HOX genes in normal, engineered and malignant hematopoiesis

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ABSTRACT  Advanced technologies and models systems are improving our understanding of developmental processes. A primary example, hematopoiesis, classically represented by a hierarchical tree with a stem cell at the apex and more lineage restricted cells following each bifurcation has recently been shown to be capable of more adaptable fate decisions. Future research will identify key molecules underpinning this more adaptable or continuous model of hematopoiesis potentially leading to improved engineering of blood cells and therapies for malignant disease. The spatio-temporal, cell specific and exquisite reliance on gene dosage attributed to the HOX family promoted them as candidate master regulators of hierarchical hematopoiesis. Recent discoveries in the need to stimulate or retain HOX expression during engineered human hematopoiesis, supported by similar studies in mice and other developmental models, reinforces their importance at the single cell level. Likewise, dysregulation of HOX in single cells can result in blood cancers such as leukemia. It will be of interest to see what additional roles HOX family members and their regulators including morphogens, epigenetic modifiers and noncoding RNAs play in this evolving field and if these master regulators can be further harnessed for clinical benefit.

KEY WORDS: HOX, hematopoiesis, stem cell, acute myeloid leukemia

Hematopoietic HOX genes

Mammalian Hox genes are a family of 39 homeodomain-containing transcription factors, organised into four distinct clusters: Hoxa, Hoxb, Hoxc and Hoxd. With the exception of the Hoxd cluster, Hox genes have key roles in hematopoiesis, particularly in regulating primitive hematopoietic cells (Thorsteinsdottir et al., 1997; Argiropoulos and Humphries, 2007). During hematopoiesis, Hox genes are mostly expressed in CD34+, hematopoietic stem cell (HSC)-enriched, populations and downregulated upon differentiation or lineage commitment (Sauvageau et al., 1994; Pineault et al., 2002).

Gain-of-function

Due to their high homology, overexpression studies in mouse have been useful in further elucidating the role of individual Hox genes in hematopoiesis. Overexpression of HOXB3 in mouse bone marrow cells resulted in impairment of T and B lymphocyte development and excessive myeloid proliferation in transplanted mice (Sauvageau et al., 1997). HOXB4 overexpression in murine bone marrow cells had a profound effect on HSC proliferation, enhancing HSC repopulating ability both in vitro and in vivo (Sauvageau et al., 1995). A similar positive regulation of hematopoietic cell growth was also seen in human cord blood stem cells, where constitutive expression of HOXB4 led to increases in CD34+ stem cell number (Buske et al., 2002). Similar to the studies of HOXB

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; AML, acute myeloid leukemia; BMI-1, B cell-specific Moloney murine leukemia virus integration site 1; BMP, bone morphogenetic protein; CDX, caudal type homeobox; EHT, endothelial-to-hematopoietic transition; ERG, ETS-related gene; ESC, embryonic stem cell; FGF, fibroblast growth factor; FLT3, FMS-like tyrosine kinase 3; HEP, hemogenic endothelium progenitor; HOX, class I homeobox; hPSC, human pluripotent stem cell; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; KDR, vascular endothelial growth factor receptor 2; LCOR, ligand dependent nuclear receptor corepressor; Meis1, myeloid ecotropic viral integration site 1; MLL/KDM5a, mixed-lineage leukaemia; MYB, myeloblastosis oncogene; NPM1, nucleophosmin-1; PBX, pre-B-cell leukemia homeobox; PRC, polycomb gene (PcG) repressor complex; PSC, pluripotent stem cell; RA, retinoic acid; RORA, retinoic acid receptor related orphan receptor A; RUNX, runt-related transcription factor; SOX, SRY-related HMG-box; SPI1, spleen focus forming virus (SFFV) proviral integration oncogene-1; TALE, three-amino-acid-looped-extension; Wnt, wingless and INT-1.

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cluster, transplantation of hoxa9-overexpressing mouse HSCs resulted in enhanced HSC self-renewal capacity and myeloipoiesis (Thorsteinsdottir et al., 2002). Overexpression of HOXA9 in human embryonic stem cells (ESCs) had a positive outcome, enhancing the generation of hemogenic endothelium progenitors and subsequently primitive and total blood cells (Ramos-Mejia et al., 2014). Whereas, ectopic expression of HOX5 and overexpression of HOXA10 in human CD34+ cord blood cells blocked erythroid differentiation and increased myeloipoiesis (Crooks et al., 1999; Buske et al., 2001).

**Loss-of-function**

While these overexpression studies indicate a key role for HOXA and HOXB cluster genes in regulating the activity of primitive hematopoietic cells, knockout studies, for the most part, do not support such a role. Despite its striking effect on HSC self-renewal in overexpression experiments, knockout of hoxb4 had no apparent effect on normal HSC activity. Moreover, knockout of the majority of the hoxb cluster, from hoxb1-b9, did not affect the repopulating activity of fetal liver cells or result in any hematopoietic defective phenotype, implying the Hox cluster is nonessential for early hematopoietic cell function (Bijl et al., 2006).

