Chapter 4
Epigenetic and Transcriptional Mechanisms Regulating the Development of the Haematopoietic System in Mammals

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Abstract The haematopoietic system is established early during embryonic development and is maintained throughout adult life by haematopoietic stem cells. The cellular intermediates leading to the formation of mature blood cells are now fairly well characterised; from the mesoderm germ layer, successive steps of commitment give rise to haemangioblast, haemogenic endothelium, and haematopoietic stem and progenitor cells. Key transcription factors, such as ETV2, SCL, GATA2 or RUNX1, have been shown to specifically control some of these cell fate decisions. However, an integrated view of the transcriptional network controlling haematopoietic specification still remains to be established. Furthermore, it has become clear over the last decade that the transcriptional control of cell fate specification is globally regulated by epigenetic mechanisms. While the chromatin landscape is starting to be unravelled in adult haematopoiesis, virtually nothing is known about the epigenetic processes regulating the onset of haematopoiesis in the developing embryo. In this chapter, we describe the current state of our knowledge on the onset of mammalian haematopoiesis, focusing on murine development as it is by far the best characterised organism.

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4.1 Ontogeny of the Haematopoietic System in Mammals

Over the last century, detailed analyses of embryonic tissues have allowed to define a spatiotemporal map of haematopoiesis during development. These analyses revealed that haematopoiesis occurs at multiple sites during development, but that only some of these sites are able to de novo generate haematopoietic cells. Despite numerous studies, the developmental origin of haematopoietic stem cells (HSCs) which maintain the haematopoietic system throughout adult life still remains unclear and controversial.

4.1.1 Anatomical Sites of Haematopoiesis During Development

In the mouse embryo, the first blood cells appear in the yolk sac (YS), an extra-embryonic supporting tissue, from embryonic day (E)7.5 before the vasculature connections are established with the embryo proper. Early haematopoietic production from the YS is referred to as the first wave of haematopoiesis or primitive haematopoiesis, as it mainly produces primitive erythrocytes (nucleated erythrocytes expressing embryonic globins), macrophages and megakaryocytes (Wong et al. 1986; Palis et al. 1999). It is believed that these cells will not contribute to the pool of haematopoietic cells found in the adult organism. A second wave of haematopoiesis arises shortly after and is called definitive haematopoiesis as it produces adult-type progenitor cells from E8.5 (Godin et al. 1995). During this second wave of haematopoiesis, HSCs which will give rise to the adult haematopoietic system are produced from E10.5. Based on milestone experiments performed with the avian model, it is now widely accepted that the first definitive HSCs originate from an intra-embryonic region (Dieterlen-Lievre 1975) and not from the YS as previously thought (Moore and Owen 1965). In the murine embryo, the AGM region (which encompasses the aorta together with the gonads and the mesonephros) was shown to contain multipotent progenitors between E9 and E12 (Medvinsky et al. 1993) and from E10 the first HSCs as defined by their ability to reconstitute the haematopoietic system of adult mouse recipients upon transplantation (Muller et al. 1994; Medvinsky and Dzierzak 1996). From E10.5 to E11, HSCs were also detected in the placenta (Gekas et al. 2005; Ottersbach and Dzierzak 2005) and the YS (Muller et al. 1994; Kumaravelu et al. 2002; Gekas et al. 2005), where they are thought to proliferate before reaching the foetal liver,

the main site of HSC expansion from E12.5, then the bone marrow from E17.5 onward (reviewed by Mikkola and Orkin 2006).

The anatomical origin of HSCs has been the subject of intensive studies. Because the YS can produce adult-type progenitors just before the blood circulation is established (Palis et al. 1999), organ explants experiments have been used to confirm the intra-embryonic origin of HSCs previously suggested in the avian model. When explanted before the establishment of blood circulation, it has been shown that the presumptive AGM region contains developmental precursors of HSCs, while the YS is devoid of HSC activity (Cumano et al. 2001). Although these results confirm the theory of the intra-embryonic origin of HSCs, it has been suggested that the early YS might independently generate precursors requiring a specialised microenvironment such as the AGM to develop into definitive HSCs (Matsuoka et al. 2001). This hypothesis has recently gained further support through lineage tracing experiments demonstrating that the early YS contains precursors of adult HSCs (Samokhvalov et al. 2007; Tanaka et al. 2012a), although technical issues surrounding the experimental strategies undermine these studies. The allantois, which gives rise to the umbilical cord, has also been proposed as a site of de novo generation of multipotent haematopoietic progenitors, either using organ explants strategies (Zeigler et al. 2006; Corbel et al. 2007), or using mouse model devoid of heart beat (Rhodes et al. 2008).

4.1.2 Cellular Origin of Haematopoiesis

The cellular origin of haematopoietic precursors has also long been a subject of controversy. A first theory was proposed in the early twentieth century based on the anatomical studies of the first erythrocytes observed within the YS. These blood cells were surrounded by endothelial cells in histological structures called "blood islands" which seem to develop from a uniform mass of mesodermal cells. This observation led to the hypothesis that haematopoietic and endothelial cells from the YS shared a common developmental precursor (Maximov 1909; Sabin 1920), later termed the haemangioblast. This precursor was eventually identified using the embryonic stem (ES) cell differentiation model in which the blast colony-forming cell (BL-CFC), the in vitro equivalent of the putative haemangioblast, was identified as a tri-potential precursor which can differentiate into haematopoietic, endothelial and smooth muscle lineages and which can be enriched based on its expression of FLK1 (VEGF-receptor 2, KDR) and brachyury (Choi et al. 1998; Ema et al. 2003; Fehling et al. 2003). Using these markers, the haemangioblast was later isolated from the gastrulating embryo (Huber et al. 2004), where it was shown to localise within the primitive streak, and not within the mesodermal masses developing into blood islands, as first hypothesised. It is now believed that the haemangioblast is a transient mesodermal precursor which develops very quickly into the haematopoietic or endothelial lineages while migrating from the primitive

streak to the extra-embryonic YS, and that as a result of both rapid commitment and migration, blood islands are polyclonal (Ferkowicz and Yoder 2005; Ueno and Weissman 2006).

