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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*

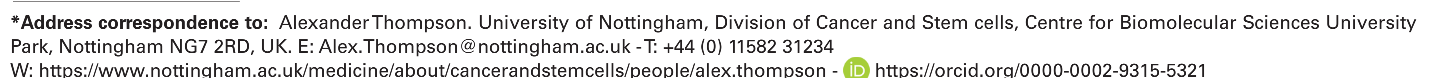
Hematopoietic HOX genes

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 hematopoeisis, *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 myelopoiesis (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-Mejía *et al.*, 2014). Whereas, ectopic expression of HOXA5 and overexpression of HOXA10 in human CD34$^+$ cord blood cells blocked erythroid differentiation and increased myelopoiesis (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, knockdown studies, for the most part, do not support such a role. Despite its striking effect on HSC self-renewal in overexpression experiments, knockdown of *hoxb4* had no apparent effect on normal HSC activity. Moreover, knockdown 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 *Hoxb* 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 knockdown of most *HOX* genes have little effect on hematopoiesis, *Hoxa9* knockdown produces the most profound effect, with disturbances in differentiation and HSC self-renewal (Alharbi *et al.*, 2013). *Hoxa9* 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 found to 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 5' *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 *HOXA* 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/β-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). Upregulation of *HOX* genes and their co-factors, such as *MEIS1*, is associated with an unfavourable outcome in AML (Andreeff *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.*, 1999). *HOXA9* 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 nucleoporin 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-

Method	Cell line	Differentiation protocol	Hematopoietic potential	Self-renewal capacity
Chadwick et al (2003)	hESCs	Promotion of hematopoietic fate + Hematopoietic cytokines + BMP4 Day 0, 1, 15 (CD45$^+$)	Form CFU-GM and BFU-E colonies in CFU assay	Form CFU-GM and BFU-E colonies in secondary CFU assay; not tested *in vivo*
Kennedy et al (2012)	hESCs & iPSCs	Mesoderm specification + Activin inhibitor; Definitive hematopoiesis specification Day 0, 2–4 (To inhibit primitive hematopoiesis), 6 (CD34$^+$ CD43$^-$), 9 (CD34$^+$ CD43$^-$), 12 (CD34$^{low/-}$ CD45$^+$ CD43$^+$)	CD34$^+$ CD43$^-$ cells (day 6 – 9) form erythroid, myeloid and mixed colonies and T cell progenitors in co-culture with OP9-DL4 stroma	Not tested
Sturgeon et al (2014)	hESCs & iPSCs	Mesoderm specification (Wnt/β-catenin, Activin-nodal); Hemogenic endothelium Day 0, 2, 3 (KDR$^+$ CD235a$^-$), 4 (CD34$^+$ CD235a$^-$), 9 (CD34$^+$ CD43$^-$)	Form erythroid and myeloid colonies in CFU assay; express embryonic globin (*HBE*); possess T-lymphoid and NK cell potential in OP9-DL4 co-culture assay	Not tested
Ng et al (2016)	hESCs	Mesoderm induction & patterning + Wnt agonist, Activin inhibitor; Hemogenic endothelium; Hemogenic endothelium & AGM-like hematopoiesis Day 0, 2, 4, 7 (↑ *CDX* & *HOXA*), 10 (CD34$^+$ SOX17$^+$), 14, 18 (CD34$^+$ SOX17$^+$ CD90$^+$), 24+	Form erythroid, myeloid and erythroid/myeloid mixed colonies in CFU assay; possess ability to generate T cells; express adult β-globin	Did not engraft when transplanted to NSG mice
Sugimura et al (2017)	hESCs & iPSCs	Mesoderm induction & patterning; Hematopoietic & endothelial specification; EHT (*ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, SPI1* transduction) Day 0, 2, 3, 4, 6, 8 (CD34$^+$ CD43$^-$), 11 (CD45$^+$), 12 → Injected NSG mice & induced TF expression for 2 weeks → Monitored engraftment for further 12 weeks	Erythroid, myeloid, B and T cells engraft in recipients at 12 weeks; mice express fetal (*HBG*) and adult (*HBB*) globin; possess mostly immature human CD4$^+$ CD8$^+$ T cells	Bone marrow transplanted from primary mice show multi-lineage engraftment up to 16 weeks