Functional redundancy within the Hox network may hamper such knockout studies and explain the lack of abnormal phenotypes. However, while knockout of most Hox genes have little effect on hematopoiesis, Hox9 knockdown produces the most profound effect, with disturbances in differentiation and HSC self-renewal (Alharbi et al., 2013). Hox9 knockout mice have depleted myeloid, erythroid and B cell progenitors in the bone marrow and a 30–40% reduction in leukocytes (Lawrence et al., 1997). HSCs obtained from hoxa9-/- mice have diminished repopulation ability in contrast to hoxa10-/- HSCs where repopulating activity was unaffected. (Lawrence et al., 2014). Additionally, the repopulating ability of HSCs derived from either hoxa9 or compound hoxa9/hoxb3/ hoxb4 null mice were similarly reduced (Magnusson et al., 2007).

Interestingly, deletion of the whole Hoxa cluster in mice reduced hematopoietic stem and progenitor cell (HSPC) proliferation in vitro and engraftment potential in vivo, however, HSPC activity could be partially restored to wild-type levels through overexpression of hoxa9 (Lebert-Ghali et al., 2016). These studies clearly demonstrate a role for Hoxa9 in regulating HSC function and in vivo engraftment.

**Embryonic hematopoietic development**

The in vitro differentiation of mouse embryonic stem cells (ESCs) to cells of the hematopoietic lineage has been largely parallel hematopoiesis in the developing mouse embryo (Keller, 1995). As such, recapitulation of this natural pathway using pluripotent stem cell (PSC) cultures is regarded as the best method to generate bona fide HSCs (Yoder, 2014). Hematopoiesis takes place in three distinct waves in the mouse embryo.

**Primitive hematopoiesis**

In the first wave, BMP4, FGF2, Wnt and Nodal signalling are crucial in patterning of the primitive streak and formation of early mesoderm. Migrating mesoderm from the early primitive streak, marked by co-expression of kinase inert domain-containing factor (KDR) and Brachyury, forms the yolk sac followed by blood islands and endothelium (Ferkowicz and Yoder, 2005; Ackermann et al., 2015). The yolk sac generates the first hematopoietic cells, primitive erythroid progenitors. These progenitors subsequently give rise to primitive erythroblasts, macrophages and megakaryocytes but do not generate HSCs or lymphoid cells. As such, the first wave is also described as ‘primitive hematopoiesis’ (Yoder, 2014). Hoxa9 and hoxc9 were identified as being highly expressed in the visceral yolk sac (E7.5) and subsequently dispersed within the yolk sac and embryo (E8.5) associated with insulin induction and primitive erythroblast formation (Mcgrath and Palis, 1997).

**Transient definitive hematopoiesis**

The second wave (E8.25–E10.5) is marked by the appearance of erythromyeloid progenitors from the hemogenic endothelium in the blood island capillaries of the yolk sac. Erythromyeloid progenitors initially develop as clusters of cells in the blood islands, which then detach and enter blood circulation (Yoder, 2014). Cells of the hemogenic endothelium are capable of generating B and T lymphoid progenitors (Böiers et al., 2013). Since erythromyeloid cells have multi-lineage differentiation potential, the second wave is considered ‘transient definitive hematopoiesis’, distinguished by specific globin expression in their progeny (McGrath and Palis, 2005). However, these transient cells lack self-renewal capacity and lymphoid potential (Kyba and Daley, 2003; Mikkola, 2006).

**Definitive hematopoiesis**

The third wave is considered ‘definitive hematopoiesis’ as it gives rise to definitive HSCs, capable of long-term repopulation and generating all hematopoietic cell types. HSCs arise from a subset of specialised hemogenic endothelial cells in the dorsal aorta of the aorta-gonad-mesonephros (AGM) through an endothelial-to-hematopoietic transition (EHT) (Ackermann et al., 2015). In the process of EHT, the hemogenic endothelium loses its endothelial potential and undergoes a commitment to the hematopoietic lineage (Swiers et al., 2013). Following specification from the hemogenic endothelium, HSCs migrate out of the dorsal aorta and eventually colonise the bone marrow. Hox co-factor Meis1 and mixed-lineage leukaemia (Mll, also known as KMT2A), a histone methyltransferase that regulates HOX genes expression through methylation of histone 3 lysine 4 residues on HOX promoters, are both expressed in the AGM and are essential for definitive hematopoiesis (Ernst et al., 2004; Azcoitia et al., 2005).

