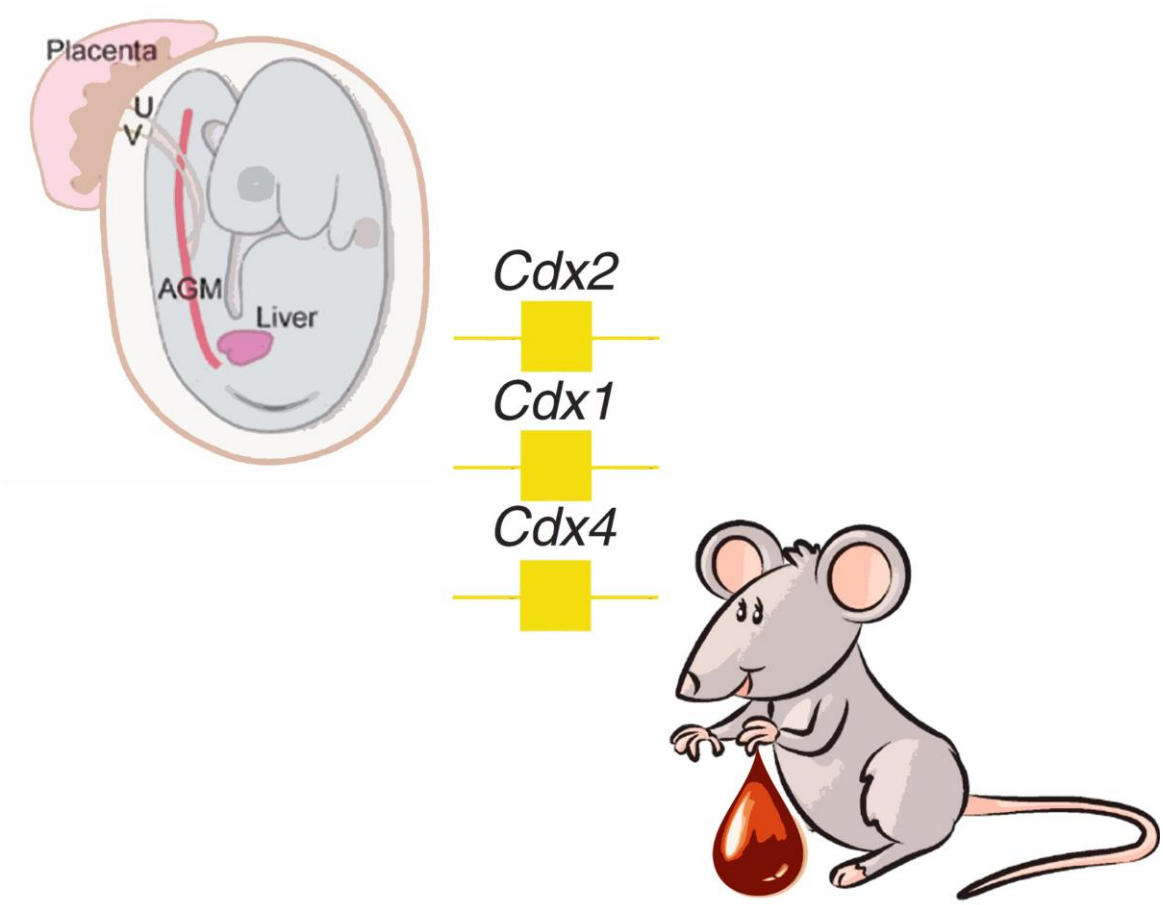
The role of *Cdx* in embryonic and adult mammalian hematopoiesis



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Colophon

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The role of Cdx *in embryonic and adult mammalian hematopoiesis*

Master thesis of the Cancer Genomics & Developmental Biology program

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On the cover

Mouse embryo illustration (adapted from Dzierzak & Speck, 2008); *Cdx* genes illustration (adapted from Young & Deschamps, 2009); adult mouse illustration; blood drop illustration. Modified with Photoshop Elements 7.0; cutout filter.

Acknowledgement

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Summary

All blood cells orginate from a common stem cell in the bone marrow: the hematopoietic stem cell (HSC), which is characterized by two main properties: self-renewal and multipotency. Hematopoiesis is a hierarchical system with the HSC at the top. During embryogenesis, hematopoietic cells and progenitors with broader potentials are progressively generated. Mammalian hematopoietic development begins in extra-embryonic structures and continues within the embryo proper. In the conceptus, the mesoderm is patterned along the anterior-posterior (A-P) axis. The positional identity of the mesoderm on the A-P axis is regulated by *Hox* genes. *Cdx* genes, another homeobox gene family, are required for the posterior growth of axial tissues during development. *Cdx* genes are known to act upstream of *Hox*. The first evidence for a role for *Cdx* in hematopoiesis was established in zebrafish. Using embryoid bodies, it was shown that the *Cdx* genes promote embryonic hematopoiesis in the mouse by upregulating target *Hox* genes. Induction of *Cdx4* promotes proliferation of hematopoietic progenitors. HSCs were more successfully derived from ESCs upon *Cdx4* and *Hoxb4* induction. *Cdx* gene deficiency jeopardizes embryonic hematopoiesis, with severe consequences for blood development upon *Cdx2* mutations. As adult *Cdx4* knockout mice only displayed minimal hematopoietic abnormalities, *Cdx4* is dispensable for adult hematopoiesis. Previous studies on *Cdx1* and *Cdx2* mutants did not report any hematopoietic malformations either. However, it was demonstrated that overexpression of *Cdx4* promotes leukemia in adult mice and that ectopic expression of *Cdx2* is a key event in myeloid leukemia. The *Cdx* genes thus act as stimulators of hematopoietic progenitor maintenance and proliferation. Loss of function of *Cdx* impairs embryonic hematopoiesis, whereas gain of function in the adult bone marrow results in overproliferation of hematopoietic progenitors, which can instigate leukemia.

Key words: hematopoiesis, mouse, *Cdx*, leukemia.

Introduction

Hematopoiesis

Adult mammalian blood contains many cell types with very diverse functions, ranging from the transport of oxygen to the production of antibodies. Blood cells have limited life spans and are produced throughout the life of an animal. They all originate from a common stem cell in the bone marrow: the hematopoietic stem cell (HSC). The HSC is characterized by two main properties: self-renewal and multipotency. Self-renewal ensures maintenance of the pool of HSC, whereas multipotency allows generation of all types of differentiated blood cells (figure 1; Alberts *et al.*, 2002; Wolpert *et al.*, 2007a).

