn repopulating preHSCs) and yolk sac (newborn repopulating preHSCs).

To confirm whether Notch could affect haematopoiesis in a cell autonomous manner, Hadland and colleagues developed chimaeras with the LacZ as the tag for Notch1$^{-/-}$ ES derived cells. They showed that Notch1$^{-/-}$ cells could also contribute to yolk sac definitive CFUs till E11.5 but dropped dramatically afterwards in yolk sac, fetal liver and bone marrow indicating Notch1 was critical for definitive haematopoiesis at later stage, which could possibly derived from long-term definitive HSCs production. To support this, they demonstrated that in Notch1$^{-/-}$ models definitive CFUs produced in yolk sac from E7 to E8.5 were not affected. This study revealed that Notch did not affect early definitive haematopoiesis as measured in yolk sac, but was critical for later definitive haematopoiesis in a cell-intrinsic way (Hadland, 2004).

Addition of inhibitor of Notch signalling pathway abrogated the emergence of haematopoietic cells from VE-cadherin^{+} haemogenic endothelium cells from the E9.5 P-Sp region (Kumano et al., 2003). Conditional knock out of Notch signaling in Tie2^{+} endothelial cells also resulted in reduced CFUs (Yoon et al., 2008). Furthermore, expression of Runx1, Gata2, and SCL, which were expressed by endothelial-like cells with potential to generate intra-aortic clusters were reduced in RBP-Jκ$^{-/-}$ P-Sp at E9.5. (Minegishi et al., 1999; North et al., 2002; Porcher et al., 1996; Robert-Moreno et al., 2005; Tsai and Orkin, 1997). These studies suggested that regulation of Notch signalling in definitive haematopoiesis was relevant to haematopoietic process post haemogenic endothelium formation.

Taking these *in vivo* studies together, in P-Sp/AGM region, Notch signaling is critical for definitive haematopoiesis which could possible mediate intra-aortic cluster formation from haemogenic endothelium activated by Jagged1. In yolk sac, early definitive CFU formation was not affected but later definitive haematopoiesis to produce newborn repopulating preHSCs and CFUs after E11.5 was impaired by Notch deficiency. Thus, role of Notch signaling pathway in haematopoiesis in vivo was spatial and temporal dependent. More likely, Notch1 is required for the

development of the newborn repopulatin preHSCs and long-term definitive hematopoietic stem cell compartment while does not affect earlier, short-term definitive hematopoietic. This was supported by Bertand's group demonstrating Notch signaling pathway distinguished two waves of definitive haematopoiesis in zebrafish model (Bertrand et al., 2010).

b) Notch signaling in haematopoietic commitments

A series of studies have reported that Notch signaling could affect fate decision of HPCs or HSCs via regulating apoptosis, proliferation, cell cycles and so on. For example, it was reported that Notch could inhibit further differentiation of haematopoietic stem and progenitor cells and maintain their self-renewal (Milner et al., 1996; Varnum-Finney et al., 2000). Co-culture of fetal liver derived HPCs with OP9 expressing ectopic Detla-like 1 could increase T cell development *in vitro* (Schmitt and Zuniga-Pflucker, 2002). Notch1 or Notch2 activation inhibited myeloid differentiation in a cytokine-dependent manner. Similarly, stimulation of Notch activity by Delta-like 1 from OP9 stromal cells resulted in inhibition of myeloid differentiation (reviewed by (Bigas et al., 2010)). As mention above, RBP-Jκ$^{-/-}$ mutation leads to higher erythroid differentiation due to reduce of apoptosis (Robert-Moreno et al., 2007). Megakaryocyte differentiation from HSCs could be promoted by Notch signaling when co-cultured on OP9 expressing ectopic Delta-like1 (Mercher T et al., 2008).

c) ES cell models to investigate Notch signaling in haematopoiesis

Because of the limitations of mouse models where deficient mice die at an early embryonic stage, *in vitro* studies using ES cells has become a major tool in the investigation of Notch signalling pathway during haematopoiesis. It was reported that activation of Notch inhibited ES cell differentiation into the mesoderm lineage (Lowell et al., 2006; Schroeder et al., 2006). On the other hand using the Notch1$^{-/-}$ ES cell line, it was suggested that Notch1 deficiency did not affect Flk1^{+} mesoderm formation (Hadland et al., 2004). These data suggested that Notch could be dispensable for mesoderm formation, while overexpression of Notch could abrogate it.

During haematopoietic differentiation of ES cells Notch1$^{-/-}$ did not affect early definitive haematopoiesis but inhibited primitive erythroid formation in suspension EBs as assessed by colony formation assays (Hadland et al., 2004). In contrast, when GSI was applied to inhibit Notch after the formation of mesoderm, the number of multi-potential CFUs from EBs co-cultured on AGM-derived stromal cells were reduced (Figure 1.6) (Gordon-Keylock et al., 2010). This could be accounted for by the fact that these are different microenvironments, which is in support of the *in vivo* studies revealing difference of Notch signaling on haematopoiesis in yolk sac and AGM (section 1.5.2.2-a). Inducible ectopic NotchIC in ES cells inhibits the generation of cardiomyocytes, endothelial cells and haematopoietic cells from mesoderm progenitor cells (Schroeder et al., 2006). However, it was also reported that ectopic Notch4 could respecify Bry^{+}Flk1^{+} haemangioblasts to a cardiac fate (Chen et al., 2008). For lineage commitment, overexpression of Notch1 in Flk1^{+} cells re-aggregation inhibited primitive erythropoiesis by interacting with Wnt signaling via Numb but this did not affect definitive haematopoiesis (Cheng et al., 2008). It was also reported that ectopic NotchIC could promote myeloid maturation and reduced immature progenitors in HPCs derived from ES cells co-culture on OP9 stromal. This observation correlated to their earlier observation in in 32D myeloid progenitor cells. In addition, reduced self-renewal of multipotent haematopoietic progenitor cells (FDCP-mix cell line) and accelerated commitments to mature myeloid cells were initiated by overexpression of NotchIC via up-regulating PU.1 (Schroeder and Just, 2000; Schroeder et al., 2003; Schroeder et al., 2006). We assumed that the role of Notch signalling pathway in haematopoiesis using ES cell system are context dependent and determined by manipulation strategies.

d) A co-culture system of EBs and AGM-derived stromal cells to investigate Notch signalling pathway

As introduced above, studies suggested regulation of Notch signalling pathway in primitive or definitive, yolk sac-derived or AGM-derived haematopoiesis could be different indicating that role of Notch signaling in haematopoiesis is microenvironment, modulation strategies and differentiation stages dependent.

By using ES cell system, it will overcome the early lethality of knock out mutation models and provide a relatively simplified microenvironment compared to mice models. However ES cell system were considered to represent the yolk sac derived haematopoiesis. Although a number of efficient ES models with addition of cytokines cocktails, gene modification or OP9 stromal co-culture have been developed to induce haematopoietic cells, these *in vitro* systems are possibly not reflecting the precise mechanisms that exist in the *in vivo* environment.

To mimic the AGM-derived haematopoiesis using ES cell system, a co-culture system of EBs with AGM-derived stromal cells without exogenous powerful induction factors has been established with suggestion the involvement of Notch signalling pathway post mesoderm formation using pharmacological approaches with GSI, though γ–secretase cleavage could happen not only to Notch receptor but also other trans-membrane proteins, such as amyloid precursor protein (APP), ErbB-4, SREBP-1, N-cadherin, and CD44 (Figure 1.6) (Gordon-Keylock et al., 2010). Based on this work, we suggested that Notch signalling pathway could regulate ES-derived haematopoiesis post mesoderm formation in the AM supporting microenvironment. A referred above, surface marker Flk1 could mark haemangioblast cells, the population formed after mesoderm and for further differentiation into haematopoietic lineages. Thus this project further specify this co-culture system and focused on the involvement of Notch signaling in $Flk1^+$ population derived haematopoiesis.

1.6 Thesis Aims

1.6.1 Hypothesis

Notch signalling pathway is involved in the ES-derived haematopoiesis in microenvironment provided by AGM-derived stromal cells.

1.6.2 Experiment strategy

To expand the work published by Gordon-Keylock in 2010 and determine how Notch signalling pathway regulates ES-derived haematopoiesis in AGM microenvironment after mesoderm formation, we carried out experiments to further

investigate the correlation between haematopoietic activity and ligands expression or Notch activity in the co-culture system. We also set up a co-culture system to focus on $Flk1^+$ haematopoiesis and determine how Notch signalling pathway affects this process using genetic modified ES cell lines.

To assess the correlation of haematopoietic activity with Notch activity in the EB/AGM-derived stromal co-culture system:

- Ligand expression in stromal cells was analyzed and compared with quantitative RT-PCR, flow cytometry, immunochemistry and western blots;
- Notch activity in EBs or stromal cells from co-cultures upon addition of GSI were analyzed with quantitative RT-PCR;
- Notch activity in EBs sorted from co-cultures was monitored throughout co-culture period with quantitative RT-PCR.

To analyze effect of Notch signalling pathway post mesoderm more specifically and avoid affecting other population or early differentiation:

- A novel co-culture system was established by co-culturing $Flk1^+$ cells derived suspension EBs on AGM-derived stromal cells with haematopoietic activity assessed by colony forming assay and surface phenotypes;

To analyze whether Notch signalling pathway could affect $Flk1^+$ derived haematopoiesis in AGM microenvironment:

- A tamoxifen inducible ES cell line, R26-NotchIC, was applied to induce Notch activity in $Flk1^+$/AM co-culture system with assessing haematopoietic activity in colony forming assay, surface phenotypes and related gene expression.

To analyze whether Notch signalling pathway is required for $Flk1^+$ derived haematopoiesis in AGM microenvironment:

- A doxycycline inducible ES cell line to knock down Notch activity was established.

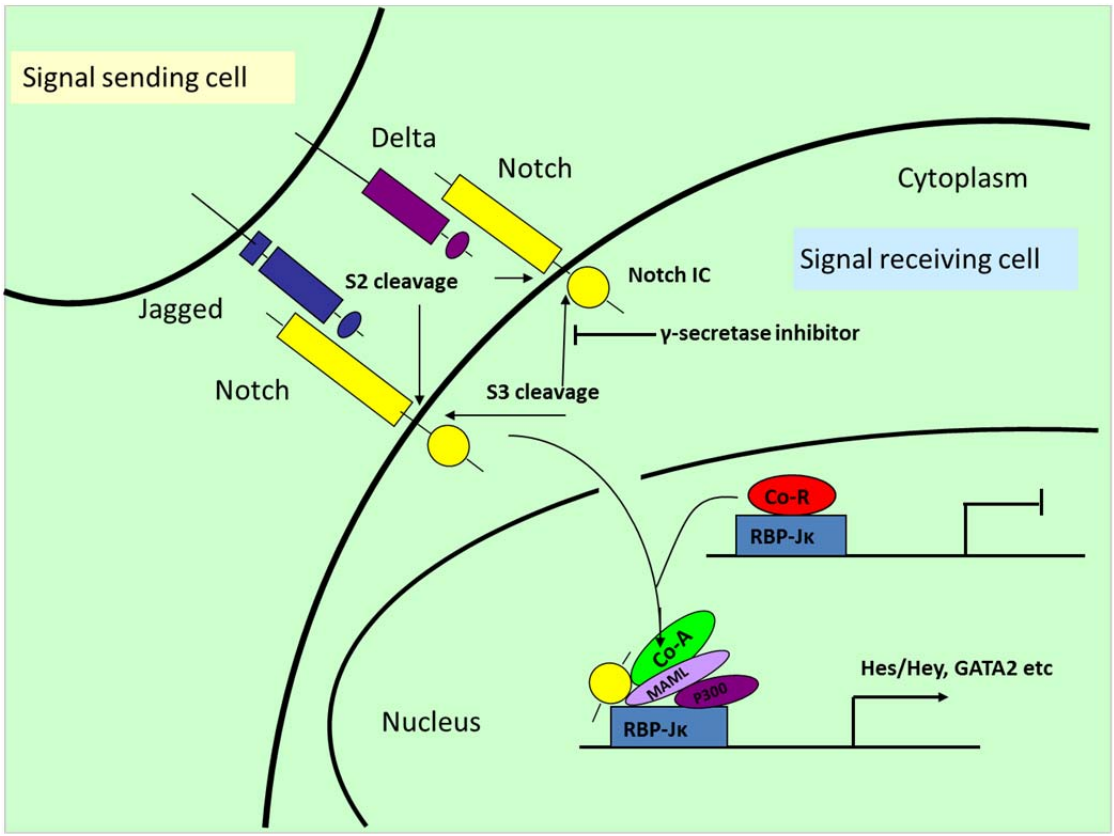

Figure 1.4 Brief summary of RBP-Jκ dependent Notch signalling pathway.
Figure adapted from review by Lai (2004)

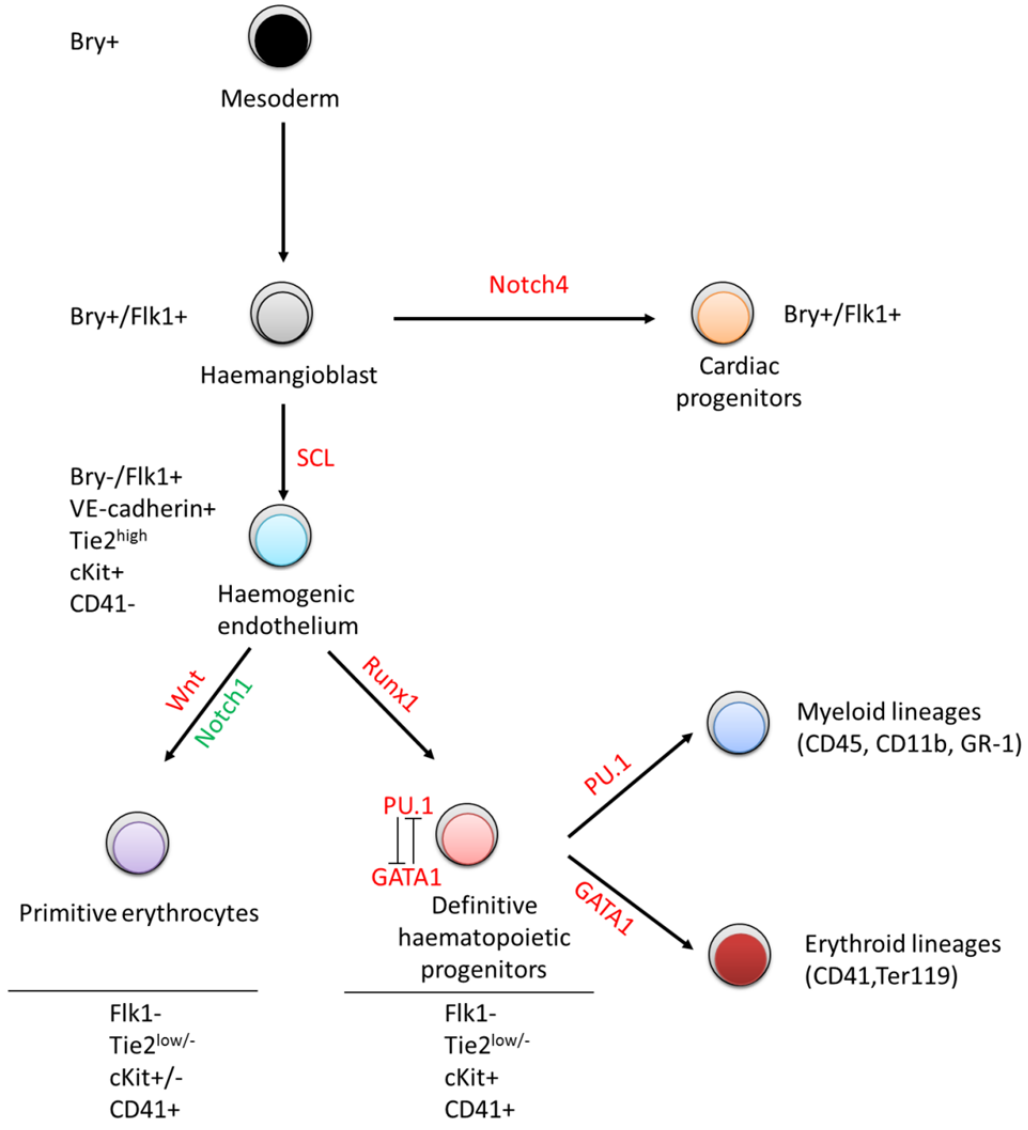

Figure 1.5 Brief summary of development of haematopoiesis established in ES cell system *in vitro* with surface phenotypes and key regulators noted.

Figure modified based on review by Lancrin et al., 2009.

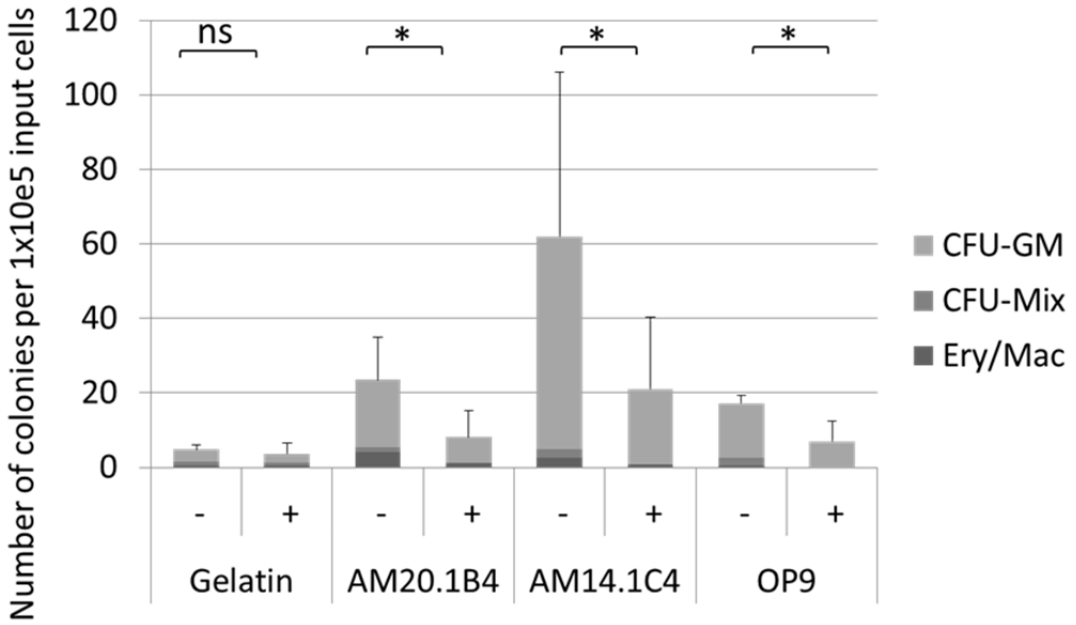

Figure 1.6 Inhibition of multipotent haematopoietic colonies in EBs differentiated to 6 days on gelatin, AM20.1B4, AM14.1C4 or OP9 by addition of γ-secretase inhibitor (GSI) between Days 4 and 6.

Error bars represent standard deviation of 3 independent experiments. P-values were calculated with Wilcoxon matched pairs tests (*$p<0.002$, ns, not significant difference). Figure reproduced with raw data supporting Gordon-Keylock et al., 2010.

Chapter 2: Materials and Methods

2.1 Molecular strategies

2.1.1 Plasmid construction

Plasmids information was listed in Appendix Table 2.1. Digestion and ligation was carried out with restriction digestion enzyme (Roche or NEB) and T4 ligase (Invitrogen) respectively according to the manual instruction.

2.1.2. Transformation of bacterial cells

For transformation, up to 1µg plasmid DNA or 1-2ul ligation product (20ul reaction volume) was added into 25µl of DH5α library Competent *E. coli* (Invitrogen) and gently mixed then incubated on ice for 30 minutes, heat shocked for 45 seconds at 42 °C followed with another incubation on ice for 2 minutes. 250µl room temperature Super Optimal broth with Catabolite repression (S.O.C.) medium (Invitrogen) was added and shaken at 225rpm at 37 °C for 1 hour. 20-100µl of the transformation reaction was plated on Luria-Bertani (LB) agar containing antibiotics (100µg/ml ampicillin or 50µg/ml kanamycin). Plates were incubated at 37 °C in incubator for 14hr – 20hr and single colony was picked up for further validation.

2.1.3 Plasmid preparation (Minipreps and Maxipreps)

A single colony was picked up and cultured in 3ml (for minipreps and starter cultures) LB containing antibiotic or 250 ml LB (for maxipreps) containing antibiotics (100µg/ml ampicillin or 50µg/ml kanamycin) then incubated overnight at 225rpm in an orbital shaker at 37 °C. The cultures were harvested using centrifuges and DNA extracted using either a miniprep kit, maxiprep kit or a HiSpeed plasmid maxi kit (Qiagen) according to manual instruction.

2.1.4 RNA extractions and cDNA synthesis for quantitative RT-PCR

Total RNA was extracted from frozen or fresh cell pellets with RNeasy Mini kit (Qiagen) following the manual instructions. DNAse I (Qiagen) was applied directly onto column to remove residual genomic DNA (Qiagen). cDNA was synthesised

using the reverse transcription Superscript III kit or SuperScript® VILO™ Master Mix (Invitrogen). The reaction was set up with up to 600 ng (for Superscript III kit) or 1.25µg (for SuperScript® VILO™ Master Mix) of RNA per reaction at 10µl then incubated at 25 °C for 10 minutes for primer annealing, then 42 °C for 60 minutes for cDNA synthesis then inactivated at 85 °C for 5 minutes. cDNA was normally stored at -20 °C.

2.1.5 Quantitative Reverse Transcriptase PCR (qRT-PCR)

QPCR primers and probes were self-designed thensynthesized by MWG Eurofins, or purchased from Aplied Biosystems. QPCR was performed on an ABI 7500 FAST qPCR machine (Applied Biosystems) with 10ng cDNA per reaction. Housekeeping gene Hprt (hypoxanthine-guanine phosphoribosyl transferase) or 18s (18S ribosomal RNA) were used as the endogenous control for relative quantitation of gene expression to the amount of cDNA loaded. Reactions were set up in triplicate in 96 well plates following the default Taqman programme for universal condition: 95 °C for 3 seconds, followed by 35- 45 cycles of 95 °C for 20 seconds to denature the cDNA and 60 °C for 30 seconds to allow annealing and extension. For sybergreen primer, a dissociation step was added up to check specificity of primer. Relative quantitation was calculated with the ΔΔCT method using SDS v1.4 software by Applied Biosystems. Using this software the gene expression in each reaction was first normalized to the endogenous control and the data was then shown as fold change to a calibrator chosen according to each experiment.

Primers and probes for qPCR were listed in Appendix Table 2.2

2.2 Cell Culture and manipulation

2.2.1 Maintenance of cells

2.2.1.1 Culture of Mouse Embryonic Stem cells (mESCs)

Mouse embryonic stem cells (mESCs) were routinely cultured on 0.1% gelatin (Sigma) coated 25cm^2 tissue culture flasks and maintained in 1 x Glasgow Minimum Essential Medium (GMEM) (Invitrogen) supplemented with 10% Fetal calf serum (Lonza), 2mM sodium pyruvate (Invitrogen), 1% non-essential amino acids (Invitrogen), and 0.1mM β-mercaptoethanol (Sigma). This media was supplemented with 100U/ml Leukaemia Inhibitory Factor (LIF). Addition of another 4mM L-glutamine (Invitrogen) is optional based on the growth and differentiating of cells. 1U/ml of LIF (Leukaemia Inhibitory Factor) was defined as the lowest concentration to maintain CP1 ES cells at undifferentiated state (Bradley et al., 1984).

LIF was prepared from the condition medium of COS7 cells transfected with the pCAGGSLIF-418 plasmid (from Professor Austin Smith). LIF concentration was determined by serial titration to test the ability to maintain CP1 ES cells at undifferentiated state according to the morphology. Batch testing for FCS chosen for maintenance and differentiation were carried out in toxicity test, self-renewal assay and mesoderm differentiation (Routinely carried out by Helen Taylor, Julie Wilson).

When ES cells were 80- 90 % confluent in culture which normally taking 48 hours, cell passage was applied. Start with gelatinizing flasks, 2ml/25cm^2 of 0.1% Gelatin in PBS (Invitrogen) was added to the flasks and left for another 5 minutes then aspirated away. Old medium was aspirated and washed with 2ml pre-warmed PBS to remove the remaining medium. Cells were then treated with 2ml trypsin solution (0.025 % trypsin (Sigma), 1 % chick serum (Gibco) and 1.3mM EDTA (BDH) in PBS) for 3-5 minutes at 37 ˚C. The flasks were tapped 5 times to lift the cells then cell suspension was added into 8ml of ES medium to neutralize the trypsin with the serum in the medium and centrifuged at 130 x g for 5 minutes. The supernatant was aspirated and the pellet was re-suspended with 10 ml fresh medium to get single cell suspension and counted using a Nebauer haemocytometer. 1 x10^6 per 25 cm^2 ES

cells were seeded onto gelatinized flask and supplemented with fresh medium to 10mls with the addition of LIF at 100U/ml then incubated at 37°C in humidified 5% CO_2 atmosphere in a Galaxy incubator (Wolf Laboratories). The ES cells number will reach up to 4-8x10^6 cells after 48 hours.

ES cell lines applied in this project were listed in Appendix Table 2.3.

2.2.1.2 Maintenance of Stroma-derived cells

a) Maintenance of embryo-derived stromal cells

Stromal cell lines used in this project were derived from haematopoietic tissues of mid-gestational mouse embryos as previously described (Oostendorp et al., 2002a; Oostendorp et al., 2002b) (Appendix Table 2. 4). Generally, AM20.1A4, AM20.1B4, UG26.1B6 and UG26.2D3 were derived from the E10 transgenic mouse embryos (C57BL/10xCBA background) carrying the temperature-sensitive SV40 T-antigen Taq (tsA58) which is active at the temperature of 33 ˚C as the immortalizing gene under the control of the β-*actin* or *PGK* (phosphoglycerate kinase) gene promoters. AM14.1C4 was derived from the AM subregion of AGM reguions from a control E11 BL1b transgenitc emryos. The BL1b was *Ly-6E (Sca-1) lacZ* transgene line (C57BL/10xCBA background). EL08.1D2 was derived from the fetal liver of BL1b E11 embryo as well.

All the stromal cell lines derived from embryo as described above were maintained on gelatinized flasks or wells in stromal medium consisting of 50% MyeloCult long-term culture medium M5300 (Stem Cell Technology), 40% alpha minimal essential medium (Invitrogen), 10% FCS (Lonza), an additional 1mM L-glutamine (Invitrogen) and 0.05 mM beta-mercaptoethanol. Cells were split at 1:2 to 1:6 ratio every 2-3 days when got around 90% confluent with trypsin solution. Stromal cell lines derived from tsA58 mice were culture at 33 ˚C while cell lines derived from BL1b were culture at 37 ˚C with a humidified 5% CO_2 atmosphere.

b) Mainteneance of bone marrow-derived OP9 stromal cells

OP9 stromal cell line was established from newborn B6C3F1 op/op mouse calvaria

do not produce functional M-CSF due to the osteopetrotic mutation in the gene encoding M-CSF (Macrophage Colony Stimulating Factor) (Kodama et al., 1994). In the presence of M-CSF, ES cells could easily differentiated into macrophages compared to other mature blood lineages. Therefore, ES cells co-cultured on the M-CSF null OP9 stromal cell monolayer could efficiently divert the differentiation into blood cells of erythroid, myeloid, and B cell lineages. OP9 stromal cell line were maintained in OP9 culture medium (80% alpha-minimal essential medium (Invitrogen) and 20% FCS, an addition 2mM L-Glutamine and 0.1mM β-mercaptoethanol), passaged every 2-3 days when got around 90% confluent and cultured at 37 ˚C with a humidified 5% CO_2 atmosphere

c) Maintenance of Cos7 cells

COS 7 cells were routinely cultured directly on 25cm^2 tissue culture flasks and maintained in 1 x ES cells culture medium and incubated at 37 ˚C with 5 % CO_2 in a Galaxy incubator (Wolf Laboratories). COS 7 were passaged as ES cells but seeded at 1:5 ratios when getting 70%-80% confluent.

2.2.2 Thawing and Freezing of cells

To thaw cells, cryovials were held in 37˚C water bath to thaw quickly and cell suspensions were transferred immediately into 8ml pre-warmed culture medium and centrifuged at 1200rpmx3mins. After aspirating medium, the cell pellet was re-suspended in 10ml fresh culture medium and transferred into a gelatinized 25cm^2 flask (plus 100U/ml LIF for ES cells). The medium was replaced around 4 hours later or the second day.

To freeze cells, cells getting 80%-90% confluent was harvested using the trypsin solution as routine and pelleted down. Cell pellet was re-suspended in the freezing medium (culture medium consisting 10% dimethyl sulphoxide (DMSO) (Sigma)). For each 25cm^2 flask, cells pellet was resuspended in 1ml freezing medium and devided into 2 cryovials. Cells were frozen in -80˚C overnight and moved into -140 ˚C or liquid nitrogen for long-term storage.

2.2.3 Differentiation of ES cells

In this project, the differentiation of ES cells were carried out in several different ways, including the formation of Embryoid bodies (EB), further culture of EBs or subpopulation from EBs in suspension or on stromal cells or gelatin.

2.2.3.1 ES cells differentiation in Embryoid body (EB) -Hanging drop method

In this hanging drop method, ES cells were differentiated in the 3-dimentioanl aggregates of uniform size. Basically, cells were passaged following the normal routine and $6x10^5$ cells were re-suspended in 20ml ES cells medium plus 100U/ml LIF. 10ul droplets (300 cells per droplet) were seeded onto the lid of square petri dishes with multi-channel pipette. Lids with droplets were turned over and placed back onto the dish bases, holding 10ml tissue culture grade water (Invitrogen). Then the hanging drops were cultured in 37 ˚C (humidified 5% CO2 atmosphere). After another 48hours, EBs were harvested by tapping the edge of lids against the surface of hood and collected with a pipette then centrifuged in 20ml universal at 1000 rpm for 3 minutes. The supernatant was aspirated and pellets of EBs were re-suspended in 20ml fresh ES cells medium without LIF. The EBs were then cultured in 90mm bacterial grade petri dish which could prevent the EBs from attach to dish and allow the EBs to differentiate in suspension. Penicillin/streptomycin was added at a dilution of 1:100 (Sigma, 2,000 units for penicillin and 2mg for streptomycin) to prevent any bacterial contamination. For every 2days, the medium was changed and EBs were transferred to a new petri dish for further differentiation.

2.2.3.2 ES cells differentiation in Embryoid Bodies (EBs) –Suspension method

The hanging drop method of EB formation could force ES cell to form aggregates of uniform size however which would take longer time to prepare and less efficiency for larger scale experiments. ES cells can form aggregates spontaneously in suspension culture, although with different sizes but could prepare higher number of cells available for further sorting.

Basically, ES cells were harvested as normal routine and $6x10^5$ cells were

re-suspended in 20ml ES cells medium then placed in a 90mm bacteriological grade peter dish and incubated at 37 ˚C (humidified 5% CO2 atmosphere). LIF was not added into the culture and the day of seeding was defined as day0. Every 48 hours, EBs were harvested and transferred into a universal tube and centrifuged at 80gx3mins. Then supernatant were aspirated and EBs pellet was re-suspend in 20ml fresh ES cell medium and placed into a new sterile petri dish.

2.2.3.3 ES cells differentiation in co-culture system of EBs or defined population derived from EBs on stromal cells

For investigation of role of stromal cells in haematopoietic differentiation, intact EBs generated using hanging drop method or defined population derived from suspension EBs were co-cultured on stromal cells for further differentiation. Labelling and irradiation of stromal cells were required.

a) Labelling of stromal cells for co-culture

Basically stromal cells were passaged several times and grown to confluence in flasks or wells for staining and irradiation. For most experiments, labeling of the stromal cells was required to distinguish ES-derived cells from stromal cells using flow cytomertry for further data analysis. The Vybrant DiD labeling system (Invitrogen) was applied throughout the project. Vybrant DiD is a carbocyanine dye with the low cytotoxicity and high resistance to intercellular transfer. It can be added directly into normal culture media to uniformly label attached culture cells with the absorption around 644nm and fluorescence Emission around 665nm, which could be easily detected in APC channel excited by 633 laser using flow cytometery. Stromal cells were stained when became confluent. Cells were washed once with PBS. After aspirating the PBS, Vybrant DiD was diluted at 1:250 in PBS. 2ml of dilution was added per 25cm^2 flak and 800ul dilution was added per well (6 well plate). The cells were incubated in the dilutions for 20min at 37 ˚C then aspirated. The cells were then washed with 5ml PBS per 25cm^2 and 2ml PBS per well for three times. Finally the PBS was aspirated and 10ml fresh ES cell medium were added and ready for irradiation.

b) Irradiation of stromal cells

The γ-irradiation (40Gy) should be carried out no longer than 48 hours prior to the co-culture. Irradiator used cesium 137 as a source of unstable atoms to decay and emit beta and gamma radiation. After the irradiation, the cells were washed with PBS and then supplemented with fresh ES medium and incubated 37 ˚C (humidified 5% CO2 atmosphere) ready for use.

c) Co-culture of EBs or defined population derived from EBs on stromal cells

Intact EBs-stroma co-culture

Intact Day 1 EBs (24 hours after the day when EBs were harvested from hanging drop and LIF was withdrawn) were picked up from suspension with yellow tips. Around 50-100 EBs per 25cm^2 were seeded directly onto stromal cells or gelatinized coated flasks as the control. After co-culture, mixture cells were harvested with trypsin solution.

Defined population-stroma co-culture using Magnetic-activated cell sorting (MACS)

Defined population was separated from EBs prepared in suspension methods using Magnetic-activated cell sorting (MACS) according to manual instruction then seeded directly onto stromal cells for further differentiation.

ES-derived Flk1$^+$ and Flk1- population were isolated from day 4 suspension EBs using anti-biotin MicroBeads (Miltenyi Biotec). Basically day 4 suspension EBs were collected into 50ml falcon tubes and centrifuged at 1000 rpm x 3mins. EB pellets were washed in PBS then treated with trypsin solution for 5mins at 37 ˚C. Trypsin solution was then quenched using ES medium and resuspended in fresh medium to get single cells and counted. Cells were pelleted down then resuspended in DPBS (PBS without Mg^+ and Ca^+) containing 10%FCS at 2x10^7/ml and incubated at room temperature for 10min to block unspecific antibody binding. Then biotin-conjugated anti-mouse-Flk1 antibody (eBioscience) were added at 1:75

dilution and incubated at 4 ˚C for 30mins with interval mix. Cells were then washed with 2ml MACS sorting buffer (DPBS containing 2% FCS, 1% Pen/strep and 2mM EDTA) per $1x10^7$ cells and ready for sorting. $Flk1^+$ and $Flk1^-$ cells were isolated using anti-biotin MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. For co-culture for another 24 to 48 hours, sorted cells were plated directly onto labeled and irradiated stromal cells or gelatin control at $4x10^4/cm^2$. For co-culture to day9, sorted cells were plated down at $1x10^4/cm^2$. For co-culture to day11, $4x10^3/cm^2$ were palted. Cells were culture in the EBD medium (IMDM supplemented with 15% FCS, 200μg/mL iron-saturated transferrin (Roche), 4.5mM monothiolglycerol (Sigma), 50μg/mL ascorbic acid (Sigma), penicillin/streptomycin (Gibco), and 2 mM glutamine) in at 37°C in 5% CO2. (Iacovino et al., 2011a)

After co-culture, mixture cells were harvested with trypsin solution for further analysis. For longer time of co-culture, cells could become sticky in which case the mechanical method would be applied to get single cell by running cell suspensions through a 23-gauge needle to disaggregate.

2.2.4 Methylcellulose-based haematopoietic colony assay and normalization

2.2.4.1 Methylcellulose-based haematopoietic colony assay

In *in vitro* system, haematopoietic progenitors could proliferate and differentiate to different mature lineages as erythroid, granulocytic, monocyte-macrophage, megakaryocyte-myelopoietic as well as lymphoid cells responding to the combination of suitable cytokines. Colony assay was widely used for detecting haematopoietic progenitors at single cell level in semi-solid medium such as methylcellulose supplemented with cytokines, in which individual progenitor called colony-forming cells (CFCs) could form cell clusters or colonies consisting of one or more types of mature haematopoietic lineages.

To set up Methylcellulose-based haematopoietic colony assay, cells from culture were harvested using trypsin solution (mechanic dissociation using needles could be applied when cells got stick after long co-culture) to get single cell suspension. $5x10^4$

or $1x10^5$ cells were plated into 35mm dishes (Stem cell technology, SCT) containing 1ml MethoCult® M3434 (SCT) (IMDM containing 1% methylcellulose, 15% FBS, 1% BSA, 10 μg/mL Insulin 200 μg/mL transferrin supplemented with 50 ng/mL rm SCF, 10 ng/mL rm IL-3, 10 ng/mL rh IL-6, 3 U/mL rh EPO, 10^{-4} M 2-Mercaptoethanol and 2 mM L-glutamine). The dishes were placed in big round dish containing an 60mm petri dish containing 10ml tissue culture grade water (Invitrogen) to prevent the methylcellulose from drying out. The dishes were incubated at 37°C with a humidified 5% CO_2 atmosphere for another 10 days. The colonies were classified based on the morphology at light microscopy and scored between day7 to day12 according to the manual.

2.2.4.2 Normalization of colony assay readout to exclude irradiated stromal cells present in cells from co-culture

When applying colony assay, the cells seeded into assays from co-culture consisted ES-derived cells and irradiated cells. It has been determined that the irradiated stromal cells could not give rise to colonies in the assays (Gordon-Keylock thesis 2009). It is necessary to normalize the percentage of ES-derived cells. After seeding cells into colony assay, rest cells were resuspended in PBS containing 2% FCS and run through BD™ FACSCalibur and BD™ LSRFortessa cell analyzer. When 7a-GFP cells were used, percentage of ES-derived cells were decided based on the percentage of GFP positive cells at fluorescence channel 1 (FL-1) in Calibur or which was excited by 488 nm laser. When wildtype ES cells like E14IV or other non-fluorescent ES cell lines were used for the co-culture, stromal cells were labeled with Vybrant DiD prior to co-culture which would be positive at FL-4 in Calibur excited by 633nm lase or R670/14 in LSRFortessa. ES could be distinguished from stromal cells by negative selection. n this project, all the numbers of colonies produced by cells from co-culture were calculated by dividing number of colonies scored by percentage of ES-derived cells in the total cells harvested from co-culture.

2.3 Construction of A2lox.DNMAL-EGFP and A2lox.EGFP ES cell lines

2.3.1 G418 concentration kill curve

To determine the optimal concentration of G418, 4 x10^3 A2lox.cre ES cells were plated into each well of 6 well plates with 5 ml of media plus LIF. The second day the media was replaced with fresh media containing different concentrations (0, 50, 150, 250, 350, 500 μg/ml) of G418 (Geneticin, PAA) and LIF. The media was replaced daily and cells monitored to determine the optimum G418 concentration. Dramatic cell death turned up at day3. 270 μg/ml G418 was used for the selection of clones. This was defined as the minimum amount of G418 required to kill all A2lox.cre ES cells and for colonies to turn up at around day7-9.

2.3.2 ES cell electroporation

A2lox.cre ES cells passaged twice before electroporation and grew at log rate. A2lox.cre ES cell line was designed to express Cre recombinase in the presence of doxycycline, therefore, co-electroporation of the Cre expression plasmid was not required. ES cells were grown in 1ug/ml doxycycline in 75 cm^2 flasks for 24 hours before electroporation with the targeting plasmid. The second day, cells were washed with PBS, trypsinised for 5 minutes at 37 ˚C, added to media and centrifuged at 1000 rpm for 5mins. Cells were then resuspended in cold PBS and counted. Cell concentration was adjusted to 1x107/ml. 30 μg (1ug/ul) of circulate plasmid p2lox.DNMAML-EGFP or p2lox.EGFP was electroporated into 1 x 10^7 cells in a 770ul volume in a electroporation cuvette using a BIORAD gene pulser electroporator (set up at 0.25KV, 900uF). The electroporated ES cells were then left in 4 ˚C for 10mins. Afterwards cell suspension was added into 20ml pre-warmed ES medium plus LIF and then aliquoted into 10 gelatinised 100 mm plates containing 10 ml ES medium plus LIF. 24 hours later the media was replaced with ES medium plus LIF and 270 ng/ ml G418 in 9 plates. The remaining plate was set up without G418 as the control. Medium was replaced every one or two days for 9 days until G418 resistant colonies turned up and grew big enough for picking up. Single ES clones

with different colony morphology were picked into to grow in 96 well gelatinised plates containing ES cell medium with LIF and G418 selection until confluent then passaged as routine into 24 well plates and finally got expanded into 25cm^2. Cells were then frozen down and 4 colonies from each electroporation were picked up and get ready for further testing. Here only two colonies were presented in the thesis.

2.3.3 Karyotyping of ES clones

ES cells were grown and passaged twice to get 70% confluent at a log growing stage. 10µl/ml KaryoMax (Colcemid) (Invitrogen) was added to ES cells (at 10µl/ml ES medium) and incubated for 3 hours at 37 ˚C to arrest cells at metaphase of mitosis. The cells were then harvested with trypsin and resuspended slowly in 8mls pre-warmed hypotonic 0.075M KCl solution. The cells were then incubated at 37 ˚C for approximately 12 minutes with interval gentle mix and then 2 ml of fresh fixative (3:1 v/v methanol/ acetic acid solution) were added. The cells were pelleted down and treated with KCl and fixative buffer for another two times then cell solutions were dropped onto cold 100% ethanol pre-treated glass slides. The slides were allowed to dry and stored at room temperature. The slides were stained with 1 µg/ml DAPI (Sigma) and observed on a Zeiss Axioskop2 microscope using a 40x or 63x objective lens. Approximately 30 spreads were photographed using a ProgRes C14 camera from Jenoptik and the numbers of chromosomes per cell counted.

2.3.4 ES cells self-renewal assay

500 ES cells were plated down in 6 well plates with LIF. The second day wells were washed with PBS twice and replaced with fresh ES cell culture medium with or without LIF. After 5 -7 days of incubation in 37 ˚C, cells were fixed and stained with Leukocyte Alkaline Phosphatase Kit (Sigma) according to the manual instruction.

2.4 Luciferase assay

To test whether DNMAML-EGFP could function properly to inhibit Notch transcriptional activity, dual luciferase assay was carried out to measure Notch downstream transcriptional factor RBP-Jk promoter transcriptional activity.

2.4.1 Plasmids co-transfection to cells

12xRBP-Jk-binding sites-luciferase reporter was a gift from Lowell, S. $2x10^5$ iDNMAML-EGFP or iEGFP cells were plated into 24 well plates minus or plus Doxycycline (1ug/ml) without LIF. The second day cells were transfected with was transfected with 0.25μg of pCAG-NotchIC plasmid to induce Notch activity. For testing Notch transcription activity, 0.75μg 12xRBPJk-luciferase plasmid plus 15ng of SV40 renilla plasmid were transfected together. For negative control, 0.75μg pGL3 plasmid plus 15ng of SV40 renilla plasmid were transfected. For positive control, pEGFP-DNMAML-N3 was co-transfected without adding Doxycycline. Transfection was carried using Lipofectmine2000 (Invitrogen)

2.4.2 Measuring luciferase activity via Dual-Luciferase Reporter Assay

Cells were collected 72 hours later after transfection and analyzed with Promega Dual Luciferase Kit (Promega) a protocol modified based on manual instruction. Basically, cells were lysated with the passive methods: 80μl 1X PLB/well was added to completely cover the cell monolayer in 24 well plate. Plates were placed rocking platform with gentle shaking at room temperature for 15mins. Lysates were transferred to a 0.5ml eppendorf vials and got frozen and thawed twice in the -80 ˚C. Luminometer was programmed to perform a 2-second premeasurement delay followed by a 10-second measurement period for each reporter assay. 30μl LARII was added into luminometer tubes. 20μl cell lysate was added into LAR II, mixed by pipetting 2 or 3 times. Place the tube in the luminometer and initiate reading then the firefly luciferase activity measurement was recorded on the printer. After that another 30μl 1x Stop & GloR reagent was added and pipetted several times to mix then initiated reading. Readout was recorded on the printer with a ratio calculated.

2.5 Flow cytometry and Fluorescent-activated cell sorting

2.5.1 Flow cytometry analysis of surface marker expression

All the flow cytometry data presented in this thesis were collected with the BD™ FACSCalibur flow cytometer (with 488nm and 633nm laser, Becton Dickinson) in Scottish National Blood Transfusion Service (SNBT) with help of Kay Samuel, or BD™ FACSCalibur and BD™ LSRFortessa cell analyzer (with 5 laster: 488nm, 561nm, 633nm, 405nm and 351nm Becton Dickinson) at Centre for Inflammation Research, Queen's Medical Research Institute (QMRI), Edinburgh.

For determining the proportion of ES-derived cells in co-culture, cells were harvested as routine and resuspended in PBS with 2% FCS then run through the FACSCalibur or LSRFortessa directly. For detecting the expression of Notch ligands and receptor expression in stromal cell lines and lineage-specific surface markers from co-culture, cells were harvested and blocked in PBS with 10%FCS for 10min at room temperature. For each FACS tube, up to $1x10^6$ cells were added and incubated with antibody with optimal dilution at 4 ˚C for 20mins. Cells then washed in 4mls FACS PBS with (PBS with 2%FCS) and centrifuged at 400g x 5min. For biotin labeled antibodies requiring streptavidin binding, cells were washed and resuspended in 150ul FACS PBS. Streptavidin conjugated with PE, APC or Percp-efluor710 (tritration for optimal dilution) was added for another 15min at 4 ˚C then wash with FACS PBS again. Samples were ready and analyzed at BD™ FACSCalibur and BD™ LSRFortessa cell analyzer. Dead and apoptotic cells were excluded from analysis using an electronic 'viable' gate on FSC and SSC. Data were analyzed using FACSDiva or flowjo.

2.5.2 Reagents for flow cytometry and apoptosis assay

Antibodies for flow cytometry and AnnexinV kit were purchased from eBioscience or BD Bioscience and used according to manual instruction.

2.5.3 Fluorescent-activated cell sorting (FACS)

Fluorescent-activated cell sorting was carried out by Shonna Johnson and Fiona Rossi at QMRI or Simon Monard at MRC Centre for Regenerative Medicine with BD™ FACSAria II cell sorter or BD FACS Jazz™.

For sorting at GFP positive ES-derived cells, cells from co-culture were harvested and resuspended at the concentration of $5x10^6$/ml in PBS with 2% FCS and filtered through the BD strainer to avoid blocking the machine. Cells were then sorted at FL1 channel at GFP expression exited at 488nm laser then harvested into PBS containing 10% FCS and pelleted down and frozen in dry ice for further analysis.

For sorting at human-CD2 (hCD2) expression, cells from co-culture were harvested and resuspended at $2x10^7$/ml in PBS with 10% FCS and 1% Pen/strep to block unspecific antibody binding at room temperature. Then anti-hCD2-PE were added at 1:40 dilution to cell suspension, mixed well and incubated at 4 ˚C for 20mins. Cells were gently mixed every 10mins. After antibody incubation, cells were washed with 4ml FACS PBS per $1x10^7$ cells and centrifuged down at 1000rpm x 5mins. Cells were then resuspended in FACS PBS with 1% Pen/Strep at $5x10^6$/ml and run through sorter. CD2 negative and positive cell population was defined at PE staining at FL2 channel which is exited at Yellow Green laser (561nm). Sorted cells were collected into FACS tube or 15ml falcon tube containing PBS with 10% FCS and 1% Pen/Strep then back to colony assay or pelleted for RNA extraction. Normally $3x10^5$ cells were collected for colony assay and 2 to $5x10^5$ cells for RNA analysis.

2.6 Protein Analysis

2.6.1 Protein extraction from cells

Cells were harvested using trypsin solution and pelleted down in 0.5ml eppendorf tubes. Cell pellets were washed with PBS and ice cold RIPA buffer (25mM tris-HCl, pH7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (ThermoScientific) was applied with addition of 1%protease inhibitor (Sigma). Typically 80ul RIPA buffer was added into confluent cells from a well in 6 well plate. Cell pellets were lysed in RIPA buffer in ice for 30mins with a short vortex every 10mins and then centrifuged at 13000rpm x 20min at 4˚C. Supernatants were transferred to a new eppendorf tube for further storage in -20˚C or determining protein concentration using standard Bradford protein assay.