**Hematopoietic differentiation of pluripotent stem cells**

Most in vitro hematopoietic differentiation protocols are based on mimicking the distinct signalling cascades which occur during hematopoiesis in vivo. BMP4, FGF2 and Wnt are known to be crucial factors in the generation of early hematopoietic progenitors during embryonic development and are also essential in initiating in vitro differentiation to hematopoietic lineage (Chadwick et al., 2003; Wang and Nakayama, 2009). As definitive hematopoietic cells arise from the hemogenic endothelium, the hemogenic endothelium has also been used as a source of definitive hematopoietic cells. Choi et al., (2012) identified hemogenic endothelium progenitors (HEPs) capable of forming definitive hematopoietic cells could be characterised by expression of VE-cadherin and lack of CD73, CD43 and CD235a. RUNX1 isoform c (RUNX1c) was similarly identified as necessary in specification of human pluripotent stem cell (hPSC)-derived HEPs. Deletion of RUNX1c did not impact the generation of HEPs from hPSCs but greatly impaired the generation
of CD45+ blood cells from HEPS (Navarro-Montero et al., 2017). This is in accordance with embryonic development where Runx1 marks HSC emergence (North et al., 2002).

**PSC-derived hematopoietic differentiation**

While, these initial PSC-derived differentiation systems showed success in generating definitive hematopoietic cells, they are still incapable of generating multi-lineage cells with long-term engraftment potential, which are core features of bona fide HSCs. Contrary to embryonic development where primitive and definitive hematopoiesis occur in three distinct waves, in PSC-derived differentiation systems these phases occur simultaneously (Ackermann et al., 2015). As both primitive and definitive systems result in the generation of CD34+ hematopoietic cells, differentiated cells cannot be distinguished based solely on CD34 positivity. However, the two programs can be distinguished based on functional end-point analyses, such as T lymphocyte formation ability, and based on responses during the differentiation process. Activin/Nodal signalling stimulates primitive hematopoiesis, therefore inhibitors of this pathway are used to initiate definitive hematopoiesis (Kennedy et al., 2012). CD235a is a marker of primitive hematopoiesis that appears to provide a means of identifying and thus discriminating the two stages. Selecting for KDR-CD235a hemogenic endothelial precursors along with Wnt signalling activation led to generation of CD45+ hematopoietic cells capable of producing primitive T lymphocytes and erythroid and myeloid cells in colony-forming unit (CFU) assays (Sturgeon et al., 2014).

**BMP/Wnt-Cdx-Hox axis**

Generation of long-term repopulating HSCs remains a major challenge for hPSC-based hematopoietic differentiation systems. Hox genes are key regulators of embryonic hematopoiesis and are implicated in HSC self-renewal. As such, induction of Hox signalling in differentiating cultures may activate key pathways responsible for development of HSCs. The caudal-type homeobox (Cdx) gene family, consisting of Cdx1-4, are upstream regulators of HOX which regulate hematopoiesis in zebrafish, mice and humans (Rawat et al., 2012). Deletion of Cdx2 in mouse ESCs compromised the formation of embryonic hematopoietic progenitors and resulted in aberrant expression of posterior or S’ Hoxa genes. Hox gene expression levels were restored by ectopic expression of Cdx4, demonstrating Hox expression is initiated and closely regulated by Cdx genes (Wang et al., 2008). Moreover, in a follow up to Sturgeon’s study, upregulation of CDX1, CDX2 and CDX4 was observed exclusively in Wnt-dependent KDR-CD235a definitive hematopoietic mesoderm populations. Temporal analysis revealed increased CDX4 expression coincided with addition of Wnt agonist CHIR99021 to the cultures. Overexpression of CDX4 in differentiating cultures yielded the same CD34+CD43 CD73 CD184 hemogenic endothelium population as Wnt activation, suggesting Wnt mediates definitive hematopoietic specification through activation of CDX4 (Creamer et al., 2017). This approach may have recapitulated a conserved BMP/Wnt-Cdx-Hox axis first identified in parallel studies performed in zebrafish embryos and murine ESCs (reviewed by Lengerke and Daley, 2012).

**Acquisition of a HOXA signature**

Recently, Ng et al., (2016) reported that acquisition of a HOXA signature during lineage specification of hPSCs may underlie the potential of subsequent hematopoietic stem and progenitor cells (HSPCs) to engraft and repopulate recipients long-term. The transcriptional profiles of hESC-derived CD34+ cells incapable of long-term engraftment were compared with long-term repopulating cord blood-derived CD34+ cells. HOXA cluster genes were identified among the genes significantly downregulated in hESC-derived CD34+ cells. In order to induce HOXA expression, hESC-derived CD34+ cells were treated with Activin inhibitor SB431542 and Wnt agonist CHIR99021, resulting in upregulation of HOXA5, HOXA9 and HOXA10 expression. Aorta-like SOX17+ cells resembling definitive hematopoietic cells of the AGM with myeloid and erythroid differentiation potential were also generated by the SB431542/CHIR99021 treated cultures. While these SOX17+ cells did not show repopulating activity, this study demonstrated that HOXA genes not only play a role in regulating definitive hematopoiesis but that acquisition of a HOXA signature signifies specification of the AGM.