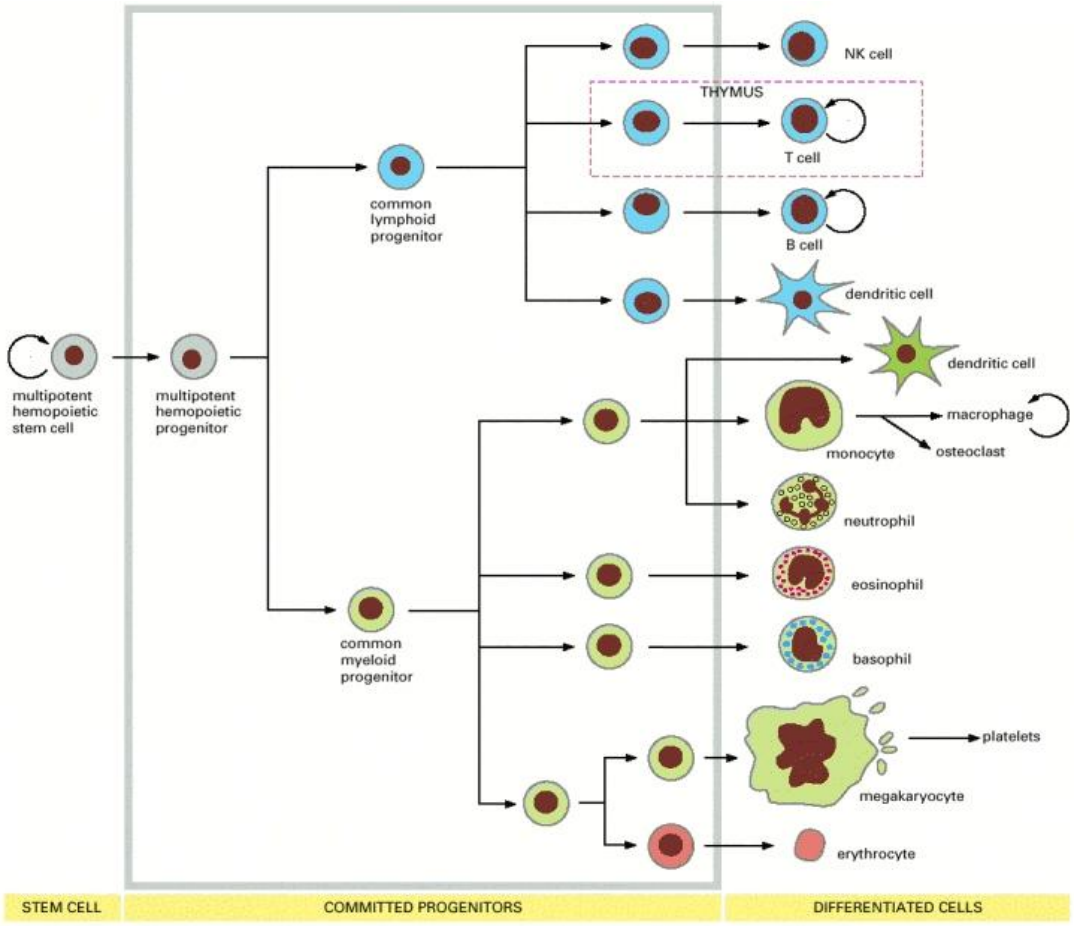

Figure 1 Overview of hematopoiesis in adult mammals. The hematopoietic stem cell divides into a HSC daughter cell and a multipotent hematopoietic progenitor. The progenitors progressively differentiate and become specialized in the range of cells they can create, as indicated by branching of the cell-lineage diagram within the gray box. Most hematopoietic cells develop in the bone marrow. However, T-cells develop in the thymus. Macrophages, osteoclasts and some dentritic cells develop within the blood from monocytes (Alberts *et al.*, 2002).

Hematopoiesis – the formation of blood cells – is a stepwise process. With every cell division, the HSC self-renews and gives rise to a multipotent hematopoietic progenitor (figure 1). Subsequently,

the hematopoietic progenitor commits to either a lymphoid of myeloid fate. The common lymphoid progenitor is capable of generating large numbers of T-cells, B-cells, natural killer (NK) cells and dendritic cells. The common myeloid progenitor creates a progeny of all other blood cells. This includes monocytes, neutrophils, eosinophils, basophils, erythrocytes and megakaryocytes, of which the latter give rise to platelets. Early commitment to the lymphoid or myeloid lineage, followed by commitment to individual lineages of blood cells, makes hematopoiesis a hierarchical system with the HSC at the top (Alberts *et al.*, 2002; Wolpert *et al.*, 2007a).

Within the bone marrow, HSCs reside in stem cell niches: specialized micro-environments that promote stem cell maintenance. In these compartments, the HSCs interact with secreted or membrane-bound factors, such as BMP4 and Wnt. These interactions influence the self-renewal, quiescence and mobilization of the stem cells. Maintenance of HSCs is thus tightly regulated. It is currently thought that the HSCs reside in two kinds of niches: the endosteal niche and the vascular niche (Oh & Kwon, 2010). These compartments lay in close proximity and contain similar structural and functional mediators. However, distinctions between the two niches are also apparent. Multiple explanations for their shared existence have been provided, yet evidence for a definitive answer is still missing.

Ontogeny of the mammalian hematopoietic system

Since adult HSCs are generated during embryonic life, HSCs are often studied in the developing embryo. During embryogenesis, members of the hematopoietic lineages appear in a reverse order compared to the adult hematopoietic hierarchy (figure 2; Dzierzak & Speck, 2008).

In the mouse conceptus, the first site for blood cell emergence is the extra-embryonic yolk sac. At embryonic day (E) 7.5, cellular aggregates develop of which the outer layer consists of endothelial cells, while the inner part contains primitive erythrocytes (Ferkowicz & Yoder, 2005). In contrast to adult erythrocytes, primitive erythrocytes are large, nucleated cells that express embryonic forms of hemoglobin (Leder *et al.*, 1992). The primitive erythrocytes serve the short term oxygen needs of the developing embryo and exist only transiently.

This first step in the ontogeny of the mammalian hematopoietic system is called primitive hematopoiesis. The cellular aggregates within the yolk sac are known as a blood islands (Haar & Ackerman, 1971). It is currently thought that the two cell lineages within the blood islands have a common mesodermal precursor: the hemangioblast (Choi *et al.*, 1998). It has been demonstrated that hemangioblasts arise in the primitive streak and subsequently migrate to the yolk sac (Huber *et al.*, 2004).

The definitive hematopoietic system arises independently from primitive hematopoiesis. At E8.25, the yolk sac and the allantois start to generate precursors with both erythroid and myeloid potential (Palis *et al.*, 1999; Rampon & Huber, 2003; Zeigler *et al.*, 2006; Corbel *et al.*, 2007; Lux *et al.*, 2007). By E9, also the placenta gives rise to erythro-myeloid progenitors (Alvarez-Silva *et al.*, 2003). These progenitors are generated *de novo*, since their appearance is already observed before the onset of blood circulation. This indicates that the progenitors cannot have developed elsewhere in the embryo and are thus newly generated in the yolk sac, allantois and placenta.

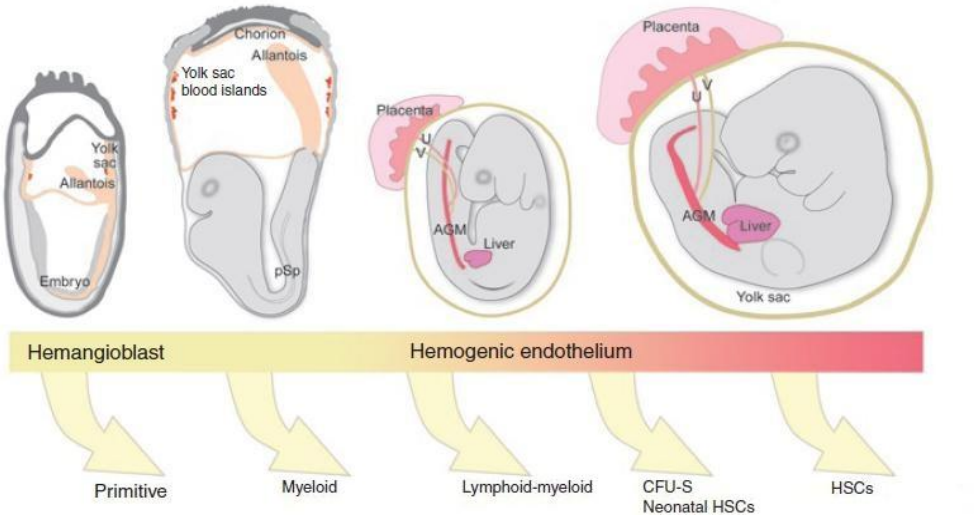

Figure 2 Generation of hematopoietic cells in the mouse conceptus. The embryo progressively generates blood cells and progenitors with a broader potential (arrows) at E7.5, E8.25, E9.0 and E10.5. Hematopoietic cells thus develop in a reverse hierarchical order (adapted from Dzierzak & Speck, 2008).

Simultaneously, progenitors with a more complex lymphoid-myeloid potential develop in the para-aortic splanchnopleura (pSp; Cumano *et al.*, 1996; Cumano *et al.*, 2001). The pSp is a caudal part of the mouse embryo and contains the dorsal aorta, the gut and the splanchnopleural lining of these tissues (Medvinsky *et al.*, 2011). In contrast to the extra-embryonic erythro-myeloid progenitors, these lymphoid-myeloid progenitors are also able to give rise to a lymphoid progeny.

At E9, neonatal HSCs (Yoder *et al.*, 1997) and colony-forming units-spleen (CFU-S; Medvinsky *et al.*, 1993) progenitors develop both in the yolk sac and in the aorta-gonad-mesonephros (AGM) region. Neonatal HSCs only reconstitute in newborn and in immunodeficient mice, but not in adult recipients. CFU-S progenitors are capable of forming hematopoietic colonies in spleens of irradiated mice. The AGM region originates from the pSp and consists of the dorsal aorta and the urogenital ridges (Medvinsky *et al.*, 2011).

At E10.5, the AGM region starts to generate adult HSCs (Muller *et al.*, 1994; Medvinsky & Dzierzak, 1996; Kumaravelu *et al.*, 2002). The HSCs develop in the ventral floor of the dorsal aorta, in intra-aortic cell clusters (North *et al.*, 2002; Taoudi & Medvinsky, 2007). Their phenotypic profile corresponds to that of the surrounding endothelial cells. Intra-aortic cell clusters are currently thought to derive from the aortic endothelium, as a result of the initiation of the hematopoietic program. Data from mouse studies collectively indicate that endothelial cells bearing hemogenic potential are the most direct precursors of adult HSCs (de Bruijn *et al.*, 2002; North *et al.*, 2002; Taoudi *et al.*, 2005). The endothelial-hematopoietic transition in the dorsal aorta is regulated by the transcription factor Runx1 (North *et al.*, 2002).

Concurrently with their development in the dorsal aorta, adult HSCs appear in the placenta (Gekas *et al.*, 2005; Ottersbach & Dzierzak, 2005) and in the umbilical and vitelline arteries (de Bruijn *et al.*, 2000). These extra-embryonic sites thus also contain HSCs, yet evidence of *de novo* generation has not been provided.

