# *Hox* genes and mesenchymal stem cells

*Hox* genen en mesenchymale stam cellen

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**Memories shape our lives.**

Memories remind us of our successes and our failures,
allow us to daydream and to drift in time.
In memories, we can revisit the beautiful places of our world
and relive experiences, which made us what we are today.

**Steve Parish**

## Contents

# 1

# General introduction

## 1.1 Introduction

The vertebrate body is a remarkable collection of different tissues and cell-types with unique functions, shapes and sizes. The diversity of cells is the result of precise regulation of gene expression during development and cell differentiation of stem cells towards their final mature state.
Stem cells are unspecialized cells that are the source of all tissues and organs that we possess during our life. The best-known stem cells are embryonic stem cells that are capable of forming all the different cell types of our body. During our life, specialized cells will often need to be replaced due to normal turnover or injury. To be able to replace the lost cells, our body possesses pools of tissue-specific stem cells and lineage committed progenitors. An organized balance between stem cells and mature cell populations is crucial for the long-term maintenance of functional tissue types. Stem cells maintain this balance via fine-tuned regulation of processes like self-renewal, proliferation, differentiation and apoptosis. This means that genes that are responsible for these processes need to be turned on and off, either in a programmed fashion or in response to the environment of the cell. If the regulation of gene expression is disturbed this can potentially lead to tissue malformation or disease.

## 1.2 Stem Cells

Stem cells are defined as cells that are clonogenic, self-renewing, and unspecialized with the capacity to differentiate into multiple cell lineages. The ability to self-renew is arguably the most important characteristic of a stem cell as it allows for extended production of differentiated cells throughout the life span of the animal. Multipotent cells with (limited) clonogenic behavior, but lacking self-renewal are termed progenitor cells. However, for a number of populations that have been termed stem cells, the criterion of self-renewal has not been determined. Because progenitor cells may closely resemble stem cells in terms of their properties, and because stem cells are not always readily testable for the above-mentioned criteria, some confusion will likely continue to exist (33,133,203,283).

Stem cells are not very easy to define, but based on their known abilities to differentiate into a particular set of cell types, they can roughly be classified in three groups. The first group is called totipotent or omnipotent and is represented by the zygote that can give rise to the complete embryo as well as the trophoblast. However, even this most primitive stem cell does not actually meet the stem cell criteria as they are poorly clonogenic and have a very limited self-renewal capacity (305). A little later in embryonic development, a second group of stem cells emerges that are called pluripotent stem cells. These cells can, similar to the zygote, differentiate towards cells of all three germ layers (endoderm, mesoderm, and ectoderm), but not to the trophoblast. The best studied pool of pluripotent cells are the embryonic stem cells, which can be derived from the inner cell mass of the blastocyst (272). The last group contains the multipotent cells, which are capable of differentiation within a single germ layer. These cells are also called tissue stem

cells, or adult stem cells, as they may be responsible for maintaining tissue homeostasis by replacing cells that are lost through normal turnover or injury (183). Tissue-specific stem cells have been described for many organs that have the capability to regenerate or repair. One well-characterized tissue stem cell type, which has been shown to meet the stem cell critera mentioned above is the hematopoietic stem cell. Hematopoietic stem cells (HSC) mainly reside in the bone marrow and are responsible for the supply off all the different blood cell lineages that are produced during our life (80,225). While studying hematopoiesis, Friedenstein and coworkers discovered that the bone marrow might contain at least two types of stem cells. It does not just contain the hematopoietic stem cells, but also contains precursors with osteogenic potential (99,239). This osteogenic precursor is a component of the bone marrow stroma. This is a supportive tissue of major importance for the formation of the hematopoietic microenvironment, in which the hematopoietic stem cells reside, proliferate and differentiate (203,307). Soon after the initial finding of the osteogenic precursors it became clear that these cells were able to differentiate towards a whole set of different mesenchymal tissues. These cells are usually described as stromal stem cells or mesenchymal stem cells (MSC).

## 1.3 Mesenchymal Stem Cells

### 1.3.1 Definition

MSC are defined as multipotent cells with mesenchymal differentiation potential towards for example cartilage, bone, fat, smooth muscle, hematopoietic supportive stroma, pericytes and tendon (69,241,288). Like for many other tissue stem cells, it is unclear whether cultured MSC should actually be regarded as stem cells or as early committed cells that have been derived from a more primitive stem cell pool (133). The arguments against the term stem cells for cultured MSC are based on the observation that only a fraction of MSC in culture retain a clonogenic potential (91). Also, they have not been properly assayed in vivo for properties like self-renewal and capacity for multilineage reconstitution, although they have been shown to contribute to bone repair upon transplantation (18). One major problem is that is it is unknown how MSC expanded in culture reflect the in vivo situation. To date, no specifically defined cell markers have been described that would allow direct identification of MSC in a tissue (see also below; CFU-F assays). However, taken the fact that mesenchymal tissues are subject to replacement and repair during the entire life of an organism, it is generally accepted that MSC exist in vivo but just lack a precise definition that would allow their isolation and comparison to their cultured derivatives (19,241).