Contemporary to the observation of the YS blood islands was the observation of blood cell clusters attaching to the endothelial wall of the dorsal aorta, which led to the theory of an endothelial origin of these haematopoietic cells (Jordan 1917). Phenotypic analysis of these clusters revealed a co-expression of both endothelial and haematopoietic markers (Garcia-Porrero et al. 1998; reviewed by Cumano and Godin 2007), suggesting a direct lineage relationship between blood and vasculature. It was later shown that sorted endothelial cells from E9.5 embryos can produce haematopoietic cells in vitro (Nishikawa et al. 1998). More convincing evidence demonstrating the production of haematopoietic cells from the endothelium in vivo came from lineage tracing experiments. The first strategy showed that Ac-LDL labelling of cells of the endothelial tree in living embryos resulted in production of labelled haematopoietic cells (Sugiyama et al. 2003, 2005). Another strategy involving inducible lineage-specific expression of a reporter gene demonstrated that part of the adult haematopoietic system was derived from an endothelial ancestor (Zovein et al. 2008). Recent technologies have now made possible the direct visualisation of “budding” haematopoietic cells from the endothelium in the AGM region (Boisset et al. 2010). This was also observed using the ES cell differentiation model (Eilken et al. 2009; Lancrin et al. 2009). The intra-aortic clusters are thought to contain the HSCs present within the AGM region, and it is hypothesised that these clusters are produced from the haemogenic endothelium although this remains to be formally demonstrated (Taoudi and Medvinsky 2007; Taoudi et al. 2008). Of interest, arterial haematopoietic activity is not restricted to the dorsal aorta as the vitelline and umbilical arteries also harbour HSCs at the same time as the aorta (de Bruijn et al. 2000) and contain intra-artery clusters (North et al. 2002). Haemogenic endothelial cells have also been isolated from the YS (Nishikawa et al. 1998; Li et al. 2005; Lancrin et al. 2009).

Although the theories of the haemangioblast and the haemogenic endothelium have been considered mutually exclusive, a unifying theory has been proposed where the haemangioblast produces haematopoietic cells through a haemogenic endothelial step (Fig. 4.1) [reviewed by Lancrin et al. (2010)]. This cellular hierarchy has been defined for the first wave of haematopoiesis; the cellular origin of the second wave of embryonic haematopoiesis is in contrast still not fully understood. It remains to be determined whether the haemogenic endothelium found within the AGM is directly derived from the first wave of already committed mesoderm or whether it is de novo specified from mesoderm via a haemangioblast intermediate. Defining the cellular origin of this second wave of haematopoiesis is critically important if one wants to understand how HSCs are generated.

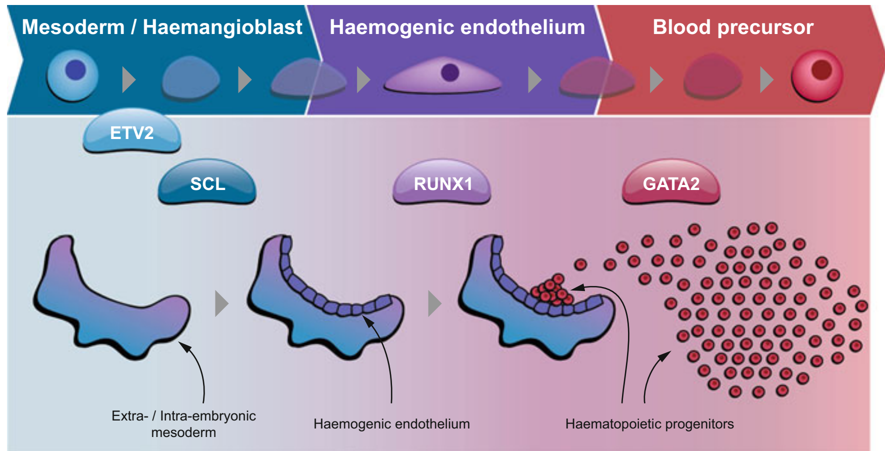

Fig. 4.1 The generation of embryonic blood cells. Mouse embryonic haematopoietic precursors are generated from extra- and intra-embryonic mesodermal cell populations through a transient haemogenic endothelium. Key transcription factors regulate the transitional steps involved in blood generation: ETV2 acts upstream of SCL and both proteins regulate the differentiation of mesoderm/haemangioblasts into haemogenic endothelial cells; RUNX1 is subsequently required for the emergence of blood precursors from the haemogenic endothelium; GATA2 is implicated in the maintenance and proliferation of the newly formed haematopoietic progenitor cells

4.2 Transcriptional Regulation of Haematopoietic Specification During Development

The spatiotemporal emergence of the haematopoietic system is a tightly controlled process orchestrated by multiple transcription factors. Several master regulators have been shown to coordinate the sequential steps leading to the production of mature blood cells. The complex transcriptional network controlling these cell fate decisions from mesoderm to haematopoietic cells is slowly been unravelled (Fig. 4.1).