Once the hematopoietic progenitors and HSCs have developed, they enter the circulation and colonize the fetal liver. This rudimentary organ is first colonized by hematopoietic progenitors at E10 (Cumano & Godin, 2007), the HSCs follow at E11 (figure 3; Dzierzak & Speck, 2008). The fetal liver provides a unique set of environmental conditions that allows the population of HSC to expand (Morrison *et al.*, 1995). Moreover, the liver rudiment promotes differentiation of erythrocytes, myeloid cells and lymphocytes (Igarashi *et al.*, 2001; Godin & Cumano, 2002).

From the fetal liver, the HSCs colonize the fetal spleen, the thymus and the bone marrow (figure 3). HSCs circulating from the fetal liver reach the fetal spleen around E13, and hematopoietic differentiation occurs there (Godin *et al.*, 1999). Alike the fetal liver, the fetal spleen is a transient hematopoietic organ. However, the HSC population within the spleen rudiment does not significantly expand (Bertrand *et al.*, 2006). Lymphocytes colonize the thymus already from E11 onwards, (Yokota *et al.*, 2006), whereas migration of HSCs and hematopoietic precursors to the bone marrow only occurs at E15 (Dzierzak & Speck, 2008).

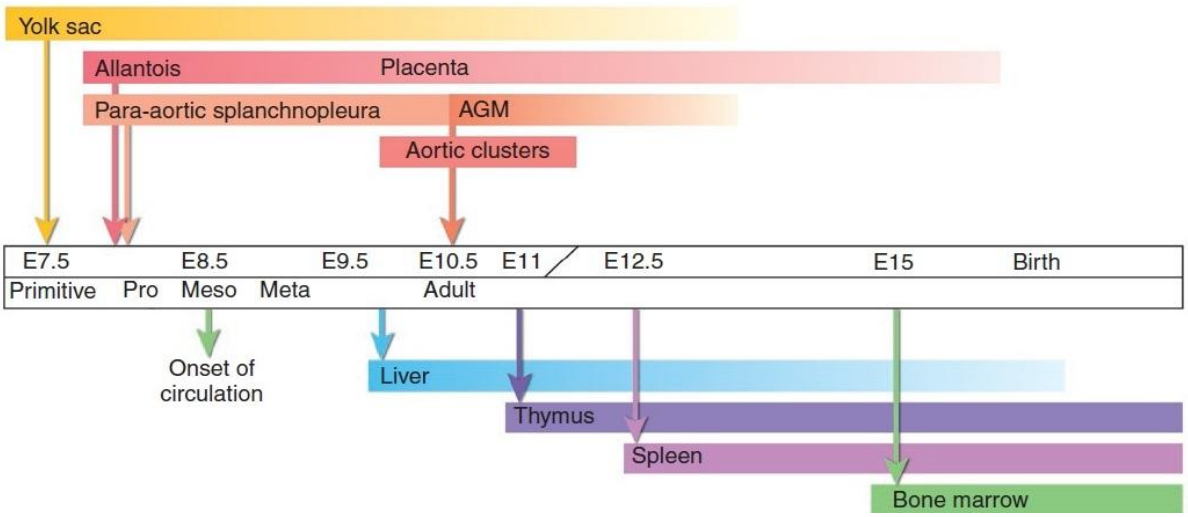

Figure 3 Timeline of hematopoietic events in the mouse embryo. Arrows above the timeline indicate tissues in which hematopoietic cells are first generated and/or appear. Circulation starts at E8.5. Arrows below the timeline indicate the earliest time of colonization (adapted from Dzierzak & Speck, 2008).

Hox and *Cdx* genes

In the mouse conceptus, the mesoderm is patterned along the anterior-posterior (A-P) axis. The positional identity of the mesoderm on the A-P axis is regulated by the *Hox* genes. The *Hox* genes encode gene-regulatory proteins that contain a DNA-binding region, known as the homeodomain. The homeodomain is encoded by the homeobox, hence the name for this gene family (Wolpert *et al.*, 2007b).

Mammalians have four clusters of *Hox* genes (figure 4; Young & Deschamps, 2009). In each *Hox* cluster, the order of the genes from 3' to 5' on the chromosome corresponds to the order in which they are expressed along the A-P axis, both spatially and temporally. The four clusters have arisen by duplications of an ancestral cluster. *Hox* genes thus resemble each other in a large extent; the

homology is most marked within the homeobox. Corresponding genes in different *Hox* clusters (e.g. *Hoxa9*, *Hoxb9*, *Hoxc9* and *Hoxd9*) belong to a paralogous group (PG).

The *Hox* clusters are thought to descend from an ancestral *ProtoHox* cluster, which also gave rise to the *ParaHox* cluster (Young & Deschamps, 2009). The *ParaHox* cluster harbors the *Gsx* and *Pdx* genes and of three paralogous *Cdx* genes: C*dx1*, C*dx2* and C*dx4*. Similarly to what is known for the *Hox* genes, the *ParaHox* cluster has a spatial transcriptional co-linearity os expression, since the *Gsx*, *Pdx*, and *Cdx* genes are expressed in domains extending less and less anteriorly.

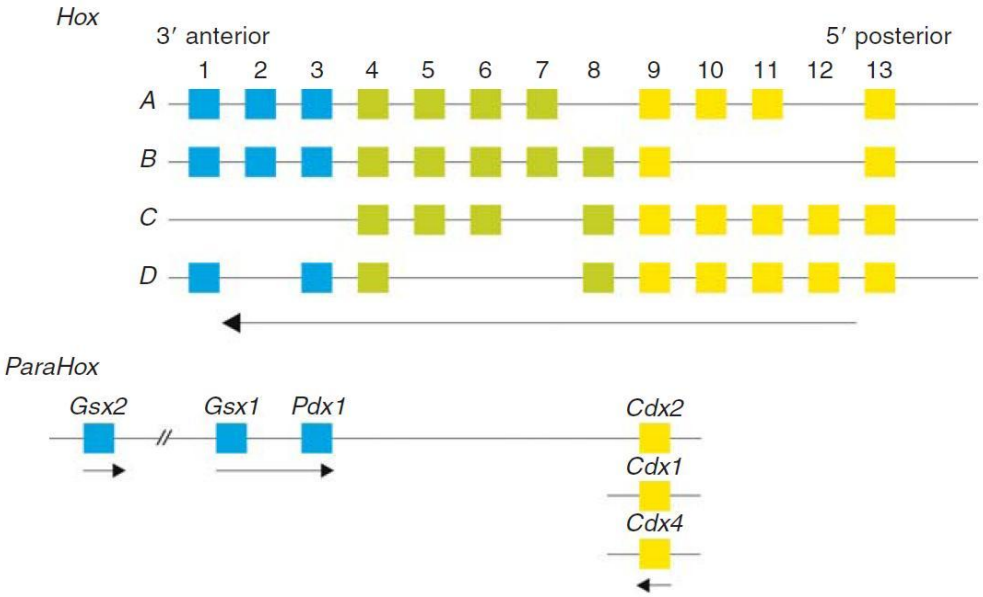

Figure 4 Schematic representation of the *Hox* and *ParaHox* genes in the mouse. The four *Hox* clusters are depicted with A-D, the numbers above the genes refer to the paralogous group (PG). The blue color represents *Hox* and *ParaHox* genes derived from anterior *ProtoHox* genes, green stands for genes derived central *Hox* genes and yellow corresponds to genes derived from the posterior *ProtoHox* gene. *Cdx* genes are thought to be distant paralogs of *Hox* PG9 to PG13. The arrows indicate the transcriptional direction of the genes (adapted from Young & Deschamps, 2009).

The *Cdx* gene family encodes a group of transcription factors which, alike Hox proteins, contain a homeodomain that is able to bind target genes. *Cdx* stands for *Caudal*-related homeobox, which refers to the posterior expressed *Caudal* gene in the *Drosophila* embryo (Wolpert *et al.*, 2007b). *Cdx* genes are required for the posterior growth of axial tissues. Elongation of the vertebrate A-P axis depends on the progressive addition of new tissues from a growth zone at the posterior end of the embryo (Mallo *et al.*, 2010).

Bialecka and colleagues demonstrated that especially *Cdx2* plays a prevalent role in axial elongation, since the loss of a single *Cdx2* allele lead to a slight posterior truncation. Homozygous *Cdx2* null mutants failed to extend the A-P axis further than the forelimb bud. Loss of *Cdx1* and *Cdx4* genes did not compromise axial elongation. However, combining mutant *Cdx1* or *Cdx4* alleles with the heterozygous *Cdx2* mutations lead to more severe truncation phenotypes (Bialecka *et al.*, 2010). These data indicate that all three *Cdx* genes contribute to axial elongation.