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-scidIL2Rγnull; 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; Slany, 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-CBP, 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' *HOXA* 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 flt3 was originally implicated in self-renewal of the mouse HSC pool, upregulation of flt3 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, retroviral 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 leukemic activity, however, *Meis1* expression was necessary to transform bone marrow cell overexpressing *Hoxa9* (Kroon

et al., 1998). The oncogencity conferred by Meis1 appears to be related to its downstream targets (Argiropoulos and Humphries, 2007). A comparison of Hoxa9/Meis1 and Hoxa9 alone immortalised progenitors, demonstrated Hoxa9 and Meis1 co-operate to induce AML and also proliferated in response to Flt3 ligand. This response and Flt3 receptor expression was observed only in Hoxa9/Meis1 transformed cells. Given the exclusivity of this expression, the authors hypothesised Meis1 confers an AML phenotype on non-leukemia Hoxa9 immortalised progenitors through activation of Flt3 (Wang *et al*., 2005). However, both *Flt3*$^{+/+}$ and *Flt3*$^{-/-}$ bone marrow progenitors developed rapid AML following Mesi1/Hoxa9 transformation, irrespective of Flt3 genotype (Morgado and Lavau, 2007).

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*-features, *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 collinear 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, 2016). 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, a lncRNA embedded between *HOXA1* and *HOXA2* termed *HOTAIRM1*, 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,

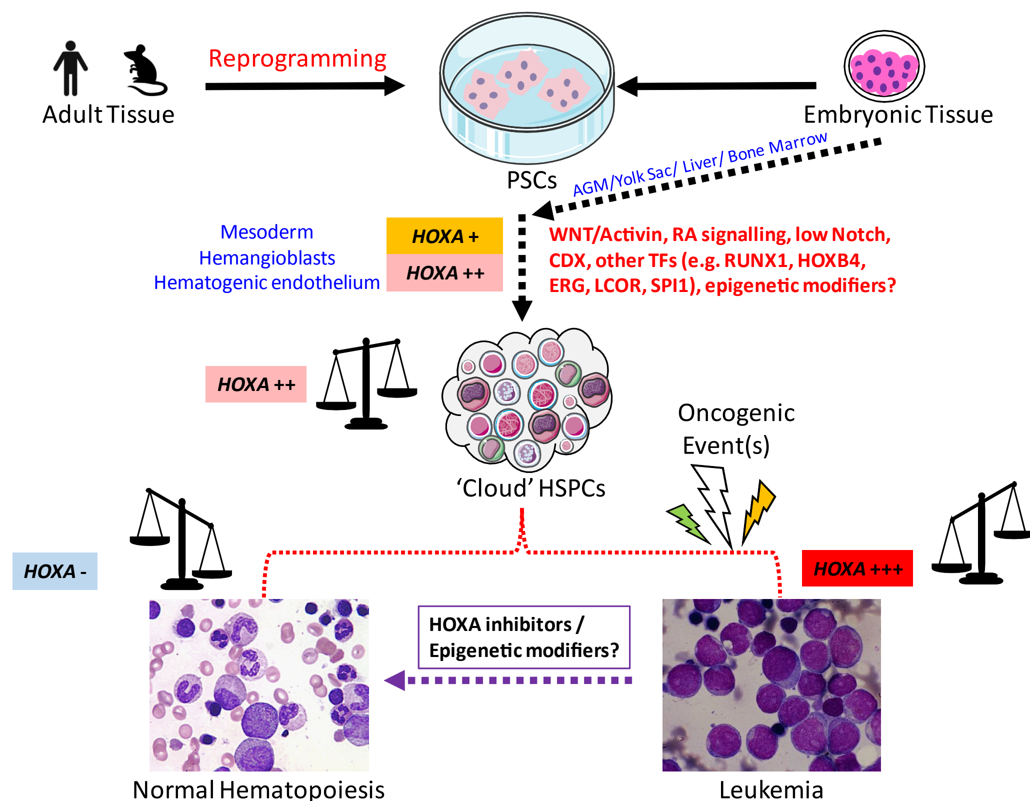

Fig. 2. A schematic of *HOXA* gene expression *in normal, engineered and malignant hematopoiesis.*

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. 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 (Velt*en 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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