### 1.3.2 Clinical applications

Because of their ability to differentiate towards several mesenchymal cell types MSC are interesting for tissue engineering, and many studies have been dedicated to the development of therapeutic (orthopaedic) strategies (81). MSC were also reported to undergo trans-differentiation towards multiple non-mesenchymal cell types (9), for example neurons (164), hepatocytes (173) and cardiac muscle (125) either upon addition of specific stimuli in vitro, or via induced injuries in vivo. However, some of the trans-differentiation results are somewhat controversial and could be caused by cell fusion and poor histological characterisation (6,43,126,187) or might actually involve different types of tissue stem cells that were co-purified from the bone marrow (167,168). Another interesting characteristic of MSC is that these cells possess immunosuppressive properties, making them a very interesting tool for therapeutic bone marrow transplantation and targeted gene-therapy (17,19,351). The use of MSC in therapeutic strategies is summarized in a large number of reviews, e.g (19,21,28,210,251,254,354).

### 1.3.3 The CFU-F assay

The main reason that MSC have not been identified in vivo is the lack of definition of a unique set of membrane epitopes or gene products that can be tagged. Several investigators have reported combinations of cell surface proteins that should be absent or present on MSC (19,74,149,210,270).However, these are often too commonly expressed on a range of mesenchymal cell types to be useful for sorting MSC. The identification of MSC has therefore relied primarily on in vitro assays. A commonly used assay to study MSC is the colony forming unit-fibroblast (CFU-F) assay (92). This assay has been established for a wide range of mammalian species and is based on the ability of MSC to form plastic or glass adherent colonies, each from a single precursor. The cells in the colonies have a fibroblast-like morphology (see Figure 1) and can be expanded in vitro while maintaining the capacity to differentiate into mesenchymal cell types. Although CFU-F cultures are usually composed of a heterogeneous cell population, this assay has been used as an in vitro correlate for MSC potential and most of the current knowledge of MSC is based on analysis of these culture-expanded cells.

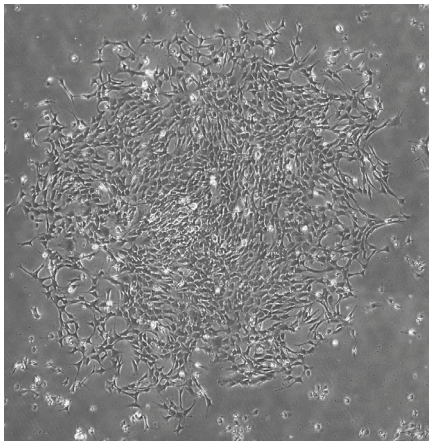

**Figure 1.**

A CFU-F colony, approximately 2 mm in diameter, from sternum bone marrow after 10 days of culture.

### 1.3.4 Tissue-specific properties.

In addition to bone marrow, MSC have been isolated via CFU-F cultures from nearly every adult tissue from adult mammals as well as from several tissues during embryonic development (65,95,136,206,264,273,324). Although the cells derived from different locations are often considered to be similar, they actually show marked phenotypic differences that are in part correlated to the tissue that they reside in. MSC from different organs have intrinsic differences in their proliferative capacity, differentiation potential and ability to support hematopoiesis. These differences are maintained during in vitro culture and also upon re-transplantation in vivo (65,94,96,98,136,273). This highly cell-autonomous behaviour of MSC again suggests that these cultured cells are a commited derivative from a still elusive parent. For example, it was shown that MSC from spleen and thymus were not able to spontaneously form fibrous tissue and bone when transplanted into the peritoneal cavity in a diffusion chamber, in contrast to MSC from bone marrow (93). Comparison between CFU-F of five different inbred mouse strains revealed enormous variation in MSC yield, the growth kinetics and the levels of alkaline phosphatase expression, suggesting a variety in osteogenic potential (244). A culture of human MSC suggested that the cells are present at various stages of differentiation and with distinct osteogenic potentials. Also, major differences in both growth rate and alkaline phosphatase activity between MSC from different individuals and even between MSC from different aspirates from the same individual were observed (245). Mesenchymal cell lines derived from different anatomical locations of the embryo have distinct expression of differentiation lineage markers. Human fetal MSC derived from BM, liver, lung and spleen were shown to have a comparable morphology and immunophenotype, but are different in their differentiation capacity (136). The basis of these differences is unknown and several researchers have embarked on doing expression studies on MSC from different sources e.g. (48,108,233,289,320,321,330,331,338). This showed clear

differences at the transcriptional level between MSC from different tissues. For instance, mouse fetal liver cell lines expressed high levels of extracellular matrix genes whereas cell lines from the aorta-gonad-mesonephros region (AGM) express more vascular smooth muscle genes (48). A serial analysis of gene expression (SAGE) of MSC derived from adult human bone marrow and umbilical cord show a lot of similarity, but expressed different combinations of differentiation markers (233). A gene expression study by Götherström et al (108) compared human fetal liver and adult bone marrow MSC. They speculated that fetal MSC have a higher proliferative capacity when compared to adult MSC as they express more genes involved in cell-cycle regulation, DNA repair and chromatin modification. Furthermore, because fetal cells expressed less lineage commitment markers they suggest that these cells are more multi potent than the adult cells.
However the key factors that define the MSC characteristics have yet to be identified. More research is needed to answer the question whether the different sources of MSC represent the same cell type, and how these are related with respect to their embryonic origin.