Young and colleagues demonstrated that in *Cdx* mutants, premature termination of axial elongation can partially be rescued by supplying canonical Wnt signals in the posterior growth zone. The results show that *Cdx* genes, together with *Hox* genes, control axial elongation by sustaining canonical Wnt signaling in the posterior growth zone. This way, *Cdx* and *Hox* genes insure a suitable environment for axial progenitors to be maintained. *Cdx* and central *Hox* genes stimulate the elongation process via a positive regulation of Wnt signaling, whereas posterior *Hox* genes inhibit this process. Moreover, Cdx proteins are key regulators of *Hox* genes. *Cdx* genes integrate posteriorizing signals from the canonical Wnt signaling pathway and transmit this information to *Hox* promoters. *Cdx* thus acts upstream of *Hox* (Young *et al.*, 2009). However, it cannot be ruled out that *Cdx* genes also play a role independent of *Hox* gene regulation during axial extension and patterning (Savory *et al.*, 2009).

Gaining evidence for the role of *cdx* in hematopoiesis: what we know from zebrafish

Zebrafish offers several advantages as a vertebrate model for hematopoietic development. First, zebrafish embryos are transparent, which allows observing *in situ* cell migration of selected groups of cells or even of single cells during development (Cumano & Godin, 2006). Zebrafish studies have therefore facilitated real-time imaging of hematopoietic development (Medvinsky *et al.*, 2011). Secondly, the zebrafish is suitable for screening mutant embryos, yielding to the identification of new genes involved in developmental process, including hematopoiesis de Jong & Zon, 2005). Genetic analysis is hence relatively easy. This property has lead to an immense availability of transgenic fish expressing fluorescent reporter proteins and lineage-tracing protocols (Cumano & Godin, 2006).

Ontogeny of the zebrafish hematopoietic system

As in other vertebrates, all of the zebrafish blood lineages are believed to originate from a pool of pluripotent, self-renewing HSCs. The zebrafish yolk sac is, however, devoid of hematopoiesis. Primitive hematopoiesis starts instead in a caudal structure named the intermediate cell mass (ICM). The ICM gives rise to mainly erythrocytes and myeloid cells. Myeloid cells are also generated in the anterior part of the embryo. From 24 hours post-fertilization (hpf) on, as circulation is established, these primitive blood cells start to circulate throughout the embryo (figure 5; Paik & Zon, 2010; Medvinsky *et al.*, 2011).

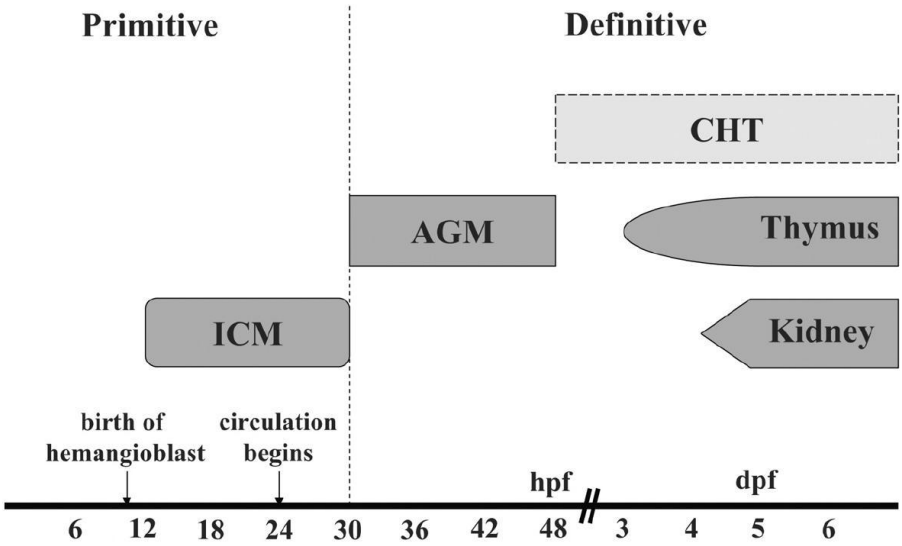

Figure 5 Timeline of zebrafish hematopoiesis. Primitive hematopoiesis starts in the ICM. Definitive hematopoiesis follows in the AGM, which comprises the ventral wall of the dorsal aorta. At 48 hpf, HSCs migrate to the caudal hematopoietic tissue (CHT) and later on to the kidney marrow. From 3 dpf days post fertilization on, lymphocyte generation is initiated in multiple thymi (adapted from Paik & Zon, 2010).

Subsequently, HSCs emerge from the ventral wall of the dorsal aorta (the zebrafish AGM region) through a process known as endothelial-hematopoietic transition, which is directed by *runx1a* (the

zebrafish equivalent of *Runx1*; Thompson *et al.*, 1998; Burns *et al.*, 2002; Kalev-Zylinska *et al.*, 2002). At 48 hpf, the HSCs enter the circulation through the cardinal vein and migrate to a posterior region in the tail: the caudal hematopoietic tissue (CHT; figure 5; Murayama *et al.*, 2006; Jin *et al.*, 2007). Lymphopoiesis occurs in the thymi from 3 dpf onwards. Eventually, by 4 dpf, the HSCs migrate to the kidney marrow, which bears a function equivalent to the mammalian bone marrow regarding hematopoiesis (Paik & Zon, 2010).

cdx4 and *cdx1a* are required for zebrafish hematopoiesis

The first evidence for a critical role for *Cdx* in hematopoiesis was established in 2003 in zebrafish. Davidson and colleagues identified the *kugelig* (*kgg*) zebrafish mutant, which exhibits tail defects and severe anemia. Linkage mapping and sequence analysis identified a mutation in the *cdx4* gene on chromosome 14. Mutant embryos showed low expression levels of two hematopoietic markers at 12 hpf: *scl* and *gata1*. The s*cl* (*stem cell leukemia*) gene plays an early but extensive role in HSC formation, both in mammals and in zebrafish. *gata1* is an erythroid-specific transcription factor. Moreover, mutant embryos displayed low expression levels of *runx1a* at 36 hfp. These results indicate a role for *cdx4* in both primitive and definitive hematopoiesis (Davidson *et al.*, 2003; Davidson & Zon, 2004).

Subsequently, Davidson and colleagues studied the expression patterns of *hox* genes in the *kgg* mutant, since these genes were known to act downstream of *cdx* genes in some instances. At least nine *hox* genes displayed a disturbed expression pattern. *hox* genes expressed in the anterior trunk showed expanded expression domains towards the posterior part of the embryo, while more caudally expressed *hox* genes displayed a reduced or absent expression. The ICM exhibited a severe reduction in hematopoietic cells, but not in angioblasts (vascular precursors), while both were known to derive from the same precursor: the hemangioblast. Rescue injections with *hoxb7a* and *hoxa9a* mRNA restored erythroid development and *scl* and *gata1* expression. However, the tail defect was still present in the *kgg* mutant. These results suggest that the hematopoietic defect in *kgg* mutants is due to the downregulation of *hox* genes in the hematopoietic cell population, rather than being an indirect defect in posterior body patterning (Davidson *et al.*, 2003; Davidson & Zon, 2004).

Members of the *cdx* familiy are known to be functionally redundant. Since the *kgg* mutant is not completely bloodless, Davidson and Zon searched for additional zebrafish *cdx* genes that may act together with *cdx4*. They demonstrated that embryos deficient of *cdx1a* and *cdx4* displayed severe disturbances in *hox* gene expression. Double mutant embryos showed fewer hematopoietic precursors within the ICM and absence of *runx1a* expression in the dorsal aorta at 36 hpf. Moreover, the mutant embryos were deficient of lymphoid cells at 4 dpf. However, endothelial cells and kidney progenitors still developed, indicating that the hematopoietic defects were not the result from a general block in posterior mesoderm differentiation. When Davidson and Zon overexpressed *hox9a* in these embryos, they observed a restoration of both ICM progenitors and presumptive AGM HSCs. This study demonstrates that *cdx1a* and *cdx4*, acting together, are required for the formation of embryonic erythroid cells and HSCs (Davidson & Zon, 2006). *cdx* genes thus play an essential role in both primitive and definitive hematopoiesis in vertebrates.

Role of *Cdx* in embryonic mammalian hematopoiesis

Using *ex vivo* systems to study hematopoiesis

Studying early hematopoietic development is challenging in mammals, since the developing embryo is difficult to access. Therefore, long-term cultures of pluripotent embryonic stem cells (ESCs) established from a variety of mammalian species, including mouse and human, are very useful *ex vivo* systems. ESCs are derived from the inner cell mass of the blastocyst and bear pluripotent characteristics similar to early epiblast cells. When reintroduced into the blastocyst, ESCs contribute to all body parts, including the germ line. *Ex vivo*, ESCs display the ability to self-renew and differentiate into numerous cell types. They are therefore a powerful tool to study developmental processes (Lengerke *et al.*, 2007; Lengerke *et al.*, 2008; McKinney-Freeman *et al*., 2008; Wang *et al.*, 2008).