2.6.2 Gel electrophoresis and western blotting

Protein samples were separated using NuPAGE® SDS-PAGE Gel System (Invitrogen) with NuPAGE® 4-12% Bis Tris Gels. Gels were run at 200 V for approximately 35-45 minutes with 1 x MOPS or MES buffer (Invitrogen) and then semidry electro-transfer onto nitrocellulose membranes was performed at 15 V for 1 hour with cold transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) The blots were then blocked for 1 hour in 5 % dried milk powder (Marvel) or 5% BSA in 1 x TBST (25mM Tris-HCl, pH 8.0, 125mM sodium chloride, 0.1% Tween-20) before hybridization with the primary antibodies in blocking solution overnight at 4 °C. After 3x 15 minute washes in TBST the membranes were then incubated with Horseradish -conjugated secondary antibody (1:1000) for 1 hour at room temperature. Equal volume of ECL solution A (0.1M Tris-HCl pH 8.6, 25mM luminal, 0.4mM coumaric acid) and solution B (0.1M Tris-HCl pH 8.6 and 0.02% hydrogen peroxide) were mixed and added to the membrane which was exposed to light-sensitive film (Kodak) to visualize the antibody-antigen reactions. Antibodies applied in this projects for western blots were purchased from Cell Signaling Technology (anti-Jagged1, anti-STAT3, anti-EGFP), Abcam (anti-Delta-like1) or Santa-Cruz (anti-Delta-like4).

2.6.3 Immunochemistry

Stromal cells were grown on glass coverslips in 24 well plates coated with 0.1% gelatin. When getting around 100% confluent, cells were irradiated with γ-irradiator as irradiation routine then incubated for another 24 hours. Cells were washed with PBS and fixed with 4 % PFA (Sigma) in PBS for 10 minutes at room temperature, followed by 3 x 5 minute PBS washes. Because we were interested in the ligands and receptors expression on the cell membrane, permeabiliztion was not required. Samples were then blocked in 5 % donkey serum (Sigma) in PBST (0.001 % TtritonX-100 in PBS) for 1 hour at room temperature. Primary antibodies were diluted at 1:100 ratio in blocking solution and incubated at 4 °C overnight in a humidified chamber. 3 x 5 minute PBST washes were applied next day followed by an incubation with a 1: 200 dilution of secondary antibodies for 1 hour at room temperature in the dark. 1μg/ ml DAPI was added into the secondary antibody incubation. After 3 x 5 minute PBST washes coverslips were mounted on glass microscope slides using a drop of Prolong Gold mounting medium (Invitrogen) and left overnight in the dark at room temperature. The slides were subsequently stored at 4 °C in the dark.

Chapter 3: Notch signalling pathway in the co-culture system of EBs and stromal cell lines

3.1 Aims

To dissect the co-culture system of stromal cell lines and EBs and investigate how Notch signalling is involved in the co-culture system

3.2 Introduction

Previous work suggested that inhibition of the Notch signaling pathway using the gamma secretase inhibitor (GSI) could abrogate the haematopoietic enhancing activity of the AM20.1B4, AM14.1C4 and OP9 stromal cell lines. Furthermore, it had been reported that direct contact was required for the haematopoietic enhancing stromal cell lines to enhance haematopoietic differentiation supporting a direct interaction between stromal cells and EBs (Figure 1.6, Gordon-Keylock et al., 2010). Taken together, these findings support the hypothesis that the Notch signaling pathway plays a role in the haematopoietic enhancing activity of the co-culture system, either in EBs or stromal cell lines, or both.

Numerous studies have also shown that the expression of Notch ligands in a stromal microenvironment can affect haematopoiesis, including proliferation, survival ability and differentiation. For example, Li L et al., found that human Jagged1 expressed by human marrow stroma inhibited differentiation of 32D myeloid progenitors through interaction with Notch1 and could be mediated to maintain haematopoietic progenitors (Li et al., 1998); Schmitt and his colleagues proved that overexpression of Delta-like1 in OP9 stromal cells could activate T cell lineage differentiation from fetal liver derived haematopoietic progenitors (Schmitt and Zuniga-Pflucker, 2002) and overexpression of Jagged2 in NH3T3 could delay differentiation of $CD34^{+}$ cells through alerting cell cycle (Carlesso et al., 1999). Especially, co-culture of AGM cells from Jagged1-/- mice on OP9 with endogenous Jagged1 could rescue the non-haematopoiesis phenotype indicated a non-cell autonomous regulation by Notch signaling (Robert-Moreno et al., 2008). Combined with the inhibitory effect of GSI on haematopoietic enhancing activity in our system, we hypothesized that the haematopoietic enhancing stromal cell lines enhanced haematopoiesis by activating higher Notch activity in the EBs, either by providing more Notch ligands directly or

by upregulating Notch signalling molecules in EBs via other factors. Furthermore, Notch activity in stromal cells could be also relevant because GSI could affect both stromal cells and EBs. Thus, experiments described here were designed to ask:

(a) whether the expression level of Notch ligands of these haematopoietic niche derived stromal cell lines correlated with their haematopoietic enhancing abilities, in which case we assumed the haematopoietic enhancing stromal cell lines could possibly provide a higher dose of Notch ligands to stimulate Notch signal in EBs;

(b) whether the Notch activity in stromal cell lines is active and responsible for the enhancing effect of EBs-derived haematopoiesis through its downstream genes or target factors;

(c) whether the haematopoietic enhancing ability of AM14.1C4 is determined by activating higher Notch activity in EBs than non-enhancing system, in which case we assumed that the Notch activity in EBs co-culture on enhancing stromal cell lines would be higher than those on non-enhancing stromal cell lines.

3.3 Experimental strategy

- To determine whether there is any correlation between the expression of Notch ligands in stromal cells and their haematopoietic enhancing abilities, flow cytometry, western blotting and immunostaining were carried to measure the ligands expression at the protein level. Quantitative RT-PCR was also carried to measure the ligand transcripts level.

- To determine whether the Notch signaling is active in stromal cell lines, Notch1 and Hey1 was analyzed in AM14.1C4, UG26.1B6 and OP9 at the RNA level. To test whether stromal cell lines could respond to GSI, co-culture of EBs with AM14.1C4 was carried out with addition of GSI from day4 to day 6. FACS was then applied to separate EBs and stromal cells at day6 then the expression of Notch downstream genes were measured in both populations.

- To determine whether the haematopoietic enhancing ability of AM14.1C4 is

determined by inducing higher Notch activity in EBs compared to the non-enhancing stromal cells, EBs were separated from stromal cells at different time points during the differentiation and quantitative RT-PCR was used to assess expression levels of the Notch ligands, receptors and known target genes.

3.4 Results

3.4.1 There were no direct correlation between Notch ligand expression and the haematopoietic enhancing abilities of stromal cell lines

In previous work, methylcellulose assays were applied to assess the ESC-derived haematopoietic activity by counting the haematopoietic colonies (CFU-GEMM, CFU-GM, Ery/Mac and CFU-M). These results suggested that OP9, AM20.1A4, AM20.1B4 and AM14.1C4 stromal cell lines (from AM region) had haematopoietic enhancing/supporting ability whereas the UG26.1B6, UG26.2D3 (from UG region) and EL08.1D2 (from fetal liver) did not (Figure 3.1) (Gordon-Keylock, et al., 2010). Here we screened the Notch ligand expression at both the RNA and protein level in these stromal cell lines to assess whether their ligand expression correlated with their haematopoietic enhancing ability.

3.4.1.1 Notch ligand mRNA could be detected in stromal cell lines but expression levels did not correlate to haematopoietic enhancing activity

Quantitative real-time RT-PCR (qPCR) was first applied to measure ligand gene transcripts. Stromal cells including AM20.1A4, AM20.1B4, AM14.1C4, UG26.1B6, UG26.2D3, EL08.1D2 and OP9 were analyzed. As a control, pooled samples from adult bone marrow and spleen (C57/B16) were measured for Jagged1 and Delta-like1 expression (data not shown). For all the experiments, UG26.1B6 was set as the calibrator. All the cDNA level raw data of the samples were expressed as fold change over the calibrator, which was assigned as a value of “1”. HPRT was used as the endogenous control.

All the stromal cell lines expressed Notch ligand: Jagged1, Jagged2, Delta-like1, Delta-like 4 (Figure 3.2). Statistically, there was no significant variance in Jagged1, Jagged2, Delta-like1 and Delta-like4 expression level among these stromal cell lines ($p=0.09337$; $p=0.3608$; $p=0.5778$; $p=0.1773$ respectively). By looking at each ligand individually, we noted several consistent trends: Jagged1 RNA level in OP9 stromal cell line was relatively higher compared to other stromal cell lines; AM14.1C4 expressed relatively higher Jagged1; For the Delta-like1, non-enhancing stromal cell

lines UG26.2D3 and EL08.1D2 expressed higher levels of Delta-like 1. Despite these differences, when correlated to their haematopoietic enhancing ability by plotting ligand expression levels to number of haematopoietic colonies in methylcellulose-based colony assay, it was found that there were no direct correlation between the ligand expression and the HPCs formation ability in the AGM-derived stromal cell lines ($p>0.5$). Although not conclusive, these data indicated the ability of the haematopoietic enhancing AM stromal cell lines to enhance ESC-derived haematopoiesis was not dependent on their expression level of Notch ligands.

However, there were several interesting points worthy of further investigation. High expression of Jagged1 in OP9 could properly reflect the difference of site of origin. In the bone marrow microenvironment, the Notch signaling pathway could be important for either enhancing or supporting the haematopoiesis via Jagged1 activation; although for each ligand, we could not observe any correlation between the enhancing ability and the ligand expression level, the complexity of the stroma suggests more possibilities. It could be the combination of the ligands or preference of one of the ligands which is really mediating the activation of Notch signaling pathway in the EBs. Alternatively, stromal cells enhance the haematopoiesis in combination with other active or repressive signaling pathways.

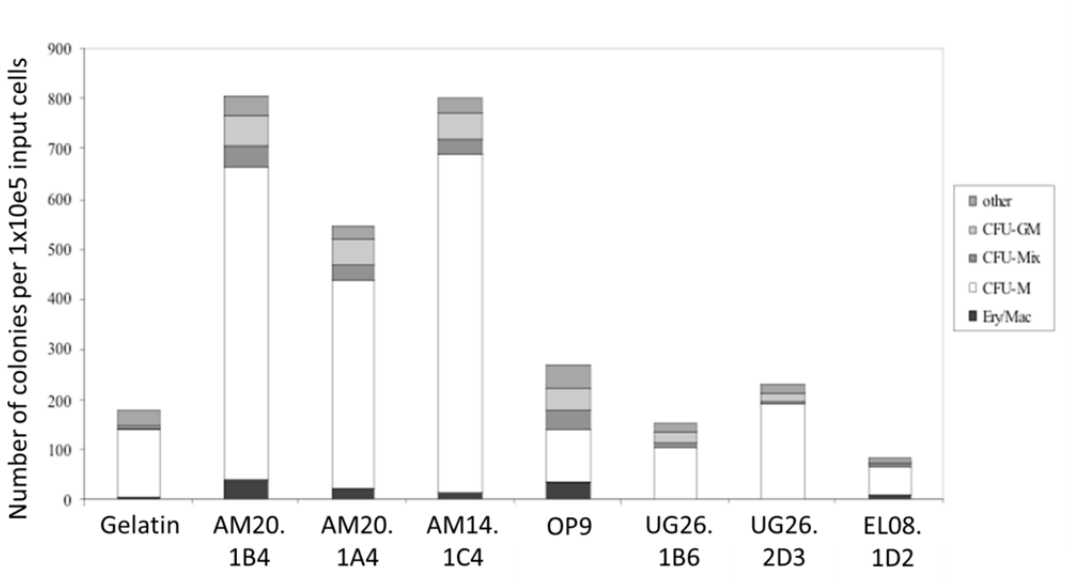

Figure 3.1 Comparison of haematopoietic enhancing ability of haematopoietic niche derived stromal cell lines in EB/stromal co-culture system.

The total number of haematopoietic colonies per 3×10^5 ES-derived cells (7a-GFP ESC) from co-cultures with stromal cell lines to 6 days differentiation was compared to gelatin control. AM20.1B4, AM20.1A4 and AM14.1C4 are derived from AM sub-region of AGM, UG26.1B6 and UG26.2D3 are derived from UG sub-region, EL08.1D2 is derived from fetal liver, OP9 is derived from from calvaria of newborn osteopetrotic op-/- mice. Data represented mean of between 3 and 11 independent experiments. Remaining colonies such as definitive erythroid and CFU-mast were categorized as "other". Figure adapted from Gordon-Keylock et al., 2010.

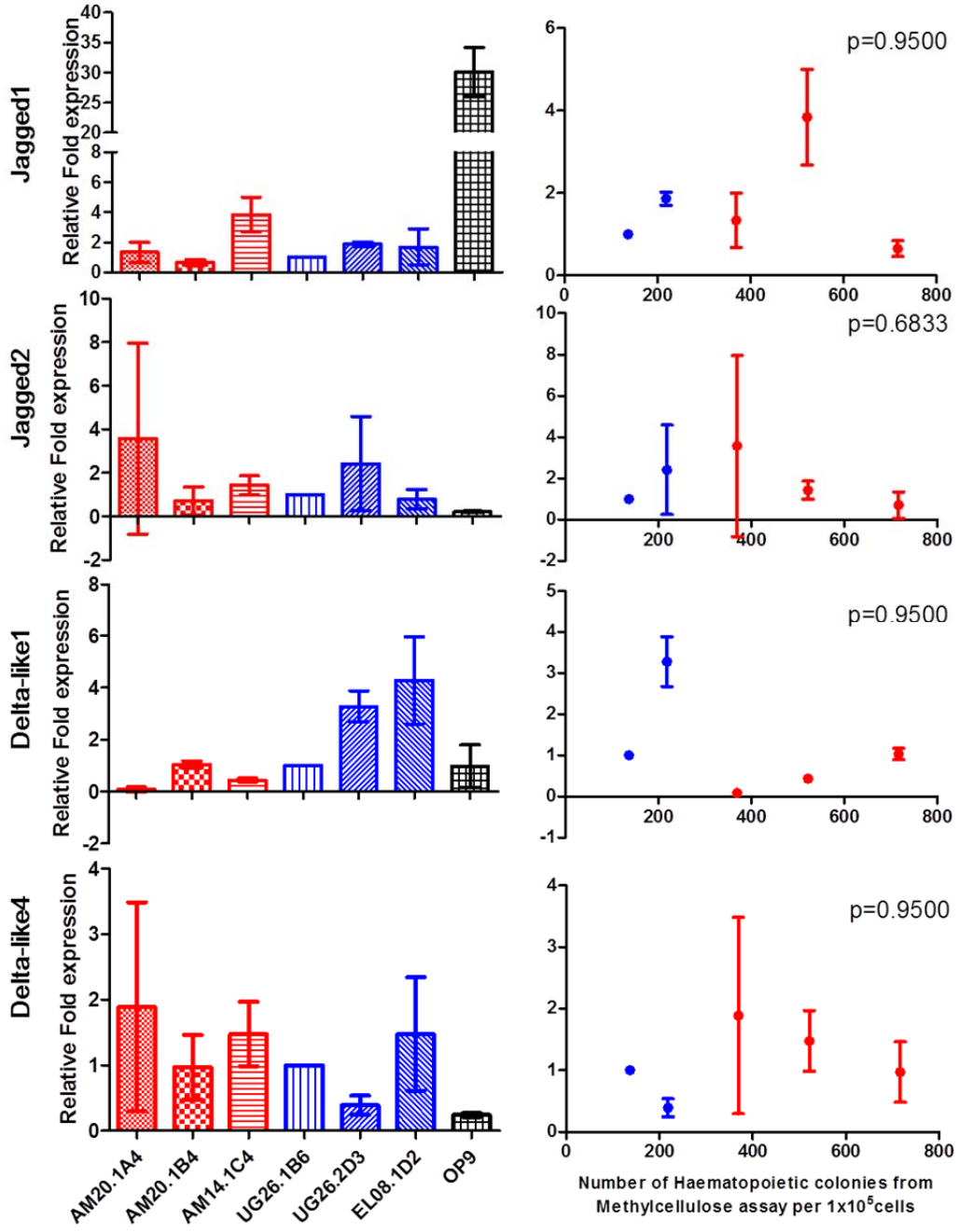

Figure 3.2 Quantitative RT- PCR measuring Notch ligand transcripts of embryonic niche -derived stromal cell lines with OP9 as a control (left panel); Correlation of ligand RNA level in embryonic niche derived stromal cell lines with haematopoietic enhancing activity (right panel).

Red bars and dots represent haematopoietic enhancing stromal cell lines, blue bars and dots represent non-enhancing stromal cell lines. Error bars represent the range of 2 independent experiments. For gene transcripts, p-values were calculated by Kruskal-Walis test. For correlation, p-values were calculated by Spearman test. Calibrator: UG26.1B6=1; Endogenous control: HPRT.

3.4.1.2 Notch ligands detected in stromal cells at protein level but their expression does not correlate to haematopoietic enhancing ability

It is possible that the gene transcripts do not reflect the active protein level exactly because of several factors such as the post-transcriptional modification, protein degradation and subcellular localisation. It is therefore necessary to measure the Notch ligand expression at the protein level. Jagged1, Delta-like1 and Delta-like 4 have been widely investigated their roles in the haematopoiesis. Here we measured their expression using immunochemistry, western blots and flow cytometry assays. Because of the limitation and sensitivity of antibodies, Jagged1 expression was measured with flow cytometry and western blot, while Delta-like 1 Delta-like 4 were measured by immunostaining.

a) Jagged1 is expressed highly in OP9 stromal cell lines

According to the qPRC data, it was suggested that Jagged1 was highly expressed in OP9 and AM14.1C4. To confirm the expression of Jagged1 in the stromal cell lines at the protein level, flow cytometry was applied to determine Jagged1 expression at the cell surface. According to the flow cytometry data, Jagged1 was expressed in all the stromal cell lines to some extent with OP9 having the highest proportion of positive cells (77.9%±2.5%) (Figure 3.3 A). Jagged1 was detected on a lower proportion of cells in all the other stromal cell lines: AM20.1A4 (16.15%), AM20.1B4 (4.4%±0.9%), AM14.1C4 (25.0%±10.1%), UG26.1B6 (18.4%±4.1%), UG26.2D3 (8.08%), EL08.1D2 (12.38%) (Fig 3.3A). The enhancing cell line AM14.1C4 and non-enhancing cell line UG26.1B6 expressed higher proportion of Jagged1 than other embryonic niche -derived stromal cell lines. Here statistics was not available because of limited repeats at 1 to 3 times.

Jagged1 expression at protein level was also determined by western blotting (Figure 3.3 B). The molecular weight shown on the blotting was around 180Kd (134Kd is the predicted MW of the unprocessed precursor), which could be down to glycosylation. Jagged1 was detected in Cos7 cells transfected with pCAG-Jagged 1 plasmid (A gift from Lowell, S.) and in OP9 cells but not in untransfected Cos7. Very weak bands were observed in the other three AGM-derived stromal cell lines, but with a much

stronger loading control indicated by GAPDH.

Taken together our Flow cytometry and Western Blots results, it was suggested that Jagged1 is widely expressed by these haematopoietic niche-derived stromal cell lines but it is not the key regulator for the enhancing AM cell lines to enhance haematopoiesis because of the relatively low expression level compared to bone marrow derived stromal cells OP9. Jagged1 could be an important regulator for bone marrow microenvironment-based haematopoiesis as previously suggested (Calvi et al., 2003).

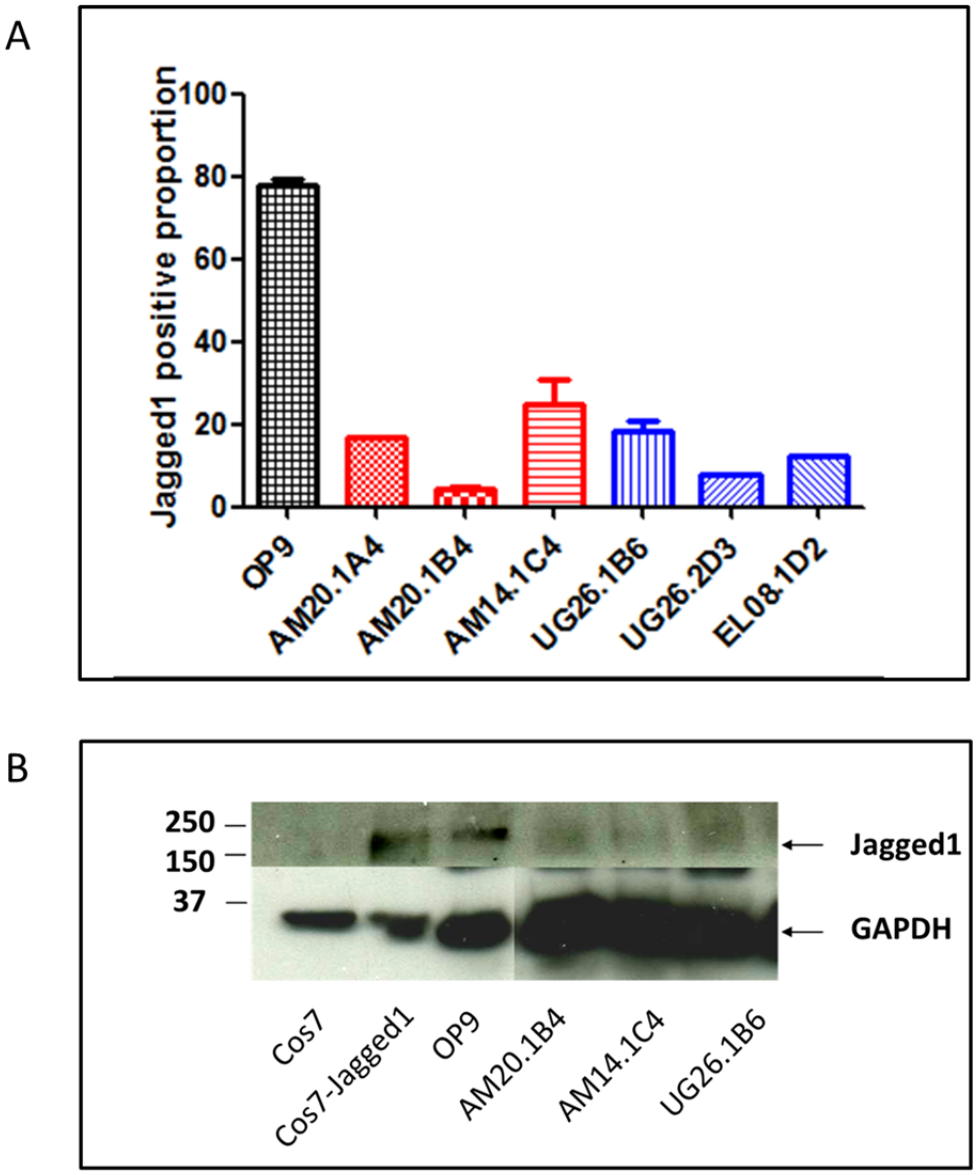

Figure 3.3 Detecting of expression of Jagged1 in stromal cell lines with flow cytometry and western blots.

(A) According to flow cytometry, Jagged1 is expressed in a higher percentage of OP9 cells compared to than other stromal cell lines. Error bars represent standard error mean (SEM) from 3 independent experiments.

(B) According to western blot, OP9 expressed higher Jagged1 than other three AGM-derived stromal cells. Cos7 trasfacted with pCAG or pCAG-Jagged1 plasmid were applied as the positive control.

b) Delta-like1 is widely expressed in stromal cell lines

Immunochemistry was carried out to detect Delta-like 1 expression and cell subcellular location in OP9, AM20.1B4, AM14.1C4 and UG26.1B6 stromal cells. Cells were grown and irradiated before immunochemistry was carried out. All four stromal cell lines expressed Delta-like 1 in the cytoplasm (Figure 3.4A). Although this assay is not quantitative, we noted that the OP9 and AM20.1B4 cell lines appeared to express higher levels of Delta-like1 compared to AM14.1C4 and UG26.1B6.

c) Delta-like4 is widely expressed in stromal cell lines

Similarly, Delta-like 4 was detected in all the four stromal cell lines at different levels but the observed level of expression was not obviously related to their enhancing ability (Figure 3.4B). It was interesting to note that in addition to cytoplasmic staining, there was bright inclusion-like staining around the nucleus. Under certain circumstance when cells do not require Delta-like 4 to be transported to the cell membrane to function as a ligand, it is possible that Delta-like 4 accumulated on the rough endoplasmic reticulum (rER) around the nucleus. This phenomenon suggests that even though the mRNA and protein are detected, the location of the protein is crucial in determining whether the ligands could functionally activate a receptor at the cell surface. Therefore, in light of this, it would be interesting to monitor the sub-cellular location of Notch ligands of the stromal cell lines during the co-culture period.

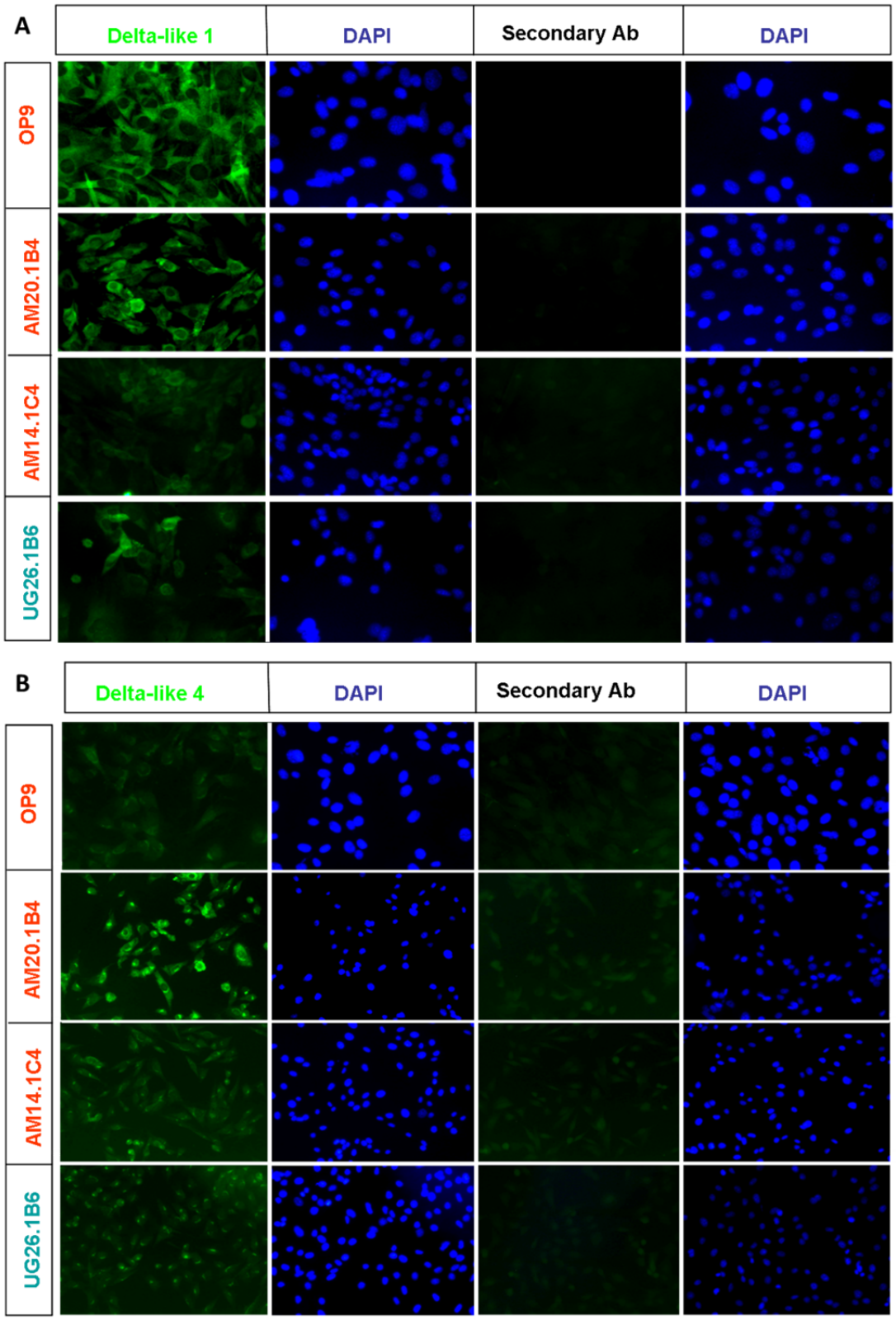

Figure 3.4 Immunochemistry of Delta-like1 (A) and Delta-like4 (B) in OP9, AM20.1B4, AM14.1C4 and UG26.1B6.

FITC staining indicated Delta-like 1 or Delta-like4 staining. DAPI stained the nucleus. Secondary antibody only was applied as the negative control.

3.4.2 Notch signaling is active in stromal cells and can be inhibited by GSI

We previously demonstrated that inhibition of the Notch pathway with GSI abrogated the haematopoietic enhancing effects of stromal cell lines. Because stromal cells and EBs in co-culture were both exposed to GSI, Notch activity was potentially inhibited in either the ESC-derived or the stromal cells or both. Alteration of cell fate in EBs could be caused by the target genes regulated by Notch within the stromal cell lines themselves. Thus, to extend the investigation of the role of Notch signalling in the co-culture, we measured the Notch activity of stromal cell lines and tested their response to the GSI compared to the EBs.

Stromal cell lines were seeded, irradiated then left for another 3 or 4 days. Cells were harvested and analyzed by quantitative RT-PCR at the expression level of Notch receptor Notch1 and downstream gene Hey1. Enhancing stromal cell line AM14.1C4 (AM14 as abbreviation) expressed a higher level of Notch1 as well as the downstream target Hey1 compared to both the non-enhancing cells line UG26.1B6 and control OP9 stromal cells (Figure3.4A). These data suggest that the higher inherent Notch activity in AM14.1C4 could in part explain its haematopoietic enhancing activity.

To determine whether Notch activity in stromal cell could also be affected by GSI in the co-culture system, we co-cultured day1 hanging drop EBs on irradiated AM14 stromal cells to day6 with treatment of GSI from day4 to day6 then harvested and FACS sorted based on GFP expression. Purified GFP^+ ES-derived cells and GFP- AM14 stromal cells were both analyzed in quantitative RT-PCR. Hes1 and Hey1 have been widely used as the downstream genes of Notch signaling pathway. Runx1 is reported to be important for definitive haematopoiesis and regulated by another Notch downstream gene GATA2. We demonstrated that the expression levels of all three genes, Hes1, Hey1 and Runx1 are inhibited by GSI in stromal cells in one preliminary experiment (Figure 3.5B). Significant inhibition of these three downstream genes was also observed in sorted EBs from EB/AM14 co-culture (Figure 3.5 C). Indeed inhibition of the direct Notch target genes Hes1 and Hey1 by GSI is greater in stromal cells compared to in ESC-derived cells. Our preliminary

data revealed that Hes1 were reduced by 90% whereas in EBs a 50% reduction in expression of these genes was observed (Figure 3.5 B, C). Inhibition of Runx1 levels was comparable in the two cell types. Based on these results, we concluded that in the co-culture system Notch signaling pathway is active in both stromal cells and EBs and this signalling could be inhibited by GSI in both cell populations. Thus the inhibitory effect of GSI on AM14 haematopoietic enhancement could be caused by inhibition of Notch signaling pathway either in EBs or AM14 stroma, or both. A further finding from our study supported the notion that Notch could be a key regulator for definitive haematopoies, which was mediated by Runx1 in the EB/AM14 co-culture system.

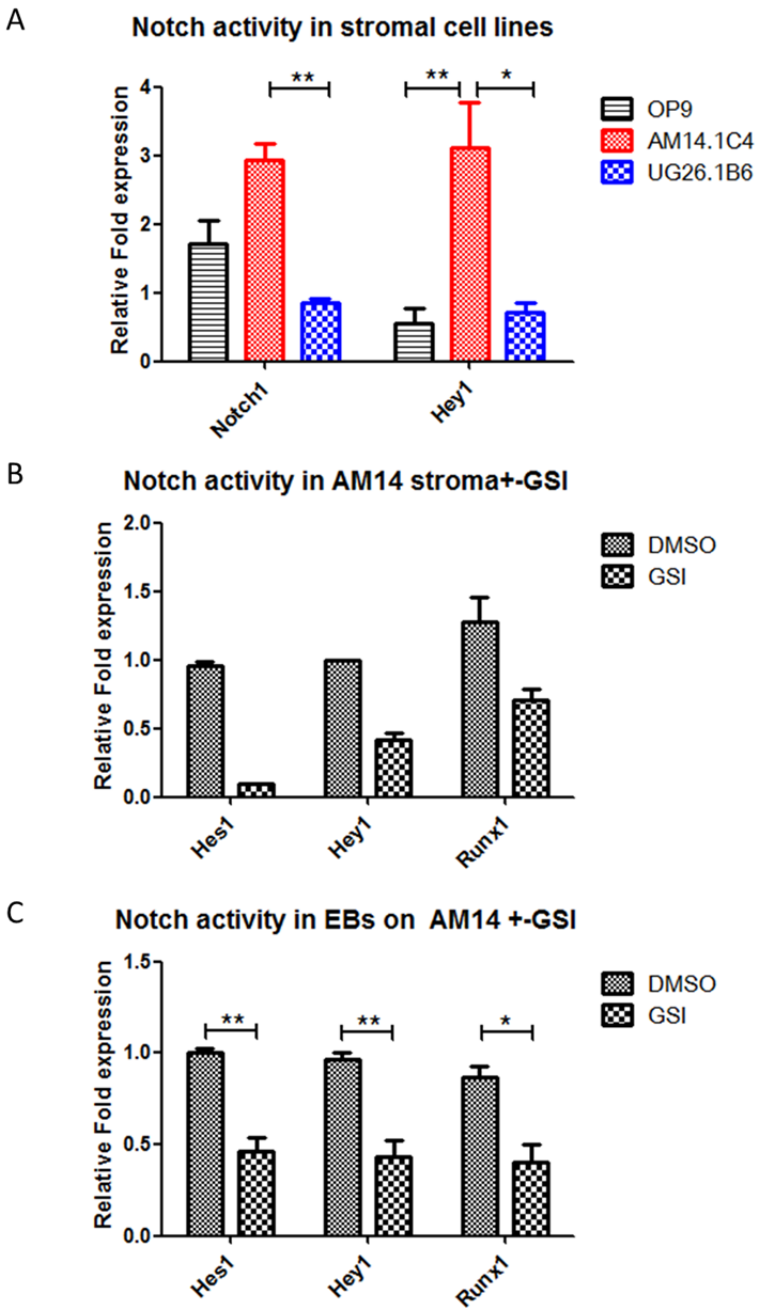

Figure 3.5 Measurement of Notch activity in AM14.1C4, UG26.1B6 and OP9 and effect of GSI on both stromal and ES-derived cells from co-culture by quantitative RT-PCR.

(A) AM14.1C4 has a higher level of Notch activity than OP9 and UG26 according to Notch1 and Hey1 expression. Calibrator: UG26.1B6. (B) Notch activity in AM14.1C4 stromal cells was inhibited by GSI according to Hes1, Hey1 and Runx1 expression; (C) Notch activity in EBs co-cultured on AM14.1C4 stroma was inhibited by GSI. Calibrator: GSI treatment sample. Endogenous control: 18s. Data represent 3 to 6 repeated reactions from 1 to 2 independent experiments. Error bars represent SEM. P-values were calculated with Mann-Whitney test. (*$p<0.05$, **$p<0.01$)

3.4.3 Investigation of Notch activity in the EBs co-cultured on the stromal cell lines during differentiation

Our analysis of Notch ligand, receptor and downstream gene expression suggested that the haematopoietic enhancing activity of AM stromal cell lines was not due to higher levels of expression of Notch ligands, but possibly related to Notch activity in the stromal cells. This led us to consider Notch activity of the ESC-derived cell populations within the co-culture system. We measured the Notch activity in the EBs from co-cultures by analysing the expression of the Notch receptors, ligands and downstream genes transcripts by quantitative RT-PCR through FACS sorting.

3.4.3.1 Confirming the activity of the Notch signaling pathway in the AM14 co-culture system

AM14.1C4 stromal cell line demonstrated the best haematopoietic enhancing ability of all cell lines tested. Although in Figure 3.4 C, we have claimed the inhibition of Notch activity by GSI on EBs from EB/AM14 co-culture, here we further monitored the Notch activity of EBs co-cultured on the AM14.1C4 through the whole co-culture period. EBs derived from ESCs that constitutively expressed GFP (7a-GFP) were co-cultured on irradiated AM14.1C4. At defined time points (Day3, 4, 5, 6), the mixture of co-culture cells including EBs and stromal cells were harvested, disaggregated into single cells then GFP-expressing ESC-derived cells were sorted by FACS (Figure 3.6). Real-time PCR was carried out to determine the levels of expression of Jag1, Notch1, Hey1 and Hes5 during the 6 day differentiation period (Figure 3.7). Notch activity of undifferentiated ES cells, day0 EBs from hanging drops, Day1 EBs were also measured and compared to the differentiated cells.

There was no variance of in the level of expression of the ligand Jag1 during differentiation ($p=0.0542$) (Figure 3.7 A) However expression of the receptor Notch1 increased during differentiation from Day0 EBs. Notch1 increased 2 fold in Day3 EBs compared to Day0 EB ($*p<0.05$) while there were no significant variance of Notch1 level from day3 to day6 ($p=0.11$) (Figure 3.7B). We also measured the Notch downstream target genes Hey1 and Hes5. Interestingly, Hey1 increased at day3

significantly compared to day0 EBs ($p=0.0005$) and remained at a stable level without significant variance ($p=0.26$), which was similar to the expression pattern of Notch1 (Figure 3.6C). In contrast, the Hes5 level was not enhanced during differentiation (Figure 3.6D). These results suggested that in the EBs co-cultured on the AM14.1C4 stromal cell line, the Notch signaling pathway is active in ESC-derived cells and increased (according to Hey1) during the co-culture period supporting previous data demonstrating the potential role of Notch in haematopoiesis (Gordon-Keylock et al., 2010). Furthermore, these data demonstrated that this increase in Notch activity is not due to an increase in expression of the ligand Jag1 ESC-derived cells. Hes5 might not be active or not be able to respond to the RBP-Jκ transcriptional activator during co-culture with AM14.1C4.

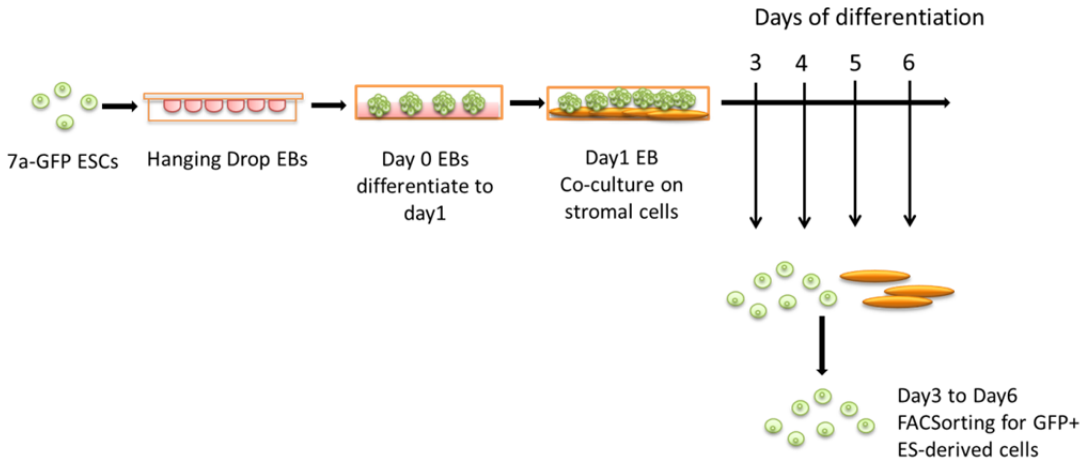

Figure 3.6 Scheme of quantitative RT-PCR to test Notch activity in ES-derived cells in EB-stromal co-culture system during the differentiation time course.

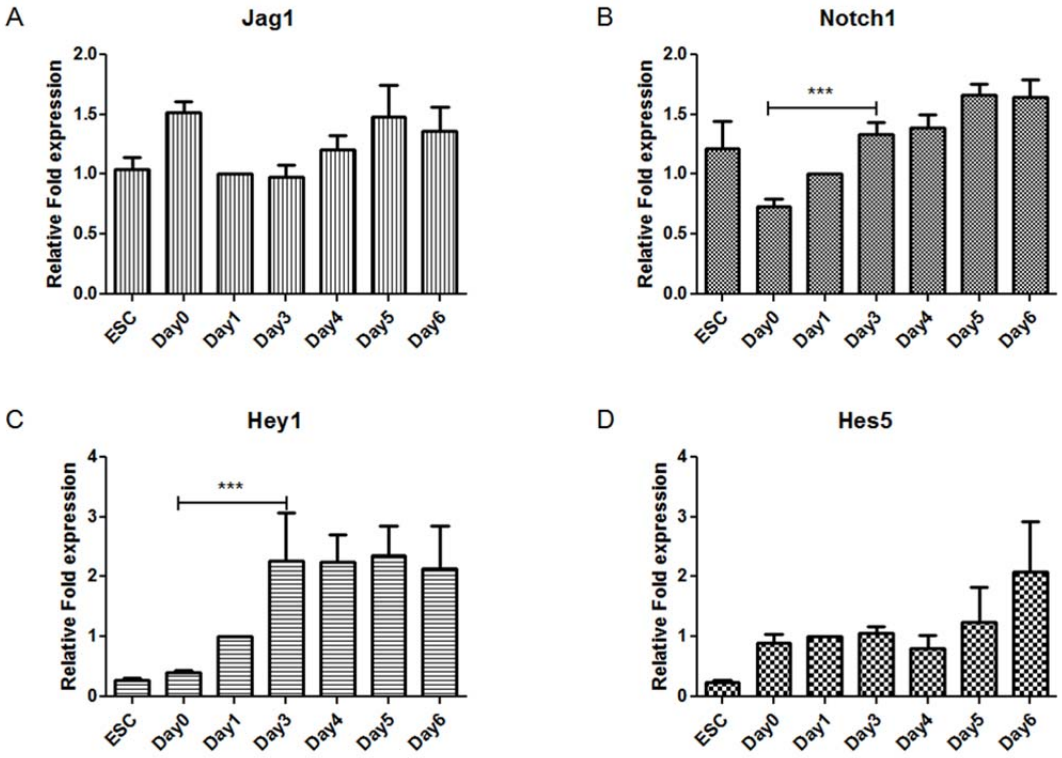

Figure 3.7 Quantitative RT-PCR analysis of Notch signaling pathway components in sorted ESC-derived cells during differentiation when co-cultured on AM14.1C4.

(A) Jag1 expressed at relative stable level during differentiation; (B) Notch1 increased from day0 to day3 then expressed at stable level; (C) Hey1 increased from da0 to day3 then expressed at relative stable level; (D) Hes5 did not change during differentiation from day0 to day6. Calibrator: day1 EBs; endogenous control 18s. Data represent 9 repeated PCR reactions from 3 independent co-culture experiments. Non-parametric Mann-Whitney tests were used to calculate p-values. Error bars represents the standard error of the mean (SEM) (***$p<0.001$).

3.4.3.2 There was no direct correlation between Notch activity in EBs throughout the co-culture period and its haematopoietic activity in the co-culture system

Our data suggested that Notch activity was active and increased during the early stages of differentiation then stablised from day 3-6 when cultured on AM14-1C4 stromal cells. In order to investigate whether this increased Notch activity in EBs was related to the haematopoietic enhancing effects of the stromal cell lines we tested Notch activity in sorted ESC-derived cells after culture in enhancing and non-enhancing conditions. EBs co-cultured on gelatin control, OP9 positive control or the non-enhancing stroma control UG26.1B6 were therefore screened for Notch1, Notch downstream genes (Hey1, Hes5) and compared to EBs in AM14.1C4 co-culture from day3 to day6. Here we noted some convincing trends in Notch1, Hey1 and Hes5 expression.

Notch1 was widely expressed in the EBs during differentiation in all 4 co-culture systems (Figure 3.8A). We noticed that the expression pattern is quite similar in the gelatin control, UG26.1B6 negative control and the AM14.1C4 co-culture system ($p>0.05$ from day3 to day6). Interestingly, EBs co-cultured on OP9 stroma expressed higher levels of Notch1, which could be caused by higher expression of Notch ligand Jagged1 in OP9 stroma that we showed previously (Figure 3.2, 3.3) (OP9 vs gelatin *$p<0.05$, ***$p<0.001$).

As for Notch downstream genes, compared to EBs from AM14.1C4 co-culture, Hey1 was expressed at relatively similar level through differentiation from day3 to day6 in these four co-culture, except at day3, EBs in gelatin had a higher level of Hey1 expression (Figure3.8 B). Although Hes5 was expressed at low or basal levels in the EBs co-culture on AM14.1C4 without any significant change throughout the co-culture period (Figure 3.6 D), Hes5 was increased significantly when co-cultured on non-enhancing UG26.1B6 compared to EBs from gelatin or AM14.1C4 co-cultures at day 5 and day6 (Figure 3.8 C).

These data demonstrated that although the Notch pathway is active in ESC-derived cells during AM14 co-culture, its activity in EBs was not higher than that from

negative control or non-enhancing stromal cell co-culture system. Thus, Notch activity in EBs and haematopoietic activity were not correlated. Interestingly, as a non-enhancing stromal cell line, UG26.1B6 was reported to support adult HSCs via Wnt5a secretion (Buckley et al., 2011). Thus, the significantly higher level of expression of Hes5 in EBs cultured on this cell line suggested a possible involvement of Notch signaling pathway of UG26.1B6 in supporting adult HSCs.

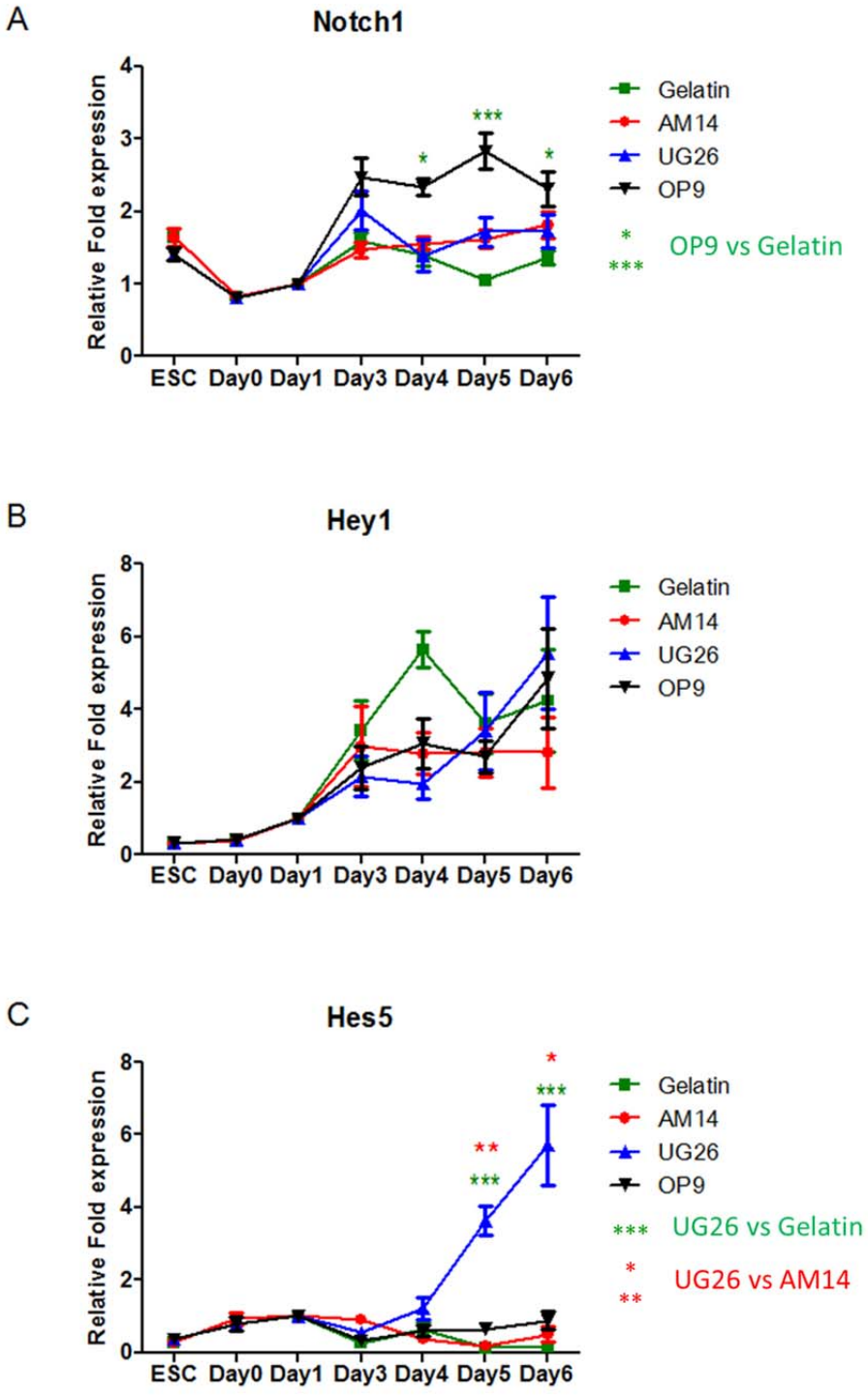

Figure 3.8 Quantitative RT-PCR to measure Notch activity in EBs co-cultured on AM14.1C4, Gelatin, UG26.1B6 and OP9 from day1 to day6.

