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Investigating the Role of Runx1 in the Specification of Haematopoietic Stem Cells from Early Precursors in the Embryo using a Runx1 Reactivatable Knockout Mouse Model

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Thesis presented for the degree of Doctor of Philosophy
University of Edinburgh
Scottish Centre for Regenerative Medicine, Institute for Stem Cell Research
2012
I declare that the work present in this thesis is my own, except otherwise stated.

Pierre Bour
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ii. **Abstract**

Runx1 is a central transcription factor in the development of the murine haematopoietic system and in the emergence and specification of its main key component, the haematopoietic stem cell. Previous studies suggested a requirement for Runx1 in a window of time stretching from mesoderm specification (E6.5) to mid-gestation (E11), but these studies did not investigate each primary haematopoietic site separately. During this PhD project, a *Runx1* reactivatable knockout mouse model was used to study the impact of the absence of Runx1 from E9.5 to E11 in primary haematopoietic sites on early precursor populations, especially PreHSC Type I and II. At E9.5, the KO conceptus was already developmentally retarded, lacking progenitors and PreHSC Type II but was not devoid of PreHSC Type I, as demonstrated by flow cytometry, thus suggesting a requirement for Runx1 in the transition from PreHSC Type I to PreHSC Type II stage. Using a novel culture system that enables the potent *in vitro* maturation of precursors of HSCs into fully mature adult-repopulating HSCs, it was found that maturation of PreHSC Type I into HSCs was hindered in KO tissues, despite the expression of *Runx1* in OP9 niche compartment, thus pointing towards a cell autonomous requirement for Runx1. In this model, the *Runx1* allele was subsequently reactivated to a functional state by tamoxifen-induced Cre-mediated recombination (CreER<sup>T2</sup> system). Tamoxifen / Cre toxicity on HSC maturation was evaluated during AGM reaggregate culture to achieve the best balance between the highest recombination levels and the lowest toxicity when Cre was induced in cell suspension prior to reaggregation. It was found that reactivation of *Runx1* at E9.5 in primary haematopoietic sites was not sufficient to rescue haematopoietic development, thus suggesting a requirement for Runx1 before E9.5.
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6 SUMMARY AND PERSPECTIVES

6.1 Summary

6.2 Perspectives

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iv. List of abbreviations

7AAD: 7-amino-actinomycin D
AGM: Aorta-Gonad-Mesonephros
CFU-C: Colony Forming Unit Cell
CFU-S: Colony Forming Unit Spleen
DA: Dorsal Aorta
dH₂O: distilled water
EB: embryoid body
ee: embryo equivalent
ER: Estrogen Receptor
ES(C): Embryonic Stem (Cell)
HIAC: Haematopoietic Intra-Aortic Cluster
HSC: Haematopoietic Stem Cell
FCS: Fetal Calf Serum
FMO: Fluorescence Minus One control
IC: isotype control
iPS: Induced Pluripotent Stem
KO: Knockout referring to Runx1\textsuperscript{LacZ/LacZ} embryo or tissue
LTR-HSC or HSC: long-term reconstituting haematopoietic stem cell
NSG: non-obese severely compromised immunodeficient IL2\textsubscript{γ} null
PBS: Phosphate Buffered Saline
PL: Placenta
P/S: Penicillin/Streptomycin
Psp: Para-aortic splanchnopleura
Re: Reactivated Runx1 tissue referring to Runx1 KO tissue that has been reactivated with tamoxifen
RT: room temperature
Solution 1: 7% FCS, 50 units/mL Penicillin/Streptomycin in Dulbecco’s Phosphate Buffered saline with ions
Solution 2: 7% heat-inactivated FCS, 50 units/mL Penicillin/Streptomycin in Dulbecco’s Phosphate Buffered saline without Calcium/Magnesium
Solution 3: 2% heat-inactivated FCS in Dulbecco’s Phosphate Buffered saline without Calcium/Magnesium

Solution 4: 2% heat-inactivated FCS 5 mM EDTA in Dulbecco’s Phosphate Buffered saline without Calcium/Magnesium

SLAM: Signalling Lymphocyte Activation Molecule
sp: somite-pairs
Tam: (Z)-4-Hydroxy-Tamoxifen
UGRs: uro-genital ridges
VC: Vascular Endothelial Cadherin
WT: Wildtype
YS: Yolk Sac
1 INTRODUCTION

The study of stem cells holds the key to regenerative medicine’s main goal: the \textit{in vitro} production of tissues to replace their \textit{in vivo} damaged counterparts. This opens new perspectives for treating conditions which remain incurable to this day such as the loss of motility after spinal cord injury.

Another crucial aspect of the field is to circumvent the issue of graft rejection by the host as donor tissue may be produced from the host stem cells.

In addition, stem cells are a powerful model to recapitulate the development of the embryo and inherited diseases. High throughput drug screening and drug discovery is another area where stem cells are useful to accelerate the discovery and the development of treatments.

A stem cell is widely defined as a cell type with the ability to undergo either self-renewal, a process through which the mother cell produces daughter cells with the same potential, or differentiation into mature cell types.

Embryonic stem (ES) cells are extracted from the inner cell mass of the pre-implantation embryo and have the unique ability to give rise to the three germ layers: endoderm, mesoderm and ectoderm (Beddington and Roberston, 1989; Bradley et al., 1984) both \textit{in vivo} and \textit{in vitro}. Their maintenance in an undifferentiated state is achieved either by culture on a fibroblast feeder layer or in the presence of leukemia inhibitory factor and bone morphogenetic protein 4 (Smith et al., 1988; Williams et al., 1988; Ying et al., 2003).

Induced pluripotent stem (iPS) cells are generated from embryonic and adult fully differentiated cell types (e.g. fibroblast, hepatocyte), which are reprogrammed into an undifferentiated ESC-like state using a combination of transcription factors. Oct4, Sox2, Klf4, c-Myc, Lin-28 and Nanog are the most commonly used (reviewed in Jaenisch and Young, 2008).
Tissue specific stem cells are found during embryonic development and in the adult organism. They can only give rise to cell lineages from the same germ layer or the same tissue.

1.1 Haematopoietic Stem Cells

A Haematopoietic Stem Cell (HSC) is defined by its ability to provide high-level peripheral blood chimerism (>5%), to achieve long-term engraftment (16 weeks) upon transplantation into a wildtype (WT) adult irradiated recipient, to self-renew and to produce multilineage progeny.

As HSCs arise de novo during embryogenesis only, embryonic HSCs are a source of great interest in stem cell research. HSC emergence is a complex process that involves a chain of spatial and temporal events and is characterized by the successive appearance of haematopoietic progenitors in extra- and intra-embryonic tissues followed by the emergence of HSCs in intra-embryonic organs. In addition, the migratory nature of HSCs makes it difficult to assess the contribution of each haematopoietic site. Also, it is still not clear whether embryonic progenitors are ontogenically distinct from the HSC lineage.

The study of the mechanisms behind HSC emergence and expansion had long been hampered by the lack of an appropriate culture technology. Indeed, once cultured by immersion (eg like ESC or iPSC), these stem cells lose their self-renewal abilities and differentiate. Fairly recently (Taoudi et al., 2008), a culture system has been developed in our laboratory that allows not only the maintenance but also the massive expansion of embryonic murine HSCs as well as the manipulation of cell compartments. This enables the identification of key cell populations involved in HSC development.

Be that as it may, much remains to be done to understand the mechanisms leading to HSC specification holds great promise for the production of such cells from ES or iPS cells as, it would make the process of obtaining donor tissue for transplantation easier and bypass the need of bone marrow donor.
1.2 The adult haematopoietic hierarchy in the bone marrow

1.2.1 The haematopoietic stem cell compartment

At the top of the adult haematopoietic hierarchy (Figure 1.1) and within the bone marrow, sits the long-term reconstituting haematopoietic stem cell (LTR-HSC), which upon transplantation into a WT adult recipient, homes to the bone marrow, gives rise to multilineage progeny for at least 16 weeks post-transplantation and contributes to more than 5% of the leukocytes in the peripheral blood. It also displays the ability to engraft adult irradiated recipients with the same multilineage potential upon serial transplantations (Szilvassy et al., 1990), which is regarded as a proof of self-renewal ability. The short-term haematopoietic stem cell (ST-HSC) displays the same properties but lacks the lifelong self-renewal capacities of its long-term counterpart.

HSCs are found within the adult bone marrow at a frequency of 1 in 10,000 nucleated cells (Kumaravelu et al., 2002). HSCs can be mobilized in the adult peripheral blood and harvested for clinical transplantation with Cyclophosphamide/G-CSF based treatment (Drize et al., 1995; Bensinger et al., 1995). In homeostasis, rare HSCs are found in the bloodstream (Mendez-Ferrer et al., 2008). Interestingly, a study showed that their release from the bone marrow to the blood is driven by circadian rhythms (Mendez-Ferrer et al., 2008) through the regulation of chemokine CXCL12 expression in the bone marrow and noradrenaline secretion by the sympathetic nervous system.

Adult HSCs switch from a predominantly proliferating to a quiescent state around the fourth week of life (Bowie et al., 2006 and 2007b). However, two populations of quiescent HSCs can be distinguished on the basis of the dividing frequency. The first population divides every 36 days whereas a more dormant population containing the majority of the HSC activity only divides every 145 days (Wilson et al., 2008). This dormant state is thought to prevent HSC exhaustion and reduce the accumulation of mutations (Orford and Scadden, 2008; Warner et al., 2004), but can be reversed to an active self-renewal state under injury or stress.
Figure 1.1: Adult haematopoietic hierarchy. Both long-term (LTR) and short-term (STR) reconstituting HSCs give rise to the entire hierarchy but are distinguished by the duration of their engraftment when transplanted into an irradiated host. Only LTR-HSCs can undergo self-renewal. CMP, common myeloid progenitors; CLP, common lymphoid progenitors (adapted from Larsson and Karlsson, 2005).
1.2.1.1 Phenotype of mouse adult HSCs

Adult HSCs can be purified by FACS sorting-based strategy on the basis of markers expression but also on their capacity to efflux Hoechst 33342 nuclear dye (Goodell et al., 1996).

Most FACS sorting strategies involve the exclusion of lineage-committed cells (i.e. expressing CD3ε, CD4, CD8, Ter119, Mac1, Gr1 or B220 markers) and the inclusion of c-Kit+Sca1+ cells. Other markers may be added to this population commonly referred to as LSK. When CD34 and Thy1.1 (Berman and Basch, 1985; Spangrude et al., 1988; Ikuta and Weissman, 1992; Osawa et al., 1996) are included in the antibody cocktail, LSK Thy1.1lo or LSK CD34lo/− fractions contain HSC at a frequency of 1 in 5 cells (Osawa et al., 1996; Wagers et al., 2002). An elegant and simple strategy based on signalling lymphocyte activation molecule (SLAM) can sort an HSC-enriched population with a frequency of 1 in 5 cells of LSK CD48CD150+ phenotype. In addition, when CD41 cells are excluded a purity of 1 HSC in 2 cells is achieved (Kiel et al., 2005).

Importantly, short-term potential is separated from the long-term on the basis of Flt3 or CD34 expression within the LSK population with LTR-HSCs being CD34−Flt3− (Yang et al., 2005; Christensen and Weissmann, 2001).

1.2.1.2 The HSC niche in the bone marrow

The term “niche” refers to a microenvironment in which the stem cell pool is maintained to prevent stem cell exhaustion and at the same time, generate differentiated progeny to supply the needs of the organism. In other words, the niche holds the key to achieving the delicate balance between self-renewal and differentiation, which is crucial for the life of the organism.

Most importantly, the investigation of the interactions between HSCs and their niches should reveal the molecular signalling involved in HSC migration, quiescence and differentiation.
Figure 1.2: Complexity and diversity of the adult HSC niche. HSCs may integrate signals from periendosteal and perivascular cells as direct cell-cell contact, intermediate cell contact or soluble secreted factors (taken from Gonneau, 2010)
To this day, many sites within the bone marrow have been reported as candidate niches for adult HSCs (Figure 1.2). Most of HSCs are located in trabecular zone of BM, but few are found in the liver and the spleen (Taniguchi et al., 1996; Wolber et al., 2002; Calvi et al., 2003; Zhang et al., 2003; Kiel et al., 2005; Kiel and Morrisson, 2008). It has been shown that endosteal cells (i.e. osteoblasts and osteoclasts) secrete factors that promote HSC maintenance (Kiel and Morrisson, 2008). Alternatively, sinusoid vascular cells and cells surrounding the perivascular environment constitutes another possible niche (Kiel and Morrisson, 2008).

A recent elegant study based on the retention of Hoechst 33342 in the bone marrow after perfusion in adult mice revealed that the most potent HSCs capable of serial transplantation are located the furthest apart from the blood flow (Winkler et al., 2010). Of note, this does not totally correlate with their distance from the vasculature. By contrast, HSCs closer to blood were only able to reconstitute primary but not secondary recipients. Interestingly, G-CSF mediated mobilization brought most potent HSCs closer to the blood flow.

1.2.2 The Colony Forming Unit Spleen

The Colony Forming Unit Spleen (CFU-S) is a short-term repopulating cell that homes to the spleen upon transplantation into an adult irradiated recipients and forms macroscopic colonies there with limited self-renewal capacity. Its multilineage capacity is restricted to the myelo-erythroid lineage. CFU-S\textsubscript{8} is detected after 8 days and become extinct before 11 days. CFU-S\textsubscript{11} is more immature, has greater self-renewal ability and remains until day 11 post-transplantation.

1.2.3 Multipotent and lineage restricted progenitors

Upon differentiation, HSCs produce multipotent and lineage restricted progenitors, which retain the ability to differentiate into mature cells types but can neither self-renew nor contribute to long-term haematopoiesis in adult irradiated recipients.
MultiPotent Progenitors (MPPs) are found within the LSK Thy1.1^Flt3{sup+} population (Christensen and Weissman, 2001) and give rise to terminally differentiated cell from all haematopoietic lineages.

Common Myeloid Progenitors (CMP) and Common Lymphoid Progenitors (CLP) both downregulate Sca1 and can be distinguished on the basis of IL-7 receptor α-chain. CMP bears a Lin^Thy1.1^bSca1^c-Kit^IL-7Rα{sup−} phenotype and the CLP a Lin^Thy1.1^bSca1^b-Kit^bIL-7Rα{sup+} phenotype (Akashi et al., 2000; Kondo et al., 1997). Both CMP and CLP can give rise to short-term engraftment in adult irradiated mice but their progeny is restricted to myeloid (Akashi et al., 2000) and lymphoid lineage (Kondo et al., 1997), respectively. *In vitro*, CMP and CLP gives rise to myeloid and lymphoid colonies, respectively.

The CMP compartment can be further divided into three distinct populations based on the expression of Fcγ receptor II-III (FcγR) and CD34 (Akashi et al., 2000). The CMP lineage of FcγR^b^CD34{sup+} gives rise to all myelo-erythroid types of colonies: Colony Forming Unit-Granulocyte/Erythrocyte/Macrophage/Megakaryocyte (CFU-GEMM), CFU-Granulocyte/Macrophage (CFU-GM), CFU-Macrophage (CFU-Mac), CFU-Megakaryocyte (CFU-Meg), Burst-Forming Unit-Erythrocyte (BFU-E). The Granulocyte Macrophage lineage-restricted Progenitor (GMP) of FcγR^hi^CD34{sup+} only produce CFU-GM and CFU-Mac colonies. The Megakaryocyte Erythrocyte lineage-restricted Progenitor (MEP) of FcγR^b^CD34{sup−} phenotype can only give rise to CFU-Meg and BFU-E. In *in vitro* colony assay, CMP gives rise to more committed GMP and MEP (Akashi et al., 2000).

Highly Proliferative Potential Colony Forming Cells (HPP-CFC, Bradley and Hodgson, 1979) are another type of progenitor which give rise to macroscopic colonies *in vitro* when exposed to appropriate factors. They can also give rise to multilineage colonies upon replating on agar medium and possess limited bone marrow repopulating abilities.
1.2.4 Fully differentiated mature haematopoietic cells

All cells from the myelolymphoid lineage express CD45 and are referred to as leukocytes. The mature cells from these lineages are all involved in immunity (Lagasse et al., 2000; Thomas, 1989).

The erythroid lineage downregulates CD45 as maturation of erythrocytes occurs. The final part of this maturation process consists of enucleation and can be followed by the differential expression of CD71 and Ter119 surface markers (Kina et al., 2000).

A table of the monoclonal antibodies used to detect haematopoietic lineages is shown in Table 1.1.

1.3 The development of the haematopoietic system in the murine embryo

The development of the haematopoietic system is a complex process that occurs in two waves and involves a chain of spatio-temporal events taking place in many extra and intra-embryonic transitory organs.

In the murine embryo, the first haematopoietic wave (also referred to as primitive haematopoiesis) involves the yolk sac only and starts around E7.5. It mainly consists of large nucleated erythrocytes produced to meet the oxygen demand of the developing embryo. These primitive erythrocytes express embryonic globins, $\beta H1$, $\varepsilon$ and $\gamma$ (Palis and Yoder, 2001; Cumano et al., 2001) and migrate to the fetal liver where they enucleate. They become undetectable after E16. Few macrophages are also produced but no HSC are produced at this stage. Interestingly, primitive erythroid cells are derived from the inner layer of the blood islands whereas the outer layer differentiates into endothelium (Palis and Yoder, 2001). Hence the model in which endothelium and haematopoietic cells arise from a common bipotential precursor referred to as the haemangioblast (Sabin, 1920). This model is discussed in more detail in paragraph 1.3.1.3.3.
<table>
<thead>
<tr>
<th>Haematopoietic lineage</th>
<th>Plasma membrane protein</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>Leucocyte</strong></td>
<td>CD45/Ly-5</td>
<td>Ledbetter and Hersenbarg, 1979; Thomas, 1989; Lagasse <em>et al.</em>, 2000</td>
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<td><strong>Erythroid</strong></td>
<td>Ter119</td>
<td>Kima <em>et al.</em>, 2000; Zhang <em>et al.</em>, 2003</td>
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<tr>
<td></td>
<td>CD71</td>
<td>Kemp <em>et al.</em>, 1987; Zhang <em>et al.</em>, 2003</td>
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<tr>
<td><strong>Lymphoid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>Krep <em>et al.</em>, 1996; Tedder <em>et al.</em>, 1994</td>
</tr>
<tr>
<td></td>
<td>CD43</td>
<td>Hardy <em>et al.</em>, 1991</td>
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<td>T-cell</td>
<td>CD38</td>
<td>Leo <em>et al.</em>, 1987; Nakano <em>et al.</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>Pierres <em>et al.</em>, 1984</td>
</tr>
<tr>
<td></td>
<td>CD8a</td>
<td>Ledbetter <em>et al.</em>, 1980; van Ewijk <em>et al.</em>, 1981</td>
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<td><strong>Natural killer</strong></td>
<td>NKI.1</td>
<td>Koo and Peppard, 1984; Yokoyama and Seaman, 1993</td>
</tr>
<tr>
<td><strong>Myeloid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mast cell</td>
<td>CD34</td>
<td>Drew <em>et al.</em>, 2002</td>
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<tr>
<td></td>
<td>c-Kit/CD117</td>
<td>Drew <em>et al.</em>, 2002</td>
</tr>
<tr>
<td></td>
<td>Sca-1</td>
<td>Drew <em>et al.</em>, 2002</td>
</tr>
<tr>
<td></td>
<td>FcγRI</td>
<td>Ishizaka and Ishizaka, 1984; Dombrowicz <em>et al.</em>, 1993; Turner <em>et al.</em>, 1999</td>
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<tr>
<td><strong>Monocyte/Macrophage</strong></td>
<td>Mac-1/CD11b</td>
<td>Springer <em>et al.</em>, 1978; Loenen <em>et al.</em>, 1994; Lagasse and Weisman, 1996</td>
</tr>
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<td></td>
<td>Gr-1</td>
<td>Hestdal <em>et al.</em>, 1991; Fleming <em>et al.</em>, 1993; Lagasse and Weisman, 1996</td>
</tr>
<tr>
<td><strong>Neutrophil</strong></td>
<td>Mac-1/CD11b</td>
<td>Lagasse and Weisman, 1996</td>
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<td>Hestdal <em>et al.</em>, 1991; Fleming <em>et al.</em>, 1993; Lagasse and Weisman, 1996</td>
</tr>
</tbody>
</table>

Table 1.1: Lineage specific markers expressed on terminally differentiated cells of erythroid, myeloid and lymphoid lineages (taken from Taoudi, 2006)
Figure 1.3: The journey of HSC through multiple sites during haematopoietic development (taken from Medvinsky et al., 2011).

Figure 1.4: Models of emergence of HSCs in the midgestation dorsal aorta (adapted from Gonneau, 2009).
By contrast, the second wave of haematopoiesis (aka definitive haematopoiesis) involves multiple sites (Figure 1.3), both extra-embryonic, with the yolk sac (Kumaravelu et al., 2002), the umbilical and vitelline arteries (North et al., 2002), the placenta (Gekas et al., 2005), and intra-embryonic with the region around the dorsal aorta of the embryo proper (Mueller et al., 1994; Medvinsky et al., 1996). Progenitors are first detected around E9.5 and the first HSC emerges around E11 in the aorta-gonad-mesonephros (AGM) region of the embryo at first, then in the yolk sac and the placenta (Kumaravelu et al., 2002; Gekas et al., 2005; Rhodes et al., 2008; Ottersbach and Dzierzak, 2005). HSCs subsequently seed the fetal liver to dramatically expand such that most of HSC activity is concentrated there by E12.5 (Kumaravelu et al., 2002; Gekas et al., 2005). Newly formed HSCs home to the bone marrow around birth where they will provide blood cells for the rest of the animal’s life.

1.3.1 The embryonic origin of the HSCs

1.3.1.1 Embryo Proper or Yolk Sac? A controversy on the source of HSCs and PreHSCs

The first HSC emerges in the AGM around E11 (Medvinsky et al., 1996; Kumaravelu et al., 2002). However, the migratory nature of HSCs (Kumaravelu et al., 2002) makes it difficult to assess whether the AGM-derived HSCs seed the yolk sac or if the yolk sac possesses the ability to generate HSC per se. Furthermore, the E10 AGM, initially devoid of HSCs (Mueller et al., 1994), has autonomous potential to initiate HSC activity in vitro, unlike the yolk sac (Medvinsky et al, 1996). This suggests that mid-gestation AGM contains both precursors of HSCs and the niche required for their maturation.

Cell fate tracking studies suggest that haematopoietic cells specified in the yolk sac contribute to adult haematopoiesis (Samokhvalov et al., 2007), although the labelling system used makes it impossible to fully exclude labelling in other organs during development.
Interestingly, coculture with AGM stromal cell line of E8 Para-aortic Splanchnopleura (Psp) and yolk sac can reveal their HSC potential (Matsuoka et al., 2001b), suggesting that PreHSCs are present in both sites.

Similarly, yolk sac and caudal part exhibit SCID-newborn repopulating activity two days before the first HSC is detected, at late E9.5 in 28 somite-pair (sp) embryos (Fraser et al., 2002). The placenta was not tested. Although the nature of the cells detected with this transplantation assay is not clear, these results indicate that both yolk sac and caudal part already contain precursors of HSCs before midgestation.

However, the culture of those E9.5 tissues with the OP9 coaggregation system (described in figure 5.1) can only mature HSC from the caudal part and not the yolk sac or the placenta (Figure S5.7), although haematopoietic progenitors expand in both organs during culture (Figure 5.2.B). Such contradictory results cannot clearly indicate the embryonic origin of the precursors of HSCs.

The study of haematopoietic potential in the Ncx1−/− embryos, which lack heartbeats, revealed that haematopoietic progenitors emerging before E10 were produced in the yolk sac but not the embryo proper (Lux et al., 2008). This was seen as a sign that all haematopoietic cells, including HSCs, produced during the second wave originate from the yolk sac. Interestingly, myeloid progenitors with multilineage potential (CFU-GEMM) could be rescued in the E9.5 Ncx1 KO caudal part when the organ was put under shear stress conditions (Adamo et al., 2009). However, the effects of shear stress were abolished when Nitric Oxide, a mediator of shear stress-induced signalling, was blocked. These results emphasize the importance of mechanical stress linked to Nitric Oxide signaling, which have been overlooked so far, in the emergence of haematopoietic cells.

Of note, a major question remains as for the inclusion of vitelline and umbilical arteries in the dissection of the yolk sac. Indeed, as these vessels exhibit repopulating activity at E11 (North et al., 2002), they could be a source of HSC contribution from the yolk sac.
1.3.1.2 The placenta as another source of HSCs

The placenta attracted attention as a possible source of HSCs when a high frequency of myeloid and pre-B progenitors were found there from E8.5 onwards (Melchers, 1979; Alvarez-Silva et al., 2003, figure 1.7). This haematopoietic potential is present in both the allantois (embryonic component) and the chorion (maternal component) and can be revealed prior to chorio-allantoic fusion at E6.5 (Zeigler et al., 2006) or prior to the establishment of circulation in the allantois around E8 (Corbel et al., 2006). These results suggest autonomous potential to initiate haematopoiesis in both chorion and allantois.

However, unlike the AGM, placenta lacks the capacity to autonomously initiate HSC formation in explant culture before late E11, unless it is of Runx1+/− genotype (Robin et al., 2006). This is more thoroughly discussed in section 1.4.4.3.

Between E11 and E13, the placenta constitutes the second largest reservoir of HSCs (after the fetal liver) in the whole conceptus (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; figure 1.6). Analysis of sections showed that the CD34⁺c-Kit⁺Sca1⁺ fraction enriched in HSCs is located within the endothelial layer of embryonic vessels from the chorionic and labyrinth regions (Ottersbach and Dzierzak, 2005; Rhodes et al., 2008).

A study using Ncx1 null embryos showed that the potential to specify progenitors does not depend on the heartbeat (Rhodes et al., 2008). However, the ability of the placenta to generate HSCs in a Ncx1 KO context remains to be assessed properly.

1.3.1.3 The multiple models of HSC emergence

HSCs have been localized to the ventral part dorsal aorta (DA), more specifically into haematopoietic intra-aortic clusters (HIAC) present in the lining of endothelium in many species including birds, mouse and human (Tavian et al., 1999; Jaffredo et al., 1998; Garcia-Porrero et al., 1998; de Bruijn et al., 2002; Taoudi et al., 2007, Rybtsov et al., 2011). It has been demonstrated that the ventral floor of the DA
is a functional niche, which can initiate and expand HSCs (Taoudi et al., 2007).

However, UGRs also demonstrated a potential after explant culture, although less important than the DA, to generate HSCs (de Bruijn et al., 2002).

HIACs express haematopoietic markers such as CD34, CD45, PECAM-1, AA4.1, Runx1 and CD41 (Reviewed in Godin and Cumano, 2005; Garcia-Porrero et al., 1998; North et al., 2002; Bertrand et al., 2005; Taoudi et al., 2005).

Despite the knowledge gained recently, the source of PreHSCs before budding of HIACs remains unclear and many models coexist so far (summarised in figure 1.4).

1.3.1.3.1 The concept of haematogenic endothelium

The observation of HIACs attached to the lumen of the dorsal aorta and vitelline and umbilical arteries has led to the theory in which HSCs are directly derived from the endothelium (Figure 1.4 and 1.5). The endothelium switches from endothelial to haematopoietic fate when exposed to appropriate environmental cues. As a matter of fact, both lineages share a variety of markers in AGM and yolk sac such as Runx1, Tie-2, PECAM-1, Vascular Endothelial-cadherin (VC), Ac-LDL, CD41, CD34, c-Kit, Sca-1 and AA4.1. (Nishikawa et al., 1998b; North et al., 2002; Taoudi et al., 2005).

Moreover, the endothelium from the embryo proper and the yolk sac harbours lymphohaematopoietic potential (Nishikawa et al., 1998b) and SCID-neonatal reconstituting activity (Fraser et al., 2002).

Lineage tracing study of VC$^+$ cells suggests that HSC in the fetal liver originates from the AGM endothelium (Zovein et al., 2008).

Live cell imaging of ES cells differentiating into the haematopoietic lineage revealed that endothelial cells (identified by morphology, VC expression, Ac-LDL uptake and Claudin-5 at cellular junction) give rise to cells expressing CD41$^+$, the earliest marker of haematopoietic commitment (Mikkola et al., 2003; Ferkowicz et al., 2003; Emambokus et al., 2003), before producing round non-adherent CD45$^+$ haematopoietic cells (Eilken et al., 2009).
Figure 1.5: Model of HSC emergence from the haematogenic endothelium of the E11 dorsal aorta (taken from North et al., 2002).
Most importantly, the excision of Runx1 in the embryonic VC+ compartment phenocopied the haematopoiesis defects observed in Runx1 KO embryo (Chen et al., 2009), especially the lack of HIACs.