4.2.1 From the Haemangioblast to the Haemogenic Endothelium

4.2.1.1 SCL

SCL (SCL/TAL1), a member of the bHLH (basic helix–loop–helix) transcription factor family, was initially identified as a target of chromosomal translocation in T-cell leukaemia (reviewed by Begley and Green 1999). In the developing embryo, SCL is expressed from E7.5 in the extra-embryonic mesoderm, and thereafter in the

haematopoietic and endothelial cells of the YS and the embryo proper (Kallianpur et al. 1994; Silver and Palis 1997; Elefanty et al. 1999), but also in some neurons of the central nervous system (van Eekelen et al. 2003). SCL-deficient embryos die around E9.5 with a complete absence of YS haematopoiesis likely to be the cause of this early embryonic lethality (Robb et al. 1995; Shivdasani et al. 1995). Analysis of the contribution of $scl^{-/-}$ ES cells to mouse chimaeras showed that they do not contribute to primitive or definitive haematopoiesis, suggesting that SCL is necessary for haematopoiesis during development (Porcher et al. 1996; Robb et al. 1996). Despite being expressed in endothelial cells, SCL does not seem to be necessary for the emergence of endothelial cells, as null embryos display a capillary network in the YS. However, these embryos lack a complete endothelial network, indicating that SCL is involved in vascular remodelling (Visvader et al. 1998; Elefanty et al. 1999). Using the in vitro model of ES differentiation, it was shown that SCL is dispensable for haemangioblast specification, but is necessary to drive the haemangioblast toward haematopoietic fate (Ema et al. 2003; D'Souza et al. 2005). More recently, it was shown that SCL is specifically required for the transition from the haemangioblast to the haemogenic endothelium stage (Lancrin et al. 2009).

Recent studies have shown that SCL is also a regulator of mesoderm patterning: SCL can induce haematopoietic specification at the expense of other lineages such as cardiac or paraxial mesoderm (Ismailoglu et al. 2008), and YS endothelial cells are mis-specified toward cardiac fate in the absence of SCL (Van Handel et al. 2012).

4.2.1.2 LMO2

LMO2 (RTBN2) is a LIM domain transcription factor involved in chromosomal translocation in T-cell leukaemia (Boehm et al. 1991; Royer-Pokora et al. 1991). During development, LMO2 displays a similar pattern of expression as SCL, being detected in the mesoderm, the haemogenic sites of the cardiovascular system (Silver and Palis 1997; Manaia et al. 2000; Minko et al. 2003) and the nervous system (Hinks et al. 1997; Herberth et al. 2005). LMO2 is also expressed transiently in some somite derivatives and in the intra-embryonic endoderm (Manaia et al. 2000). Embryos deficient for LMO2 present a phenotype similar to SCL null embryos. They die around E10.5 from the absence of YS erythropoiesis (Warren et al. 1994), and null ES cells do not contribute to adult haematopoiesis cells in chimaera experiments (Yamada et al. 1998). Interestingly, LMO2 does not bind to DNA directly but has been shown to interact with transcriptional regulatory complexes involved in haematopoietic differentiation (Warren et al. 1994; Wadman et al. 1997; Rabbitts 1998; Xu et al. 2003). More specifically, a transcriptional complex involving LMO2, SCL, GATA-1, LDB1 and E2A was described in erythroid lineage. It is proposed that LMO2 acts as a scaffold to link SCL and GATA factors both of which are involved in DNA binding (Wadman et al. 1997). Since LMO2 is required for haematopoietic emergence, it is very likely that this

factor is also involved in transcriptional complexes regulating haematopoietic specification, as it was recently suggested (Nottingham et al. 2007; Landry et al. 2008; Wilson et al. 2010).

4.2.1.3 ETV2

ETV2 (ETSRP71) is a member of the ETS family of transcription factors which was recently identified in zebrafish (Sumanas et al. 2005), mouse and human (Sumanas et al. 2008). ETV2 is expressed transiently in the developing embryo; it is first detected in the extra-embryonic mesoderm around E7.0, in the endothelial and haematopoietic cells of the blood island at E8.5, then in the endothelial cells of the developing vasculature of the embryo proper but is virtually absent after E11.5 (Lee et al. 2008; Kataoka et al. 2011; Koyano-Nakagawa et al. 2012; Wareing et al. 2012a).

Ectopic expression of ETV2 during ES cell differentiation promotes the formation of endothelial and haematopoietic progenitors (Lee et al. 2008; Koyano-Nakagawa et al. 2012; Liu et al. 2012). Embryos deficient for ETV2 die by E10.5 with a complete absence of blood progenitors and severe vascular defects (Lee et al. 2008; Kataoka et al. 2011; Koyano-Nakagawa et al. 2012; Wareing et al. 2012b), and null ES cells do not contribute to haematopoiesis or endothelial cells in chimaera experiments (Liu et al. 2012). Taken together, these data suggest a requirement of ETV2 in the establishment of haematopoietic and endothelial lineages. Supporting this theory, endogenous ETV2 expression is correlated with haematopoietic potential in the embryo and in the ES differentiation model (Kataoka et al. 2011; Koyano-Nakagawa et al. 2012; Wareing et al. 2012a). In particular, ETV2 expression marks the haemogenic endothelium population in vitro and in vivo and is required for its formation (Wareing et al. 2012a).

Interestingly, ETV2-deficient ES cells display increased cardiogenic potential (Liu et al. 2012) and ETV2 overexpression supresses development of the cardiac lineage (Rasmussen et al. 2011), a phenotype reminiscent of SCL activity. In line with these findings, it has been shown that SCL is a direct transcriptional target of ETV2 (Kataoka et al. 2011; Wareing et al. 2012b). Considering that ETV2 is expressed in primitive mesodermal precursors and only required until FLK1 expression as shown by conditional deletion experiments (Wareing et al. 2012a), it is proposed that ETV2 directs early mesoderm to differentiate toward haematopoietic and endothelial lineages and that its action is mediated by the induction of SCL.