Day1 EBs were used as the calibrator, assigned as "1". Endogenous control: 18s. Error bar represent SEM from 6 repeated reactions from two independent experiments. P-values were calculated with Kruskal-Wallis test with Dunn's multiple comparison tests. (*p<0.05, **p<0.01, ***p<0.001)

3.5 Conclusion

The aim of this chapter was to explore any correlation between Notch signaling pathway and haematopoietic enhancing ability in stromal cell lines or EBs co-cultured on stromal cell lines. To address this question, we applied FACSorting to fraction the mixture of ESC-derived cells and stromal cells in the co-culture and assessed components of the Notch signaling pathway including ligands, receptors, downstream genes as well as responding ability to GSI in each. Based on the results, we concluded that:

- Notch ligand expression in stromal cell lines did not correlate with their haematopoietic enhancing ability (Figure 3.2-3.4);

- Stromal cell line AM14 with haematopoietic enhancing ability displayed higher Notch activity than non-enhancing stromal cell line UG26 and also responded to GSI indicating the correlation between Notch activity in stromal cells with haematopoietic enhancing ability. Thus Notch activity in stromal cell lines could determine their haematopoietic enhancing ability on ES cells (Figure 3.5).

- Notch activity increased in EBs co-cultured on AM14 stromal cells during differentiation. Definitive haematopoietic marker Runx1 was inhibited when Notch activity blocked by GSI. These together suggested that Notch signaling was required in haematopoietic differentiation in EBs co-cultured on AM14 (Figure 3.5, 3.7).

- No correlation found between Notch activity in EBs and haematopoietic differentiation ability. This suggested that stromal cell lines did not determine EB-derived haematopoiesis via modulating Notch activity in EBs. Other key signaling pathways are likely to be involved and responsible for haematopoietic enhancing ability of stromal cells (Figure 3.8).

3.6 Discussion

3.6.1 Notch Ligands in stromal cell lines-- different target cell population; coordination with other signaling pathways and dose of expression level.

In this thesis, we measured the ligand expression pattern in the stromal cells at RNA and protein level. However it is well recognized that level of RNA may not accurately reflect the level of functional protein within a cell. The immunostaining assay or flow cytometry is necessary to measure the level of protein but not particularly sensitive nor quantitative. In contrast quantitative RT-PCR has the strength to measure very small differences and is more sensitive compared to the other assays. Our findings suggested that the haematopoietic-enhancing AM stromal cell lines did not express the Notch ligands at a higher level comparing to the non-enhancing stroma. This could be explained by:

(a) The expression of Notch ligands in haematopoietic enhancing stromal cell lines does not determine their haematopoietic enhancing activity;

(b) Signal Coordination, different microenvironment and target population: Although isolated stromal cell lines are better simplified and specified for investigation of factors controlling cell decisions compared to an explant culture, there still represent a combination of several regulators including cellular and ECM (Extracellular Matrix) interactions, secreted factors, direct cell-cell contact or short-distance factors. These factors could have already altered the fate of co-cultured EBs or their ability to respond to the Notch ligands; or the Notch ligand should coordinate with these factors to function. In this case, we could assume that under some circumstances, the Notch ligands expressed by certain stromal cells are functional while some stromal cell lines expressed the ligands are abundant, reflecting their origin difference or even colony difference. Schmitt et al., found that when Delta-like1 was expressed in OP9 and S17, they behaved differently to induce the T cell differentiation from HPCs, suggesting that although derived from similar niche and expressed similar level of ectopic Delta-like1, OP9 provide a better microenvironment for Delta-like1 to mediate

induction of T cell lineage commitment (Schmitt et al., 2002). Thus, in our system, although not expressing higher dose of ligands, the ligands expressed in the haematopoietic enhancing stromal cell lines could be more efficient than the non-enhancing ones;

(c) Dose issue. Contradict to our finding that Delta-like1 is expressed in OP9 using both quantitative RT-PCR and immunostaining assay, Schmitt's et al reported that Delta-like 1 could not be detected in OP9 by RT-PCR and when ectopic Delta-like 1 was expressed they observed the phenotype (Schmitt et al., 2002). Not considering about the sensitivity of detecting technique, this could support our theory that the dose of Notch ligands is important and that low levels of expression of ligands might not be functional.

(d) Preference to different ligands. A series of studies have focused on the difference of Notch ligands effects on haematopoiesis (Neves et al., 2006). The RNA and protein measurement in our study revealed these four ligands were widely expressed which further study is necessary to figure out the functional and critical ligand, which could be different among these four co-culture systems.

Effect of Jagged1 in the co-culture system

Jagged1 is highly expressed in OP9 stromal cell lines by both quantitative RT-PCR and protein assays. This finding is also observed in Schmitt's work demonstrating that Jagged1 and Jagged2 were expressed in OP9. It is also consistent with previous studies demonstrating that Jagged1 is expressed in primary bone marrow stroma and in bone marrow stromal lines including PA6 from new born mice calvaria andHS-27a from human bone marrow. Jagged1 could support or promote the proliferation of haematopoietic progenitors through the interaction with Notch1 (Jones et al., 1998; Varnum-Finney et al., 1998) (Li et al., 1998; Carlesso et al., 1999; Neves et al., 2006). According to our qPCR results, AM14.1C4 also expressed a relatively high level of Jagged1. Although this is not observed in AM20.1A4 or AM20.1B4, it is possible that AM14.1C4 is derived from AGM region of E11 wildtype mice C57BL/6, which is different from AM20 lines. Charbord and colleagues (2002) have already

compared a panel of stromal cell lines derived from different developmental niches and found that these AM lines did differ and represented different stages from mesenchymal to vascular smooth muscle cells (VSMCs) pathway (Charbord et al., 2002). A series of work by Oosterdorp et al have also screened a batch of stromal cell lines from AGM and fetal liver and clarified many differences in surface phenotype and cytokine expression (Oostendorp et al., 2002a; 2002b; 2005). Recent work by Robert-Moreno in 2008 claimed that Jagged1 is important for haematopoiesis in AGM region (Robert-Moreno et al., 2008). In light of these work, we could predict that Jagged1 expressed by AM14.1C4 could be one key factor to enhance haematopoietic differentiation.

3.6.2 Notch activity in EBs and stroma in co-culture system

We could detect the Notch ligand Jagged1, receptor Notch1 and two downstream genes Hey1 and Hes5 in the EBs co-cultured on AM14.1C4. Surprisingly, Hey1 was expressed at similar level across the four co-culture systems. However it should be noted that the co-culture system is a mixture of stromal cell lines and ES-derived cells at different stages and cell fates. It is highly possible that certain population in the EBs maintains higher Notch activity while other populations have lower activity.

High Notch activity in EBs from non-enhancing co-culture

Interestingly, although being non-enhancing stromal cells, Hes5 was found to be expressed higher in EBs co-cultured on UG26.1B6 co-culture from day 5. Higher Notch activity in non-enhancing population seem to contradict several studies claiming that Notch signaling pathway activated by Jagged1 could promote haematopoietic progenitor formation or proliferation (Milner et al., 1996; Robert-Moreno et al., 2008; Varnum-Finney et al., 2000). There are several explanations for this:

(a) In the EBs of mix of cells, Hes5 expression is not contributed by expression in haematopoietic progenitors, but in the stromal-like cells or other cell population;

(b) It is possible that a high level of Hes5 expression affected the ability of seeded cells to respond to the colony assay. According to studies by Kawamata and colleagues, overexpression of Hes1 or Hes5 can function in a comparable manner to

Notch1 to prevent the myeloid maturation from bone marrow haematopoietic progenitors (Kawamata et al., 2002). Thus, the high level of Hes5 at late stage (day5 to day6) just before seeding into the colony assay may inhibit myeloid progenitors to mature into colonies upon cytokines stimulation as suggested by Kawamata;

(c) Controversially, Schroeder et al., in 2000 published that mNotch1 overexpression could inhibit the proliferation but promote maturation of myeloid progenitors (Schroeder et al., 2000). According to this, higher Hes5 could have promoted maturation of progenitors before being assayed in the colony assay so less colonies are detected;

(d) Alternatively, there are other signaling pathways in the UG26.1B6 which could compensate the effect of Hes5. In addition, others have reported that the UG26.1B6 cell line has the ability to support the haematopoietic stem cells proliferation, probably through the secreted Wnt5a (Oosendorp et al., 2002; Buckley et al., 2011). In light of this, the increment of Hes5 indicated a potential role of Notch in the haematopoietic stem cell maintenance, probably via interaction with Wnt5a.

Notch activity in microenvironment affected HPCs formation

We found that the Notch signaling pathway was active in both EBs co-culture on AM14.1C4 or AM14.1C4 itself and that both cell populations responded to the inhibitor. Furthermore, the Notch1 and Hey1 were expressed at higher level in AM14.1C4 compared to the UG26.1B6. This result implied a potential effect of stroma-derived Notch activity on haematopoietic enhancement.

So far, most research working on haematopoiesis claiming the importance of Notch signaling pathway are based on *in vivo* study or *in vitro* study of mixed cell populations. However it is possible that it is not the Notch activity in the "pre-HSCs/HPCs" cells that is important, but rather the Notch activity of the surrounding cells or supporting stromal cell like cells. More intriguingly, as described in the introduction, in embryonic haematopoiesis, it was still unclear whether HSCs emerge from haemogenic endothelium, haemangioblast, or mature from a precursor at the mesenchyme and then migrate through the aorta wall. Recently, some studies have focused on the role of mesenchymal cells supporting the haematopoiesis because the

secreted factors by mesenchymal did affect haematopoiesis. In addition, the Notch signaling pathway has been found to affect the integrin family so it is also possible that AM14.1C4 can alter or affect cell adherence which could ultimately affect haematopoietic differentiation (Hodkinson et al., 2007). In future studies, it would be interesting to knock down Notch activity in the AM14.1C4 to see whether its ability to enhance haematopoiesis could be abrogated.

Chapter 4: Investigation of response of ES-derived Flk1^{+} cells to haematopoietic niche derived stromal cell lines

4.1 Aim

To test whether AM stromal cell line could affect haematopoietic commitment of $Flk1^+$ cells.

4.2 Introduction

The *in vitro* enhancing AM stromal cell line (AM14.1C4) provides a haematopoietic enhancing microenvironment that mimics AGM-derived haematopoiesis *in vivo*. In this EB/stroma co-culture system, inhibition of Notch activity from day4 to day6 abrogated haematopoietic differentiation implicating the involvement of Notch signaling in the enhancement by AM stromal cells post mesoderm formation. However, this EB/stroma co-culture system is complex, consisting of stromal cells and ES-derived cells of different cell fates making it difficult to dissect the underlying mechanisms. Furthermore it has been demonstrated by a number of studies that the Notch signaling pathway can function differently throughout the differentiation stages and is cell type dependent. In light of several models proposed by other groups focusing on the origins of HSCs in the AGM region *in vivo*, it is widely accepted that the AGM region could provide the microenvironment for the further differentiation of post-mesoderm cells into HSCs. It is also known that mesoderm derived from primitive streak could commit to different sub-regions including chordamesoderm, intermediate mesoderm as well as lateral plate mesoderm where haematopoietic cells are derived from and paraxial mesoderm that gives rise to somites and mesenchymal cells that ultimately form the haematopoietic niche. Several genes have been known to emerge sequentially during mesoderm development: Brachyury, a primitive streak mesoderm marker expressed in all nascent mesoderm (Herrmann, 1991; Kispert and Herrmann, 1994); Flk1 (VEGFR2), a receptor of VEGF, a marker of lateral plate mesoderm and the haemangioblast (or haemogenic endothelium) defined as the common precursor of haematopoietic and endothelial cells. To better understand how Notch signaling pathway is involved in haematopoietic differentiation in the AGM region, we chose to focus on the time window before mesoderm formation in the first instance and to ask whether AM stromal cells could affect haematopoietic differentiation after this stage.

A previous study in our lab suggested that the co-culture system could not affect commitment to mesoderm cells from ES cells by assessing early mesoderm marker Brachyury expression by flow cytometery using a Brachyury reporter cell line. Instead AM stromal cells were able to induce haematopoiesis derived from Brachyury^{+} fraction (Gordon-Keylock et al., 2011). To focus on the effect of co-culture on mesoderm formation, we measured the effects of stromal cells on mesoderm commitment and assessed the expression of Brachyury and Flk1 using quantitative RT-PCR analysis. We then established a Flk1^{+}/stroma co-culture system to test the effect of haematopoietic niche-derived stromal cell on the fate of Flk1^{+} cells. Experiments were carried out to answer:

(a) Whether the enhancing AM stromal cell line affects the commitment of ES cells to Brachyury and Flk1 mesodermal fates

(b) Whether the AM stromal cell line could modulate the later haematopoietic differentiation from Flk1^{+} cells.

4.3 Experimental approach

- To determine whether the enhancing AM stromal cell line affects the commitment of ES cells to mesodermal fates, differentiating EBs were co-cultured on AM stromal cell line then FACS sorted and the expression of Brychyury and Flk1 was analyzed using quantitative RT-PCR; Flk1 expression was also analyzed with flow cytometry during differentiation.

- To determine whether AM stromal cells affect cell fate decision after Flk1^{+} formation, Flk1^{+} cells were differentiated and isolated from EBs then co-cultured on several different haematopoietic niche derived stromal cell lines including: AGM-derived enhancing cell line AM (AM14.1C4), AGM derived non-enhancing cell line UG (UG26.1B6) and the bone marrow derived OP9. Haematopoietic colony formation activity, haematopoietic surface marker expression, haematopoietic progenitors and pan-haematopoietic cell proliferation and survival were measured using methylcellulose assays and flow cytometry.

4.4 Results

4.4.1 Kinetics of mesodermal marker expression in co-culture

To test whether the haematopoietic enhancing AM stromal cell line enhanced haematopoiesis by promoting mesoderm formation or post mesoderm differentiation, we carried the co-culture of EBs with AM or gelatin as the control. One day old hanging drop EBs with 7a-GFP ES cell line were co-cultured on gelatin or AM stromal cell line to day6. Between day3 and day6, EBs were separated from stromal cells by FACS and Brychyury and Flk1 was assessed by quantitative RT-PCR analysis (Described in Figure 3.4). Purified EBs were analyzed using quantitative RT-PCR and gene expression level was expressed as fold increase relative to a calibrator (day1 old EBs), which was assigned a value of "1".

Brachyury expression increased significantly in EBs from both gelatin controls and AM co-cultures at day3, 4 and 5 compared to undifferentiated ESC ($*p<0.05$) (Figure 4.1 A). However when comparing these two co-culture systems at day 3, EBs on gelatin showed a higher (3 fold) level of Brachyury expression than EBs on AM stromal cell lines ($*p<0.02$). There was no significant difference between gelatin and AM condition at day 4 and 5 ($p>0.05$) (Figure 4.1 A). A possible explanation could be that the microenvironment provided by the AM stromal cells promoted the Brachyury positive cells to leave the early mesoderm stage and enter the later differentiated state but without changing the overall kinetics of Brachyury. Brachyury is a relatively early marker when we considered the haematopoietic related mesoderm so next we focus on the Flk1 expression kinetics which was more related to haematopoiesis.

Flk1 expression kinetics in EBs cultured on gelatin and AM stromal cells displayed comparable patterns from ESC to day5, with a peak at day4. This result suggested that AM did not affect the formation of $Flk1^+$ formation in the co-culture. However at day 6, EBs cultured on gelatin expressed $Flk1^+$ significantly higher (2 fold) than EBs on AM stromal cell lines, suggesting that AM stromal cell line could possibly force the further differentiation into haematopoietic commitment from $Flk1^+$ after day4

(*p<0.05) (Figure 4.1 B). In conclusion, the screen of mesodermal like genes suggested that $Flk1^+$ formation were not affected in the microenvironment provided by AM stromal cell line, which indicated that AM may provide the haematopoietic enhancing microenvironment for the later haematopoietic differentiation from haemangioblast.

To further confirm the conclusion based on the quantitative RT-PCR analysis, we analyzed the expression Flk1 at the protein level using flow cytometry. One day hanging drop EBs generated from E14IV ES cells were co-cultured on irradiated Vybrant DiD labeled stromal cells to day6. From day3 to day8, cells from co-culture were harvested and analyzed by flow cytometry. ES-derived cells which should be negative at FL-4 channel in FACS Calibur or R670/14 in BDFortessa were distinguished from the Vybrant DiD-stained stromal cells. From day3 to day5, the $Flk1^+$ expression kinetics in both conditions had a similar pattern: there was no significant difference of $Flk1^+$ proportion between gelatin control and haematopoietic enhancing AM co-culture prior to day6 which supported the notion that AM microenvironment did not enhance or block the emergence of $Flk1^+$ cells. However at day6, $Flk1^+$ expression in cells cultured on gelatin was slightly higher compared to AM co-culture then at day8 this difference was more pronounced (*p<0.05) (Figure4.1 C). Thus the difference in Flk1 expression in the two culture systems is observed at both the RNA and protein level with the difference in protein being detected slightly later. This could be explained by the timing difference between RNA level and protein level. Protein difference could appear later than the transcripts. It is possible that at the later stages the AM microenvironment may be promoting ES-derived cells $Flk1^+$ cells to further differentiate possibly into haematopoietic lineage suggesting a later effect on haematopoiesis by AM stromal cells.

Alternatively, AM could maintain microenvironment consisted of non-$Flk1^+$ cells, for example the paraxial mesoderm niche, to support the haematopoiesis after the formation of $Flk1^+$, but not to affect the haematopoiesis from $Flk1^+$ directly. These two possibilities could also function together to promote ES-derived haematopoiesis. This point will be borne in mind but this project will first focus on the effect of AM

stromal cells on $Flk1^+$ further differentiation.

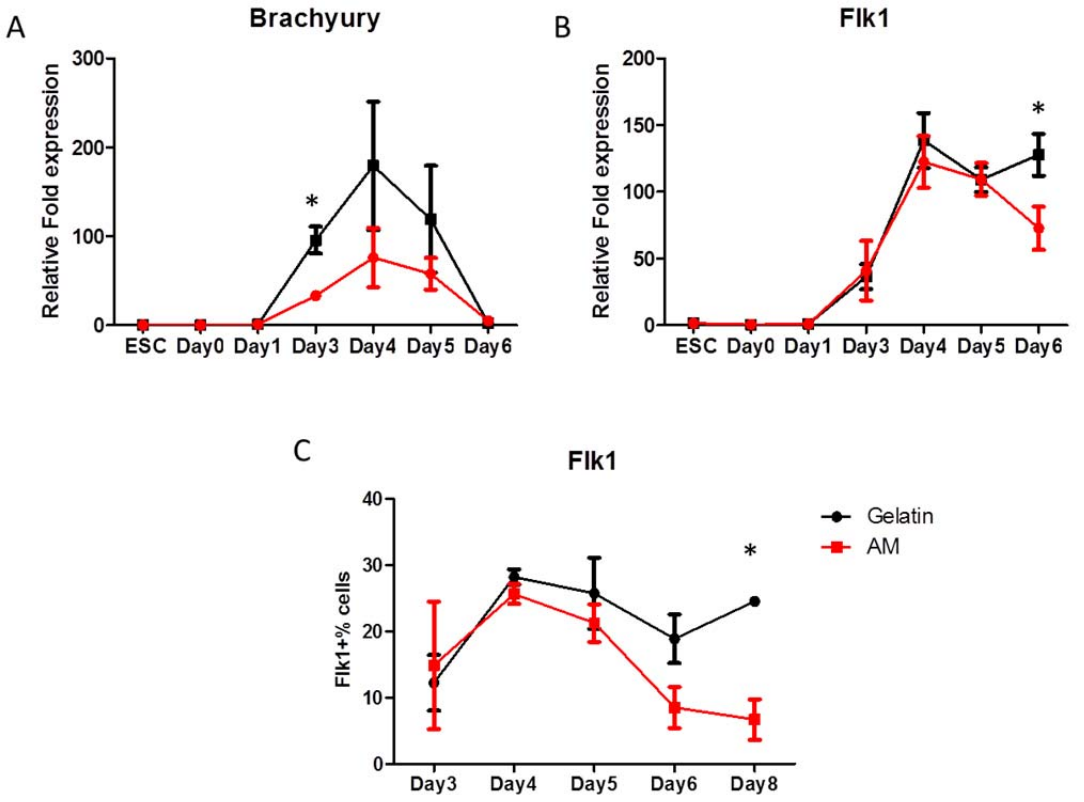

Figure 4.1 Quantitative RT-PCR and flow cytometry analysis to compare mesodermal gene expression kinetics in EBs cultured in haematopoietic enhancing AM microenvironment and Gelatin control.

One day hanging drop EBs were co-cultured to day6 and sorted from AM stroma or gelatin by FACS and qRT-PCR carried out for Brachyury (A), Flk1 (B) expression kinetics. One day EBs were used as calibrator (value assigned as 1); 18s was used as the endogenous control; Flk1 expression at the protein level was analysed with flow cytometry (C). Day 1 hanging drop EBs were co-cultured on gelatin and AM stroma then analyzed by flow cytometry at days 3, 4, 5, 6 and 8 of differentiation. Data represent 3 independent experiments. Error bars represented SEM. P-values were calculated with Friedman test or Mann-Whitney test.

4.4.2 Investigation of Flk1$^+$ haematopoietic differentiation on AGM-derived stromal cell lines

In order to address the role of Notch signaling pathway on haematopoietic differentiation in the AM microenvironment (AM14.1C4), it is necessary to specify the population responsive to AM stromal cell line. According to the results suggesting that AM stromal cell line did not affect the Flk1$^+$ formation, we asked the question whether the AM stroma promoted haematopoiesis after the stage. To test this, Flk1$^+$ were differentiated in suspension EBs. Flow cytometry was applied to monitor the emergence of Flk1$^+$ cells during differentiation. Flk1 positive and negative cells were purified by MACS sorting from day 4 suspension EBs and co-cultured on gelatin control or stromal cell lines. Methylcellulose assay and flow cytometry were applied to assess the haematopoietic enhancing effect of stromal cell lines on Flk1 positive and negative cells. We carried out these tests with wildtype ES cell line E14IV and a genetically modified ES cell line R26-NIC-C5, a tamoxifen inducible ES cell line. When tamoxifen was applied, ectopic NotchIC in ROSA26 locus will be expressed. This cell line is widely applied in this project and will be described in detail in Chapter5.

4.4.2.1 Differentiation of Flk1$^+$ in suspension Embryoid Bodies.

Choi and colleagues carried out a kinetics analysis and demonstrated that blast colony-forming cell (BL-CFC), the *in vitro* equivalent to haemangioblast, expressed Flk1 and was present in the EBs between day 2.5 and 4 of differentiation (Choi et al., 1998). In light of this, we simply differentiated ES cells in suspension EBs and monitored emerge of Flk1$^+$ cells.

Flk1$^+$ cells were generated in both differentiating E14IV and R26-NIC-C5 ESC cell lines with similar kinetics. During differentiation, Flk1$^+$ emergence peaked at day4. Statistically, for E14IV ES cell line, around 55% ES-derived cells expressed Flk1, which was significantly higher than day2 at around 2% (*$p<0.03$) (Figure 4.2 A). The NIC-C5 ES cell line generated a higher proportion of Flk1$^+$ at 60% than day2 (*$p<0.03$) (Figure 4.2 B).

The surface marker CD41 and cKit have been used together to define ES cell-derived definitive haematopoietic progenitors *in vitro* (Mikkola et al., 2003; Mitjavila-Garcia et al., 2002). Low levels (<1%) of $CD41^{+}cKit^{+}$ cells were present at day 4 and began to increase to 2-3% at day 5 (Figure 4.2 C, D). Therefore, these results suggest that $Flk1^{+}$ could be differentiated in suspension EBs and day 4 would be a suitable time point to purify $Flk1^{+}$ population with a low committed haematopoietic potential as determined by $CD41^{+}cKit^{+}$.

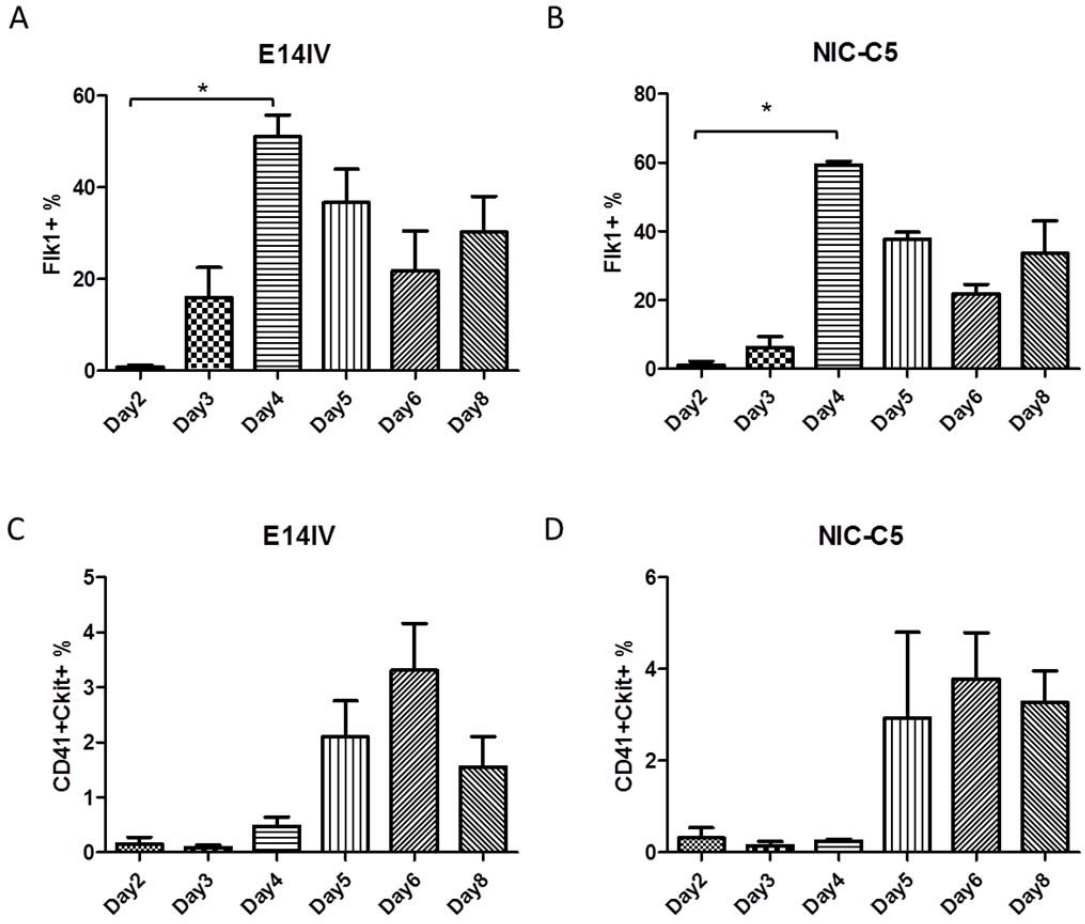

Figure 4.2 E14IV and NIC-C5 ES cell lines produced Flk1$^+$ and CD41$^+$cKit$^+$ with similar kinetics.

Data represented 3 independent experiments. Error bars represented SEM. P-values were calculated with Friedman test with post paired test (*$p<0.05$).

4.4.2.2 Investigation of colony formation ability of Flk1$^+$ derived cells co-cultured on AGM-derived stromal cell lines.

a) Haematopoietic progenitors with colony formation ability are enriched in cells derived from Flk1$^+$ population

The methylcellulose assay is a colony-forming assay consisting of methylcellulose, serum and a series of cytokines (EPO, IL-3, IL-6, SCF, insulin etc) and is widely used to measure haematopoietic activity *in vitro*. When single cells are seeded into the methylcellulose medium, haematopoietic progenitors respond to the cytokines and form colonies. Colonies can be categorized according to the morphology and cell types existing in the colonies: CFU-GEMM (granulocytes, erythrocytes, macrophages and megakaryocytes) (Figure 4.3 A), CFU-GM (granulocytes and macrophages) (Figure 4.3 B), BFU-E (bigger red colonies of erythrocytes with high proliferation ability) (Figure 4.3 C), CFU-E (smaller red or brown colonies of erythrocytes with low proliferation ability), CFU-M (macrophages) (Figure 4.3 D) and Mac/Ery (macrophages and erythrocytes) (not shown). These colonies can be easily identified with white light microscopy and haematopoietic progenitors can thus be functionally quantified.

To investigate the effect of stromal cells on the cell fate of Flk1$^+$, we co-cultured Flk1$^+$ cells on stromal cells and assessed the emergence of haematopoietic colonies. Many previous studies have demonstrated that Flk1$^+$ was a convincing marker of haemangioblast. To confirm this in our system we separated Flk1$^+$ and Flk1$^-$ compartments from day 4 EBs and performed co-culture with stromal cells or gelatin control then assessed haematopoietic activity in methylcellulose assay (Figure 4.4 A). Basically, Flk1$^+$ or Flk1$^-$ were purified by MACS sorting (Figure 4.4 B) then co-cultured at the density of $4x10^4$cells/cm^2 on Vybrant DiD pre-stained irradiated stromal cells or gelatin control. At specific time points, cells were harvested and assayed in methylcellulose. Colonies were scored and haematopoietic activity was measured by counting the number of each type of haematopoietic colony. In addition, haematopoietic activity was also assessed using flow cytometry and quantitative RT-PCR (Figure 4.4 A). In preliminary experiments it was observed that at day6 (48

hours post co-culture), both in gelatin or stromal cell line co-culture systems, $Flk1^+$ derived cells could commit to more haematopoietic progenitors than $Flk1^-$ control (Figure 4.5). Thus, as expected, haematopoietic progenitors were enriched in the $Flk1^+$ compartment. Flk1- cells produced a very low baseline number of haematopoietic colonies but this level was not altered significantly by co-culture on any of the stromal cell lines.

b) Haematopoietic progenitors with colony formation ability derived from $Flk1^+$ population peaked at day6

To determine the kinetics of haematopoietic progenitor formation from $Flk1^+$ cells in co-culture we carried out the methylcellulose assay to measure haematopoietic activity at day4, day6 and day9. E14IV ES cells were differentiated in suspension EBs. After 4 days of differentiation, $Flk1^+$ cells were isolated and plated in methylcellulose colony assays at the density of $1x10^5$ per dish. $Flk1^+$ cells were also co-cultured to day6 and day9 then seeded into colony assay (Figure 4.6). Compared to day4 $Flk1^+$, cells derived from $Flk1^+$ at day6 showed higher haematopoietic colony production. At day9, the colony activity of $Flk1^+$ derived cells was reduced compared to day6, even lower than day4 in gelatin control. This indicated that progenitors with colony formation ability derived from day 4 $Flk1^+$ cells peaked at day6. More importantly, this result demonstrated that there was no difference in the kinetics of haematopoietic activity in the four co-culture systems. These data led us to measure the haematopoietic activity with colony assay and compare different co-culture systems at day6 in all further experiments.

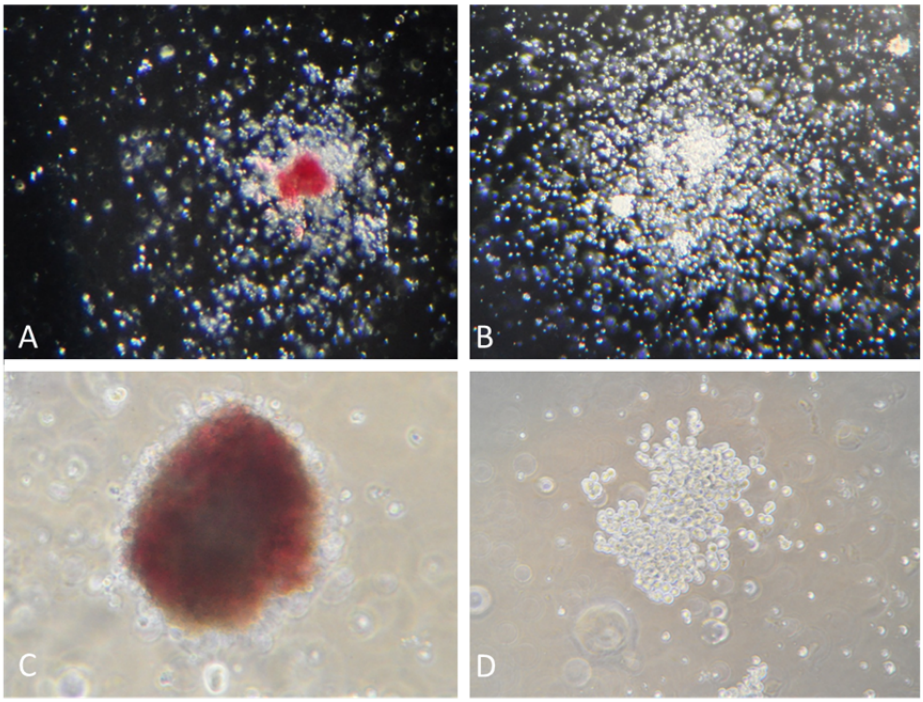

Figure 4.3 Examples of colonies observed in the methylcellulose colony formation assay.

(A) CFU-GEMM colony containing granulocytes, erythrocytes, macrophages and megakaryocytes (X10); (B) CFU-GM containing granulocytes and macrophages (X10); (C) BFU-E containing erythrocytes with high proliferation ability (X20); (D) CFU-M containing macrophages (X20).

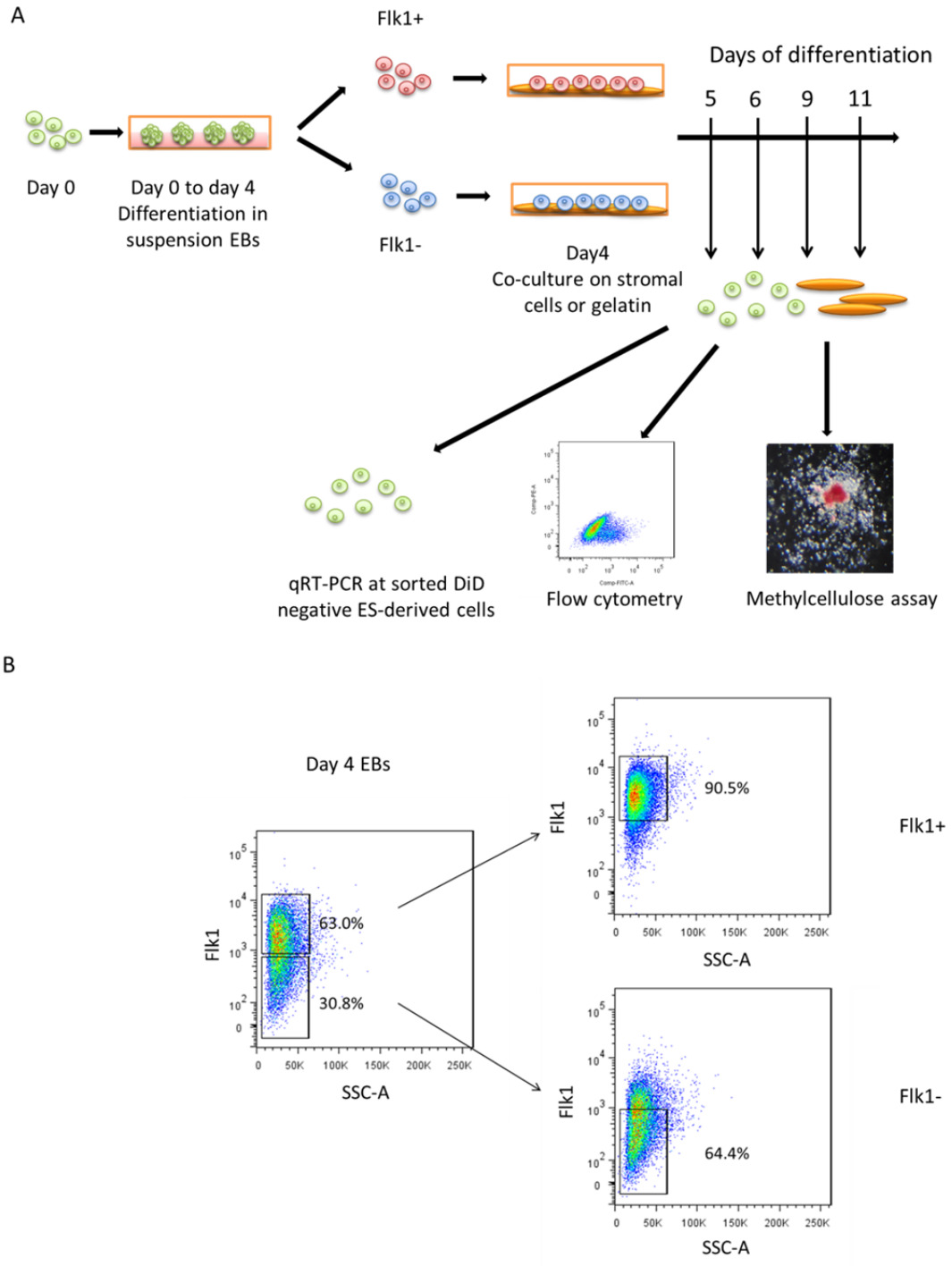

Figure 4.4 Scheme of differentiation of Flk1$^+$ and Flk1$^-$ compartments from day 4 suspension EBs co-cultured on stromal cells or gelatin control.

(A) Flk1$^+$ and Flk1$^-$ population further co-cultured and assessed by methylcellulose assay, flow cytometry or qRT-PCR post FACSorting at haematopoietic activity; (B) Flk1$^+$ and Flk1$^-$ population were fractioned from day 4 suspension EBs with MACS sorting.

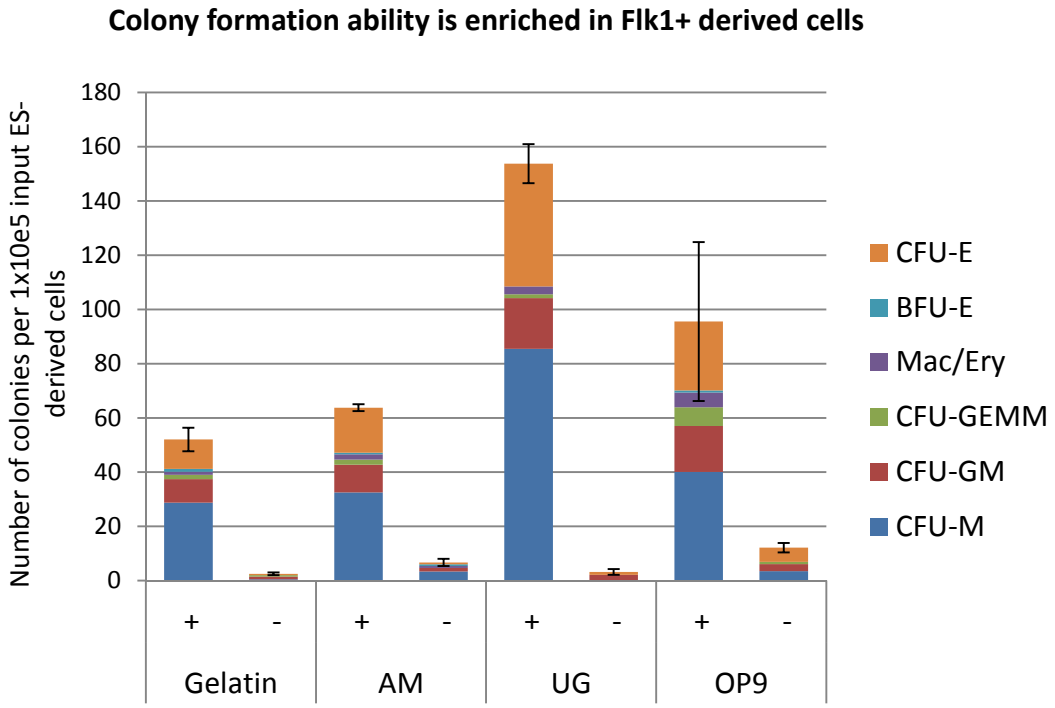

Figure 4.5 Haematopoietic progenitors with colony formation ability is enriched in Flk1^{+} population at day6.

Flk1^{+} maintained higher haematopoietic colony formation ability than Flk1^{-} in both gelatin control and stromal co-culture systems. Data represents one experiment. Error bars represented deviations of duplicate dishes.

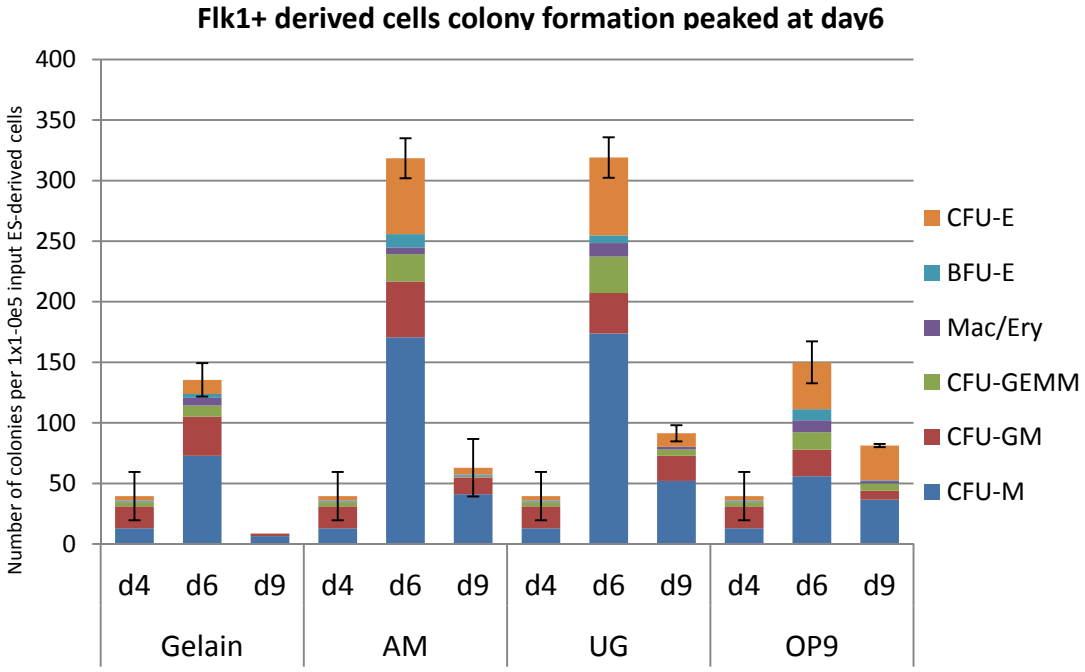

Figure 4.6 Methylcellulose-based haematopoietic colony assays for day4 Flk1$^+$ fraction and Flk1$^+$ derived cells after co-culture with AM UG, OP9 and Gelatin to day 6 and day 9.

Flk1$^+$ cells were purified from day4 suspension EBs using E14IV ES cell line then co-cultured on gelatin control or stromal cells to day6 and day9. Data represents 1 experiment. Error bars represented the deviation of duplicate dishes in the same experiment.

c) AM stromal cell lines could partially enhance haematopoietic colony formation ability of Flk1$^+$ derived cells

In order to determine whether the AM enhancing cell line could also enhance haematopoietic activity of Flk1$^+$ cells, purified Flk1$^+$ cells from day4 suspension EBs were co-cultured on irradiated AM stromal cells. Cells were harvested and seeded into methylcellulose colony assay at day6. Ten days later colonies were scored and compared to gelatin control. Effects of stromal cell lines on EBs could be different from their effects on the Flk1$^+$ cells. Thus, non-enhancing cell line UG and positive OP9 stromal cell line defined in the EB/stroma system were included here and screened for enhancing activity as well. In light of many previous work claiming that ESC differentiation *in vitro* was ES cell line dependent, two ES cell lines, E14IV and NIC- C5 were both tested in the Flk1$^+$ /stroma co-culture system. Because of the large variation observed in the differentiating efficiency between experiments, Wilcoxon matched pairs test was applied to compare number of colonies in pairs from the same experiment to exclude the effect of experiment variability.

HPCs formation with E14IV cell line (Figure 4.7; 4.8):

Compared to gelatin control, no significant difference was found in the frequency of CFU-M, CFU-GEMM or Mac/Ery in the Flk1$^+$/ stroma co-culture systems ($p>0.05$) (Figure 4.7 A, C, D); Statistically, both AM and UG line could promote higher CFU-GM production than gelatin (*$p<0.05$); BFU-E frequency was enhanced in the UG co-culture system compared to gelatin control (*$p<0.05$) (Figure 4.7 E); CFU-E frequency was enhanced in the AM and OP9 co-culture systems compared to gelatin control (*$p<0.05$) (Figure 4.7 F). Thus assessment of the numbers of the different types of colonies indicated that AM and UG could provide the microenvironments to enhance both myeloid and erythorid progenitors differentiated from Flk1$^+$.

To address the haematopoietic enhancing capacities of AM, UG and OP9 stromal cell lines on Flk1$^+$ cells in a more direct way, all the types of colony were added up and compared to gelatin control and to each other (Figure 4.8). It was found that only AM could enhance the overall number of haematopoietic CFU over that of gelatin control at day6 (422±407 for AM vs 119±76 for gelatin, n=6, *$p<0.05$). However no

difference was observed when CFU frequencies in UG (257±250) and OP9 (190±162) co-cultures were compared to gelatin control. Furthermore, the CFU frequencies among AM, UG and OP9 co-cultures were comparable. These data demonstrated that AM stromal cells could enhance haematopoietic differentiation from $Flk1^{+}$ cells compared to gelatin, at a moderate level.