Stimulating HOXA gene expression during AGM development may therefore provide a means of generating self-renewing HSCs from PSCs. HOXA genes, in particular HOXA5 and HOXA7, were found to be highly expressed in fetal liver-derived HSPCs while suppressed in hESC-derived HSPCs which lacked repopulation activity. Overexpression of HOXA5 and HOXA7 in hESC-derived CD34+ cells did not enhance HSPC expansion in vitro, however, activation of retinoic acid (RA) signalling during EHT generated HSPCs with enhanced proliferation and also induced expression of HOXA4 genes. Thus, acquisition of a HOXA signature is developmental stage specific, depends on the presence of HOXA regulatory factors and acts in parallel with other pathways (Dou et al., 2016).

**Wnt and RA signalling**

RA has previously been demonstrated to promote HSC development and is essential for development of hemogenic endothelium in mouse embryos (Goldie et al., 2008). In AGM-derived hemogenic endothelium cultures activation of RA signalling promoted HSC development through downregulation of the Wnt/beta-catenin pathway (Chanda et al., 2013). However, Ng et al., (2016) and Sturgeon et al., (2014) both utilised Wnt signalling activation to govern specification of the hemogenic endothelium in PSC-based studies. Thus the requirement for Wnt signalling is evidently stage-dependent.

**Transcription factors**

In an alternative transcription factor-driven approach to hematopoietic differentiation, Doulatov et al., (2013) demonstrated transfection of hPSC-derived CD34+CD45+ hematopoietic progenitors with five transcription factors (HOXA9, ERG, RORA, SOX4 and MYB) produced hematopoietic cells capable of short-term myeloid and erythroid engraftment in vivo. Following on from this study, Sugimura et al., (2017) achieved long-term myeloid, B and T cell engraftment of HSPCs generated from hemogenic endothelium transfected with seven transcription factors (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1 and SPI1). Both of these papers demonstrate the importance of HOXA genes, in particular that of HOXA9, in generating HSPCs with long-term multi-lineage repopulating abilities. Furthermore, HOXA9 occupies the promoters of ERG, MYB, SOX4 and SPI1 (Huang et al., 2012), suggesting it is a crucial factor in obtaining repopulating activity.

As models have become more defined our understanding of the molecular basis for HSPC production has improved (Fig. 1.) Although further follow-up analyses are required, recent approaches
by the Daley (Sugimura et al., 2017) and Elefanty (Ng et al., 2016) laboratories along with studies on Cdx-Hox expression (Rawat et al., 2012) provide strong support that acquirement of a HOXA signature is a key process in definitive hematopoiesis. Interestingly, acquisition and retention of a HOXA signature also appears to be key in malignant hematopoiesis.

**HOX genes in acute myeloid leukemia**

HOX genes are frequently dysregulated in leukemias. In acute myeloid leukemia (AML), HOX genes have been shown to induce or promote AML by forming oncogenic fusion proteins or collaborating with other AML-inducing mutations (Alharbi et al., 2013). Uprogation of HOX genes and their co-factors, such as MEIS1, is associated with an unfavourable outcome in AML (Andreff et al., 2008). In particular, expression of HOXA9 was found to be the main determinant of poor prognosis in a cohort of AML patients (Golub et al., 2008). In particular, expression of HOXAXA9 is overexpressed by 2 – 8 fold in approximately 50% of all AML cases (Li et al., 2013; Collins et al., 2014). As HOXA9 has many downstream targets, which also confer poor prognosis in AML, it is unclear how central a role HOXA9 plays in the outcome of the disease phenotype (Collins and Hess, 2016). In terms of a direct role of HOXA9 in leukemogenesis, HOXA9 expression alone does not appear to be sufficient. Hoxa9 overexpression failed to transform mouse bone marrow cells, however, co-overexpression with three-amino-acid-looped-extension (TALE) co-factor genes MEIS1 or PBX3 produced rapid leukemic transformation (Li et al., 2016). In fact, HOXA9 and MEIS1 are frequently co-expressed in myeloid leukemias (Lawrence et al., 1999). Therefore, it appears Hoxa9 does not solely induce leukemogenesis but requires other collaborating factors, such as Meis1 or fusion to nucleopomin 98 (NUP98).