4.2.2 From the Haemogenic Endothelium to the Haematopoietic Progenitors

4.2.2.1 RUNX1

RUNX1 (Acute Myeloid Leukaemia (AML-1), CBFα) is a member of the family of Runt-domain transcription factors and heterodimerizes with CBFβ to form the core-binding factor (CBF). Both subunits have been frequently found involved in chromosomal translocation in leukaemia and myelodysplasic syndromes (Look 1997). RUNX1 is expressed from E7.5 in the YS mesoderm and blood islands, and in haemogenic sites in the embryo later on. In the embryo proper, RUNX1 is expressed in endothelial cells of the aorta and vitelline artery as early as E8.5 and in the endothelial cells and haematopoietic clusters of the aorta and umbilical artery at E10.5 (North et al. 1999; Lacaud et al. 2002).

Embryos deficient for RUNX1 die around E12.5–E13.5, most likely due to haemorrhages in the central nervous system. Primitive erythropoiesis is observed in the YS, but no definitive haematopoiesis is present in the foetal liver (Okuda et al. 1996), suggesting a role for RUNX1 in the establishment of definitive but not primitive haematopoiesis. This hypothesis was confirmed using the ES cell differentiation model, where it was shown that *runx1*$^{-/-}$ ES cells generate primitive haematopoiesis in vitro (Lacaud et al. 2002), but do not contribute to definitive haematopoiesis in vivo in chimaeras embryos (Okuda et al. 1996). Deficient embryos are devoid of HSCs in the foetal liver and the YS but also in the AGM region (Cai et al. 2000; Mukouyama et al. 2000), suggesting a requirement of RUNX1 during the early steps of HSCs specification. This hypothesis was supported by the observation that intra-aortic clusters, believed to contain the emerging HSCs, are absent in RUNX1-deficient embryos (North et al. 1999). Further studies showed that RUNX1 is actually necessary for the generation of haematopoietic cells from the haemogenic endothelium. Indeed, endothelial cells sorted from E10.5 *runx1*$^{-/-}$ embryos do not generate haematopoietic cells in vitro (Yokomizo et al. 2001). Moreover, using a conditional KO mouse model, it was shown that RUNX1 expression is required in endothelial cells for HSCs emergence, but dispensable in cells already engaged in the haematopoietic lineage (Li et al. 2006; Chen et al. 2009). In vitro, it was shown that RUNX1 is required for the transition from the haemogenic endothelium population to haematopoietic cells (Lancrin et al. 2009).

Altogether, these studies revealed that RUNX1 is necessary for the emergence of definitive HSCs from the haemogenic endothelium during development. Furthermore, RUNX1 activity seems to be dose dependent since haploinsufficient embryos display HSC activity from E10, 1 day earlier than WT embryos. But at E11.5, although *runx1*$^{+/-}$ AGM contain the same number of HSCs, they are not able to amplify during ex vivo culture, suggesting that RUNX1 could also regulate the maintenance/expansion of HSCs in the embryo (Cai et al. 2000).

4.2.2.2 GATA2

GATA2 is a member of the zinc finger transcription factor and is part of the GATA family. During development, GATA2 is expressed at E7.5 in extra- and intra-embryonic mesoderm and in the YS blood islands (Minegishi et al. 1999; Kobayashi-Osaki et al. 2005). Later during development, GATA2 is expressed in the endothelial and haematopoietic cells of the AGM and foetal liver (Minegishi et al. 1999, 2003; Kobayashi-Osaki et al. 2005). GATA2 is also expressed in non-haematopoietic tissues such as the central nervous system and the uro-genital compartment (Nardelli et al. 1999; Siggers et al. 2002).

GATA2-deficient embryos die in utero around E10.5 with severe anaemia (Tsai et al. 1994). They display a markedly reduced primitive haematopoiesis. In chimaera experiments, null ES cells do participate in primitive haematopoiesis, although in a very low proportion, but are unable to contribute to definitive haematopoiesis (Tsai et al. 1994). Interestingly, deficient ES cells can produce definitive haematopoietic cells in vitro, but GATA2 is likely involved in the subsequent survival and/or proliferation of these haematopoietic progenitors (Tsai et al. 1994; Tsai and Orkin 1997). This hypothesis was further supported when it was shown that GATA2 haploinsufficiency leads to a decrease in HSCs number in the AGM, YS and foetal liver between E10 and E12 and that these HSCs display reduced proliferation during AGM explant culture. Compensatory mechanisms are likely to rescue GATA2 deficiency since older embryos and adult *gata2*$^{+/-}$ mice have normal numbers of HSCs. These HSCs, however, have a proliferative defect revealed in non-steady state physiology such as competitive engraftment experiments (Ling et al. 2004; Rodrigues et al. 2005).

4.2.3 Reconstruction of Gene Regulatory Networks

As described above, the emergence and specification of haematopoietic cells during development is regulated by transcription factors. Using loss and gain of function approaches, the systematic analysis of individual transcription factor has allowed to identify master regulators of this process and to understand at which specific time and stages they are acting. However, how these factors interact together within wider regulatory networks to orchestrate the tightly regulated process of haematopoietic development is still largely unknown. Gene regulatory networks are described as the functional interplay between transcription factor proteins and the ‘*cis*-regulatory modules’ (CRM) associated with their target genes (reviewed by Pimanda and Gottgens 2010). Current strategies to decipher regulatory networks fall into two categories: the ‘bottom-up’ and the ‘top-down’ approaches.