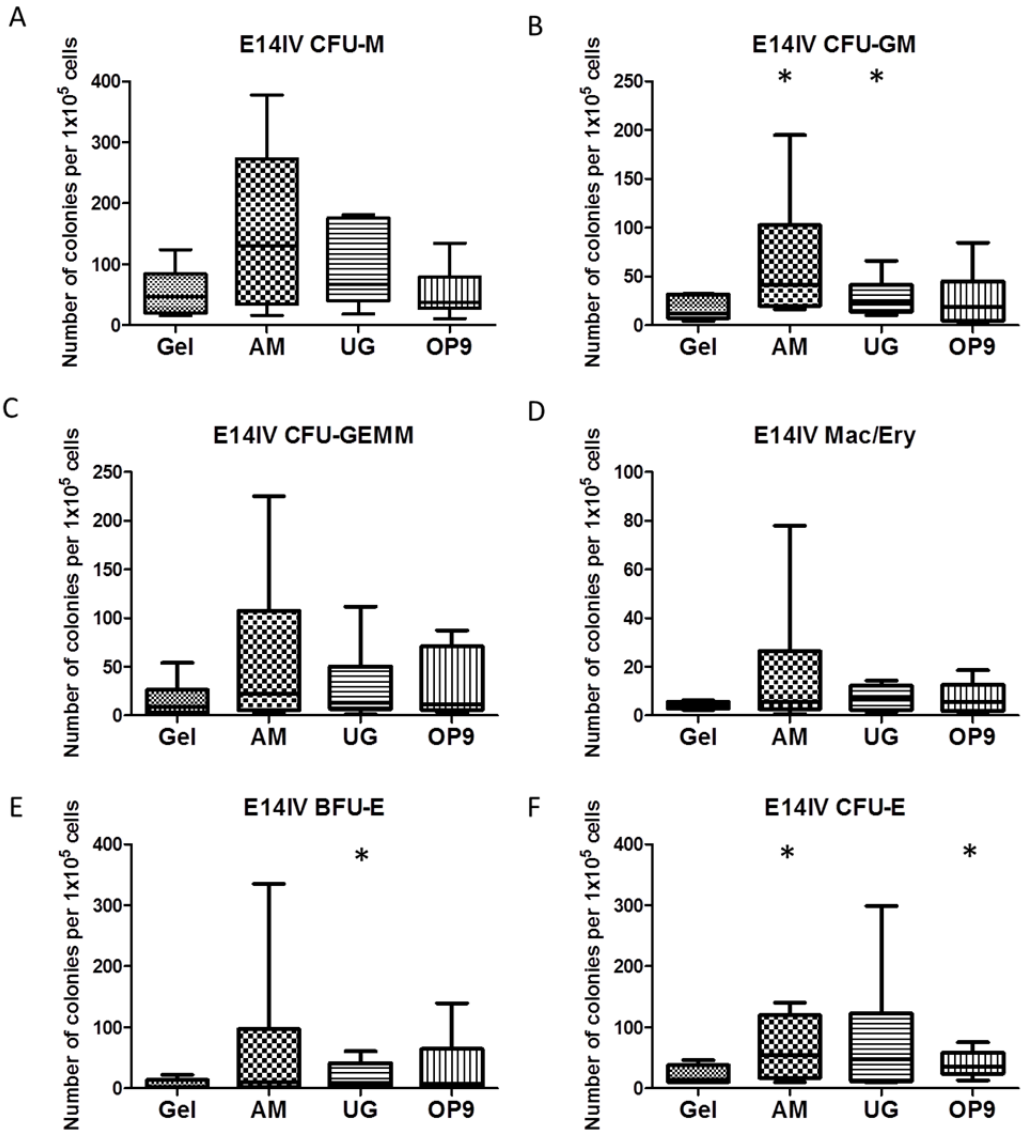

Figure 4.7 Haematopoietic colonies formed by Flk1^{+} derived cells (E14IV ES cell line) co-cultured to day6.

(A) CFU-M; (B) CFU-GM; (C) CFU-GEMM; (D) Mac/Ery; (E) BFU-E; (F) CFU-E. Data represent 6 independent experiments. Error bars represent the min to max. P-values were calculated with Wilcoxon matched pairs test (*$p<0.05$ compared to gelatin control).

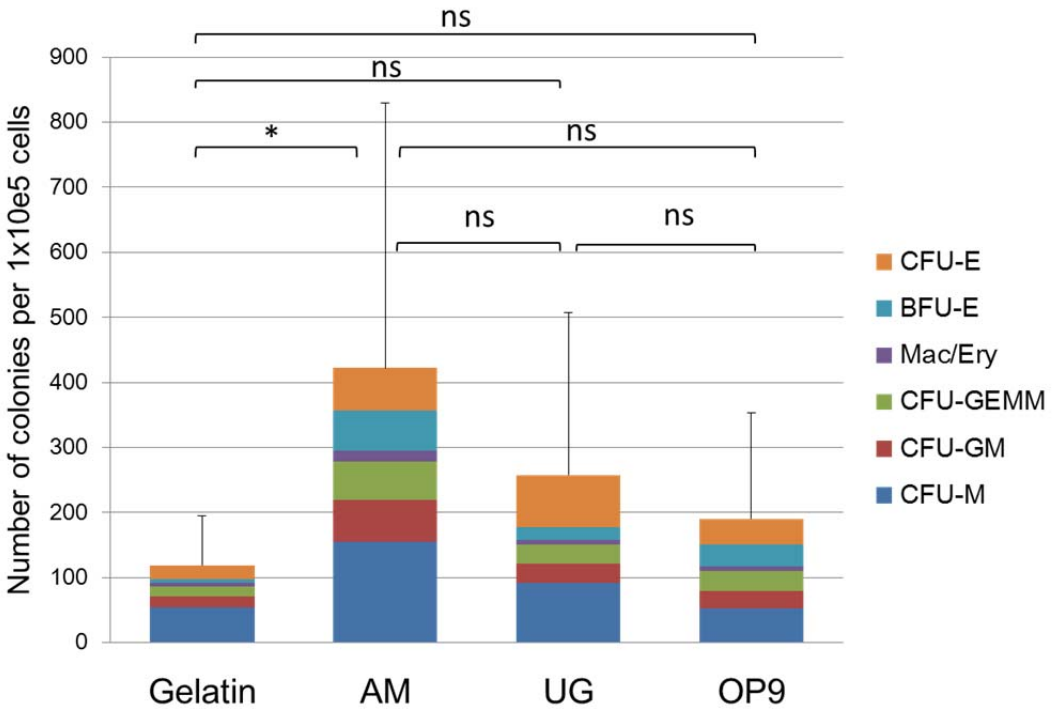

Figure 4.8 Total number of haematopoietic colonies in methylcellulose assay by Flk1$^+$ derived cells (E14IV ES cell line) in co-culture at day6.

AM stromal cells enhanced Flk1$^+$ derived haematopoiesis at moderate level compared to gelatin control. UG and OP9 did not enhanced haematopoiesis significantly compared to gelatin. Data represent 6 independent experiments. Error bars represent standard deviation. P-values were calculated with Wilcoxon matched pairs tests (*$p<0.05$, ns: not significant different).

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HPCs formation with NIC- C5 ES cell line (Figure 4.9; 4.10):

Similarly, the frequency of CFU-M in OP9 co-culture was comparable to gelatin control ($p>0.05$), which was consistent to the EB/stromal system that OP9 inhibit the formation of CFU-M. Both AM and UG cell line could enhance CFU-M production significantly higher than gelatin (**$p<0.05$). (Figure 4.9 A); UG stromal cell line could enhance a significant greater frequency of CFU-GM than gelatin control (Figure 4.9 B); Compared to gelatin control, AM could provide microenvironments to promote CFU-GEMM production (*$p<0.05$) (Figure 4.9 C). No differences were observed in frequencies of Mac/Ery obtained from co-culture with AM, UG or OP9 stromal cell line compared to gelatin ($p>0.05$) (Figure 4.9 D); Frequency of BFU-E were both enhanced in the AM and UG stromal co-culture systems compared to gelatin control (*$p<0.05$) (Figure 4.9 E). Frequency of CFU-E were enhanced in the AM and OP9 stromal co-culture systems compared to gelatin control (*$p<0.05$) (Figure 4.9 F). According to the comparison of individual colonies frequencies to gelatin, it is suggested that AM could enhance the multi-potential haematopoietic progenitors from $Flk1^+$ cells; AM and UG stromal cell line could promote the frequency of haematopoietic progenitors which could commit to myeloid or erythroid lineages. OP9 could promote the frequency of erythroid progenitors.

All these colonies were piled up and statistically compared in pairs as shown in Figure 4.10. It is showed that both AM and UG stromal cell line enhanced the number of the overall haematopoietic colonies over that of gelatin control at day6 (AM vs Gel: 500±423 vs 165±86; UG vs Gel: 372±195 vs 165±86; n=7, *$p<0.05$). OP9 could not further enhance $Flk1^+$ derived haematopoiesis compared to gelatin (223±165 vs 165±86, n=7, $p>0.05$). AM microenvironment enhanced significantly greater haematopoietic activity than OP9 (*$p<0.05$), while with a comparable capacity to UG stromal line. The number of CFU present in UG co-culture was comparable to OP9 co-culture ($p>0.05$). These data demonstrate that both AM stromal cells and UG stromal cells could enhance haematopoietic differentiation from $Flk1^+$ cells compared to gelatin. Combing data generated from the two ES cell lines, it is confirmed that AM stromal cell line could enhance $Flk1^+$ derived haematopoiesis. The reason that UG haematopoietic enhancing effect was not

observed using E14IV cell line could be accounted for the limited number of experiments or variance of haematopoietic enhancement efficiency in repeats. More repeats will be necessary for further statistical analysis.

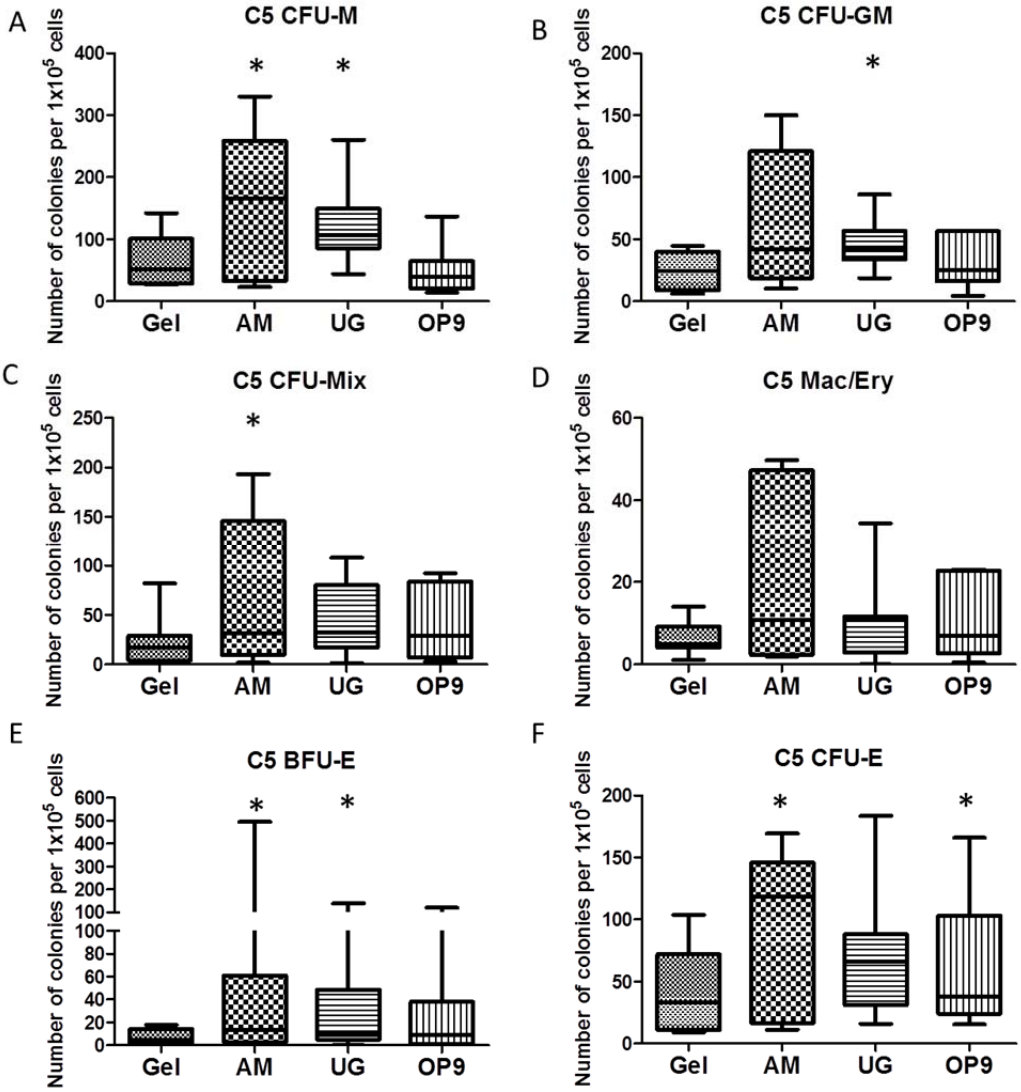


Figure 4.9 Haematopoietic colonies formed by Flk1$^+$ derived cells (NIC- C5 ES cell line) co-cultured to day6.

(A) CFU-M; (B) CFU-GM; (C) CFU-GEMM; (D) Mac/Ery; (E) BFU-E; (F) CFU-E. Data represent 7 independent experiments. Error bars represent min to max value. P-values were calculated with Wilcoxon matched pairs test (*$p<0.05$ compared to gelatin control).

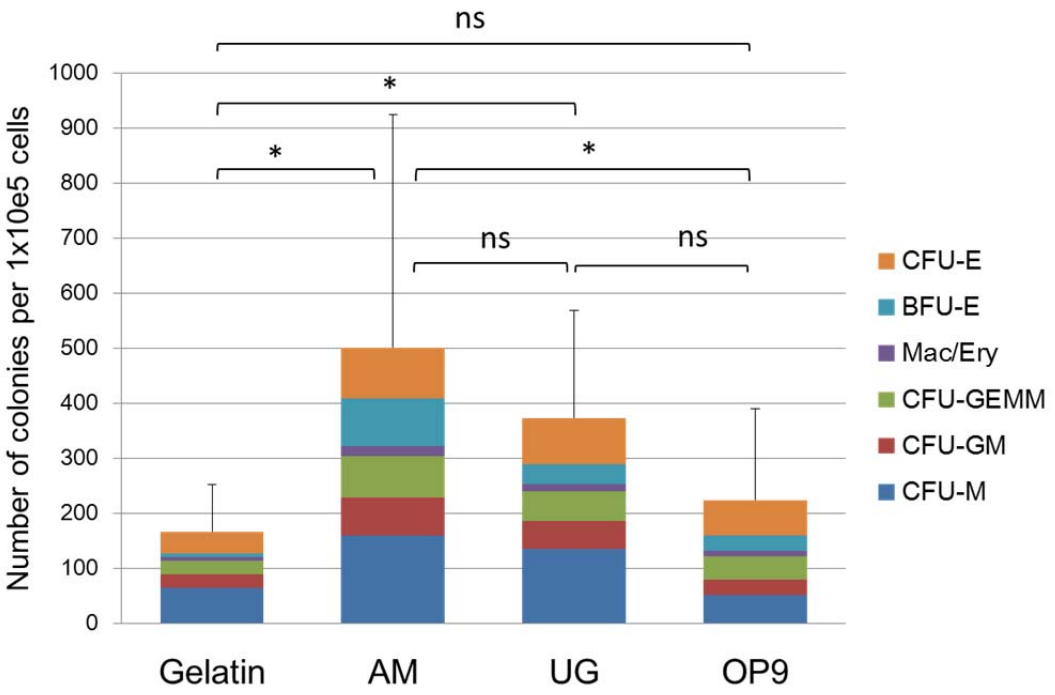


Figure 4.10 Total number of haematopoietic colonies in methylcellulose assay by Flk1^{+} derived cells (NIC- C5 ES cell line) in co-culture at day6.

AM and UG stromal cells enhanced Flk1^{+} derived haematopoiesis at moderate level compared to gelatin control. OP9 did not enhance haematopoiesis significantly compared to gelatin.Data represent 7 independent experiments. Error bars represent standard deviation. P-values were calculated with Wilcoxon matched pairs tests (*$p<0.05$, ns: not significant different).

d) Measurement of haematopoietic progenitors and pan-haemaotpoietic cells production in Flk1$^+$/stroma co-cultures by surface markers with flow cytometry analysis

AM stromal promote frequency of CD41$^+$cKit$^+$ haematopoietic progenitors from Flk1$^+$ cells

To further characterize the haematopoietic activity in the Flk1$^+$/stroma co-culture system, cells from co-cultures were analyzed by flow cytometry at defined time points during differentiation. CD41$^+$cKit$^+$ was used to define haematopoietic progenitors and CD45 as a marker for pan-haematopoietic cells which was not expressed by mature erythrocytes. E14IV ES cell line and NIC-C5 ES cell line were both applied for the analysis. Basically, Flk1$^+$ cells were fractioned from day 4 suspension EBs then co-cultured on gelatin control or stromal cell lines at 1x10^4/cm^2. Cells were harvested at day6, day9 and day 11 then analyzed by flow cytometry. It was noted that the peak of emergence of CD41$^+$cKit$^+$ at day6 and CD45 at around day8 to day9 in co-cultures (data not shown). At day 6, by using either E14IV or NIC- C5 ES cell line there were no significant differences in the proportion of Flk1$^+$ derived cells co-expressing CD41 and cKit in the UG or OP9 co-culture systems compared to gelatin control ($p>0.05$), while AM stromal cell lines enhanced the proportion of CD41$^+$cKit$^+$ progenitors significantly up to 2 fold in both ES cell lines (*$p<0.05$) (Figure 4.11 A, B). Flow cytometry analysis at a later differentiation stage (day9) revealed that co-culture with stromal cell lines did not increase the proportion of CD45-expressing haematopoietic cells compared to gelatin ($p>0.05$) (Figure 4.11 C, D). Thus, surface marker expression suggested that the AM stromal cells could further promote the differentiation of haematopoietic progenitors at a moderate level, which is consistent with the the CFU enhancement observed in colony assay by AM stromal cells. However the CD41$^+$cKit$^+$ compartments produced in each co-culture systems has not been tested in functional assay. To further determine this, it will be interesting to purify CD41$^+$cKit$^+$ from each co-culture and measure their colony formation ability in methylcellulose assay.

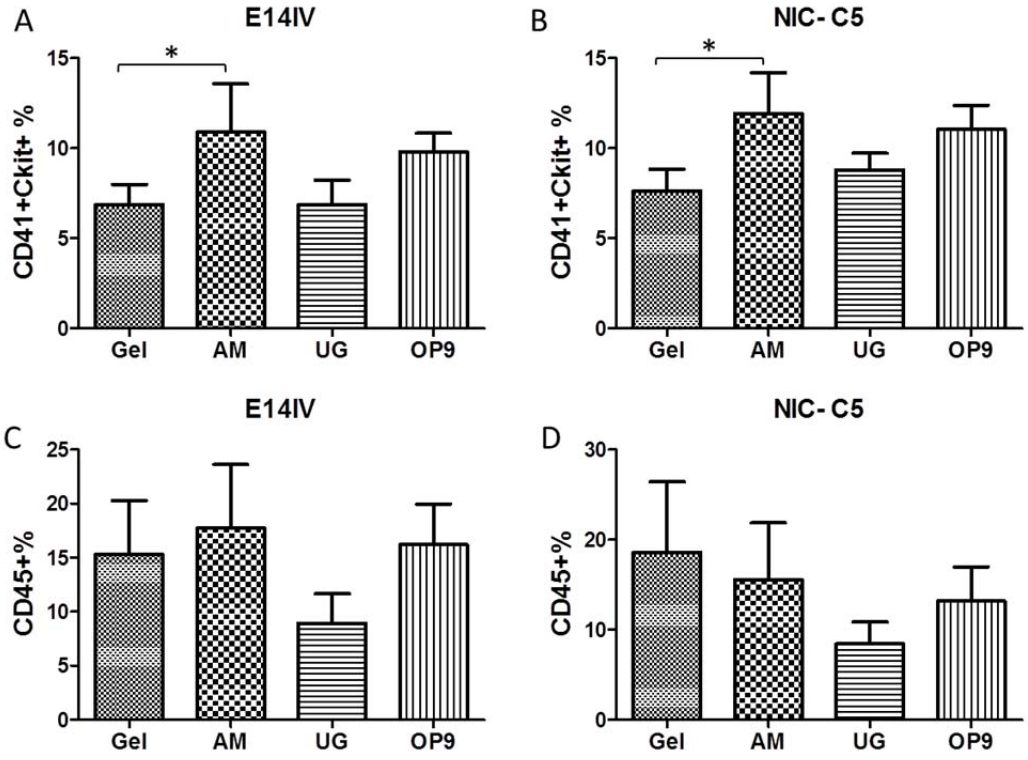


Figure 4.11 Flow cytometry analysis of $CD41^{+}cKit^{+}$ haematopoietic progenitors and $CD45^{+}$ pan-haematopoietic cells production in $Flk1^{+}$/stroma co-culture system.

This data represent between 3 and 7 independent experiments. Error bars represented SEM. P-values were calculated with Wilcoxon matched pairs test (*$p<0.05$).

AM and OP9 co-culture increased the number of Flk1$^+$ derived cells.

To further investigate the effect of stromal cell lines on Flk1$^+$ cells, we assessed the proliferation and survival of Flk1$^+$ derived cells. Flk1$^+$ were differentiated on Vybrand DiD stromal cells or gelatin control then harvested and counted at the defined time points. After normalization to the Flk1$^+$ derived cells percentage to exclude stromal cells, the number of Flk1$^+$ derived cells was recorded and plotted (Figure 4.12).

At day6, the total number of cells derived from both ES cell lines in OP9 co-culture was higher than the gelatin control (*$p<0.05$). At day9, the total number of cells derived from E14IV Flk1$^+$ cells in AM co-culture and UG co-culture was significantly higher than that of gelatin control (*$p<0.002$) (Figure 4.12 A). For NIC-C5, number of cells derived from Flk1$^+$ cells in AM co-culture was significant higher than gelatin control (*$p<0.002$) (Figure 4.12 B). This result suggested that AM line could promote proliferation or survival of the cells derived from Flk1$^+$ cells at later stage.

AM could increase number of Flk1$^+$ derived progenitors (day6) or pan-haematopoietic cells (day9) in co-culture

To determine whether the proliferation or survival ability of haematopoietic progenitors and haematopoietic cells could also be promoted by co-culturing with stromal cells, we calculated the exact cell number of CD41$^+$cKit$^+$ at day6 and CD45$^+$ at day9 from each culture (Figure 4.12 C-F). AM and OP9 stromal cell lines could enhance the number of CD41$^+$cKit$^+$ cells compared to gelatin control (*$p<0.01$), in both ES cell lines (Figure 4.12 C, D). Combined with the frequency shown in Figure 4.11 and the overall cell number in Figure 4.12 A-B, it is suggested that at day6 both AM and OP9 could enhance the number of CD41$^+$cKit$^+$ cells, but with different mechanisms. AM stromal cells promoted the CD41$^+$cKit$^+$ exact numbers by promoting the CD41$^+$cKit$^+$ percentage, while OP9 stromal cells promoted the CD41$^+$cKit$^+$ exact number by enhancing the overall cell proliferation.

At day9, it is found that AM could promote the CD45$^+$ proliferation or survival

ability significantly higher than gelatin control in both ES cell lines (*$p<0.01$). However there were no difference by comparing UG or OP9 co-cultures to gelatin control ($p>0.05$). Overall, microenvironment provided by AM stromal cells could promote formation and proliferation/survival of $CD41^{+}cKit^{+}$ at day6 and proliferation/survival of pan-haematopoietic cells ($CD45^{+}$) at day9. This could be mediated by apoptosis, which will be discussed later.

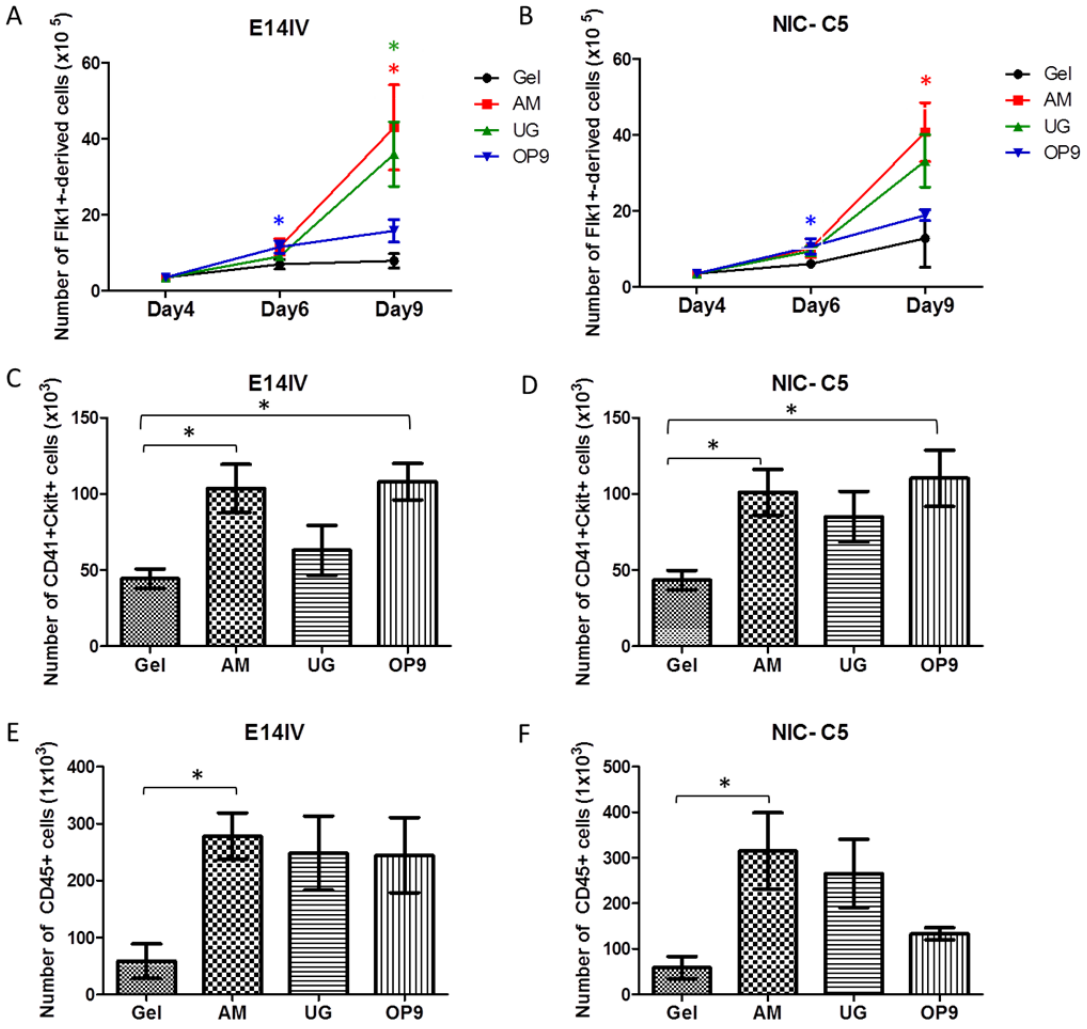


Figure 4.12 Analysis of total cell numbers and exact numbers of CD41^{+}cKit^{+} at day6 and CD45^{+} cells at day9 from Flk1^{+}/stroma co-culture system.

This data represent between 3 and 7 independent experiments. Error bars represented SEM. P-values were calculated with Friedman test with post paired test or Wilcoxon matched pairs test (*p<0.05).

4.4.2.3 Inhibition effect on Flk1$^+$ cells formation from ES cells by UG26.1B6

Of note, although not significant, UG stomal cells have shown a limited enhancement of haematopoiesis from Flk1$^+$ cells compared to gelatin control (Figure 4.8, 4.10). However, in EB/stromal co-culture system, UG did not enhance haematopoietic differentiation compared to gelatin control (Krassowska et al., 2006; Gordon-keylock et al., 2010). To better analyze this, we measured the early effect of UG stromal cells on ES-derived differentiation by detecting Flk1$^+$ expression kinetics on UG stromal cells. Day1 EBs by E14IV ES cells was plated on gelatin control and UG stromal cells to day8. Flk1 expression was measured with flow cytometric analysis. Interestingly, it was shown that UG stromal cell line inhibited Flk1$^+$ formation significantly at day4 and day5 compared to gelatin control (Figure 4.13). This result suggested that in EB/stromal co-culture system, UG stromal cells inhibited haematopoietic differentiation from ES cells via inhibiting Flk1$^+$ formation while enhanced haematopoiesis from Flk1$^+$ cells at limited level, which supported the notion that the effect of stromal cell lines was spatiotemporal and targeting population dependent.

It was reported that UG26.1B6 could expressed high level of Wnt5a to support the self-renewal of HSCs (Buckley et al., 2011). The Wnt5a is known to inhibit the canonical Wnt signaling pathway, a key regulator for mesoderm formation. In light of this, it is highly possible that UG26 stromal cell line inhibited Flk1$^+$ formation via inhibiting Wnt signaling pathway. Furthermore, one novel enhancer of Flk1$^+$ has been identified which could respond to canonical Wnt signals (Ishitobi et al., 2011). Therefore, secretion of Wnt5a to inhibit canonical Wnt signals could be an essential reason for the inhibition of Flk1$^+$ formation in EB/UG co-culture system.

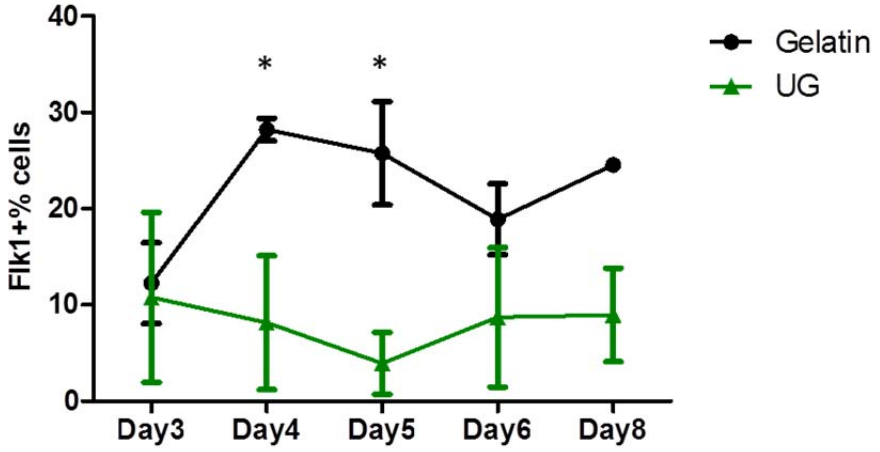


Figure 4.13 UG26.1B6 inhibited Flk1$^+$ formation in EB/UG co-culture system compared to gelatin control.

Day 1 haning drop EBs by E14IV ES cells were co-cultured on Vybrant-DiD pre-stained and irradiated UG26.1B6 (UG) stromal cells or gelatin control to day8. Co-cultures from day3, 4, 5, 6, and 8 were harvested for flow cytometric analysis at Flk1$^+$ frequency Error bars represented SEM. P-values were calculated with Mann-Whitney test (*$p<0.05$).

4.5 Conclusion

To set up a more specific co-culture system to ask how Notch signaling pathway is involved in haematopoiesis post mesoderm formation, $Flk1^+$ cells were co-cultured on AM14.1C4 (AM) stromal cell line. Results in this chapter suggested that:

- AM co-culture did not affect commitment to $Flk1^+$ from day1 EBs in EB/stromal system (Figure 4.1).

- Flk1/AMl system was established with moderate haematopoietic enhancing ability according to CFU formation (Figure 4.7-4.10).

- AM stromal cells could promote formation and expansion of $CD41^+cKit^+$ (day6) and $CD45^+$ expansion (day9) derived from $Flk1^+$ cells (Figure 4.11-4.12).

- Though not enhance $CD41^+cKit$ frequency or exact number, comparable enhancing effects to AM stromal cells according to CFU formation indicated UG stromal cells was also supportive on $Flk1^+$ derived haeamtopoiesis (Figure 4.9-10, 4.11-12).

- UG stromal cells inhibited commitment to $Flk1^+$ cells from ES cells in EB/stromal co-culture system, however, it enhanced $Flk1^+$ derived CFU formation suggesting effects of stromal cells are spatiotemporal and target dependent (Figure 4.9, 410, 4.13).

4.6 Discussion

4.6.1 Two possible models for AM stromal cell lines to promote haematopoietic differentiation in EB/stroma system?

Previous studies revealed that primary E10.5 AGM explants and stromal cell lines derived from the aorta and surrounding mesenchyme (AM subregion of AGM region) could promote haematopoietic differentiation from ES cells (Krassowska et al., 2006; Gordon-Keylock et al., 2011). In light of this we carried out further investigation on when and how the AM stromal cell lines promoted haematopoiesis to define a time window and specific responding population for further analysis of the molecular mechanisms. Using quantitative RT-PCR and flow cytometry, we noticed that expression of mesodermal related genes in EBs co-cultured on AM stromal lines was comparable to the gelatin control. Although Brachyury expression in AM co-culture was lower than gelatin control at day3, Gordon-Keylock et al., 2011 suggested that the co-culture with AM stromal cell line did not change the Brachyury expression pattern using flow cytometry at the protein level. Quantitative RT-PCR analysis to investigate the expression is more sensitive but does not reflect the real functional protein level. Although a difference was detected at day3, the later expression pattern in AM co-culture was comparable to the gelatin control indicating the overall differentiation from ESC to early mesoderm was not significantly affected (Figure 4.1 A).

Furthermore, it makes more sense to focus on the Flk1 expression which marks the haemangioblast representing a later stage than Brachyury. Based on the result that commitment to Flk1 from ES cells was not affected by AM co-culture, we suggested one model that the haematopoietic enhancing effect of AM stromal cell line could be acting on $Flk1^+$ cells. Although it was confirmed both in qRT-PCR and flow cytometry that $Flk1^+$ was not affected by AM stromal cell line, it did not reflect whether the function of $Flk1^+$ cells were comparable. We therefore tested the ability of $Flk1^+$ cells to differentiate further by simply seeding the sorted $Flk1^+$ population from each EB co-culture system into methylcellulose colony assay and used more surface markers to define and compare the $Flk1^+$ function between AM co-culture

and gelatin (Figure 4.1 B, C).

Our data suggest that AM could directly enhance haematopoiesis from $Flk1^+$ cells. However, to explain the mechanism of AM to enhance haematopoiesis from ESCs in EB/AM system, we cannot exclude the possibility that the $Flk1^-$ cells produced in the EB/AM co-culture could also provide the microenvironment to support haematopoietic differentiation of the $Flk1^+$ population. In light of *in vivo* studies demonstrating that HSCs emerge at the ventral endothelia layer in the dorsal aorta, the haematopoietic enhancing effect of the AM stromal cell line could be reflecting its region of origin in AGM from where HSCs emerge. Unknown factors or surface ligands could be the key regulators to directly induce HSCs emergence or maturation, While alternatively, the AM stromal cell line could support or enhance a haematopoietic niche consisting of ES-derived non-haematopoietic cells such as mesenchymal cells or endothelial cells. The higher expression of paraxial mesoderm related genes such as Delta-like 3 and HoxB4 could support this hypothesis (Appendix Figure S4.1). It will be interesting to look at other paraxial mesoderm or endothelial related genes especially at protein level. Moreover, it would be informative to screen gene expression within the Flk1- fraction from co-culture.

4.6.2 Reliable system to enhance $Flk1^+$ by co-culturing with AM stroma cells, but not as efficient as EB system

In $Flk1^+$/stroma co-culture using the NIC-C5 ES cell line, the increase in the number of haematopoietic colonies by comparing AM co-culture to gelatin was around 2.6±1.5 fold. The UG stromal cell line could also enhance the overall haematopoietic activity by around 2.3±0.4 fold. However OP9 did not have the enhancing effect on $Flk1^+$ derived haematopoiesis compared to gelatin. In the previously published work using EB/stroma using 7a-GFP ES cell line, it was claimed that AM stromal cell line could enhance haematopoietic activity with a higher efficiency which was comparable to OP9. UG stromal cell line was non-enhancing (Gordon-Keylock et al., 2011). Though the difference could be caused by the responsive ability of different ES cell line, the more plausible reason could be the difference between these two systems.

Thus, to better compare the enhancing efficiency of EB/stroma system with Flk1$^+$/stroma system, we also carried out the EB/stroma co-culture using NIC-C5 and E14IV ES cell line. Thereafter, we simply compared the AM stroma haematopoietic enhancing effect on frequency of haematopoietic progenitors (CFU) (measured by normalization number of CFU formed in AM co-culture to CFU formed in gelatin control) present in Flk1$^+$/stroma with EB/stroma co-culture system (Appendix Figure S4.2). For NIC- C5 ES cell line, we noted that the effect of AM stromal cell line on Flk1$^+$ was significantly lower than that in EB/stromal system, while E14IV was relatively similar. This comparison implied the possible requirement of the Flk1- which could be the haematopoietic supporting niche for further haematopoietic differentiation on Flk1$^+$ fraction. It has been predicted that in the *in vivo* environment, mesodermal-like cells are able to migrate through the surrounding mesenchyme then to the endothelial layer of dorsal aorta in the AGM region, which supported the notion that cells without haematopoietic commitment in the *in vitro* EB system were important for haematopoiesis. It is also reported that VEGF was important for the hematopoietic induction of Flk1$^+$ cells especially in the serum free system (Nostro et al., 2008) (Choi et al., 1998; Park et al., 2004; Pearson et al., 2008). It would be necessary to test VEGF expression by AM stromal cell line and by the Flk1- cells from EB/AM co-culture.

OP9 could not enhance Flk1$^+$ haematopoietic differentiation in the Flk1$^+$/stromal system. However it has been shown in several studies that OP9 could induce haematopoiesis from ES cell stage (Nakano et al., 1994; Gordon-Keylock et al., 2011). One possibility is that OP9 induces haematopoietic differentiation by providing a supporting enviroment for ES-derived Flk1- cells. Thus Flk1$^+$ cells may not repond directly to OP9 but rather to the to the Flk1- ES-derived niche. In contrast, Nishikawa and colleagues in 1998 reported that OP9 could enhance Flk1$^+$ derived haematopoietic differentiation effciently (Nishikawa et al., 1998a). By comparing the haematopoietic enhancing efficiency published in our lab system in 2011 by Gordon-Keylock with the data published by Nakano in 1994 or Nishikawa in 1998, we noticed that the OP9 haematopoietic enhancing efficiency is much lower in our lab system. This difference could indicated that OP9 was not working at the best

status in our hands.

4.6.3 Heterogenety of Flk1$^+$ cells in ES differentiation.

In our study, we investigated role of Notch signaling pathway in haematopoiesis in the specified Flk1$^+$ population, in which the side-effects from Flk1- fraction for example, paraxial mesoderm, ectoderm or endoderm were excluded. However Flk1$^+$ population from day4 EBs was still a heterogenic population with several cell types. As demonstrated by a series of studies charactizing Flk1$^+$ expression during ES cell differentiation, Flk1 could mark different population in combination with other marker. Flk1 was reported to widely express on lateral mesoderm-like cells, which could further differentiate into Flk1$^+$/SCl$^+$ precursor for haematopoietic/endothelial cell lineages and Flk1$^+$/SCL- precursor for endothelial/ smooth muscle cell lineages. (Chung et al., 2002; Ema et al., 2003; Nishikawa et al., 1998a) Flk1$^+$ was also applied to mark haemangioblast from day2.5 to day4 EBs (Bry$^+$/Flk1$^+$) and haemogenic endothelium (Flk1$^+$/Tie2$^+$/cKit$^+$ or Flk1$^+$/VE-cad$^+$) during EB differentiation (Fehling et al., 2003; Iacovino et al., 2011b; Lancrin et al., 2009; Nishikawa et al., 1998a). In addition, Flk1$^+$ was an endothelial cell marker and also reported to be present on haematopoietic precursors in day 4 and day6 suspension EBs (Hirai et al., 2003; Kabrun et al., 1997). Thus, Flk1$^+$ sorted from day4 EBs in our system could possibly represent a mix population including lateral mesoderm, haemangiolbast, haemogenic endothelium, early haematopoietic progenitors as well as endothelial cells.

To better clarify the Flk1$^+$ fraction we purified from day4 EBs in our system, we measured CD41$^+$cKit$^+$ frequency and haematopoietic colony formation ability in which low CD41$^+$cKit$^+$ frequency and low colony numbers indicated that Flk1$^+$ cells at day4 were still enriched by earlier progenitors (Figure 4.2, 4.6). Therefore, Flk1$^+$ from day 4 EBs possibly represented lateral mesoderm, haemangioblast or haemogenic endothelium. It will be necessary to apply BL-CFC assay to quantify the proportion of haemangioblast in the day4 EBs derived Flk1$^+$ cells and include other markers like Brachyury, SCL, Tie2 or VE-Cad to further characterize and purify Flk1$^+$ population for co-culture.

4.6.4 Do Flk1$^+$ cells seeded on stroma from day 4suspension EBs represent YS-derived or AGM-derived haematopoiesis?

A series of *in vitro* studies have investigated Flk1$^+$ derived haematopoiesis, although at a different time window compared to this study. For example it was suggested that day3.25 Flk1$^+$ cells derived from EBs represent the primitive (yolk sac derived) haematopoiesis while day5.25 Flk1$^+$ cells more closely resemble AGM-derived haematopoiesis. These two processes could be distinguished by Sox17 expression in Flk1$^+$ or CD93 co-expressing CD41 post aggregation culture of Flk1$^+$ cells (Fehling et al., 2003; Irion et al., 2010; Bertrand et al., 2005a; de Bruijn et al., 2002). It was noted in our previous study that the timing to the peak Brachyury expression is one day later than that published by Fehling's et al. (Fehling et al., 2002; Gordon-Keylock et al., 2011) using the same ES cell line, revealing distinct differences between the two systems. It is therefore difficult to address here whether the Flk1$^+$ at day 4 using E14IV ES cell line in our system represented YS-derived or AGM-derived haematopoiesis. Screening the Sox17 expression in purified Flk1$^+$ cells during the differentiation might address this issue. By using a powerful induction system of combination of activin A, BMP4 and VEGF (AVB), CD93 could be detected from Flk1$^+$ aggregation (Irion et al., 2010). CD93 has been tested in our system at day6 and day9 post co-culture of day 4 Flk1$^+$ with stromal cells. However very weak expression of CD93 was detected which indicated that the yolk-sac haematopoiesis could be the dominant process in our culture. Alternatively, the stromal cell lines could not provide the microenvironment for haematopoiesis as efficient as the serum-free system with AVB cytokines.

4.6.5 Mechnism of effects of AM on proliferation/survival of Flk1$^+$ derived cells

Promotion of haematopoietic activity by colony assay and flow cytometry was observed in AM stromal cell line. We also observed that AM and OP9 could promote Flk1$^+$ derived cells proliferation/survival compared to gelatin control. Thus we measured whether this could be initiated by apoptosis. In preliminary experiment, AnnexinV/DAPI double staining on the overall cells from co-culture has shown that Flk1$^+$ derived cells from gelatin control committed a more extensive apoptosis

process than cells from AM co-culture, which indicated that the AM could possibly enhance haematopoietic progenitors and cells by increasing cell survival ability (Figure S4.3).

4.6.6 $CD41^{+}cKit^{+}$ function? Definitive? Primitive?

We notice UG26 could also enhance CFU formation using NIC-C5, while no enhancement was observed according to the frequency of $CD41^{+}cKit^{+}$. In light of this, $CD41^{+}cKit^{+}$ formed in different co-culture may have different ability to form colonies in the methylcellulose assay, and so it would be worthwhile to test this possibility.

In addition, many studies have been using $CD41^{+}cKit^{+}$ mostly based on the *in vitro* work with ESC system to measure definitive haematopoiesis. Both the primitive and definitive progenitors could respond to the methylcellulose colony assay, which could explain the difference between surface marker expression and colony formation ability in $Flk1^{+}$/UG co-culture.

Chapter 5: Investigation of effects of ectopic NotchIC on Flk1^{+} cells derived haematopoiesis in Flk1^{+}/AM co-culture

5.1 Aim

To investigate how ectopic NotchIC affects haematopoietic differentiation of $Flk1^+$ derived cells in the AM microenvironment.

5.2 Introduction

A co-culture system of $Flk1^+$ cells and AM stromal cells (AM14.1C4) derived from the AGM region has been set up to promote haematopoietic progenitor production. This system could provide a powerful platform to investigate the molecular mechanisms that regulated haematopoiesis in the AGM region. In light of previously published work that ES-derived haematopoiesis in the EB/AM co-culture system was abrogated by blocking Notch signaling with the γ-secretase inhibitor post mesoderm, probably via Runx1 (Gordon Key-lock et al., 2011), we further determined whether Notch signaling was involved in the $Flk1^+$ derived haematopoiesis in the $Flk1^+$/AM co-culture system. As discussed in Chapter 3, γ-secretase inhibitor (GSI) could affect Notch activity in both ES-derived cells and in stromal cells, so the abrogation effect could be caused by inhibition of Notch activity in either cell population or both. To determine the effect of Notch signaling in ES-derived cells more specifically, a genetic modified ES cell line, R26-NotchIC, was used to overexpress the Notch intracellular domain (NotchIC) to activate ectopic Notch activity (Lowell et al., 2006). This ES cell line was established based on an E14Tg2a derivative, $R26CreER^{T2}$ ES cell line, which was set up by Grotewold, L.

In this cell line, one allele of the ROSA26 locus was targeted with $Cre\text{-}ER^{T2}$ (Cre recombinase fused to a mutated ligand-binding domain of the human estrogen receptor) (Vallier, 2001) (Grotewold, L. unpublished data). A floxed triple-polyA termination sequence under PGK promoter followed by NotchIC sequence and IRES- human CD2 (hCD2) was then targeted into the other ROSA26 allele in a Rosa26 targeting construct (Zambrowicz et al., 1997). When 4'hydroxy-Tamoxifen (4-OHT) was added into the culture, the termination sequence was excised by Cre-mediated recombination and the expression of ectopic NotchIC was driven under the Rosa26 promoter with hCD2 as a tag (Figure 5.1). As described in Chapter 4,

Flk1$^+$ cells formed by this ES cell line were able to respond to the AM stromal cell stimulation and haematopoietic differentiation was increased compared to gelatin control, as assessed by both cell surface phenotype and colony formation assay. However in this system, constitutive expression of NotchIC after treatment of 4-OHT is irreversible because importing of Cre-ER^{T2} into the nucleus results in the excision of the termination sequence. Daughter cells therefore express NotchIC with hCD2 and the effect of NotchIC cannot be withdrawal. To overcome this issue and to determine the role of NotchIC signaling in Flk1$^+$ derived haematopoiesis in a more specific spatiotemporal window, 4-OHT was added at different time windows. Overall, experiments were designed to answer the following questions:

(a) Whether the R26-NotchIC ES cell line could respond to 4-OHT and express NotchIC together with hCD2 leading to increased Notch activity;

(b) Whether ectopic NotchIC affect haematopoietic differentiation of Flk1$^+$ cells.

5.3 Experimental approach

- To determine whether R26-NotchIC ES cell line could respond to 4-OHT and result in enhanced Notch activity, Notch transcription activity was measured using a luciferase assay and by quantitation RT-PCR of downstream gene expression. Two sub-clones, R26-NotchIC-B5 and R26-NotchIC-C5, were tested to determine whether human CD2 expression could be detected by flow cytometry upon 4-OHT treatment and to optimize the dose and time length of addition;

- To determine whether ectopic NotchIC could affect Flk1$^+$ derived haematopoiesis by surface marker expression, Flk1$^+$ cells were purified from day4 suspension EBs and co-cultured on AM stromal cells with or without 4-OHT to day 6 and Day9 followed with flow cytometric analysis;

- To assess the effects of ectopic NotchIC on Flk1$^+$ derived haematopoiesis including number of HPCs in co-cultures and subsequent proliferation and

differentiation, haematopoietic colony assays were performed in the presence and absence of 4-OHT at different time windows.

- FACS sorting was applied to purify the $hCD2^-$ and $hCD2^+$ fraction from the same co-culture treated with 4-OHT. Comparisons of these two populations were carried out to measure the effect of NotchIC using flow cytometry analysis, haematopoietic colony assay and quantitative RT-PCR.