1.3.1.3.2 The non-haematogenic PreHSC theory

Most of the studies mentioned above did not take into account the possibility that a precursor of HSC, which could have been specified from a non-endothelial compartment, migrates to and inserts itself into the endothelium (Taoudi et al., 2008). It may do so by upregulating VC, thereby explaining why the HSC lineage is labelled in VC lineage tracing experiments (Zovein et al., 2008; figure 1.4).

Interestingly, Oregon Green (OG) labelling of the E11 DA (Rybtsov et al., 2011) and subsequent repopulation assays showed that some PreHSCs are located both in the aortic and the subaortic layers of the endothelium and migrate centripetally towards the dorsal aorta. This might indicate an extraendothelial origin of the PreHSCs.

In addition, Runx1 is reportedly expressed in the sub-aortic mesenchyme (North et al., 1999), which is compacted in the KO embryo and thus, may hamper PreHSC migration towards the DA (North et al., 1999; Bee et al., 2010).

1.3.1.3.3 The haemangioblast theory

The close association of haematopoietic and endothelial cells within the E7 YS has led to a model in which both haematopoietic and endothelial lineages arise from a common bipotential precursor, the haemangioblast (Sabin, 1920; figure 1.4). This concept is supported by the analysis of the embryoid body (EB) differentiation model from ES cells. In this model, blast colony forming cells (BL-CFC) of Flk1+Brachyury+ phenotype that have endothelial and haematopoietic potentials arise before the onset of in vitro haematopoiesis. As an analogous in vivo population of the same phenotype and same potency was isolated from the primitive streak in the E7
embryo (Huber et al., 2004), these BL-CFCs were thought to be the in vitro equivalent of the haemangioblast (Choi et al., 1998).

However, the presence of smooth muscle in the colonies produced from BL-CFC indicates that it could represent an uncommitted mesodermal progenitor population (Choi et al., 1998; Fehling et al., 2003).

1.3.1.3.4 Subaortic patches

HSCs found in E11 AGM differ from those found earlier in the mouse conceptus. The former achieve high-level repopulation upon transplantation into an adult recipient (bona fide HSCs) but the latter found in both E9 YS and E8 P-Sp (Yoder et al., 1997a and b; Cumano et al., 2001) can only achieve low-level repopulation (PreHSCs). Hence the theory that early HSCs might need maturation to acquire a high-level repopulating potential (Medvinsky and Dzierzak, 1999).

Cells expressing HSC markers such as Lmo2, GATA2, AA4.1, c-Kit, CD31, CD41 and low levels of CD45 are localized in transient subaortic structures, termed subaortic patches (SAPs) (Manaia et al., 2000; Bertrand et al., 2005; Figure 1.4). This observation led to the idea that these SAPs constitute a pool of PreHSCs. Interestingly, SAPs and HIACs disappear concomitantly around E13 when the AGM HSC activity ceases (Bertrand et al., 2005).

1.3.1.4 A role for primordial germ cells

The colonization of E10.5 UGRs by totipotent germ cells have led to the hypothesis in which primordial germ cells (PGCs) might be a source of HSCs (Rich, 1995). PGCs arise from the base of the allantois at E6.5 from the ectodermal layer (Ginsburg et al., 1990). They express alkaline phosphatase and generate erythroid cells in vitro and cobblestone areas and therefore may have the potential to form HSCs (Rich, 1995), although functional evidence remains to be published to confirm this hypothesis.
1.3.2 Polarization of the haematopoietic development in the E11 dorsal aorta

A growing body of data suggests that the development of HSCs and haematopoietic progenitors occurs in a polarized fashion within the AGM region around midgestation. The initial clue came from the observation of HIACs on the ventral wall on the dorsal aorta (DA) exclusively. Two main axes of polarization are considered: an axio-lateral polarization that separates the DA from the urogenital ridges (UGRs) and a dorso-ventral bisection of the DA yielding a ventral aspect (AoV) and a dorsal aspect (AoD). The AoD was also considered separately from the dorsal tissues (dT) including the notochord, which has been shown to secrete factors (e.g. Sonic Hedgehog, VEGF) influencing HSC development in vitro (Pardanaud and Dieterlen-Lievre, 1999; Peeters et al., 2009).

1.3.2.1 Axio-lateral polarization

HSCs localize solely to the DA of the E11 AGM region (de Bruijn et al., 2000b; Rybtsov et al., 2011; Taoudi and Medvinsky, 2007). However, by E12.5, UGRs contain HSCs, which could be either the result of HSC migration from the DA or the autonomous potential of UGR to support HSC emergence. To this day, there is no clear answer, since contrary results about the HSC generating potential of E11 UGRs have been published. E11 UGRs seem to have a potential in explant (de Bruijn et al., 2000b) but not in reaggregate culture (Gonneau, 2009) although statistics were more robust in the study using explants. This suggests that UGRs may have potential but the maturation of HSCs occurs at lower frequency in UGR than in DA.

The E11 DA contains PreHSCs but UGRs are required for optimal expansion in vitro (Gonneau, 2009).

No axio-lateral polarity for CD45⁺ haematopoietic cells and progenitors was observed in fresh and reaggregated E11 AGM (Gonneau, 2009).
1.3.2.2 Dorso-ventral polarization

Intra-aortic clusters, considered to be the morphological manifestation of HSC development, are found attached to the endothelial lining of the E11 DA not only ventrally but also more occasionally laterally and dorsally (Dieterlen-Lievre et al., 1981; Medvinsky et al., 1996; Garcia-Porrello et al., 1995; Tavian et al., 1996; Bertrand et al., 2005; Taoudi and Medvinsky, 2007; Gonneau, 2009). Haematopoietic CD45+ cells and progenitors are distributed evenly between AoD and AoV domains in fresh E11 DA. Likewise, there is an even distribution of Runx1 expression in fresh E11 as assessed with Runx1-GFP reporter.

By contrast, HSCs mainly located in fresh E11 AoV and AoV has autonomous potential to induce HSC formation in explant culture and is a functional niche for HSCs (Taoudi and Medvinsky, 2007). However, AoD also has PreHSCs but these can be matured in reaggregate culture only and take longer to mature (5 days) than their AoV counterparts (4 days).

Interestingly, dorsal tissue displays an inhibitory effect on PreHSC maturation in AGM explants (Gonneau, 2009; Peeters et al., 2009). On the contrary, dT do not affect the production of haematopoietic and progenitor cells in explant culture.

1.3.2.3 Hierarchical organisation and early precursors specification in the AGM region

Thank to the development of the reaggregation system and its derivative, the OP9 coaggregation (Rybtsov et al., 2001; Sheriden et al., 2009; Taoudi et al., 2008), which enable the manipulation of cell populations to investigate their role in HSC specification and emergence, precursors of HSCs have been thoroughly characterized in terms of phenotype, hierarchical organisation to HSCs and spatial localization (Rybtsov et al., 2011; Taoudi et al., 2008).

In the OP9 coaggregation system, the stromal component of the E11 AGM, which enables the potent maturation of HSCs from a VC+CD45+ PreHSC Type II
population (Taoudi et al., 2008), is replaced with an equivalent amount of OP9 cells (e.g. 100,000 cells per embryo equivalent). This manipulation allows HSC to mature from a more immature type of PreHSC, the VC⁺CD45⁺CD41low PreHSC Type I population (Rybtsov et al., 2011).

The OP9 stromal cell line was derived from the bone marrow of osteopetrotic M-CSF null newborn mice and potently support myeloerythroid haematopoietic differentiation (Nakano et al., 1994).

A body of evidence suggested that HSCs arise directly from the endothelial, which is in agreement with the haematogenic endothelium model (North et al., 2002, Zovein et al., 2008; figure 1.5). However, a recent study in our laboratory elegantly showed that HSC specification might follow a more complex model (Rybtsov et al., 2011). With Oregon green (OG) staining of the DA, it was shown that the lower layers of the aorta contained two types of PreHSCs that migrate to the upper layer of the DA and to the HIACs. Importantly, those two types share a hierarchical connection: PreHSCs Type I (VC⁺CD45⁺CD41low) mature into HSCs via a PreHSC Type II (VC⁺CD45⁺) stage (Rybtsov et al., 2011; Taoudi et al., 2008). Interestingly, HSCs solely localise into the HIACs found in the ventral wall of the DA.

PreHSCs Type I arise at late E10 (36-39 sp) in the AGM but cannot be detected by functional assay in the placenta or the yolk sac (although cells of that surface phenotype are present). This highlights the specific potency of the AGM to mature PreHSCs into bona fide adult repopulating HSCs.

A population with a PreHSC Type I phenotype already exists in early E10 AGM (30-35 sp). However, it requires a longer culture period to mature into HSC (7 days instead of 5). It is therefore referred to herein as ProHSC.

ProHSCs are found from E9.5 (24-29 sp) in the caudal part (embryo proper cut below the heart) but not in the placenta or the yolk sac (Figure 5.2.A and S5.7). However, technical limitations due to the loss of activity after sorting make the phenotypic characterization of this population impossible at the moment.

Of note, both PreHSCs Type I and mature VC⁺CD45⁺ HSCs stay within the CD41low population in the AGM region (Rybtsov et al., 2011). Therefore, it is likely that PreHSC Type II also express CD41 at low levels even if this needs to be formally assessed.
1.3.2.4 Phenotypic characterization of embryonic HSCs and their precursors

If adult and embryonic HSCs share common markers such as CD45 and c-Kit (Sanchez et al., 1996), embryonic LTR-HSCs harbours several phenotypes that make their isolation to a high degree of purity hard to achieve.

The first HSCs detected in the E11 AGM and E12 yolk sac are all of VC⁺CD45⁺ phenotype (North et al., 2002; Taoudi et al., 2005). VC is subsequently downregulated during the migration of HSCs from the primary haematopoietic sites to the fetal liver, such that HSC activity of the fetal liver is equally divided between the VC positive and negative fractions (Taoudi et al., 2005).

All HSCs in the embryonic sites of emergence are of CD34⁺c-Kit⁺Sca1⁺ phenotype (Gekas et al., 2005; Ottersbach and Dzierzak, 2005; Sanchez et al., 1996; Yoder et al., 1997a) indicating similar mechanisms for their specification. CD34 expression is required for fetal liver expansion of HSCs but it downregulated thereafter (Matsuoka et al., 2001).

In the E13 to E14 fetal liver, HSCs all express Mac1, a mature lineage marker for macrophages (Morrisson et al., 1995). However, only a tiny fraction expresses Mac1 in E11 fetal liver and AGM HSCs are found in both Mac1 positive and negative fraction (Sanchez et al., 1996). Likewise, AA4.1⁺B220⁺ defines a population enriched in HSCs in the fetal liver but not in the adult bone marrow.

1.3.3 Quantitative analysis of HSCs during embryonic development as a model for hepatic colonisation

Quantitative studies of the HSC pool in the midgestation embryo revealed a dynamic process of seeding of the fetal liver in HSC by consecutive waves from the AGM, yolk sac and placenta (Ema and Nakauchi, 2000; Gekas et al., 2005; Kumaravelu et al., 2002). This process occurs mainly between E11 and E13 (Figure 1.6).
Figure 1.6: Dynamics of HSC contents throughout the second wave of haematopoiesis in primary (AGM, Yolk Sac, Placenta) and secondary (Fetal Liver, Bone Marrow) haematopoietic sites (taken from Gekas et al., 2005).
Evidence of a seeding process was shown by a concomitant expansion of the HSC pool in the liver and increasing numbers of HSCs in the embryonic circulation (Kumaravelu et al., 2002). In addition, lineage tracing of the VC\(^+\) cells supports an AGM origin for haematopoietic cells found in the fetal liver (Zovein et al., 2008)

Evidence of the seeding in consecutive waves is supported by:

- The consistent low numbers of HSCs (1-3 HSCs, Gekas et al., 2005, Kumaravelu et al., 2002) detected in the AGM between E11 and E13 despite a significant capacity of this organ for HSC expansion (Kumaravelu et al., 2002; Medvinsky and Dzierzak, 1996; Taoudi et al., 2008).

- The low numbers observed in the yolk sac at all times, which becomes competent for HSC expansion at E12 and contains 7 HSCs (Kumaravelu et al., 2002).

- The large amounts of HSC detected in the placenta between E11 and E13.5 and the decrease of the placental HSC pool after E13.5 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

As HSC emergence occurs after the onset of circulation, the origin of HSCs and the importance of the contribution of each site remain extensively discussed within the scientific community. The best way to evaluate the contribution of each site to the hepatic HSC pool would be to use site-specific reporters.

1.3.4 Developmental relationships between embryonic progenitors and HSC

It is still unclear whether embryonic progenitors and HSC arise from a common ancestor or if these lineages are segregated early during the second wave of haematopoiesis. Indeed, progenitors and precursors of HSC emerge in a close spatio-temporal relationship, which makes it difficult to separate these lineages.

In fact, progenitors and precursors of HSC appear both around E9.5 in caudal part (Rybtsov et al., unpublished and figure S3.11). At E10, the PreHSC Type I (VC\(^+\)CD45\(^-\)CD41\(^{low}\)) population harbours both HSC and progenitor potentials (Rybtsov et al., unpublished). A recent study based on the rescue of Cbfβ deficient
embryos by Tek or Sca1-driven expression of Cbfβ (the subunit forming with Runx1 the core binding factor complex binding to DNA) suggests that erythro-myeloid progenitors and HSC emerge in the yolk sac and placenta from distinct lineages, respectively from Tek⁺Sca1⁻ and Tek⁻Sca1⁺ ancestors (Chen et al., 2011).

Expression of Cbfβ was required in both Tek and Sca1 compartments for the HSC to emerge from the AGM and the vitelline and umbilical vessels. Interestingly, more HSCs were produced from rescued yolk sac and placenta when Cbfβ expression was rescued in both Tek and Sca1 compartments indicating a positive impact of progenitor lineage on HSC development.

1.4 Runx1 ranks as a central transcription factor in the emergence of HSCs and the homeostasis of the haematopoietic system

Runx1/AML1/PEBPα2/CBFα2 first attracted attention as a gene often displaying chromosomal translocations involved in many forms of leukaemia (AML stands for Acute Myeloid Leukemia; Liu et al., 1993; Miyoshi et al., 1995; Speck and Gilliland, 2002). Since then, Runx1 has been under great scrutiny as a gene and a transcription factor.

More generally, Runx genes owe their name to the sequence the Runx proteins bind to, the runt homology domain based on the homology to Drosophila pair-rule protein runt.

Runx1 belongs to a family of transcription factors of 3 members (Runx1, Runx2 and Runx3) with a high degree of homology of their coding regions (Bae, 1993; Ogawa, 1993a). Runx2 play a role in osteogenesis and null embryos die around birth, probably because of a malformed rib cage that prevents normal breathing (Komori et al., 1997). Runx3 is expressed in bones, and is crucial for the development / survival of dorsal root ganglia neurones (Levanon et al., 2002; Levanon et al., 2001a). Of note, Runx3 expression partially overlaps Runx1 during haematopoietic ontogenesis with the possibility of a cross-regulation during embryogenesis (Levanon et al., 2001a; Spender et al., 2005).
1.4.1 Structure and regulation of Runx1

1.4.1.1 Gene structure

The murine Runx1 gene is a 224 kb gene located on chromosome 16 (analogous to chromosome 21 in human). It is composed of 7 exons (Figure 1.7). Runx1 expression is driven by two promoters, a distal P1 and a proximal P2 (Figure 1.7), which are differentially active during haematopoietic development. P2 is mostly active during the first wave of haematopoiesis whereas P1 is active during the second wave in the fetal liver and in adult HSCs (Bee et al., 2009b; Challen and Goodell, 2010; Fujita et al., 2001; Sroczynska et al., 2009; Telfer and Rothenberg, 2001).

Alternative P1 and P2 usage and alternative splicing give rise to multiple isoforms, of which 2 have mainly been characterized: Runx1b and Runx1c (Figure 1.9). Their role and function is discussed in the next paragraph.

Runx1 expression is tightly regulated by a large number of transcription factors such as, Gata family members, Ets and SCL (Landry et al., 2008), which binds the +23 bp enhancer in the Runx1 locus (Nottingham et al., 2007; figure 1.8). Interestingly, hsp68 promoter-driven GFP reporter including the +23 bp enhancer recapitulate Runx1 expression in the sites of HSC emergence (Bee et al., 2009a), indicating that the +23 bp enhancer confers the haematopoietic specificity to the Runx1 gene.

Moreover, the presence of a Runx binding site in the Runx1 locus indicates that Runx family members regulate Runx1 gene expression (reviewed in Swiers et al., 2010).

The most frequent translocations leading to leukaemia involve fusions with ETO, TEL1 and ET6 (Mangan and Speck, 2011). The effects of the fusion proteins produced from chromosomal translocations on adult haematopoiesis are complex and mostly lineage and stage specific. As they are beyond the scope of this introduction, they are summarised in figure 1.10.
Figure 1.7: Structure of the murine Runx1 gene with its two promoters P1 (distal) and P2 (proximal) (Swiers et al., 2010).

Figure 1.8: Runx1 expression is regulated by Scl, Ets and Gata-2 transcription factors through the +23 bp enhancer, which confers the haematopoietic specificity to Runx1 (taken from Swiers et al., 2010).
Figure 1.9: Alternative splice forms of Runx1 produced from P1 and P2 promoters. White boxes: coding sequences; grey: untranslated regions (adapted from Gonneau, 2010).
Figure 1.10: Lineage- and stage-specific effects of deregulated Runx expression in the hemopoietic system. Arrows and block arrows indicate the promotion and inhibition of differentiation, while - and + symbols indicate whether these effects are mediated by a deficit (-) or an excess (+) of Runx activity (taken from Cameron and Neil, 2004)
1.4.1.2 Protein structure

The Runx1 protein forms with its binding partner Cbfβ a heterodimeric transcription factor which binds to the consensus sequence G/AACCG/AC/A (Kitabayashi et al., 1998; Zhang et al., 1996; Giese et al., 1995). Upon dimerisation with Cbfβ, allosteric changes in the Runx1 conformation occur (Yan et al., 2004), which enhance Runx1 binding affinity to the Runx binding site. From a molecular perspective, Cbfβ replaces negative regulatory domain for DNA binding (NRDB)n and c responsible for the low affinity of Runx1 towards DNA. Cbfβ−/− embryos phenocopy the Runx1 null embryo with E12 embryonic lethality (Okuda et al., 1996; Sasaki et al., 1996), highlighting the poor affinity of Runx1 for DNA in absence of Cbfβ. Interestingly, Cbfβ deficiency can be rescued by overexpression of Runx1 in Cbfβ−/− embryos (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996a).

The onset of Runx1 expression is regulated by BMP proteins and SCL transcription factor (Pimanda et al., 2007). The Runt domain contains a nuclear localization signal and Runx1 is localized inside the nucleus (Lu et al., 1995). By contrast, Cbfβ is mainly located in the cytoplasm, indicating that Runx1 function is regulated by the translocation of Cbfβ into the nucleus (Lu et al., 1995; Tanaka et al., 1997). Moreover, the binding of Runx1 to Cbfβ protects the former from ubiquitin-proteasome-mediated degradation (Huang et al., 2001). All the aforementioned processes are recapitulated in figure 1.11.A.

1.4.1.3 Runx1-mediated transcriptional regulation

Runx1-mediated transcriptional activation occurs upon binding to DNA when Runx1 recruits various co-factors (Durst and Hiebert, 2004; Lutterbach et al., 2000; figure 1.11.B). Runx1 activates many haematopoietic genes: interleukin-3 (IL3) (Uchida et al., 1997), granulocyte macrophage colony stimulating factor (GM-CSF) (Zhang et al., 1996), defensin protein NP3 (Westendorf et al., 1998). Interestingly, the lack of haematopoiesis in E9.5 Runx1 KO embryo proper can be rescued with
A. Repression of target genes
B. Expression of target genes
C. Repression of target genes
Figure 1.11: Overview of Runx1 dynamics inside the cell. A. Journey and regulation of Runx1 from the gene to the protein. Once synthetized, Runx1 is translocated to the nucleus and binds there to NRDBn and NRDBc domains responsible for its poot affinity towards DNA. In absence of Cbfβ, Runx1 is degraded through the ubiquitin-proteasome pathway. When cytoplasmic Cbfβ is translocated to the nucleus, dimerization with Runx1 occurs and the complex binds to the Runt domain of target genes to influence their transcription. B. Runx1 recruits with partners such as ETS1, Myb, C/EBP and P300/CBP to mediate target gene expression. C. When interacting with partners such as Groucho, mSin3A, histone deacetylases (HDACs) and methyltransferases (e.g. SUV39H1), Runx1 mediates target genes repression (adapted from Gonneau, 2010).
viral transduction of the transcriptionally active form of Runx1 (i.e. which possesses the AD domain, figure 1.12) (Goyama et al., 2004).

Runx1 also mediates transcriptional inactivation by interacting with Groucho via its VWRPY motif (Aronson et al., 1997; figure 1.11.C), histones acetylases (e.g. mSin3A) and methyltransferase (e.g. USV39H1) via its inhibitory domains (ID) (Lutterbach et al., 2000; Reed-Inderbitzin et al., 2006; figure 1.11.C). It is also possible that Runx1 inactivates genes simply by occupying the Runt domain with a short isoform lacking the transactivation domain (Figure 1.10 and 1.12).

Runx1 represses genes such as CD4 and Tcrd (T-cell receptor delta chain) (Durst and Hiebert, 2004).

1.4.2 Runx1 expression during steady-state haematopoiesis

Studies using reporters for Runx1 activity highlighted the lineage and maturation stage-specific expression of Runx1 in the murine adult bone marrow (BM) and peripheral blood (PB) (North et al., 2004, Lorsbach et al., 2004; Gonneau, 2009).

All adult HSCs and CFU-S11 and the vast majority of bone marrow CFU-Cs express Runx1. Of note, overall decreased cellularity was observed in BM and thymus but not in the spleen of Runx1LacZ/+ animals (North et al., 2004). Interestingly, the phenotypic impact of a hemizygous dosage of Runx1 in the mouse is mild compared to that in the human (platelet defect, myeloid leukemia) (Song et al., 1999).

Runx1 is expressed by all HSCs and CFUs but Runx1 expression is downregulated upon erythroid maturation and is variable during lymphocyte maturation in the thymus (Figure 1.13, North et al., 2004; Lorsbach et al., 2004). Runx1 is also expressed in the myeloid compartment of the adult BM with the strongest expression reported in immature myeloid cells. Overall, Runx1 expression, which starts at the time of HSC emergence, tends to decrease during differentiation, hence the idea that Runx1 is strongly correlated with the stemness of the HSCs.
Figure 1.12: Structure of Runx1b long isoform and 521-41 short isoform. Runt: Runt DNA-binding domain; m: binding region for mSin3A; AD: transactivation domain; ID: inhibitory domain; VWRPY: VWRPY motif (adapted from Goyama et al., 2004).
Figure 1.13 A. Evolution of Runx1 expression during T-cell maturation. Pie charts represent individual maturation stages; grey shaded areas represent the average percentages of Runx1- expressing cells within each cell population. The percentages of Runx1+ cells (±SD) within the T-cell lineage are shown below the pies for thymic DN1 lymphoid progenitors (CD3-CD4-CD8-CD44+CD25-), DN2 pro-T (CD3-CD4-CD8-CD44*CD25+), DN3 early pre-T (CD3-CD4-CD8-CD44*CD25+), DN4 late pre-T (CD3-CD4*CD8*CD44*CD25+), DP (CD4*CD8+), CD4 SP (CD4*CD8+), CD8 SP (CD4-CD8+), spleen CD4 SP, spleen CD8 SP, thymic NK cell (CD3-CD4-CD8+NK1.1+), and γδ T cells (CD4-CD8*TCRγδ+). B. Percentages of Runx1+ cells (±SD) within B-cell differentiation are shown for pre-pro-B (B220*CD43*HSA+), pro-B (B220*CD43*HSA+*BP1-), large pre-B (B220*CD43*HSA+*BP1-), small pre-B (B220*CD43*HSA+*BP1-), immature B (B220*CD43*HSA+IgM+IgD-), mature B (B220*CD43*HSA+IgM+IgD+), and spleen mature B (B220*CD43*HSA+IgM+IgD+) cells (adapted from North et al., 2004).
In PB and the spleen, Runx1 is more strongly expressed in mature CD4 lymphocytes than in mature CD8 counterparts. Interestingly, mature CD4 lymphocytes are more sensitive to Runx1 dosage (Lorsbach et al., 2004).

Importantly, the designs of published Runx1 reporters may introduce a bias in the reported Runx1 expression. In one case, one of the Runx1 WT alleles was targeted with a β-galactosidase reporter, a non-functional Runx1 allele, thus creating a heterozygote environment (North et al., 2004). However, this approach allows an amplification of the Runx1 signal through the enzymatic activity of the β-galactosidase. In another study (Lorsbach et al., 2004), one Runx1 WT allele was targeted with a murine Runx1b cDNA, a truncated version of Runx1 (from exon 4 to stop codon), flanked by an IRES-GFP sequence at the 3’ end of the gene and an active selection marker. Consequently, the only isoform expressed is the long isoform Runx1b, which may alter the normal course of adult haematopoiesis. In addition, the intensity of GFP expression is relatively weak, thus hindering the detection of Runx1 low-expressing cells. In the third case, an IRES-GFP cassette was knocked at the 3’ end of the Runx1 WT locus just after the last exon (Suleman, 2007; Gonneau, 2009). The main advantage of this approach is that the pattern of Runx1 isoforms remains presumably undisturbed compared to WT. However, the levels of intensity of GFP fluorescence reported are modest to poor. Ideally, one wants to combine a bright reporter with an undisturbed pattern of Runx1 isoforms. This was achieved by combining the +23 bp enhancer of the Runx1 gene, which confers the haematopoietic specificity to the Runx1 gene, in an hsp68 promoter-driven GFP reporter (Bee et al., 2009b).

1.4.3 Runx1 requirements during steady-state haematopoiesis

Expression of Runx1 in the most immature compartment of the adult BM (e.g. HSCs, CFU-Ss and CFU-Cs) along with the embryonic lethality observed in Runx1 KO embryos led to the idea that Runx1 must be essential for the maintenance of the HSC pool during adult life.

Several studies using Runx1 conditional KO revealed that Runx1 is in fact mainly dispensable for the maintenance and the homing of HSCs to the BM upon
transplantation. However, Runx1 deleted adult animals display differentiation defects in several lineages (Ichikawa et al., 2004; Growney et al., 2005). B and T-cell maturation is impaired and the production of common lymphocyte progenitor is dramatically reduced. Megakaryocytic maturation is inhibited although megakaryocytic precursors are generated. Consequently, there is a bias towards the myeloid lineage, which is not affected by the loss of Runx1. Of note, the myeloproliferative phenotype reported after Runx1 deletion in both studies may be the result of pIpC-based induction of the Mx1-Cre deletor mouse as interferon has been shown to induce proliferation in the bone marrow (Kuehn et al., 1995).

The effect of Runx1 dosage on the adult HSC pool remains unclear to this date as two studies reported contradictory results. In one study (Sun and Downing, 2004), a 50% decrease in the HSC pool was reported whereas in another study (Ichikawa et al., 2004), an increase was demonstrated. The main difference between these two studies is that Ichikawa et al. used a Mx1-Cre deletor mice to conditionally delete one Runx1 allele whereas Sun and Downing mice were Runx1+/− from the embryo conception. This indicates that a full dosage of Runx1 is necessary to establish a normal pool of HSCs. In an attempt to resolve these discrepancies, a third study, using both an inducible Mx1-Cre and a Vav-deletor (excision occurs around the time of emergence of the first HSC) approaches, showed that loss of Runx1 had only little or no effect on the numbers of adult HSC (Cai et al., 2011). However, excision of Runx1 affected the expression of stem cell markers within the HSC pool (HSC shifted from the CD48− to the CD48+ compartment), which could account for previously reported decreases (Sun and Downing, 2004).