4.2.3.1 Bottom-up Approaches

Bottom-up approaches start from one identified component of a specific network, such as a CRM or a transcription factor, and reconstruct the regulatory mechanisms upstream of this component within the network. An example of a bottom-up approach is the study of the transcriptional regulation of the *Scl* gene during haematopoietic development. Using in vivo transgenic reporter assays, several enhancers have been identified in the *Scl* locus, some of which have been shown to specifically direct the expression of a reporter gene in the haematopoietic and endothelial cells during development (Sanchez et al. 1999; Gottgens et al. 2002, 2004; Silberstein et al. 2005). Interestingly, the +19 enhancer, a 600-bp core sequence 19 kb downstream of the transcription start site, was shown to contain three binding motifs which were necessary for its activation (Gottgens et al. 2002). These motifs, two conserved ETS-binding sites and one conserved GATA-binding site, were bound by FLI1, ELF1 and GATA2 and it was shown that these three transcription factors formed a transcriptional complex regulating SCL expression. This *Ets/Ets/Gata* motif was the first CRM identified in the haematopoietic specification process. This CRM was then used in a genome-wide screening to identify new enhancers of other genes putatively involved in haematopoietic development, three of which (namely *Fli1*, *Hex* and *Smad6*) were found to display an expression pattern similar to the *Scl* +19 enhancer in vivo (Donaldson et al. 2005; Pimanda et al. 2007a).

Further studies of the *Fli1* enhancer lead to the identification of the first fully connected triad of HSC transcription factors, the SCL/FLI1/GATA2 triad (Pimanda et al. 2007b). This triad consists of genes encoding transcription factors regulating each other, thus forming a powerful feedforward loop stabilising the stem cell state. This activation is likely to take place early during haematopoietic specification as all three factors are co-expressed in presumptive HSCs of the AGM intra-aortic clusters. A network model was recently proposed where the triad is initiated by NOTCH and BMP4 signalling and modulated by RUNX1 in the haemogenic endothelium (Narula et al. 2010, 2013).

4.2.3.2 Top-Down Approaches

Top-down approaches are used to identify downstream targets of specific transcription factors. They are usually based on genome-wide expression profiling of cell populations following induction or inactivation of a transcription factor of interest. This approach generates a list of potential transcriptional targets based on functional relevance and the presence of predicted binding sites in the regulatory sequences of the candidates (as performed for ETV2 in Wareing et al. 2012b). Combining analyses for multiple transcription factors should then allow to highlight interconnections between transcriptional programmes and help reconstructing regulatory networks.

The main drawbacks of such approaches are the need to validate the direct binding of the transcription factor on its target and the risk of missing potential candidates because of lack of proper annotations of genomic sequences. These limitations are now being bypassed with the use of the ChIP-seq technology (chromatin immunoprecipitation combined with high-throughput sequencing). It is not yet possible to use this technology with low number of cells, thus precluding analysis of rare cell populations in the developing embryos. Nonetheless, this strategy was recently used to identify members of the transcriptional network controlled by SCL during haematopoietic development. A screen of direct transcriptional targets of SCL was conducted using a haematopoietic stem/progenitor cell line and 11 of these candidates were validated in vivo using ChIP and transgenic reporter assays for the corresponding enhancer region (Wilson et al. 2009). Using this strategy, the same group generated genome-wide binding profiles for ten major haematopoietic transcriptional regulators: SCL, LYL1, LMO2, GATA2, RUNX1, MEIS1, PU.1, ERG, FLI-1 and GFI1b (Wilson et al. 2010). This study suggested the existence a heptamer complex (containing SCL, LYL1, LMO2, GATA2, RUNX1, FLI-1 and ERG) acting to regulate the haematopoietic programme. Of interest, a collaboration involving RUNX1 with SCL, LYL1, LMO2 or GATA2 had not been described previously.

More recently, two studies have investigated the direct transcriptional targets of RUNX1 during haematopoietic development. In the first study, the combination of gene expression profiling and ChIP-seq analysis in differentiating ES cells identified a small number of direct transcriptional targets of RUNX1 during the haematopoietic specification. Among those, only 29 genes were shown to be affected by the loss of RUNX1 during haematopoietic emergence in the E7.5 YS (Tanaka et al. 2012b). Interestingly, 23 of these genes were previously described as direct transcriptional targets of the SCL/FLI1/GATA2 triad, thus reinforcing the hypothesis of transcriptional collaboration of these factors within an early haematopoietic regulatory network. In another study, the binding profile of SCL, FLI1 and RUNX1 were compared during the endothelial to haematopoietic transition in the ES cell differentiation model (Lichtinger et al. 2012). This study suggested that the binding profiles of SCL and FLI1 were altered during the transition, although most of the redistribution occurred within the same gene locus or in its vicinity; this shift in binding activity was shown to be in part a consequence of RUNX1 binding at the same locus. This study along with previous work from the same group demonstrated the implication of RUNX1 in modulating chromatin remodelling and epigenetic changes at the onset of haematopoiesis (Hoogenkamp et al. 2009; reviewed by Lichtinger et al. 2010).

While transcription factors implicated in haematopoietic development are further characterised, their role defined and their places within transcriptional networks uncovered, it becomes essential to integrate this wealth of knowledge into the higher levels of regulation encompassing the epigenetic control of genome-wide chromatin landscape.

4.3 Epigenetic Regulation of Transcription

Epigenetics describes the chromatin-based events regulating gene expression in a heritable manner. These events include DNA methylation and histone modifications. Changes in the DNA methylation pattern or alterations in the histone modifications landscape can modulate gene expression, either directly or by facilitating the recruitment of additional chromatin-modifying enzymes. The key role of chromatin modifying enzymes in haematopoiesis is highlighted by the fact that the function or expression of several of these enzymes is deregulated in leukaemia and other haematological malignancies (Fig. 4.2).