When ESCs are plated at high-density nonadhesive substrates, they develop into cystic embryoid bodies (EBs), which will differentiate into tissues from all three germ layers and in which hematopoietic cells develop. Various studies collectively indicate that this *ex vivo* system reproduces early hematopoiesis. The hemangioblast was even first identified in this system, and later on validated in the mouse conceptus (Choi *et al.*, 1998). Hence, the ESC/EB system is an effective model to explore molecular pathways underlying hematopoietic development, which would otherwise be difficult to investigate *in vivo* (Lengerke *et al.*, 2007; Lengerke *et al.*, 2008; McKinney-Freeman *et al*., 2008; Wang *et al.*, 2008).

Cdx genes promote mouse embryonic hematopoiesis

Next to studying expression patterns of hematopoietic genes and *hox* genes in the *kgg* mutant, Davidson and colleagues explored whether retroviral expression of *Cdx4* in mouse HSCs or multipotential progenitors enhances their self-renewal and proliferation, since this was already demonstrated for *Hoxb4*. Murine EB-derived hematopoietic cells were transfected with retroviral *Cdx4* and assayed for effects on hematopoietic colony formation. *Cdx4* induced a marked expansion of hematopoietic progenitors, including a 13-fold increase in CFU-GEMM (colony forming unit-granulocyte/erythroid/macrophage/megakaryocyte) colonies and an 11-fold increase in CFU-GM (colony forming unit-granulocyte/macrophage) colonies. The strongest effect of *Cdx4* on colony formation was found between days 4 and 5 of EB differentiation (Davidson *et al.*, 2003).

Subsequently, Davidson *et al.* examined alterations in *Hox* gene expression in *Cdx4* transduced cells. *Cdx4* induced a pronounced enhancement in *Hox* expression levels, especially for *Hoxb4* (30-fold), *Hoxb3* (19-fold), *Hoxb8* (5-fold) and *Hoxa9* (4.1-fold; Davidson *et al.*, 2003), all of which previously have been involved in HSC or hematopoietic progenitor expansion (Perkins & Cory, 1993; Thorsteinsdottir *et al.*, 2002; Björnsson, 2003). These results demonstrate that *Cdx4* acts at early stages of hematopoietic development to promote the proliferation of hematopoietic progenitors by upregulating the expression of target *Hox* genes (Davidson *et al.*, 2003).

Wang and colleagues investigated the effect of *Cdx4* expression on hematopoiesis by using a murine ESC line with tetracycline-inducible *Cdx4*. Upon incubation with the tetracycline analogue

doxycycline, increased numbers of primitive erythroid and multipotential hematopoietic colonies were observed. Consistent with this finding, elevated expression levels of both early (*Scl*, *Gata1* and *β-H1* globin) and definitive (c-myb and *AML1*) hematopoietic genes were detected after *Cdx4* induction. Moreover, *Cdx4* induction resulted in an increased expression of posterior *Hox* genes (*a6*, *a7*, *a9*, *a10*, *b9* and *c6*) in hematopoietic populations. These results indicate that *Cdx4* induction stimulates both primitive and definitive hematopoietic progenitor formation by influencing *Hox* gene expression during hematopoietic development (Wang *et al.*, 2005).

Moreover, Wang *et al.* demonstrated that induction of both *Cdx4* and *Hoxb4* enables engraftment of ES-derived hematopoietic progenitors in lethally irradiated mice. These recipients reconstituted myeloid, lymphoid and erythroid lineages at multiple hematopoietic sites, including the bone marrow. Serial transplantation of the bone marrow from these recipients into lethally irradiated secondary mice showed a successful reconstitution of multiple lineages of hematopoietic cells. These data indicate successful derivation of HSCs from ESCs after *Cdx4* and *Hoxb4* induction (Wang *et al.*, 2005).

McKinney-Freeman and colleagues explored the role of all three *Cdx* genes during hematopoietic development by inducing ectopic expression of *Cdx* in EBs. The three *Cdx* genes turned out to differentially influence early blood development. *Cdx4* induction enhanced the formation of hematopoietic progenitors from day 2 to day 4 of EB differentiation, whereas induction of *Cdx1* had an increasing effect from day 4 to day 6. On the contrary, *Cdx2* induction inhibited nearly all hematopoietic activity in EBs, probably by blocking progenitor differentiation. Expression levels of hematopoietic lineage-specific genes (*Runx1*, *Scl* and *Gata1)* supported these observations. Moreover, all three *Cdx* genes upregulated the expression of posterior *Hox* genes; and *Cdx2* more prominently than *Cdx1* and *Cdx4* (McKinney-Freeman *et al.*, 2008).

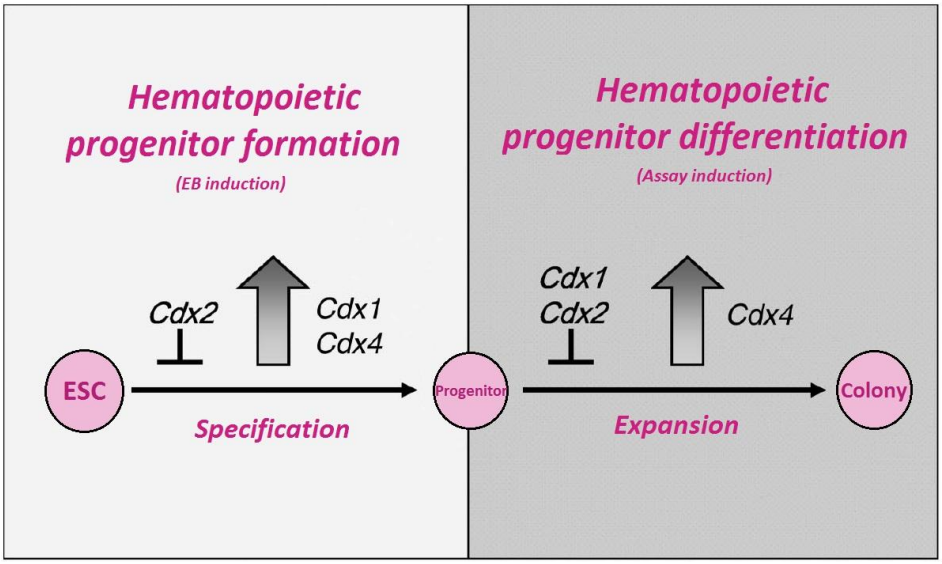

Figure 6 Model for the effects of *Cdx* genes in ESC-derived hematopoiesis. *Cdx* genes differentially regulate the formation of hematopoietic progenitors during EB induction and subsequent differentiation of these progenitors during assay induction (adapted from McKinney-Freeman *et al.*, 2008).

McKinney-Freeman *et al.* next studied the influence of ectopic *Cdx* expression on the colony forming ability of EB-derived hematopoietic progenitors. *Cdx4* induction strongly enhanced the formation of CFU-GEMM colonies. *Cdx2* induction suppressed all hematopoietic colony formation. Surprisingly, *Cdx1* induction also inhibited this process. Ectopic *Cdx4* expression thus consistently enhanced hematopoietic potential both during EB and colony formation, whereas *Cdx2* consistently suppressed blood formation. *Cdx1* displayed different effects, first enhancing blood formation within EBs but suppressing hematopoietic colony formation. This suggests that *Cdx1* may inhibit the maturation of hematopoietic progenitors. Taken together, these results demonstrate different effects of the *Cdx* genes on hematopoietic progenitor formation and differentiation (figure 6; McKinney-Freeman *et al.*, 2008).

Cdx deficiency jeopardizes embryonic hematopoiesis

All of the aforementioned evidence indicates a crucial role for the *Cdx* genes in hematopoietic development. However, no significant hematopoietic defects were previously observed in *Cdx* mutant embryos (Subramanian *et al.*, 1995; Chawengsaksophak *et al.*, 1997; Chawengsaksophak *et al.*, 2004; Van Nes *et al.*, 2006). Therefore, Wang and colleagues investigated the hematopoietic potential of *Cdx*-deficient mouse ESCs and mouse embryos.

Cdx4-deficient ESCs displayed a slight decrease in the number of erythroid colonies and multiple progenitor colonies. In *Cdx4*-deficient embryos, primitive erythroid colony formation was reduced from E7.5 until E9. These data suggest that primitive hematopoiesis is somewhat impaired upon *Cdx4* deficiency. As for *Cdx1*-deficient ESCs, the formation of hematopoietic progenitors was reduced. However, no obvious blood defects were observed in *Cdx1*-deficient mice, indicating that *Cdx1* presumably plays a role in early hematopoiesis, but with a compensatory mechanism. Moreover, hematopoietic cells of both *Cdx1*- and *Cdx4*-deficient 6 day EBs displayed a reduced expression of posterior *Hoxa* genes, indicating that *Cdx1* and *Cdx4* induce expression of these *Hox* genes in these cells (Wang *et al.*, 2008).