### 1.3.5 Residence and origin

It is unclear whether all MSC originate from a common precursor population in the embryo and later colonize different organs, or arise locally from organ primordia.
One possible pool of pluripotent cells from which MSC could be derived during adult life, are the MAPC, which stands for the somewhat confusing name multipotent adult progenitor cells (145). The isolation of MAPC relies on the same sources and the same adherent properties as that of MSC although additional purification steps are added to enrich this extremely small population of cells. In contrast to MSC, MAPC are not restricted to mesenchymal lineages, but differentiate in vitro also into cells of visceral mesoderm, neuroectoderm, and endoderm. When injected into an early blastocyst, a single MAPC contributes to most, if not all, somatic cells (144). MAPC also seem to meet the criterion of self-renewal as they proliferate extensively for more than 100 population doublings without obvious senescence or loss of differentiation potential (325). However, no direct lineal relationship is known between MAPC and MSC and these are therefore treated as two independent stem cell populations. Detailed microscopic analysis led to the hypothesis that MSC or their derivatives reside in the blood vessel walls as multipotent pericytes (288,353). Phenotypical and morphological data from several researchers may also indicate that BM MSC are identical to the BM stromal supportive cells termed adventitial reticular cells (ARC) (149). Already in 1975 it was shown that ARC likely originate from the already preformed cartilage where these cells seem to prepare the microenvironment to which the hematopoietic cells migrate via newly formed blood vessels (54). It even remains possible that ARC, bone lining osteoblasts and pericytes, that all form part of an extended stromal marrow network all possess the multipotent capacity and could all be identified as MSC (149).

Alternatively, MSC might arise in a central location and later colonize different organs. During fetal development, CFU-F can be found at sites of active hematopoiesis. The appearance of MSC in the hematopoietic sites slightly precedes the migration of the HSC from the fetal liver to the bone marrow cavity (324). However, these experiments do not actually prove that the MSC colonize the tissues via migration.

### 1.3.6 Circulation

One of the reasons that colonization of MSC in the different tissues is not likely caused via migration in the blood stream is that the migratory properties of MSC via the circulation are rather poor. Although several researchers have reported the isolation of MSC from the adult bloodstream, others contradict this observation (172,190,246,326,337). However, it is possible that fetal MSC migrate more extensively (38,206).
Because of the therapeutic interest, several researchers have addressed the possibilities of MSC homing to either their original site of isolation or to injured tissue. MSC freshly prepared from the bone marrow home to the bone marrow when transplanted back into the mouse. These homing properties however become lost after culture for both human (146) and mouse (270)) MSC. More recent work suggests that targeting of MSC to specific tissues can be influenced via homing signals expressed by for example an injured tissue (22,169). To date, the question of MSC circulation is still a matter of controversy and more work is needed to solve this question.

### 1.3.7 Hox genes and MSC

It is still largely unknown what gene expression programs and external cues are responsible for regulating proliferation, self-renewal, homing, differentiation and tissue-specific differences of MSC. We specifically became interested in the question how tissue-specific properties become established in vivo and how they are maintained in vitro and in vivo. An interesting group of candidate genes that might play a role in this establishment of tissue specific cell identity are the *Hox* genes. *Hox* genes are widely expressed in mammals in a variety of tissues and organs, during both embryonic development and adult life (reviewed by Morgan (216)). They play important roles in tissue specification and cell identity during embryonic development, and likely also continue this role during adult life. Fibroblasts from different parts of the body express specific sets of *Hox* genes (47,266). It was suggesteds that combinations of *Hox* genes may provide cells in the adult with a lasting topographic “address”, and control region-specific functions in the adult body, analogous to their embryonic function. *Hox* genes are expressed in both fetal and adult derived MSC (2,108,243,331) and it is conceivable that they might be involved in encoding tissue-specific properties of MSC.

## 1.4 *Hox* Genes

*Hox* genes encode a family of transcription factors, and are organized in evolutionarily conserved clusters in the genome. They can be found in many species and have been identified in all studied bilateral animals and cnidarians. *Hox* genes were first recognized for their important functions during embryonic development. This was initially studied in Drosophila melanogaster by discovery of the Homeotic complex (*HOM-C*). Incorrect expression of *HOM-C* genes can lead to drastic changes in body morphology (homeotic transformations), in which one specific body segment or structure transformed into the likeness of another (186).
The *HOM-C* is the Drosophila homolog of the mammalian *Hox* clusters, and the chromosomal positioning of the *HOM-C* genes is strikingly similar to the *Hox* genes in mammals. However, whereas Drosophila only contains one *HOM-C*, split over two clusters, mammalian genomes contain four copies of its *Hox* cluster (*A-D*), thought to be derived through cluster duplication during evolution with some loss of individual genes (166). These four clusters contain a total of 39 genes subdivided in 13 paralogue groups (Figure 2).
*Hox*/*HOM* genes play an instrumental role during embryonic development. Despite large differences that exist between Drosophila and mammalian embryonic development, a lot of knowledge concerning gene structure, expression and function of mammalian *Hox* genes is derived from studying the role of HOM-C genes in anterior-posterior axis formation in Drosophila (185,204). Like HOM-C genes, *Hox* genes play an important role in embryonic patterning in which embryonic cells form ordered arrangements of differentiated cells to construct functional tissues and organs.