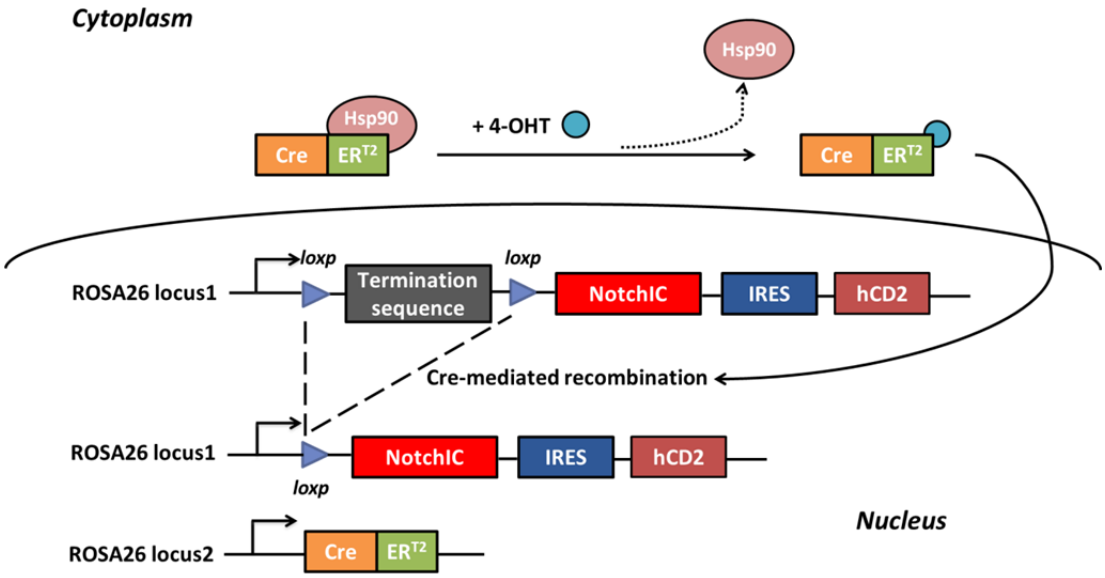


Figure 5.1 Construction of 4-OH-Tamoxifen inducible RC-NotchIC ES cell line. One allele of the ROSA26 locus is targeted with Cre-ER^{T2} and the other is targeted with a cassette containing a floxed transcription termination sequence followed by NotchIC sequence and IRES- human CD2 (hCD2). In Cre-ER^{T2} system, Cre-ER^{T2} protein is constitutively expressed in cytoplasm and inactive by binding to heat shock protein (Hsp90). When 4-OH-Tamoxifen (4-OHT) is added into the culture, Hsp90 protein is dissociated and Cre-ER^{T2} protein is released from the inactive complex and imported into nucleus to mediate the subsequent excision of loxP-flanked termination sequence. Finally expression of ectopic NotchIC was driven under the Rosa26 promoter together with hCD2 (Lowell et al., 2006).

5.4 Results

5.4.1 Validation of RC-NotchIC ES cell line

As described in Figure 5.1, RC-NotchIC ES cell line is predicted to express NotchIC-IRES-hCD2 upon the addition of 4-OHT. To confirm this, we tested the effect of 4-OHT on RC-NotchIC ES cell line. We first tested the enhancement of Notch activity using a luciferase assay for transcription activity and by quantitative RT-PCR of Notch downstream target genes. In luciferase assays, 12xRBP-Jκ binding site Luciferase reporter (a gife from Lowell, S.) and renilla internal control vector were co-transfected into RC-NotchIC-B5 ES cell line. The luciferase readout was compared between minus 4-OHT and plus 4-OHT. After 72 hours of treatment, there was a 2.5-fold increase of RBP-Jκ transcriptional activity in the ES cells treated with 1μM 4-OHT comparing to the control culture (Figure 5.2 A, Black column). As the positive control, a pCAG-NotchIC expression vector, which constitutively expressed NotchIC at a high level, was co-transfected into cells with the 12xRBP-Jκ binding site Luciferase reporter. A 1200 fold increase in the transcriptional activity was observed in this positive control culture (Figure 5.2 A, Red column). These results indicated that addition of 4-OHT is able to enhance the Notch activity in RC-NotchIC-B5 ESC at a moderate level. It has been suggested in several previous studies a high level of ectopic Notch activity could be toxic or could not reflect physiological conditions, so this moderate enhancing system might provide a better platform for the further investigation of Notch signaling pathway in the haematopoiesis. In quantitative RT-PCR analysis, the Notch downstream target genes Hes1 and Hes5 were increased upon addition of 4-OHT by 2 folds and 6 folds, respectively, compared to the control culture (Figure 5.2 B).

4-OHT was added to two sub-clones, RC-NotchIC-B5 and RC-NotchIC-C5 at 0μM, 0.25μM or 1μM for 1 day, 2 days and 5 days to determine the optimal conditions required for induction of NotchIC expression in undifferentiated ES cells (Figure 5.2 C, D). As the human CD2 (hCD2) marks NothIC expression we used the detection of CD2 expression by flow cytometry to monitor the realtime induction of Cre-mediated excision upon 4-OHT. For RC-NotchIC-B5 ES cell line, the proportion

of $hCD2^+$ cells increased to around 20% after 24 hours and 35% after 48 hours. Longer treatment to 5 days did not have any significant effect. For RC-NotchIC-C5, the proportion of $hCD2^+$ cells increased to 40% then reached 80% after two days. Longer addition of 4-OHT did not further increase the frequency either. It was noted with both ES cell lines that 0.25μM and 1μM had the same efficiency of induction. This result suggested that addition of 0.25μM of 4-OHT for 48 hours was sufficient to induce optimal frequency of NotchIC expression. RC-NotchIC-C5 had better induction efficiency than RC-NotchIC-B5. Thus, the majority of overexpression experiments were carried out with this ES cell line.

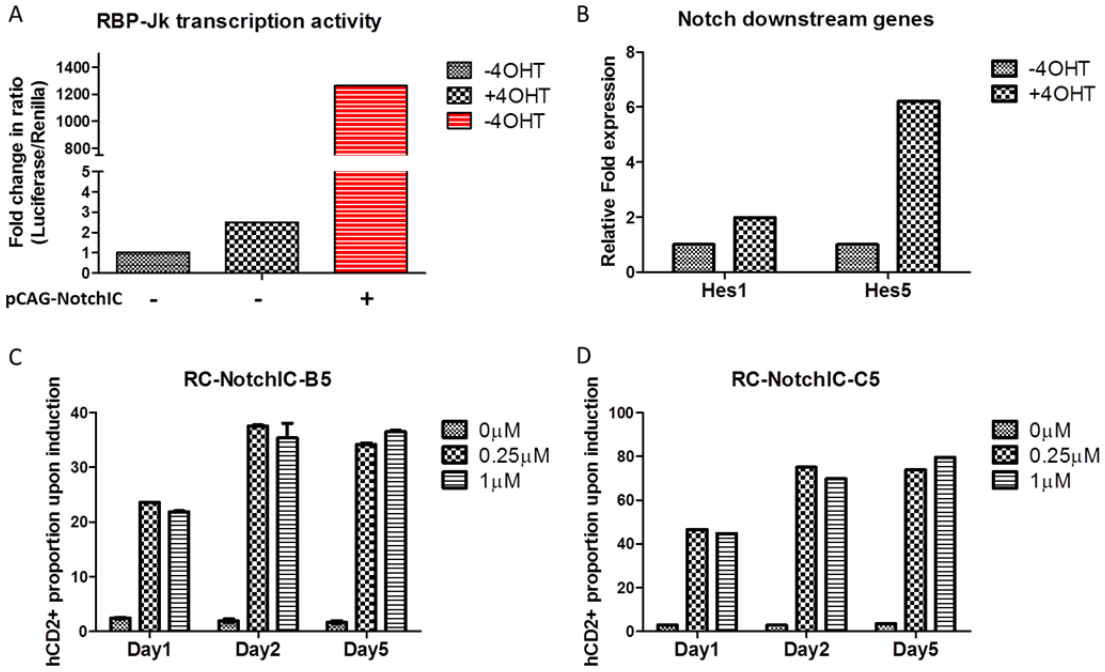


Figure 5.2 Validation of RC-NotchIC ES cell line.

(A) RBP- Jκ transcription activity was enhanced upon addition of 4-OHT by 2.5 fold (black columns). Positive control pCAG-NotchIC enhanced transcription activity by 1200 fold (red column); (B) Hes1 and Hes5 were enhanced upon addition of 4-OHT in quantitative RT-PCR; (C, D) Two sub-clones could respond to 4-OHT according to hCD2 expression. 4-OHT dose at 0.25 μM for 2 days was sufficient to reach optimum of $hCD2^+$ cells.

5.4.2 Overexpression of NotchIC inhibited the formation of $CD41^{+}cKit^{+}$ haematopoietic progenitors and myeloid differentiation

To analyze how Notch signaling is involved in the haematopoietic differentiation of $Flk1^{+}$ cells in the AM microenvironment, we activated ectopic NotchIC expression in the $Flk1^{+}$/AM co-culture. RC-NotchIC-C5 ES cells were differentiated in suspension EBs to day4, $Flk1^{+}$ cells were purified and then co-cultured on irradiated AM stromal cells with or without 4-OHT to day6, day9 and day11. Flow cytometry for cell growth rate, surface marker expressions and methylcellulose assays for haematopoietic colony formation abilities were assayed (Figure 5.3). The E14IV ES cell line was used as a control.

5.4.2.1 Effect of addition of 4-OHT on cell growth rate of $Flk1^{+}$ derived cells

To measure whether the addition of 4-OHT would affect cell growth, we quantified the exact number of cells derived from $Flk1^{+}$ population at day6, day9 and day11. In both ES cell lines, no significant difference was observed between minus and plus 4-OHT to day6 and day9 ($p>0.05$). However when 4-OHT was added to day11, we noticed that addition of 4-OHT start to inhibit the growth of C5 ES cell line (Figure 5.4). Although experiments were carried out only twice at day11, we observed a reduction on cell number in both experiments at around 20% and 25% (data not shown). 4-OHT did not affect the growth of control cell line E14IV. This result suggested that addition of 4-OHT at 0.25 μM for up to 5 days (ie from day 4-9) did not affect the cell growth rate, either by inhibiting cell proliferation or promoting cell apoptosis. The inhibition of growth by longer exposure to 4-OHT could be caused by overexpression of NotchIC or by importing of $Cre\text{-}ER^{T2}$ into the nucleus.

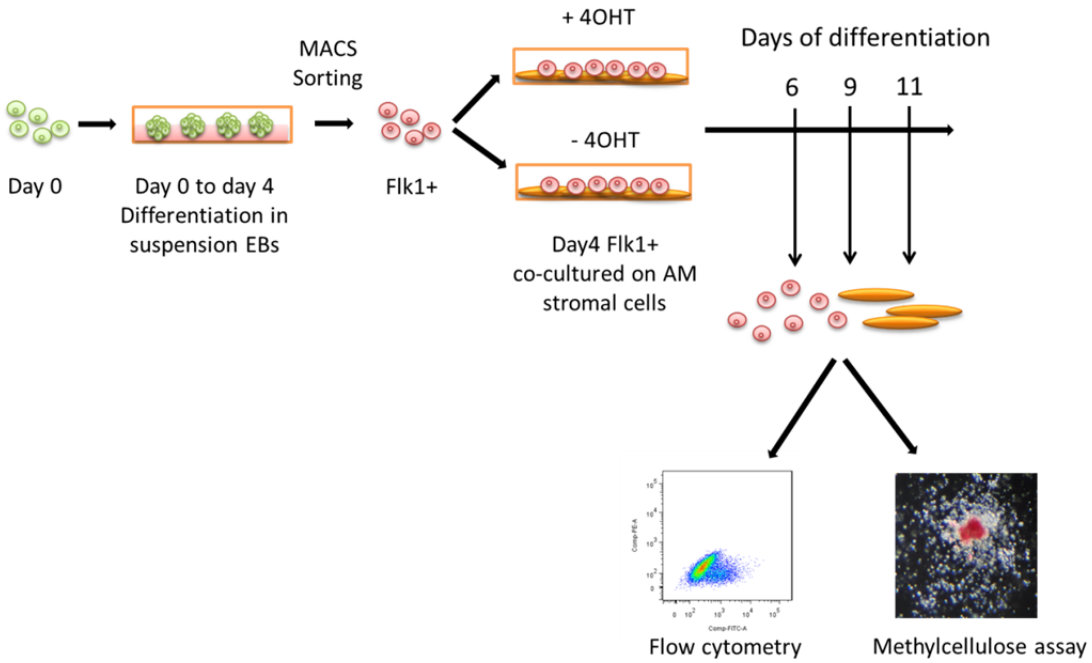


Figure 5.3 Scheme of ectopic NotchIC expression in Flk1^{+}/AM co-culture system. ES cells were differentiated in suspension EBs to day4. Flk1^{+} cells were purified and co-cultured on AM stromal cells. 4-OHT were added into co-culture to day 6, day9 and day11 followed with flow cytometry and methylcellulose assay analysis.

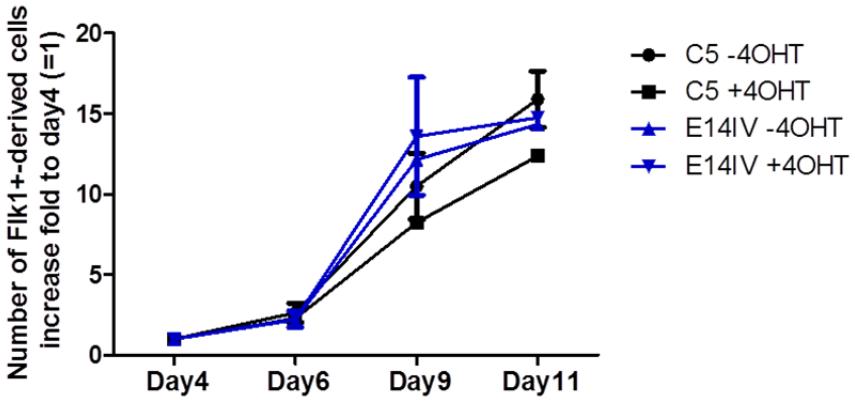


Figure 5.4 Growth rates of R26-NotchIC-C5 and E14IV Flk1^{+}/AM co-culture with or without addition of 4-OHT at 0.25µM.

Cell growth was not affected by 4-OHT to day9. There was a slight inhibitory effect of 4-OHT on cell number on day11 in R26-NotchIC-C5 ES cell line but not in the E14IV control cell line.

5.4.2.2 Ectopic NotchIC reduced the proportion of $CD41^+cKit^+$ at day6

A series of studies initiated by Mikkola et al (2003) demonstrated that the $CD41^+cKit^+$ population formed in the ES cell differentiation system represented the definitive haematopoietic progenitors consisting of common multi-potential progenitors, myeloid progenitors, and erythroid progenitors. We therefore assessed the production of this cell type by flow cytometry to measure the effect of ectopic NotchIC in our co-culture system. $Flk1^+$ cells were co-cultured on Vybrant DID stained and irradiated AM stromal cells at day4 to day6 and day9. Flow cytometry was applied to screen for the co-expression of CD41 and cKit to measure the haematopoietic progenitor formation at day6, which was suggested previously to be the peak of $CD41^+cKit^+$ production (data not shown). We observed that the addition of 4-OHT to $Flk1^+$/AM co-culture with NIC-C5 ES cell line significantly inhibited the production of $CD41^+cKit^+$ cells by approximately 30% percent (*$p<0.05$) (Figure 5.5 A). This result indicated that ectopic NotchIC reduced the number of haematopoietic progenitors determined by $CD41^+cKit^+$ from $Flk1^+$ cells at day6, at a moderate level (Figure 5.5 B).

To characterize the effect of NotchIC on later stages of haematopoietic differentiation, CD45, CD11b and Ter119 expression at day 9 were assessed for the production of pan-haematopoietic cells, myeloid lineages and erythroid lineages, respectively. Overexpression of NotchIC did not affect the frequency of $CD45^+$, $CD45^+CD11b^+$ and $Ter119^+$ (Figure 5.6). Interestingly, although $CD41^+cKit^+$ formation was abrogated by ectopic NotchIC, no effect of myeloid lineages was detected according to CD45 and CD11b expression.

Nonetheless, although not statistically significant, ectopic NotchIC inhibited $Ter119^+$ formation in 3 independent experiments at 30%, 64% and 75% (data not shown), while no difference was observed in E14IV control cell lines (Figure 5.6). This result indicated an inhibiting effect of NotchIC on erythroid lineage commitment. The reason for the lack of statistical significance could be that the inhibition effects varied between experiments, limited number of repeats or more likely, the low production of $Ter119^+$ in this system lacking exogenous EPO.

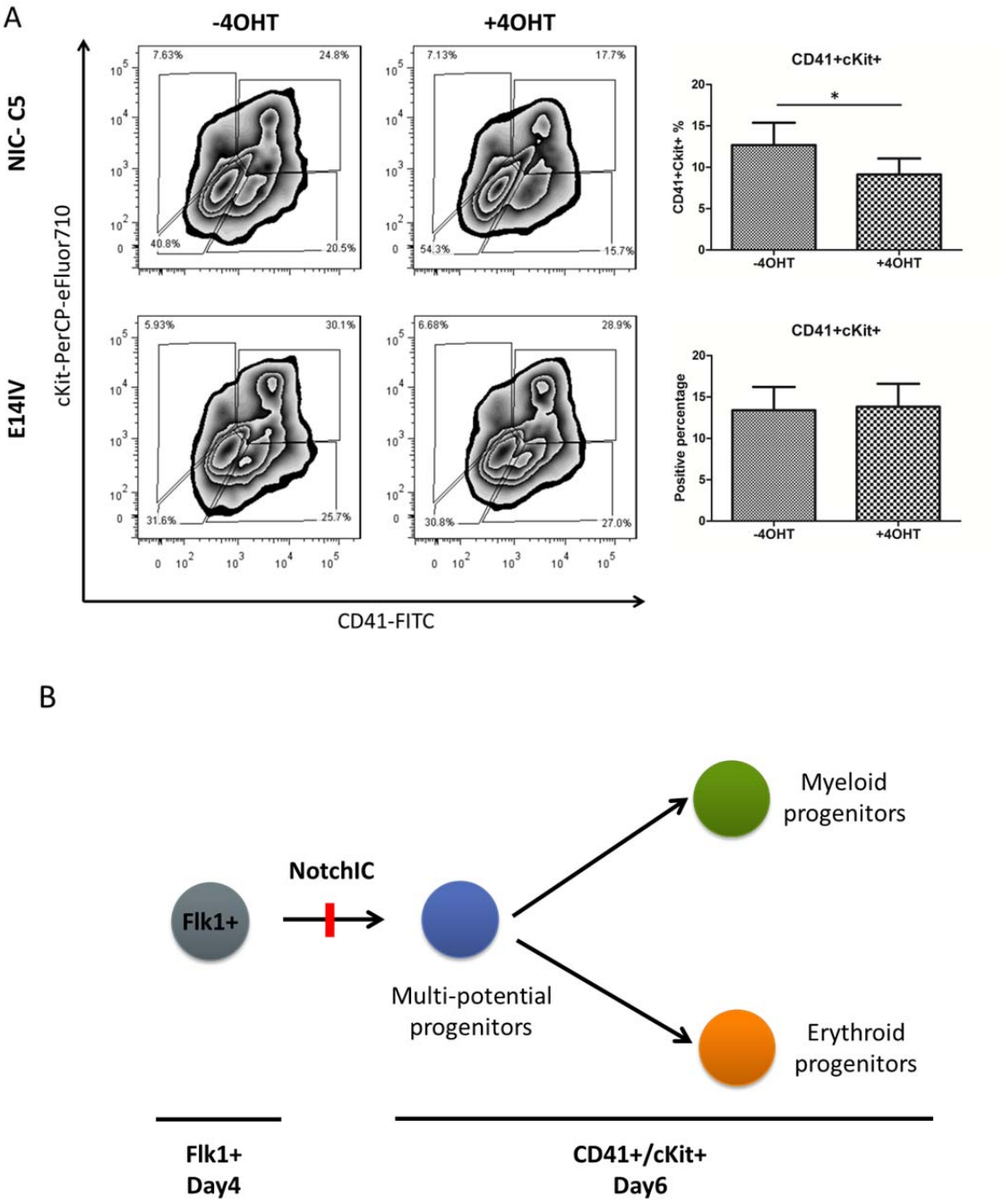


Figure 5.5 Ectopic NotchIC inhibited CD41$^+$cKit$^+$ formation from Flk1$^+$ in co-culture with AM stromal cells.

(A) Frequency of CD41$^+$cKit$^+$ was inhibited significantly when 4-OHT was applied to co-culture from day 4 to day 6. E14IV was used as the control. (B) A model for inhibition on CD41$^+$cKit$^+$ from Flk1$^+$ by ectopic NotchIC. CD41$^+$cKit$^+$ represented a mix population of haematopoietic progenitors (Multi-potential progenitors, myeloid progenitors and erythroid progenitors). Data represented 7 independent experiments. Error bars represented SEM. P value was calculated with Wilcxon matched pairs test. (*$p<0.05$)

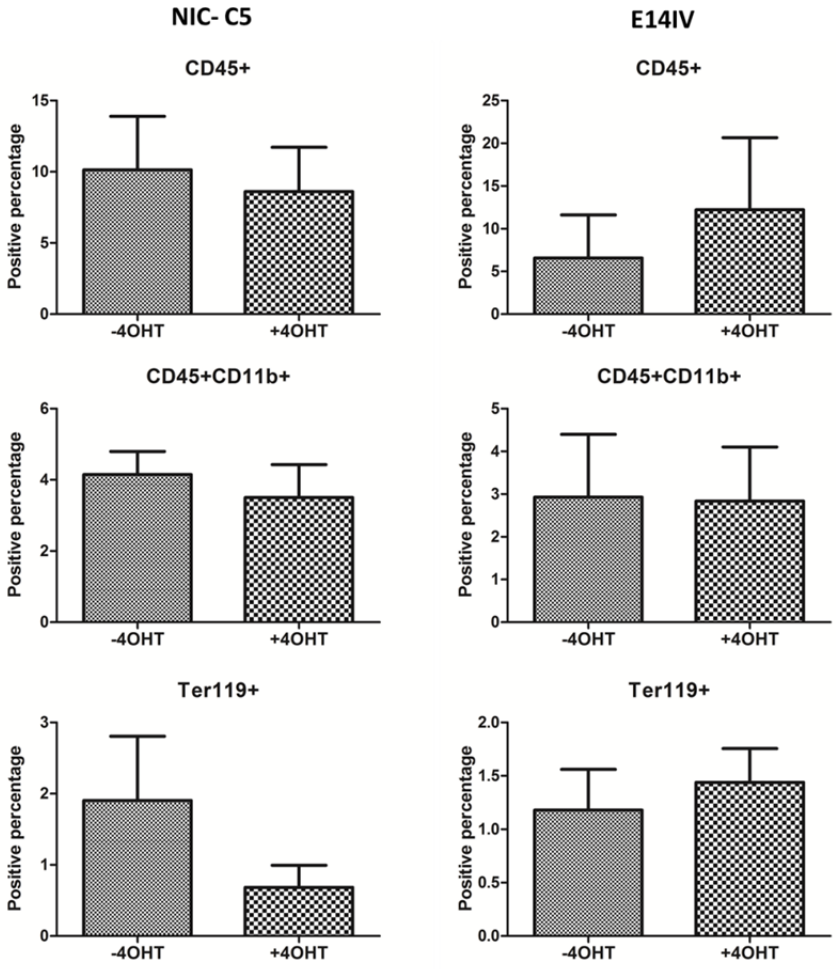


Figure 5.6 Effect of ectopic NotchIC on myeloid and erythroid differentiation from Flk1$^+$ cells in Flk1$^+$/AM co-culture.

At day9, ectopic NotchIC did not affect formation of CD45$^+$ or CD45$^+$CD11b$^+$ cells but did inhibit Ter119$^+$ cells production. Data showed average of 3 to 5 independent experiments. Error bars represent SEM. P value was calculated with Wilcxon matched pairs test (not significant when $p>0.05$).

5.4.2.3 Ectopic NotchIC reduced number of myeloid progenitors derived from the Flk1$^+$ cells at day6

Although analysis of haematopoietic surface marker expression pattern has been a critical assay to determine the haematopoietic differentiation, it could not be assumed that the ES-derived HSCs or HPCs shared the exact same pattern as it was discovered *in vivo* systems. Therefore, functional assays ultimately provide a better definition of the cell fate and function. In our system, characterization of the surface phenotype of cells derived from Flk1$^+$ by overexpression of NotchIC demonstrated the inhibition effect of ectopic NotchIC on haematopoietic progenitor formation defined by CD41$^+$cKit$^+$. To determine how NotchIC affected Flk1$^+$ derived cells haematopoiesis functionally, cultures were seeded into methylcellulose assay to measure number of HPCs including myeloid progenitor (CFU-M, CFU-GM), erythroid progenitor (BFU-E, CFU-E) and multi-potential progenitor (CFU-GEMM, Mac/Ery). Of note, with this strategy the whole differentiation process included formation of HPCs (also defined as colony forming units/CFUs) in co-cultures and subsequent colony formation in colony assay determined by HPCs abilities to proliferate and differentiate to terminal lineages. Thus, 4-OHT was added at different time points to determine the spatiotemporal effects of NotchIC as followed:

a) To understand how ectopic NotchIC affected production of HPCs (CFUs) from Flk1$^+$ cells in co-cultures and subsequent proliferation and differentiation of these HPCs in colony assay, 4-OHT was added to the co-culture for 2 days then withdrawn in colony assay (Figure 5.7 A);

b) To understand how ectopic NotchIC affected ability of HPCs from co-cultures to proliferate and differentiate to form colonies in the colony assay, 4-OHT was only added in the colony assay (Figure 5.7 B);

c) To overcome the irreversible effect of NotchIC in colony assay and understand how ectopic NotchIC affected production of haematopoietic progenitors from Flk1$^+$ cells in co-cultures, cells from co-cultures with or without 4-OHT were both seeded into colony assay with 4-OHT, in which case, the later irreversible effects of NotchIC in colony assay could be counteracted (Figure 5.7 C).

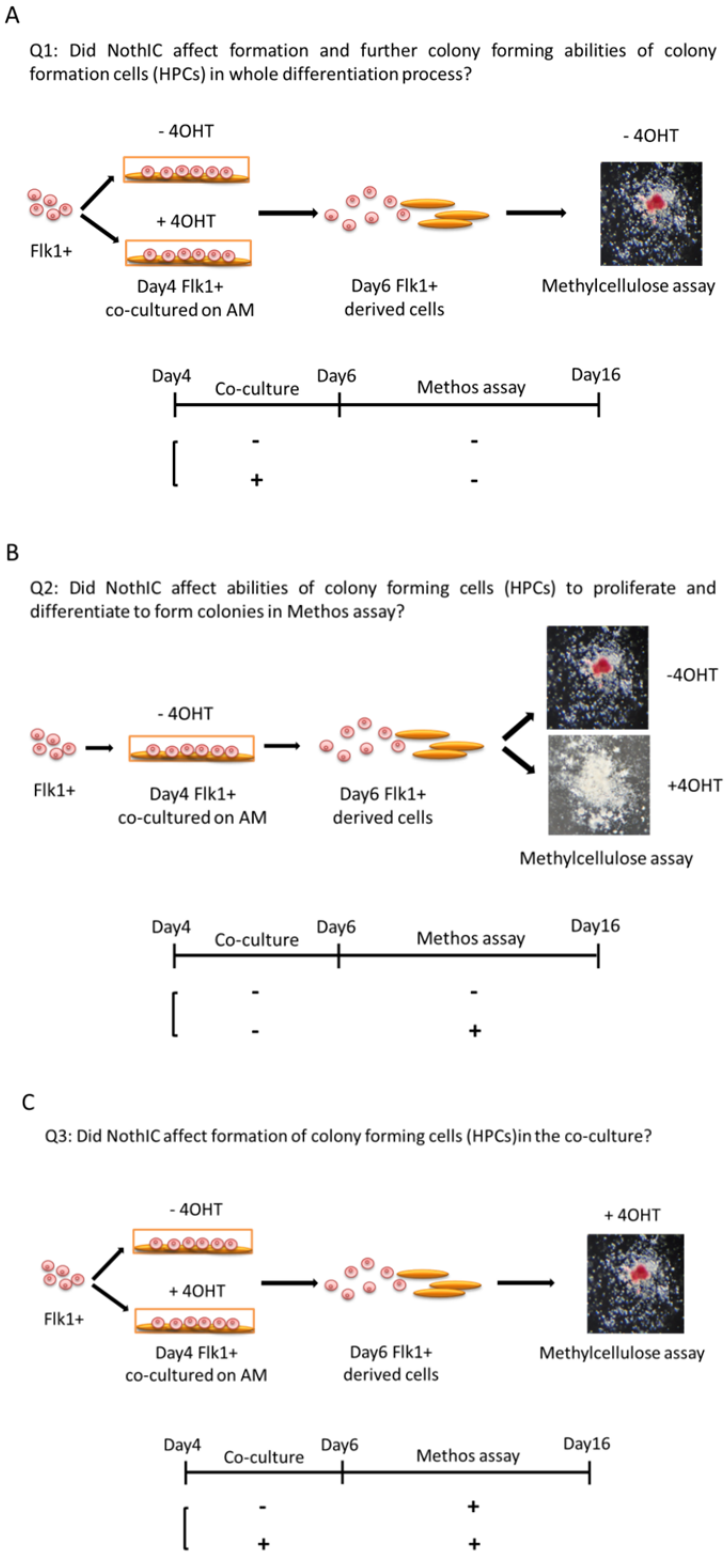


Figure 5.7 Scheme of addition of 4-OHT at different time points during differentiation including HPCs (CFUs) production in co-culture and later colony formation in colony assay.

a) Overexpression of NotchIC prevented both myeloid and erythroid differentiation from Flk1$^+$ cells

To determine the effect of ectopic NotchIC on haematopoiesis of Flk1$^+$ cells to terminal lineages, we activated NotchIC overexpression from the Flk1$^+$ stage. Flk1$^+$ cells were co-cultured on AM stromal cells with or without 4-OHT for 2 days (day6), and then seeded into methylcellulose assay at $1x10^5$/dish without 4-OHT for another 10 days (Figure 5.8 A). E14IV ES cell line was used as the control.

It was noted that the number of overall haematopoietic colonies including multi-potent and uni-potent was reduced significantly upon activation of NotchIC (Figure 5.8 B, *$p<0.05$). When CFU-GEMM and Mac/Ery colonies that had both myeloid and erythroid potential were stacked, we noted that ectopic Notch could inhibit the number of these multi-potential colonies (Figure 5.8 C, *$p<0.05$). No difference was observed in E14IV control (Figure 5.8 B, C). Ectopic NotchIC inhibited myeloid differentiation or proliferation according to the reduction of CFU-M and CFU-GM (Figure 5.8 D, *$p<0.05$). Overexpression of NotchIC also significantly decreased the number of erythroid colonies at a very limited level (Figure 5.8 E, $p>0.05$). Therefore, these results suggested that during the Flk1$^+$ derived haematopoiesis including haematopoietic progenitors production and subsequent colony forming in colony assay, ectopic NotchIC expression could inhibit both myeloid and erythroid differentiation.

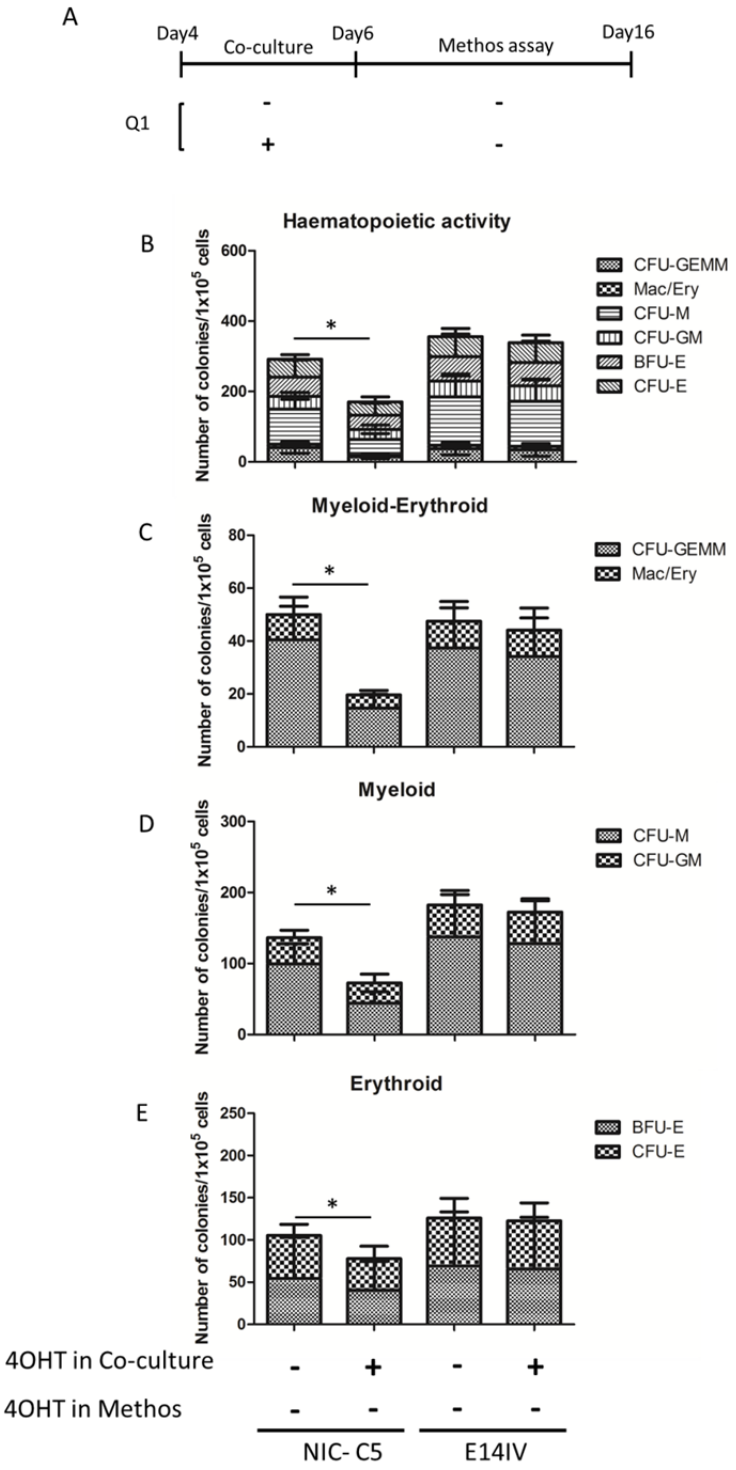


Figure 5.8 Ectopic NotchIC expression inhibited myeloid and erythroid commitment of Flk1$^+$ cells.

(A) Scheme of inducing NotchIC expression with 4-OHT in Flk1$^+$/AM co-culture. Significant reduction of number of haematopoietic colonies (B), multi-potential colonies (C), myeloid colonies (D) and limited reduction of erythroid colonies (E) by ectopic NotchIC in co-culture. Error bars represented SEM from 7 independent experiments. P values were calculated by Wilcoxon matched pairs test (*p<0.05).

b) Overexpression of NotchIC did not affect myeloid colony formation but inhibited erythroid colony formation ability from haematopoietic progenitors

Next, we assessed the effect of NotchIC on abilities of haematopoietic progenitor to proliferate and differentiate in methylcellulose assay. $Flk1^{+}$ was cultured on AM stromal cells without 4-OHT addition for 2 days to produce haematopoietic progenitors then seeded into colony assay with or without 4-OHT (Figure 5.9 A).

In this cytokine-induced haematopoietic environment (ie the methylcellulose-based colony forming assay), the number of overall colonies was reduced by 28% (Figure 5.9 B). Multi-potent progenitors (CFU-GEMM, Mac/Ery) were reduced by 60% (Figure 5.9 C). Although the difference was not significant, the inhibition was observed in all 5 independent experiments with various levels of inhibition (data not shown). Interestingly, addition of 4-OHT in the colony assay did not abrogate the number of myeloid colonies formed by myeloid progenitors as mentioned above (Figure 5.9 D). Statistically, significant inhibition of erythroid colonies formed by erythroid progenitors was observed (Figure 5.9 E, $*p<0.05$). This result suggested that addition of 4-OHT on haematopoietic progenitors from co-cultures could inhibit the colony formation of erythroid but not myeloid progenitors in these colony assays. Thus, number of multi-potential progenitors (CFU-GEMM and Mac/Ery) reduction observed in this circumstance could be accounted for the inhibition on erythroid colony forming potential.

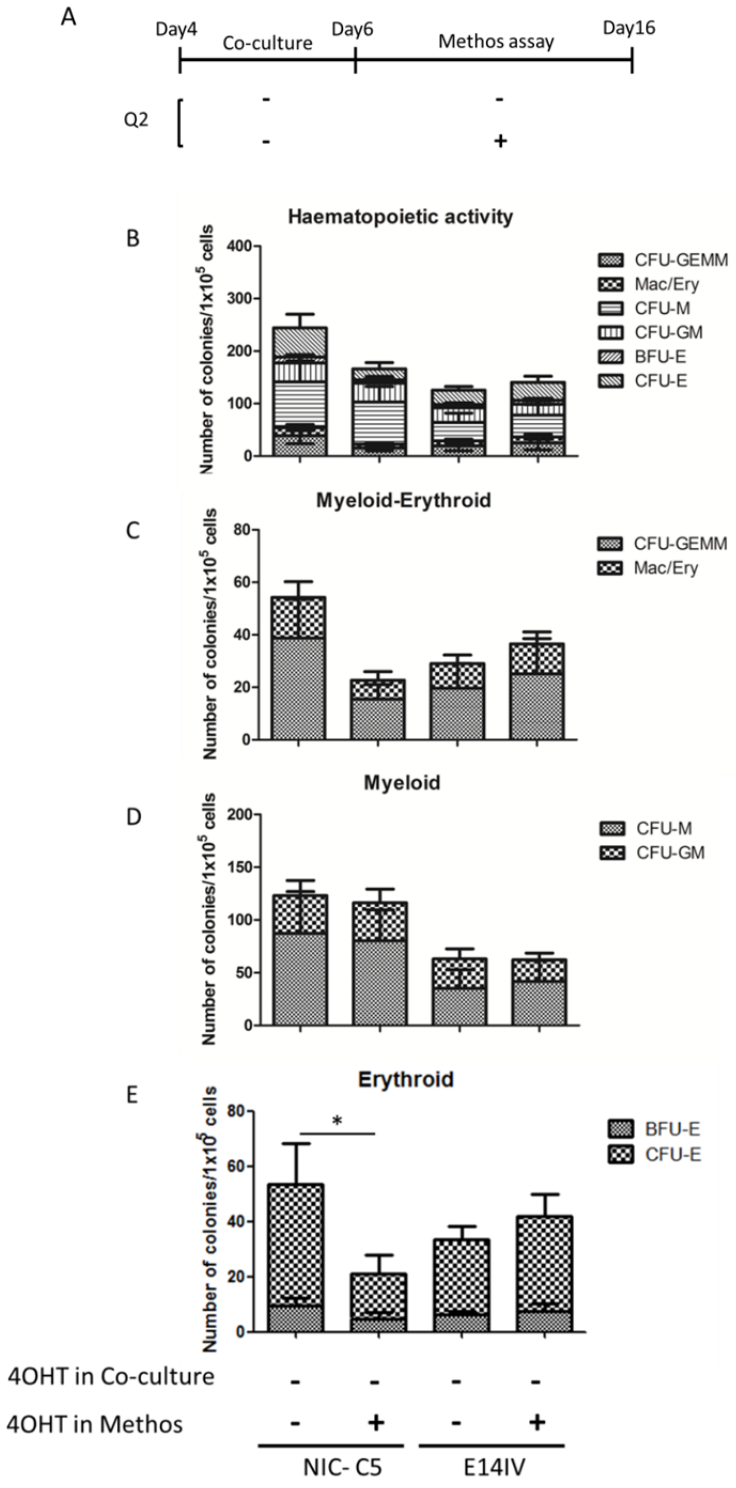


Figure 5.9 Effects of ectopic NotchIC on colony formation ability from haematopoietic progenitors.

(A) Scheme of NotchIC induction with 4-OHT in colony assay. NotchIC inhibited proliferation/ differentiation of haematopoietic progenitors (B), multi-potential progenitors (C) and erythroid progenitors (E) but not affected proliferation/differentiation of myeloid progenitors in colony assay. Error bars represented SEM for 5 independent experiments. P values were calculated by Mann-whitney test (*p<0.005)

c) Overexpression of NotchIC could prevent myeloid progenitor formation from Flk1$^+$ cells

In this enhancing system, NotchIC is expressed constitutively upon 4-OHT addition and therefore when 4-OHT is added at the Flk1$^+$ stage, the effect of NotchIC would be present in the co-culture and persist in the methylcellulose colony assay. To better elucidate the effect of NotchIC on haematopoietic progenitor formation ability from Flk1$^+$ in the co-culture, we compared the haematopoietic activity when 4-OHT was present throughout the co-culture AND the methylcelllose assay with conditions where 4-OHT was added to the methylcellose culture alone (Figure 5.10 A).

Experiments were only carried out twice so statistic comparison was not available. However, in both experiments (Experiment1 and Experiment2) we observed that addition of 4-OHT could inhibit the number of myeloid colonies (CFU-GM and CFU-M). Because the irreversible effect of ectopic NotchIC in methylcellulose assay has been overcome by adding 4-OHT to both cultures, it could be assumed that ectopic NotchIC reduced number of myeloid progenitors from Flk1$^+$ in the co-culture (Figure 5.10 B).

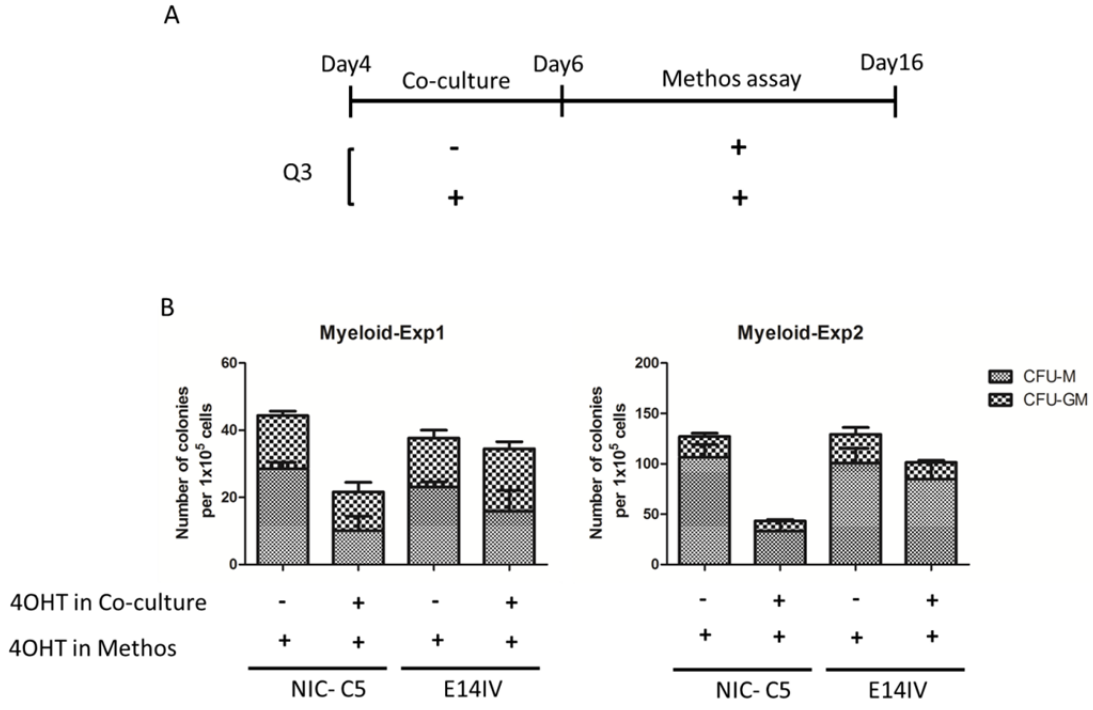


Figure 5.10 Effects of ectopic NotchIC on myeloid progenitor formation from Flk1^{+} cells in co-cultures.

(A) Scheme of ectopic NotchIC induction with 4-OHT addition in both co-cultures AND colony assay. (B) NotchIC inhibited myeloid commitment in repeated experiments. Data represent 2 independent experiments. Error bars represent SEM of duplicate dishes.

5.4.3 Overexpression of NotchIC inhibited myeloid differentiation in hCD2$^+$ fraction compared to hCD2$^-$ fraction in the same culture microenvironment

As described in Figure 5.2, the NotchIC induction efficiency of 4-OHT was around 70%. However in the co-culture system, it was found that the induction efficiency was around 50% (Figure 5.11 A). Therefore, after treatment of 4-OHT in the co-culture, Flk1$^+$ derived cells consisted of 2 fractions: one with ectopic NotchIC -hCD2 expression, the other without ectopic NotchIC being hCD2 negative. The data described so far on the effects of ectopic NotchIC was determined by comparison between the treated group and untreated group (Figures 5.5 to Figure 5.10). Nevertheless the excision efficiency cannot be assumed as 100% in every system. The ectopic NotchIC in hCD2$^+$ population could potentially change the whole microenvironment of the co-culture system by secreting factors or ligands and in turn modulate the fate of hCD2$^-$ population. It is also possible that the hCD2$^-$ fraction in the 4-OHT treated culture could also change cell fate and contribute to the myeloid differentiation inhibition observed above. To better elucidate the difference between NotchIC$^-$ (hCD2$^-$) and NotchIC$^+$ (hCD2$^+$) fraction, we compared haematopoietic activities directly between NotchIC- and NotchIC$^+$ populations (based on hCD2 expression) which were derived from the same co-culture treated with 4-OHT.

5.4.3.1 Ectopic NotchIC inhibit CD41$^+$cKit$^+$ formation and further myeloid differentiation

It has been demonstrated previously that in the surface phenotype analysis at day6, CD41$^+$cKit$^+$ population was moderately reduced by ectopic NotchIC, while no significant change was observed for later haematopoietic marker CD45 or CD11b at day9. To better compare the NotchIC negative and positive population at haematopoietic differentiation, we stained the Flk1$^+$ derived cells from co-culture treated with 4-OHT with PE-conjugated hCD2 antibody to distinguish these two fractions. Further flow cytometric analysis of surface markers were carried out with these two populations from the same co-culture (Figure 5.11 A). At day6, it was found that the frequency of CD41$^+$cKit$^+$ in hCD2$^+$ population (NotchIC$^+$) was

significantly lower (25%) than that of hCD2$^-$ population (NotchIC-) (Figure 5.11 B, *p<0.05). When the co-culture was treated with 4-OHT longer to day9, it was revealed that expression of CD45 was reduced to around 60% in hCD2$^+$ compared to the hCD2$^-$ population, suggesting that ectopic NotchIC could inhibit formation of CD45 (Figure 5.11 C, *p<0.05). Therefore, these results indicated that overexpression of NotchIC could inhibit the formation of CD41$^+$cKit$^+$ haematopoietic progenitor formation and pan-haematopoietic cells (CD45$^+$) formation. Because CD45 is expressed by pan- hematopoietic cells except mature erythrocytes and platelets, in which case CD45 could more likely represent the myeloid direction. It will be interesting to look at the co-expression of CD45 with other myeloid markers like CD11b, GR1, F4/80 or lymphoid markers.

5.4.3.2 Ectopic NotchIC abrogated myeloid differentiation via inhibiting myeloid progenitor formation

Our result demonstrating that haematopoietic differentiation of the NotichIC-expressing, hCD2$^+$ fraction was lower than that of the hCD2$^-$ fraction, which was consistent with our previous flow analysis of whole cultures (Figure 5.5). In light of this, we carried out methylcellulose assays to measure whether hCD2$^+$ would give rise to less haematopoietic cells than hCD2$^-$. In addition, quantitation RT-PCR was applied to determine whether related haematopoietic genes were affected. Thus, Flk1$^+$ cells were co-cultured on Vybrant DiD stained AM stromal cells with the addition of 4-OHT to induce NotchIC$^+$ (hCD2$^+$) and NotchIC$^-$ (hCD2$^-$) population. At day6, stromal cells were excluded according to the APC channel then NotchIC- and NotchIC$^+$ fractions were separated by FACS based on CD2 expression. The purified fractions were then seeded into methylcellulose assay or for quantitation RT-PCR analysis (Figure 5.12).

When hCD2$^-$ and hCD2$^+$ cell populations were seeded into methylcellulose assay, it was found that hCD2$^+$ fraction (with higher Notch activity) formed significantly less myeloid colonies (Figure 5.13 A, *p<0.05). To elucidate whether the inhibition on myeloid differentiation was caused by inhibition of myeloid progenitor formation in co-cultures or colony formation ability of these progenitors, we tested the effect of

ectopic NotchIC on the colony formation ability in $hCD2^-$ fraction. Interestingly, ectopic NotchIC expression did not affect the myeloid colony formation ability in colony assay indicating that NotchIC abrogated myeloid differentiation via inhibiting the formation of myeloid progenitors (Figure 5.13 B). To confirm this, $hCD2^-$ and $hCD2^+$ fractions were seeded into colony assay with addition of 4-OHT to counteract the effect of NotchIC in methylcellulose assay. Similarly to Figure 5.13 A, the number of myeloid colonies were reduced significantly (Figure 5.13 C, $*p<0.05$). Therefore, in the co-culture treated of 4-OHT, the myeloid differentiation ability of the $hCD2^+$ fraction was reduced compared to $hCD2^-$. This inhibition effect of ectopic NotchIC took place at the stage of myeloid progenitor formation from $Flk1^+$ cells in the $Flk1^+$/AM co-culture system.