**NUP98-fusion proteins**

HOX fusions with NUP98 were first reported to be involved in leukemia with the identification of NUP98-HOXA9 fusion gene in AML patients. Subsequently, 28 other distinct fusion partner genes were identified in patients with leukemia, including six from the HOX family, demonstrating a direct link between HOX genes and leukemia (Gough et al., 2011). The most commonly occurring NUP98 fusion, NUP98-HOXA9, was shown to be directly involved in the pathogenesis of leukemia, producing a myeloid-proliferative disease, which progressed into AML in mice transplanted with NUP98-HOXA9-transduced bone marrow cells (Kroon, 2001). 5' HOX genes, including HOXA11, HOXA13, HOXC13 and HOXD13, have all been identified as fusion partners with NUP98 (Gough et al., 2011), suggesting the ability of NUP98-HOX fusions to form leukemia is restricted to posterior HOX genes. Using novel NUP98-

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**Fig. 1. Methods of pluripotent stem cell differentiation to hematopoietic stem and progenitor cells (HSPCs).** Summary of key differentiation methods from the literature are outlined, with illustrations focusing on the most important features of each differentiation method. The hematopoietic potential and self-renewal capacity of the resulting HSPCs from each method is also highlighted. Abbreviations: CFU, colony-forming unit; EB, embryoid body; EHT, endothelial-to-hematopoietic transition; hESC, human embryonic stem cell; HSPC, hematopoietic stem and progenitor cell; iPSC, induced pluripotent stem cell; NSG, NOD/LtSz-scidIL2Rnull; PSC, pluripotent stem cell.
HOX fusions not detected in humans, leukemia was induced in murine transplant models with HOXA10 and HOXB3 as fusion partners, but not HOXB4 (Pineault et al., 2004). Interestingly, co-expression of the Hox co-factor Meis1 with all NUP98-HOX fusions tested accelerated the development of AML. This indicates all HOX genes, not just 5’ HOX genes, possess an intrinsic ability to become leukemogenic (Kroon, 2001; Pineault et al., 2004).

**MLL-fusion proteins**

MLL rearrangements are found in over 70% of infant leukemias and approximately 10% of adult and therapy-related AMLs. They are formed by gross chromosomal translocations at the 11q23 locus, producing fusion genes which comprise of the N-terminus of MLL fused to the C-terminal of its fusion partner gene (Krivtsov and Armstrong, 2007). MLL has over 88 different fusion partners, however, over 80% of MLL fusion genes result from translocation with AF4, AF9, ENL, AF10, ELL or AF6 (Meyer et al., 2017). A partial tandem duplication (PTD) in the N-terminus of MLL can also take place, occurring in approximately 12% of AML cases (Basecke et al., 2006; Meyer et al., 2017). Aberrant HOX gene expression is implicated in all MLL-rearranged myeloid and lymphoblastic leukemias (Armstrong et al., 2002). In particular, upregulation of HOXA9 and MEIS1 solely in MLL-rearranged subtype of leukemias suggests they are directly involved in MLL-induced leukemogenesis (Yeoh et al., 2002).

As MLL regulates HOX genes expression, dysregulated HOX expression in response to MLL fusion genes is expected. Interestingly, the SET domain responsible for MLL methylation activity is lost during translocation. However, fusion partners AF4, AF9, AF10 and ENL all coordinate with histone methyltransferase DOT1L, an activity which is maintained following translocation. Therefore, as some MLL fusion proteins are chromatin modifiers themselves, it is proposed that MLL fusion proteins mediate their effects on HOX expression via DOT1L (Krivtsov and Armstrong, 2007; Slayny, 2009).

Several studies in mice and immortalised leukemic cell lines have indicated the acquisition and retention of a 5’ Hoxa signature may be a requirement in MLL-mediated leukemogenesis (Horton et al., 2005). Mouse primary myeloid progenitor lines immortalized with MLL oncogenes (MLL-ENL, MLL-AF6, MLL-GBP, MLL-ELL and MLL-AF10) displayed a 5’ Hoxa profile, whereby 5’ Hoxa genes, including Hoxa7 and Hoxa9, were expressed in all lines while 3’ Hoxa genes were expressed less regularly. Moreover, in contrast to wild-type cells, Hoxa9+/− bone marrow cells transduced with MLL-ENL displayed severely impaired replating ability and failed to generate leukemia following transplantation into mice. This indicates a crucial role for Hoxa9 both in maintenance and initiation of MLL-ENL-mediated leukemogenesis (Ayton and Cleary, 2003). Contrary to this finding, Kumar et al., (2004) observed no deficiency in leukemia initiation or latency in Hoxa9−/− mice following knock-in of oncogene MLL-AF9. The leukemogenic potential of Hoxa9−/− mice was equivalent to wild-type, although mice deficient in Hoxa9 showed a more immature myeloid phenotype. However, elevated 5’ Hoxa levels was observed in all mice, consistent with a role of Hox genes in MLL-mediated leukemia. Mice transplanted with Hoxa9-deficient human MLL-rearranged SEMK2 cells also exhibit reduced leukemia burden, implicating Hoxa9 in leukemia survival in vivo (Faber et al., 2009). The differences in outcomes from these experimental models may reflect a combination of the complexity in HOX regulation with cell-of-origin and cellular context. Whether HOXA9 or other 5’ HOX genes are essential for all MLL-rearranged leukemogenesis or not, they do appear to play a significant role in initiation and maintenance of the disease phenotype.