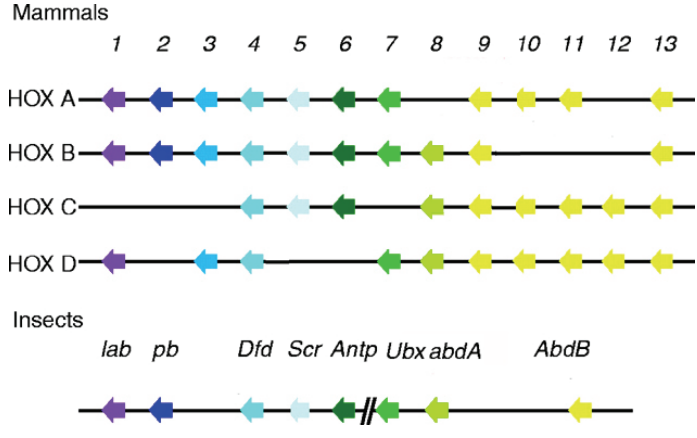


**Figure 2. The *Hox*/*Hom* clusters**
A schematic view of the 4 mammalian *Hox* clusters in comparison to the single homologous Drosophila HOM-C. Arrows represent *Hox* genes and their direction of transcription. The colors correspond to the various paralogy groups. These genes are most closely related in sequence, and are derived from a common ancestral *Hox* gene.

## 1.5 Colinearity

Looking at the genomic arrangement of the *Hox* clusters, a colinear relationship can be recognized between the physical gene order along the chromosome and their anterior boundary of expression and function along the developing embryonic body axis (77,111,158,186). In mammals, this colinear gene activation results in combinatorial expression of subsets of *Hox* genes in specific areas along the body axes that are translated into a regional identity. This specific regional expression coincides with a functional hierarchy, in which the products of more posteriorly expressed genes are often functionally dominant over the anteriorly expressed genes. This phenomenon is referred to as posterior prevalence (75). The combination of functionally active *Hox* genes that specifies a specific tissue or (vertebral) segment, is often referred to with the term: *Hox* code (157).
Several types of colinear expression have been described (158). First, spatial colinearity refers to the ordered array of spatially restricted domains in for example the paraxial mesoderm (77,111), neural tube (42), limbs (148,348), Müllerian system (312) and gastrointestinal tract (156,249), in which 3' genes (*Hox*1, 2, 3 etc) are expressed more anteriorly than the 5' genes (*Hox*11, 12, 13) of the gene clusters. Second, temporal colinearity refers to the timing of gene expression during embryogenesis in which 3' genes are expressed earlier than the 5' genes of the gene clusters (140).
The *Hox* code is not only determined by the presence or absence of *Hox* proteins, but also by their relative expression levels (112). During limb development, 5' *Hox*d genes show an increasingly higher level of expression than the subsequent neighboring 3' genes. This phenomenon is termed quantitative (reverse) colinearity (159).

## 1.6 Gene Regulation

Within the *Hox* gene clusters, the tight genomic organization is extremely important for correctly coordinated regulation of gene expression. It is also suggested that the clusters are kept together due to sharing of evolutionarily conserved regulatory elements that are instrumental for expression of the genes (76). Despite tremendous advances in the field, several questions on how gene expression is exactly initiated and maintained are still unanswered.
Gene transcription is a complex multi-level process that is not only dependent on the availability of transcription factors and RNA polymerase II, but also by the context of the chromatin and the cell-cycle stage. Eukaryotic genomes are organized into condensed, heterogeneous chromatin fibers throughout most of the cell cycle. Nucleosomes are the fundamental structural units of chromatin. They are comprised of a core histone octamer (H2A, H2B, H3, and H4), around which the associated DNA is wrapped. In order for the transcriptional machinery to access the DNA template, the packaging of eukaryotic DNA into nucleosomal arrays must be modified towards a transcriptionally active or 'open' state. Dynamic changes in chromatin folding can influence the availability of a gene to the transcription

machinery. The chromatin state is directly influenced by post-translational modifications of the amino-terminal tails of histones by remodeling enzymes that control the dynamics of chromatin folding (131,263).After a gene has been released from its 'closed' chromatin state, transcription is influenced by changes in nuclear concentrations of activator and repressor proteins that bind the DNA at specific binding sites. In mammals, every gene usually has its own promoter. This is a stretch of DNA sequence that is found directly upstream of the coding DNA and to which specific factors required for gene transcription bind. Transcription starts with the binding of the pre-initiation complex (PIC) to the promoter, followed by RNA polymerase II recruitment, which is responsible for the production of the messenger RNA. The PIC is a collection of transcription factors that assembles and stabilizes on specific recognition sequences that are present in the promoter (217). Promoter sequences often display little or no tissue specificity. Expression specificity is usually regulated by specialized cis-regulatory elements that either enhance the basal transcription of a promoter (enhancers) or repress the transcription (silencers/ repressors). These regulatory elements are basically a collection of protein recognition sites to which transcription factors can bind. Structurally they are very similar to promoters but lack a transcription start site. Enhancers do not follow a simple rule with respect to their location relative to the promoters. They can be found upstream (5'), downstream (3'), overlapping with promoters or in the intron of a gene. Enhancers can be very promiscuous in their promoter activation, but they can also be highly specific for a promoter and/or tissue (67,89,297).
To investigate *Hox* gene regulation, numerous transgenic and deletion experiments in the mouse have revealed the presence of both local and long distant cis-regulatory elements. These elements are involved in the control of tissue and cell specific expression, and can be responsible for the establishment of collinear expression by specification of the expression boundaries in the developing embryo (202,299,300). Most of this research has focused on elucidating the regulation of *Hox* gene expression during development. *Hox* gene regulation in the adult mammal on the other hand, is still an unexplored field.