1.4.4 Runx1 expression and requirements during embryonic development

The study of transgenic embryos highlighted the crucial role played by Runx1 for the emergence of HSCs and progenitors during the development of the haematopoietic system.
1.4.4.1 *Runx1* is expressed in the sites of HSC emergence

The first signs of *Runx1* expression are detected in the yolk sac and placenta during the first wave of haematopoiesis from E7.5 onwards (North et al., 1999; Zeigler et al., 2006). Additionally, *Runx1* expression has been observed in the major arteries of the conceptus (umbilical and vitelline arteries and the dorsal aorta) around E11 (Figure 1.14). Expression of *Runx1* in the subluminal layers of the dorsal aorta and the HIACs (North et al., 2002; figure 1.15) was seen as the confirmation of the endothelial origin of the HSCs. However, the labelling of the sub-aortic mesenchyme also points towards a broader mesodermal origin of HSCs.

1.4.4.2 Lessons from the *Runx1* KO embryo

*Runx1* plays a quite unique role in the development of the haematopoietic system as its loss only mildly affects the first wave by reducing the production of macrophages and erythrocytes. Moreover, produced erythrocytes are deformed and exhibit reduced expression of erythroid lineage-specific markers such as Ter119, KLF1 and Gata1 (Yokomizo et al., 2008).

On the contrary, the loss of *Runx1* totally blocks the onset of the second wave of haematopoiesis, which ends up by the embryonic lethality reported around E12. The most striking manifestation of the lethality consists of an extensive necrosis in the embryo (Wang et al., 1996a) with anaemia and a haemorrhaging pattern that follows the neural tube from the tail to the head (Figure 1.16, Samokhvalov et al., 2006). The disappearance of haemorrhages upon reactivation of *Runx1* in the Tie2 embryonic haemopoietic/vascular compartment (Liakhovitskaia et al., 2009) suggests a vascular origin of those haemorrhages rather than a nervous defect. The pale fetal liver of the KO embryo indicates a lack of haematopoiesis normally found at that stage in its WT counterpart. Most importantly, the *Runx1* KO embryo and its yolk sac are totally devoid of HSC activity (Figure 3.2) and progenitor activity is reportedly missing from E9.5 onwards in the embryo proper (Goyama et al., 2004; Wang et al., 1996) and from E10.5 in the yolk sac (Wang et al., 1996).
Figure 1.14: Runx1 expression in the E11 embryo as reported by LacZ staining. Runx1 is expressed in the major haematopoietic sites: yolk sac (ys), aorta-gonad-mesonephros (AGM), umbilical (u) and vitelline arteries (v), foetal liver (fl) (taken from North et al., 1999).

Figure 1.15: Expression of Runx1 (blue, A and B) and Flk1 (brown, B) in E11 Runx1LacZ/+ dorsal aorta sections. Arrows indicate HIACs. (taken from North et al., 2002).
Figure 1.16: E12 wildtype (left) and Runx1\(^{-/-}\) (right) embryos. Notice the extensive haemorrhages in the central nervous system of the KO embryo. Arrowheads indicate the foetal liver, pale in the KO embryo, consistent with a lack of haematopoiesis (taken from Samokhvalov et al., 2006).

Figure 1.17: Runx1 expression in the vasculature (as observed by LacZ staining in heterozygote Runx1\(^{LacZ/WT}\) in A.) is rescued in Tie2-reactivated embryos (as observed by the absence or a significant attenuation of LacZ staining in B.). Arrows: Umbilical Cord; arrowhead: Placenta. (taken from Liakhovitskaia et al., 2009).
Interestingly, the E10 KO embryo lacks intra-aortic clusters in the dorsal aorta (Yokomizo et al., 2001), which have been directly correlated to HSC activity in E11 WT AGM (Rybtsov et al., 2011).

A compaction of the mesenchyme underlying the DA has been reported in the E11 Runx1 KO AGM (Bee et al., 2010), suggesting that the migration of precursors of HSCs to the lumen of the DA (Rybtsov et al., 2011), and thus, the maturation process that occurs along the way may be hampered. It is also possible that Runx1 expression in the precursors is required for their migration through the mesenchyme towards the DA.

The WT allantois and chorion, which merge together to give rise to the placenta have haematopoietic potential per se when isolated before circulation or chorio-allantoic fusion (Zeigler et al., 2006). Very interestingly, in the case of a Runx1 KO conceptus, both allantois and chorion displayed a pronounced reduction of their haematopoietic potential before circulation or fusion, even though the chorion carried a functional allele of Runx1 as the mother is of Runx1+/− phenotype. This suggests that the haematopoietic potential of the maternal component of the placenta before fusion depends of the expression of Runx1 in its embryonic counterpart, indicating a probable paracrine signaling between the allantois and chorion.

Zebrafish studies also indicate that Runx1 marks primitive and definitive haematopoietic sites (Lam et al., 2009).

In terms of Runx1 requirements, excision of Runx1 in the embryonic VC compartment leads to the ablation of HSC development. However, excision of Runx1 in the Vav1 embryonic compartment, Vav1 being one the first pan-haematopoietic genes expressed, does not abolish HSC emergence (Chen et al., 2009). These results suggest that the time window when Runx1 is required starts with VC expression in the developing embryo (potentially after mesoderm specification at E6.5) and stops at E11 with the emergence of the first HSC.
1.4.4.3 Lessons from the *Runx1* heterozygote embryo

A hemizygote dosage of *Runx1* on haematopoietic development totally upsets the spatio-temporal pattern reported in the WT embryo. Firstly, HSCs in the *Runx1*\(^{+/−}\) conceptus emerge at E10 (Cai et al., 2000), that is one day before its WT counterpart.

Moreover, HSCs are first detected in the yolk sac, then in the AGM, which is the opposite sequence of events compared to the WT.

In addition, the *Runx1*\(^{+/−}\) fetal liver (FL) already contains many HSCs by E11 unlike its WT counterpart, which only has comparable number of HSCs in its FL by E12. In other words, the sequence of events that leads to the emergence of HSCs and their migration to the FL takes place 24h earlier in the *Runx1* heterozygote conceptus compared to the WT, with the first HSC being detected in the yolk sac.

Furthermore, sections of the E11 *Runx1*\(^{+/−}\) AGM revealed that clusters are less defined in this organ, indicating a delay in AGM HSC specification compared to the *Runx1*\(^{+/−}\) yolk sac. Alternatively, immature HSCs may still arise in the *Runx1*\(^{+/−}\) AGM but cannot be retained there and mature on their way to the yolk sac. To test this hypothesis, it would be interesting to assess the HSC content of the embryonic blood from the E10 and E11 *Runx1* heterozygote embryo.

Explant culture revealed a shift from the E11 *Runx1*\(^{+/−}\) AGM to the yolk sac and placenta in their ability to mature HSCs (Robin et al., 2006). This indicates either a shift in PreHSC numbers from the AGM to the Yolk sac and placenta or a modification of the niche of those organs, which gives yolk sac and placenta earlier competence for maturation of HSCs.

Surprisingly, the reported decreased in E11 *Runx1*\(^{+/−}\) AGM HSC activity can be rescued by the addition of IL3 during explant culture (Robin et al., 2006), a known downstream target of Runx1 (Lantz et al., 1998). Of note, addition of IL3 in E11 yolk sac and placenta explants from *Runx1* heterozygote embryos also increases their HSC output (Robin et al., 2006).

*Runx1* dosage also affects the distribution of HSCs between CD45 positive and negative compartments (North et al., 2002). This suggests a role for Runx1 in the upregulation of CD45 from precursors of HSCs and therefore, a possible requirement in the transition between PreHSC Type I and PreHSC Type II phenotypes.
1.4.4.4 Lessons from Runx1 rescue in the embryo

In rescue studies, it is crucial to identify the cell population, which is rescued in terms of surface markers and haematopoietic potential in order to better describe the steps precursors go through before becoming HSCs.

The expression of Runx1 driven by Gata1 regulatory elements in Runx1 KO conceptus leads to a partial rescue of the clonogenic progenitors from the second wave of haematopoiesis. More specifically, only the erythroid precursors are rescued and no intra-aortic clusters are detected (Yokomizo et al., 2007). Interestingly, Gata1-rescued embryos show no haemorrhages at E12 and survive until E14.5.

Lentiviral transfection of Runx1 KO E9.5 Para-aortic splanchnopleura (Psp, which becomes the AGM by E10) rescues both progenitors and haematopoietic cells but rescue at later stage for HSC activity was not assessed (Mukouyama et al., 2000; Goyama et al., 2004). This suggests that in absence of Runx1, the embryo proper already contain precursors of haematopoietic cells that require the expression of Runx1 to mature. Alternatively, virus-based transfection may have forced cells to differentiate along the haematopoietic lineage. However, this hypothesis has not been tested by assessing, for instance, the endothelial potential of Runx1-transfected versus mock-transfected cells.

Two elegant studies (Liakhovitskaia et al., 2009 and unpublished) using deleter mice carrying reactivatable Runx1 KO alleles (Samokhvalov et al., 2006) suggest that reactivation of Runx1 in either Tie2 or CD41 embryonic compartments is sufficient to rescue the HSC and progenitor lineages. Tie2 being expressed in the majority of the VC compartment from E8.5 to E11 (Liakhovitskaia et al., 2009; Taoudi et al., 2005), Runx1 is rescued in a broad compartment (the whole vasculature of the embryo) with this approach (Figure 1.17). By contrast, CD41 is a haematopoietic marker and the successful rescue of HSC lineage in this compartment suggests that Runx1 is required when haematopoietic commitment had already taken place. This result goes against the model in which Runx1 is required for the endothelial to haematopoietic transition but not thereafter (Chen et al., 2009). Of note, the earlier CD41-rescued cells are detected around E8 in the allantois and the
yolk sac (Rybtsov et al., 2011) and assessment of the endothelial potential of such cells is under way in our laboratory. This will determine whether those cells are endothelial, haematopoietic or bipotential precursors.

Importantly, full rescue of the HSC lineage is achieved when pregnant dams carrying tamoxifen-inducible Runx1-CreER\textsuperscript{T2} Runx1 Reactivatable KO embryos are injected intraperitoneally with tamoxifen at E7.5. However, the rescue fails if tamoxifen is injected at later stages (Nishikawa et al., unpublished), thus confirming that Runx1 expression is required around E8. However, the time needed for tamoxifen to reach the target population can be highly variable when using this strategy as tamoxifen is diluted in the maternal and embryonic bloodstreams. Therefore, the latest embryonic stage when Runx1 reactivation can rescue the HSC lineage remains unclear.

1.5 Runx1 is involved in the development and the homeostasis of neural, gastrointestinal and bone systems

Studies of the intestinal, neural systems and cartilage and bone development have shown that Runx1 plays a role in the development and the homeostasis of these tissues (Lian et al., 2003). The persistent neonatal lethality and sternum ossification defects observed after the successful Tie2 or CD41-driven rescue of the HSC lineage in Runx1 KO embryos strongly suggests that Runx1 is required for proper development of the rib cage (Liakhovitskaia et al., 2009 and unpublished; Liakhovitskaia et al., 2010). More generally, Runx1 and Runx2 have been shown to cooperate in the skeletal development with a possible role for Runx1 in mediating endochondral and intramembranous bone formation (Smith et al., 2004).

Recently, Runx1 was identified as a safekeeper of the mouse gastrointestinal tract integrity via its tumor suppressor activity (Fijnemann et al., 2011).

Runx1 also plays a role in the development of the mammalian nervous system via its repression activity, especially on the specification of olfactory receptor neurons (Thierault et al., 2005). By contrast, in Runx1 KO embryos, hindbrain branchiovisceral motor neuron precursors of the cholinergic lineage are correctly specified but then fail to progress to a more differentiated state and undergo
increased cell death, resulting in a neuronal loss in the mantle layer (Thierault et al., 2004). Loss of Runx1 also affects the sensitivity of KO animals to thermal and neuropathic pain and overexpression of Runx1a isoform leads to increased threshold for mechanical sensitivity, retarded growth, pigment defects, dystrophic root ganglia and megacolon (Kanaykina et al., 2010).

1.6 Other key genes involved in the development of the haematopoietic system

Many genes have been involved in the development of the haematopoietic system. However, detailing each of them is beyond the scope of this introduction. Only the most relevant genes are detailed below. For the rest of them, a table recapitulates their role and corresponding KO phenotype (Table 1.2).

1.6.1 The role of CD41 in developmental haematopoiesis

CD41 (also known as glycoprotein IIb or integrin aIIb) is an integrin, a family of proteins capable of binding to extracellular matrix. First considered as a megakaryocyte-platelet specific marker involved in coagulation, CD41 emerged as a broader haematopoietic marker expressed on myeloid progenitors and adult BM CFU-S (Berridge et al., 1989). Likewise, the notion of megakaryocyte-platelet lineage restricted marker was challenged by a large body of data suggesting that CD41 is actively involved the ontogeny of the haematopoietic system (Mikkola et al., 2003; Ferkowicz et al., 2003; Emambokus et al., 2003).

Functionally, CD41 forms with CD61 a complex in the presence of calcium, that binds to a variety of factor including fibrinogen, fibronectin, von Willebrand factor and vitronectin (Shattil et al., 1998).
Table 1.2: Genes involved in haematopoietic ontogeny during the first and the second wave of haematopoiesis (taken from Gonneau, 2010).

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Time of death</th>
<th>YS haematopoiesis</th>
<th>FL haematopoiesis</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tal1/SCL</td>
<td>9-10.5</td>
<td>markedly reduced</td>
<td>absent</td>
<td>Robb et al., 1995; Shivdasani et al., 1995</td>
</tr>
<tr>
<td>Lmo-2</td>
<td>9-10.5</td>
<td>markedly reduced</td>
<td>absent</td>
<td>Warren et al., 1994; Yamada et al., 1998</td>
</tr>
<tr>
<td>GATA-1</td>
<td>10.5-11.5</td>
<td>markedly reduced</td>
<td>absent</td>
<td>Pevey et al., 1991</td>
</tr>
<tr>
<td>Flk-1</td>
<td>8.5-9.5</td>
<td>markedly reduced</td>
<td>absent</td>
<td>Shalaby et al., 1997; Shalaby et al., 1995</td>
</tr>
<tr>
<td>Tie-2</td>
<td>8.5-9.5</td>
<td>markedly reduced</td>
<td>absent</td>
<td>Takakura et al., 1998</td>
</tr>
<tr>
<td>CBP</td>
<td>8.5-10.5</td>
<td>reduced</td>
<td>absent</td>
<td>Oike et al., 1999</td>
</tr>
<tr>
<td><strong>Genes affecting both primitive and definitive haematopoiesis only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML1/Runxl</td>
<td>11.5-12.5</td>
<td>normal</td>
<td>blocked</td>
<td>North et al., 1999; Okuda et al., 1996; Wang et al., 1996</td>
</tr>
<tr>
<td>CEBPb</td>
<td>11.5-14</td>
<td>normal</td>
<td>blocked</td>
<td>Wang et al., 1996b</td>
</tr>
<tr>
<td>GATA-2</td>
<td>10.5-11.5</td>
<td>reduced</td>
<td>markedly reduced</td>
<td>Tai et al., 1994</td>
</tr>
<tr>
<td>c-myb</td>
<td>15</td>
<td>normal</td>
<td>decreased</td>
<td>Mucenski et al., 1991</td>
</tr>
<tr>
<td>c-kit</td>
<td>at birth</td>
<td>decreased erythropoiesis</td>
<td>blocked erythropoiesis</td>
<td>Bernex et al., 1996; Ogawa et al., 1993</td>
</tr>
<tr>
<td>Pu.1</td>
<td>18.5</td>
<td>normal</td>
<td>reduced</td>
<td>Scott et al., 1997</td>
</tr>
<tr>
<td>Ikaros</td>
<td>viable</td>
<td>normal</td>
<td>reduced</td>
<td>Georgopoulos et al., 1994; Nichogiannopoulou et al., 1999</td>
</tr>
</tbody>
</table>
1.6.1.1 CD41 expression in the developing embryo

The earliest CD41 expression is detected in trophoblasts of the embryo at the implantation stage (Rout et al., 2004). It has been demonstrated that CD41 plays a role in the adhesion and migration of mouse trophoblast cells. However, this early expression has not been correlated to haematopoiesis so far.

CD41 has been identified as the first missing haematopoietic markers in SCL/Tal-1⁻ EBs, which indicates that CD41 is a downstream target of the SCL/Tal-1 gene (Mikkola et al., 2003). Furthermore, clonogenic precursors are detected in both CD45⁺ and CD45⁻ compartment in E9.5 YS but all express CD41 (Mikkola et al., 2003).

CD41 expression was reported in the early YS from E7 to E9.5 (Ferkowicz et al., 2003), in the P-Sp from E9.5 (Ferkowicz et al., 2003) until at least E11.5 (Taoudi et al., 2005), in the vitelline and umbilical arteries from E9.5 (Ferkowicz et al., 2003) and in the pre-fusion allantois (Corbel et al., 2007). Moreover, CD41 expression often co-localizes with HIACs in the Psp/AGM region (Emambokus et al., 2003; Taoudi et al., 2005; Corbel and Salaun, 2002). Thus, CD41 rather than CD45 marks the haematopoietic compartment in the early embryo. Of note, the majority of the results concerning CD41 population were obtained with a FITC-conjugated antibody.

Significant discrepancies in the resolution of CD41 negative and low-expressing populations have been observed when comparing PE and FITC-conjugated antibody from the same clone, with the former giving much stronger signal (Taoudi et al., 2005; Rybtsov et al., 2011). Therefore, it is quite likely that CD41 expression has been underestimated both in terms of proportion and first appearance in the primary haematopoietic sites.

1.6.1.2 Haematopoietic potential of CD41⁺ cells in the conceptus

In the E9.5 yolk sac and embryo proper, haematopoietic potential of CD41 compartment is associated with the c-Kit⁺CD34⁺ fraction and is enriched for progenitor cell activity (Mikkola et al., 2003).
Almost the entire c-Kit⁺CD34⁺ compartment, which marks HSC potential (i.e., putative PreHSCs) from E9.5 to E13, expresses CD41 in the E9.5 yolk sac. Accordingly, the vast majority of the myelo-erythroid potential, including HPP-CFC, CFU-GM, BFU-E, and CFU-Mix potential is in the c-Kit⁺CD34⁺CD41⁺ population. This compartment also exclusively harbours neonatal reconstituting activity compared to its counterpart c-Kit⁺CD34⁺CD41⁻ (Ferkowicz et al., 2003; Mikkola et al., 2003).

In E9.5 Psp, myelo-erythroid progenitors, including CFU-GM, BFU-E, CFU-Mix and HPP-CFC, are found in both CD41 positive and negative population whereas the vast majority of c-Kit⁺CD34⁺ compartment expresses CD41. This suggests that in E9.5 Psp, CD41 expression is not specific to progenitor population. No transplantation data are available for CD41⁺ E9.5 P-Sp (Ferkowicz et al., 2003).

In E12.5 fetal liver, significant amount of progenitors, with the same potential as those found in E9.5 P-Sp, fall into the CD41⁻/lo fraction. Low-level long-term reconstitution into adults is achieved with Kit⁺CD34⁺CD41⁺ cells and higher levels are reached with Kit⁺CD34⁺CD41⁻ population, which indicates a downregulation of CD41 on reconstituting haematopoietic cells. However, FL was depleted from Mac1⁺ population in these experiments, which could have dramatically reduced the number of HSCs in each transplanted population. In the adult BM, repopulating activity was enriched in the Kit⁺CD34⁺CD41⁻ fraction suggesting that CD41 is downregulated in adult LTR-HSCs (Ferkowicz et al., 2003).

### 1.6.1.3 CD41 role in haematopoietic cell regulation

CD41 binding function through the CD41/CD61 receptor complex has been shown for haematopoietic cells and may contribute to regulate their number in haematopoietic organs (Emambokus et al., 2003). Surprisingly, mutation in either CD41 or CD61 does not impair embryonic haematopoietic development or adult homeostasis but results in a non-lethal bleeding disorder known as Glanzmann’s thrombasthenia (Bellucci and Caen, 2002). This suggests that CD41 is dispensable for haematopoietic development. Recent lineage tracing experiments showed that HSC are mainly specified through a CD41⁺ stage (Rybtsov et al., 2011). However,
the presence of unmarked HSCs in the adult bone marrow indicates a possible CD41-independent pathway for HSC specification.

1.6.2 Genes involved in ex vivo expansion of HSCs

1.6.2.1 Stem Cell Factor / c-Kit ligand

The Stem Cell Factor (aka Steel factor or c-Kit ligand) binds to c-Kit to promote cell survival and is expressed on the surface of HSCs throughout development (Gekas et al., 2005; Morrisson et al., 1995; Sanchez et al., 1996; Yoder et al., 1997a).

Alternative splicing of the SCF mRNA results in membrane-bound and secreted forms of SCF (Toksoz et al., 1992). Mutations in the SCF/c-Kit system often result in infertility due to spermatogenesis defects (reviewed in Mauduit et al., 1999). Of note, recipients homozygote for a specific mutation in the c-Kit receptor called W41 remain fertile but are mildly compromised, which makes them useful tool to investigate haematopoiesis in a c-Kit deficient environment (Geissler and Russell, 1983a).

SCF production is increased after irradiation of adult recipients, which is thought to influence the outcome of transplantation-based repopulation assays (Brehm et al., 2012).

Interestingly, it was shown recently that transgenic expression of membrane-bound SCF enhanced the engraftment of human HSC in non-irradiated non-obese severely compromised immunodeficient IL2rγ null (NSG) mice (Brehm et al., 2012).

Most of the cytokine combinations used for ex vivo expansion of HSCs include SCF (Zhang and Lodish, 2008). SCF is a key component of the cytokine cocktail in the reaggregate and the OP9 coaggregate cultures for HSC maturation and expansion (Taoudi et al., 2008; Rybtsov et al., 2011 and unpublished).
1.6.2.2 Interleukin 3

There have been contradictory reports of the effects of interleukin 3 (IL3) on adult HSCs expansion \textit{ex vivo} (Bryder and Jacobsen, 2000; Peters et al., 1996; Yonemura et al., 1996). However, a body of data suggests a positive effect on emergence and expansion of embryonic HSCs (Robin et al., 2006).

Adult steady-state haematopoiesis remains fairly unaffected by the disruption of IL3 or the IL3 receptor. Moreover, IL3 deficient mice have normal blood cell counts, CFU-Cs and HSCs (Lantz et al., 1998; Mach et al., 1998). \textit{In vitro} mast cell differentiation from IL3 null bone marrow cells was impaired when SCF only was present, but rescued upon addition of exogenous IL3, emphasizing the synergistic action of IL3 and SCF (Lantz et al., 1998).

Most importantly, IL3 receptor is not expressed in \textit{Runx1} null embryos, which are devoid of HSCs and is reduced in \textit{Runx1} heterozygote embryos (Robin et al., 2006), which could explain the decrease reported in AGM HSCs. These results also strongly suggest that \textit{IL3} is a downstream target of \textit{Runx1}. Interestingly, E11 \textit{Runx1}^{+/−} AGM HSC deficiency can be rescued by supplementing the AGM with exogenous IL3 during explant culture (Robin et al., 2006).

Addition of exogenous IL3 leads to early emergence of HSC from early E10 WT AGM, yolk sac and placenta (30-34 sp) (Robin et al., 2006) as well as increased proliferation and survival of populations enriched in HSCs (Sca1^{+}c-Kit^{+} (de Bruijn et al., 2002) and CD34^{+}c-Kit^{+} (Sanchez et al., 1996)).

IL3 is a key component of the cytokine cocktail in the reaggregate and the OP9 coaggregate cultures for HSC maturation and expansion (Taoudi et al., 2008; Rybtsov et al., 2011 and unpublished).

Taken together, these results highlight the importance of IL3 during haematopoietic development, especially on the emergence, proliferation and survival of the first embryonic HSCs.
**1.6.2.3 Flt3-Ligand**

Fms-like tyrosine kinase 3 ligand (Flt3-L) binds to the Flt3 receptor (also known as Flk2), which is expressed in the bone marrow microenvironment as well as in the myeloid and lymphoid lineage (Gilliland and Griffin, 2002).

Upon injection of a mouse recipient with Flt3-L, haematopoietic progenitors in the bone marrow and the spleen reportedly expand and HSCs are mobilized to the peripheral blood (Brasel et al., 1996).

Surprisingly, Flt3-L null animals do not display any obvious defects or phenotype. They have a normal HSC pool but B cell progenitors, dendritic cells and natural killers cell numbers are reduced *in vivo* (McKenna et al., 2000; Sitnicka et al., 2002).

However, the expansion reported after *ex vivo* culture of adult bone marrow HSCs with SCF and Flt3-L strongly indicates that Flt3-L synergizes with other cytokines to enhance HSC function (Buza-Vidas et al., 2009; Diehl et al., 2007; Gilliland and Griffin, 2002).

Interestingly, Ftl3-L receptor is not expressed in the E9.5 yolk sac, E11.5 AGM and fetal liver of the Runx1 KO embryos (Okada et al., 1998), suggesting a role for Flt3-L and its receptor in HSC emergence.

The study of embryonic haematopoiesis suggests that both Flt3 receptor and ligand are dispensable for normal HSC function in the E14.5 fetal liver. This includes steady state haematopoiesis, fetal and post-transplantation expansion (Buza-Vidas et al., 2009). However, to date, no study has addressed the potential role of Flt3 receptor and ligand in HSC emergence at earlier stages in primary haematopoietic sites (AGM, extra-embryonic vessels, yolk sac and placenta).

Flt3-L is a key component of the cytokine cocktail in the reaggregate and the OP9 coaggregate cultures for HSC maturation and expansion (Taoudi et al., 2008; Rybtsov et al., 2011 and unpublished).
1.7 The tamoxifen-inducible Cre/loxP system, an valuable tool in the investigation of the mechanisms underlying HSC specification

Conditional gene deletion is a powerful approach to study the role of a gene in the adult life of the animal, especially when gene loss leads to embryonic lethality. Gene deletion strategies often involve the Cre enzyme (Cre stands for Catalyzer of Recombination), a 38kD integrase, which comes from the P1 bacteriophage and catalyses the recombination between two of its recognition sites, called \(\text{loxP}\) (Hamilton and Abremski, 1984).

\(\text{loxP}\) sites consist of a 34 bp consensus sequence with a core spacer of 8 bp and two 13 bp palindromic flanking sequence. \(\text{lox511}\) is an alternative recognition site, which only differs slightly from \(\text{loxP}\) in the spacer sequence. Nonetheless, Cre only catalyses deletion between two homologous \(\text{loxP}\) sites. Many studies use insertion strategy based on heterologous \(\text{loxP}\) sites (reviewed in Nagy, 2000).

Temporal control is another crucial aspect when one wants to investigate the aftermaths of gene excision from a precise developmental stage. To this end, inducible forms of the Cre protein were developed either by cloning Cre downstream of an inducible Mx promoter or by fusing Cre with a mutated Estrogen Receptor (ER\(^{\text{T2}}\)) domain. The resulting forms of Cre are inducible with interferon/pIpC (Mx1-Cre) and tamoxifen (CreER\(^{\text{T2}}\)), respectively (reviewed in Nagy, 1999). Importantly, the mutation of the ER domain resulted in a decreased affinity for estrogen-like molecules, which are produced naturally in mammals and may be found in FCS. Therefore, any potential leakage in Cre activity should be strongly attenuated. This issue is discussed further and leakage during reaggregate culture is assessed in Chapter IV.

The tamoxifen-inducible CreER\(^{\text{T2}}\) system is recapitulated in figure 1.18. Briefly, CreER\(^{\text{T2}}\) is sequestrated in the cytoplasm by heat shock protein in absence of tamoxifen. Upon addition of tamoxifen, CreER\(^{\text{T2}}\) is translocated into the nucleus where it recombines the target gene of interest.

Inducible-Cre systems have proven invaluable tools to decipher the role of Runx1 during adult haematopoiesis in the bone marrow (Ichikawa et al., 2005;
Figure 1.18: Tamoxifen-mediated CreER$^{T2}$ gene excision mechanism. A. In absence of tamoxifen, Cre-ER$^{T2}$ fusion protein (CreER$^{T2}$) is sequestrated in the cytoplasm by heat shock proteins (Hsp) bound to the Estrogen Receptor domain. In the nucleus, the floxed STOP cassette prevent any expression of the gene of interest. B. When tamoxifen (Tam) is added, it binds to CreER$^{T2}$ with greater affinity than Hsp and allow the translocation of CreER$^{T2}$ into the nucleus where it can recombine target genes by removing the fragment located between two loxP sites, here a STOP cassette. Of note, CreER$^{T2}$ also catalyses the opposite reaction, that is to say the insertion of a cassette in the locus of interest, albeit at much lower efficiency.
Growney et al., 2005), as well as in lineage tracing studies in the embryo (Samokhvalov et al., 2007; Zovein et al., 2008).