As *Cdx2* null mutants die between E3.5 and E5.5, Wang *et al.* subsequently analyzed the differentiation of *Cdx2*-deficient EBs, which displayed a decreased number of hematopoietic progenitor colonies. A genome-wide microarray expression analysis on *Cdx2*-deficient day 6 EBs showed, next to alterations in *Hox* gene expression, a reduced expression of several hematopoietic lineage-specific genes, including *Runx1*, *Scl*, *Gata1* and *β-H1* globin, of which the latter three are markers for primitive hematopoietic development. These results support an essential role for *Cdx2* in early embryonic hematopoiesis (Wang *et al.*, 2008).

Since the *Cdx* genes contribute to axial elongation in a dose-dependent manner (Bialecka *et al.*, 2010), Wang and colleagues next explored the effect on hematopoiesis of *Cdx1* and *Cdx2* knockdowns in *Cdx4*$^{-/-}$ embryos. The results indicate that *Cdx* family members are also functionally redundant in this developmental process: the dosage of *Cdx* genes is an important factor in hematopoietic specification, with an essential role for *Cdx2*. Moreover, ectopic *Cdx4* expression significantly upregulated the expression of posterior *Hox* genes (*a7*, *a9*, *b8* and *b9*). Overexpression of a *Hoxb4* transgene rescued impaired hematopoietic progenitor formation in *Cdx2*-deficient EBs, suggesting that *Hoxb4* is downstream of *Cdx2*. Taken together; Wang *et al.* demonstrated that *Cdx*

genes are required for primitive hematopoiesis during embryonic development, with a crucial role for *Cdx2*. The *Cdx* family members act redundantly during blood development by activating overlapping posterior target *Hox* genes (Wang *et al.*, 2008).

The results from Wang *et al.* (2008) are contradictory to the findings of McKinney-Freeman *et al.* (2010), since the latter show that *Cdx2* has an inhibiting effect on both hematopoietic progenitor formation and differentiation, and *Cdx1* suppresses hematopoietic progenitor differentiation in EBs. It is at present incomprehensible why these results do not correspond and wherefore *Cdx* genes have an antagonistic function during development.

Regulation of *Cdx* in embryonic hematopoiesis

Since little is known about the genetic pathways upstream of *Cdx*, Lengerke and colleagues explored the molecular mechanisms underlying hematopoietic specification during mouse embryogenesis. They demonstrated that the growth factor BMP4 plays two distinct and sequential roles during blood formation in ESCs. During the first 2 days of EB development, BMP4 mediated dorsal-ventral patterning and induces ventral-posterior mesoderm. Subsequently, BMP4 promoted blood specification from preformed mesodermal cells by first inducing the formation of hemangioblasts and later stimulating the maturation of hematopoietic progenitors. Moreover, the data indicate that BMP4 accomplishes hematopoietic specification by activating Wnt3a (Lengerke *et al.*, 2008).

Lengerke *et al.* next investigated the effects of BMP and Wnt signaling on the *Cdx-Hox* pathway by differentiating EBs in the presence of these signals and analyzing RNA levels. They discovered that BMP4 upregulates *Cdx1* and *Cdx4* expression via Wnt3a and induced expression of posterior *Hoxa* and *Hoxb* genes. When BMP4 signaling was inhibited, enforced expression of either *Cdx1* or *Cdx4* rescued hematopoietic development, suggesting that BMP4 signaling is upstream of the *Cdx-Hox* pathway. Moreover, BMP and Wnt regulate *cdx4* expression in the zebrafish, indicating that this pathway is evolutionary conserved. In conclusion, the linear BMP-Wnt-*Cdx-Hox* pathway is required during hematopoietic development (Lengerke *et al.*, 2008).

Role of *Cdx* in adult mammalian hematopoiesis

Cdx4 is dispensable for adult hematopoiesis but promotes leukemia

As described in the previous chapter, quite some research has been performed on the role of the *Cdx* genes in embryonic mammalian hematopoiesis. Koo and colleagues decided to study the role of *Cdx4* in adult mammalian hematopoiesis by using null and conditional *Cdx4* mice knockout models. Surprisingly, both *Cdx4* knockout models did not display major hematopoietic defects. Apart from an increase in the number of lymphocytes and a decrease in pre-B colony forming activity in bone marrow cells, loss of *Cdx4* did not cause any hematologic abnormalities. Moreover, *Cdx4* deletion did neither alter the number nor the repopulating abilities of HSCs and hematopoietical progenitors. These data indicate that *Cdx4* is not essential for the establishment and preservation of adult hematopoiesis (Koo *et al.*, 2010).

Abnormal *Hox* gene expression has been linked to the pathogenesis of human leukemia (Perkins *et al.*,1990; Sauvageau *et al.*, 1997; Thorsteinsdottir *et al.*, 1997; Pineault *et al.*, 2003; Soulier *et al.*, 2005). Since this deregulation might involve upstream master regulators, Bansal and colleagues investigated the role of *Cdx4* in acute myeloid leukemia (AML). Mouse transplantation assays showed that overexpression of *Cdx4* in bone marrow cells causes AML when grafted into lethally irradiated recipients. In addition, $Cdx4^{+}$ bone marrow cells from leukemic mice displayed an upregulation of a specific subset of *Hox* genes, namely *a6*, *a7*, *a9*, *b4*, *b8* and *c6*. These results demonstrate that overexpression of *Cdx4* in bone marrow cells deregulates expression of posterior *Hox* genes, with the potential to instigate leukemia (Bansal *et al.*, 2006).

Ectopic expression of *Cdx2* is a key event in myeloid leukemia

Among mutations affecting hematopoiesis, chromosomal translocations are quite frequent. Chromosomal translocations can instigate malignancy of the blood cells via two different mechanisms. Juxtaposition of a potent enhancer or promoter can cause deregulation of the expression of a proto-oncogene. Secondly, chromosomal translocations can lead to the formation of fusion genes with oncogenic potential. Creation of fusion genes is often a key event in acute myeloid leukemia (AML; Look, 1997; Rowley, 1999; Bohlander, 2000; Rawat *et al.*, 2004).

A gene frequently involved in chromosomal translocations is the oncogene *EVT6* (also known as *TEL*), which is located on the short arm (p) of chromosome 12. Chase and colleagues reported three cases with breakpoints in the *ETV6*, causing the chromosomal translocation t(12;13)(p13;q12) and subsequent AML. In one of these patients, the translocation led to a fusion of *ETV6* with *CDX2*, which was already known to be located on 13q12. The translocation breakpoint left the *CDX2* gene intact (Chase *et al.*, 1999).

cDNA analysis from the patient's bone marrow revealed *ETV6-CDX2* fusion transcripts in leukemic cells. Surprisingly, also normal *CDX2* transcripts were detectable in these cells, while *CDX2* expression is usually restricted to the intestinal epithelium (James & Kazenwadel, 1991). Neither normal nor fusion transcripts were detected in the other two patients, indicating that this translocation is heterogenous at the molecular level. In addition, Chase *et al.* discovered aberrant expression of *CDX2*

in a patient suffering from chronic myeloid leukemia (CML). This patient did not display any karyotypical abnormalities on chromosome 12 and 13. The results of this study indicate that deregulation of *CDX2* can be a key event in myeloid leukemia (Chase *et al.*, 1999).

To elucidate this issue, Rawat and colleagues established a mouse model that reflects t(12;13)(p13;q12) human AML and analyzed whether expression of the *ETV6-Cdx2* fusion protein or ectopic expression of *Cdx2* is able to malignantly alter early hematopoietic progenitors *in vivo*. Lethally irradiated recipient mice transplanted with bone marrow cells that overexpress *Cdx2* were dying 90 days posttransplantation. On the contrary, mice transplanted with bone marrow cells expressing the fusion gene did not became moribund. Mice overexpressing *Cdx2* displayed an increase (3.8-fold) of white blood cells, which is a hallmark of leukemia. Immunohistochemistry stainings revealed that the malignant cells beared an undifferentiated myeloblastic phenotype that is characteristic for AML (Rawat *et al.*, 2004).

Serial transplantation of bone marrow cells of diseased *Cdx2* animals into 11 lethally irradiated secondary recipients demonstrated that the *Cdx2*-induced AML is transplantable, since these animals died within 24 days posttransplantation. Moreover, Rawat *et al.* transplanted a mixture of *ETV6-Cdx2* expressing cells, *Cdx2* expressing cells, and *Cdx2* and *ETV6-Cdx2* co-expressing cells into 13 mice. Administration of *Cdx2* and *ETV6-Cdx2* co-expressing cells did not lead to a more advanced or alternated disease phenotype compared to the injection of only *Cdx2* expressing cells (Rawat *et al.*, 2004).