### 1.6.1 Regulation of colinear expression

*Hox* gene expression can roughly be resolved into three overlapping phases: initiation, establishment and maintenance (71). Transcription initiation is thought to involve the binding of factors in the proximity of the *Hox* clusters that enables chromatin opening, and allows gene transcription. After initiation the *Hox* genes are sequentially activated to form well established expression domains, which are subsequently maintained through epigenetic mechanisms. Because most factors, as described below, that are thought to initiate *Hox* gene expression also play a role in establishment of the expression domains these phases are discussed together.

#### *Initiation and establishment*

It has been suggested that the *Hox* clusters undergo a progressive decondensation of the chromatin structure from 3′ to 5′ via, for example the progressive release from a

silenced state. This would allow increasing numbers of *Hox* genes to be activated while preventing posterior genes from being expressed too early during embryonic development. The precise nature of the underlying mechanisms however, is still to be established (46,158).
The most extensively studied potential activator of *Hox* gene expression initiation in the fetal hindbrain is retinoic acid (RA) (105). *Hox* genes in both embryonic presomitic mesoderm as well as cell lines respond with a colinear sensitivity in level and time to increased RA levels (175,200,234,247,290,291). However, whether retinoids are actually involved in the initial activation of the *Hox* genes in the primitive streak or only act in a later stage to regulate mesoderm segmentation is not completely clear (200,214,269).
Retinoids may act as ligands to activate two families of nuclear receptors, the RA receptors (RARα, β and γ), and the retinoid X receptors (RXRα, β and γ).
These nuclear receptors recognize cis-acting DNA sequences called retinoic acid response elements (RAREs) and heterodimerize on these target sites (115). Several clusters of RAREs can be found in the vicinity of 3' *Hox* genes (196) and it has been speculated that these play a role in the initiation of *Hox* gene expression by inducing chromatin decondensation and nuclear re-organization by extrusion of the genes out of their chromosome territory (46). However, for most of the potential RAREs their direct involvement in regulation of *Hox* gene expression has not been studied yet.

Recently it had been described that transcription in the *Hox* clusters shows a high complexity in both human and mouse. Unannotated non-coding transcripts can represent up to 60% of the total transcriptional output of a cluster and can be found on both sense and antisense strands (197). Addition of RA to cultured cells triggers this transcription of non-coding RNA (ncRNA) within the *Hox*A cluster (44,284). It has been hypothesized that production of non-coding transcription in regulatory regions of the *Hox*A cluster is involved in a RA-induced transcription activation process. The transcribed sequences are not conserved in evolution, and the transcribed sequences themselves do not have a direct regulatory effect on *Hox* gene activation (284). After chromatin decondensation, ncRNA expression within the clusters might play a role in transcription establishment of *Hox* genes by forcing an open chromatin state and allowing the activation of cis-regulatory elements (26,267,274,281,284), although transcriptional interference of ncRNA has also been reported (240,267). The actual function of these transcripts is still to be resolved.
In contrast, naturally occurring microRNAs (miRNAs) constitute a powerful route to dynamically silence specific gene expression programs by either translational inhibition or direct RNA degradation. Two conserved miRNA loci have been identified in the *Hox* clusters and their target sequences are present in the 3′-UTR of neighboring *Hox* genes. These miRNAs are expressed in embryos in a spatial pattern that is suggestive for a role opposing *Hox* gene expression (198,344). However, the actual functional contribution of miRNAs to *Hox* regulation has not been demonstrated yet.

Several other proteins that play an important role in the anteroposterior patterning of the embryo have been implicated in *Hox* gene activation. These include factors like

FGF, WNT and CDX proteins. The pathways in which these proteins act seem to contribute to RA receptor activation during somitogenesis, and could potentially influence the expression boundaries of *Hox* genes (24,72,79). Transcription of *Hox* genes in the presomitic mesoderm shows a coincidence with the dynamic expression of members of the Notch signaling pathway and it has been suggested that *Hox* genes are under influence of the segmentation clock (78,349). However there is as yet very little evidence of factors that directly interact with *Hox* genes.

### *Maintenance*

As mentioned previously, the context of the chromatin is very important in the process of gene expression. Chromatin structure is predominantly regulated through a set of posttranslational histone modifications such as methylation, acetylation, phosphorylation and ubiquitination (165). Studies of such histone modifications have suggested that the chromatin state contributes to the specification and maintenance of cell identity.

Posttranslational histone modifications catalyzed by trithorax group (trxG) proteins or
Polycomb group (PcG) proteins play important roles in dividing the genome into transcriptionally active and silent areas, respectively. These proteins work antagonistically via PcG or trxG response elements (PRE/TRE), which are specific DNA binding sites for these protein complexes (25) (265,298). Their enzymatic activities place epigenetic marks that are thought to propagate transcriptional memory from one cell generation to the next. PcG and TrxG proteins are found in multi-protein complexes, containing both histone methyltransferase activity, as well as proteins that bind methylated histone lysine residues. There are at least two distinct PcG complexes in mice. PcG repressive complex 1 (PRC1) contains Cbx, Mph, Ring, Bmi1, and Mel18. PRC2 (also termed EED-EZH2) contains Ezh2, Eed, and Su(z)12 (188).
Mutations in PcG genes lead to ectopic *Hox* gene expression and consequent posterior homeotic transformations in both Drosophila and vertebrates. Long-term repression of *Hox* genes is modulated by PRC2 mediated histone H3 methylation. Subsequent recruitment of PRC1 members then promotes condensation of the chromatin structure in which ubiquitination of histone H2 by PRC1 is supposed to be an essential step in *Hox* gene silencing (40).
Recently, a new family of histone H3 demethylases that oppose PcG mediated silencing was identified at *Hox* promoters during differentiation of stem cells. The recruitment of UTX and JMJD3 demethylases to the promoters of *Hox* genes seems to be required either for the transcriptional activation during differentiation of stem cells, or for the maintenance of expression in lineage-committed cells (3,4).