In the present work, tamoxifen-inducible Cre expression was driven by the Rosa26 promoter for ubiquitous CreER\(^{T2}\) expression. Thus, \textit{Runx1} reactivation becomes potentially achievable in haematopoietic compartment but also in non-haematopoietic compartments, which may play a role in HSC development.
1.8 Goals of the present work

The hypothesis underlying the present work is that the reactivation of the Runx1 locus at E9.5 followed by an in vitro culture period will rescue the development of HSCs in the primary haematopoietic sites (AGM/Caudal Part, Yolk Sac and Placenta).

Now that precursors of HSCs, namely PreHSC Type I and II, have been identified (Rybtsov et al., 2011), the tamoxifen-reactivatable Runx1 KO model (Samokhvalov et al., 2006) offers a unique opportunity to investigate the role of Runx1 in these immature populations. Moreover, tracking these populations constitutes a precious indicator of the sequence of events that ultimately leads to the emergence of HSCs.

That is why the present work focused on the roles and requirements of Runx1 in the mouse conceptus from E9.5 to E11 and followed three main lines of investigation:

1. Investigate the cell populations (i.e. PreHSC Type I and II, endothelium and more mature cell populations such as VC’CD45+ and Ter119+) produced in absence of Runx1 and the level of progenitor activity present. This was performed in the fresh Runx1 KO conceptus from E9.5 to E11 using a reactivatable Runx1 transgenic model.

2. The study of tamoxifen/Rosa26-CreERT2 system toxicity on HSC maturation during reaggregate culture of E11.5 AGM. This should shed light on the impact of the tamoxifen/Rosa26-CreERT2 system on results from culture of Runx1 reactivated tissues.
   a. The first goal was to define a set of doses of tamoxifen and a way of performing induction that gives the best balance between HSC output and recombination of the sGFP reporter locus.
b. The second goal was to evaluate the levels of recombination achievable on the *Runx1* locus during OP9 coaggregation culture with the established doses.

3. The study of the haematopoietic potential of E9.5 *Runx1* KO and reactivated (Re) tissues when haematopoietic development is recapitulated *in vitro* using the OP9 coaggregate culture.
   a. The first goal was to determine whether the culture of E9.5 KO organs (i.e. caudal part, yolk sac and placenta) with OP9 could produce HSC, progenitors and the populations absent in the fresh tissues.
   b. The second goal was to evaluate whether the reactivation of *Runx1* in these E9.5 KO organs followed by culture with OP9 could rescue the HSC lineage, progenitor activity and the cell populations missing in the fresh KO tissues.
2 MATERIALS AND METHODS

2.1 Animal crossings

Mice were housed and bred in animal facilities at the University of Edinburgh in compliance with Home Office regulations. Wild type embryos were generated from the pairing of C57BL/6 females and males. Transgenic [Runx1\(^{LacZ/LacZ}\); Rosa26\(^{CreERT2/CreERT2}\)] embryos were obtained by crossing compound [Runx1\(^{LacZ/GFP}\); Rosa26\(^{CreERT2/CreERT2}\)] females and males and were used in Chapter III and first half of Chapter V (Culture of Runx1 KO tissues). Transgenic [Runx1\(^{LacZ/LacZ}\); Rosa26\(^{CreERT2/WT}\)] embryos were obtained by crossing [Runx1\(^{LacZ/GFP}\); Rosa26\(^{CreERT2/CreERT2}\)] males with [Runx1\(^{LacZ/WT}\); Rosa26\(^{WT/WT}\)] females in last half of Chapter V (Culture of Reactivated Runx1 tissues). Runx1-LacZ allele was described previously (Samokhvalov et al., 2006) and is summarized in Fig 2.1. Runx1-GFP allele is described in Fig 2.2. Transgenic [sGFP\(^{+}\); Rosa26\(^{CreERT2/WT}\)] embryos were obtained by crossing compound [sGFP\(^{-/-}\); Rosa26\(^{CreERT2/WT}\)] males with [sGFP\(^{+/+}\); Rosa26\(^{WT/WT}\)] females. Silent GFP allele was described previously (Gilchrist et al., 2003) and is summarized in Fig 2.3.

The morning after discovering a vaginal plug was designated as day 0.5. Developmental stage of embryos was determined according to Theiler’s criteria and somite pair (sp) counting. Briefly, embryos with 24 to 29 sp were considered E9.5; embryos with 30 to 39 sp were considered E10; embryos with 40 to 49 sp were considered E11.
A. Knocked-out Runx1 locus:

![Diagram of Runx1-LacZ knock-in reporter locus with LoxP sites and LacZ-STOP cassette]

B. Reactivated fully functional Runx1 locus:

![Diagram of Reactivation via Cre-mediated recombination]

Figure 2.1: A. Runx1-LacZ knock-in reporter locus. The Runx1 locus has been targeted at its endogenous IRES following the proximal promoter P2 with a floxed LacZ-STOP cassette containing a splice acceptor. Thus, LacZ reports activity from both P1 and P2 promoters. B. Upon Cre-mediated recombination, the Runx1 locus is reactivated and produce fully functional isoforms of Runx1 proteins (adapted from Samokhvalov et al., 2006). Primers used for genotyping shown in A. yield a 350 bp and in B. a 200 bp.
Figure 2.2: Runx1-GFP reporter locus (Suleman, 2007). The Runx1 locus has been targeted at the Exon 6 with an IRES-GFP containing vector. As a result, all cells expressing long isoforms of Runx1 are labeled in green.

Figure 2.3: Silent GFP locus (Gilchrist et al., 2003). Constitutive PGK-promoter driven GFP expression is hampered by a floxed Puro-STOP cassette that can be removed by the Cre recombinase. Transgenic cell lines and animals were obtained by random insertion of this transgene.
2.2 Making of solutions used to process biological tissues

Biological tissues were processed using mainly 4 solutions. Solution 1 was made of 7% Fetal Calf Serum (FCS) and 50 units/mL Penicillin/Streptomycin in Dulbecco’s Phosphate Buffered Saline (PBS) with Calcium/Magnesium. Solution 2 was made of 7% heat-inactivated FCS, 50 units/mL Penicillin/Streptomycin in Dulbecco’s PBS without Calcium/Magnesium. Solution 3 was made of 2% heat-inactivated FCS in Dulbecco’s PBS without Calcium/Magnesium. Solution 4 was made of 2% heat-inactivated FCS, 5 mM EDTA in Dulbecco’s PBS.

2.3 Isolation of embryonic tissue and preparation of single cell suspension

Embryonic tissues of interest were dissected in solution 1 (7% FCS, 50 units/mL Penicillin/Streptomycin (P/S) in Dulbecco’s Phosphate Buffered saline with Calcium / Magnesium) using sharpened tungsten needles under LEICA MZ8 microscope. Caudal Parts were obtained by cutting the embryo proper horizontally below the heart as described in figure 2.4, Yolk Sacs were dissected free of large umbilical and vitelline vessels. The Placenta was dissected as previously described (Taoudi et al., 2005). Tissues were subsequently digested in collagenase/dispase (Roche) at 1 mg/ml final concentration in solution 1 for 40 min at 37°C in waterbath under gentle shaking. Collagenase/dispase was then neutralized with 2 mL of solution 1, tissues were spun down for 5 min at 1500 rpm (GS-6R centrifuge, Beckmann), supernatant was discarded and cells were resuspended in 1 mL of solution 2 (7% heat-inactivated FCS, 50 units/mL P/S in Dulbecco’s Phosphate Buffered saline without Calcium/Magnesium) by gentle pipeting up and down no more than twenty times until a homogenous suspension was produced. If cell
Figure 2.4: Schematic of E9.5 Caudal Part dissection. Embryo is cut below the heart (dashed white line) in two pieces. The lower part, the Caudal Part, is used for experiments. The upper part, the Rostral Part, is used for genotyping.
suspension was not immediately used cells were spun for 5 min at 1500 rpm, 4°C and left on ice or in the fridge at 4°C.

2.4 Genotyping of transgenic embryos

Runx1 Transgenic embryos were tested for Runx1-GFP expression by flow cytometry using an aliquot of living yolk sac cells. Runx1-GFP positive embryos were then discarded. Heads from Runx1 transgenic embryos litters were lysed overnight into 100 µL of lysis buffer (composition per 1 mL: 45 µL of 10% Tween 20; 45 µL of NP40; 20 µL of Proteinase K 10 mg/mL; 100 µL of 10X Qiagen PCR Buffer) at 65°C and shortly spun down at 13,000 rpm with a benchtop centrifuge (Biofuge, Heraeus). Then, PCR for Runx1 was performed using 2 µL (for E10.5 and E11.5 tissues) or 5 µL (for E9.5 tissues) of the supernatant with Taq and Q-Buffer (Qiagen) according to the manufacturer instructions except for primer concentration. Each reaction contained three primers to distinguish between KO, Het and WildType (WT), Rx1LacZ_1 5’GACAAGCTGGACAGAAAGGT3’ at 1 µM, Rx1LacZ_3 5’CACTGGACAAACAGTGGCTG3’ at 1 µM, Rx1LacZ_5 5’GATGATCTTCTCCTCAACGTC3’ at 1 µM. The following program was used:
94°C 4 min
94°C 30s
58°C 30s
x35
72°C 1 min
72°C 10 min
PCR products were then separated on a 2% agarose gel (Figure 2.5). When needed, genotype was further confirmed by Southern Blotting with A-probe on Runx1 locus as previously described (Samokhvalov et al., 2006).

Before dissection, littermates from [sGFP+/--; Rosa26CreERT2/WT] x [sGFP+/++; Rosa26WT/WT] crossing were isolated with their yolk sac intact and looked under a MZFLIII dissecting microscope (Leica) for green fluorescence. All GFP positive embryos were discarded and GFP negative embryos were used for next steps.
Figure 2.5: Genotyping of Runx1 KO with Runx1 PCR. Runx1\textsuperscript{LacZ/LacZ} (KO) embryos (3,4) yielded a single band of 350 bp whereas Runx1\textsuperscript{+/+} and Runx1\textsuperscript{GFP/GFP} (WT) embryos (7,8) produced a single band of 200 bp. Runx1\textsuperscript{LacZ/GFP} and Runx1\textsuperscript{LacZ/+} (Het) embryos (1,2,5,6) produced both bands. No template control (-) produced no band as expected.

Figure 2.6: Genotyping of CreERT2\textsuperscript{+} embryos with Cre PCR. CreERT2\textsuperscript{+} embryos (2,3,4,6) yielded a 300 bp and CreERT2\textsuperscript{−} embryos (1,5) did not yield any band. No template control (-) produced no band as expected.
Heads from [sGFP⁺; CreERT2⁺/-] embryos were lysed overnight into 100 µL of lysis buffer. Then, PCR for Cre was performed using 2 µL (E11.5 tissues) or 5 µL (for E9.5 tissues) of the supernatant with Taq and Q-Buffer (Qiagen) according to the manufacturer instructions using a final concentration of 5 µM per primer. 

(CreF: 5′TCGATGCAACGAGTGATGAG3′; CreR: 5′AGTGCGTTCGAACGCTAGAG3′).

The following program was used:

94ºC 3 min
94ºC 30s
56ºC 30s
72ºC 1 min
72ºC 10 min

x40

PCR products were then separated on a 2% agarose gel (Figure 2.6).

2.5 AGM reaggregation culture

After dissection and dissociation, when appropriate, cells were counted by flow cytometry using counting beads (Perfect Count Microspheres, Ref: CYT-PCM-100, Cytognos) and 7-amino-actinomycin D (7AAD, eBioScience) to discard dead cells. To this end, 0.01 ee of cells was used along with 10,000 beads. Absolute number of cells was obtained using the following formula:

\[
\text{Total live cell number} = \frac{10,000}{\text{Number of bead events}} \times 100 \times \text{Number of live cell events}
\]

Cells were reaggregated as described (Sheridan et al., 2009, Taoudi et al., 2008) except that washed 0.8 µM Millipore membranes were used (catalogue number: AAW0P2500, Millipore). Prior to culture, membranes were washed at room temperature for 10 min in 100 units/mL P/S and 2x10 min in 50 units/mL P/S then air-dried. Reaggregates were cultured for 5 days at the gas/liquid interface in IMDM (Invitrogen) supplemented with murine IL-3, SCF and Flt-3L at 100 ng/ml each (all from Peprotech), 20% heat-inactivated FCS, 4mM glutamine and 50 units/mL P/S (referred as to IMDM⁺) at 37°C within a humidified 5% CO₂ incubator (Heraeus...
Instruments). 1 ee was used per 1 reaggregate. After culture, reaggregates with their membrane were dissociated in solution 1 with 1% collagenase/dispase for 40 min at 37°C in waterbath under gentle shaking. Collagenase/dispase was then neutralized with 2 mL of solution 1, tissues were spun down for 5 min at 1500 rpm (GS-6R centrifuge, Beckmann), supernatant was discarded and cells were resuspended in 1 mL of solution 2 by gentle pipeting up and down no more than twenty times until a homogenous suspension was produced. If cell suspension was not immediately used cells were spun for 5 min at 1500 rpm, 4°C and left on ice or in the fridge at 4°C.

2.6 OP9 coaggregation culture

After dissection and dissociation, cells were treated as described in the previous section (AGM reaggregate) except that 100,000 of freshly dissociated OP9 cells were added per embryo equivalent of fresh E9.5 tissue (24-29 sp). The coaggregates hence obtained were cultured in IMDM+ for 24h. Then, the medium was changed and cells were further cultured for 6 days.

2.7 Tamoxifen induction

When required, cells were incubated with tamoxifen to induce Cre-mediated recombination (step referred to as Tamoxifen induction). Stock solution of 5 mM Tamoxifen was obtained by dissolving 4-hydroxytamoxifen powder (Sigma, catalogue reference: H7904) in absolute methanol. Tamoxifen induction was performed in two different ways. Tamoxifen was added at the beginning of the culture period for a limited period of time in the culture medium (IMDM+) but not to the cells and then, this medium was replaced with fresh tamoxifen-free medium for the rest of the culture. Alternatively, a single cell suspension was incubated before culture in polystyrene 5 mL FACS tube containing 300 µL of IMDM+ medium (similar to IMDM+ but without cytokines) and tamoxifen for 1h30 in incubator at 37°C, 5% CO₂ and gently shaken every 30 min to keep cell suspension homogenous. Tamoxifen was subsequently diluted with 3-4 mL of solution 2, cells were spun
down and supernatant was discarded before the cells were reaggregated or coaggregated in IMDM.

2.8 Maintenance of OP9 stromal cells

OP9 stromal cells were maintained in IMDM supplemented with 20% FCS, 4mM glutamine and 50 units/mL P/S. Before passaging, OP9 cells were checked for absence of osteoblastic morphology and adherence to the flask under the microscope and then, detached with Trypsin (Invitrogen).

2.9 In vitro haematopoietic progenitor assay

Clonogenic progenitor assay was carried out in duplicate using semisolid M3434 complete medium (Stem Cell technologies) according to manufacturer instruction, supplemented with 50 units/mL P/S. Haematopoietic colonies were scored after 8-11 days of incubation. If a sample of initial cell suspension was used, average and standard deviation between two dishes were calculated. Average values with a standard deviation higher than 20% were excluded. If the entirety of the cell suspension was plated, all colonies found in the dishes were added up.

2.10 Flow cytometry

Staining was performed in U-bottom 96-well plate. Typically, a single cell suspension was spun down and resuspended in 25 µL of anti-mouse CD16/32 (FcBlock, eBioscience) diluted to 1:500 in solution 2 then incubated for 5 min on ice. 25 µL of solution containing appropriate antibodies in solution 2 was added and cells were incubated for 30 min. When required, cells were then spun down at 1500 rpm for 5 min, supernatant was removed and cells were incubated with streptavidin-APC for 20 min. After that, cells were washed twice to remove excess antibody. The table below shows antibodies used with their corresponding isotype and supplier. Flow
cytometry was performed on a LSR II Fortessa or FACS calibur (both from BD Bioscience). Dead cells were excluded on the basis of 7AAD uptake. When applicable, OP9 cells were excluded on the basis of forward / side scatter and forward side scatter vs 7AAD. Antibodies used in this work are shown in table 2.1.

### 2.11 Short and long term reconstitution assay

For injection in primary recipients, fresh or cultured *Ly5.2/5.2* embryonic cells were mixed with 100,000 *Ly5.1/5.2* adult nucleated bone marrow carrier cells per recipient in ice cold solution 3 (2% heat-inactivated FCS in Dulbecco’s Phosphate Buffered saline without Calcium/Magnesium).

For injection in secondary recipients, 10⁶ nucleated bone marrow cells were prepared from the primary recipient according to the procedure described in paragraph 2.12.

Prior to transplantation, *Ly5.1/5.1* recipients were irradiated with a split dose of 930 rad (2 doses were given 3 hours apart). No more than 300 µL were injected per recipient into the lateral tail vein (according to the regulations of the Animal Scientific Procedures Act, UK, 1986) using a 30-gauge syringe needle (BD Plastipak). After 6 weeks for short-term and 16 weeks for long-term, blood was harvested from recipients through tail vein bleeding (see Isolation of adult tissue section for details). Recipients showing at least 5% of peripheral blood leukocyte chimerism were considered successfully reconstituted.

Where appropriate, the percentage of attenuation for HSC activity (% Attenuation) was obtained using the following formula:

\[
\text{% Attenuation} = 100 - \frac{\text{HSC output condition}}{\text{HSC output WT-T}} \times 100
\]

### 2.12 Isolation of adult tissues

When required, adult animals were sacrificed according to Schedule 1 method of cervical dislocation. Peripheral blood from adult recipient was collected from the tail vein (2-3 drops of blood per recipient) directly into 0.5 mL of solution 4
<table>
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<th>Conjugate</th>
<th>Isotype</th>
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Table 2.1: List of antibodies and conjugates used in this work with their corresponding isotypes, clone identifier and supplier.
(2% heat-inactivated FCS, 5 mM EDTA in Calcium/Magnesium free Dulbecco’s PBS). Bone marrow cell suspension was obtained by flushing femurs into solution 2 using a 26-gauge syringe needle (BD Microlance). Spleen and thymus were dissected free of fat and connective tissues and directly put into solution 2 solution. Single cell suspension from these organs was obtained by pressing grossly cut organs into a 96-well U-bottom plate with a 1 mL syringe plunger (BD Plastipak) in presence of solution 2. The cell suspension was subsequently filtered through a 40-µm cell strainer (BD Falcon) to exclude cell clumps.

2.13 Erythrocyte depletion of adult tissue-derived single-cell suspensions

A single-cell suspension was centrifuged at 2000 rpm for 5 min, resuspended in 1 mL of PharM Lyse solution (10X stock solution diluted to 1/10 in dH2O) and incubated for 15 min in the dark at RT. After that, the suspension was centrifuged at 2000 rpm for 5 min and resuspended in solution 2.

2.14 Statistical analysis

Statistical analysis was performed with Graphpad Prism 5 software. In chapter III, data obtained from separate embryos where tested for statistical significant difference between wildtype (WT) and transgenic groups using a Mann-Whitney test (test for non-parametrical and unpaired data). In chapter IV and V, the means of tested conditions were tested against the mean of a WT control group using an unpaired t test. The variances between those two groups were also tested with a F-test. Difference in the mean was considered statistically significant when the $p$-value was lower than 0.05 and indicated by an *. A $p$-value lower or equal to 0.01 was indicated by **. Difference in the variance was considered statistically significant when the $p$-value was lower than 0.05 and indicated by an §. A $p$-value lower or equal to 0.01 was indicated by §§.
3 CHARACTERIZATION OF THE HAEMATOPOIETIC DEVELOPMENT IN ABSENCE OF RUNX1 IN THE REACTIVATABLE KNOCKOUT RUNX1 MOUSE MODEL

Runx1 is required during the second wave of haematopoiesis (starting around E9) for haematopoietic stem cells to emerge within the conceptus. In its absence, this process fails, but the developmental stage at which Runx1 is needed has not been clearly identified, although it has been suggested that Runx1 is required during the endothelial to haematopoietic cell transition (Chen et al., 2009).

It has been shown that HSCs develop through a multistep process from precursors of defined phenotypes. Briefly, PreHSC Type I cells (bearing the VC+CD45-CD41low phenotype) develop into PreHSC Type II cells (VC+CD45+), which in turn mature into fully functional HSCs (Rybtsov et al., 2011). To date, these populations have not yet been analyzed in detail throughout the haematopoietic development of the Runx1 KO embryo.

Haematopoietic progenitor activity was only partly analyzed in the Runx1 KO conceptus during the 2nd wave of haematopoiesis. Therefore, it is not known whether the absence of progenitors at E10 in KO Yolk Sac and AGM (Wang et al., 1996) extends to earlier stages. In addition, progenitor activity in the KO Placenta from E9.5 to E11 remains largely uncharacterized to this day.

The Runx1 Reactivatable KO model used in this study (also known as Runx1LacZ/LacZ KO, Samokhvalov et al., 2006) has been partly characterized already and showed the expected signs of haemorrhage around E12 followed by embryonic lethality. Furthermore, Runx1 KO ES cells were unable to produce haematopoietic cells. However, HSC and progenitor activities have not been assessed so far.

The goals of this chapter are to shed light on the role of Runx1 in the development of haematopoietic cells by:

- Identifying key developmental stages and cell populations that are Runx1-dependent
- Assessing progenitor activity from E9.5 to E11 in Caudal Part, Yolk Sac and Placenta
- Assessing the HSC activity in the major haematopoietic sites at E11 in the Runx1 KO model developed in our laboratory.

To achieve this, Runx1\textsuperscript{LacZ/GFP} females were crossed with Runx1\textsuperscript{LacZ/GFP} males to produce Runx1\textsuperscript{LacZ/LacZ} knockout (KO) embryos. All embryos were homozygote for Cre\textsubscript{ER\textsuperscript{T2}} (also referred to as Rosa26\textsuperscript{CreERT2/CreERT2}). Of note, each embryo equivalent of KO tissue was processed individually and haemorrhaging E11 embryos were discarded.

Phenotypes of populations of interest and the gating strategy used in this chapter are indicated in Figure 3.1. Gating strategy for intact cells, counting beads and 7AAD\textsuperscript{-} cells are shown in appendix (Figure S3.1). The number of repeats for each stage, the total number of ee used and the number of somite-pairs of the embryos used in this study are reported in Table 3.1.

### 3.1 HSC content in fresh KO haematopoietic tissues at E11

E11 Runx1 KO AGMs, Yolk Sacs and fetal livers were dissected, dissociated into a single cell suspension and injected into irradiated adult recipients. Engraftment was assessed 16 weeks post-transplantation.

No HSC activity was detected in the E11 Runx1 KO conceptus at 16 weeks post-transplantation (Figure 3.2). This is in line with previous observations and previous Runx1 KO models (Wang et al., 1996; Speck et al., 1999).
Figure 3.1: Gating strategy of populations of interest. Viable cells (gates shown in Figure S3.1) are gated for VC and CD45 expression (A) and Ter119 expression (B). VC^+CD45^- cells are then subsequently gated according to their levels of CD41 expression (C). The phenotypes of the gated populations of interest are shown in D.
<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Type of analysis (Number of repeats)</th>
<th>Genotype of tissues</th>
<th>Number of ee</th>
<th>Number of somite-pairs</th>
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<tr>
<td>E9.5</td>
<td>FACS (N=3)</td>
<td>WT</td>
<td>5</td>
<td>24;24;25;25;27</td>
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<tr>
<td></td>
<td></td>
<td>KO</td>
<td>4</td>
<td>23;25;27</td>
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<td></td>
<td>Colony assay (N=3)</td>
<td>WT</td>
<td>6</td>
<td>17;20;23;24;26;28</td>
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<td></td>
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<td></td>
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</tr>
<tr>
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<td>FACS (N=3)</td>
<td>WT</td>
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<td>KO</td>
<td>4</td>
<td>40;42;43;46</td>
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<tr>
<td></td>
<td></td>
<td>KO</td>
<td>5</td>
<td>40;42;42;44;45</td>
</tr>
</tbody>
</table>

Table 3.1: Numbers of embryo equivalent for WT and KO tissues used to produce the results presented in this chapter and number of somite-pairs of the embryos they relate to.
Figure 3.2.: Absence of HSC activity in E11 Runx1 KO AGM, Yolk Sac, and Fetal liver. Each dot represents the engraftment in a single adult irradiated recipient after 16 weeks. Each recipient was given 1 ee of KO tissue or 2 ee of WT control AGM. Hemorrhaging embryos were discarded. Data are cumulative of 3 independent experiments.
3.2 Dynamics of haematopoietic content in fresh KO Caudal Part/AGM, Yolk Sac and Placenta between E9.5 and E11

In order to determine from where the lack of HSC activity in E11 KO conceptus originates, KO haematopoietic organs were analyzed from E9.5 (around the onset of the second wave of haematopoiesis) to E11 (emergence of the first HSC). To this end, Caudal Parts, Yolk Sacs and Placentas were dissected from KO and WT concepti, dissociated into a single cell suspension and stained with antibodies for flow cytometry analysis. The following cells populations were tracked, as they share hierarchical developmental relations:

- **Endothelium (VC⁺CD45⁻CD41⁻)**
- **PreHSC Type I (VC⁺CD45⁺CD41<sub>low</sub>)**
- **PreHSC Type II (VC⁻CD45⁻)**
- **Mature haematopoietic cells (VC⁻CD45⁺)**
- **Erythrocytes (Ter119⁺)**

Progenitor activity was also assessed from E9.5 to E11 by methylcellulose culture.

In this chapter, the following fluorochromes were used: CD45-V450, CD41-PE; VC-A647; Ter119-PE.

3.2.1 *Runx1* KO Caudal Parts / AGMs have reduced number of cells with PreHSC Type I and II phenotypes

When comparing knockout (KO) and wildtype (WT) tissues, it is important to first assess cellularity and cell viability, as any reduction in populations of interest could be explained by a general reduction in cell numbers in KO tissues.

There was no significant difference observed between KO and WT groups in terms of total number of live cells from E9.5 to E11 although the KO average values were consistently lower than their WT counterparts (Figure 3.3.A).

For the percentage of live cells the situation is different. No differences were found between KO and WT groups from E9.5 to E10, but from E11 there were
A. # Live cells

B. % Live cells

C. Endothelium

D. PreHSC I

E. PreHSC II

F. Mature VC-CD45+
Figure 3.3.: Impaired haematopoietic development in fresh KO Caudal Part (E9.5 and E10.5) and AGM (E11). KO Live cells are no affected in numbers (A, assessed by flow cytometry and counting beads) and in percentage (B), except at E11. The loss of Runx1 reduces precursors, mature haematopoietic cells (C,D,E,F) but not erythrocytes (G). Progenitors (H) remain inexistent. Each dot represent the mean value for a given stage. Error bars shows standard deviation between at least 3 independent experiments. The difference between the two groups is considered significant if the $p$-value < 0.05 (indicated by *). A $p$-value $\leq 0.01$ is indicated by **. Data cumulative of 10 independent experiments. Representative FACS plots are shown in figure 3.4 for C and D, in figure 3.5 for E and F and in figure 3.6 for G.
statistically more 7AAD\(^+\) cells (\(p\)-value = 0.03) in the KO group (75±3\%) than in the WT group (90±2\%) (Figure 3.3.B). This may reflect an increased level of apoptosis, which was previously reported in dying Runx1 KO embryos (Wang et al., 1996), which will eventually die by E12, although this needs to be confirmed, for example by Annexin V staining.

Interestingly, the number of cells with endothelial phenotype (VC\(^+\)CD45\(^-\)CD41\(^-\)) was similar in KO compared to WT groups at all stages except E9.5 (Figures 3.3.C, 3.4 gated on corresponding plots in figure 3.5). The 2.5-fold reduction observed at E9.5 in the KO group (672±160 cells per ee in KO, 1,650±750 cells per ee in WT, \(p\)-value = 0.03) could be explained by the lack of production of VC\(^+\)CD45\(^-\)CD41\(^-\) cells that may become PreHSC Type I by subsequently upregulating CD41. In other words, less haemogenic endothelium is produced in E9.5 KO Caudal Part.