4.3.1 Post-translational Histone Modifications

The complexity of histone code is highlighted by the large range of possible modifications including acetylation, methylation, phosphorylation, ubiquitylation and SUMOylation, amongst others. Combinations of different modifications are thought to induce structural changes in the chromatin, and therefore modify the accessibility of transcription factors to regulatory sequences, allowing the regulation of gene expression in a time and tissue-specific manner. Our understanding of the specific functions of the proteins that direct chromatin modifications at different stages of the development or maintenance of the haematopoietic system still remains very limited.

4.3.1.1 Histone Acetyltransferases and Histone Deacetylases

The transfer of an acetyl group to specific lysine residues located at the histone tails is catalysed by histone acetyltransferases (HATs). This modification reduces the stability of the interaction between histone and DNA (Hong et al. 1993; Puig et al. 1998) relaxing the chromatin structure and thereby affecting gene expression (Shogren-Knaak et al. 2006; Campos and Reinberg 2009). In addition, this acetylated residue could also act as a docking site for bromodomain containing regulatory factors.

Among this group, the Monocytic Leukaemia Zinc Finger protein (MOZ, MYST3 or KAT6) is a member of the MYST family of HATs and transcription co-activators and was first identified as a translocation partner in various forms of AML (Borrow et al. 1996; Carapeti et al. 1998; Chaffanet et al. 2000; Esteyries et al. 2008). MOZ is the catalytic component of a large multi-subunit protein complex also harbouring ING5 (Inhibitor of Growth 5), the bromodomain PHD-finger protein (BRPF1) and EAF6 (Esa1-associated factor 6) (Doyon et al. 2006). Genetic deletion of MOZ results in severe defects in the development and maintenance of HSCs and the development of erythroid cells thus leading to

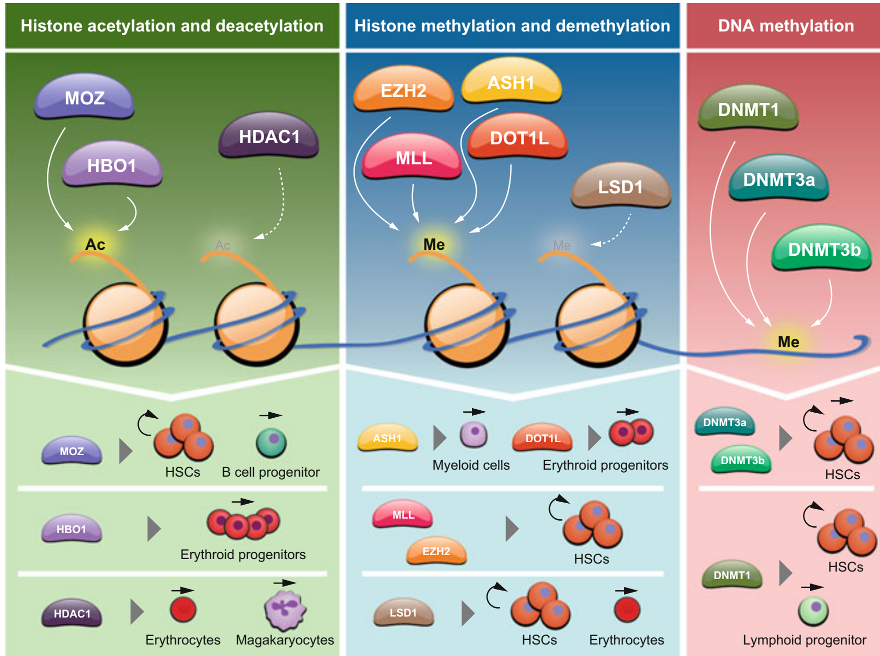

Fig. 4.2 Epigenetic modulators of haematopoietic development. Histone and DNA-modifying enzymes tightly control the proliferation and differentiation of haematopoietic cells. Histone acetylation: while MOZ acetylates lysine (K) residues of histone tails to promote haematopoietic stem cells (HSCs) proliferation and lymphoid differentiation, HBO1-mediated acetylation is required for erythroid progenitor differentiation; on the other hand, HDAC1 removes acetyl groups from histone tails and regulates erythroid and megakaryocyte differentiation. Histone methylation: ASH1 and DOT1L catalyse the methylation of histone 3 (H3) K36 and K79 and are involved in myeloid and erythroid progenitor differentiation, respectively; EZH2 and MLL methylate H3K27 and H3K4, respectively, and play crucial roles in HSCs proliferation; LSD1 removes methyl groups from H3K4 and H3K9 and it has been associated with HSCs proliferation and erythroid differentiation. DNA methylation: DNMT enzymes are responsible for the cytosine methylation in CpG islands of DNA; whilst DNMT3a and DNMT3b have been implicated in HSCs proliferation and differentiation, DNMT1 regulates HSCs proliferation and the differentiation of lymphoid cells. *White solid* and *dashed arrows* indicate addition and removal of modification groups, respectively; *black straight* and *circular arrows* indicate differentiation and proliferation of haematopoietic cells, respectively

embryonic lethality (Thomas et al. 2006; Katsumoto et al. 2008). A more specific abrogation of the HAT activity of MOZ (*MOZ*$^{HAT-/-}$) indicated that the HAT activity of MOZ is critical for HSCs function (Perez-Campo et al. 2009). *MOZ*$^{HAT-/-}$ embryos also display defects in the numbers and functionality of HSCs and committed progenitors as well as lower numbers of immature B cells (Perez-Campo et al. 2009). This phenotype is a direct consequence of the inability of HSCs and progenitor cells to proliferate, suggesting that MOZ-driven acetylation controls the appropriate balance between proliferation and differentiation in HSCs and progenitor cells. Beside this function, MOZ also acts as a co-activator for

several transcription factors with haematopoietic specificity, such as RUNX1 (Kitabayashi et al. 2001; Holbert et al. 2007), MLL1 (Paggetti et al. 2010) and PU.1 (Katsumoto et al. 2006), further highlighting the relevance of this HAT in the control of haematopoiesis (Perez-Campo et al. 2013).