5.4.3.3 Ectopic NotchIC inhibited the molecular characteristics of myeloid differentiation, without affecting early haematopoietic markers

Furthermore, we applied quantitative RT-PCR on the sorted $hCD2^-$ and $hCD2^+$ fraction to screen a series of critical genes related to haematopoiesis and cell fate decisions. The Notch downstream gene, Hey1, was confirmed to be increased 8 fold in the $hCD2^+$ fraction compared to the $hCD2^-$ fraction. Gata2 and Runx1, which are reported to be downstream of Notch signaling and regulate definitive haematopoiesis in the AGM region, were not affected (Figure 5.14 A). It could be possible that Gata2 and Runx1 do not function as the downstream genes in the molecular network existing in this $Flk1^+$/AM co-culture system or the moderate enhancement of Notch activity as indicated in Figure 5.2 could not reach the threshold to affect Gata2 or Runx1. Thus, ectopic NotchIC in the $Flk1^+$/AM system did not affect $Flk1^+$ haematopoiesis via Gata2 or Runx1. Furthermore, detection of Gata2 and Runx1 as well as an increase of Runx1 at day6 compared to $Flk1^+$ cells at day4 (data not shown) suggested that definitive haematopoiesis existed in this $Flk1^+$/AM co-culture system.

In light of the effects of ectopic NotchIC on myeloid and erythroid differentiation, we measured the other haematopoietic progenitor and lineage related genes. PU.1, a haematopoietic-specific ETS family transcription factor that regulates many

lymphoid and myeloid-specific gene promoters was inhibited by ectopic NotchIC as well (Figure 5.14 B). SCL is involved in a complex with Lmo2, Gata1, E2A, and Ldb1 to form a complex which is critical for erythroid differentiation. Here we tested the gene expression by qPCR at SCL, Lmo2, Gata1 to check effects of ectopic NotchIC on erythroid differentiation. Hemoglobin β-H1 and β-major were also included. Interestingly, we noticed that these myeloid and erythroid related genes decreased significantly in $hCD2^+$ fraction compared to $hCD2^-$ (Figure 5.14 B). Thus, this result supported that ectopic NotchIC upon 4-OHT addition could abrogate further myeloid and erythroid differentiation from $Flk1^+$ cells. In addition, detection of β-H1 and β-major with a sharp increase compared to day4 $Flk1^+$ (data not shown) indicated that both primitive and definitive haematopoiesis existed in this $Flk1^+$/AM co-culture system.

In addition to regulate erythroid differentiation, SCL is an important transcription factor for early embryonic haematopoiesis and could identify the sub-population of mesoderm which was reported to determine the haemogenic endothelium formation from haemangioblast cells. Thus the inhibition of SCL suggested that haematopoietic inhibition in our system could be inhibited at an early stage when $Flk1^+$ formed the haemogenic endothelium.

To investigate the underlying mechanisms of the inhibition effect of ectopic NotchIC, we screened other genes which were related to haematopoietic differentiation. We first hypothesized that the suppression of haematopoietic differentiation from $Flk1^+$ could be compensated by converts to other lineages. For the haemogenic endothelium cell fate, Flk1 and VE-Cad were not affected. Cell fates were not converted to paraxial mesoderm according to Tbx6 and Dll3 expression. Sox17, which was not affected either, was also tested because it was a marker for endoderm and found to be critical to distinguish the AGM-derived haematopoiesis from the yolk sac (Irion et al., 2010) (Figure 5.14 C). This result indicated that ectopic NotchIC did not maintain $Flk1^+$ cells at haemangioblast or haemogenic endothelium stage or convert cell fate to the paraxial mesoderm. Interestingly, we noticed that Wnt5a, which has been demonstrated to maintain HSCs /HPCs survival and

proliferation (Austin et al., 1997), was reduced at a moderate level. This implied a possibility that ectopic NotchIC could inhibit haematopoietic progenitor proliferation/survival via inhibiting Wnt5a, which caused reduction of number of myeloid progenitors (Figure 5.14 C). Further rescue experiment by adding Wnt5a into the co-culture will be necessary to verify this possibility.

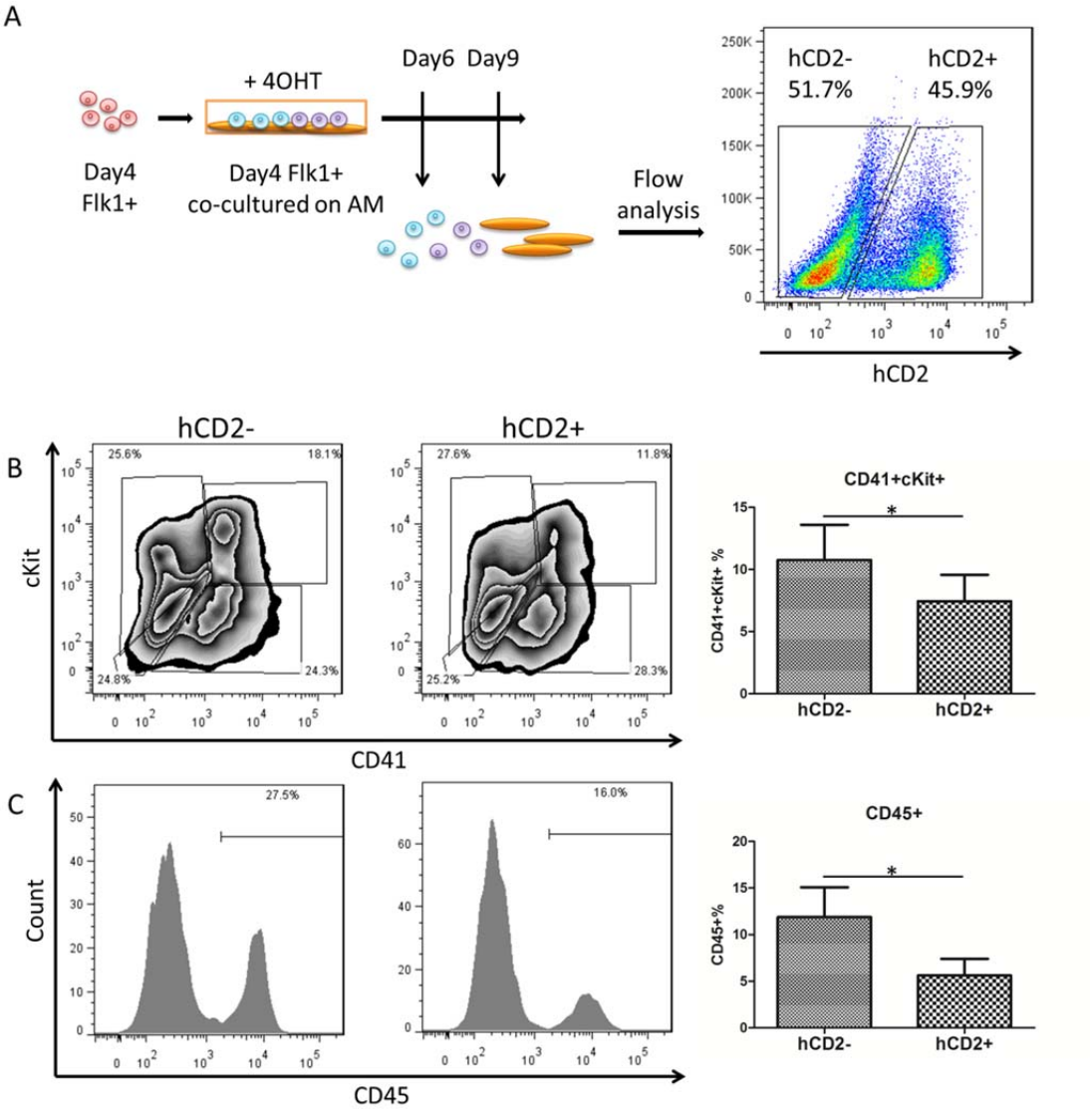


Figure 5.11 Ectopic NotchIC inhibited CD41^{+}cKit^{+} formation and further myeloid differentiation.

(A) Scheme of flow analysis of surface phenotypes of Flk1^{+} derived cells in hCD2^{-} and hCD2^{+} fraction from co-culture at day6 and day9. (B, C) CD41^{+}cKit^{+} and CD45^{+} frequency was reduced in hCD2^{+} compared to hCD2^{-} fraction. Data represented 6 and 7 independent experiments. Error bars represented SEM. P values was calculated with Wilcoxon matched pairs test (*p<0.05).

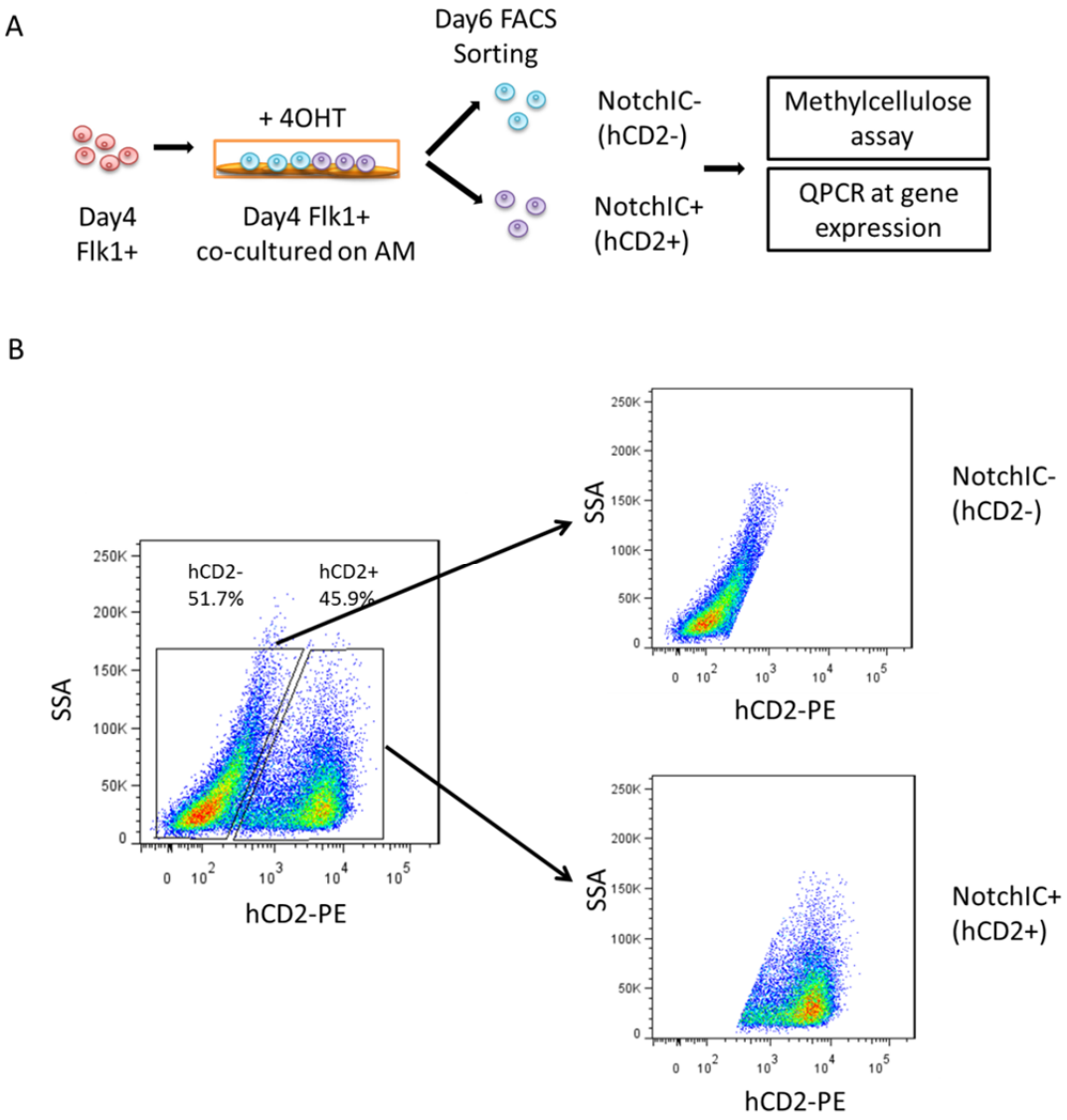


Figure 5.12 Scheme of ectopic NotchIC induction with 4-OHT in co-culture (A) with following FACS sorting on hCD2$^-$ and hCD2$^+$ fraction for methylcellulose assay and quantitative RT-PCR at day6 (B).

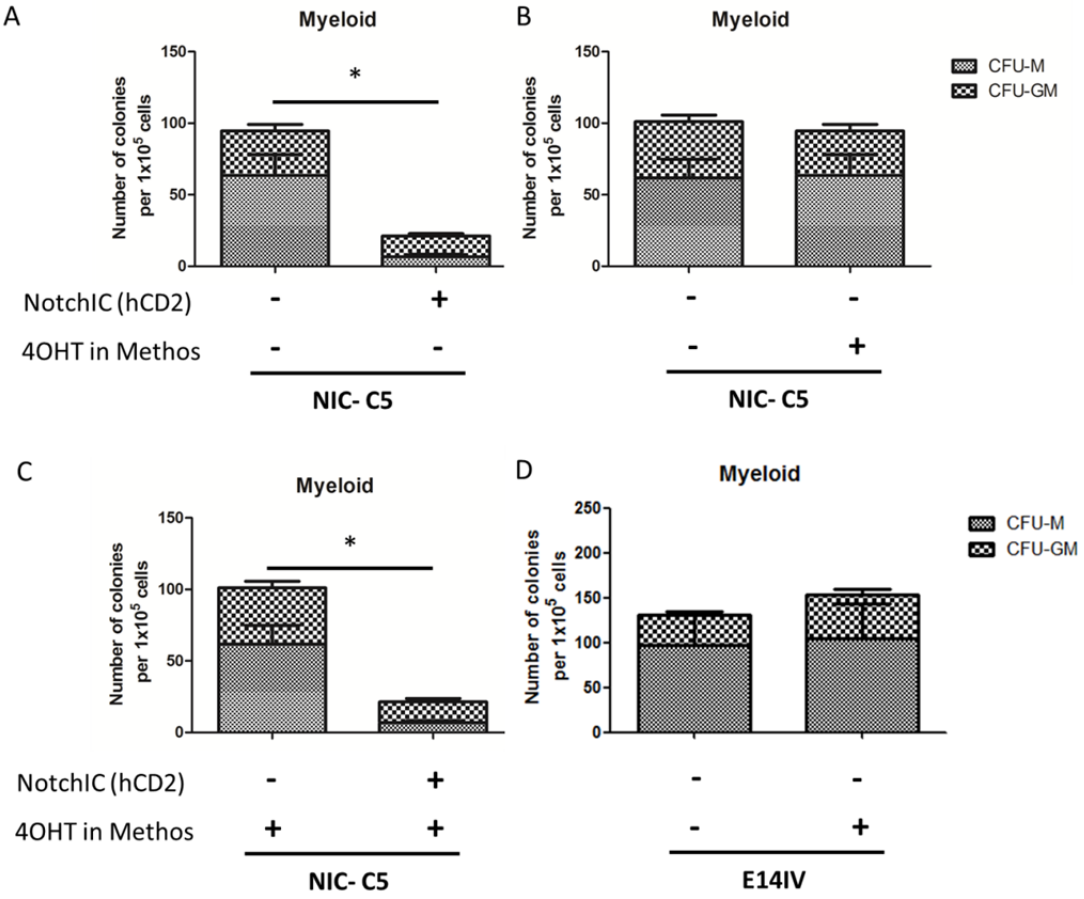


Figure 5.13 Comparison of Flk1$^+$ derived myeloid differentiation in sorted hCD2$^-$ and hCD2$^+$ fraction.

(A) NotchIC inhibited myeloid commitments; (B) NotchIC did not affect colony formation ability of myeloid progenitors; (C) NotchIC inhibited myeloid progenitors forming from co-culture; (D) No effect observed in E14IV control cells. Data represented 3 independent experiments. Error bars represented SEM. P value was calculated with Mann-Whitney test (*$p<0.01$)

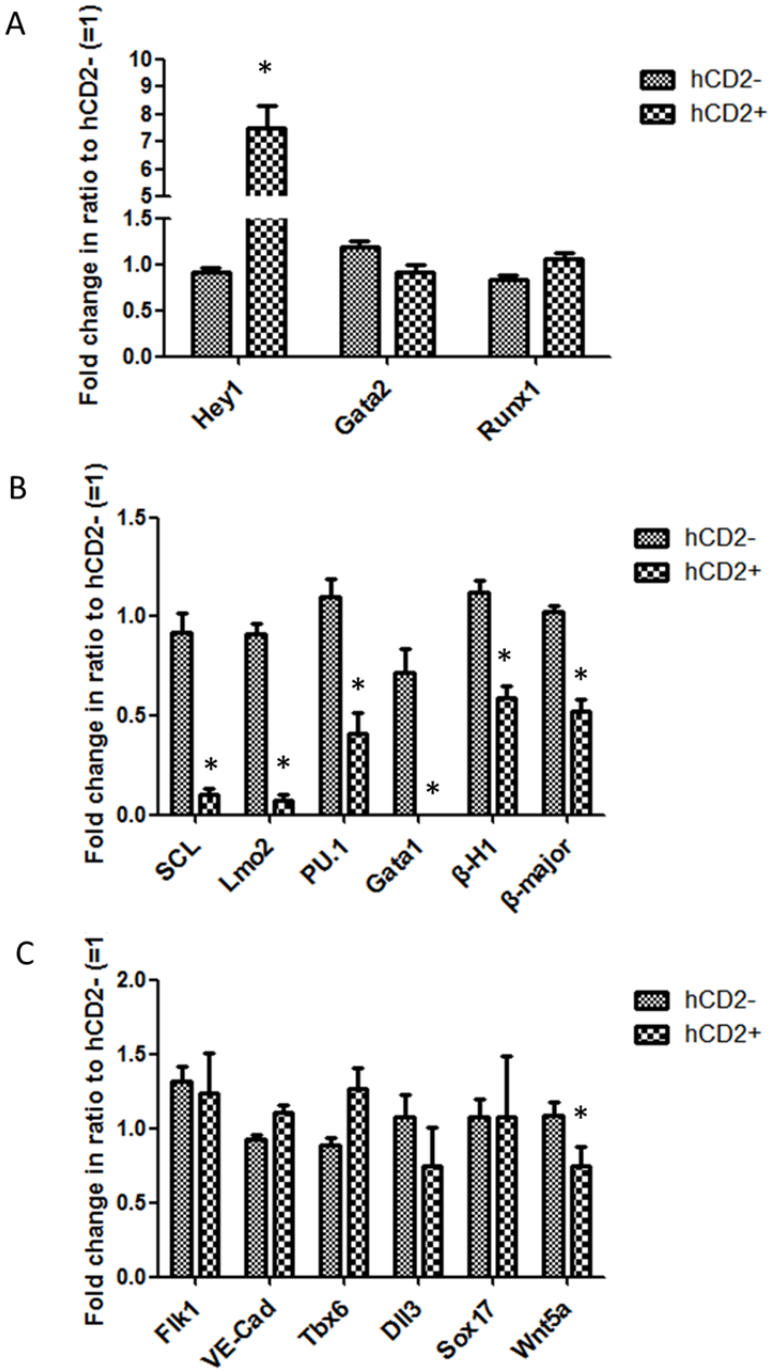


Figure 5.14 Quantitative RT-PCR at gene expression in hCD2$^-$ and hCD2$^+$ fraction.

(A) Hey1 was upregulated in hCD2$^+$. Gata2 and Runx1 were not affected. (B) Myeloid related gene PU.1 and erythroid related genes SCL, Lmo2, Gata1, β-H1 and β-major were suppressed. (C) Comparison of genes of other lineages in hCD2$^-$ and hCD2 fraction. Wnt5a was suppressed. Gene expressions in hCD2$^+$ fraction were related to hCD2$^-$ fraction, assigned as "1". HPRT was used as the endogenous control. Data represented 3 independent experiments. Error bars represented SEM. P values were calculated with Mann-Whitney test (*$p<0.05$)

5.4.4 Models of modulation of ectopic NotchIC at Flk1$^+$ derived haematopoiesis

In light of the results above, ectopic NotchIC could potentially function differently on the myeloid and erythroid differentiation. Here we summarize the cell response upon addition of 4-OHT at different time windows and draw a brief conclusion (Figure 5.15). Induction of ectopic NotchIC in co-culture from day4 to day6 inhibited CD41$^+$cKit$^+$ frequency at morderate level (Figure 5.5). Further sorting to purify hCD2$^-$ and hCD2$^+$ fraction supported that the inhibition effect was specific to NotchIC induced population (hCD2$^+$) (Figure 5.11). Inhibition on CD41$^+$cKit$^+$ formation suggested inhibitory effect of NotchIC on Flk1$^+$ derived haematopoiesis in co-culture. Of note, CD41$^+$cKit$^+$ could be co-expressed on a mix population of haematopoietic progenitors including multi-potential (CFU-GEMM, Mac/Ery), myeloid progenitor (CFU-GM, CFU-M), erythroid progenitor (BFU-E, CFU-E) and etc. However, no difference of definitive haematopoietic genes Gata2 or Runx1 indicated the inhibition could possibly occurred at later stage when myeloid progenitor or erythroid progenitor formation, not multi-potential progenitors (Figure 5.14).

For the myeloid lineage, ectopic NotchIC had an inhibitory effect on myeloid differentiation from Flk1$^+$ cells when 4-OHT was added into the co-culture then withdrawn from the colony assay (Figure 5.8). This was also confirmed by a decrease in the proportion of CD45$^+$ cells observed by comparing sorted hCD2$^-$ and hCD2$^+$ compartments (Figure 5.12). However, later differentiation and proliferation of myeloid progenitors to form colonies (CFU-GM, CFU-M) in colony assay was not affected (Figure 5.9). Furthermore, addition of 4-OHT into the colony assay to counteract the effect of NotchIC in colony assay revealed that ectopic Notch inhibited the production of myeloid progenitors from Flk1$^+$ (Figure 5.10). Comparison of purified hCD2$^-$ and hCD2$^+$ compartments in colony formation assays as well as down-regulation of PU.1 at day6 from co-culture supported this hypothesis (Figure 5.13). Thus, ectopic NotchIC abrogate myeloid differentiation from Flk1$^+$ by inhibiting myeloid progenitor formation in co-culture (Figure 5.15).

For the erythroid lineage, ectopic NotchIC inhibited erythroid differentiation from

Flk1^{+} cells at very limited level when 4-OHT was added into co-culture then withdrawn from the colony assay (Figure 5.8). However, a more prounced inhibitory effect was observed in the later colony formation by erythroid progenitors in colony assays (Figure 5.9). Thus, ectopic NotchIC abrogated erythroid differentiation, specifically by inhibiting the ability of erythroid progenitors to proliferate or differentiate and to form colonies in the methylcellulose assay. The early effect of NotchIC on erythroid production still needs to be elucidated (Figure 5.15). Downregulation of erythroid differentiation genes Gata1, SCL/Lmo2 and hemoglobin at day6 from co-culture also supported the inhibitory effect of ectopic NotchIC on erythroid differentiation as observed from colony assay. Of note, inhibition caused by toxicity of Cre-ER^{T2} will be discussed later in discussion part.

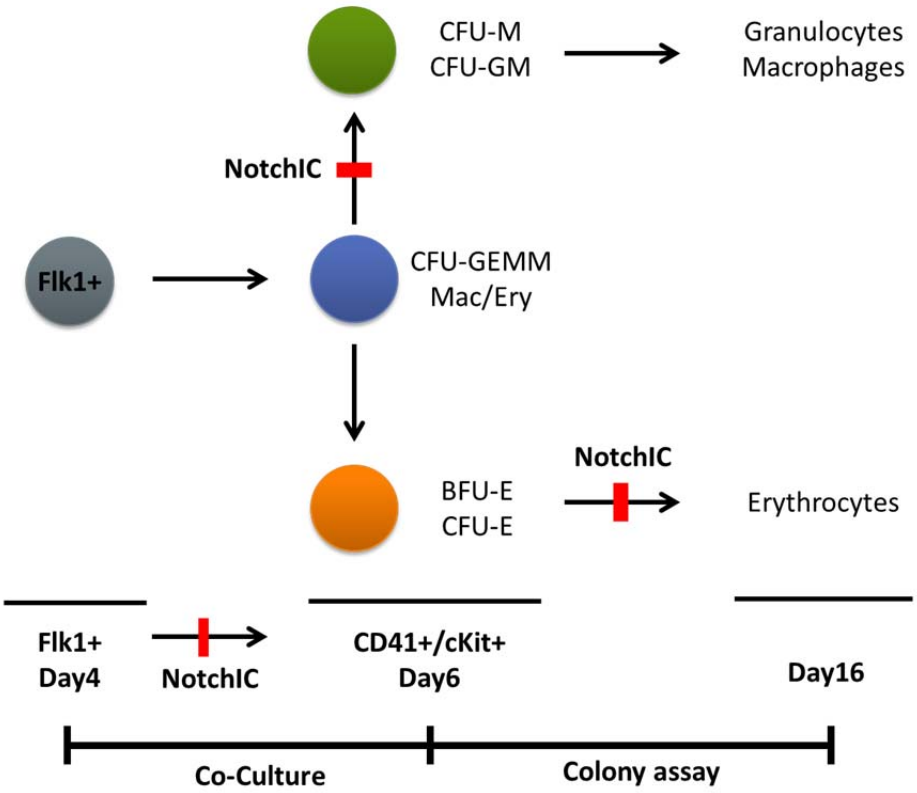


Figure 5.15 Modulation models of ectopic NotchIC on haematopoietic differentiation from Flk1$^+$ cells

Ectopic NotchIC inhibited CD41$^+$cKit$^+$ formation in co-culture to day6 at morderate level (indicated by thin red bar). NotchIC inhibited myeloid differentiation via myeloid progenitor formation in co-culture and erythroid lineage termination at later stages in colony forming assay (indicated by thick red bar).

5.5 Conclusion

In this chapter, we applied a tamoxifen inducible system to overexpress ectopic NotchIC and measure its effect on $Flk1^+$ derived haematopoiesis according to CFUs, surface markers and gene expression in $Flk1^+$/AM co-culture system. The results in this section indicated that:

- Ectopic NotchIC inhibited $CD41^+cKit^+$ haematopoietic progenitor formation from $Flk1^+$ cells (Figure 5.5).

- Ectopic NotchIC inhibited myeloid differentiation via inhibiting myeloid progenitor (CFU-GM/CFU-M) formation from $Flk1^+$ at day6 while not affecting colony forming ability of myeloid prognitors (Figure 5.8-5.10, 5.13)

- Ectopic NotchIC inhibited erythroid differentiation from $Flk1^+$ cells (Figure 5.9, 5.14).

- Comparing $hCD2^-$ and $hCD2^+$ fraction, inhibition effects on $CD41^+cKit^+$ and $CD45^+$ frequency, myeloid and erythroid differentiation were enriched in $hCD2^+$ ($NotchIC^+$) population (Figure 5.11, 5.13,5.14).

5.6 Discussion

5.6.1 A moderate inducible system to express ectopic NotchIC

Advantages and disadvantages of 4-OHT inducible system

So far, a series of gain- of- function systems have been developed to express ectopic NotchIC to investigate Notch signaling at different aspects in development. In this chapter, we have applied a 4-OHT inducible system to overexpress NotchIC during differentiation (Lowell et al., 2006). By comparing to other systems, this system is characterized by a very low background that ectopic NotchIC was repressed efficiently in the absence of 4-OHT and could be activated once 4-OHT was added at relatively high recombination efficiency (R26-NotchIC-C5); Secondly, targeting of Cre-ER^{T2} and NotchIC into each allele of Rosa26, a locus displayed ubiquitous activity during embryonic development assured the expression of both during differentiation in our system without silencing. Zambrowicz and his colleagues also confirmed that Rosa26 locus was active for haematopoietic differentiation *in vivo* during development (Friedrich and Soriano, 1991; Zambrowicz et al., 1997). Furthermore, targeting into Rosa26 locus avoided affecting other gene expression which have been discovered in other studies by random integrations; Thirdly, expression of NotchIC was under regulation of Rosa26 promoter, which was constitutively active during differentiation and moderate compared to other combination of promoters like CAG, in which case expression of NotchIC was moderate and not toxic during differentiation (Lowell et al., 2006). Fourthly, expression of hCD2 as a tag enabled us to monitor induction efficiency, as well as separate the hCD2^{-} and hCD2^{+} fraction and investigated effect of ectopic NotchIC on each, which provided a platform to further understand intrinsic or extrinsic way of Notch signaling to modulate haematopoiesis. Overall, in our differentiating system, we achieved stable and moderate ectopic NotchIC expression being controlled tightly in response to 4-OHT. Nevertheless, the shortfall of this system is that ectopic NotchIC is irreversible once activated by 4-OHT, which was overcome to some extent in our study. In addition, moderate expression level of NotchIC by Rosa26 promoter could possibly not reach the threshold to stimulate a strong phenotype upon induction.

Dosage of ectopic NotchIC induction

Thus, characteristics of this system, likely explains why the phenotypic changes observed in this study were moderate compared to other studies. Of note, compared to the constitutively ectopic NotchIC expression under the CAG promoter, the transcription activity in 4-OHT inducible cell line under ROSA26 promoter displayed a limited enhancement (Figure 5.2), which was also confirmed by Lowell and her colleagues (Lowell et al., 2006). The reason for the partial inhibition on $CD41^{+}cKit^{+}$ at 30% as observed in Figure 5.4 could also be accounted for the limited enhancement of Notch activity regulated by ROSA26 promoter.

Furthermore, emerging evidence suggests that the "dosage" of Notch signaling influences haematopoietic precursor cell-fate outcomes (Dallas et al., 2005; Delaney et al., 2005). We have tested whether NotchIC could inhibit the formation of $Flk1^{+}$ from ES cells as reported by Schroeder's study using the OP9/ES cell co-culture system (Schroeder et al., 2006a), in which case no change in $Flk1^{+}$ formation from suspension EBs was observed in our system (data not shown). This was not surprising because in Schroeder's study, ectopic NotchIC was driven under the promoter of CAG to yield a high level of robust NotchIC expression. The influence on $Flk1^{+}$ could be caused by either inhibitory effects on mesoderm formation by NotchIC, or the extremely high NotchIC levels interfering with the early differentiation potential of ES cells as discussed by Lowell and Schroeder (Lowell et al., 2006; Schroeder et al., 2006a).

5.6.2 Effect of ectopic NotchIC on primitive and definitive haematopoiesis in $Flk1^{+}$/AM co-culture

Embryonic haematopoiesis can be defined into three stages: primitive erythropoiesis for primitive erythrocytes, megakaryocytes and myeloid cells in yolk sac; the first wave of definitive haematopoiesis in early AGM and yolk sac with limited self-renewal capacity but multi-potent including myeloid, definitive erythroid and/or lymphoid differentiation and second definitive haematopoiesis for long-term definitive HSCs production in AGM region with a later migration to fetal liver. Based on the results of detection of $CD41^{+}cKit^{+}$ population, expression of Runx1,

β-H1 and β-major expression at day6 with an increase compared to day4 $Flk1^+$ cells (data not shown), we proposed that primitive and definitive co-existed in our system. Whether long-term HSCs exist in our system is still ambiguous until transplantation is carried out. So far studies on Notch signaling on haematopoiesis suggested complicated involvements of Notch signaling in primitive, definitive and long-term haematopoiesis. Due to high complexity of the Notch signaling pathway and interacting with other pathways, the effects of Notch varies in different systems based on experimental models, timing, signal strength and developmental context.

According to our results, ectopic NotchIC did not affect Gata2 or Runx1 expression but inhibited critical genes for later commitment. Furthermore, we also suggest that inhibition on definitive haematopoiesis defined by $CD41^+cKit^+$ could be initiated by a later inhibition effect on myeloid or erythroid progenitor numbers. Thus, it is possible that $Flk1^+$ derived definitive haematopoiesis in AM microenvironment was not affected by ectopic NotchIC at early stage (ie formation of CMP from haemangioblast/haemogenic endothelium), while later formation of both primitive and definitive terminal lineages were abrogated.

This assumption correlates to work published by Ganapati et al by overexpressing NotchIC in a Tet-off system finding that ectopic NotchIC could inhibit formation of $CD34^+cKit^+$ followed with lower myeloid marker CD11b expression in OP9 co-culture system (Ganapati et al., 2007). In convert, Hadland and his colleagues' work using knock down system claiming that Notch1 deficiency in EB differentiation did not affect the production of definitive colony formation cells but increase CFU-EryP (Hadland et al., 2004). However Kumano and colleagues demonstrated that Notch1 deficiency could interrupt AGM-derived haematopoiesis but not yolk sac-derived haematopoiesis in colony forming assay with an inhibition on Runx1, Gata2 at RNA level (Kumano et al., 2003). Accordingly, Robert-Moreno and his colleagues also revealed the absence of haematopoietic cells but increase of endothelial cells in RBP-Jκ mutant in AGM region by downregulating GATA2, Runx1 and SCL expression (Robert-Moreno et al., 2005). These two loss- of function systems emphasized the critical role of Notch for definitive haematopoiesis, which

seemed to be controversial to our result. However these reported abrogation effect on AGM-derived haematopoiesis or definitive transcription factors could be accounted by inhibition on long-term HSCs potential (the second definitive wave), which was not determined yet in our system. Moreover, gain-of function could be not opposite to loss- of function.

Alternatively, ectopic NotchIC did affect definitive haematopoiesis at early stage (ie formation of CMP from haemangioblast or haemogenic endothelium) while not via Gata2 or Runx1 because these two genes were not function as Notch downstream target in this molecular network in co-culture, or ectopic NotchIC was too moderate to reach the threshold to affect Gata2 or Runx1.

5.6.3 Inhibition effect of ectopic NotchIC on myeloid differentiation via abrogating MYELOID PROGENITOR formation from Flk1$^+$ cells

In our system, we noted that ectopic NotchIC abrogate myeloid differentiation via inhibiting the number of MYELOID PROGENITOR but not affecting later differentiation or proliferation of MYELOID PROGENITOR in colony assay. This inhibitory effect was also confirmed by CD41$^+$cKit$^+$ and CD45 expression and could possibly via inhibiting PU.1. This conclusion agreed with some previous studies but also controversial to other studies, though most of which were based on the adult haematopoiesis system. A recently published work suggested that ectopic NotchIC could suppress RNA level of several critical myeloid transcription factors including PU.1 in mouse HSCs (Klinakis et al., 2011). de Pooter et al demonstrated that co-culture with OP9-Delta like1 could inhibit maturation of myeloid cells from both ES cells and primary haematopoietic progenitors mediated by GATA2 (de Pooter et al., 2006). Walker also proved that stimulation of Jagged1 could inhibit myeloid colony formation from CD34$^+$ cells in the absence of cytokine stimulation (Walker et al., 1999). To better understand the reason for inhibitory effect, increasing evidences suggested Notch signaling favored progenitor proliferation over differentiation. Siter reported that activation of NotchIC in bone marrow derived HSCs could increase HSC self-renew *in vivo* and favour T lineage commitment but inhibit myeloid differentiation (Stier, 2002). Carlesso and Buono demonstrated that increase or

accumulation of Notch activity could promote haematopoietic progenitor proliferation and inhibit further differentiation (Buono et al., 2010; Carlesso et al., 1999)

Although inhibition on myeloid differentiation has been widely reported, the exact stage when inhibitory effect takes place is still of controversial. In our system, Notch inhibited myeloid differentiation via abrogating MYELOID PROGENITOR formation. However, as reported by other groups, Notch signaling was proposed to interrupt myeloid differentiation via promoting myeloid progenitor accumulation and inhibiting later terminal differentiation or maturation (Bigas et al., 1998; Kawamata et al., 2002b; Qyang et al., 2004; Saleem and Conrad, 2011). In light of this, it will be necessary to check whether colonies in colony assay formed by the induced population were more immature compared to the untreated population in our system.

In contrast, several studies using overexpression systems to activate Notch signaling by either exogenous stimulation of ligands or ectopic NotchIC in bone marrow derived HSCs or HPCs (FCDP-mix cells or 32D) suggested that Notch signaling pathway could promote myeloid differentiation or maturation but inhibited haematopoietic progenitor proliferation, possibly via upregulating PU.1 expression and other transcription factors critical for proliferation, differentiation or apoptosis (Kawamata et al., 2002a; Schroeder, 2003; Schroeder and Just, 2000a; Schroeder and Just, 2000b; Schroeder et al., 2003; Schroeder et al., 2000; Schroeder et al., 2006b; Schwanbeck et al., 2008). This observation correlates to suppression on myeloid differentiation to GR-1 derived from Notch1$^{-/-}$ ESC *in vitro* (Yan et al., 2010; Zhou, 2012). Therefore, the role of Notch signaling in MYELOID PROGENITOR formation and later commitment is still controversial and context, timing, cytokines, and modulation strategy dependent. More important, embryonic sites of hematopoiesis represented environments distinct from adult hematopoiesis, with unique regulatory requirements. Thus, ectopic NotchIC modulation on myeloid differentiation in our system provided a novel idea about the potential of Notch signaling in the myeloid differentiation in AGM-derived microenvironment without addition of exogenous cytokines.

5.6.4 Erythroid differentiation in Flk1$^+$/AM co-culture system

To better understand how ectopic NotchIC affects Flk1$^+$ derived haematopoiesis and to consider the complexity of Notch signaling, we analysed the effects on both myeloid and erythroid. As summarized in Figure 5.15, when 4-OHT was added to the co-culture and withdrawn two days later, an inhibition effect was observed but at a very limited level (Figure 5.8 E). We noted a larger inhibitory effect of NotchIC on erythroid progenitor to form colonies in the colony assay compared to Figure 5.8 E, which indicated ectopic NotchIC could abrogate proliferation and differentiation ability of erythroid progenitors (Figure 5.9 E). Thus, ectopic NotchIC showed an inhibitory effect on erythroid differentiation. However it was difficult to distinguish how NotchIC acted between the process of erythroid progenitor formation in co-culture and later colony formation by erythroid progenitorss. Comparison was carried out between culture treated with 4-OHT in colony assay with culture treated in co-culture AND colony assay, which displayed a better inhibitory in longer treatment as well in Appendix Figure S5.1. It was highly possible that induction of Notch activity in co-culture stimulated the responding ability of erythroid progenitors in colony assay to form colony or simply increased number of erythroid progenitors.

Effect of ectopic NotchIC on erythroid differentiation

As we noted, NotchIC inhibited erythroid differentiation, more likely at terminal commitment. This inhibitory effect was also confirmed by quantitative RT-PCR at SCL, Lmo2, Gata1 and β-globin level decrease upon induction of NotchIC. So far, a series of studies investigating effect of Notch signaling have been reported but are also controversial. Comparable to our study in ES system, it was found that Notch1-/- ES cells increased numbers of primitive erythroid colony forming progenitors (EryP) in EBs *in vitro* culture, but not *in vivo* (Hadland et al., 2004). Similarly, overexpression of NotchIC abrogated EryP formation from EBs but not affect definitive colonies (Cheng et al., 2008). This was also supported by several studies using adult HSCs models *ex vivo* or *in vivo* demonstrating the inhibitory effect of Notch signaling on erythroid differentiation and maturation or apoptotic inducing effect, possibly via suppressing Gata1 activity through Hes1 (Elagib et al., 2004; Ishiko et al., 2005; Lam et al., 2000; Maeda et al., 2009; Okuhashi et al., 2010;

Robert-Moreno et al., 2007; Ross et al., 2012; Tachikawa et al., 2006). Conversely, several other groups suggested the induction effects of Notch signaling on erythroid differentiation and maturation (Henning et al., 2008; Henning et al., 2007; Sugimoto et al., 2006). The controversies are probably not surprising because of the differences between the systems with different cytokine stimulation or types of progenitors. For example, Notch signaling could regulate erythroid differentiation via modulating cytokine-dependent signal pathways like EPO.

Toxicity of Cre on erythroid differentiation

Inhibition of erythroid differentiation was demonstrated in our studies by using flow cytometry at mature erythrocyte marker Ter119 expression and BFU-E and CFU-E formation in colony assay as well as inhibition of erythroid related genes. However, toxicity of Cre has been reported in several studies on several cell types, in which case toxicity could be another potential reason for the abrogation of erythroid differentiation in our system. It was reported that Cre recombinase could cause growth inhibition and DNA damage (Loonstra et al., 2001; Silver and Livingston, 2001).

To determine whether this suppression was caused ectopic NotchIC or Cre, we simply seeded the FACS sorted $hCD2^{+}$ fraction in to the methylcellulose assay with or without 4-OHT. In theory, loxp -termination sequence- loxp in $hCD2^{+}$ cells have been removed with Cre so that further addition of 4-OHT into cells should not drive more NotchIC expression. $hCD2^{-}$ fraction and E14IV were used as the control. As it has been shown previously, addition of 4-OHT could inhibit erythroid colony formation in $hCD2^{-}$ fraction. However this inhibition effect was observed in $hCD2^{+}$ fraction as well. Addition of 4-OHT did not affect colony formation by E14IV (Appendix Figure S5.2). Therefore, by taking suppression of mature erythrocytes marker Ter119 and critical genes for erythrocytes commitment into account together, it could be explained as the importing of Cre into nucleus causing the inhibition of erythroid differentiation at terminal stage. Introducing another control cell line with Cre-ER^{T2} targeted into the ROSA26 locus will be critical and necessary.

5.6.5 Possible explanation for suppression of Flk1$^+$ derived haematopoiesis

During differentiation, multi-potential hematopoietic progenitors could undergo a series of commitment decisions to choose between survival and apoptosis, between proliferation and lineage commitment, or between differentiation directions.

Survival and proliferation of Flk1$^+$ derived HPCs and haematopoietic cells

Increasing evidence has emerged to suggest that Notch signaling is involved in haematopoiesis via regulating cell survival, proliferation and differentiation. In our system, Flk1$^+$ differentiation into myeloid progenitors or erythroid lineages was abrogated. This result could be caused by the cell survival and proliferation ability. Preliminary data of apoptosis analysis on day6 and day8 indicated a slight higher percentage of AnnexinV$^+$DAPI- staining in Flk1$^+$ derived cells (Appendix Figure S5.3). It will be necessary to measure the total number of Flk1$^+$ derived haematopoietic progenitors and cells to measure whether overexpression of NotchIC could inhibit myeloid and erythroid differentiation via affecting cell survival or proliferation ability, probably through apoptosis of the Flk1$^+$ derived HPCs and haematopoietic cells. In light of Schroeder's studies, it will also be interesting to check whether ectopic NotchIC could affect cell cycle of HPCs and haematopoietic cells in our system (Schroeder and Just, 2000a).

Potential of Flk1$^+$ cells to other lineages including lymphoid, cardiac, endothelial and VSMCs development

A series of studies have demonstrated that activation of Notch signaling could prime haematopoietic differentiation into T lineages (de Pooter and Zuniga-Pflucker, 2007; Henning et al., 2007; Jaleco et al., 2001; Kutleša et al., 2009; Mohtashami et al., 2010; Sambandam et al., 2005; Schmitt and Zuniga-Pflucker, 2002; Stier et al., 2002). Although no measurement of T lineages has been carried out in our system, it will be interesting to investigate whether ectopic NotchIC favours haematopoietic differentiation to lymphoid lineage over myeloid and erythroid lineages.

Except for cell survival, proliferation ability or converting to lymphoid lineages from haematopoietic progenitors, ectopic NotchIC could possibly convert cell fate to other

lineages from Flk1$^+$ cells. We noted that in our system, ectopic NotchIC could inhibit SCL/Lmo2 expression, which was critical for formation of haemogenic endothelium from Flk1$^+$ haemangioblast cells (Lancrin et al., 2009). Thus Notch could possibly affected early commitment of Flk1$^+$ cells to haemogenic endothelium by inhibiting SCL in our system, though no difference of Flk1 or VE-Cadherin RNA level was affected. It is worthwhile to include other markers for early haematopoisis in flow cytometry analysis.

It has been reported by a series of studies that Flk1$^+$ cells were able to differentiate to haematopoietic, endothelial, vascular smooth muscle as well as cardiac lineages (Ema et al., 2006; Huber et al., 2004; Ishitobi et al., 2011; Kattman et al., 2006; Kouskoff et al., 2005; Lugus et al., 2009; Wu et al., 2006; Yang et al., 2008). For example, ectopic Notch4 has been reported to respecify Flk1$^+$ haemangioblast cells to a cardiac fate over a haematopoietic fate (Chen et al., 2008). In our system, Flk1$^+$ cells by Notch-C5 ES cell lines were also co-cultured on OP9 stroma to induce cardiomyocytes in this project. However in our co-culture system of Flk1$^+$ with OP9 cells, beating colonies were observed but no difference was detected when 4-OHT was added to induce ectopic NotchIC. This could be explained that ectopic NotchIC was moderate compared to Chen's system. Alternatively, induction using serum-free system with addition of cytokines determined the different molecular network different from the OP9 co-culture system in our system. In addition, Notch1 and Notch4 could function differently. In the same research, overexpression of Notch4 was also found to moderately increase the level of genes related to endothelial and VSMCs development including Flk1, VE-Cadherin, SM22 and pDGF-β from Flk1$^+$ haemangioblast (Chen et al., 2008). Notch activation mediated by Cre recombination in specific lineages also supported the involvement of Notch signaling in definitive haematopoiesis by regulating the cardiac, endothelial and VSMCs differentiation. (Tang et al., 2012; Venkatesh et al., 2008). Thus, based on the inhibitory effect of NotchIC on haematopoietic differentiation from Flk1$^+$ cells, it would be interesting to further investigate whether Notch could convert cell fate to cardiac, endothelial or VSMCs fate.

5.6.6 Microenvironment for haematopoiesis affected by ectopic NotchIC

As discussed in 5.6.5, ectopic NotchIC could potential convert the fate decision from $Flk1^+$ cells. Moreover, the function of non-haematopoietic priming cells to alter the haematopoietic microenvironment could be affected by Notch activation. For example, Tang and colleagues noted that Notch1 activation in endothelial cells (VE-$cadherin^+$) could abrogate haematopoiesis (Tang et al., 2012). It is plausible as they addessed that activtion of Notch1 imbalanced the fate decision of endothelial/haematopoietic from endothelial cells, or alternatively structural endothelial cells which are also VE-$cadherin^+$ overexpressing NotchIC provide an inhibitory microenvironment for haematopoietic differentiation. Thus, modulation of Notch activity in more specified population like endothelial cells or haemogenic endothelium cells and check its effect on haematopoietic differentiation will be interesting.

Chapter 6: Investigate whether Notch signalling pathway is required for Flk1^{+} cells derived haematopoietic differentiation in Flk1^{+}/AM co-culture

6.1 Aim

To set up a system to measure whether Notch signaling was involved during the haematopoietic differentiation of $Flk1^+$ cells in AM supporting microenvironment.

6.2 Introduction

In previous chapters, we demonstrated that AM stromal cells could enhance haematopoietic differentiation of $Flk1^+$ cells. Of note, overexpression of Notch intracellular domain (NotchIC) to activate Notch signaling abrogated formation of $CD41^+cKit^+$ haematopoietic progenitors and myeloid differentiation. To further understand the involvement of Notch signaling pathway during haematopoietic differentiation in the $Flk1^+$/AM co-culture, we knocked down Notch signaling using a dominant negative strategy. In many studies, knock down or knock out of the components of Notch signaling have been used to modulate Notch activity. Dominant-negative MAML1 (DNMAML1 or DML), a truncation of MAML1 which functions as a component to form the RPB- Jκ co-activator, provides the binding site for NotchIC but does not recruit other co-activators. So far several studies have used DNMAML1 (DML) to inhibit the Notch signaling pathway (Gonzalez-Garcia et al., 2009; Maillard et al., 2008). As reported in several published studies, the Notch signaling pathway controls cell fate decisions at different stages during development both *in vivo* and *in vitro*. To investigate the effect of Notch signaling on cell fate at a particular stage on specific cells, we set out to establish a system whereby we could knock down Notch signaling using DNMAML1 at defined time windows in ESC-derived cells without an inhibitory effect on stromal cells. We chose the doxycycline inducible system (Iacovino et al., 2011a). To set up this inducible system, we carried out the following experiments:

(a) ES cell clones expressing doxycycline-inducible DML-EGFP or EGFP alone were generated;
(b) Each ES cell clone was tested for Doxycycline-inducibility and screened in a series of assays designed to assess self-renewal and differentiation;
(c) The efficiency of inhibition of Notch activity was measured;
(d) The effect of DML-EGFP on haematopoietic differentiation of $Flk1^+$ cells in

AM supporting microenvironment was tested.