Dynamics of PreHSC Type I (VC\(^+\)CD45\(^-\)CD41\(^{low}\)) content in the KO group was similar to the WT group with a peak at E10 and a drop at E11 (Figure 3.3.D). However, all numbers were strongly attenuated in the KO with a 8.5-fold reduction at E9.5 (75±55 cells per ee in KO, 640±320 cells per ee in WT, \(p\)-value = 0.02, figure 3.4) and E10 (2,231±1,100 cells per ee in KO, 620±380 cells per ee in WT, \(p\)-value = 0.02). The results suggest an impaired ability of KO Caudal Part/AGM to produce such cells. Given these results, the lack of Runx1 has already impacted the PreHSC Type I population by E9.5, suggesting a requirement of Runx1 before E9.5 in the production of PreHSC Type I cells.

PreHSC Type II population (VC\(^+\)CD45\(^+\)) could only be detected in the KO group around E10 (Figure 3.3.E) in 3 out of 6 Caudal Parts (89±170 cells per ee in KO, 1,700±700 cells per ee in WT, \(p\)-value = 0.002, figure 3.5). As this population presumably develops from PreHSC Type I, the results indicate that the lack of Runx1 blocks the transition from PreHSC Type I to PreHSC Type II. In addition, PreHSC Type I and II may have a direct effect on the number of endothelial cells in the niche, explaining the reduction observed in the KO endothelial compartment at E9.5.
Figure 3.4.: Representative FACS plots of PreHSC Type I (VC^+CD45^-CD41^{low}) for Caudal Part/AGM gated on VC^+CD45^+ population. For FMO minus CD41 control see Figure S3.3. Similar number of events is shown between WT and corresponding KO dot plots.
Figure 3.5.: Representative FACS plots of PreHSC Type II (VC⁺CD45⁺) and mature VC⁺CD45⁺ haematopoietic cells for E9.5 and E10 Caudal Part and E11 AGM in WT and KO. For isotype control see Figure S3.2. Similar number of events is shown between WT and corresponding KO dot plots.
3.2.2 **Runx1** KO Caudal Parts / AGMs lack haematopoietic progenitors and have reduced numbers of mature cells but not erythrocytes

To assess the progenitor activity, single-cell suspensions from WT and KO Caudal Parts were put in methylcellulose-based assay and colony formation was scored after 8-11 days.

CFU-Cs were previously shown to be missing in the E9.5 KO Caudal Part (Goyama et al., 2004). The **Runx1** KO model used in the present study was devoid of any progenitor in the E9.5 KO Caudal Part as expected. Therefore, the absence of progenitor observed at later stages (E10 and E11) (Figure 3.3.H) originates from E9.5 and Runx1 is required for the progenitor activity to develop in the embryo.

In the WT group, progenitors appeared between 20 and 23 sp (Figure S3.11.A) to peak at E10 with 630±120 CFU-Cs per ee. The comparison between E11 and previous stages is biased since, for E11, AGM only was used.

In line with the lack of PreHSC type I and II and progenitors, mature VC⁻CD45⁺ haematopoietic cell numbers were significantly reduced in the KO group at E9.5 (166±150 cells per ee in KO, 3,000±1,750 cells per ee in WT, *p*-value = 0.02, figure 3.3.F and 3.5), E10 (6,000±6,000 cells per ee in KO, 36,000±21,000 cells per ee in WT, *p*-value = 0.004) and E11 (1,000±375 cells per ee in KO, 7,000±2,300 cells per ee in WT, *p*-value = 0.03). This points towards an impaired development of mature VC⁻CD45⁺ haematopoietic cells, which could be a consequence of the lack of development of PreHSC Type II. However, it is possible that other precursors could produce mature haematopoietic cells in a Runx1-dependent manner. Noticeably, at E9.5, VC⁻CD45⁺low cells were only detected in KO which may reflect a developmental delay.

Of note, those VC⁻CD45⁺ cells found in the KO Caudal Part may have been produced in the Yolk Sac during the first wave of haematopoiesis around E7.5.
Figure 3.6.: Representative FACS plots of Ter119+ erythrocyte population for E9.5 and E10 Caudal Part and E11 AGM in WT and KO. For isotype control see Figure S3.3.C. Similar number of events is shown between WT and corresponding KO dot plots.
No significant difference was observed between KO and WT group in Ter119+ content from E9.5 to E11, although the average value in WT group was systematically higher than its KO counterpart (Figure 3.3.G and 3.6). The results suggest that Runx1 is not required for erythroid development in the embryo.

3.2.3 Runx1 KO Yolk Sacs have reduced number of cells with PreHSC Type II but not with Type I phenotypes

When Runx1 is present in heterozygous state, the spatio-temporal pattern of HSC emergence is disturbed and HSCs are detected first is the Yolk Sac around E10 (Cai et al., 2000). Also, the loss of Runx1 affects only mildly the first wave of haematopoiesis but totally abrogates the emergence of progenitors in the Yolk Sac during the second wave (Wang et al., 1996; Yokomizo et al., 2008). Therefore, analysis of the KO Yolk Sac may shed more light on the role of Runx1 in this organ.

KO and WT groups showed similar trends in terms of total number of live cells from E9.5 to E11 with no statistical difference observed although the WT average was consistently higher than its KO counterpart (Figure 3.7.A).

The same held true for the percentage of live cells (Figure 3.7.B).

Unlike the E9.5 KO Caudal Part, no significant difference was observed between KO and WT groups in endothelial cell (VC+CD45−CD41−) content in the KO Yolk Sac (Figure 3.7.C and 3.8 gated on corresponding plots in figure 3.9). This finding suggests that if PreHSC Type I cells develop from endothelial cells around E9.5, this process is Runx1-independent in the KO Yolk Sac but not in the KO Caudal part.

No significant difference was observed between KO and WT groups in endothelial cell content from E10 to E11.
A. # Live cells

B. % Live cells

C. Endothelium

D. PreHSC I

E. PreHSC II

F. Mature VC-CD45^+
Figure 3.7.: Impaired haematopoietic development in fresh KO Yolk Sac (E9.5 to E11.5). KO Live cells are no affected in numbers (A, assessed by flow cytometry and counting beads) and in percentage (B). The loss of Runx1 does not impact endothelium (C) and PreHSC I (D) but only reduces PreHSC II (E), mature haematopoietic cells (F). Progenitors (H) remain inexistent. Erythrocytes are only affected at E9.5 (G). Each dot represent the mean value for a given stage. Error bars shows standard deviation between at least 3 independent experiments. The difference between the two groups is considered significant if the $p$-value $< 0.05$ (indicated by *). A $p$-value $\leq 0.01$ is indicated by **. Data cumulative of 10 independent experiments. Representative FACS plots are shown in figure 3.8 for C and D, in figure 3.9 for E and F and in figure 3.10 for G.
Figure 3.8.: Representative FACS plots of PreHSC Type I (VC<sup>+</sup>CD45<sup>-</sup>CD41<sub>low</sub>) for Yolk Sac gated on VC<sup>+</sup>CD45<sup>+</sup> population. For FMO minus CD41 control see Figure S3.6. Similar number of events is shown between WT and corresponding KO dot plots.
Unlike the Caudal Part, analysis of KO Yolk Sac revealed no statistical differences in the PreHSC Type I (VC^+CD45^-CD41^low) compartment between KO and WT groups from E9.5 to E11 (Figure 3.7.D and 3.8) although the WT average was consistently higher than that of KO. These results indicate that Runx1 is dispensable for the development of PreHSC Type I in the KO Yolk Sac. These results also argue against the notion that Runx1 is required for endothelial to haematopoietic transition as PreHSC Type I is a cell type already committed to the haematopoietic lineage.

By contrast to PreHSC Type I population, the dynamics of PreHSC Type II (VC^+CD45^+) content were extremely different in KO and WT groups between E9.5 and E10 (Figure 3.7.E and 3.9). At E9.5, the PreHSC Type II content in the WT group peaked at 5,000±2,200 cells per ee, which is 167-fold more than E9.5 WT Caudal Part (30±17 cells per ee), indicating that such cells may have emerged earlier in the Yolk Sac than in the Caudal Part. Alternatively, those cells could be more proliferative in the Yolk Sac than in the Caudal Part.

Subsequently, WT numbers decreased to 3,100±1,000 cells per ee at E10 and increased again to 4,900±1,600 cells per ee at E11. The inverse is seen in the KO group where the PreHSC Type II content at E9.5 was 100±80 cells per ee, then it peaked at E10 to reach 1,700±400 cells per ee and finally decreased to 157±200 cells per ee at E11. Therefore, the absence of Runx1 leads to an overall attenuation of the number of PreHSC Type II in the KO Yolk Sac. Taken together, the data indicates that Runx1 is required around E9.5 for proper development of PreHSC Type II in the Yolk Sac. The results also suggest a requirement for Runx1 in transition from PreHSC Type I to PreHSC Type II. Alternatively, Runx1 may be required for the migration of PreHSC Type II to Yolk Sac from other tissues.
Figure 3.9.: Representative FACS plots of PreHSC Type II (VC⁺CD45⁺) and mature VC⁺CD45⁺ haematopoietic cells for Yolk Sac at E9.5, E10 and E11 in WT and KO. For isotype control see Figure S3.5. Similar number of events is shown between WT and corresponding KO dot plots.
3.2.4 *Runx1* KO Yolk Sacs lack haematopoietic progenitors and have reduced numbers of mature cells and erythrocytes

As expected, E10 and E11 KO Yolk Sac showed no progenitor activity (Figure 3.7.H). More surprisingly, E9.5 KO Yolk Sacs did not show any sign of progenitor activity except in 1 out of 6 ee tested where only one colony was produced. More Yolk sacs may need to be tested to really confirm the low frequency or the absence of progenitor activity. These results indicate that progenitors from the first wave already disappeared by E9.5 in the Yolk Sac. Whether this premature extinction is due to the lack of Runx1 or occurs independently of Runx1 would require additional experiments with the ability to separate progenitors from the 1st and 2nd waves in WT Yolk Sac.

At E9.5, the WT Yolk Sac already contained 447±320 CFU-Cs per ee whereas the WT Caudal Part harbourcd only 50±60 CFU-Cs per ee (Figure S3.11.B). This could be explained by an early emergence (before the Caudal Part) of 2nd wave progenitors from the Yolk Sac. Alternatively, Yolk Sac and Caudal Part progenitors emerged at the same time but proliferated more actively in the Yolk Sac.

Similarly to the KO Caudal Part, the KO Yolk Sac showed a dramatically reduced content of VC−CD45+ cells compared to its WT counterpart (Figure 3.7.F and 3.9) with a 24-fold reduction at E9.5 (416±600 cells per ee in KO, 9,900±3,500 cells per ee in WT, *p*-value = 0.02), a 4.7-fold reduction at E10 (3,400±1,900 cells per ee in KO, 16,100±7,100 cells per ee in WT, *p*-value = 0.002) and E11 (900±400 cells per ee in KO, 16,000±6,000 cells per ee in WT, *p*-value = 0.03). Thus, it appears that the development of VC−CD45+ cells at E11 is impaired in the Yolk Sac in absence of Runx1.

At E9.5, the Ter119+ content in the KO group was reduced by 2.7-fold (30,000±13,000 cells per ee in the KO group, 82,000±32,000 cells per ee in the WT group, *p*-value = 0.03, figure 3.7.G and 3.10). However, no statistical differences were observed between KO and WT groups from E10 to E11, although the average value in the WT group was consistently higher than its KO counterpart. These results
Figure 3.10.: Representative FACS plots of Ter119⁺ erythrocyte population for Yolk Sac at E9.5, E10 and E11 in WT and KO. For isotype control see Figure S3.7. Similar number of events is shown between WT and corresponding KO dot plots.
suggest a requirement for Runx1 in the development of erythrocytes around E9.5 but not at later stages. Alternatively, the reduction observed in erythrocytes numbers at E9.5 could originate from events occurring during the first wave of haematopoiesis (E7.5 to E9) when Runx1 has been shown to impact erythrocyte development (Yokomizo et al., 2008). These results imply that erythrocyte production at E10 and E11 may be Runx1-independent in the Yolk Sac, even though no CFU-C activity was detected.

3.2.5 Runx1 KO Placentas have retarded development at E9.5 but not at E10 and show an accumulation of PreHSC Type II at E11

Due to the chimerical maternal-embryonic nature of the Placenta, it is important to distinguish cells of embryonic origin from these of maternal origin. This was achieved by using a Runx1-GFP reporter (Gonneau, 2009; Suleman, 2007). The Runx1<sup>GFP</sup> allele is a fully functional allele with an IRES-GFP cassette knocked in at the 3’ end of the Runx1 locus. With the mother being of Runx1<sup>LacZ/GFP</sup> genotype, most of maternal haematopoietic cells express GFP.

Unlike the Caudal Part/AGM and the Yolk Sac, analysis of the KO E9.5 Placenta showed reduced cellularity when compared to its WT counterpart (25,000±13,000 cells per ee in the KO group, 182,000±46,000 cells per ee in the WT group, \( p \)-value = 0.02). This difference was not observed at E10 and E11 suggesting a delay in the development of the Placenta (Figure 3.11.A).

Interestingly, the percentage of cell viability in KO Placenta exceeded WT Placenta at E9.5 (82±3% for the KO group, 72±2% for the WT group, \( p \)-value = 0.02) (Figure 3.11.B). Such a difference was not observed at later stages. Given these results, there is a delay in the placental development due to a possible delay in haematopoietic commitment.

The endothelial cell (VC<sup>+</sup>CD45<sup>-</sup>CD41<sup>-</sup>) content followed the same trend as the total number of live cells with reduced numbers at E9.5 in the KO group (1,100±550 cells per ee in the KO group, 24,150±7,800 cells per ee in the WT group,
A. # Live cells

B. % Live cells

C. Endothelium

D. PreHSC I

E. PreHSC II

F. Mature VC-CD45⁺
Figure 3.11.: Impaired haematopoietic development in fresh KO Placenta (E9.5 to E11.5). KO Live cells are only impacted at E9.5 with lower numbers (A, assessed by flow cytometry and counting beads) but higher viability (B). The loss of Runx1 reduces endothelium (C), PreHSCs (D,E) and mature cells (F) at E9.5 but not at E10 and increases these compartments at E11. Erythrocytes are reduced until E10 but not at E11. Progenitors (H) remain inexistent. Each dot represent the mean value for a given stage. Error bars shows standard deviation between at least 3 independent experiments. The difference between the two groups is considered significant if the p-value < 0.05 (indicated by *). A p-value <= 0.01 is indicated by **. Data cumulative of 10 independent experiments. Representative FACS plots for C and D are shown in figure 3.12 for E9.5, figure 3.13 for E10 and figure 3.14 for E11. Representative FACS plots for E and F are shown in figure 3.15 for E9.5, in figure 3.16 for E10 and in figure 3.17 for E11. Representative FACS plots for G are shown in figure 3.18.
\( p\text{-value} = 0.01 \), figure 3.11.C and figure 3.12 gated on figure 3.15.A for WT and B for KO) but not at later stages (Figure 3.16.A and B for E10 WT and KO respectively, and figure 3.17.A and B for E11 WT and KO). E9.5 KO Placenta also showed reduced percentages of endothelium (6.5±2% in the KO group, 9.9±1.2% in the WT group). Like in the E9.5 KO Caudal Part, reduced numbers of endothelial cells could be explained by the lack of production of \( \text{VC}^+\text{CD45}^-\text{CD41}^- \) cells that would presumably become PreHSC Type I by subsequently upregulating CD41.

The trend observed for PreHSC Type I (\( \text{VC}^+\text{CD45}^-\text{CD41}^- \)) was similar to the trend observed in the total number of live cells. KO Placenta had reduced numbers of PreHSC Type I at E9.5 (145±70 cells per ee in the KO group, 3,100±800 cells per ee in the WT group, \( p\text{-value} = 0.02 \), figure 3.11.D and 3.12.A for WT and B for KO) but not at later stages, suggesting that the absence of Runx1 delays in haematopoietic development of the Placenta. E9.5 KO Placenta also showed reduced percentages of PreHSC Type I (0.6±0.2% in the KO group, 1.8±0.1% in the WT group). Maternal Runx1-GFP expression was clear at E9.5 (Figure 3.12.C and D) and E10 (Figure 3.13.C and D) but less obvious at E11 (Figure 3.14.C and D) where only a few events were GFP⁺.

In line with the observation made for PreHSC Type I, E9.5 KO Placenta was entirely depleted in PreHSC Type II (\( \text{VC}^+\text{CD45}^+ \)) (0±0 cells per ee in the KO group, 19,000±12,000 cells per ee in the WT group, \( p\text{-value} = 0.02 \), figure 3.11.E and 3.15.A for WT and B for KO) but such a difference was not observed at E10 (Figure 3.11.E). E9.5 KO Placenta also showed reduced percentages of PreHSC Type II (0.6±0.5% in the KO group, 3.5±0.3% in the WT group). The situation observed in the E11 KO Placenta is quite unique compared to age-matched KO Caudal Part and Yolk Sac. There was indeed an accumulation of PreHSC Type II cells in the E11 KO Placenta (78,000±12,000 cells per ee in the KO group, 23,000±12,500 cells per ee in the WT group, \( p\text{-value} = 0.03 \)). Maternal Runx1-GFP expression was detected at E9.5 (Figure 3.15.C and D), E10 (Figure 3.16.C and D) and E11 (Figure 3.17.C and D). These results suggest that in the Placenta, the absence of Runx1 leads firstly to a
Figure 3.12.: FACS analysis of E9.5 KO Placenta revealed that all KO PreHSC I (VC⁺CD45⁻CD41low gated on VC-CD45⁺ population) are of maternal origin. Representative FACS plots of PreHSC Type I for E9.5 Placenta in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO (shown as histogram in C and as dot plot in D); For FMO minus CD41 control see Figure S3.9. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
Figure 3.13.: FACS analysis of E10 KO Placenta revealed that all KO PreHSC I (VC\(^+\)CD45\(^-\)CD41\(^{low}\) gated on VC\(^-\)CD45\(^+\) population) are of embryonic origin. Representative FACS plots of PreHSC Type I for E10 Placenta in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO (shown as histogram in C and as dot plot in D); For FMO minus CD41 control see Figure S3.9. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
Figure 3.14.: FACS analysis of E11 KO Placenta revealed that all KO PreHSC I (VC⁺CD45⁻CD41low gated on VC·CD45⁺ population) are of embryonic origin. Representative FACS plots of PreHSC Type I for E11 Placenta in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO (shown as histogram in C and as dot plot in D); For FMO minus CD41 control see Figure S3.9. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
Figure 3.15.: FACS analysis of E9.5 KO Placenta revealed that all KO PreHSC II (VC<sup>+</sup>CD45<sup>-</sup>) and mature cells (VC<sup>-</sup>CD45<sup>+</sup>) are of maternal origin. Representative FACS plots of PreHSC Type II (VC<sup>+</sup>CD45<sup>-</sup>) and mature VC<sup>-</sup>CD45<sup>+</sup> haematopoietic cells for Placenta at E9.5 in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO PreHSC Type II (C,D) and mature cells (E,F). For isotype control see Figure S3.8. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
Figure 3.16.: FACS analysis of E10 KO Placenta revealed that a fraction of KO PreHSC II (VC+CD45+) are of maternal origin. Mature cells (VC-CD45+) do not exhibit clear Runx1-GFP expression and are therefore of embryonic origin. Representative FACS plots of PreHSC Type II (VC+CD45+) and mature VC-CD45+ haematopoietic cells for Placenta at E10 in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO PreHSC Type II (C,D) and mature cells (E,F). For isotype control see Figure S3.8. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
Figure 3.17.: FACS analysis of E11 KO Placenta revealed that this organ contains KO PreHSC II (VC⁺CD45⁺) and mature cells (VC⁻CD45⁺) of maternal origin. Representative FACS plots of PreHSC Type II (VC⁺CD45⁺) and mature VC⁻CD45⁺ haematopoietic cells for Placenta at E11 in WT (A) and KO (B) with Maternal Runx1-GFP expression in KO PreHSC Type II (C,D) and mature cells (E,F). For isotype control see Figure S3.8. Similar number of events is shown between WT and corresponding KO dot plots except for GFP plots where all acquired events are shown.
delay in the development of PreHSC Type II cells from E9.5 to E10, then secondly to an accumulation of these cells at E11.

3.2.6 *Runx1* KO Placenta lack haematopoietic progenitors, have reduced numbers of erythrocytes and show an accumulation of mature cells at E11

Surprisingly, no CFU-C activity was detected in the KO Placenta (Figure 3.11.H and Figures S3.11.C, S3.12.C, S3.13.C). This is unexpected as the maternal component of the Placenta carries a functional Runx1\textsuperscript{GFP} allele therefore, one may expect signs, albeit reduced, of CFU-C activity.

In the WT group, the progenitor content of the Placenta displayed an upward trend with 100±60 CFU-Cs per ee at E9.5, 433±210 CFU-Cs per ee at E10 (4.3-fold increase between E9.5 and E10) and 2,900±800 CFU-Cs per ee at E11 (6.7-fold between E10 and E11).

The mature VC\textsuperscript{CD45}\textsuperscript{+} haematopoietic cell compartment followed the same trend as the PreHSC Type II compartment (Figure 3.11.F). This compartment was depleted at E9.5 (7,200±5,000 cells per ee in the KO group, 86,000±44,000 cells per ee in the WT group, \(p\)-value = 0.02, figure 3.15.A for WT and B for KO), showed similar numbers with the WT group at E10 and finally (Figure 3.16.A for WT and B for KO), accumulated at E11 in the KO Placenta (320,000±33,000 cells per ee in the KO group, 120,000±42,000 cells per ee in the WT group, \(p\)-value = 0.03, figure 3.17.A for WT and B for KO). E9.5 KO Placenta also showed reduced percentages of mature VC\textsuperscript{CD45}\textsuperscript{+} haematopoietic cell (13±4.2\% in the KO group, 35±3.5\% in the WT group). Maternal Runx1-GFP expression was detected at E9.5 (Figure 3.15.E and F) and E11 (Figure 3.17.E and F), although at lower levels, but was not obvious at E10 (Figure 3.16.E and F). These results suggest that in the Placenta, the absence of Runx1 leads firstly to a delay in the development of mature haematopoietic cells from E9.5 to E10, then secondly to an accumulation of these cells.
Figure 3.18.: Representative FACS plots of Ter119+ erythrocyte population for Placenta at E9.5, E10 and E11 in WT and KO. For isotype control see Figure S3.10. Similar number of events is shown between WT and corresponding KO dot plots.
The KO Ter119+ compartment was depleted at both E9.5 (6,400±1,400 cells per ee in the KO group, 66,000±14,000 cells per ee in the WT group, \( p \)-value = 0.02, figure 3.18) and E10 (106,000±34,000 cells per ee in the KO group, 460,000±104,000 cells per ee in the WT group, \( p \)-value = 0.02) compared to its WT counterpart (Figure 3.11.G) but not at E11. These results indicate that Runx1 is required in erythrocyte production at E9.5 and, unlike in the KO Yolk Sac, at E10 as well. It could also mean that erythrocytes produced in E10 KO Placenta accumulate in the Caudal Part and Yolk Sac.

### 3.3 Discussion

In the Caudal Part/AGM, the fact that PreHSC type I population is severely reduced at E9 and E10 in a Runx1 KO context, but not at E11 suggests that such cells mainly develop before E11. Interestingly, Runx1 absence does not abrogate the development of such cells but delays it and strongly reduces their numbers. Whether this process occurs through reduced cell-cycling activity (Runx1 is involved in cell-cycling, Bernardin and Friedman. 2002) or reduced survival remains to be properly determined by staining, for instance, with BrdU for cell-cycle analysis and with Annexin V for apoptotic assays.

Interestingly, the KO endothelial compartment is only reduced at E9.5 in the Caudal Part indicating that if PreHSC Type I cells are specified from the endothelium, this event exclusively takes place between E9.5 (or before) and E10 and stops thereafter.

The KO PreHSC Type II population development is also strongly impaired. Such cells are only detectable at E10 (which explains the absence of HSC activity at E11, as HSC are also VC\(^+\)CD45\(^+\)). They also come possibly from the Yolk Sac and the Placenta but are presumably of embryonic origin as suggested by the absence of maternal Runx1-GFP expression. This suggests that the absence of Runx1 strongly delays the emergence of those cells and reduces their number. As such cells presumably develop from PreHSC Type I phenotype (as shown functionally at E10 in Rybtsov et al., 2011), this strongly indicate that Runx1 is required in the transition from a PreHSC Type I to a PreHSC Type II stage.
Likewise, the development of mature VC^+CD45^+ haematopoietic cells in Caudal Part/AGM is severely compromised in absence of Runx1. As the majority of haematopoietic cells have a VC^+ ancestry (Zovein et al., 2008), this falls in line with the developmental defects observed in PreHSC Type I and II compartments.

Those observations are somewhat reminiscent of the Runx1 KO zebrafish model where cells undergoing endothelial to haematopoietic transition were observed at a highly reduced frequency and were followed by the bursting of such cells (Kissa and Herbomel, 2010).

The lack of progenitors observed in KO Caudal Part/AGM at all stages is in line with the reduced numbers of PreHSC Type I and II and VC^CD45^+ cells observed and previous studies (Goyama et al., 2004). Intriguingly, even if progenitors are absent, putative progenitor-containing populations, that are PreHSC Type I and II and VC^CD45^+ populations are present at some point. This may suggest that some progenitors are present but the methylcellulose-based medium may not contain the optimal combinations of cytokines to allow them to form colonies. The results for Caudal Part/AGM are summarised in table 3.2.

Unlike their KO Caudal Part counterparts, PreHSC Type I cells in the KO Yolk Sac are not significantly impacted which suggests a more Runx1-independent developmental program, may those cells be specified *in situ*.

In line with this observation, no difference could be detected between KO and WT endothelial compartments in the KO Yolk Sac at E9.5 (unlike E9.5 KO Caudal Part) suggesting a Runx1-independent specification of PreHSC Type I from the endothelium.

In contrast, the PreHSC Type II population is strongly depleted in absence of Runx1, which clearly indicates that in the Yolk Sac, Runx1 is required for the transition from a PreHSC Type I to a PreHSC Type II phenotype. In line with the lack of KO PreHSC Type II cells observed, mature VC^+CD45^+ haematopoietic cell development is impaired in the KO Yolk Sac.

Interestingly, erythropoiesis in KO Yolk Sac is only impacted at E9.5 but not at later stage indicating that the absence of Runx1 delays erythropoiesis in the Yolk Sac, may erythrocytes detected in the KO Yolk Sac be produced *in situ*.
<table>
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<tr>
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<th>E10</th>
<th>E11</th>
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<td>normal</td>
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<tr>
<td></td>
<td>PreHSC I</td>
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<tr>
<td></td>
<td>Mature VC-CD45+</td>
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<td></td>
<td>Erythrocytes</td>
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<td></td>
<td>CFU-Cs</td>
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Table 3.2.: Summary of chapter results of KO Caudal Part/AGM compared to its WT counterpart.
The total lack of progenitors observed in the KO Yolk Sac from E10 to E11 is somewhat expected as those time points are well distinct from the first wave of haematopoiesis starting around E7.5. However, the lack of progenitors observed in E9.5 KO Yolk Sac is much more surprising as one expects to see at least frequent remnants of the Runx1-independent first wave in terms of progenitor activity. When such remnants are observed, they appear at low frequency (1 out 6 E9.5 KO Yolk Sac contained 3-4 CFU-Cs / ee, the same frequency was observed at E10). The low frequency observed suggests that the first wave of haematopoiesis stops well before E9.5.

The lack of HSC activity at E11 is expected and in line with the lack of progenitors observed in the KO conceptus.

The results for Yolk Sac are summarised in table 3.3.

The study of the KO Placenta reveals unique features in terms of Runx1 requirements.

Firstly, it is the only organ studied that shows a significantly reduced cellularity combined with a increased percentage of live cells at E9.5 but not a later stages which both point toward a delay in the development of the whole organ. This is all the more striking because the KO Placenta, due to its chimeric nature, is the only organ to carry one functional allele of Runx1 brought by the mother (of Runx1^LacZ/GFP genotype). As expected, this decreased cellularity observed at E9.5 translates in all cell compartments analyzed. Interestingly, the decrease observed in PreHSC Type I and II and mature haematopoietic cells is not only due to the decreased cellularity of the E9.5 KO Placenta but also to a decrease in the percentages of those populations. This suggests that a reduced fraction of the Placenta undergoes haematopoietic differentiation when Runx1 is absent in the embryonic compartment. Of note, the absence of significant difference observed at E10 between KO and WT Placenta in terms of cellularity also translates in all populations analyzed, which shows no significant differences between KO and WT groups.
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<tr>
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<tr>
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<td>CFU-Cs</td>
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Table 3.3.: Summary of chapter results of KO Yolk Sac compared to its WT counterpart.
Secondly, even if the cellularity and cell viability is similar in KO and WT groups at E10, the erythroid compartment remains depleted until E11. As the Placenta is the main site of $O_2$ exchanges, reduced erythroid content strongly indicates a reduction in the oxygen supply among the whole conceptus, which may also be held partly responsible for the delays and defects observed in a Runx1 KO developmental context.