Another member of the MYST family of HATs, HBO1 (HAT Bound to ORC 1, MYST2 or KAT7), has a key role in the regulation of genes responsible for embryonic patterning and foetal erythropoiesis (Kueh et al. 2011). Similarly to MOZ, HBO1 is also able to form a complex with a bromodomain-containing protein, BRD1. Highlighting the importance of these bromodomain-containing proteins for the activity of HAT complexes, BRD1 null embryos, also displayed an important defect in foetal liver erythropoiesis, similar to that of embryos lacking the HBO1 protein. This defect is due to the inability of HBO1 to bind to its target genes (such as GATA1, GATA2, SCL/TAL1, STAT5a and ETO2), as BRD1 is thought to act as a bridging protein between HBO1 and its activator protein ING4 (Mishima et al. 2011) to form an active HAT complex. In the absence of BRD1, the complex is not functional, resulting in a substantial decrease in H3K14 acetylation levels at target promoters and the subsequent defects in foetal liver haematopoiesis.

Histone deacetylases (HDACs) are in charge of reversing the activity of HAT proteins (Yang and Seto 2008). In mammals, there are four groups of HDACs based on sequence and domain similarities. Of those, group I (HDACs1, 2, 3 and 8) encompasses proteins involved in the growth and differentiation of mammalian cells (Lagger et al. 2002). The expression of group I HDACs is very low in haematopoietic progenitor cells, but is induced in more differentiated progenitors and then either down-regulated during myeloid differentiation or retained during erythroid and megakaryocytic differentiation (Wada et al. 2009). Transcription of HDAC1 has been shown to be regulated by haematopoietic transcription factors. Indeed, GATA1 mediates the transcription of HDAC1 driving the differentiation of myeloid progenitors into erythroid–megakaryocytic lineages. In contrast, when HDAC1 transcription is down-regulated by members of the C/EBP transcription factors, myeloid progenitors differentiate into myeloid cells (Wada et al. 2009). Therefore, HDAC1 is implicated in early cell fate decisions during haematopoiesis. Due to the negative role of HDAC1 on myeloid differentiation, inhibitors of HDACs have been used, in conjunction with other agents, with good results to treat certain haematological disorders (Quintas-Cardama et al. 2011).

4.3.1.2 Histone Methyltransferases and Histone Demethylases

Histone methylation can take place on both lysine (K) and arginine (R) residues (Zhang and Reinberg 2001; Martin and Zhang 2005). Two groups of Histone Methyltransferases (HMTs) with opposing activities have a crucial role in the regulation of haematopoiesis and HSCs proliferation. One of them, the polycomb family (PcG) carries out the methylation at lysine 27 of histone H3 (H3K27), a mark linked to gene silencing, whereas the other group, the Trithorax (TrxG)

methylates H3K4, a mark related to gene activation. These two groups of proteins regulate the expression of HOX genes among other targets.

EZH2 (Enhancer of Zeste homolog 2), a component of the Polycomb Repressor Complex 2 (PRC2), catalyses the di- and tri-methylation of H3K27. This modification is then bound by the Polycomb Repressor Complex 1 (PRC1), resulting in the transcriptional repression of the targeted genes. Ezh2 is essential for foetal liver HSCs proliferation and erythropoiesis, whereas its role in adult HSCs seems less important (Mochizuki-Kashio et al. 2011). Conversely, it has been shown that the forced expression of EZH2 enhances the number and proliferative potential of HSCs (Herrera-Merchan et al. 2012). A more detailed description of polycomb function in adult haematopoiesis and development can be found in the following Chaps. 5 and 6.

With the exception of MLL (Mixed Lineage Leukaemia) proteins, little is known about the role of TrxG genes in haematopoiesis. MLL plays a crucial role in the proliferation and differentiation of haematopoietic progenitors and maintains appropriate expression level of genes such as HOXA7 and HOXA9 during embryonic development. It has been proposed that the role of MLL in maintaining rather than in initiating HOX genes expression could be related to the fact that binding of MLL to specific clusters of CpG residues within HOXA9 locus protects this area from DNA methylation and subsequent silencing (Erfurth et al. 2008). Besides its role as a regulator of HOX genes expression, MLL can interact with other proteins with important roles in haematopoiesis, such as RUNX1 (Huang et al. 2011) or MOZ (Paggetti et al. 2010). Another member of the TrxG family, MLL5, has been shown to have an important role in haematopoiesis and HSC self-renewal (Heuser et al. 2009; Madan et al. 2009). In MLL5 null mice, HSCs display multiple haematopoietic defects, such as impairment in neutrophil function and in erythropoiesis, but more importantly in competitive repopulation capacity. Interestingly, HSCs from MLL5 null mice have a dramatically increased sensitivity to DNA demethylation-induced differentiation with 5 azadeoxycytidine indicating that MLL5 could be implicated in the regulation of HSC proliferation by a mechanism that involves DNA methylation (Heuser et al. 2009). For more details about MLL function in haematopoiesis, see the Chap. 7.

ASH1 (Absent small and homeotic disks protein 1 homologue) is a unique HMT that catalyses the methylation of H3K36 (Tanaka et al. 2007; An et al. 2011; Yuan et al. 2011). ASH1 is specifically expressed in HSCs in the bone marrow (Sung et al. 2006) and undifferentiated precursors of T cells in the thymus (Tanaka et al. 2008). ASH1 was recently shown to regulate HOX gene transcription synergizing with MLL, although this regulator activity seems to be independent of the HMTs catalytic domain (Tanaka et al. 2011). Knockdown of ASH1 in murine HSCs results in decreased number of macrophages and granulocytes, a phenotype similar to that induced by loss of MLL1 function indicating that this protein is a key epigenetic regulator of normal haematopoiesis.