**NPM1**

Nucleophosmin-1 (NPM1) is a ubiquitous nuclear chaperone protein that shuttles between the cytoplasm, nucleoplasm and nucleolus. Translocations within the reading frame at the C-terminus of NPM1 impair the NPM1 protein’s nuclear shuttling abilities and result in accumulation of NPM1 in the cytoplasm. Mutations in NPM1 occur in approximately 35% of adult AML cases (Falini et al., 2009). NPM1-mediated AML also exhibits a HOX signature, though distinct from that of MLL-mediated AML. Gene expression analysis revealed elevated HOXA9, HOXA10, HOXB2, HOXB6 and MEIS1 levels in pediatric AML patients. However, a comparison of pediatric NPM1-mediated and MLL-rearranged leukemias showed an upregulation of HOXB genes, in particular HOXB2 and HOXB6, exclusively in NPM1-mutated AML (Mullighan et al., 2007). In a different study, a similar signature was observed in adult AML patients, with elevated levels of HOXA and HOXB genes, as well as co-factors MEIS1 and PBX3 (Verhaak et al., 2005).

**FLT3 and MEIS1**

Mutations in the FMS-like tyrosine kinase 3 (FLT3) gene are the most frequent genetic aberration seen in AML, occurring in approximately 25 – 45% of patients. The most common mutation, present in 15-35% of AML cases, is an internal tandem duplication (ITD) caused by a duplication of the juxtamembrane domain, while the second most common mutation is a missense point mutation in exon 20 of the tyrosine kinase domain, which occurs in 5 – 10% of AML patients. FLT3 ligand is expressed by most hematopoietic organs, while the FLT3 receptor is predominantly expressed in primitive myeloid and lymphoid progenitors (Stirewalt and Radich, 2003). FLT3 has been proposed to play a role in adult HSC self-renewal. Expression of FLT3 on human CD34+ cord blood cells is necessary for in vivo myeloid and lymphoid reconstitution (Sitnicka et al., 2003). Interestingly, although IT3 was originally implicated in self-renewal of the mouse HSC pool, upregulation of it3 in HSC-enriched mouse bone marrow fractions was subsequently found to reduce HSC self-renewal capabilities (Adolfsson et al., 2001).

High HOX expression in AML patient samples is correlated with the presence of NPM1 mutations (Verhaak et al., 2005) and also with elevated levels of FLT3 (Roche et al., 2004). Additionally, NPM1-mutated leukemias themselves are strongly associated with higher frequency of FLT3 mutations (Alcalay et al., 2005). In mice studies, Npm1c/Flt3-ITD transgenic mice rapidly developed leukemia in contrast to Npm1c knock-in mice where a significantly longer latency was observed, signalling collaboration between mutant Npm1 and Flt3-ITD proteins in leukemogenesis (Vassiliou et al., 2011; Mupo et al., 2013). Similarly, wild-type Flt3 co-ordinated with NUP98-HOX fusions in co-transduced bone marrow cells to initiate an aggressive AML upon transplantation to mice. In addition, retrofit transduction of pre-leukemic NUP98-HOX myeloid lines with Meis1 led to leukemic conversion and also a 5 – 7 fold increase in Flt3 protein levels (Palmqvist et al., 2012).

Meis1 is widely implicated in Hox-mediated leukemia. Meis1 itself has no leukemogenic activity, however, Meis1 expression was necessary to transform bone marrow cell overexpressing Hoxa9 (Kroon et al., 2004).
Regulation of hematopoietic HOX genes

As for other gene families, our understanding of the regulation of HOX genes has been improved recently by advanced technologies such as high throughput sequencing. Along with confirmation of upstream molecules including WNT, RA and CDX (above) cis-factors, trans-factors and epigenetic modifiers have been identified that help untangle some of the complexity associated with HOX expression.