Thirdly, the E11 KO Placenta shows an accumulation of PreHSC Type II and VC$^+$CD45$^+$ although total cellularity is similar to its WT counterpart. Such an accumulation can have many explanations:

- Runx1 is involved in the migration through the whole conceptus of haematopoietic cells produced in the Placenta.
- Runx1 expression regulates haematopoietic cells numbers in the Placenta throughout development.
- The embryo starts dying around E11 due to the absence of Runx1. In this context, the only site where Runx1 is expressed is the mother component of the KO Placenta. Therefore, KO cells produced in AGM or Yolk Sac are attracted and migrate towards the only healthy niche that remains in the KO conceptus. In other words, AGM and Yolk Sac are seeding the Placenta with haematopoietic cells.
- Alternatively, the E11 KO Placenta somehow senses the coming haemorrhages and necrosis observed around E12, and thus, makes an effort to produce more haematopoietic cells, in an attempt to jugulate those haemorrhages.
- The hemizygous dosage of Runx1 in KO Placenta, which resulted in a delay in the development of the Placenta at E9.5, induces a high level of proliferation at E11 leading to the results observed.

The results for Placenta are summarised in table 3.4.

Surprisingly, Runx1 KO Placenta does not harbour any progenitor activity although it carries a functional Runx1 allele. This suggests that progenitors found in the WT Placenta may originate solely from the embryonic part of the Placenta. Alternatively, if the maternal part produces progenitors in a WT situation, it requires
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<tr>
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<td>accumulation</td>
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<tr>
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<td>accumulation</td>
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<tr>
<td>CFU-Cs</td>
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Table 3.4.: Summary of chapter results of KO Placenta compared to its WT counterpart.
signalling from the embryonic part to do so. These results point towards an exclusive requirement of Runx1 in the embryonic component for the generation of progenitors and development of haematopoietic potential in the whole organ, which is in line with previous results (Zeigler et al., 2006). Of note, fresh E11 KO Placenta was not tested for HSC activity in this study although the absence of progenitor activity makes it likely that such a test would be negative.

In summary, the lack of Runx1 impacts the KO conceptus 2 days before the embryo starts showing any signs of haemorrhages and it does so on mainly three key populations in the development of the haematopoietic system that are PreHSC Type I and II and VC$^{+}$CD45$^{-}$ populations. In addition to that, the lack of progenitor in Runx1 KO conceptus indicates that Runx1 is required for the onset of the second wave of haematopoiesis in the whole conceptus. Of note, the presence of PreHSC Type I by phenotype does not mean they are functional. Also, PreHSCs Type I require 5 days of maturation in OP9 culture. Such cells are not found in the WT Caudal Part at E9.5 yet (Rybtsov et al., 2011). However, VC$^{+}$CD45$^{-}$CD41$^{low}$ precursors from early (30-35 sp) E10 WT Caudal Part can be matured into repopulating HSCs when cultured for 7 days. Consequently, those precursors cannot be called PreHSCs Type I but are referred to as ProHSCs.
4 ASSESSMENT OF TAMOXIFEN-MEDIATED REACTIVATION OF SILENT GFP REPORTER AND TOXICITY DURING REAGGREGATE CULTURE

The main goal of this project is to address the role of Runx1 through the reactivation of its locus using the tamoxifen-inducible Cre/LoxP technology (Rosa26-CreERT2). As this system was shown to exhibit toxicity on adult haematopoietic differentiation (Higashi et al., 2009), its toxicity needs to be assessed on haematopoietic development during reaggregate culture. The other parameter to test is the efficiency of recombination of the gene of interest that can be achieved using this strategy.

In order to facilitate this study, a compound transgenic model that is heterozygote for CreERT2 (Rosa26CreERT2+/+) and silent GFP (or sGFP, previously described in Gilchrist et al., 2003) was used. The transgenic AGMs bearing both CreERT2 and sGFP transgenes are termed “Cre” and the wildtype AGMs, as “WT” hereafter.

In addition, toxicity was assessed on E11.5 AGM cultured for 5 days as reaggregate even if in the next chapter, Runx1 reactivation was subsequently performed on E9.5 Caudal Part. The main reason for that is OP9 coaggregation with E9.5 WT Caudal Part only produces 1 HSC per ee whereas reaggregation of E11.5 WT AGM produces approximately 150 HSCs per ee from an organ that originally contained only 1 HSC (Taoudi et al., 2008). This gives a dynamic range on which to assess quantitatively the toxicity of tamoxifen and the Cre recombinase in terms of attenuation of HSC output after culture. Importantly, HSCs are generated by maturation in both coaggregation and reaggregation systems.

It is also important to distinguish between two sources of toxicity, possibly cumulative, when using the tamoxifen-inducible CreERT2 system:
- The toxicity of tamoxifen as a chemical (referred to as tamoxifen toxicity hereafter), which was reported to be an intramembranous inhibitor of lipid
peroxidation, to exhibit peroxyl radical scavenging activity (Custodio et al., 1994) and to decrease mitochondrial ATP levels (Cardoso et al., 2003).

- The toxicity due to Cre-mediated illegitimate recombinations (referred to as tamoxifen-Cre toxicity hereafter) between cryptic (i.e. endogenous) and transgenic loxP sites. Indeed, cryptic loxP sites are present in the mouse genome at a frequency of 1.2 per megabase and are homogenously distributed throughout the genome (Higashi et al., 2009).

To this end, two additional controls were included:
- A WT AGM induced with tamoxifen
- An uninduced Cre control in the second part of this chapter.

Of note, all transgenics used in this chapter carried two functional wildtype alleles of Runx1. In this chapter, the following fluorochromes were used: CD45-PE and VC-A647.

4.1 Tamoxifen induction during reaggregate culture hampers haematopoietic development

4.1.1 Experimental design

In a first approach, Cre recombination was induced during the first six hours of the 5-day culture period (Figure 4.1) as it was reported to be an efficient dose to achieve high level recombination and low level of toxicity in flat culture system (Higashi et al., 2009). WT and transgenic E11 AGM were dissected and dissociated into a cell suspension. Cells were then reaggregated in IMDM+. Tamoxifen was added to the culture medium (IMDM+) but not to the reaggregated AGM cells, so that when the medium was replaced, the tamoxifen would be supposedly entirely removed from the cell environment. At the end of the induction period, culture medium was replaced with a tamoxifen-free IMDM+ medium and reaggregates cultured for a total of 5 days and subsequently analyzed using flow cytometry (staining for haematopoietic markers
Figure 4.1: Experimental design for tamoxifen induction during reaggregate culture.
CD45 and endothelial marker VC), *in vitro* colony forming assay and *in vivo* transplantation assay.

4.1.2 All tested doses recombine most of the cells in the Cre reaggregates

Tamoxifen induction with both 1 and 5 \(\mu\)M for 6h was sufficient to recombine most of the cells in Cre reaggregates as indicated by the GFP expression (Figure 4.2.A and 4.5.A and B) with 92±6% and 93±1.4% of GFP\(^+\) cells respectively. Of note, 100% of GFP\(^+\) cells was never achieved which could be explained by the silencing of the sGFP locus in some cells, making it unreachable to the Cre recombinase. This silencing may also occur once the sGFP locus has been activated. Another possible explanation is a negative selection against GFP\(^+\) cells during the culture as such cells may accumulate illegitimate recombination events and be somehow impaired (e.g. cell cycle) in their development *in vitro*.

4.1.3 Tamoxifen induction and Cre recombination are toxic for HSC development

When Cre reaggregates were induced with tamoxifen, HSC development was totally abolished revealing a dramatic level of toxicity due to tamoxifen-Cre (Figure 4.2.B).

In induced WT reaggregates, the HSC output was reduced to approx. 25 HSCs per reaggregate for both doses tested (6-fold reduction compared to 150 HSCs per uninduced WT reaggregate) indicating that in the absence of Cre activity, tamoxifen is also toxic *per se* for HSC maturation.
Figure 4.2: Tamoxifen induction during culture recombines most of the cells but hampers haematopoietic development. A. Percentage of GFP positive cells after culture (representative FACS histograms are shown in Figure 4.5). B. Peripheral blood chimerism of irradiated adult recipients after 16 weeks. Each recipient received 0.03 ee of reaggregated AGM. Data are cumulative of 3 independent experiments. C. Number of CFU-Cs per ee after culture. In A and C, bars show average between 3 independent experiments and error bars represent standard deviation. Each repeat had at least 3 reaggregates per condition.
4.1.4 Tamoxifen induction and Cre recombination are toxic for progenitor development

In tamoxifen-induced Cre reaggregates, the progenitor output was decreased to 1,480±1,300 CFU-Cs per ee with 1 µM and to 1,420±60 CFU-Cs per ee with 5 µM (Figure 4.2.C) whereas uninduced WT reaggregates produced 7,740±950 CFU-Cs per ee. Interestingly, tamoxifen did not significantly reduce the CFU-C output from WT reaggregates (6,030±1,700 CFU-C per ee with 1 µM, 5,620±1,000 CFU-C per ee with 5 µM). Taken together, this indicates that tamoxifen as an inducer of Cre activity, but not as a chemical, is toxic for CFU-C expansion in vitro.

When tamoxifen induction is performed during culture, there is a clear correlation between reduced HSC output and reduced CFU-C outputs.

4.1.5 Toxicity on haematopoietic and endothelial cell populations

Tamoxifen-induced Cre reaggregates did not show a significant difference compared to their uninduced WT counterparts in terms of cellularity (Figure 4.3.A) or percentage of live cells (Figure 4.3.B) after culture suggesting that Cre recombination does not significantly impact cell growth during culture. Tamoxifen-induced WT reaggregates behaved similarly to their uninduced counterparts indicating that the tamoxifen itself did not have an impact on the growth of the reaggregate. Of note, cellularity was similar in fresh E11 Cre and WT AGMs (Figure S4.6).

All conditions tested showed no significant reduction in terms of mature VC− CD45+ haematopoietic cell output except for 5 µM induced Cre reaggregates (33,280±4,600 cells per ee in this condition, 114,670±42,330 cells per ee in the WT), (Figure 4.3.C, 4.4.C for Cre 5 µM Tam and 4.4.E WT not induced WT). This suggests that the VC−CD45+ compartment is less sensitive to Cre activity than CFU-Cs.
Figure 4.3: Tamoxifen induction during culture impairs mature haematopoietic cell production without clearly impacting viability (A. Absolute number of live cells after culture per ee as assessed by flow cytometry and counting beads. B. Percentage of live cells (7AAD−) after culture per ee) and other cell populations (C. Number of haematopoietic VC−CD45+ cells per ee after culture. D. Number of endothelial VC−CD45− cells per ee after culture. E. Number of double positive VC−CD45+ cells per ee after culture). All values shown are average between 3 independent experiments and error bars represent standard deviation. The difference between averages in one group and the WT -T condition was considered significant if the p-value < 0.05 (indicated by * above the group tested).
Figure 4.4: Representative FACS plot of reaggregates after culture stained for VC and CD45. A. Cre reaggregate induced with 1 μM tamoxifen. B. WT reaggregate induced with 1 μM tamoxifen. C. Cre reaggregate induced with 5 μM tamoxifen. D. WT reaggregate induced with 5 μM tamoxifen. E. WT reaggregate not induced.
Figure 4.5: FACS-assessed GFP expression (green line) after culture in Cre reaggregates induced with A. 1 µM tamoxifen and B. 5 µM tamoxifen. Negative gate (gray line) was obtained using WT uninduced reaggregates. Representative FACS histograms of 3 independent experiments.
No clear differences were detected in terms of endothelial VC\(^+\)CD45\(^-\) cell output among the tested conditions (Figure 4.3.D).

No clear differences were found in terms of VC\(^+\)CD45\(^+\) cell output among the tested conditions (Figure 4.3.E).

No clear correlation was found between the absence of HSC in Cre reaggregates and any of the populations assessed when tamoxifen induction was performed at the beginning of the culture.

### 4.2 Tamoxifen induction before culture significantly reduces toxicity and preserves some HSC activity after culture

All doses tested in the previous section showed a high level of recombination coupled with a dramatic level of toxicity that completely abolished HSC maturation and led to a decreased CFU-C output during culture. In order to achieve a good efficiency of recombination and retain the ability of the culture system to mature HSC, a modified approach to tamoxifen induction was designed. In addition to that, an uninduced Cre control (referred to as Cre \(\sim\)T) was added to assess the impact of the presence of the constitutively expressed Cre on HSC development \textit{in vitro}. This control was also used to assess any leakage in GFP expression in uninduced Cre reaggregates after culture as fetal calf serum might contain oestrogen-like molecules capable of binding to CreER\(^T2\) protein.

#### 4.2.1 Improved experimental design

Tamoxifen induction was performed before the culture in a polystyrene FACS tube in IMDM\(^\ast\) (similar to IMDM\(^\ast\) but without cytokines) at 37\(^\circ\)C for 1h30 (Figure 4.6 and Materials and Methods). This approach allows a better control of several parameters. First of all, tamoxifen can be washed away from the cells properly and any possibility that tamoxifen might remain stuck to or inside the
Cell suspension

5 days

Induction in cell suspension for 1h30 in IMDM

Reaggregate in tamoxifen-free IMDM

Flow Cytometry GFP, CD45, VC

Colony assay

Transplantation assay

Wash tamoxifen away

5 days

E11.5 AGM WT or sGFP

Cell suspension

+Tamoxifen

Figure 4.6: Improved experimental design for tamoxifen induction before the start of the culture.
nitrocellulose acetate membrane used for culture is ruled out. Secondly, the reduced incubation period may help reducing illegitimate recombination events. Thirdly, the absence of cytokines may prevent the Cre recombinase to access genes involved in HSC maturation and function. Fourthly, the process of the reaggregate reorganization taking place at the beginning of the culture may be less disturbed by ongoing recombination.

4.2.2 Tamoxifen induction before culture achieves high level of recombination of sGFP locus

When cells were induced with 1 µM tamoxifen prior to the culture, the level of recombination achieved was variable with 64.3±33.8% of GFP+ cells at the end of the culture (Figure 4.7.A and 4.15.A). When induction tamoxifen was performed with 5 µM, the vast majority of the cells (93.3±5.1%) were GFP+ after culture (Figure 4.15.B). These results show that maximum recombination is best achieved with 5 µM tamoxifen.

Of note, no GFP leakage was observed in uninduced Cre reaggregates (Figure 4.15.C).

4.2.3 Tamoxifen induction before culture reduced the toxicity on HSCs

When Cre was induced before the culture with either 1 or 5 µM, HSC could be maturated in both conditions but with a different HSC output (Figure 4.7.B) in each case. 1 µM allowed the production of approximatively (approx.) 15 HSCs per ee, which represents a 1.4-fold decrease compared to the uninduced Cre condition (21 HSCs per ee). When 5 µM was used, only 3 HSCs per ee could be detected (7-fold decrease compared to the uninduced Cre condition) demonstrating a higher toxicity on HSC maturation during culture. These results suggest an opposite correlation between the level of recombination and the HSC output.
Figure 4.7: Tamoxifen induction before the culture leads to reduced impairment of haematopoietic development but reduced levels of GFP recombination. A. Percentage of GFP positive cells in Cre reaggregates after culture, representative FACS histograms are shown in Figure 4.15. Error bars show standard deviation between 3 independent experiments. B. Contribution to peripheral blood of irradiated adult recipients after 16 weeks. Each dot represents a recipient which received 0.03 ee of cultured AGM. C. Contribution of GFP+ donor HSCs into the blood of the recipients after 16 weeks. Data are cumulative of 3 independent experiments. Error bars represent standard deviation between 3 independent experiments. All values shown are average between 3 independent experiments. The difference between averages in one group and the WT -T condition was considered significant if the p-value < 0.05 (indicated by * above the group tested). 3 ee were used for each condition.
Of note, all recipients successfully reconstituted (at least 5% blood chimerism) with induced Cre HSCs produced a majority of GFP+ progeny inside the recipients (Figure 4.7.C) except for two recipients that produced only GFP− progeny. This observation could be explained by either the silencing of the activated GFP locus or HSCs somehow escaped recombination.

Interestingly, both induction protocols tested gave similar fold of reduction in HSC output from tamoxifen-induced WT reaggregates (approx six-fold reduction in WT reaggregates treated with either 1 or 5 µM, although more recipients transplanted with 1 µM WT reaggregates would be required to confirm this result). These findings suggest that even a short incubation (1h30) with tamoxifen is sufficient to affect HSC expansion *in vitro*.

### 4.2.4 Multilineage potential of tamoxifen-induced Cre and WT HSCs and engraftment in secondary recipients

It is important to assess the multilineage potential of tamoxifen-induced Cre HSCs because it can reveal an impaired ability of HSCs to differentiate along a particular lineage due to recombination. Moreover, *in vitro* B cell but not myeloid differentiation from adult bone marrow is reportedly affected by tamoxifen-Cre induction (Higashi et al., 2009).

All Cre and WT reaggregate-derived HSCs (tamoxifen-induced and non-induced) showed multilineage potential in the peripheral blood (Figure 4.8), bone marrow (Figure 4.9), spleen (Figure 4.10) and thymus (Figure 4.11). Given these results, tamoxifen induction and Cre expression do not affect multilineage potential of reaggregate-derived HSCs.

Of note, the thymus of recipients that received HSCs from induced reaggregates (WT or Cre) displayed a bias in T cell differentiation towards a CD4+CD8− phenotype (15±4% CD4+CD8− cells for induced group, 4±2% for uninduced group, *p*-value = 0.02 obtained using a Mann-Whitney test).
Blood

Cre 1 µM

WT 1 µM

Cre 5 µM

WT 5 µM

CD45.1

CD45.2

Myeloid

Gr1

Mac1

Lymphoid

B220

CD3

CD45.2

CD45.1

CD3

CD45.2

CD45.1

CD3

WT 5 µM

WT 1 µM

Cre 5 µM

Cre 1 µM

Blood

Myeloid

Lymphoid

WT 1 µM

WT 5 µM

Cre 1 µM

Cre 5 µM
Figure 4.8: Long-term multilineage (myelo-lymphoid) haematopoietic reconstitution in the peripheral blood by HSCs generated from E11.5 Cre and WT AGM reaggregates. Data were collected from high-level (>5%) reconstituted recipients injected with 0.03 ee. Plots are representative of 3 recipients mice. Quadrants are based on appropriate isotype controls (Figure S4.2).
Figure 4.9: Long-term multilineage (myelo-lymphoid) haematopoietic reconstitution in the bone marrow by HSCs generated from E11.5 Cre and WT AGM reaggregates. Data were collected from high-level (<5%) reconstituted recipients injected with 0.03 ee. Plots are representative of 3 recipients mice. Quadrants are based on appropriate isotype controls (Figure S4.3).
Spleen

Cre 1 μM

WT 1 μM

Cre 5 μM

WT 5 μM

Lymphoid

CD45.1

B220

CD45.2

CD3
Figure 4.10: Long-term multilineage (myelo-lymphoid) haematopoietic reconstitution in the spleen by HSCs generated from E11.5 Cre and WT AGM reaggregates. Data were collected from high-level (<5%) reconstituted recipients injected with 0.03 ee. Plots are representative of 3 recipients mice. Quadrants are based on appropriate isotype controls (Figure S4.4).
Thymus

Cre 1 μM

WT 1 μM

Cre 5 μM

WT 5 μM

Lymphoid

CD45.2

CD45.1

CD8

CD4
Figure 4.11: Long-term multilineage (myelo-lymphoid) haematopoietic reconstitution in the thymus by HSCs generated from E11.5 Cre and WT AGM reaggregates. Data were collected from high-level (<5%) reconstituted recipients injected with 0.03 ee. Plots are representative of 3 recipients mice. Quadrants are based on appropriate isotype controls (Figure S4.5).
A.

<table>
<thead>
<tr>
<th>Primary recipients</th>
<th>Secondary recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Peripheral blood chimerism</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- Cre 1 µM
- Cre 5 µM

B.

<table>
<thead>
<tr>
<th>Blood</th>
<th>Myeloid</th>
<th>Lymphoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre 1 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45.1: 55.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr1: 87.7% 9.87%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220: 1.68% 0.670%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cre 5 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45.2: 2.01%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mac1: 0.196% 0.114%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3: 2.63% 97.4%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.12: A. Secondary recipient engraftment of E11.5 Cre AGM reaggregate-derived HSCs. B. Long-term multilineage (myelo-lymphoid) haematopoietic reconstitution in the peripheral blood by HSCs generated from E11.5 Cre AGM reaggregates. Plots are representative of 2 recipients mice. Quadrants are based on appropriate isotype controls (Figure S4.2).
HSCs produced from Cre reaggregates induced with 1 μM tamoxifen yielded high level engraftment Figure (4.12.A) and bloodmultilineage potential in the peripheral blood of secondary recipients (Figure 4.12.B). HSCs produced from Cre reaggregates induced with 5 μM tamoxifen failed to repopulate secondary recipients to high levels and did not generate multilineage progeny at least in the blood (no B220 expression could be detected). This indicates that a dose of 1 μM tamoxifen does not affect HSC multilineage potential and self-renewal. The failure of Cre HSCs induced with 5 μM tamoxifen to reconstitute secondary recipients can be explained by the normal drop observed in the level of engraftment between the first (6%) and the second (ranging from 0 to 2%) recipients.

4.2.5 Tamoxifen induction before culture reduced the toxicity on progenitors

Unlike with the first induction protocol, no clear-cut difference was observed among all conditions tested when tamoxifen induction was performed before culture (Figure 4.13.A). This suggests that the tamoxifen doses tested are not significantly toxic for progenitor development during culture. These results also suggest that developing HSCs are more sensitive to tamoxifen / Cre than developing progenitors.

4.2.6 Impact on haematopoietic and endothelial cell populations when tamoxifen induction is performed before culture

Surprisingly, a significant difference in cellularity was observed between both WT and Cre reaggregates induced with 1 μM tamoxifen and WT –T (239,250±32,970 cells per ee in WT 1 μM, 246,400±11,930 cells per ee in Cre 1 μM and 398,990±5,180 cells per ee in WT -T, respectively) whereas no difference was found in conditions treated with 5 μM (Figure 4.13.B). Of note, the same treated conditions showed no differences when compared to Cre –T.
Figure 4.13: Tamoxifen induction before culture has no clear effect on progenitor development (A.) and has a milder and dose-dependent effect on reaggregate populations. B. Total number of cells per ee after culture as assessed by flow cytometry and counting beads. C. Percentage of live (7AAD⁻) cells after culture. D. Number of haematopoietic VC−CD45⁺ cells per ee after culture. E. Number of endothelial VC⁺CD45⁻ cells per ee after culture. F. Number of double positive VC⁺CD45⁺ cells per ee after culture. Error bars represent standard deviation between 3 independent experiments. All values shown are average between 3 independent experiments. The difference between averages in one group and the WT -T condition was considered significant if the p-value < 0.05 (indicated by * above the group tested). For each conditions, at least 3 ee per experiment were used.
No differences were observed in terms of percentage of live cells among all conditions tested (Figure 4.13.C).

WT 1 µM condition showed a decrease in mature VC^CD45^ haematopoietic cell output with 102,820±22,740 versus 161,560±1,500 cells per ee in the WT –T (Figure 4.13.D, for FACS plots see figure 4.14.B for WT 1 µM Tam and 4.14.F for WT-T). In contrast, WT 5 µM tamoxifen showed an increased (295,810±43,630 cells per ee, figure 4.14.D) in VC^CD45^ cell output. These results suggest that the effect of tamoxifen on the production of mature haematopoietic cell in WT reaggregates is dose-dependent.

No clear differences were detected in terms of endothelial VC^CD45^- cell output among the tested conditions (Figure 4.13.E).

Interestingly, all Cre conditions showed a significantly reduced VC^CD45^ content compared to WT –T (Figure 4.13.F, 4.14.A, C and E). Cre 1 µM had 440±220 cells per ee, Cre 5 µM had 570±90 cells per ee and Cre –T had 480±530 cells per ee versus 1,670±320 cells per ee for WT –T. This indicates that reduced numbers of HSCs in Cre reaggregates may be due to decreased numbers of PreHSC Type II in the fresh E11.5 Cre AGM. This also indicates that the decrease observed in the VC^CD45^ population from Cre reaggregates is the consequence of constitutive Cre expression (even if CreER^T2 remains sequestrated inside the cytoplasm) rather than tamoxifen induction.

Results from sections 4.1 and 4.2 are summarised in table 4.1.

4.3 Recombination of Runx1 locus achieved with 1 and 3 µM tamoxifen

As Runx1 reactivation will be performed in E9.5 haematopoietic organs, which will be subsequently cultured using the OP9 coaggregate system – a powerful system that support potent maturation of early precursors of HSC into repopulating
Figure 4.14: Representative FACS plot of reaggregates after culture stained for VC and CD45. A. Cre reaggregate induced with 1 µM tamoxifen. B. WT reaggregate induced with 1 µM tamoxifen. C. Cre reaggregate induced with 5 µM tamoxifen. D. WT reaggregate induced with 5 µM tamoxifen. E. Cre reaggregate not induced. F. WT reaggregate not induced.
Figure 4.15: FACS-assessed GFP expression (green line) after culture in Cre reaggregates induced with A. 1 µM tamoxifen, B. 5 µM tamoxifen and C. no tamoxifen. Negative gate (gray line) was obtained using WT uninduced reaggregates. Representative FACS histograms of 3 independent experiments.
Table 4.1: Summary of chapter results regarding *in vitro* haematopoietic development during reaggregation culture of Cre and WT E11 AGM. N means normal; R, reduced; Accu, accumulation, all relatively to the WT -T control (last column on the right).

% Attenuation is obtained using the following formula: \[ \text{% Attenuation} = \frac{100 - \frac{\text{HSC output condition}}{\text{HSC output WT - T}}}{1} \times 100 \]
HSCs from 24-29 sp embryos - it is important to assess the levels of recombination that can be achieved in these conditions.

Recombination of the Runx1 locus was assessed in tamoxifen-induced E9.5 Runx1 KO Caudal Part, Yolk and Placenta (all tissues were Rosa26\textsuperscript{CreERT2/+} and were obtained by crossing Runx1\textsuperscript{LacZ/+} Rosa26\textsuperscript{+/-} females with Runx1\textsuperscript{LacZ/GFP} Rosa26\textsuperscript{CreERT2/CreERT2} males) after 24h of OP9 coaggregate culture (for experimental design, see Figure 5.8 in chapter V).

As 5 µM tamoxifen resulted in a severe reduction of HSC production during E11 AGM reaggregate culture, a maximal dose of 3 µM was tested instead. A minimal dose of 1 µM was also tested as it resulted in reduced recombination levels.

Because recombination was assessed after coaggregation with OP9 cells, which carry two Runx1\textsuperscript{WT} alleles, it was necessary to confirm the KO genotype of the Runx1 transgenic tissues used. This was achieved by blotting the fresh rostral part of KO embryos (Figure 4.16.A and B lane 1).

With the coaggregated Caudal Part, 1 and 3 µM tamoxifen achieved similar levels of recombination (75±5% with 1 µM and 80±5% with 3 µM, figure 4.16.A and B lane 2). 1 µM was therefore retained as the optimal dose for the Caudal Part to minimize any toxicity.

With the coaggregated Yolk Sac, 1 µM tamoxifen only achieved poor levels of recombination but 3 µM achieved 3-fold higher levels of recombination (5±2% and 20±5% respectively, figure 4.16.A and B lane 3). 3 µM was therefore retained as the optimal dose for the Yolk Sac.

With coaggregated Placenta, 1 and 3 µM tamoxifen achieved similar levels of recombination (20±4% with 1 µM and 20±5% with 3 µM, figure 4.16.A and B lane 4). 1 µM was therefore retained as the optimal dose for the Placenta to minimize any toxicity.

Of note, the low levels of recombination achieved in the Yolk Sac and the Placenta strongly suggest that these organs are more resistant to recombination than the Caudal Part. These findings are quite unexpected for the E9.5 KO Yolk Sac as its WT counterpart produces many haematopoietic cells and progenitors, which
Figure 4.16: Recombination levels of Runx1 locus assessed by Southern blot in E9.5 KO tissues induced with 1 µM (A) and 3 µM (B) of tamoxifen for 1h30 and coaggregated with OP9 for 24h.