6.3 Experimental approach

- To generate the doxycycline inducible DML-EGFP ES cell line (iDML) and control EGFP cell line (iEGFP), DML-EGFP and EGFP in p2lox plasmid were electroporated into A2lox.cre parental ES cell line;

- To validate ES clones, western blot and flow cytometric analysis were applied to assess the expression of DML-EGFP and EGFP expression. The dose of doxycycline was optimized. Karyotyping and self-renewal assays were carried out to characterize these ES cell clones for ES cells properties;

- To determine whether DML-EGFP could abrogate Notch signaling, Notch transcription activity was assessed using the luciferase reporter system and quantitative RT-PCR of Notch downstream genes.

- To measure whether iDML and iEGFP ES clones could respond to AM stromal cells in EB/AM and $Flk1^{+}$/AM co-cultures, methylcellulose assays were applied to determine the haematopoietic activity;

- To test whether DML-EGFP could affect $Flk1^{+}$ derived haematopoiesis, DML-EGFP was induced in $Flk1^{+}$/AM co-culture system and haematopoietic activity were measured in both colony assay and flow cytometry.

6.4 Results

6.4.1. Generation of doxycycline inducible DNMAML1-EGFP ES cell lines for knocking down Notch activity

6.4.1.1 Construction of two Doxycycline inducible ES cell lines iDNMAML1-EGFP (iDML) and iEGFP.

DNMAML1-EGFP fragment was excised from pEGFP-DNMAML1-N3 plasmid (a kind gift from Maillard, I.) with BglII and NotI sites then cloned into p2lox-EGFP plasmid digested with XhoI and NotI with blunting strategy. According to sequence of p2lox plasmid and DML-EGFP, the correct clone should only contain one EcoRI site. Clones were screened by EcoRI digestion (Figure 6.1 A). The p2lox-DML-EGFP plasmid was also sequenced after construction to confirm the correct integration of DML-EGFP.

A2lox.cre parental cell line (kindly provided by the Kyba lab) was constructed based on A17 ES cell line, a derivative of E14Tg2a ES cell line, in which reverse tetracycline-controlled transcriptional activator (rtTA) has been inserted into the Rosa26 locus. This A17 ES cell line was then targeted in the HPRT locus with tetracycline- responsive promoter (TRE) followed with a Cre transgene flanked by two incompatible loxP and loxM sites together with Δneo, a G418 resistance gene lacking ATG start codon and promoter to construct the A2lox.cre ES cell line (Iacovino et al., 2011a). To obtain inducible DML-EGFP ES cell lines, 1μg/ml Doxycycline was added to induce Cre expression 24 hours prior to electroporation of p2lox-DML-EGFP, a plasmid bearing PGK promoter and two heterozygous loxP sites followed with inserted DNMAML1-EGFP fragment. In correct clones, DML-EGFP expression was regulated by the TRE promoter so when doxycycline is added, the rtTA in Rosa26 locus binds to TRE promoter then drives the expression of DML-EGFP. The PGK promoter with start codon ATG is then able to drive expression of Δneo gene resulting in G418 resistance. The Cre transgene with two loxM sites is then excised at this point (Figure 6.1 B). We also established control cell lines with inducible EGFP by electroporation of p2lox-EGFP. With this strategy, we established two types of cell lines: iDML-EGFP (41 clones obtained) and iEGFP

(57 clones obtained).

6.4.1.2 Validation of iDML and iEGFP ES clones by western blots and flow cytometry.

To determine whether DML-EGFP could be expressed from the TRE promoter upon addition of doxycycline (Dox), two chosen iDML ES clones: iDML.1 and iDML.2 (named as A2 and A10 in the original work) were treated with 5μg/ml Doxycycline for 48 hours in the presence of LIF. Cos7 cells were transfected with pEGFP-DML-N3 or pCAG-EGFP plasmid as positive controls. Two control ES cell lines iEGFP.1 and iEGFP.2 (named as C8 and H8 in the original work) as well as A2lox.cre parental cell line were also treated with Dox as controls. Western blot showed that upon addition of Dox, iDML.1 and iDML.2 expressed the 38 kD DML-EGFP and the iEGFP.1 and iEGFP.2 cell lines expressed the 30kD EGFP. No expression was detected in A2lox.cre parental cell line. STAT3 (79kD) was used as the endogenous control (Figure 6.2A). Because EGFP was used as a tag in the system, it was also possible to monitor the expression of DML-EGFP or EGFP by flow cytometry. iDML clones and iEGFP clones was treated with 5μg/ml Dox for 48 hours in the presence of LIF. It was shown that EGFP in all the four chosen clones could be detected. The induction efficiency was around 90% (iDML.1 92.8%; iDML.2 90%; iEGFP.1 88.6%; iEGFP.2 85.8%) (Figure 6.2 B). This result suggested that iDML and iEGFP could respond to Dox and express DML-EGFP or EGFP as detected by both western blot and flow cytometry at the protein level.

6.4.1.3 Optimization of dose of Doxycycline to induce DML-EGFP expression

To optimize the dose of doxcycyline to induce DML-EGFP expression, we used western blotting and flow cytometry to monitor EGFP expression upon addition of dox at different doses using the iDML.1 ES clone. ES cells were treated with dox and analysed by western blotting using an anti- EGFP antibody. There was no detectable expression of DML-EGFP in the absence of doxycycline. By adding doxycycline at concentration ranging from 0.5μg/ml to 14μg/ml for 48 hours, expression of DMl-EGFP was detected. The induction efficiency of doxycycline was comparable

from 0.5μg/ml to 8μg/ml. Concentration of 14μg/ml had a lower level of induction. An EGFP band at 30kd was detected in the blot indicating possible degradation (Figure 6.3 A). Flow cytometry was also applied to measure induction of DML-EGFP in differentiating cells. ES cells were differentiated into day4 suspension EBs then disaggregated into single cells then cultured on gelatin with addition of dox. No DML-EGFP was detected in the absence of Dox. Induction efficiency was comparable among 1μg/ml to 5μg/ml. 0.5μg/ml had a slightly lower level of induction efficiency (Figure 6.3 B). Thus, a dose of 1μg/ml was applied to induce DML-EGFP in iDML.1 ES clone both in stem cells and in differentiating cells

6.4.1.4 Karyotyping of iDML and iEGFP ES clones

It is widely acknowledged that karyotypes of ES cells could become abnormal after long term of culture with a trisomy of chromosomes being the most commonly observed aberration (Liu et al., 1997); (Rebuzzini et al., 2008). Therefore, we carried out karyotyping assay to determine whether the chosen clones contained a normal chromosome numbers by counting DAPI stained chromosome spreading on slides. Clones with more than 80 % of randomly picked up cells containing 39-40 chromosomes were defined as normal karyotyping. E14 IV ES cells, a widely used ES cell line in our lab with a more stable normal karyotype compared to other ES cell lines were checked here as the positive control. It was found that A2lox.cre parental ES cell line and the four chosen inducible ES cell lines possessed the acceptable percentage of cells with correct chromosome number (Table 6.1). Interestingly, iEGFP.2 contained higher percentage of cells with 39 or less chromosomes than other clones. This was not conclusive because it could be caused by bad spreading or overlapping of chromosomes in the preparation for this ES cell line. Thus it is necessary to repeat or apply more sensitive strategies to confirm this.

6.4.1.5 Self-renewal ability of iDML and iEGFP clones

To measure whether the four chosen ES clones could function as ES cells with self-renewal ability in the presence of LIF and differentiation ability in the absence of LIF, we carried out clonal density, self-renewal assays of A2lox.cre parental ES

cell line, two iDML and two iEGFP ES clones. Briefly, 500 ES cells were plated in two gelatinized wells in 6 well plates in the presence of LIF. The following day cells were washed with PBS twice then cultured in ES medium with or without LIF separately for another 6 days. Then cells were fixed and stained for alkaline phosphatase, which was expressed by undifferentiated ES cells. Stem cell colonies with a tight round morphology and pink undifferentiated ES cells, mixed colonies with a pink centre and white edges and differentiated colonies consisting of only white differentiated cells were identified by microscopy and scored. In the presence of LIF, the majority of cells formed stem cell colonies or mixed colonies. Rare differentiated colonies were observed (Figure 6.4 A). In the absence of LIF the majority of the cells formed mixed colonies and differentiated colonies (Figure 6.4 B). This result suggested that the parental ES cell line, iDML ES cell lines and iEGFP ES cell lines could undergo self-renew in the presence of LIF and differentiate upon withdrawal of LIF under these conditions.

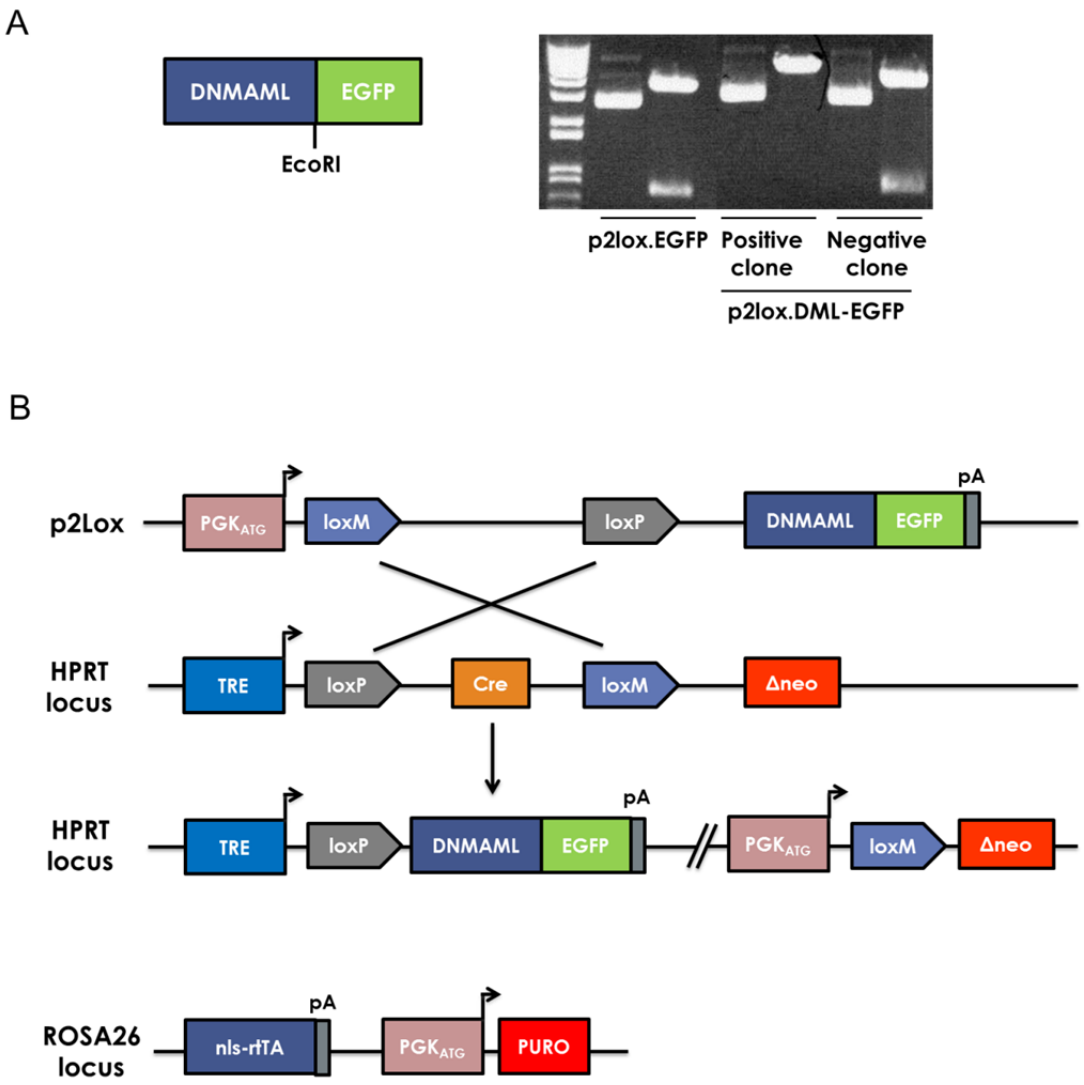


Figure 6.1 Construction of Doxycycline inducible DNMAML1 ES cell lines (iDML).

(A) The positive p2lox.DNMAML-EGFP was confirmed with one ECORI digestion site. (B) The incoming plasmid p2lox shown above is inserted with DNMAML-EGFP fragment for further integration into A2lox.cre ES cell line. In A2lox.cre ES cell line Cre is flanked by heterologous loxP/loxM sites at downstream of a TRE promoter and followed with Δneo for selection in HPRT locus. The heterologous loxP/loxM sites on the incoming plasmid are in the opposite orientation compared to targeting cassette in HPRT locus, in which case the Cre recombinanse induced by addition of Dox 24 hours earlier could catalyze the integration of the DNMAML-EGFP under TRE promoter and PGK promoter to enable Δneo expression for further selection. In correct recombinated ES cell line, addition of Dox could enable rtTA to activate TRE promoter and DNMAML-EGFP expression.

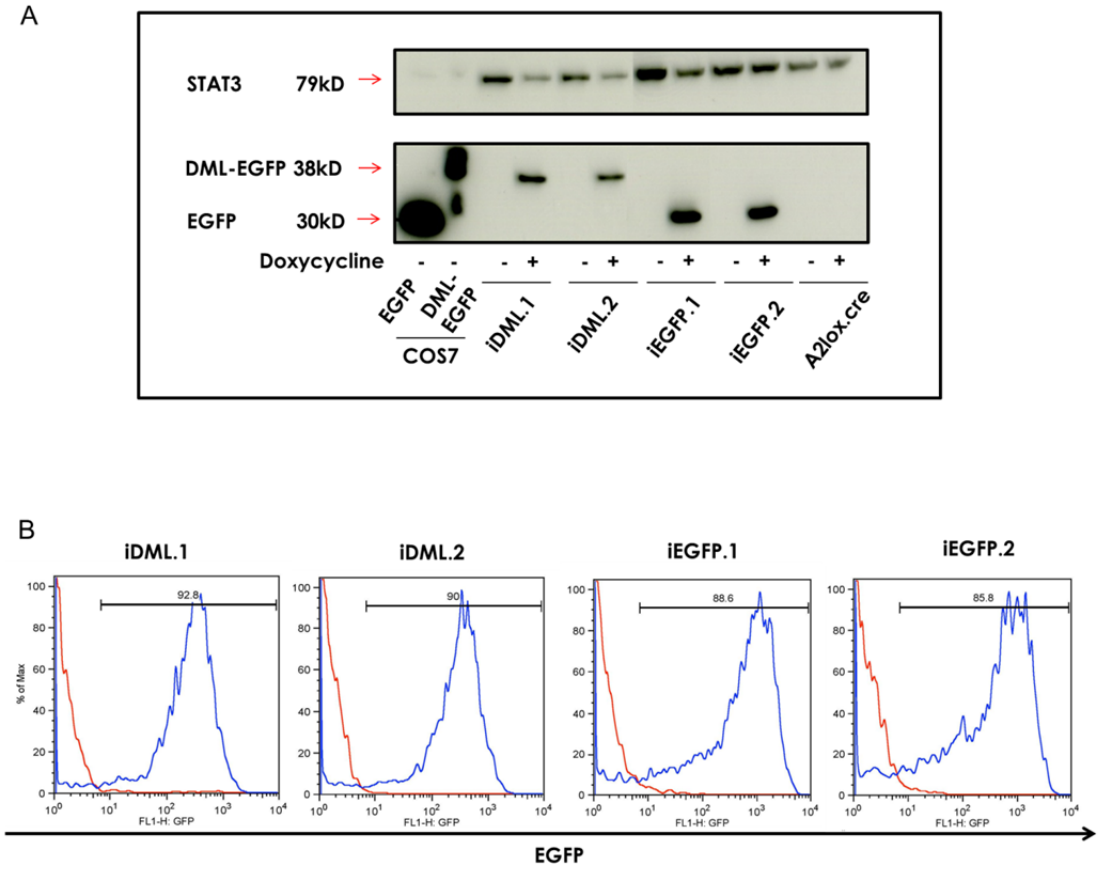


Figure 6.2 Validation of DML-EGFP and EGFP expression by western blot and flow cytometry analysis.

(A) Expression of DML-EGFP and EGFP were confirmed by western blotting using an anti-EGFP antibody. An anti-STAT3 antibody was used as the endogenous loading control. Cos7 cells were transfected with pCAG-EGFP or pEGFP-DML-N3 plasmid as positive controls. (B) Expression of DML-EGFP or EGFP was confirmed by flow cytometric analysis in FL1 channel. Red represented untreated samples. Blue represent treated smaple with Doxycycline at 5 μg/ml.

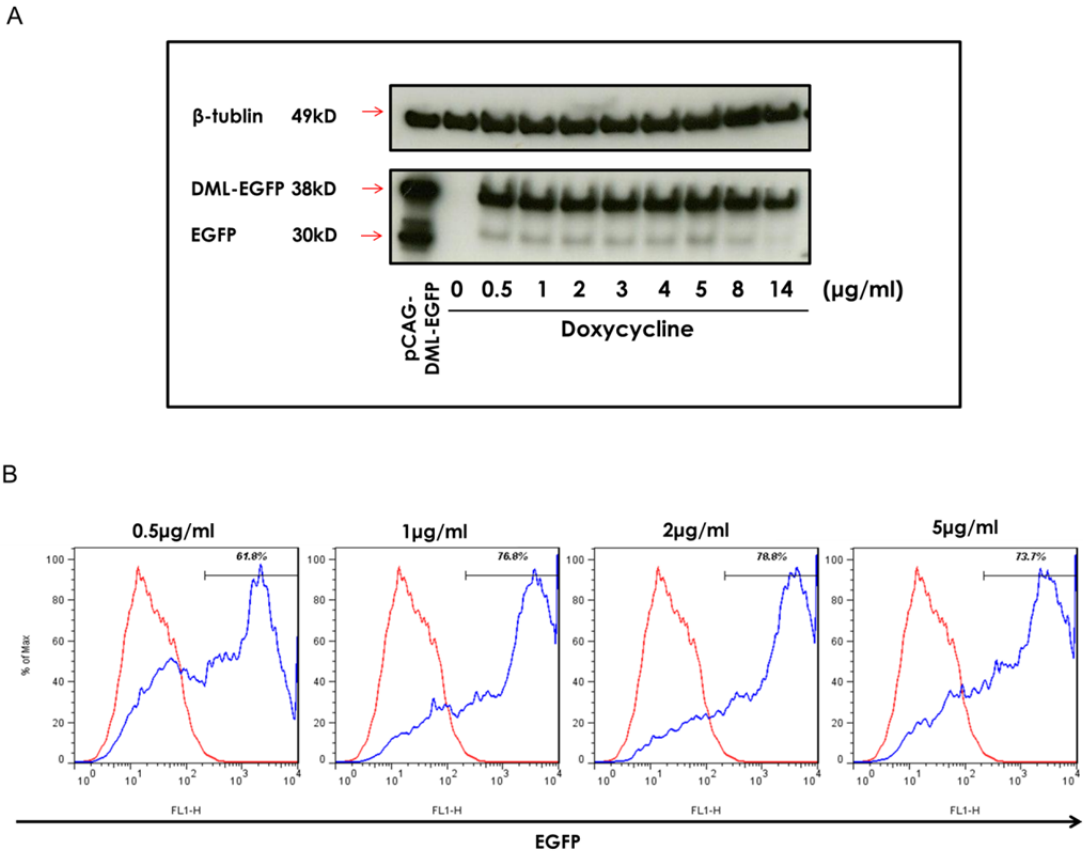


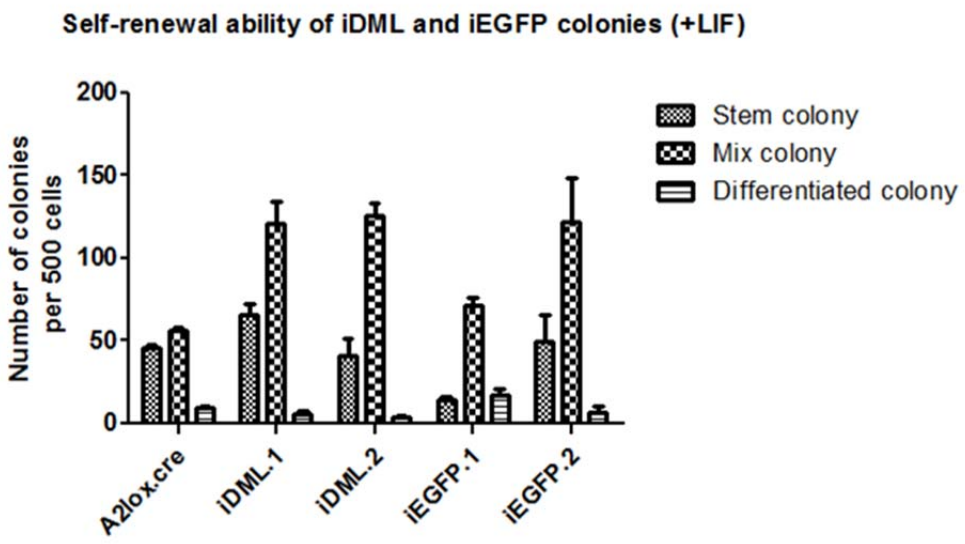
Figure 6.3 Optimization of dose of doxycycline used to induce DML-EGFP expression in iDML.1 ES clone with western blot and flow cytomteric analysis.

(A) Dose optimization was determined by western blot against EGFP antibody. β-tublin was used as the endogenous control. Cos7 cells were transfected with pEGFP-DML-N3 plasmid as the positive control. Doxycycline was added at the concentration ranging from 0.5 to 14 μg/ml. (B) Optimization of doxycycline dose were carried out in cells from day4 suspension EBs to day6 using iDML.1 ES clone. Doxycycline was added at the concentration ranging from 0.5 to 5 μg/ml. EGFP was detected by flow cytometric analysis in FL1 channel. Red represented untreated samples. Blue represent treated smaple with Doxycycline.

ES cell lines	Percentage of cells with normal karyotype of 39-40 chromosomes (Number of counted cells)
E14IV	86.7% (15)
A2lox.cre	94.4% (36)
iDML.1	92.1% (38)
iDML.2	88.9% (45)
iEGFP.1	92.1% (38)
iEGFP.2	85.0% (20)

Table 6.1 Karyotype of *iDML and iEGFP ES clones*.
All the clones were in the acceptable range of chromosome numbers (39-40).

A



B

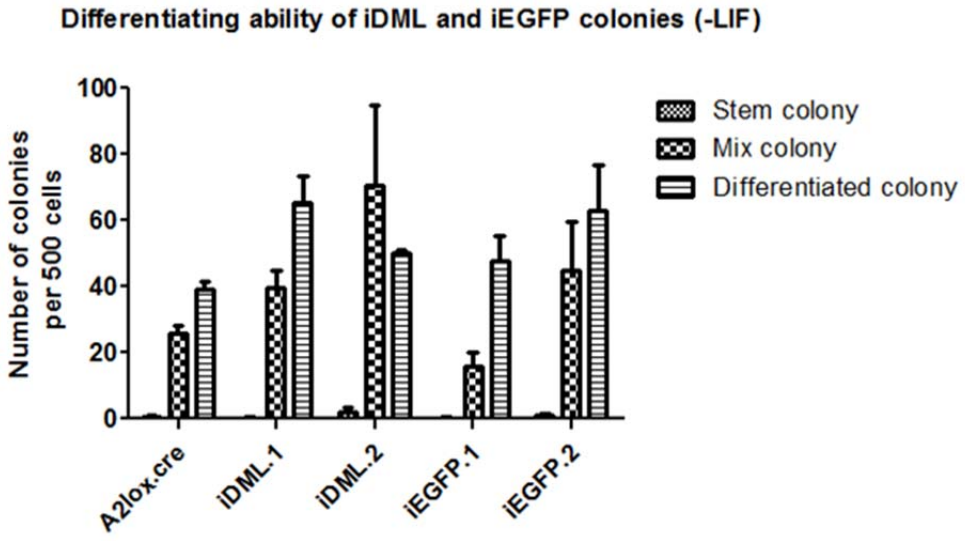


Figure 6.4: Self-renewal ability of parental ES cell line, iDML and iEGFP ES clones.

Numbers of stem colony, mix colony and differentiated colony were scored and shown in the presence (A) or absence (B) of LIF. Data represents 4 repeats from 2 independent experiments. Error bars represent SEM.

6.4.2 Validation of inhibition activity of DML-EGFP on Notch activity

6.4.2.1 DML-EGFP could inhibit exogenous Notch activity

To determine whether DML-EGFP expressed by iDML ES cell lines could inhibit Notch activity efficiently, we used the luciferase assay with 12xRBP-Jκ binding site luciferase reporter to measure Notch transcriptional activity upon expression of DML-EGFP. A2lox.cre parental ES cell line and two clones each of iDML and iEGFP were tested. Briefly, $1x10^5$ ES cells were plated into 24 well plates with or without addition of Dox in the absence of LIF. The next day the pCAG-NotchIC plasmid was transfected into all the ESCs to activate a high level of Notch activity then the RBP-Jκ luciferase reporter plasmid was co-transfected with the endogenous control renilla. As the positive control, pEGFP-DML-N3 plasmid was co-transfected with RBP-Jκ luciferase reporter and renilla without Dox addition (Figure 6.5 A). After 48 to 72 hours, cells were lysed and tested in the Dual-Luciferase® Reporter Assay System. The positive control, pEGFP-DML-N3 could inhibit Notch activity almost at 100% in all ES cell lines. Upon adding Dox, Notch activity in the two iDML ES clones were inhibited to approximately 50% while no effects were observed in the iEGFP ES clones or A2lox.cre parental ES cell line (Figure 6.5 B). According to these data, we concluded that DML-EGFP expressed by the iDML ES clones was able to inhibit exogenous NotchIC expression.

6.4.2.2 Effects of DML-EGFP on endogenous Notch activity

As described above, DML-EGFP is able to inhibit exogenous Notch activity conferred by NotchIC overexpression, which suggests that the DML-EGFP is functional. To address whether DML-EGFP could inhibit endogenous Notch activity during ES differentiation, we carried out quantitative RT-PCR to measure downstream gene expression of Notch signaling, including Hey1, Hes5 and Gata2. Differentiations were carried out in both EB system and $Flk1^+$ system on gelatin control.

a) **DML-EGFP inhibited endogenous Notch activity in day6 EBs**

Briefly, day1 EBs generated from A2lox.cre, iDML.1, iDML.2, iEGFP.1 and

iEGFP.2 ES cells were co-cultured on gelatin to day6. Doxycycline was added at 5μg/ml from day4 to day6. At day6, cells were harvested for quantitative RT-PCR at Hey1, Hes1 and Gata2. It was observed that addition of dox could inhibit Hey1 expression significantly on iDML.1 and iDML.2 ES cell line while no statistical difference were observed in parental ES cell line or iEGFP ES clone (Figure 6.6 A, **$p<0.01$, *$p<0.05$). Thus, this result indicated that DMl-EGFP induced by dox could efficiently inhibit Notch activity. Hes1 was also inhibited in iDML.1 and iDML.2 as well at 60% and 20% respectively, while Gata2 was not affected (Figure 6.6 B, C). This result was only done once so is not conclusive but it might indicate that Gata2 is not active or not regulated by Notch signaling in this EB differentiation system. iDML.1 ES clone seemed to have a better potential to inhibit Notch activity according to the inhibition percentage of Hey1 and Hes1, and so this cell line was used for further experiments.

b) **DML-EGFP did not affect endogenous Notch activity in Flk1$^+$ derived cells at day6**

To investigate how Notch affected haematopoetic differentiation of Flk1$^+$ cells, we further determined whether Notch activity in Flk1$^+$ derived cells could be abrogated by DML-EGFP. Thus, Flk1$^+$ cells were isolated from day4 suspension EBs generated from iDML.1 and iEGFP.1 ES cell line then cultured on gelatin to day6. We noticed that compared to day4 Flk1$^+$ cells, Notch activity decreased significantly at day6 in both iDML.1 and iEGFP.1 ES clones, which indicated that Notch activity in day6 Flk1$^+$ derived cells possessed lower Notch activity (Figure 6.7 A, **$p<0.01$). Based on this, Dox was added at 1μg/ml from day4 to day6 then cells were analysed at day6 with quantitative RT-PCR at Hey1 expression. GSI were added as the positive control with DMSO as the diluent control. Compared to DMSO diluent control, GSI inhibited Hey1 expression significantly (**$p<0.01$). Statistically, no difference of Hey1 expression was observed between minus Dox and plus Dox in either iDML.1 or iEGFP.1 ES cell line (Figure 6.7 B). In light of the Figure 6.6 A, no effect of Hey1 expression could be explained by the possibility that Notch activity in the Flk1$^+$ derived cells was not active enough to respond to DML-EGFP, or the expression level of DML-EGFP was not powerful enough to affect Notch activity in this system.

However, it will be necessary to screen more downstream genes to confirm this point. In addition, this test was carried out on $Flk1^+$ cells cultured on gelatin. To better understand effect of DML-EGFP on haematopoiesis in AM supporting microenvironment, FACSorting and qRT-PCR at downstream genes will be applied to purified $Flk1^+$ derived cells cultured on AM stromal cells in the future.

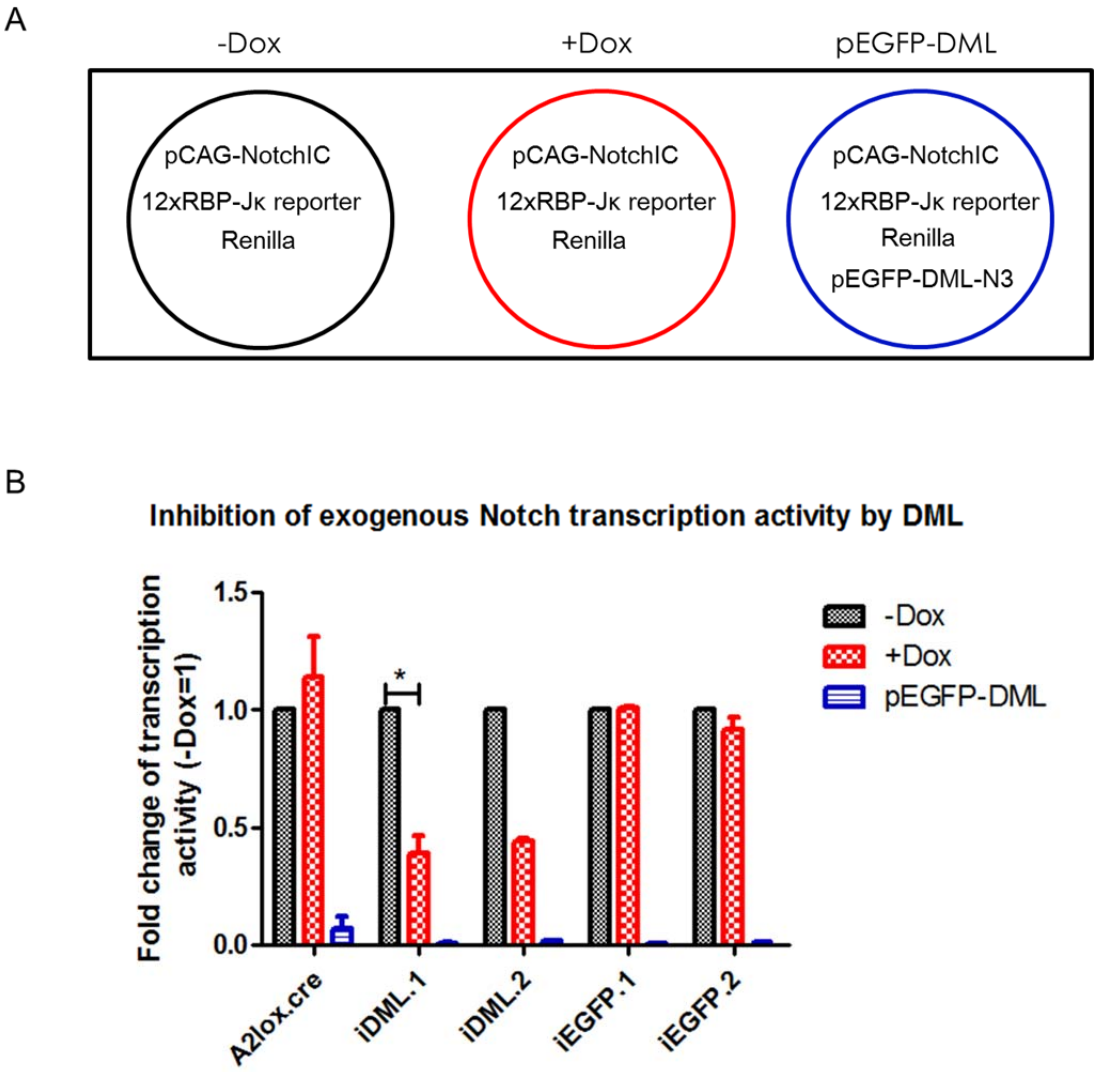


Figure 6.5 Inhibition of exogenous Notch activity by DML induced by addition of Dox.

(A) Scheme of transfection: pCAG-NotchIC was transfected to induce exogenous Notch activity with luciferase reporter and endogenous control. –Dox, $^{+}$Dox and co-transfection of pEGFP-DML-N3 groups were labeled with different colors; (B) Transcriptional activity was calculated as the ratio of luciferase to renilla readout. Then transcriptional activity of $^{+}$Dox (Red column) or pEGFP-DML (Blue column) were calculated as fold change to –Dox (Black column), which was assigned as "1". Data represented 3 independent experiments for A2lox.cre, iDML1 and iEGFP2 clones; 1 experiment for iDML2 and iEGFP1 ES clones. Error bars represents SEM. P value was calculated by Mann-Whitney test (*$p<0.05$).

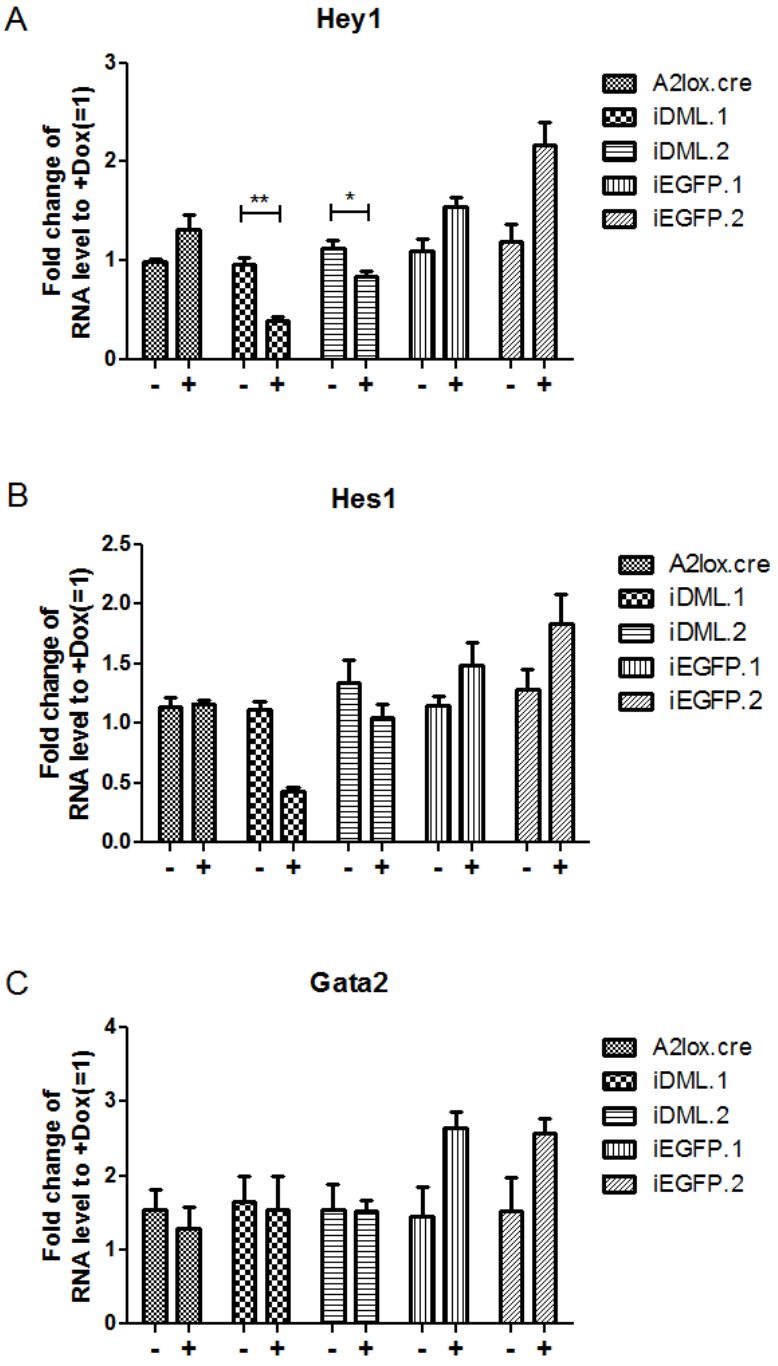


Figure 6.6 Inhibition of endogenous Notch activity by DML upon addition of Dox.

(A) Induction of DML by dox inhibited Hey1 expression significantly in day6 EBs on gelatin formed by iDML.1 and iDML.2 while no effects observed in EBs formed by either parental cells or iEGFP ES clones; (B) DML inhibited Hes1 expression in iDML.1 and iDML.2 ES clone at 60% and 20% respectively; (C) Gata2 was not affected by DML. Data represented 1 or 2 independent experiments. Error bars represents SEM from readouts of 6 PCR amplification wells. P value was calculated by Mann-Whitney test. (**$p<0.01$, *<0.05).

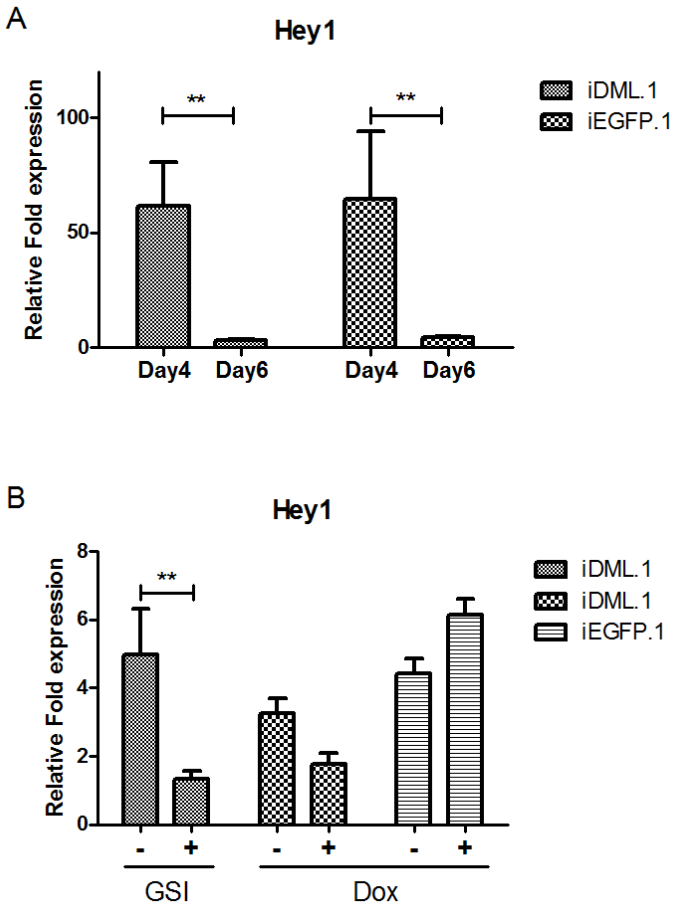


Figure 6.7 Effects of DML on Notch endogenous activity in Flk1$^+$ derived cells cultured on gelatin.

(A) According to Hey1, Notch activity decreased significantly at day6 compared to day4 in Flk1$^+$ derived cells in both iDML.1 and iEGFP.1 ES clones; (B) GSI could inhibit Hey1 expression in Flk1$^+$ derived cells at day6. No significant difference was observed when iDML or iEGFP alone was induced by Dox. Experiments represented 2 independent experiments. Error bars represented SEM from readouts of 6 PCR amplification wells. P value was calculated by Mann-Whitney test. (**p<0.01).

6.4.3 iDML and iEGFP ES cells could respond to AM stromal cell lines in EB/AM and Flk1$^+$/AM co-culture system

As described above, iDML and iEGFP ES clones were able to express DML or EGFP upon induction of dox. These ES cell lines could self-renew and differentiate under proper circumstances. Furthermore, DML could functionally inhibit Notch activity. To investigate effects of DML in ES-derived haematopoiesis, we first tested whether these ES cell lines could respond to the stimulation of AM stromal cell lines as well. Day1 EBs generated from iDML.1 and iEGFP.1 ES cells were co-cultured on gelatin control or irradiated AM stromal cells. After another 5 days of co-culture, cells were harvested and then seeded into methylcellulose assay to measure production of HPCs. It was found that AM stromal cells could enhance haematopoietic differentiation of iDML.1 by 3.5 fold compared to gelatin control (*$p<0.05$). Although not statistically significant, an increase in the number of HPCs after AM co-culture was observed twice using iEGFP.1 ES cell line by 2.8 fold on average. This result suggested that both iDML.1 and iEGFP.1 could respond to AM in EB/AM co-culture system (Figure 6.8 A).

To determine whether Flk1$^+$ cells derived from iDML.1 and iEGFP.1 ES cells could respond to AM stromal cells, Flk1$^+$ were purified from day4 suspension EBs then co-cultured on irradiated AM stromal cells or gelatin control. At day6, cells were harvested and seeded into methylcellulose assay to measure haematopoietic activity. In this preliminary experiment, it was found that AM stromal cells could enhance haematopoietic activity of Flk1$^+$ cells derived from both iDML.1 and iEGFP.1 ES cell lines by 6.8 fold and 3.1 fold respectively (Figure 6.8 B). Flk1$^-$ fraction was co-cultured on AM stromal cells and few HPCs were formed in methylcellulose assay (Data not shown). This result indicated that Flk1$^+$ population from both ES cell lines could respond to AM stromal cells in the Flk1$^+$/AM co-culture system. According to this, we suggested that iDML.1 and iEGFP.1 ES cell lines could be applied in further experiments to investigate the effects of DML on the haematopioetic differentiation of Flk1$^+$ cells in the microenvironment provided by AM stromal cells.

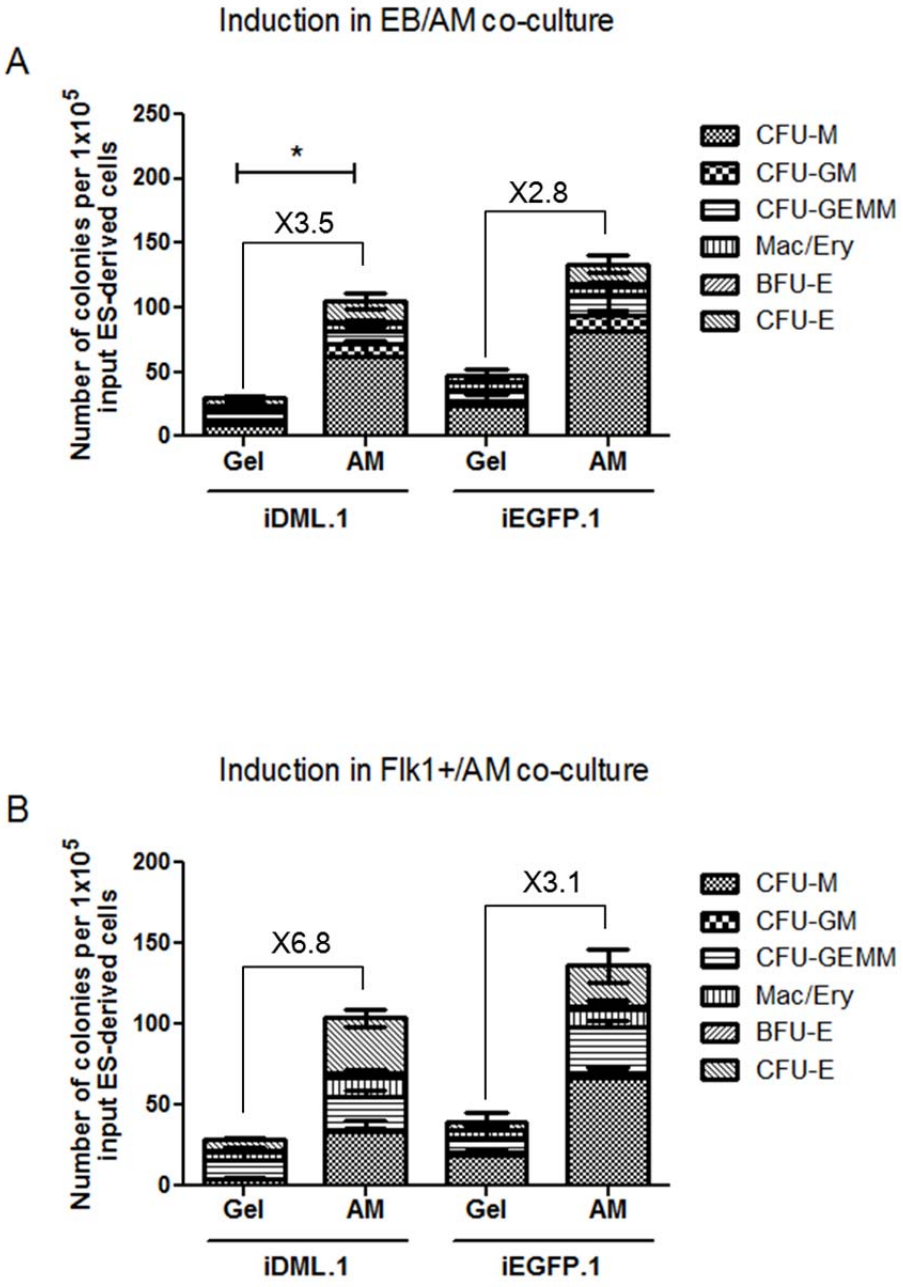


Figure 6.8 AM stromal cell line could enhance haematopoietic differentiation of iDML.1 and iEGFP.1 ES cells in EB/AM and Flk1^{+}/AM co-culture systems.

(A) In EB/AM co-culture system, AM stromal cells enhanced haematopoietic differentiation of iDML.1 by 3.5 folds and iEGFP.1 by 2.8 fold; (B) In Flk1^{+}/AM co-culture system, AM stromal cells enhanced haematopoietic differentiation of iDML.1 by 6.8 folds and iEGFP.1 by 3.1 fold. Data represented 1 to 3 independent experiments. Error bars represented SEM. P values were calculated by Mann-Whitney test (*$p<0.05$).

6.4.4 Effect of DML-EGFP on haematopoietic differentiation of Flk1$^+$ cells in Flk1$^+$/AM co-culture system

To analyze how DML-EGFP affected haematopoiesis in the microenvironment provided by AM stromal cells, we added Dox into Flk1$^+$/AM co-culture to induce DML-EGFP expression using iDML.1 ES cell line. EGFP was also induced in iEGFP.1 ES cell line as the control. Methylcellulose assay was applied to measure haematopoietic activity. It was found that there was no effect in total number of colonies upon treatment of doxycycline in either iDML.1 ES cell line or iEGFP.1 control ES cell line (Figure 6.9 A). This result implies that expression of DML-EGFP does not affect haematopoietic progenitor formation from Flk1$^+$ cells in the Flk1$^+$/AM co-culture system.