DOT1L (disruptor of telomere silencing 1-like or KMT4) was shown to specifically methylate H3K79 as knockdown of this protein in mice results in a total loss of H3K79 methylation (Jones et al. 2008). Dot1L null mutant mice die between

E10.5 and E13.5 with severe and selective defects in erythroid, but not myeloid, differentiation (Feng et al. 2010). DOT1L methylation is a critical regulator of GATA2 and PU.1 transcription; mice lacking this HMT show an increase in PU.1 levels together with a decrease in GATA2 levels. Therefore, DOTL1 has a key role in early haematopoiesis, controlling the numbers of erythroid and myeloid cells (Feng et al. 2010). The role for DOT1L in erythropoiesis is also supported by a previous report showing that H3K79 methylation is enriched at the promoter of the β-globin locus (Im et al. 2003). DOT1L also seems to play a role in the development of leukaemia harbouring translocations of the MLL gene, as targets of the MLL-AF9 translocation display an alteration of their histone methylation pattern, specifically affecting the dimethylation of H3K79 (Bernt et al. 2011).

Histone lysine methylation is a dynamic process also regulated by the action of histone demethylases (HDMTs). LSD1 (Lysine-specific demethylase 1) which catalyses the demethylation of both H3K4 and H3K9, was the first HDMTs to be identified. Depletion of LSD1 in mice not only exacerbates the proliferation of HSCs and progenitor cells but also leads to severe defects in the differentiation of erythroid cells (Sprussel et al. 2012). LSD1 has also been shown to cooperate with the transcriptional repressor GFI1 to regulate differentiation of diverse haematopoietic lineages (Saleque et al. 2007).

4.3.2 DNA Methylation

DNA methylation consists in the addition of a methyl group to cytosines that precede guanines (CpGs). This process is catalysed by DNA methyltransferases (DNMTs) using S-adenosyl-methionine as donor of methyl groups (Hermann et al. 2004). A recent study showed that DNA methylation was extensively reprogrammed during early development in mammals with important changes taking place in the transition from the blastocyst to the post-implantation epiblast (Borgel et al. 2010). De novo methylation in the epiblast would be targeted to lineage-specific haematopoietic genes (such as Pou2af1, a gene that encodes a transcriptional co-activator involved in B-cell development, or Cytip, a gene expressed in leukocytes, among others). The promoters of these lineage-specific genes would be subsequently de-methylated during terminal differentiation. Both DNMT3a and DNMT3b are considered to be responsible for "de novo" methylation as they act preferably on un-methylated DNA substrates (Jaenisch and Bird 2003). DNMT3a and DNMT3b show different expression patterns and targets (Jaenisch and Bird 2003) and DNMT3b seems to be the main enzyme required for promoter methylation during implantation (Borgel et al. 2010). Initial studies on DNMT3a indicated that *Dnmt3a Dnmt3b* double-deficient HSCs, but not *Dnmt3a* or *Dnmt3b* single-deficient HSCs, were incapable of long-term reconstitution in transplantation assays (Tadokoro et al. 2007). However, in contrast to these studies where *Dnmt3a*-null HSCs contributed normally to haematopoiesis, the use of a conditional knock-out model to study the functions of DNMT3a (Challen et al. 2012) revealed that this

protein is required to silence the HSCs self-renewal programme, thus permitting haematopoietic differentiation.

The importance of DNA methylation during haematopoiesis was initially suggested by gene deletion studies targeting *Dnmt1*. DNMT1 reproduces cytosine methylation patterns from a hemi-methylated substrate after DNA replication, and therefore is considered responsible for the maintenance and propagation of the methylation pattern (Jaenisch and Bird 2003). The ablation of DNMT1 expression in HSCs resulted in these cells undergoing apoptosis, whereas the expression of a hypomorphic allele lead to reduced repopulation capacity and decreased production of lymphoid progenitors while the development of myelo-erythroid progenitors remained normal (Broske et al. 2009). There is also evidence that DNMT1 interacts with GATA1, GFI1 and ZBP-89 in mouse erythro-leukemic cells (MEL).

Recent studies analysing DNA methylation during HSCs ontogeny have shown that, although overall DNA methylation landscape is essentially maintained during this process, the largest number of genes undergoing changes in their methylation pattern were highly expressed in downstream progenitors but not in HSCs. Indeed, only a few genes expressed in HSCs were differentially methylated (Beerman et al. 2013). These latest results suggest that DNA methylation in HSCs regulates the expression of genes that are activated during the differentiation of HSCs to defined lineages.

4.4 Concluding Remarks

The molecular and cellular control of haematopoietic development is an intricate process regulated at multiple levels. In this chapter, we have discussed the cellular complexity of haematopoietic specification, the transcriptional control regulating key steps of this differentiation process and how transcription factors may integrate into wider regulatory networks. To date, little is known about the epigenetic regulation of embryonic haematopoiesis and our understanding is mostly limited to the description of the phenotypes observed upon gene deletion of specific chromatin modifier proteins. Furthermore, the inaccessibility of the developing embryo and the low number of cells undergoing haematopoietic specification in these embryos hamper the biochemical study of the complexes of transcription factors and epigenetic regulators orchestrating haematopoietic development and maintenance. Devising novel technologies and experimental strategies will allow us to further push the boundaries to decipher the chromatin landscape during blood cell formation. By integrating together these multiple layers of regulation, we will further our understanding of haematopoietic specification. Ultimately, for a complete picture, this knowledge will have to be generated at the single cell level and linked to the extrinsic signals provided by the micro-environment which guides and instructs the developing blood cells.

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