Lanes: 1. Rostral part used for genotyping 2. Coaggregated Caudal Part (Coagr CP) 3. Coaggregated Yolk Sac (Coagr YS) 3. Coaggregated Placenta (Coagr PL). Average percentage of recombination (ratio between Re and KO bands) ±standard deviation between 2 experiments (at least 3 KO ee per experiments) were determined with AIDA Analyzer software and are indicated below each lane.
supposedly express Runx1. Alternatively, these organs may not develop well during culture as the system has been optimized mainly for Caudal Part / AGM.

In the case of the KO Placenta, if this organ is made up of half maternal and half embryonic cells and that maternal cells do not express R26-CreER\textsuperscript{T2}, the highest level of recombination achievable is 50%. Therefore, the tested doses achieved recombination of either both Runx1 alleles in half of the embryo-derived placental cells or of one allele only but in the totality of these cells.

All the levels of recombination achieved with the Runx1 locus were inferior to those achieved with the sGFP locus, even if the tissues were not age-matched. This indicates that the Runx1 locus is more resistant to recombination than the sGFP locus. This may be explained by the ubiquitously expressed locus were the sGFP transgene is inserted (matrulin-2 gene, Liakhovitskaia et al., unpublished) whereas Runx1 is not expressed in all cells of the organs tested and is therefore not accessible for the Cre.

4.4 Discussion

The results reported in this chapter strongly suggest that HSC precursors are more sensitive to Cre toxicity than progenitors precursors. This is somehow reminiscent of previous findings (Higashi et al., 2009) where haematopoietic immature proliferating cells of adult bone marrow were found to be more sensitive to Cre activity. However, one would expect progenitors to be more sensitive to Cre activity as they cycled more often during reaggregate culture (five to seven divisions) than HSCs (one to four divisions) (Taoudi et al., 2008) and thus, progenitors DNA should be more accessible to Cre. One possible explanation is that genes involved in HSC homing to bone marrow and/or self-renewal are somehow more sensitive to Cre activity than genes involved in differentiation (expressed in both HSCs and progenitors). This is further supported by the fact that HSC generated from induced Cre reaggregates gave rise to multilineage myelo-lymphoid progeny once transplanted.
Tamoxifen induction had no impact on endothelial VC\textsuperscript{−}CD45\textsuperscript{−} population but showed an effect on mature VC\textsuperscript{−}CD45\textsuperscript{−} and more immature VC\textsuperscript{−}CD45\textsuperscript{+} populations depending on the way the induction was performed. Induction during culture reduced only the most mature VC\textsuperscript{−}CD45\textsuperscript{−} population of Cre reaggregates when the highest dose tested (5 \(\mu\text{M}\)) was used. By contrast, induction before culture did not impact this population in the Cre reaggregates but only in their WT counterparts with a dose-dependent effect.

According to the results in this chapter, a balance must be found between recombination and HSC output when the CreER\textsuperscript{T2} system is used in combination with the reaggregate culture system. The present work focuses on reactivating Runx1 in the relevant target population that is the precursors of HSCs. As it is unknown whether this target population is still prone to Cre-mediated recombination at E9.5, two doses were used in the next chapter, one low (1 \(\mu\text{M}\)) and one higher (3 \(\mu\text{M}\)).

The nature of tamoxifen-CreER\textsuperscript{T2} toxicity has been reported to consist of attenuated proliferation and increased apoptosis in thymus and spleen (Higashi et al., 2009).

It is known that the expansion of the HSC compartment during reaggregate culture occurs mainly by maturation and has only a minor proliferation component (one to four cell divisions) (Taoudi et al., 2008). The six-fold reduction of the HSC output in tamoxifen-induced WT reaggregates suggests that the tamoxifen suppresses that proliferation component and also affect the maturation process, although this would require the use of Carboxyfluorescein Succinimidyl Ester (CFSE) to be confirmed.

Surprisingly, tamoxifen induction before culture of Cre reaggregates with 1 \(\mu\text{M}\) hardly resulted in any reduction in the HSC output (compared to uninduced Cre control) whereas the use of 5 \(\mu\text{M}\) resulted in a seven-fold reduction. This may be explained by most of the tamoxifen molecules binding to the CreER\textsuperscript{T2} protein when 1 \(\mu\text{M}\) is used and thus, tamoxifen cannot disturb cell metabolism. With 5 \(\mu\text{M}\), all Cre-bound estrogen receptors may be saturated and the excess of tamoxifen could then disturb cell metabolism.
Toxicity could also be explained by hampered cell migration. During the first 48h of reaggregation culture, HSC numbers remain constant and cellularity first drops then starts to increase (Taoudi et al., 2008). This suggests a reorganization of the reaggregate before the massive HSC expansion can take place. The toxicity observed may result from the inhibition of this process. At the gene level, illegitimate recombination resulting from Cre activity may lead to the silencing, excision or aberrant expression of genes involved in cell migration.

The results obtained in this chapter reported a reduced ability of the Rosa26-CreER\textsuperscript{T2} AGM to expand HSCs during reaggregate culture. However, limiting dilution analysis should be performed to quantify this reduction precisely.
5 ROLES AND REQUIREMENTS OF RUNX1 DURING 
IN VITRO MATURATION OF PROHSCs AND 
PROGENITORS FROM E9.5 HAEMATOPOIETIC 
tISSUES.

In this chapter, the haematopoietic potential of Runx1 KO and reactivated (Re) primary haematopoietic sites was tested at E9.5 using a novel culture system that support the potent maturation of precursors, termed ProHSCs, into repopulating HSC in 7 days of culture. This system offers a unique opportunity to assess haematopoietic and HSC potentials from tissues as early as E9.5 making it a very powerful tool, especially in the case of embryonic lethal transgenic models such as the Runx1 KO model.

This system produces one HSC per ee of E9.5 WT Caudal Part (24-29 sp) after 7 days of culture (Rybtsov et al., unpublished), which is the equivalent of AGM HSC content at E11.5 (Kumaravelu et al., 2002). Consequently, this system was considered to recapitulate in vitro the haematopoietic development that occurs in vivo in the embryo proper between E9.5 and E11.

In this chapter, the following fluorochromes were used: CD45-V450, CD41-PE; VC-A647; Ter119-PE.

5.1 In vitro haematopoietic development in absence of Runx1 reactivation (-Tamoxifen)

Before dealing with reactivation, it is important to assess the ability of E9.5 Runx1 KO tissues to generate HSCs in order to evaluate how far these tissues can go into haematopoietic development without Runx1. The use of the OP9 coaggregate system makes it possible to:

- Investigate the haematopoietic potential of each organ separately.
- Bypass the necrosis and embryonic lethality observed *in vivo* within the Runx1 KO embryos around E11.
- Assess whether the pronounced reduction of VC⁺CD45⁻CD41low population observed in fresh E9.5 Runx1 KO Caudal Part and Placenta (see Chapter III) correlates with a reduced potential of these tissues to mature ProHSC into repopulating HSC.
- Investigate whether Runx1 expression in the OP9 niche is sufficient to achieve maturation of ProHSCs as Runx1 expression has been reported in OP9 cells (Sugiki et al., 2007).

5.1.1 Experimental design

The culture system used is based on the OP9 coaggregate system (Rybtsov et al., 2011) with some modifications that enable the robust maturation of ProHSC into repopulating HSC from E9.5 embryos (> 23 sp). Reactivatable Runx1 knockout (KO) embryos were obtained by crossing Runx1LacZ/GFP Rosa26CreERT2/CreERT2 female with Runx1LacZ/GFP Rosa26CreERT2/CreERT2 males. Before dissection, the number of somite-pairs (sp) in each embryo was counted and all embryos and related tissues of either less than 24 sp or more than 29 sp were discarded. Transgenic E9.5 embryos were staged by counting somite-pairs and tissues (Caudal Part, Yolk Sac and Placenta) were dissected, processed, tamoxifen-induced and coaggregated separately (Figure 5.1). WT tissues were treated likewise, except they were pooled after dissection.

When transplantation was performed, each coaggregated Runx1 KO organ was injected separately so that any engraftment may be traced back to a given staged tissue. In addition, each irradiated adult recipient received 1 embryo equivalent (ee) of cultured KO tissue. WT tissues were pooled together and injected at a dose of 1 ee per recipient.

When *in vitro* analyses were performed, all cultured KO tissues were pooled and split equally between FACS and colony assay in order to collect as many events as possible during Flow Cytometry analysis. Colony forming assay was performed in duplicate and each dish received 0.25 ee.
Figure 5.1: Experimental design for OP9 coaggregation of E9.5 Runx1 KO tissues (No induction).
A total of four experiments were performed: two repeats with \textit{in vivo} transplantation assays and two repeats with \textit{in vitro} assays.

5.1.2 \textit{Runx1} KO Caudal Part, Yolk Sac and Placenta are unable to maturate ProHSCs

None of the E9.5 \textit{Runx1} KO tissues could produce HSC during the culture (Figure 5.2.A). Many explanations are possible:

- ProHSCs are present in fresh E9.5 KO organs, as shown by phenotype in Chapter III, but Runx1 is required for their \textit{in vitro} maturation into repopulating HSCs. In this case, reactivation of \textit{Runx1} in KO organs should lead to the production of HSC. Also, this points towards a cell autonomous requirement of Runx1 for ProHSC maturation, as \textit{Runx1} expression in the OP9 niche is not sufficient. However, testing this hypothesis would require sorting of the ProHSC population to a high level of purity, excision of \textit{Runx1}, coaggregation of deleted tissues with OP9 and transplantation into adult irradiated recipients.

- Runx1 is required for the production of functional ProHSCs from early precursors despite the presence of ProHSC by phenotype in fresh E9.5 KO tissues as assessed in chapter III.

- ProHSCs may be present in the KO Yolk Sac and/or Placenta but cannot mature because the coaggregate system has not been optimized for those tissues. In this case, there is a possibility that \textit{in vivo}, ProHSCs migrate from Yolk Sac and Placenta to Caudal Part. However, OP9 coaggregation of E9.5 WT Yolk Sac and Placenta did not produce any HSC (Figure S5.7), suggesting that functional ProHSCs are not present in these tissues, unless culture conditions are not sufficient to support their maturation.
Figure 5.2: Coaggregate culture is not sufficient for KO tissues to develop any haematopoietic activity.

A. Engraftment of E9.5 coaggregated KO tissues after 16 weeks. Each recipient received 1 ee of tissue individually processed from dissection to transplantation. E9.5 coaggregated WT Caudal Part was used as a positive control. Data are cumulative of 3 independent experiments.

B. CFU-C activity in E9.5 coaggregated WT and KO tissues.

C. Growth fold (ratio between cellularity in fresh tissue and cultured tissue as assessed by flow cytometry with counting beads, OP9 excluded) of E9.5 WT and KO tissues after culture. Tissues above the dotted line grew during the culture, those below declined.

D. Percentage of live (7AAD−) cells after culture. For B to D, at least 2 ee of KO tissue were used per experiment, each bar shows the average and error bars show standard deviation between 2 independent experiments.
5.1.3 *Runx1* KO Caudal Part, Yolk Sac and Placenta lack progenitors after coaggregate culture

None of the E9.5 *Runx1* KO tissues exhibited any clonogenic activity after culture (Figure 5.2.B). This points towards a cell autonomous requirement of Runx1 for progenitor development although deletion of *Runx1* in conditional *Runx1* KO tissues followed by OP9 coaggregation culture would be required to confirm this hypothesis.

The absence of progenitors suggests that Runx1 is required either for transition between the progenitor and a more differentiated stage (i.e. cells the colonies are made up of) or between a more immature precursor stage and progenitor.

WT Caudal Part progenitors expanded 170-fold during culture (50±60 CFU-Cs per ee in the fresh, 8,470±1,410 CFU-Cs per ee after culture). Interestingly, E9.5 WT Yolk Sac progenitors also expanded during culture although more modestly (from 447±320 CFU-Cs per ee in fresh, 27,500±11,300 CFU-Cs per ee after culture, a 60-fold expansion). Likewise, WT Placenta progenitors expanded but less than WT Yolk Sac (100±60 CFU-Cs per ee in fresh, 2,600±600 CFU-Cs per ee after culture, a 26-fold expansion), strongly suggesting that WT Yolk Sac and Placenta haematopoietic compartments are stimulated during culture even though no ProHSC can be matured from those tissues (Figure S5.7). The inhibition potential of Yolk Sac and Placenta on Caudal Part ability to mature ProHSCs is being tested in the lab.

Given these results, it is possible that WT Yolk Sac and Placenta may be biased towards progenitor production instead of ProHSC maturation during OP9 coaggregation culture.
5.1.4 Haematopoietic content in Runx1 KO Caudal Part, Yolk Sac and Placenta after coaggregate culture

Despite the lack of HSC and CFU-C production, KO Caudal Part expanded during OP9 coaggregation culture to a similar extent in the WT (growth fold of 2.7±0.7 in KO, 3.3±0.7 in WT, Figure 5.2.C). The percentage of live (7AAD-) cells was slightly but significantly (p-value = 0.04) higher in KO Caudal Part than in its WT counterpart (93.7±1% live cells in KO, 89.5±0.7% live cells in WT, Figure 5.2.D). This might be a sign of reduced apoptosis usually observed with reduced differentiation although Annexin-V staining would be required to confirm this.

The endothelial (VC\(^+\)CD45\(^-\)CD41\(^-\)) compartment was not significantly different after culture between KO and WT Caudal Part although the average was higher in KO (2,628±1,495 cells per ee in KO, 425±363 cells per ee in WT, Figure 5.3.A and 5.4.A for WT and 5.4.B for KO). This might indicate a tendency towards accumulation of endothelial cells in KO Caudal Part, however more experiments may be required to see a clear difference.

The trend observed in ProHSC / PreHSC Type I-like compartment was much clearer: those cells accumulated in KO Caudal Part (590±117 cells per ee in KO, 33±1 cells per ee in WT, Figure 5.3.B and 5.4.C for WT and 5.4.D for KO) during culture. However, no PreHSC Type II (Figure 5.3.C and 5.4.B) or mature VC\(^-\)CD45\(^+\) haematopoietic cells (Figure 5.3.D and 5.4.B) could be detected. Interestingly, the number of KO ProHSC / PreHSC Type I cells increased 8-fold during culture (75±55 cells per ee in fresh, 590±117 cells per ee after culture) whereas those values dropped 19-fold in the WT after culture (640±320 cells per ee in fresh, 33±1 cells per ee after culture). Many explanations are possible:

- The absence of Runx1 blocks the transition between ProHSC / PreHSC Type I and PreHSC Type II stages.
- ProHSC generation is already delayed in fresh E9.5 KO Caudal Part and after 7 days of culture, KO Caudal Part advance to the stage of fresh E9.5 WT Caudal Part, at least in terms of ProHSC / PreHSC Type I. In this case, Runx1 expression may be required before E9.5 and the beginning of the second wave of haematopoiesis.
Figure 5.3: Coaggregate culture is not sufficient for KO tissues to develop produce haematopoietic cells. A. Number of endothelial cells (VC+CD45−CD41−) per ee after culture. B. Number of PreHSC Type I (VC+CD45−CD41low) cells per ee after culture.. C. Number of PreHSC Type II (VC+CD45+) cells per ee after culture. D. Number of VC−CD45+ mature haematopoietic cells per ee after culture. E. Number of erythroid Ter119+ cells per ee after culture. At least 2 ee of KO tissue were used per experiment, each bar shows the average and error bars show standard deviation between 2 independent experiments.
Figure 5.4: Representative FACS plot showing populations of interest after culture in E9.5 Caudal Part. Mature haematopoietic cells (VC^-CD45^+) and PreHSC Type II (VC^-CD45^+) in WT (A) and KO (B). Endothelial (VC^-CD45^-CD41^-) and PreHSC Type I cells (VC^-CD45^-CD41^low) in WT (C) and KO (D). Erythroid (Ter119^+) cells in WT (E) and KO (F). For corresponding isotype and FMO controls, see Figure S5.1.
Most of the Ter119\(^+\) cells found in fresh E9.5 KO Caudal Part disappeared during culture (16,400±7,000 cells per ee in fresh, figure 3.3.G, 247±31 cells per ee after culture, Figure 5.3.F and 5.4.E for WT and 5.4.F for KO). Interestingly, no erythroid colony could be detected in WT Caudal Part after culture (Figure 5.2.B), even if higher numbers of Ter119\(^+\) cells were found in this organ (25,000±9,000 cells per ee in fresh, figure 3.3.G, 19,921±17,500 cells per ee after culture). This indicates that the culture system may be biased toward myeloid differentiation at the expense of the erythroid lineage.

Unlike the Caudal Part, KO Yolk Sac clearly degenerated during culture whereas WT Yolk Sac expanded (growth fold 0.07±0.06 in KO, 2.7±0.9 in WT, \(p\)-value = 0.001, Figure 5.2.D). Likewise, most of populations assessed were practically undetectable after culture of KO Yolk Sac:

- The frequency of ProHSC / PreHSC Type I-like cells was 1,250±950 cells per ee in fresh (Figure 3.7.D) and 140±190 cells per ee after culture (Figure 5.3.B and 5.5.D).
- The frequency of PreHSCs Type II was 100±80 cells per ee in fresh (Figure 3.7.E) and only 5±7 cells per ee after culture (Figure 5.3.C and 5.5.B).
- Mature VC\(^+\)CD45\(^+\) content was 416±600 cells per ee in fresh (Figure 3.7.F) and 20±10 cells per ee after culture (Figure 5.3.D and 5.5.B).
- Ter119\(^+\) population was 30,000±13,000 cells per ee in fresh (Figure 3.7.G) and only 1,020±1,390 cells per ee after culture (Figure 5.3.F and 5.5.F).

The endothelial output in cultured KO Yolk Sac was more variable with 210 cells per ee after culture in one experiment and 1030 cells per ee after culture in another experiment (Figure 5.3.A) (430±530 cells per ee in fresh KO Yolk Sac, Figure 3.7.C).

Taken together, these results suggest that \textit{Runx1} expression within the Yolk Sac is an indispensable component for Yolk Sac maintenance and expansion during coaggregate culture. To a certain extent, this is unexpected as the haematopoietic program in the Yolk Sac is thought to be more Runx1-independent, Runx1 being
Figure 5.5: Representative FACS plot showing populations of interest after culture in E9.5 Yolk Sac. Mature haematopoietic cells (VC\(^{-}\)CD45\(^{+}\)) and PreHSC Type II (VC\(^{+}\)CD45\(^{+}\)) in WT (A) and KO (B). Endothelial (VC\(^{+}\)CD45\(^{-}\)CD41\(^{-}\)) and PreHSC Type I cells (VC\(^{+}\)CD45\(^{-}\)CD41\(^{\text{low}}\)) in WT (C) and KO (D). Erythroid (Ter119\(^{+}\)) cells in WT (E) and KO (F). For corresponding isotype and FMO controls, see Figure S5.2.
dispensible for the first wave of haematopoiesis and fresh E9.5 KO and WT Yolk Sacs showing same numbers of PreHSC Type I (Figure 3.7.D).

Both KO and WT Placenta degenerated during culture as illustrated by their growth fold (0.6±0.06 and 0.5±0.07 respectively, Figure 5.2.C), thus demonstrating the non-ideal conditions for this organ in the OP9 coaggregation system.

The endothelial output from culture was significantly reduced in KO Placenta (1,710±2,270 cells per ee in the KO, 11,850±5,250 cells per ee in WT, \( p\)-value = 0.02, figure 5.3.A and 5.6.A for WT and 5.6.B for KO).

The culture yielded variable amounts of ProHSC / PreHSC Type I-like cells in both KO and WT Placenta (Figure 5.3.B). In one experiment, there were 200 cells per ee in KO and 210 cells per ee in WT. In another experiment, there were no cells in KO and 530 cells per ee in WT.

Likewise, the PreHSC Type II output was variable in WT Placenta but not in KO (Figure 5.3.C and 5.6.D). KO Placenta yielded 260±70 cells per ee, although most of them expressed Runx1-GFP indicating a maternal origin (Figure 5.7.A). WT Placenta yielded 214 cells per ee in one experiment and 764 cells per ee in another experiment.

Surprisingly, mature VC-CD45+ cells were present in cultured KO Placenta (Figure 5.3.D and 5.6.B). However, all expressed Runx1-GFP indicating a maternal origin (Figure 5.7.B).

KO Placenta produced less Ter119+ cells after culture than its WT counterpart although the difference was not significant (3,710±3,200 cells per ee in KO, 22,580±17,470 cells per ee in WT, figure 5.7.E).

It is difficult to draw conclusions from these results because the culture system has not been optimized for the Placenta. However, the reduced endothelial content and the absence of Runx1-GFP negative VC-CD45+ mature cells point towards a requirement of Runx1 in the development of ProHSC/PreHSC Type I cells from the endothelium.
Figure 5.6: Representative FACS plot showing populations of interest after culture in E9.5 Placenta. Mature haematopoietic cells (VC−CD45+) and PreHSC Type II (VC+CD45+) in WT (A) and KO (B). Endothelial (VC+CD45−CD41−) and PreHSC Type I cells (VC+CD45−CD41low) in WT (C) and KO (D). Erythroid (Ter119+) cells in WT (E) and KO (F). For corresponding isotype and FMO controls, see Figure S5.3.
Figure 5.7: All CD45+ expressing cells were from maternal origin in the E9.5 KO Placenta after culture as demonstrated by their maternal Runx1-GFP expression. A. PreHSC Type II (VC+CD45+) cells, B. VC-CD45+ mature haematopoietic cells. There were not enough events (<20) to plot Maternal Runx1-GFP expression in the PreHSC Type I (VC+CD45-CD41<sub>low</sub>) compartment.
5.2 *In vitro* haematopoietic development in *Runx1* reactivated tissues

5.2.1 Experimental design

At this point of the investigation, it was shown in the laboratory that less HSCs could be produced during OP9 coaggregation from tamoxifen-induced E9.5 Caudal Parts that were homozygote for Rosa26-CreER\(^T2\) compared to heterozygote counterparts (Rybtsov et al., unpublished). Therefore, a different setup was designed to obtain embryos that were *Runx1* KO and Rosa26-CreER\(^T2\) heterozygote: *Runx1\(^{LacZ/GFP}\) Rosa26\(^{CreERT2/CeERT2}\) males were crossed with *Runx1\(^{LacZ/WT}\) Rosa26\(^{WT/WT}\) males. Transgenic E9.5 embryos were staged by counting somite-pairs and tissues were dissected, processed, tamoxifen-induced and coaggregated separately (Figure 5.8). WT tissues were treated the same way, except they were pooled after dissection.

When transplantation was performed, each coaggregated *Runx1* reactivated (Re) organ was injected separately into two irradiated adult recipients so that any engraftment may be traced back to a given staged tissue and HSC output quantified using limiting dilution. WT Caudal Parts were pooled together and transplanted at a dose of 1 ee per recipient as a control.

When *in vitro* analyses were performed, all cultured KO tissues were pooled and split equally between FACS and colony assay in order to collect as many events as possible during Flow Cytometry analysis. Colony forming assay was performed in duplicate and each dish received 0.25 ee.

A total of 8 experiments were performed. For each dose tested (i.e. 1 and 3 \(\mu\)M tamoxifen), two experiments with transplantation and two experiments with *in vitro* assays were performed. When *in vitro* analyses were performed, Re tissues were compared to WT tissues induced with 3 \(\mu\)M tamoxifen.
Figure 5.8: Experimental design for OP9 coaggregation of E9.5 Runx1 Reactivated tissues.
5.2.2 No HSCs are produced in reactivated Caudal Part, Yolk Sac or Placenta after coaggregate culture

None of the reactivated organs could mature any HSC after culture, be it with 1 or 3 µM tamoxifen (Figure 5.9.A, B and C).

It is unlikely that tamoxifen-induction is toxic for ProHSC maturation, since tamoxifen-induced WT controls produced high-level repopulating HSCs with both doses used. Furthermore, ProHSC maturation following tamoxifen induction (1 µM Tam for 1h30) has been achieved in the laboratory using the Rosa26-CreERT² system coupled with other conditional KO genes (Rybtsov et al., unpublished).

Another explanation may be that tamoxifen-induction delays ProHSC maturation such that seven days of culture are not sufficient to mature ProHSCs into repopulating HSCs. However, this is unlikely as such a delay has not been observed with other conditional KO genes.

Alternatively, if ProHSCs are not yet formed in E9.5 KO tissues, seven days of culture may not be enough to both generate ProHSCs and then mature them into HSCs. In this case, the possibility remains that tamoxifen induction may be toxic for precursors of ProHSCs.

5.2.3 No progenitors are produced in reactivated Caudal Part, Yolk Sac or Placenta after coaggregate culture

Reactivated Caudal Part, Yolk Sac and Placenta were devoid of progenitor activity after culture (Figure 5.10.A).

Tamoxifen induction showed no significant reduction in progenitor output from WT and Cre reaggregates (Figure 4.13.A) although a potential toxicity on precursors of progenitors cannot be ruled out.

Another explanation might be that Runx1 is required for the maturation of progenitors from immature precursors and this process may take more than seven days in vitro.
A. 

Reactivated Caudal Part

Embryo #:

Tamoxifen concentration: 1 µM 3 µM 1 µM 3 µM 0 µM

B. 

Reactivated Yolk Sac

Embryo #:

Tamoxifen concentration: 1 µM 3 µM 1 µM 3 µM 0 µM

C. 

Reactivated Placenta

Embryo #:

Tamoxifen concentration: 1 µM 3 µM 1 µM 3 µM 0 µM
Figure 5.9: Reactivated E9.5 KO tissues did not contain any HSC after culture as shown by the absence of engraftment after 16 weeks. A. Caudal Part, B. Yolk Sac, C. Placenta. Each recipient received 1 ee of tissue processed individually from dissection to transplantation. E9.5 coaggregated WT Caudal Part +/-Tamoxifen were used as a positive control. Data are cumulative of 4 independent experiments.
Figure 5.10: Reactivated E9.5 KO tissues did not contain any progenitor after culture, had normal growth-fold (except for YS) and normal cell viability. A. CFU-C activity in E9.5 coaggregated WT and Re tissues. B. Growth fold (ratio between cellularity in fresh tissue and cultured tissue, as assessed by flow cytometry with counting beads, OP9 excluded) of E9.5 WT and Re tissues after culture. Tissues above the dotted line grew during the culture, those below declined. C. Percentage of live (7AAD⁻) cells after culture. At least 2 ee of KO tissue were used per experiment, each bar shows the average and error bars show standard deviation between 2 independent experiments for each dose of tamoxifen tested and 4 independent experiments for 3 µM Tam WT.
5.2.4 Haematopoietic content in reactivated Caudal Part, Yolk Sac and Placenta after coaggregate culture

Re and WT Caudal Parts displayed similar growth-fold after culture (4.2±0.7 with 1 µM Tam Re, 4.1±0.4 with 3 µM Tam Re and 4±0.7 for 3 µM Tam WT, figure 5.10.B). This suggests that tamoxifen induction and Runx1 reactivation did not reduce the ability of the Caudal Part to expand during culture.

No difference was observed between Re and WT Caudal Parts in terms of cell viability after culture (Figure 5.10.C), unlike with KO Caudal Part. This might point towards more differentiation in Re Caudal Part than in its KO counterpart.

Both 1 and 3 µM Re Caudal Part showed a significant accumulation (p-value = 0.03) of endothelial (VC^CD45^-CD41^) cells after culture (Figure 5.11.A) with 8,500±1,495 cells per ee in 1 µM Tam Re (Figure 5.12.B and E), 7,000±3,000 cells per ee in 3 µM Tam Re (Figure 5.12.C and F) and 3,490±363 cells per ee for 3 µM Tam WT (Figure 5.12.A and D). The same tendency was observed between KO and WT Caudal Parts after culture (Figure 5.3.A, 5.4.A and C for WT-T, 5.4.B and D for KO).

Yet, no accumulation of ProHSC / PreHSC Type I was detected in Re Caudal Parts (figure 5.11.B) (190±40 cells per ee in 1 µM Tam Re, figure 5.12.E, 297±100 cells per ee in 3 µM Tam Re, figure 5.12.F, 233±50 cells per ee in 3 µM Tam WT, figure 5.12.D) as opposed to KO Caudal Part (Figure 5.3.B). Given these results, reactivation of Runx1 in the Caudal Part leads to an accumulation of more immature cells (i.e. haemogenic endothelium) but not of ProHSC / PreHSC Type I.