In comparison to PcG proteins relatively little is known about the mammalian TrxG protein complexes. Several complexes that contain TrxG proteins have been purified from Drosophila embryos, all with different chromatin-modifying properties involving methylation of H3 (195,293). In mice and humans however, there is only

limited knowledge involving some members of the SWI/SNF complex and the Mll protein (also named Mll1, All-1 and Hrx). The human SWI/SNF complex has been implicated in gene activation by a variety of activators, suggesting a broad function in control of gene expression (11) but so far no direct effect on *Hox* gene regulation has been described.
Mll is the homolog of Drosophila trx and can be part of in a huge protein complex with more than 20 partners (222). In contrast to SWI/SNF members, Mll has been implicated in *Hox* maintenance, and loss of the Mll protein is associated with a loss of *Hox* gene expression (121,250,342,346). MLL is able to bind specifically to *Hox* promoters and activates expression (208,222). On the other hand it also binds to a large domain within the transcriptionally active region of the *HOX*A cluster, which is thought to function in *Hox* gene maintenance and cell identity (116).

## 1.7 Function of Hox proteins

A common feature of homeobox proteins is the presence of the homeodomain. The *Hox* gene family belongs to the superclass of homeobox genes. The human genome contains over 200 predicted functional homeobox genes divided between 102 gene families (129). The homeodomain is a 60-amino-acid motif, which is highly conserved in evolution and present in proteins of fungi, plants, and animals. Proteins containing a homeodomain are usually classified as transcription factors.
Homeobox proteins modulate transcription through specifically recognised DNA recognition sites to which the homeodomain binds (101,102). However, Hox proteins display surprisingly weak DNA binding properties to the predicted recognition sites. In order to exert their function, Hox proteins often need to interact with other DNA-binding proteins, which act as cofactors. The cofactors that have been identified so far all belong to the PBC and MEIS classes of TALE (Three Amino acid Loop Extension) homeodomain proteins (212). It has turned out to be very difficult to predict potential interactions between *Hox* proteins and their cofactors. In addition, cofactor dependence also complicates the possibility to predict potential Hox target genes on the basis of protein recognition sites in the DNA. As a result only very few direct interactions of Hox proteins with target genes have been reported (182,306). However, despite the lack of knowledge with regards to target genes a substantial body of knowledge has been accumulated about the roles of Hox proteins within many pathways during both embryonic development (as described above) as well as some functions in the adult animal.

### 1.7.1 Function in adulthood

*Hox* genes continue to be expressed in the adult (216) where they seem to have partially maintained a spatial colinearity in which posterior tissues express more *Hox* genes compared with anterior tissues (47,266,308,343). For example during adult life, *Hox* genes are essential for processes like cyclic endometrial development and for endometrial receptivity in which also some colinear expression was reported (5,312). *Hox* proteins may be involved in skin repair (191) and it was hypothesized that they contribute to the positional identity that upon injury, influences the regeneration of the correct skin type in a certain location (268,282). Besides

functioning in (colinear) patterning processes, their function also extends to some non-colinear processes related to cell-identity during differentiation. This can for example be seen in hematopoiesis (1,193,316) and hair follicle morphogenesis (14) in which several Hox proteins play a role in the regulation of normal cellular proliferation and differentiation. As expected from proteins involved in proliferation, deregulation of *Hox* gene expression in adult tissues is often correlated with cancer (114), which has mostly been studied for hematopoietic malignancies (232). However, the function of most *Hox* gene expression detected in adult cell types and organs is not known. It is also unclear whether the *Hox* expression in adult mammals is the maintenance of some embryonic patterns, or is established postnatally to accomodate Hox functions in specific adult processes. Extensive regulatory and functional studies are required to answer this question.

## 1.8 Outline of this thesis

We hypothesised that Hox proteins may play a role in the establishment of regional differences between different MSC isolates. The first step in understanding the potential function and regulation of *Hox* genes in MSC is to establish their exact expression patterns. We have mapped *Hox* gene expression in MSC from different sites of the body. The resulting expression profiles could then be compared to their anatomical origin and their differentiation potential. This showed that *Hox* genes in MSC are expressed in region specific patterns (**Chapter 2**). Having established the expression patterns for all *Hox* genes in the MSC, we attempted to identify the responsible regulatory elements. These elements may be located either outside of or within the *Hox* clusters. By expressing differently sized *Hox* constructs we have narrowed down some regions that might play a role in activation and regulation of *Hox* gene expression in MSC (**Chapter 3**). In a parallel approach, we have attempted to identify enhancer elements responsible for *Hox* gene expression in hematopoietic cells. Hox proteins play an important role in proliferation and differentiation of a variety of hematopoietic lineages. We showed that the elements that are responsible for expression of *HOXA* genes in hematopoietic cells are most likely located at a remote position outside the cluster. Also we developed an assay, which could potentially lead to the identification of these elusive elements (**Chapter 4**).