To better confirm this, flow cytometric analysis was applied to screen co-expression of CD41 and cKit. Correlating to the colony forming assay result, addition of Dox did not affect the frequency of CD41$^+$cKit$^+$ haematopoietic progenitor in this preliminary experiment (Figure 6.9 B). Thus, this experiment indicated that DML-EGFP does not affect the haematopoietic differentiation from haemangioblast like cells Flk1$^+$ in the AM supporting microenvironment. However repeats are necessary to confirm this. In light of the data represented in Figure 6.7, it could be explained that the Notch activity in Flk1$^+$derived haematopoiesis in Flk1$^+$/AM co-culture was at a basal level, which could not be affected by DML-EGFP. Nevertheless, to draw this conclusion, it is required to measure the Notch activity of Flk1$^+$ derived cells at day6 in AM co-culture and compared to day4. In addition, including GSI as the control will be necessary.

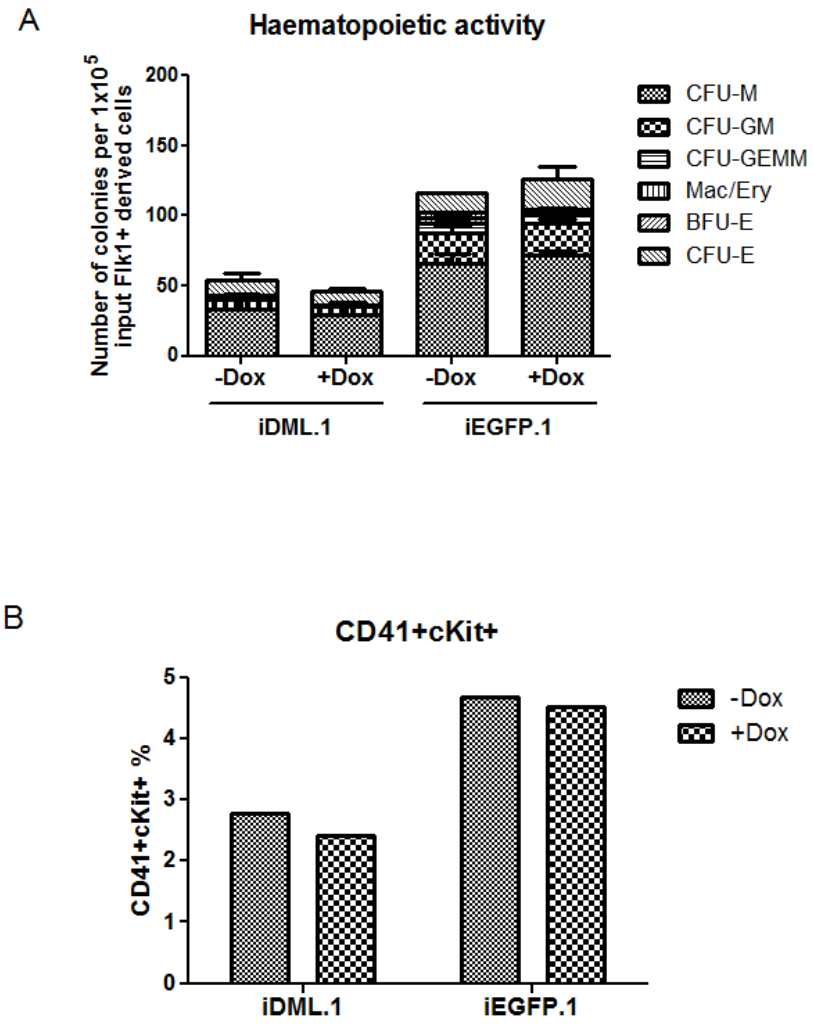


Figure 6.9 DML-EGFP did not affect Flk1$^+$ derived haematopoiesis in Flk1$^+$/AM co-culture system.

(A) Haematopoietic colony formation abilities of cells derived from Flk1$^+$ cells by both iDML.1 and iEGFP.1 ES clones were not affected by addition of Dox in Methylcellulose assay; (B) Frequency of CD41$^+$cKit$^+$ haematopoietic progenitors by iDML.1 and iEGFP.1 ES clones were not affected by addition of Dox. Data represented 1 independent experiment. Error bars represented SEM of readouts from two plating dishes.

6.5 Conclusion

- Doxycycline inducible DML-EGFP ES cell lines with normal karyotypes and self-renewal ability were established.

- iDML and iEGFP ES clones were able to respond to AM stromal cells in both EB/AM and $Flk1^+$/AM co-culture system showing increased haematopoietic differentiation.

- DML-EGFP expressed by iDML ES cell clone could inhibit exogenous Notch transcriptional activity induced by NotchIC and endogenous Hey1 and Hes1 RNA level in EBs.

- Notch activity decreased during differentiation from day 4 $Flk1^+$ cells; Notch activity in $Flk1^+$ derived cells at day6 could not be further inhibited by DML-EGFP.

- In one preliminary experiment, expression of DML-EGFP did not affect $Flk1^+$ derived haematopoiesis in $Flk1^+$/AM co-culture system indicating a basal level of Notch activity in $Flk1^+$ derived cells.

6.6 Discussion

6.6.1 Inhibition effect of dominant negative MAML1 (DML)

We noticed that the inhibition efficiency on RBP-Jκ transcription activity by DML-EGFP upon dox addition was relatively low compared to co-transfection of pEGFP-DML-N3 plasmid, which was near 100% (Figure 6.5). Expression level of DML could also be critical to knock down Notch activity to a certain level. Furthermore, although GSI could inhibit endogenous Hey1 expression in $Flk1^{+}$ derived cells, DML-EGFP did not inhibit Hey1 significantly (Figure 6.7). Thus, we suggest that the effect of dominant negative MAML1 on Notch signaling transduction was more moderate compared to GSI or Notch activity dependent. Here we discuss the potential factors which could affect the inhibition effect of DML-EGFP on Notch activity.

Human dominant negative MAML in murine system

The Mastermind gene was first identified in Drosophila as a neurogenic gene (Smoller et al., 1999). The family of human mastermind-like genes (MAML) was identified later in 2000 revealing a biological function of MAML genes in Notch signaling pathway (Wu et al., 2000; Wu et al., 2002). Based on this, Weng and his colleagues found a truncated form of human MAML1 called dominant negative MAML1 which could abolish Notch signaling and suppress the growth of pre-T acute lymphoblastic leukemia cells, (Weng et al., 2003). This was the source of the DML-EGFP that was used in this project and so in fact we used the human DNMAML1 homologue in our murine ES cell system.

In 2004, Wu and colleagues cloned the murine mastermind-like 1 (MAML1) gene which shared 85% of amino acid sequence identity to human MAML1. The mouse MAML1 contained the basic domain at the N terminus which was similar to human MAML1 for interaction with Notch intracellular domain and two acidic domains with transcriptional activities. They also confirmed the interaction between MAML1 and Notch receptor 1-4 and found MAML1 could function as the transcription co-activator in Notch signaling pathway by activating Hes1 promoter in human U20S

cells exposed to NIH3T3 overexpressing Jagged2 (Wu and Griffin, 2004). Thus, in mouse development, murine MAML1 was also a critical transcriptional co-activator for Notch signaling pathway. Although human MAML1 was applied here in murine system, it has been demonstrated by several groups that dominant negative human MAML1 could efficiently abrogate Notch activity in the murine system including T-lymphoblastic cell line BW5147, T-ALL cell line, megakaryocyte development *in vivo*, bone marrow derived LSK exposed to Delta-like 1 and lymphoid differentiation. We are the first group however to apply that human DNMAML1 in a murine ES system.

Compensation by murine MAML2 and MAML3

So far, the MAML family was found to consist of 3 members including MAML1, MAML2 and MAML3 in both human and murine system. These three human MAML genes are highly homologous to their murine homologies with sequence identity ranging from 85 to 90%. For human homologies, human MAML1, MAML2 and MAML3 shared conserved protein sequence at basic domain to bind NotchIC (Wu and Griffin, 2004). Although most studies have focused on MAML1, it has been shown that both MAML2 could also interact with Notch receptor 1-4 while MAML3 function more efficiently with Notch4. MAML1 and MAML2 could both function as co-activators of Notch signaling while MAML3 has weaker transcription activation capacity in Notch signaling pathway. Similarly in murine work, it is found that murine MAML1, MAML2, and MAML3 were involved in the Notch signaling (Oyama et al., 2011; Sasaki et al., 2011; Wu and Griffin, 2004). Thus, although only human dominant negative MAML1 was applied here, it should be able to interfere the interaction of murine MAML2 and MAML3 with Notch receptors. Nevertheless, in addition to the basic region at N-terminus of MAML to bind Notch receptor, the function of rest regions of murine MAML2 and MAML3 has not been well clarified. It would be possible that they have other potential abilities to affect Notch activation which would compensate the effect of human DNMAML1. To better understand how human DNMAML1 affects Notch signaling in this murine system, it will be necessary to check expression of mouse MAML1, MAMl2, and MAML3 expression during differentiation.

Induction expression level and efficiency by addition of doxycycline

The inhibitory effect of DNMAML1 was found to correlate to the expression level (Weng et al., 2003). Under regulation of TRE promoter, the expression level of DML-EGFP was also dependent on the dose of Dox. Here it was determined that 1μg/ml of Dox was enough to induce best DML-EGFP expression. Higher dose of Dox was not recommended because other groups have claimed the toxicity of high dose of Dox (Das et al., 2010). Of note, by comparing Figure 6.2 and Figure 6.3, the induced proportion of DML-EGFP decreased during differentiation according to flow cytometric analysis. It could be explained that ROSA26 locus or HPRT locus is silenced during differentiation. This situation needed to be verified for further experiments.

6.6.2 Threshold of Notch activity to respond to DNMAML1

We noticed that Notch activity decreased significantly during differentiation of $Flk1^+$ cells from day4 to day6 (Figure 6.9A). Hey1 expression was not affected by addition of dox while it was significantly inhibited by GSI. When strong Notch activity was induced by pCAG-NotchIC, DML-EGFP could inhibit transcription activity efficiently. Similarly, when DML-EGFP was induced in EBs, Hey1 could also be inhibited. As introduced previously in chapter3, Hey1 expression was quite consistent during EB differentiation from day3 to day6 on gelatin, which was different from the decrease in $Flk1^+$ derived cells. Thus we suggest that the level of Notch activity is a pivotal factor in determining whether DNMAML1 could abrogate Notch activity or not. GSI was more efficient compared to DNMAML1 which could even knock down already low levels Notch signaling. This is not surprising because it has also been demonstrated in several studies using this dominant negative strategy. Maillard and his colleagues reported that Notch signaling was dispensable for maintenance of the adult haematopoietic stem cells. They found that bone marrow derived LSK progenitors were exposed to a lower intensity of Notch signaling compared to progenitors according to receptors and downstream gene expression, in which case induction of DNMAML1 did not change the expression of Hes1, Dtx1 or Runx1. However after exposure to the exogenous Delta-like 1 by OP9-DL1 stromal cells, Hes1 and Dtx1 expression were induced and could be inhibited by DNMAML1

(Maillard et al., 2008). Similarly, it was also claimed that DNMAML1 could abrogate megakaryocyte development. In this study, megakaryocyte-erythroid progenitors possessed higher Notch activity compared to granulocyte-macrophage progenitors or earlier common progenitors (Mercher et al., 2008). Therefore, we suggested that to inhibit Notch activity DNMAML1 requires a higher signal level of Notch, which could explain why inhibition was observed in luciferase assay and EBs but not in $Flk1^+$ derived cells in our study. Nevertheless, except for Hey1, more downstream genes of Notch signaling still need to be screened to measure effect of DNMAML1.

6.6.3 Advantages and disadvantages of this dox dependent DML-EGFP inducible ES cell line

Specificity of DNMAML1 on Notch activity

So far, multiple strategies have been developed to knock down or knock out Notch signaling pathway. At first, strategies to abrogate Notch signaling were non-specific enough or could be compensated by redundant effects by other Notch components, including ADAM inhibitors, γ-secretase inhibitors (GSI), and deficiency of receptors or ligands (Duncan et al., 2005; Mancini et al., 2005; Radtke et al., 1999). Hence, specific modulation of Notch signaling became critical in recent studies. For examples, introducing dominant-negative form of RBP-Jκ, inactivation of RBP-Jκ gene, overexpression of Numb or Deltex1 have been applied to block Notch (Cheng et al., 2008; Duncan et al., 2005; Han et al., 2002; Maillard et al., 2004; Tanigaki et al., 2002). Here, the system we set up could also inhibit Notch more specifically compared to the inhibitors or inactivation of receptors or ligands.

Although this system was more specific to modulate Notch signaling compared to previous studies, the possibility that DNMAML1 might interfere with other Notch-independent pathways cannot be ruled out. As Numb was involved in Wnt signaling pathway, Deltex1 was involved in BMP pathways, MAML1 was reported to interact with MEF2C, p53, β-catenin and Mesp2 (Donner et al., 2007; Firestein and Hahn, 2009; Sasaki et al., 2011; Shen et al., 2006). This was not surprising because MAML1 contains conserved domains that bind ankyrin repeats and other

co-activators, which coupled with the tissue-specific transcription factors and downstream genes to make it possible for Notch signaling pathway to regulate diverse processes of development.

Context-dependent

The efficiency and context to function also needs to be borne in mind. As it was found that overexpression of Deltex1 to antagonize Notch signaling were discovered in the T-cell differentiation, DNMAML1 also had limitations. Data represented here showed that DNMAML1 could only function when exposed to strong Notch signaling, in which case, utilization of this strategy is context dependent. In spite of this, this doxycycline-inducible ES cell line could provide a powerful platform for molecular investigation of Notch signaling pathway in many fields during development. It will be interesting to add Notch ligands like Jagged1 into the $FLk1^{+}$/AM co-culture system and measure whether DML-EGFP could inhibit downstream genes efficiently.

Spatio-temporal possibility

This doxycycline inducible system has advantages over constitutive or inducible systems like Cre, ER (estrogen receptor) system, which makes it possible to answer the question in more defined spatio-temporal window. In the future, we still need to further confirm the reversible ability of these ES cell lines and optimize the time length of doxycycline pulse.

Chapter 7: Summary and Perspectives

7.1 Summary

As described in the introduction, regulation of Notch signaling in primitive and definitive haematopoiesis is likely to be different dependent on their origins of yolk sac and intra-embryonic P-Sp/AGM region. The ES cell *in vitro* system has been widely considered to represent haematopoiesis of the yolk sac. To better understand how Notch regulated the AGM-derived haematopoiesis and overcome the difficulties of *in vivo* studies such as the effects of Notch in other tissues during development or early embryonic lethality, we established this co-culture system of AGM-derived stromal cells with ES cells. Intrigued by previously studies of co-culture system of EBs on AGM-derived stromal cell lines and the GSI experiment revealing the involvement of Notch signaling pathway, we further developed a $Flk1^+$/AM co-culture system to investigate the role of Notch signaling in $Flk1^+$ derived haematopoiesis in the AM14.1C4 microenvironment.

In EB/stroma co-culture system, we noted that there was no direct correlation between the levels of Notch ligand expression in the different stromal cell lines and their induction ability, or correlation between Notch activity in EBs with their haematopoietic activity. Thus, Notch signaling pathway in EBs was required in the enhancing co-culture to enhance haematopoiesis, but not further increased compared to the non-enhancing ones. Of note, Runx1 was reduced upon GSI addition from day4 to day6 in EBs. This confirmed Runx1 could be a downstream target gene of Notch signaling and Notch signaling regulated definitive haematopoiesis via Runx1 from day4 to day6, which was post mesoderm formation. In addition, the haematopoietic enhancing stromal cell line AM14.1C4 had a higher level of Notch activity suggesting the inherent Notch activity in stromal cells could be the key regulator to determine the microenvironment for enhancing haematopoiesis in EBs in co-culture.

As noted, the complexity of the EB/stroma co-culture system makes it difficult to analyze the underlying molecular mechanism. AM co-culture did not affect commitment to $Flk1^+$ from day1 EBs in EB/AM system. Thus, $Flk1^+$ cells were co-cultured directly onto stromal cells and AM14 displayed a moderate enhancing

effect on haematopoiesis. AM stromal cells could promote formation and expansion of $CD41^+cKit^+$ (day6) and $CD45^+$ expansion (day9) derived from $Flk1^+$ cells. In this $Flk1^+$/AM co-culture system, reduction of $CD41^+cKit^+$ haematopoietic progenitors by activating NotchIC in $Flk1^+$ cells indicated an inhibitory effect of ectopic NotchIC on haematopoiesis, which contradicted to the EB/AM co-culture system that Notch signaling pathway was required for the haematopoietic enhancing activity of AM stroma. This inhibitory effect was further confirmed by colony assay and revealed inhibition of MYELOID PROGENITOR formation from $Flk1^+$ cells. Ectopic NotchIC also inhibited erythroid differentiation from $Flk1^+$ cells, but at a more terminal stage. Finally, doxycycline inducible DML-EGFP ES cell lines were established and able to respond to AM stromal cells in both EB/AM and $Flk1^+$/AM co-culture system showing increased haematopoietic differentiation. DML-EGFP expressed by iDML ES cell clone could inhibit exogenous Notch transcriptional activity and endogenous Hey1 and Hes1 RNA level in EBs. However Notch activity in $Flk1^+$ cells could not be further inhibited by DML-EGFP, which could be accounted for the low Notch activity in $Flk1^+$ derived cells.

7.2 Discussion and Perspectives

By pooling together data from this project, we considered several interesting points which are worthy of further investigation.

7.2.1 Application of NIC-C5 and iDML-EGFP ES cell line in alternative haematopoietic differentiation systems.

As referred, several systems have been developed for ES-derived haematopoietic differentiation *in vitro* in which the molecular networks are system-dependent. These systems also vary in inducing/enhancing efficiency, type and function of HPCs and HSCs for example, repopulating capacity. In this project, 4-OHT inducible NIC-C5 and doxycycline inducible iDML-EGFP ES cell lines were applied to spatiotemporally induce or knock-down Notch activity in $Flk1^+$ derived haematopoiesis in AM supporting microenvironment, which possibly provided an enhancing/supporting signals in AGM region *in vivo*. Of note, it is worthwhile to

apply these two inducible ES cell lines in other published systems to characterize involvements of Notch signaling pathway.

It is easier to start with a simple and routine differentiation system using suspension EBs without addition of stromal cells or cytokines. In addition to produce three germlayer like cells, suspension EB differentiation system *in vitro* was widely applied in haematopoietic differentiation and suggested to differentiate in parallel to yolk sac derived haematopoiesis *in vivo*, in which mesoderm, haemangioblast, primitive/definitive HPCs and haematopoietic cells are formed but not repopulating HSCs. By forming suspension EBs using these two ES cell lines to induce ectopic NotchIC or DNMAML temporally, it will be interesting to measure how Notch signaling pathway affect mesoderm formation, primitive and definitive haematopoietic differentiation. Furthermore, because suspension EBs were widely applied amoung different groups thus this will lead us to compare our inducible ES cell lines to published tools established to modulate Notch activity in suspension EBs.

7.2.2 Investigation of haematopoietic enhancing effects of AM14 stromal cells on Flk1$^+$ cells at cellular level

Interestingly, AM14.1C4 (AM14) stromal cells did not affect Flk1$^+$ formation in the EB/AM co-culture system which indicated a potential to enhance haematopoietic differentiation from Flk1$^+$ cells. It will be necessary to monitor the cell types formed in AM14 co-culture between Flk1$^+$ and HPCs and compared to gelatin control and other stromal cells. This will lead us to understand the differentiation roadmap from Flk1$^+$ to HPCs and HSCs in the AGM microenvironment.

7.2.3 Niche requirements for haematopoiesis and role of Notch signaling is niche dependent

In the EB/AM co-culture system, Runx1 was down regulated when GSI was applied to inhibit Notch signaling, which indicated Notch could regulate definitive haematopoiesis in AM14 microenvironment. However, in the Flk1$^+$/AM co-culture

system, Gata2 and Runx1 were not up regulated by ectopic NotchIC expression. This contradicts the studies of Robert-Moreno (AGM haematopoiesis and 32D cell line) and Burns (zebrafish model) claiming the close association of Notch signaling pathway with Gata2 and Runx1 which also focused on the haematopoiesis in AGM region. Here we consider another possibility that the way Notch regulated the definitive haematopoiesis is niche-dependent.

For example, AM14, the stromal cell line derived from dorsal aorta and surrounding mesenchymal area of AGM region at E11, was reported to be vascular smooth muscle cell-like and thus derived from mesenchymal cells (Charbord et al., 2002). The surrounding mesenchyme area has been suggested to be a potential niche for the further differentiation of the haemangioblast to pre-HSCs, haemogenic endothelium formation or pre-HSCs maturation. Although AM14 was applied in both co-culture systems, the AM14 stromal cells function as the inducing factor (Gordon-Keylock et al., 2011) in EB/AM co-culture while for $Flk1^{+}$/AM co-culture, AM14 stroma function more like a supporting factor according to preliminary apoptosis assay. Thus, other niche-like cells could be required for mimicking the *in vivo* definitive haematopoiesis.

It was noted that paraxial mesoderm could function as the supporting/inducing microenvironment for haematopoiesis via HoxB4 overexpression (Jackson et al., 2012). In our system, Delta-like3 and HoxB4 were increased at certain time points in EB/AM co-culture compared to gelatin suggesting an involvement of paraxial mesoderm in regulation of haematopoiesis. In addition, according to *in vivo* data or ES model, VEGF secreted by endoderm was reported to be critical for further endothelial and haematopoietic differentiation of $Flk1^{+}$ cells P-Sp/AGM (reviewed by (Cumano and Godin, 2007)). Although VEGF expression has not been validated in AM14 stromal cells, we assumed that the endoderm-like cells in EBs could be a potential source for VEGF secretion. Thus, endoderm should be involved in regulating the microenvironment for AGM-derived haematopoiesis.

Therefore, $Flk1^{+}$/AM co-culture was designed to ask how Notch regulated $Flk1^{+}$

derived haematopoiesis in a specific way avoiding the effect of Notch in non-haematopoietic cells. However to better define the role of Notch signaling in AGM-derived haematopoiesis *in vitro*, a chimeric cell mixing experiment to construct the intact microenvironment will be necessary to and reflect the *in vivo* situation. This experiment would involve generating EBs with 7a-GFP ES cell line which expressed GFP constitutively and R26-NIC-C5 ES cell lines. The Flk1- (non-hameatopoietic) fraction from 7a-GFP day 4 EBs and the $Flk1^+$ fraction from R26-NIC-C5 day 4EBs would then be mixed and co-cultured on AM14 together so that Flk1- by 7a-GFP ES cells and AM14 stromal could construct the niche for the $Flk1^+$ fraction derived from the R26-NIC-C5 ES cell line. 4-OHT would then be added and further characterization of effect of ectopic NotchIC in haematopoiesis of R26-NIC-C5 ES cell line would be carried out using CFUs, $\%CD41^+cKit^+$ as well as Gata2 and Runx1 expression and compared to untreated samples, or just compared the $hCD2^-$ and $hCD2^+$ population (Appendix Figure S7.1 A).

In addition, Notch signaling in the microenvironment could be critical to determine its effect on haematopoiesis. For example, Notch activity in AM14 stroma could be involved in the haematopoietic regulation as revealed by GSI experiment in EB/AM co-culture (Gordon-Keylock et al., 2011). Thus, another two experiments could be applied to modify Notch signaling in the microenvironment. One would be to down-regulate by introducing pCAG-DNMAML or up-regulate by introducing pCAG-NotchIC into the AM14 stromal cells then co-culture carried out with EBs or $Flk1^+$ cells. The other is to do the mix co-culture by mixing $Flk1^+$ fraction from 7a-GFP cells with Flk1- fraction from R26-NIC-C5 ES cells to investigate effect on $Flk1^+$ derived haematopoietic by activating Notch signaling in Flk1- microenvironment (Appendix Figure S7.1 B).

To conclude, by setting up this *in vitro* co-culture system of AM14 with mixtures of different ES-derived population, it will be more flexible to introduce Notch signaling modification and ask specific questions.

7.2.4 Measurement of effect of ectopic Notch in haematopoiesis in specifically defined population

It was well acknowledged that different cell types emerged in ES cell differentiation. Even though cell sorting for particular population like $Flk1^+$ was widely applied to investigate underlying molecular mechanism in this specific population, the timing and mix population issue that cells derived from $Flk1^+$ could not be at the identical stage or direction during differentiation increased the complexity of system. Plus, gene expression could be present in different population with different function. Thus, it will be worthwhile to compare haematopoietic related genes like SCL, Gata2, and Runx1 in sorted $hCD2^+$ and $hCD2^-$ population from a better defined cellular population such as the haemogenic endothelium.

Furthermore, single cell PCR strategy will be another option to monitor the effect of NotchIC on single cell decision in haematopoietic differentiation. Based on the purified population (ie haemogenic endothelium) during differentiation, PCR screen of related genes on single cells derived from the purified population will be more informative and accurate, though technically more difficult and higher throughput screening required.

7.2.5 Dissect differentiation process and determine the population first affected by ectopic NotchIC

As it was suggested in chapter5, inhibition of several genes related to haematopoiesis including Gata1, PU.1, SCL/Lmo2 and hemoglobin, but n Gata2 or Runx1 was not inhibited upon ectopic Notch activation. This data suggested a possibility that inhibition of ectopic NotchIC took place at haematopoietic lineage determination stage. However SCL/Lmo2 could also regulate the haemogenic endothelium formation from haemangioblast. Thus, it is necessary to trace back and determine whether an earlier stage is inhibited before the HPCs formation. For example, $Tie2^{high}cKit^+CD41$- or $Flk1^+$ VE-$Cadherin^+$ population will be first checked to see whether haemogenic endothelium from $Flk1^+$ is abrogated. In addition to identify the population which could be affected by ectopic NotchIC, it is critical to understand

the underlying mechanism. Thus, assays for apoptotic, proliferate and survival ability will be further checked on the affected population or its precursors.

7.2.6 Interaction of Notch signaling with other haematopoietic regulators

HoxB4 is a transcription factor, which has been found in $CD34^+$ haematopoietic precursors derived from human bone marrow (Sauvageau et al., 1994). In addition, it was reported that HoxB4 could enhanced ES-derived haematopoiesis in a non-cell autonomous way in EB differentiation system (Jackson et al., 2012). Published data has revealed the regulation of Notch signaling by HoxB4 in microarray data (Schiedlmeier et al., 2007). Thus it will be worthwhile to investigate the interaction of Notch signaling and HoxB4 in ES-derived haematopoiesis. We have established a ES cell lines in which HoxB4-ERT2 was introduced to iDML-EGFP ES cell line with a random integration under pCAG promoter. This ES cell line will provide a platform to further understanding whether Notch signaling is involved in the regulation by HoxB4 in haematopoiesis.

7.2.7 *In vivo* study

In this project, R26-NIC-C5 ES cell line to induce moderate Notch and iDML-EGFP ES cell line to inhibit Notch specifically were applied to investigate effects of Notch signaling in HPCs formation from $Flk1^+$ population or EBs in microenvironment supported by AM14. However, the most direct strategy to understand role of Notch signaling in AGM region will be *in vivo* models. Thus, it will be useful to establish mice models using these two ES cell lines, respectively. HPCs and HSCs production could be measured in these two models with or without treatment of 4-OHT and doxycycline.

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Appendix

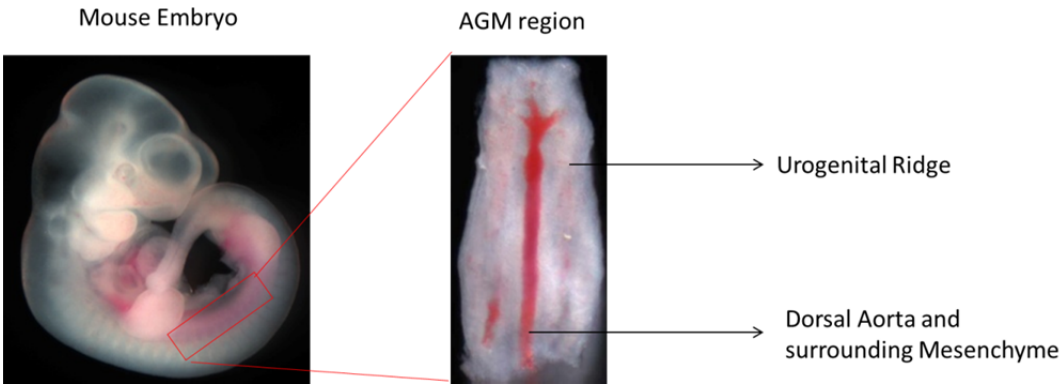

Figure S1.1 Stromal cell lines were derived from AGM sub-regions in mid-gestational (E10/E11) mouse embryos.

Dosrasal Aorta and surrounding mesenchyme (AM), Urogenital Ridge (UGR/UG). Pictures provied by Gordon-Keylock.

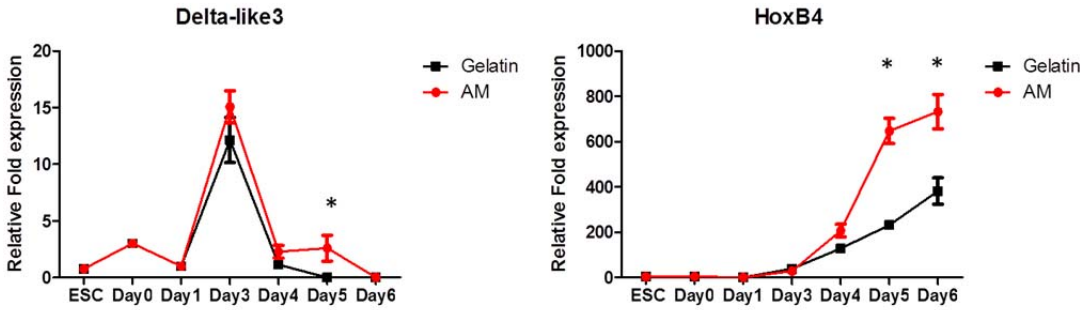

Figure S4.1 Expression pattern of genes related to paraxial mesoderm in EBs co-cultured on gelatin and AM stromal cell lines.

One day hanging drop EBs were co-cultured to day6 and sorted from AM stroma or gelatin by FACS and qRT-PCR carried out for Delta-like3 (A), HoxB4 (B) expression kinetics. One day EBs were used as calibrator (value assigned as 1); 18s was used as the endogenous control; Dela-like3 in EBs co-cultured on AM stromal cells was expressed significantly higher than gelatin at day5. AM14 stromal cells enhanced HoxB4 expression significantly compared to gelatin at day5 and 6. Data represent 3 independent experiments. Error bars represented SEM. P-values were calculated with Mann-Whitney test (*$p<0.05$).

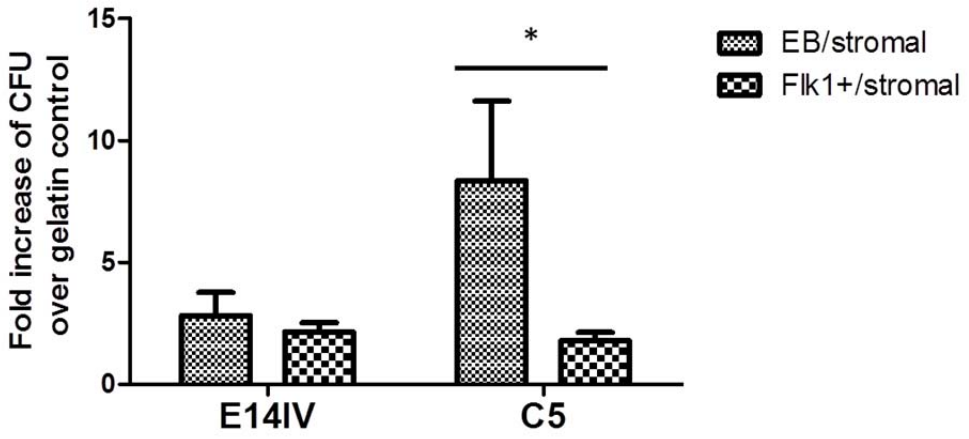

Figure S4.2 Comparison of haematopoietic enhancing efficiency of EB/AM and Flk1$^+$/AM co-culture system.

Enhancement fold was calculated as CFU formed in AM co-culture normalized to CFU formed in gelatin control. For E14IV ES cells, effeiciency of EB/AM and Flk1$^+$/AM co-culture systems were comparable. For C5 ES cells, AM could enhance EBs haematopoiesis significantly higher than Flk1$^+$. Error bars represented SEM. P-values were calculated with Mann-Whitney test (*$p<0.05$).

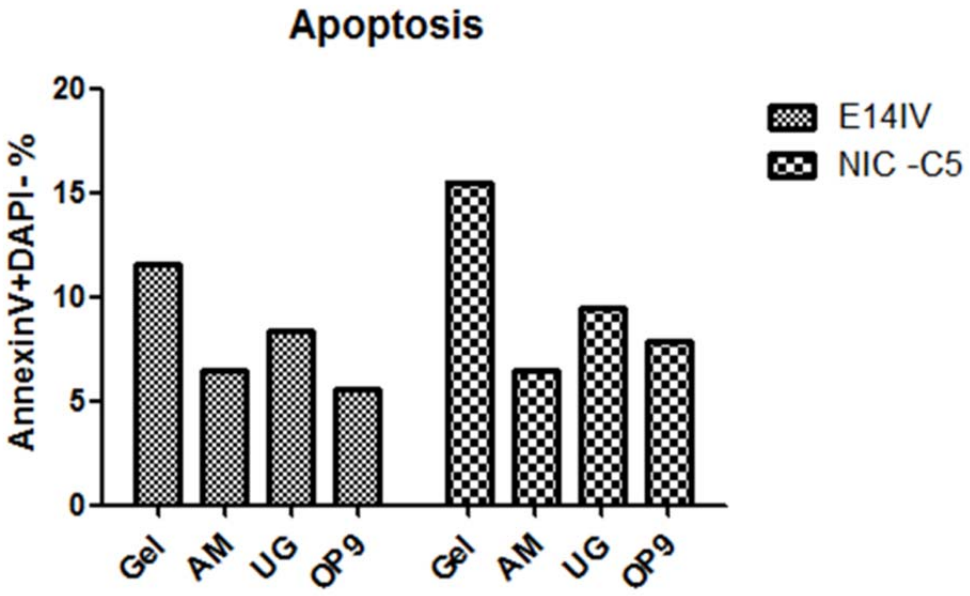

Figure S4.3 Co-culture with stromal cells promote proliferation/survival ability of Flk1$^+$ cells.

Flk1$^+$ cells differentiated from E14IV or NIC-C5 ES cell lines wre co-cultured on gelatin control or AM14.1C4, UG26.1B6 and OP9 stromal cells to day 6. Cells were then harvested and analyzed in apoptosis assay using flow cytometry. Figure presented the frequency of cells proceeding apoptosis. In this preliminary experiment, co-culture of Flk1$^+$ cells on stromal cells could promote cell proliferation/survival ability via reducing apoptotic cells (AnnexinV$^+$DAPI-) compared to gelatin control.

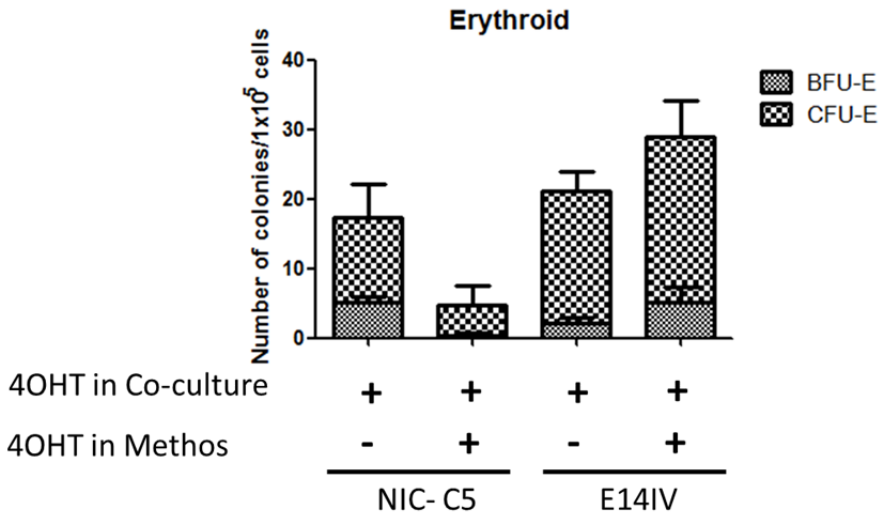

Figure S5.1 Longer treatment of 4-OHT into methylcellulose further inhibited formation of erythroid colonies from Flk1^{+} cells.

Addition of 4-OHT into both co-culture to day 6 AND methylcellulose assay could further inhibit number of erythroid colonies compared to the one only added in co-culture. No difference was observed in E14IV. Data represent 2 independent experiments.

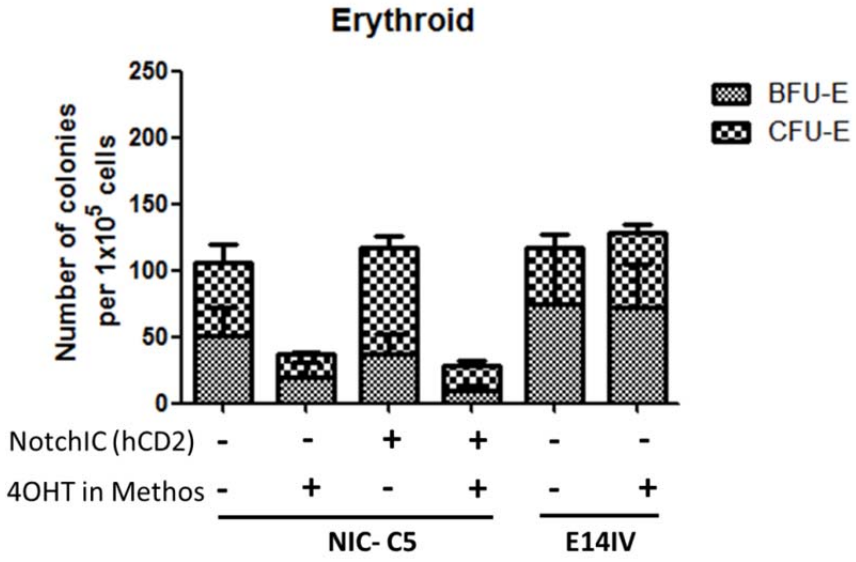

Figure S5.2 Addition of 4-OHT inhibited number of erythroid colonies in both hCD2$^-$ and hCD2$^+$ fraction indicating a toxic issue.

hCD2$^-$ and hCD2$^+$ fraction from co-culture with addition of 4-OHT were separated by FACS sorting and plated into methylcellulose assay with addition of 4-OHT. hCD2$^+$ fraction, which should not further be affected by ectopic NotchIC, displayed an inhibition on erythoid colonies indicated a toxic issue. No difference was observed in E14IV control. Data represent 2 independent experiments.

Apoptosis increased by ectopic NotchIC
(AnnexinV+DAPI-%)

	E14IV		NIC -C5	
	-4OHT	+4OHT	-4OHT	+4OHT
Day6	4.55%	4.63%	2.57%	3.25%
Day9	4.1%	4.15%	4.48%	5.93%

Figure S5.3 Activation of ectopic NotchICl slightly increased apoptosis of Flk1$^+$ derived cells.

Flk1$^+$ cells were purified and co-cultured on AM14.1C4 to day6 with or without addition of 4-OHT. Flow cytometry was carried out at day6 and day9 to measure apoptotic percentage. E14IV was applied as the control. Data represent one experiment.

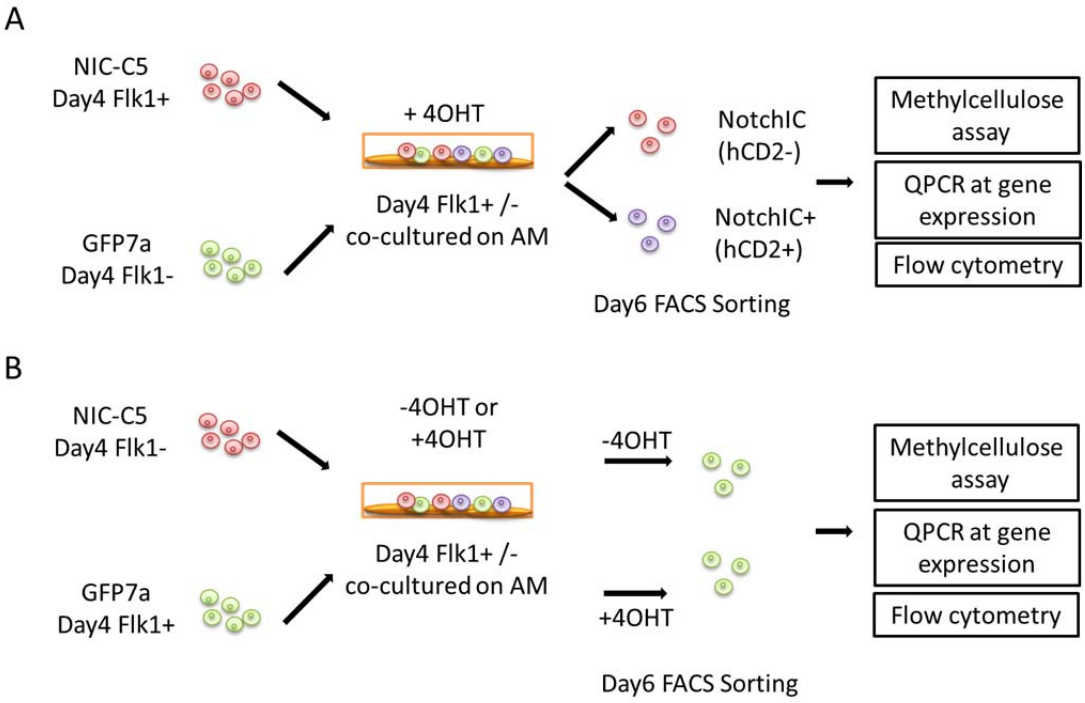

Figure S7.1 Scheme of a chimeric cell mixing experiment to construct the intact microenvironment for Notch signaling analysis.

(A) Flk1^{+} fraction by NIC-C5 were mixed with Flk1- fraction by 7a-GFP and co-cultured on AM14 stromal cells with addition of 4-OHT. At day6 or later hCD2^{-} and hCD2^{+} were separated and haematopoietic activity or other lineages assessed in CFUs, qPCR and flow cytometry analysis. (B) Flk1^{+} fraction by 7a-GFP were mixed with Flk1- fraction by NIC-C5 and co-cultured on AM14 stromal cells with or without addition of 4-OHT. At day6 or later 7a-GFP ES-derived cells were separated and haematopoietic activity or other lineages assessed in CFUs, qPCR and flow cytometry analysis.

	Usage	Construction	Resource
p2lox.EGFP	Introducing EGFP into HPRT locus in A2lox.cre cell line		Iacovino et al., 2011a
p2lox.DML-EGFP	Introducing DML-EGFP into HPRT locus	DML-EGFP was digested from pEGFP-DML-N3 plasmid using BglII (blunted) and NotI to XhoI (blunted) /NotI site in p2lox.EGFP	
pEGFP-DML-N3	Cloning DML-EGFP into p2lox backbone		Weng et al., 2003 Maillard et al., 2004
pCAG -NotchIC	Overexpressing NotchIC in A2lox.cre, A2lox.DML EGFP and A2lox.EGFP cell line for luciferase assay		Lowell et al.,2006
pCAG –Jagged1	Overexpressing Jagged1 in Cos7 cells as positive control		Lowell et al.,2006
Renilla	Internal control of Notch activity luciferase assay		Lab stock
pGL3	Negative control of Notch activity luciferase assay		Lab stock

Table 2.1 Information of plasmids applied in this project.

Gene	Forward Primer	Probe	Reverse Primer
Dll3	GCCTGATGGCCTCGTACGT	TTTCAATGACGAGGGAGAAGGT	TGCCCTTCCGCGATG
Flk-1	CAAAAACCAATATGCCCTGATTG	GCTGACACGTTGGCAGCTT	GACCAGCGTACTTACAGTTTT
Tbx6	TGTTAAGCTCACCAACAGCACACT	AGGCTGGTACTTGTGCATCGA	CCCCCATGGCCACC
β-major	GACAAGCTGCATGTGGATCCT	GGTGGTGGCCCACAATCA	AGAACTTCAGGCTCCTGG
β-H1	GAGAAGGCAGCTATCACAAGCA	ATCAGGAGCCTTCCCAGAGTT	CTGGGATAAAGTGGACTTG
Hprt	GCTCGAGATGTCATGAAGGAGA	AAAGAACTTATAGCCCCCCTTGA	CCATCACATTGTGGCCCTCTGTGTG
Hoxb4	CCTGGATGCGCAAAGTTCA	GTCAGGTAGCGATTGTAGTGAAACTC	CCAGCAGGTCCTGGAG
Hey1	GCAGGAGGGAAAGGTTATTTTGA	CGAAACCCCAAACTCCGATAG	CGCCCTGGCTATGG
Hes5	TGCTCAGTCCCAAGGAGAAAA	CGGTCCCGACGCATCTT	ACTGCGGAAGCCGGT
Notch1	TGCATGGATGTCAATGTTCGA	ACTGCAGGAGGCAATCATGAG	ACCAGATGGCTTCACAC
Gata2	CCCAAGCGGAGGCTGTCT	CTGCCAGAAGAGCGG	TCGTCTGACAATTTGCACAACA
Runx1	GATTCAACGACCTCAGGTTTGTC	TAGAGGCAAGAGCTTCA	TTGTAAAGACGGTGATGGTCAGA
Gata1	TTTCTCCCTCCTCTTAGAGCCA		ATCTTTAAGGGTGCAGGGCA
SCL	CTTTGCAGCTTCACTGGGATAA		TACGGACCCAATGGACTTCC
Lmo2	CGGATCCGTGCCTATGAGA		AGGCGGCGCATTTGAA

Wnt5a	CGAAGCAAACCAGCTCACCACATAGA		CAGAAGGCTACCAAGCCCATGAA
Jag1	CATCGTACTGCCTTTCAGTTTCG	AGGCCTCCACCAGCAAAGT	CTGGCCGAGGTCC
Sox17	AGCTCCAGAAACTGCAGACCAGAA		TCCATGAGGTGACATGCTGAGGTT
VE-Cadherin	TGGACAGACTGCAGTGGAGAGA	CCTTCGTGGAGGAGCTGATC	CCTTCTGCTCACGGAC
PU.1	ATGGAAGGGTTTTCCCTCACCGCC		GTCCACGCTCTGCAGCTCTGTGAA
Brachyury	ABI gene expression assay Mm00436877_m1		
18s	ABI gene expression assay Hs99999901_s1		
Hes1	ABI gene expression assay		

Table 2.2 List for primers and probes for quantitative RT-PCR in this project.

Self-design Tapman Primer efficiencies were calculated using the slope of the standard curve generated from qPCR of serial template cDNA dilutions and calculated at primer efficiency calculator at http://www.finnzymes.com/java_applets/qpcr_efficiency.html. Sybergreen primier were adapted from published paper. Further validation is recommended.

ES cell lines	Origin	Usage	Reference
R25-NIC-B5 ES cells R25-NIC -C5 ES cells	Lowell's lab	Tamoxifen inducible ectopic NotchIC	Lowell et al., 2006
A2lox.CRE ES cells	Kyba's lab	Parental cell line for construction of doxycycline inducible cell line (constructed based on A17 ES cell line)	Iacovino et al., 2011a
iDML.1 (A2) iDML.2 (A10) iEGFP.1 (C8) iEGFP.2 (H8)	Developed in the lab based on A2lox.CRE	Doxycycline inducible overexpression of DNMAML-GFP or GFP	
7a-GFP ES cells	laboratory stocks	ES cell line constitutively expresses eGFP	Gilchrist et al., 2003
E14 IV ES cells	laboratory stocks	Wild-type ES cell line	

Table 2.3 ES cell lines applied in this project.

Stromal cell line	Derived tissue	Embryonic day	Transgenic Embryo
AM20.1A4	Aorta-mesenchyme of AGM	E10	tsA58
AM20.1B4	Aorta-mesenchyme of AGM	E10	tsA58
AM14.1C4	Aorta-mesenchyme of AGM	E11	BL1b
UG26.1B6	Urogenital ridges of AGM	E11	tsA58
UG26.2D3	Urogenital ridges of AGM	E11	tsA58
EL08.1D2	Foetal liver	E11	BL1b

Table 2.4 Embryonic haematopoietic niche derived stromal cell lines applied in this project.