PreHSC Type II cells were undetectable in both 1 and 3 µM tamoxifen-induced Re Caudal Parts (Figure 5.11.C, 5.12.B for 1 µM Tam Re and 5.12.C for 3 µM Tam Re).

However, 3 µM but not 1 µM Tam Re Caudal Parts surprisingly yielded VC^-CD45^ haematopoietic cells after culture (Figure 5.11.D), although significantly less than the wildtype (3,980±300 cells per ee in 3 µM Tam Re, figure 5.12.C, 190,540±34,900 cells per ee for 3 µM Tam WT, figure 5.12.A, p-value = 0.001). This is unexpected as no sign of their precursors, that is to say PreHSC Type II, was
Figure 5.11: Reactivated Runx1 E9.5 tissues were mostly devoid of CD45$^+$ haematopoietic cells (Placental CD45$^+$ cells might be of maternal origin). A. Number of endothelial cells (VC$^+$CD45$^-$CD41$^-$) per ee after culture. B. Number of PreHSC Type I (VC$^+$CD45$^-$CD41$^{low}$) cells per ee after culture. C. Number of PreHSC Type II (VC$^+$CD45$^+$) cells per ee after culture. D. Number of VC$^-$CD45$^+$ mature haematopoietic cells per ee after culture. E. Number of erythroid Ter119$^+$ cells per ee after culture. At least 2 ee of KO tissue were used per experiment, each bar shows the average and error bars show standard deviation between 2 independent experiments for each dose of tamoxifen tested and 4 independent experiments for 3 µM Tam WT.
Figure 5.12: Representative FACS plot showing populations of interest after culture in E9.5 Caudal Part. Mature haematopoietic cells (VC<sup>+</sup>CD45<sup>+</sup>) and PreHSC Type II (VC<sup>+</sup>CD45<sup>+</sup>) in 3 μM Tam WT(A) and Re (B. 1 μM, C. 3 μM). Endothelial (VC<sup>+</sup>CD45<sup>-</sup>CD41<sup>-</sup>) and PreHSC Type I cells (VC<sup>+</sup>CD45<sup>+</sup>CD41<sup>low</sup>) in 3 μM Tam WT (D) and Re (E. 1 μM, F. 3 μM). Erythroid (Ter119<sup>+</sup>) cells in 3 μM Tam WT (G) and Re (H. 1 μM, I. 3 μM). For corresponding isotype and FMO controls, see Figure S5.4.
detected. Importantly, these VC$^{+}$CD45$^{-}$ cells were not clonogenic, as indicated by the absence of progenitor activity in 3 µM Re Caudal Part (Figure 5.10.A). Also, this suggests that a dose of 3 µM Tam recombines Runx1 in a population that is not reached when 1 µM Tam is used.

Reactivation of Runx1 led to an increase in the production of Ter119$^{+}$ cells in Caudal Part after culture (Figure 5.11.E) (4,200±30 cells per ee in 1 µM Tam Re, figure 5.12.H, 4,800±100 cells per ee in 3 µM Tam Re, figure 5.12.I, 247±30 for KO, figure 5.4.F) although the erythroid content was still significantly reduced compared to its WT counterpart (11,314±3,000 cells per ee in 3 µM Tam WT, p-value = 0.03, figure 5.12.G). It is important to notice that Ter119$^{+}$ cells were present in higher amount in reactivated Caudal Part than in KO. Indeed, it was reported that tamoxifen induction completely abolished the in vitro production of Ter119$^{+}$ cells from Rosa26-CreER$^{T2}$ bone marrow (Higashi et al., 2009). This may indicate that the system is robust enough to withstand the toxicity originating from tamoxifen / CreER$^{T2}$.

Taken together, these data render the first signs of rescued haematopoietic development from endothelium in the Re Caudal Part that may lead to the generation of HSCs if the culture is prolonged.

The reactivation of Runx1 in the Yolk Sac did not lead to any improvement concerning the growth-fold, which remains inferior to 1, thus demonstrating a degeneration (growth-fold: 0.1±0.07 in 1 µM Tam Re, 0.3±0.1 in 3 µM Tam Re, 0.07±0.06 in KO, 3±0.9 in 3 µM Tam WT, p-value = 0.001 between 1 or 3 µM Tam Re and 3 µM Tam WT, Figure 5.10.B).

No difference was observed between Re and WT Yolk Sacs in terms of cell viability after culture (Figure 5.10.C).

In accordance with the growth-fold, the endothelial population was reduced in Re Yolk Sacs after culture when compared to WT (Figure 5.11.A) (328±110 cells per ee in 1 µM Tam Re, figure 5.13.B and E, 400±57 cells per ee in 3 µM Tam Re, figure 5.13.C and F, 967±1,340 cells per ee in KO, figure 5.5.B and D, and 2,450±120 cells per ee in 3 µM Tam WT, figure 5.13.A and D, p-value = 0.02 between 1 or 3 µM Tam Re and 3 µM Tam WT), but gave more reproducible results
Figure 5.13: Representative FACS plot showing populations of interest after culture in E9.5 Yolk Sac. Mature haematopoietic cells (VC⁺CD45⁺) and PreHSC Type II (VC⁺CD45⁺) in 3 µM Tam WT (A) and Re (B. 1 µ M, C. 3 µ M). Endothelial (VC⁺CD45⁻CD41⁻) and PreHSC Type I cells (VC⁺CD45⁻CD41⁺low) in 3 µ M Tam WT (D) and Re (E. 1 µ M, F. 3 µ M). Erythroid (Ter119⁺) cells in 3 µ M Tam WT (G) and Re (H. 1 µ M, I. 3 µ M). For corresponding isotype and FMO controls, see Figure S5.5.
than with KO Yolk Sacs as indicated by the reduced standard deviation. This might indicate that the Yolk Sac behaves more stably in culture after Runx1 reactivation.

The PreHSC Type I compartment was also reduced in Re Yolk Sacs after culture (78±40 cells per ee in 1 µM Tam Re, figure 5.13.E, 120±20 cells per ee in 3 µM Tam Re, figure 5.13.F, 140±187 cells per ee in KO, figure 5.5.D, 896±300 cells per ee in 3 µM Tam WT, figure 5.13.D, p-value = 0.03 between 1 or 3 µM Tam Re and 3 µM Tam WT, figure 5.11.B). Likewise, reactivation of Runx1 in Yolk Sac led to more stable cell numbers than in KO after culture.

Re Yolk Sacs were devoid of PreHSC Type II (Figure 5.11.C, 5.13.B for 1 µM Tam Re and 5.13.C for 3 µM Tam Re) as well as mature VC-CD45+ cells (Figure 5.11.D).

Reactivation of Runx1 did not lead to an increase in Ter119+ cell production from cultured Yolk Sacs when compared to KO (929±400 cells per ee in 1 µM Tam Re, figure 5.13.H, 2,000±230 cells per ee in 3 µM Tam Re, figure 5.13.I, 1,020±1,390 cells per ee in KO, figure 5.5.F, and 19,443±6,415 cells per ee in 3 µM Tam WT, figure 5.13.G, p-value = 0.01 between 1 or 3 µM Tam Re and 3 µM Tam WT, figure 5.11.E), although it led to more stable cell numbers.

In summary, the reactivation of Runx1 did not lead to any sign of rescue of the haematopoietic development in the Yolk Sac. Nevertheless, cell numbers generated were more reproducible in reactivated than in KO Yolk Sac even though only two repeats were performed with KO and Re tissues, suggesting a more stable behaviour during culture after Runx1 reactivation.

Interestingly, Re and WT+T Placentas were maintained during culture after reactivation of Runx1, as demonstrated by a growth-fold close to 1 (1.2±0.2 in 1 µM Tam Re, 1.1±0.2 in 3 µM Tam Re and 0.9±0.1 in 3 µM Tam WT, figure 5.10.B, 0.6±0.06 in WT-T, 0.5±0.07 in KO respectively, Figure 5.2.C). This suggests that tamoxifen induction supports the maintenance of Placenta during coaggregation culture.

No difference was observed between Re and WT Placentas in terms of cell viability after culture (Figure 5.10.C).
Figure 5.14: Representative FACS plot showing populations of interest after culture in E9.5 Placenta. Mature haematopoietic cells (VC$^+$CD45$^+$) and PreHSC Type II (VC$^+$CD45$^+$) in 3 µM Tam WT (A) and Re (B, 1 µM, C, 3 µM). Endothelial (VC$^+$CD45$^-$CD41$^-$) and PreHSC Type I cells (VC$^+$CD45$^+$CD41$^{low}$) in 3 µM Tam WT (D) and Re (E, 1 µM, F, 3 µM). Erythroid (Ter119$^+$) cells in 3 µM Tam WT (G) and Re (H, 1 µM, I, 3 µM). For corresponding isotype and FMO controls, see Figure S5.6.
The endothelial compartment of Re Placenta showed a significant accumulation of cells after culture (Figure 5.11.A) with 46,000±2,270 cells per ee in 1 µM Tam Re (Figure 5.14.B and E), 39,000±2,000 cells per ee in 3 µM Tam Re (Figure 5.14.C and F) and 9,930±5,250 cells per ee in 3 µM Tam WT (Figure 5.14.A and D), \( p \)-value = 0.02 between 1 or 3 µM Tam Re and 3 µM Tam WT. Of note, the trend was the opposite to that observed in the KO Placenta (figure 5.3.A).

PreHSC Type I also accumulated in Re Placenta after culture (Figure 5.11.B) with 2,500±120 cells per ee in 1 µM Tam Re (Figure 5.14.E), 1,900±300 cells per ee in 3 µM Tam Re (Figure 5.14.F), 85±120 cells per ee in KO (Figure 5.6.D) and 550±230 cells per ee in 3 µM Tam WT (Figure 5.14.D), \( p \)-value = 0.03 between 1 or 3 µM Tam Re and 3 µM Tam WT.

Unlike Re Caudal Part and Yolk Sac, Re Placenta contained PreHSC Type II and mature VC’CD45+ cells after culture (Figure 5.11.C). However, these two cell populations were also found in KO Placenta after culture at similar numbers for PreHSC Type II and increased numbers for mature haematopoietic cells. For PreHSC Type II, there were 520±5 cells per ee in 1 µM Tam Re (Figure 5.14.B), 100±20 cells per ee in 3 µM Tam Re (Figure 5.14.C), 260±70 cells per ee in KO (Figure 5.6.B) and 5,240±390 cells per ee in 3 µM Tam WT (Figure 5.14.A), \( p \)-value = 0.02 between 1 or 3 µM Tam Re and 3 µM Tam WT. For mature VC’CD45+ cells (Figure 5.11.D), there were 3,820±250 cells per ee in 1 µM Tam Re, 2,000±300 cells per ee in 3 µM Tam Re, 20,940±680 cells per ee in KO and 63,930±6,280 cells per ee in 3 µM Tam WT, \( p \)-value = 0.007 between 1 or 3 µM Tam Re and 3 µM Tam WT.

The erythroid compartment increased after culture in Re Placentas compared to their KO counterparts (Figure 5.11.E): 9,000±1,000 cells per ee in 1 µM Tam Re (Figure 5.14.H), 17,000±230 cells per ee in 3 µM Tam Re (Figure 5.14.I), 3,710±3,200 cells per ee in KO (Figure 5.6.F) and 10,560±1,230 cells per ee in 3 µM Tam WT (Figure 5.14.G). Moreover, 3 µM Re Placenta produced significantly more Ter119+ cells than WT or 1 µM Re counterparts (\( p \)-value = 0.04 between 3 µM Tam WT or 1 µM Tam Re and 3 µM Tam Re).

Despite the reactivation of RunxI, more mature cells (PreHSC Type II), already present in KO cultured Placenta, were not produced in higher quantities except for VC’CD45+ compartment. However, the reactivation of RunxI in the
coaggregated Placenta led to the accumulation of immature cells (endothelial and PreHSC Type I) and to an increase in erythrocyte production. This may be the first sign of haematopoiesis and prolonged culture may produce progenitors and HSCs. Of note, the animal crossing setup did not lend itself to distinguishing between cells of maternal and embryonic origin.

5.3 Discussion

The reported results in this chapter showed different aspects of Runx1 requirement during haematopoietic development, the first being the time window in which Runx1 reactivation rescues the haematopoietic lineage. The production of mature haematopoietic cells in Re Caudal Part and the maintenance of Re but not KO Placenta during culture both suggest that the target population where Runx1 is normally expressed is still present at E9.5. Therefore, it should be still possible to rescue HSCs at E9.5 in these organs. This would require the length of the culture to be increased, and Re coaggregates to be transplanted into irradiated adult recipients. Alternatively, transplantation into newborn or NSG mice may reveal the potential of Re organs after 7 days of culture.

The failure of both KO and Re Yolk Sacs to expand and even be maintained during culture strongly suggests that the target population in which Runx1 expression is crucial is no longer present at E9.5. Alternatively, this target population may come from Caudal Part or Placenta and its migration towards the Yolk Sac is Runx1-dependent.

Results of this chapter are summarised in table 5.1 for Caudal Part, in table 5.2 for Yolk Sac and in table 5.3 for Placenta.

Another important aspect is the stages at which Runx1 is required. The absence of HSCs after reactivation and culture in Re Caudal Part indicates that ProHSCs were not fully specified in the fresh KO organ, probably from earlier precursors (e.g. haemogenic endothelium). Consequently, Runx1 is required for the specification of ProHSCs in the Caudal Part. Alternatively, ProHSCs may migrate from Yolk Sac or Placenta to the Caudal Part in a Runx1-dependent manner.
Table 5.1.: Summary of chapter results from cultured Runx1 KO and Re Caudal Parts/AGMs compared to their WT counterpart. Normal means there is no significant difference between the condition tested and the corresponding control. Accu means accumulation of cells.

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## Yolk Sac after culture

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</tr>
<tr>
<td>PreHSC II</td>
<td></td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>Mature VC-CD45+</td>
<td></td>
<td>rare</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td></td>
<td>rare</td>
<td>reduced</td>
<td>reduced</td>
</tr>
<tr>
<td>CFU-Cs</td>
<td></td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
</tbody>
</table>

Table 5.2.: Summary of chapter results from cultured Runx1 KO and Re Yolk Sacs compared to their WT counterpart. Normal means there is no significant difference between the condition tested and the corresponding control.
### Placenta after culture

<table>
<thead>
<tr>
<th>Parameters</th>
<th>-T KO</th>
<th>1 µM Re</th>
<th>3 µM Re</th>
</tr>
</thead>
<tbody>
<tr>
<td># HSC Culture output / ee</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Growth rate</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Endothelial</td>
<td>reduced</td>
<td>accu</td>
<td>accu</td>
</tr>
<tr>
<td>PreHSC I</td>
<td>normal</td>
<td>accu</td>
<td>accu</td>
</tr>
<tr>
<td>PreHSC II</td>
<td>normal</td>
<td>reduced</td>
<td>rare</td>
</tr>
<tr>
<td>Mature VC·CD45⁺</td>
<td>normal</td>
<td>reduced</td>
<td>reduced</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>normal</td>
<td>normal</td>
<td>accu</td>
</tr>
<tr>
<td>CFU-Cs</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
</tbody>
</table>

Table 5.3.: Summary of chapter results from cultured Runx1 KO and Re Placentas compared to their WT counterpart. Normal means there is no significant difference between the condition tested and the corresponding control. Accu means accumulation of cells.
Unfortunately, it is not possible to assess the ProHSC potential in Yolk Sac and Placenta at the moment with this culture system.

Reactivation of Runx1 does not rescue progenitors after culture in all organs tested suggesting that Runx1 expression is necessary for the specification of progenitors. Indeed, if progenitors were already present in KO tissues but only lacked Runx1 to form colonies, such a potential should have been revealed after reactivation and coaggregate culture.

More generally, the lack of HSC and progenitor activities after reactivation suggests that Runx1 is required before E9.5 for haematopoietic development in the conceptus. This is in line with previous studies where Runx1 reactivation driven either by Tie2- or CD41-Cre led to successful in vivo rescue of HSC and progenitors (Liakhovitskaia et al., 2007 and unpublished). In those studies, the use of Tie2- or CD41-Cre,sGFP embryos showed that the GFP expression was already detected at E8, suggesting a very early requirement for Runx1. Of note, CD41-Cre recombines Runx1 locus much weakly (around 10% in tail of E11.5 embryo) than Rosa26-CreER\textsuperscript{T2}. This rules out the possibility that CD41-Cre reaches a cell population that Rosa26-CreER\textsuperscript{T2} would miss. In addition, full rescue of the haematopoietic development is achieved when Runx1 is reactivated following tamoxifen injection into pregnant dams at E7.5 of gestation. The rescue fails if the injection is performed at E8.5 (Nishikawa et al., unpublished).

The third aspect concerns the requirement of Runx1 in the OP9 niche compared to the tissues cultured. The lack of HSCs and progenitors in KO tissues after culture points towards a cell autonomous requirement of Runx1 expression for haematopoietic development. However, to assess the importance of Runx1 expression in the niche, reverse experiments where WT organs are coaggregated with a Runx1 KO or knockdown OP9 cell line should be performed.
6 SUMMARY AND PERSPECTIVES

6.1 Summary

This work shed a new light on the role of Runx1 in the specification of early haematopoietic precursors.

In Chapter 3, the study of precursors populations in the major primary haematopoietic sites of Runx1 KO conceptus revealed that Runx1 expression is required mainly around E9.5 for a correct transition between ProHSC / PreHSC Type I and PreHSC Type II stages. These results suggest a role for Runx1 in the upregulation of CD45 during maturation of haematopoietic precursors. This aspect is consistent with the CD45 negative HSC phenotype reported in Runx1 heterozygote embryos (North et al., 2002). The study of progenitor activity revealed that Runx1 expression is necessary for progenitors to emerge at E9.5 in the Yolk Sac but more surprisingly, in the Placenta too. Results also confirmed that the Runx1 KO model developed in our laboratory (Samokhvalov et al., 2006) lack progenitors in the embryo from E9.5 to E11, and HSC in E11 conceptus (although the Placenta was not assessed) as reported in previously published Runx1 KO models (Wang et al., 1996; Cai et al., 2000).

In Chapter 4, the study of tamoxifen induction on the maturation of WT and CreER\textsuperscript{T2}-expressing HSCs during reaggregate culture revealed that maturation of HSC is very sensitive to both tamoxifen as a chemical and the Cre-mediated recombination. The study also showed that progenitors are generally less affected than HSCs, which is in line with previous reports suggesting that immature cells are more sensitive to tamoxifen induction (Higashi et al., 2009). In addition, the best balance between HSC output and sGFP recombination is achieved when tamoxifen induction is performed in IMDM for 1h30 before the culture with doses of tamoxifen comprised between 1 and 5 µM. As 5 µM of tamoxifen almost completely abolished HSC maturation, doses of 1 and 3 µM were selected to reactivate the Runx1 locus in E9.5 Caudal Part, Yolk Sac and Placenta.
In Chapter 5, the study of the haematopoietic potential after OP9 coaggregation of E9.5 KO Caudal Part, Yolk Sac and Placenta revealed that the maturation of ProHSCs into HSCs is Runx1-dependent and Runx1 expression is probably required in a cell-autonomous manner for this process to occur. Likewise, the lack of progenitors after OP9 co-culture confirms that Runx1 expression is required in a cell autonomous manner for the emergence of progenitors. In line with results from Chapter III, Runx1 is required for the specification of phenotypical PreHSCs Type II from E9.5 Caudal Part and Yolk Sac, but not from Placenta, during culture.

The lack of HSCs and progenitors reported in reactivated tissues after culture suggests that Runx1 is required for the specification of their precursors, ProHSCs and “Pre”-progenitors, respectively, at least in the Caudal Part. The presence of mature haematopoietic cells in reactivated Caudal Part with 3 µM tamoxifen and the increased production of erythrocytes (compared with KO) in both reactivated Caudal Part and Placenta after 7 days of culture may well constitute the first signs of a rescued haematopoietic development. However, this observation requires an extension of the culture to be confirmed. The very fact that 7 days are not sufficient to properly rescue the HSC and progenitors lineages and to produce PreHSCs Type II strongly suggests that Runx1 expression is required before E9.5 in the Caudal Part. The reported degeneration of the reactivated E9.5 Yolk Sac during culture also indicates that Runx1 needs to be expressed at an earlier stage in this organ for haematopoietic development to occur.

6.2 Perspectives

The requirement of Runx1 for the transition between PreHSC Type I and PreHSC Type II stages is being confirmed in the laboratory at the moment using a conditional Runx1 KO model (aka Timer-AML1, Growney et al., 2005). This is tested by sorting PreHSC Type I from E10 AGM, deleting Runx1 with tamoxifen induction, coaggregating with OP9 and transplanting into adult irradiated recipients. Likewise, requirement of Runx1 is being tested between PreHSC Type II and HSC
stages with the same strategy but PreHSC Type II population is sorted from late E10 AGM instead. At this stage, the VC^+CD45^+ phenotype represents PreHSC II as HSCs have not emerged yet.

The possible rescue through the reactivation of Runx1 in E9.5 KO tissues will need to be tested by extending the culture up to 14 days, as floating nitrocellulose membranes tend to fall apart after 2 weeks of cultures (D. Paruzina, personal communication). If this approach remains unsuccessful, another approach which combines in vivo reactivation of Runx1 by injecting pregnant dams with tamoxifen followed by OP9 coaggregate culture of E9.5 reactivated tissues may shed more light on the time window when Runx1 is required for haematopoietic development. However, the injection of pregnant dams with tamoxifen adds a factor of uncertainty as for the time window. Indeed, the time needed to reach embryonic target cells in those conditions may vary significantly from one dam to another thus yielding variable results. To bypass this issue, in utero injection of tamoxifen is best suited and currently under development in our laboratory.

Alternatively, a multistep culture system, which enables the ex vivo maturation of HSCs from E8 conceptus is currently being developed in the lab and may prove useful to investigate the role of Runx1 in the presomic embryo.

In addition, it is of prime importance to determine the phenotype and the endothelial potential on a clonal level of the precursors from which the rescue occurs in Runx1 reactivated embryos. Indeed, if these precursors already express CD41 and have lost their potential to form endothelial colonies before Runx1 starts being expressed, it suggests that haematopoietic commitment, possibly from the endothelium, occurs before Runx1 starts to be expressed. In other words, Runx1 would be required after the endothelial to haematopoietic transition.

Most importantly, dissecting biological process will contribute to understand the molecular clues involved in HSC specification in order to faithfully recapitulate haematopoietic development and produce HSCs from alternative sources such as ES and iPS cells. Much remains to be done although engraftment into the bone marrow of ES-derived haematopoietic cells carrying a HoxB4 inducible transgene has been
achieved (Matsumoto et al., 2009). However, this approach is not suitable for medical purposes as the transgene was integrated in the genome and may therefore trigger abnormal proliferation, paving the way to cancer. Moreover, continuous induction was necessary to maintain high-level engraftment and HoxB4 ectopic expression in the adult bone marrow hampered lymphoid differentiation. One must also bear in mind that approaches developed on short lifespan animals (eg mouse, zebrafish) may not be fully transposable to human whose longer lifespan may give more time for cell abnormalities to develop.

Another significant caveat is the use of fetal calf serum in protocols developed with murine models. This is currently a bottleneck, which hampers the translation to clinical studies (so-called bench to bedside process). Much remains to be done to move HSC research towards a serum-free, xeno-free (free of animal compounds, 100% recombinant) environment.

In summary, very significant progress has been made in the last 10 years in understanding the molecular clues and the mechanisms involved in the generation, maintenance and expansion of haematopoietic stem cells. However, producing bona fide haematopoietic stem cells from wildtype embryonic and induced pluripotent stem cells remains to be achieved.
7 Supplementary figures
**A.**

- **FSC vs. SSC**
  - Counting beads
  - GFP
  - Live cells
  - Percentages:
    - Counting beads: 98.4%
    - Live cells: 8.2%
    - 7AAD:
      - 26.3

**B.**

- **SSC vs. FSC**
  - Counting beads
  - Live cells
  - Percentages:
    - Counting beads: 53.9%
    - Live cells: 203%
Figure S3.1: Gating strategy of counting beads and live cells. Counting beads (A) were separated from cells and then from debris on the basis of forward and side scatter. Counting beads are made of two populations equal in proportion as shown on the plot named “Counting beads”. Caudal Part / AGM (B), Yolk Sac (C) and Placenta (D) cells were separated from debris on the basis of forward and side scatter. Dead cells were then excluded on the basis of 7AAD uptake. Numbers show percentage within each gate.
Figure S3.2.: Isotype controls for VC/CD45 staining in Caudal Part at E9.5 (A), E10 (B) and in AGM at E11 (C).
Figure S3.: FMO for VC^CD45^CD41^low staining in **Caudal Part** at E9.5 (A), E10 (B) and in **AGM** at E11 (C).
Figure S3.4.: Isotype controls for Ter119+ staining in Caudal Part at E9.5 (A), E10 (B) and in AGM at E11 (C).
Figure S3.5.: Isotype controls for VC/CD45 staining in Yolk Sac at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.6: FMO for VC^+CD45^+CD41^{low} staining in Yolk Sac at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.7: Isotype controls for Ter119$^+$ staining in Yolk Sac at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.8.: Isotype controls for VC/CD45 staining in Placenta at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.9.: FMO for VC^+CD45^+CD41^{low} staining in Placenta at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.10.: Isotype controls for Ter119\(^+\) staining in *Placenta* at E9.5 (A), E10 (B) and at E11 (C).
Figure S3.11: Average number of progenitors per ee in fresh E9.5 Caudal Part (A), Yolk Sac (B) and Placenta (C). Data are cumulative of 3 independent experiments. $p$-value $<=$ 0.01 is indicated by **.
Figure S3.12: Average number of progenitors per ee in fresh E10 Caudal Part (A), Yolk Sac (B) and Placenta (C). Data are cumulative of at least 3 independent experiments. $p$-value $\leq 0.01$ is indicated by **.
Figure S3.13: Average number of progenitors per ee in fresh E11 AGM (A), Yolk Sac (B) and Placenta (C). Data are cumulative of at least 3 independent experiments. $p$-value $\leq 0.01$ is indicated by **.
Figure S4.1.: Isotype control for VC/CD45 staining in reaggregates after culture with tamoxifen induction performed during culture (A) or before culture (B).

Figure S4.2.: Isotype control for multilineage staining in the peripheral blood.
Figure S4.3.: Isotype control for multilineage staining in the bone marrow.

Figure S4.4.: Isotype control for multilineage staining in the spleen.
Figure S4.5.: Isotype control for multilineage staining in the thymus.

Figure S4.6.: Average cellularity in fresh E11 WT and Cre AGM. Each bar shows the average and error bars show standard deviation between 3 independent experiments with at least 2 ee of each genotype per experiment. Dead cells were excluded on the basis 7AAD and live cells were counted by flow cytometry using counting beads.
Figure S5.1.: Isotype controls for A. VC/CD45 and C. Ter119 staining and FMO control for PreHSC Type I (VC^CD45^CD41^{low}) staining in E9.5 KO Caudal Part after culture.
Figure S5.2.: Isotype controls for A. VC/CD45 and C. Ter119 staining and FMO control for PreHSC Type I (VC<sup>+</sup>CD45<sup>-</sup>CD41<sup>low</sup>) staining in E9.5 KO Yolk Sac after culture.
Figure S5.3.: Isotype controls for A. VC/CD45 and C. Ter119 staining and FMO control for PreHSC Type I (VC⁺CD45⁻CD41low) staining in E9.5 KO Placenta after culture.
Figure S5.4.: Isotype controls for A. VC/CD45 and C. Ter119 staining and B. FMO control for PreHSC Type I (VC<sup>+</sup>CD45<sup>-</sup>CD41<sup>low</sup>) staining in E9.5 Re Caudal Part after culture.
Figure S5.5.: Isotype controls for A. VC/CD45 and C. Ter119 staining and B. FMO control for PreHSC Type I (VC$^+$CD45$^-$CD41$^{low}$) staining in E9.5 Re Yolk Sac after culture.
Figure S5.6.: Isotype controls for A. VC/CD45 and C. Ter119 staining and B. FMO control for PreHSC Type I (VC⁺CD45⁻CD41^-low) staining in E9.5 Re Placenta after culture.
Figure S5.7.: Engraftment of E9.5 WT Yolk Sac and Placenta after 7 days of OP9 coaggregation culture. Engraftment was assessed 16 weeks post-transplantation. Data are cumulative of 3 independent experiments.
8 References


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