# 2

# Mesenchymal stem cells from different organs are characterized by distinct topographic *Hox* codes.

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## 2.1 Summary

Mesenchymal stem cells (MSC) are multipotent cells found as part of the stromal compartment of the bone marrow and in many other organs. They can be identified in vitro as CFU-F (colony forming unit-fibroblast) based on their ability to form adherent colonies of fibroblast-like cells in culture. MSC expanded in vitro retain characteristics appropriate to their tissue of origin. This is reflected in their propensity for differentiating towards specific lineages, and their capacity to generate, upon retransplantation in vivo, a stroma supporting typical lineages of hematopoietic cells. *Hox* genes encode master regulators of regional specification and organ development in the embryo and are widely expressed in the adult. We investigated whether they could be involved in determining tissue-specific properties of MSC. *Hox* gene expression profiles of individual CFU-F colonies derived from various organs and anatomical locations were generated, and the relatedness between these profiles was determined using hierarchical cluster analysis. This revealed that CFU-F have characteristic *Hox* expression signatures that are heterogeneous but highly specific for their anatomical origin. The topographic specificity of these *Hox* codes is maintained during differentiation, suggesting that they are an intrinsic property of MSC. Analysis of *Hox* codes of CFU-F from vertebral bone marrow suggests that MSC originate over a large part of the anterioposterior axis, but may not originate from prevertebral mesenchyme. These data are consistent with a role for *Hox* proteins in specifying cellular identity of MSC.

## 2.2 Introduction

Adult mammals have limited capacity for regenerating lost tissues. Yet, many organs contain populations of multipotent stem cells that are capable of regenerating specialized cell types under certain conditions and that might offer perspectives for regenerative medicine, although relatively few of these have established roles in normal tissue homeostasis and repair. In addition, essentially all organs and tissues tested contain so-called mesenchymal stem cells (MSC). MSC were first identified by the pioneering work of Friedenstein and coworkers as a small population of osteoblast progenitor cells in bone marrow, which are normally quiescent but can undergo self-renewal in vivo (reviewed by Phinney (241)). They could be assayed in vitro as CFU-F (colony-forming unit-fibroblast), based on their ability to form colonies of adherent fibroblastoid cells in culture (92). Subsequently, work by several groups showed that CFU-F colonies contain multipotent cells that can be expanded in vitro while retaining the potential to differentiate into several mesenchymal cell types including osteoblasts, chondrocytes, and adipocytes (reviewed by Prockop (253)). Because of this capacity and because of their immunomodulatory properties (see review by Rasmusson (260)), MSC have great clinical potential for tissue engineering and repair, and as a vehicle for gene therapy strategies (reviewed by Prockop (254)).

MSC display remarkably tissue-specific regenerative potential when tested in appropriate in vivo assays. Thus, bone marrow MSC transplanted under the kidney

capsule can generate a bone and marrow organ that supports hematopoiesis by invading host-derived myeloid progenitors. In contrast, MSC from spleen generate a reticular stroma that primarily supports lymphopoiesis (94). Furthermore, bone marrow but not thymus MSC spontaneously differentiate into bone when transplanted intraperitoneally in diffusion chambers (90). In vitro, MSC from different organs also exhibit intrinsic differences in proliferative capacity and in the efficiencies with which they can be induced to differentiate towards specific mesenchymal lineages (65,136,273).

It is unclear how tissue-specific differentiation programs are regulated in MSC. Prime candidates are the members of the *Hox* family of transcription factors, which are master regulators of regional specification during embryonic development (reviewed by McGinnis and Krumlauf (204)). The 39 mammalian *Hox* genes are organized in four paralogous gene clusters located on different chromosomes. They are expressed during embryogenesis in a coordinated manner, resulting in overlapping, regionally restricted domains of expression. These expression patterns are characterized by an evolutionarily conserved correlation, termed colinearity, between the physical order of the genes within the clusters, and their anterior limits of expression. Thus, genes located more 3' in the cluster are expressed more anteriorly than more 5' genes (reviewed by McGinnis and Krumlauf (204)). *Hox* genes continue to be expressed in the adult in many organs (reviewed by Morgan (216)). Also, fibroblasts from different parts of the body express specific sets of genes including *Hox* genes (47,266). These data suggest that combinations of *Hox* genes may provide cells in the adult with a lasting topographic "address", and control region-specific functions in the adult body, analogous to the embryonic "*Hox* codes" (157) specifying regional differences in development.

The developmental origin of MSC is largely unexplored. In the mouse embryo, CFU-F can be found as early as embryonic day (E)11.5 (206,324), but lineal relationships between embryonic and adult CFU-F have not been established. As *Hox* genes are expressed in fetal as well as adult MSC (108,243,331), we embarked on a profiling study of *Hox* gene expression in CFU-F colonies derived from different organs, expecting to gain insight into (i) the lineage relationships among MSC, and (ii) the putative role of *Hox* proteins in modulating tissue-specific properties of MSC. In this study we show that CFU-F-derived fibroblasts have specific *Hox* gene expression signatures that correlate with their anatomical origin and suggest that CFU-F originate along a large part of the anteroposterior axis. We investigate how *Hox* gene expression is influenced by cell-extrinsic factors in CFU-F cultures, and find that CFU-F *Hox* codes are a largely intrinsic property of MSC. These data are consistent with a role for *Hox* proteins in determining cellular identity of MSC.