Topology

Integrative epigenomic analysis, including development of assays for transposase-accessible chromatin using sequencing (ATAC-seq, Buenrostro et al., 2013) and chromosome conformation capture-based (HiC) approaches (reviewed by Denker and De Laat, 2016), have been used to map gene clusters. Pioneered by the Dubuole laboratory, the differential topology, chromatin state and gene-enhancer contacts of Hox loci have been identified during embryonic development (between E8.5 and E12.5). As predicted, the chromatin structure is highly dynamic during cellular expression of HOX genes. Initiation of transcription resulted in a switch from a single to a bimodal 3D organization whereby newly activated HOX genes progressively clustered into a transcriptionally active compartment (Noordermeer et al., 2011). This spatio-temporal organization coincided with active chromatin marks including H3K4me3 that may underpin collinear expression of HOX clusters. It was later discovered that HoxA and HoxD clusters lie at the junction of two topologically associating domains, which may also help explain the bimodal expression of these clusters during development (Dixon et al., 2012).

Noncoding RNAs

Long noncoding RNA transcripts (lncRNAs) affect diverse biological processes through regulation of mRNA stability, RNA splicing, chromatin structure, and sequestration of regulatory molecules including DNA, protein and micro-RNA (miR). Mechanisms of action of lncRNAs which are associated with cell fate decisions in normal and malignant hematopoiesis may be therapeutically targetable (reviewed by Alvarez-Dominguez and Lodish, 2017).

Transcription of lncRNAs within HOX gene clusters (Rinn et al., 2007), along with key non-coding miRs, is now well described with functional significance attributed (reviewed by Kumar and Krumlauf, 2007), along with key non-coding miRs, is now well described with functional significance attributed (reviewed by Kumar and Krumlauf, 2007). Interestingly, these lncRNAs affect gene regulation (positively and negatively) both in cis and in trans on a wide range of Hox and non-Hox genes. Alternative start sites, extensive RNA splicing and expression from either coding or non-coding strands results in multiple isoforms of these elements. Comparative analysis between mouse and human loci indicates more noncoding transcripts (including miRs) within or flanking the HoxA cluster relative to other clusters (De Kumar and Krumlauf, 2016). Of particular interest here, an lncRNA embedded between HOXA1 and HOXA2 termed HOTAIR1, was initially identified as being myeloid-specific and upregulated during granulocyte differentiation (Zhang et al., 2009) when HOX genes are downregulated. In mouse, Hotairm1 and another isoform Hotairm2 are rapidly upregulated in the presence of RA during myelopoiesis and ESC differentiation (De Kumar et al., 2015).

Additional lncRNAs embedded within the hematopoietically active HOXA locus include HOTTIP which is located 330 bp upstream of HOXA13 and displays bivalent (H3K4me3 and H3K27me3) epigenetic marks (Wang et al., 2011). Hottip may modulate posterior HoxA gene expression by directly binding WDR5–MLL complexes.
Hematopoietic HOX genes

providing a means for localizing histone methyl transferase activity.

**Epigenetic modifiers**

The balance between self-renewal and differentiation of HSCs is regulated by epigenetic mechanisms. HOX genes that evolved from the homeotic selector genes (HOM-C) classically retain a reliance on the balanced regulation by multi-subunit complexes containing MLL (trithorax ortholog) and polycomb gene (PcG) repressor complex (PRC) proteins such as BMI-1 (B cell-specific Moloney murine leukemia virus integration site 1). PcGs were first identified as negative regulators of HOM-C genes in *Drosophila melanogaster* whilst trithorax was identified as a positive regulator maintaining HOM-C expression. As previously mentioned, MLL plays a major role in HOX expression in normal hematopoiesis and dysregulated MLL is associated with aggressive leukemia. Key roles for PRC components in hematopoiesis are also emerging but similar phenotypic outcomes in both gain- and loss-of-function studies demonstrate a significant degree of complexity (Vidal and Starowicz, 2017; Sashida and Iwama, 2017). The two main complexes PRC1 and PRC2 catalyze repressive histone modifications e.g. methylation of histone H3 at lysine-27 (H3K27me).

BMI-1 forms the core of the PRC1 complex and plays a significant role in HOX gene regulation. Knockdown of BMI-1 results in upregulation of HOX genes (Cao et al., 2005) and synergizes with the lysine acetyltransferase KAT6A (MOZ) in maintaining adult HSCs through altered quiescence and senescence (Sheikh et al., 2017). At least six mammalian PRC1 sub-complexes have been identified that have different subunit compositions. BCOR, a component of PRC1.1 was recently identified as a critical regulator of hematopoiesis by inhibiting myeloid cell proliferation and differentiation. Loss of BCOR resulted in upregulation of key hematopoietic HOX genes including HOXA5, HOXA7 and HOXA9 possibly by loss of promoter recruitment or regulation of H2A ubiquitination (Cao et al., 2016).