These results demonstrate that ectopic expression of the proto-oncogene *Cdx2*, and not expression of the fusion gene *ETV6-Cdx2*, is the key transforming event in AML upon the chromosomal translocation (12;13)(p13;q12). Rawat and colleagues yet emphasize that the mechanism of transcriptional induction that leads to ectopic expression of *Cdx2* is not precisely known. A plausible explanation could be that after the translocation, *Cdx2* is under the control of *ETV6* enhancers, since it was demonstrated that the chromosome 13 breakpoint lies upstream of *Cdx2* (Rawat *et al.*, 2004).

To clarify to what extent ectopic expression of *CDX2* has a role in myeloid leukemia, Scholl and colleagues systematically analyzed *CDX2* expression in a large cohort of patients with myeloid leukemia. *CDX2* transcripts were detectable in the bone marrow and blood of 90% of the patients with AML (153 out of 170). Moreover, 2 out of 10 patients with CML, 2 out of 5 myelodysplastic syndrome cases and 8 out of 15 human myeloid leukemia cell lines contained *CDX2* transcripts. However, *CDX2* expression was not detectable in HSCs and hematopoietic progenitors derived from healthy individuals (Scholl *et al.*, 2007).

To further study the leukemogenic potential of *Cdx2 in vivo*, Scholl *et al.* performed a murine bone marrow transplantation assay. Primary murine hematopoietic progenitors transfected with *Cdx2* caused AML in transplanted recipients after a median of 187 days. Histopathological analysis revealed that a population of immature myeloid cells extensively infiltrated the bone marrow, liver and spleen of the recipients. Both *Cdx2* transfected hematopoietic progenitors and *Cdx2* expressing immature myeloid progenitors displayed deregulated expression of Hox family members. An upregulation (25-fold) of *Hoxb8* was detectable in the *Cdx2* transfected hematopoietic progenitors, as well as a decreased expression of *Hoxa10*. Spleen cells demonstrated an enhanced expression (3-

fold) of *Hoxb6*, and a downregulation of *Hoxa7* and *Hoxa9*. Together, these results demonstrate that aberrant expression of *CDX2* in the hematopoietic compartment is a common event in the pathogenesis of myeloid leukemia. In addition, *CDX2* is to a certain extent responsible for the altered *HOX* gene expression observed in the majority of patients suffering from AML (Scholl *et al.*, 2007).

Conclusion and discussion

Are *Cdx* genes important for hematopoiesis?

Davidson *et al.* (2003) were the first to identify a critical role for *cdx4* in hematopoiesis. Subsequently, these authors provided evidence that *cdx1a* is also essential for this process (Davidson & Zon, 2006). *cdx1a* and *cdx4* are jointly required for both primitive and definitive zebrafish hematopoiesis and achieve this by inducing *hox* gene expression in the hematopoietic cell population.

As for the mammalian embryo, Davidson *et al.* (2003) showed that *Cdx4* acts at early stages of mouse hematopoietic development to promote the proliferation of hematopoietic progenitors. These results are supported by Wang *et al.* (2005), who moreover provides evidence that pluripotent, self-renewing HSCs can be more successfully derived from ESCs upon *Cdx4* and *Hoxb4* induction. McKinney-Freeman *et al.* (2008) found that ectopic expression of the *Cdx* genes in EBs differentially influence early blood development (figure 6). Together, these studies indicate that induction of *Cdx* genes regulates the maintenance and proliferation of hematopoietic cells by influencing the expression of target *Hox* genes. Table 1 shows that alterations in *Cdx* gene expression on the *Hoxa* and *Hoxb* clusters mostly affect posterior *Hox* genes.

When Wang *et al.* (2008) explored the hematopoietic potential of ESCs and embryos upon *Cdx* deficiency, it was demonstrated that both *Cdx1* and *Cdx4* play a modest role in primitive hematopoiesis, whereas *Cdx2* is essential for this process. Thus, the *Cdx* genes act redundantly during early hematopoietic development, with a crucial role for *Cdx2*. In addition, Lengerke *et al.* (2008) showed that the linear BMP-Wnt-*Cdx*-*Hox* pathway is required during hematopoietic development.

As for the mammalian adult, Koo *et al.* (2010) demonstrated that *Cdx4* is not essential for the establishment and preservation of adult mouse hematopoiesis. Together, the studies of Wang *et al.* (2008) and Koo *et al.* (2010) indicate that *Cdx4* probably plays a modest role in early hematopoietic development, but that its function in adult hematopoiesis is completely redundant with the other *Cdx* genes. As Koo *et al.* (2010). Wang *et al.* (2008) suggested, definitive hematopoiesis is probably less sensitive to *Cdx* gene expression and bears a more powerful compensatory mechanism than primitive hematopoiesis, since previous studies with *Cdx* knockout mice have not documented any hematopoietic defects (Subramanian *et al.*, 1995; Chawengsaksophak *et al.*, 1997; Chawengsaksophak *et al.*, 2002; van den Akker *et al.*, 2002), except for placental vasculature malformations (van Nes, *et al.* 2002).

When expressed aberrantly, *Cdx* genes contribute to leukemogenesis. Bansal *et al.* (2006) demonstrated that ectopic expression of *Cdx4* in bone marrow cells lead to enhanced proliferative and clonogenic capacities of hematopoietic progenitors that are characteristic for AML. Rawat *et al.* (2004) and Scholl *et al.* (2007) provided evidence for an equivalent role of *Cdx2*. *Cdx2* strongly inhibits the maturation of hematopoietic progenitors (McKinney-Freeman *et al.* 2008). In the adult bone marrow, this effect most likely contributes to the role of *Cdx2* in the pathogenesis of AML (Scholl *et al.*, 2007). Comparing these studies suggests that the role of the role of *Cdx2* as an inducer

of leukemia is more potent than the role of *Cdx4*, which is alike their effectiveness in hematopoietic ontogeny.

In conclusion, the *Cdx* genes act as stimulators of hematopoietic progenitor maintenance and proliferation in a dose-dependent manner. Loss of function of *Cdx* jeopardizes embryonic hematopoiesis, whereas gain of function in the adult bone marrow results in overproliferation of hematopoietic progenitors, which can instigate leukemia.

***Cdx* gene**	**Assay**	**Cell population**	**Effect on *Hox* genes**	**Reference**
Cdx4	Retroviral transfection of EBs	Whole EBs	Upregulation of *Hoxa6, Hoxa9, Hoxb3, Hoxb4, Hoxb8*	Davidson *et al.* (2003)
Cdx4	Tetracycline-inducible EBs	Hematopoietic cells ($CD41^+$)	Upregulation of *Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxb9*	Wang *et al.* (2005)
Cdx1	Ectopic induction in differentiating EBs	Whole EBs	Upregulation of *Hoxa4, Hoxa6, Hoxa7, Hoxa9, Hoxb6, Hoxb7, Hoxb8, Hoxb9*	McKinney-Freeman *et al.* (2008)
Cdx2	Ectopic induction in differentiating EBs	Whole EBs	Upregulation of *Hoxa4, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxb7, Hoxb8, Hoxb9*	McKinney-Freeman *et al.* (2008)
Cdx4	Ectopic induction in differentiating EBs	Whole EBs	Upregulation of *Hoxa6, Hoxa7, Hoxb9*	McKinney-Freeman *et al.* (2008)
Cdx1	Gene deficiency in EBs	Hematopoietic cells ($CD41^+$)	Downregulation of *Hoxa2, Hoxa3, Hoxa4, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxb1, Hoxb3, Hoxb6, Hoxb9*	Wang *et al.* (2008)
Cdx2	Gene deficiency in EBs	Whole EBs	Downregulation of *Hoxa2, Hoxa3, Hoxa4, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxb3, Hoxb4, Hoxb5, Hox6a, Hoxb7, Hoxb8, Hoxb9*	Wang *et al.* (2008)
Cdx4	Gene deficiency in EBs	Whole EBs	Downregulation of *Hoxa3, Hoxa4, Hoxa6, Hoxb3, Hoxb5*	Wang *et al.* (2008)
Cdx4	Gene deficiency in EBs	Hematopoietic cells ($CD41^+$)	Downregulation of *Hoxa2, Hoxa3, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxb1, Hoxb3, Hoxb9*	Wang *et al.* (2008)

Table 1 **Effect of *Cdx* genes on *Hoxa* and *Hoxb* clusters in EBs.** This overview shows that modifications in *Cdx* gene expression during EB differentiation and hematopoietic development affect a broad range of *Hox* genes along the *a* and *b* clusters, but is most sensitive to posterior *Hox* genes.