Histone modifications are closely linked with DNA methylation state. While MLL is best characterised as a histone methyltransferase, it also contains a DNA methyltransferase homology domain, CxxC, in its N-terminus which binds to unmethylated CpG residues (Slany, 2009). Mll CxxC domain binds to CpG clusters in the Hoxa9 locus, preventing DNA methylation. In the presence of MLL fusion proteins, MLL-AF4 and MLL-AF9, a subset of CpG residues continue to be protected, while the remainder become methylated. Furthermore, MLL fusions increase Hoxa9 expression regardless of whether CpGs became methylated or not (Erfurth et al., 2008), demonstrating other factors besides epigenetic dysregulation are responsible for Hoxa9 upregulation in leukemia. MLL also possesses a SET domain with H3K4 methylation activity in its C terminus. While MLL is best characterised as a histone methyltransferase, it also contains a DNA methyltransferase homology domain, CxxC, in its N-terminus which binds to unmethylated CpG residues (Slany, 2009). Mll CxxC domain binds to CpG clusters in the Hoxa9 locus, preventing DNA methylation. In the presence of MLL fusion proteins, MLL-AF4 and MLL-AF9, a subset of CpG residues continue to be protected, while the remainder become methylated. Furthermore, MLL fusions increase Hoxa9 expression regardless of whether CpGs became methylated or not (Erfurth et al., 2008), demonstrating other factors besides epigenetic dysregulation are responsible for Hoxa9 upregulation in leukemia. MLL also possesses a SET domain with H3K4 methylation activity in its C terminus. While MLL is best characterised as a histone methyltransferase, it also contains a DNA methyltransferase homology domain, CxxC, in its N-terminus which binds to unmethylated CpG residues (Slany, 2009). Mll CxxC domain binds to CpG clusters in the Hoxa9 locus, preventing DNA methylation. In the presence of MLL fusion proteins, MLL-AF4 and MLL-AF9, a subset of CpG residues continue to be protected, while the remainder become methylated. Furthermore, MLL fusions increase Hoxa9 expression regardless of whether CpGs became methylated or not (Erfurth et al., 2008). Histone modifications are closely linked with DNA methylation state. While MLL is best characterised as a histone methyltransferase, it also contains a DNA methyltransferase homology domain, CxxC, in its N-terminus which binds to unmethylated CpG residues (Slany, 2009). Mll CxxC domain binds to CpG clusters in the Hoxa9 locus, preventing DNA methylation. In the presence of MLL fusion proteins, MLL-AF4 and MLL-AF9, a subset of CpG residues continue to be protected, while the remainder become methylated. Furthermore, MLL fusions increase Hoxa9 expression regardless of whether CpGs became methylated or not (Erfurth et al., 2008), demonstrating other factors besides epigenetic dysregulation are responsible for Hoxa9 upregulation in leukemia. MLL also possesses a SET domain with H3K4 methylation activity in its C terminus. Loss of the SET domain leads to defects in monomethylation of H3K4 and also in DNA methylation at the same Hox loci (Terranova et al., 2006). Therefore, histone modifications influence DNA accessibility and methylation resulting in regulated gene expression.

Methylation of CpG islands is linked to dysregulated gene expression in leukemia (Bullinger and Armstrong, 2010). CpG islands in the proximal promoters of HOX genes are frequently methylated in lymphoid and myeloid leukemia patients. Methylation of CpG islands is commonly associated with gene silencing implicating HOX downregulation in development of leukemia. However, HOXA9, HOXA10 and HOXB4 are rarely methylated in AML patients, consistent with a role for overexpression of these HOX genes in AML pathogenesis (Saraf et al., 2006; Strathdee et al., 2007). This may be due to aberrant DNA methylation in HOX promoters contributing to different leukemia phenotypes (He et al., 2011). Alternatively, methylation status at CpG shores within coding regions and/or at remote locus control regions may have a stronger influence over HOX expression than at proximal promoter elements.

**Summary and perspectives**

The association of HOX genes with normal and malignant hematopoiesis is long standing and more recently the importance of maintaining HOX expression during the engineering of HSPCs from PSCs has been reported from independent groups. The role of morphogens including RA, WNT, CDX and epigenetic modifiers is well documented. Regeneration and retention of strict spatiotemporal HOX expression may be essential for the in vitro production and maintenance of bona fide HSCs. In addition, targeted repression of specific HOX genes may be critical for curative therapy in malignant hematopoiesis (Fig. 2).

Advanced technologies are increasing our understanding of the spatiotemporal expression of HOX and potential roles of the developmental master regulators they encode. Recent studies using combined single cell tracking and molecular profiling challenges the traditional hierarchical model of hematopoiesis (Velten et al., 2017; Karamitros et al., 2018). This newly proposed continuum in blood cell development creates the potential for further discovery of the criticality of hematopoietic HOX factors throughout this process. However, lack of HOX-specific tractable models and quality antibodies remain to hamper functional studies. Attaining and retaining appropriate HOX expression in parallel with functional studies in clinically relevant models will underpin future research into normal, engineered and malignant hematopoiesis.

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