Orthology between mouse and zebrafish *Cdx* genes

The results of Wang *et al.* (2008) and Koo *et al.* (2010) contradict those of Davidson *et al.* (2003), who observed severe hematopoietic defects in *cdx4* mutant zebrafish. However, whereas mammals have three *Cdx* genes that are known to be involved in hematopoiesis, zebrafish only have two, since *cdx1b* is only known to play a role in the zebrafish intestine (Cheng *et al.*, 2008; Flores *et al.*, 2008; Chen *et al.*, 2009). This suggests that the functions of the zebrafish *cdx* genes are, with regard to hematopoiesis, less redundant and more critical than mammalian *Cdx* genes. Koo *et al.* (2010) propose that it is therefore likely that the crucial functions of *cdx1a* and *cdx4* genes in zebrafish can be extended to the mammalian homolog *Cdx2*. However, Flores *et al.* (2008) reason that *cdx1b* is functionally equivalent of *Cdx2*.

Nowadays, the orthology between mouse and zebrafish *Cdx* genes is still a matter of debate. When exploring orthologous genes, comparisons between gene sequences is not sufficient. One should also investigate in which developmental processes the gene is activated within the organism. Obviously, mice and zebrafish do not employ *Cdx* genes in an identical manner.

Cdx genes bear reminiscent functions in diverse developmental processes

The function of the *Cdx* genes in hematopoiesis appears to be reminiscent of other function of these genes in the developing embryo. The *Cdx* genes are also required for correct patterning of the A-P axis and for posterior tissue expansion during embryogenesis (Van den Akker *et al*., 2002; Chawengsaksophak *et al*., 2004, Young *et al.*, 2009). Inactivation studies with *Cdx1* and *Cdx4* showed that neither single nor simultaneous reduction of these genes jeopardizes axial elongation (Subramanian *et al*., 1995; Van Nes *et al*., 2006). However, *Cdx2* heterozygosity in the *Cdx4* null background led to a severe truncation and thus a premature arrest of axial extension (Young *et al.*, 2009).

Wang *et al.* (2008) demonstrated that combined inactivation of *Cdx1* and *Cdx4* decreased only the formation of erythroid progenitors more significantly than single inactivation of *Cdx1* or *Cdx4*. Simultaneous reduction of *Cdx2* and *Cdx4* significantly reduced hematopoietic progenitor formation. *Cdx1* and *Cdx2* knockdowns in *Cdx4*$^{-/-}$ embryos almost eradicated all blood development. These results indicate an essential role for *Cdx2* and more redundant roles for *Cdx1* and *Cdx4* in both axial elongation and hematopoiesis in the murine embryo.

In both these developmental processes, the *Cdx* genes signal by activating certain *Hox* genes. Young *et al.* demonstrated that in Cdx2$^{+/-}$/Cdx4$^{-/-}$ mutants, gain of expression of *Hoxb8* significantly restored the length of the axial skeleton. Also *Hoxa5* was able to rescue the posterior truncation of these mutants, suggesting that both *Hox* genes act downstream of *Cdx* in axial elongation. As for hematopoiesis, ectopic expression of *Hoxb4* could rescue the *Cdx2* null mutant phenotype (Wang *et al.*, 2008). Moreover, *Cdx4* activates *Hoxb4* in hematopoietic progenitors, indicating that this *Hox* gene is downstream of the *Cdx* genes during hematopoietic ontogeny (Wang *et al.*, 2005). In addition, induction and transfection studies with *Cdx* genes have shown that they act in embryonic hematopoiesis by influencing the expression of target *Hox* genes (Davidson *et al.*, 2003; Wang *et al.*, 2005; McKinney-Freeman *et al.,* 2008).

A third comparison between hematopoietic ontogeny and axial extension with regard to the role of *Cdx* is the regulation by upstream molecules. Several studies demonstrated that Wnt signaling acts upstream of *Cdx* gene expression during A-P patterning (Pilon *et al.*, 2006; Ikeya & Takada, 2001; Forlani *et al.*, 2003). Lengerke *et al.* (2008) provided evidence that a linear BMP-Wnt-*Cdx*-*Hox* pathway is required during hematopoietic development. Together, these data indicate that *Cdx* is under the control of Wnt signaling during diverse developmental processes. However, a remaining question hampers the comparison between the involvement of *Cdx* genes in axial growth and hematopoiesis: does gain of function of Wnt signaling rescue the hematopoietic defects in *Cdx* mutants, as was demonstrated for axial extension (Young *et al.*, 2009).

To what extent do embryoid bodies represent embryonal hematopoiesis?

Since the developing embryo is difficult to access, early hematopoietic development is often studied in *in vitro* ESCs, which differentiate into cystic EBs. This system has proven to be a powerful tool to explore molecular pathways underlying hematopoietic development, including research on the role of the *Cdx* genes on embryonic hematopoiesis described in this thesis (Davidson *et al.*, 2003; Wang *et al.*, 2005; Lengerke *et al.*, 2008; McKinney-Freeman *et al*., 2008; Wang *et al.*, 2008).

Although this *in vitro* system is an effective model, EBs develop quite differently from *in utero* embryos. EBs consist of semi-organized tissues of all three germ layers, but lack embryonic patterning (Daley, 2003). Therefore, when studying embryonic hematopoiesis *in vitro*, one should take into account that hematopoiesis in EBs may not faithfully recapitulate *in vivo* blood formation.

The first wave of hematopoiesis in the EB resembles the initial blood cell emergence in the developing embryo. Both in the yolk sac and in the EB, the first hematopoietic cells that arise are primitive erythrocytes. These cells exist only temporarily. They are followed both in the yolk sac and in the EB by adult enucleated erythrocytes and multipotential myeloid progenitors emerge. The most primitive hematopoietic progenitor in EBs is the blast colony-forming cell, which is capable of generating definitive erythrocytes and multilineage myeloid colonies in a colony-forming assay. Multipotential lymphoid-myeloid progenitors only arise in the embryo proper. Without induction of hematopoietic genes in ESCs, lymphoid precursors do not develop in this *in vitro* system. Thus, hematopoiesis in EBs is most similar to blood development in the embryonic yolk sac (Daley, 2003).

Lengerke and colleagues compared the *in vitro* colony-forming ability and *in vivo* repopulation capacity of *Cdx4/Hoxb4*-treated ESC-derived hematopoietic progenitors with adult bone marrow cells. ESC-derived hematopoietic progenitors have more potent *in vitro* clonogenic properties than adult bone marrow, since they displayed an enrichment in early colony-forming progenitors, with an 8-fold increase in CFU-GEMM colonies and a 10-fold increase in myeloid colonies. However, ESC-derived hematopoietic progenitors showed a lower repopulation capacity when transplanted *in vivo*: ESC-derived hematopoietic cultures resulted in a 16-fold decrease in CFU-S than adult bone marrow. According to Lengerke *et al.*, this difference could be explained either by a reduced proliferative capacity of ESC-derived progenitors or by an impaired ability to engraft the adult bone marrow (Lengerke *et al.*, 2007).

Therapeutic approaches for *Cdx*

Transplantation of bone marrow derived HSCs is the standard treatment for leukemia and genetic blood disorders. However, HLA-matching bone marrow donors are not always available and the procedure is agonizing. Therefore, *in vitro* culture of genetically matched HSCs would be a welcome alternative. The results from Wang *et al.* (2005) indicate that induction of *Cdx4* and *Hoxb4* in ESCs generates self-renewing, multipotential HSCs. Use of similar application for the derivation of HSCs out of human stem cells could provide treatment for these diseases. Utilizing induced pluripotent stem cells will circumvent ethical aspects of this specific treatment.

Ectopic expression of *Cdx4* in bone marrow cells caused AML (Bansal *et al.*, 2006), yet loss of this gene resulted in minimal hematopoietic impairment (Koo *et al.*, 2010). These results imply that *Cdx4* is in principle an attractive therapeutic target for curing AML (Koo *et al.*, 2010).

Suggestions for future research

This thesis shows that lots of research has been performed on the role of the *Cdx* genes in embryonic mammalian hematopoiesis. Koo *et al.* (2010) and previous studies (Subramanian *et al.*, 1995; Chawengsaksophak *et al.*, 1997; Chawengsaksophak *et al.*, 2002; van den Akker *et al.*, 2002; van Nes, *et al.* 2002) demonstrated that, with regard to adult blood formation, loss of these genes marginally affects hematopoiesis and vasculature formation.

Bansal *et al.* (2006) studied the role of *Cdx4* in AML, whereas Rawat *et al.* (2004) and Scholl *et al.* (2007) performed this for *Cdx2*. Currently, the role of *Cdx1* in malignant hematopoiesis is under exposed. Investigating this function by overexpressing *Cdx1* in the adult bone marrow and subsequent transplantion assays can elucidate if *Cdx1* has a similar role in leukemia as *Cdx2* and *Cdx4*. In case of evidence for a role of *Cdx1* in malignant hematopoiesis, one should critically reinvestigate the effect of *Cdx1* on adult blood formation. When this, alike *Cdx4*, results in minimal hematopoietic impairment, *Cdx1* is also in principle an attractive therapeutic target for curing leukemia.

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