## 2.4 Results

***Distinct Hox gene expression signatures in CFU-F colonies from different locations***

To gain insight into the putative role of *Hox* proteins in establishing tissue-specific properties of MSC, and to obtain clues about the origin of MSC in the embryo, we investigated whether *Hox* genes are differentially expressed by CFU-F from different organs and anatomical locations. For this study we used primary CFU-F colonies, which, though comprising a heterogeneous collection of CFU-F-derived progeny with an admixture of non-fibroblastic, primarily hematopoietic cells (discussed by Prockop (253) and Prockop et al (254); and see below), represent the closest approximation to the individual CFU-F that is amenable to gene expression profiling. Seeding densities and CFU-F frequencies for the tissues analyzed are listed in Table 1.

**Table 1.** Plating conditions and CFU-F frequencies.

| Tissue of origin | Seeding density (x$10^4$ nucleated cells /$cm^2$) | Number of CFU-F colonies per 20$cm^2$ plate ¶ | Average CFU-F frequency (/$10^6$ nucleated cells) |
|---|---|---|---|
| Lung | 10 | 2; 5 | 2 |
| Thymus | 100 | 19; 24 | 1 |
| BM - sternum | 5 | 16; 16; 18; 9 | 15 |
| BM - forelimb | 5 | 4; 8; 10; 12 | 9 |
| | 10 | 13; 32 | 11 |
| BM - femur | 10 | 21; 23; 24; 28; 35 | 13 |
| BM - tibia | 10 | 18; 21 | 10 |

¶ numbers presented are a typical set of data obtained in a single experiment (i.e., experiment 2 described here)

Initially, we derived an average expression profile by pooling cDNA from several colonies (pools P1 and P2 representing two independent experiments, see Fig. 1). This analysis revealed that genes from all four *Hox* clusters are expressed in CFU-F colonies, in complex patterns that are different according to their origin (Fig. 1). These differences were reproducible, as P1 and P2 profiles of colonies from the same location were largely in agreement. Since clonally expanded CFU-F from the same tissue can exhibit considerable heterogeneity in differentiation potential and gene expression (93,171,189), we investigated whether differences between P1 and P2 could be due to these pools having been sampled from a population of CFU-F colonies that is heterogeneous also with respect to *Hox* gene expression.

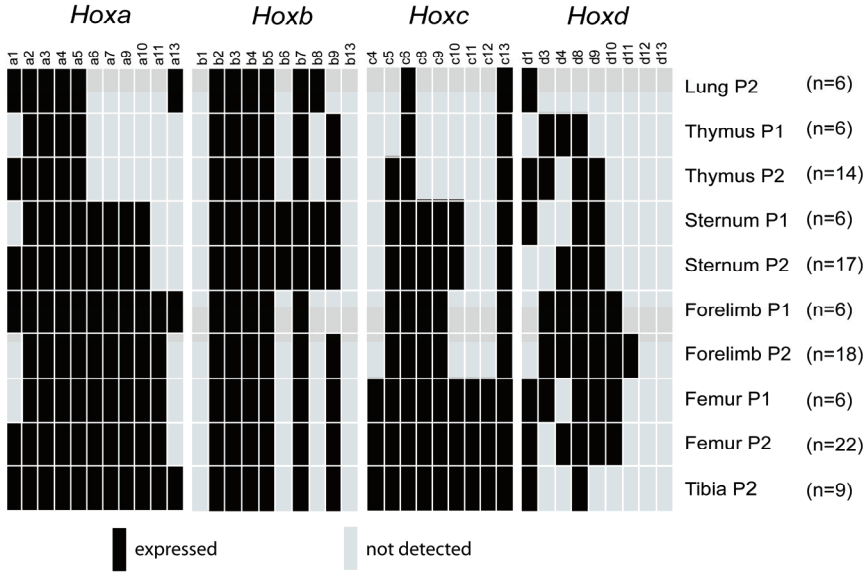



**Figure 1. *Hox* gene expression profiles of CFU-F from different tissues.**
Gene expression was analyzed in cDNA pools comprising six to twenty-one individual colonies as indicated (n=..), P1 and P2 representing pools of colonies isolated in independent experiments.

Thus, we determined *Hox* gene expression profiles for each individual CFU-F colony represented in the P2 pools. To objectively assess the degree of similarity between these profiles, hierarchical clustering was applied to the single-colony profiles as well as the averaged profiles P1 and P2, resulting in the dendrogram shown in Fig. 2. This analysis revealed significant heterogeneity among CFU-F colonies from each of the different locations; nevertheless, the vast majority of expression patterns segregated into coherent groups representing different tissues of origin. This was evident when the dendrogram was cut at a level yielding five clusters, comprising four major clusters representing sternum, forelimb, hindlimb and lung/thymus (Fig. 2).
A few profiles ended up in the "wrong" cluster (Thymus 10, Thymus 7 and Forelimb 13 in the sternum cluster) or outside any of the four main anatomical clusters (Femur 1 and Femur 2). Whether this is due to natural variation in gene expression, or rather to technical issues, cannot be determined based on the present data. Due to the limited amount of cDNA obtained from individual colonies, replication of PCR assays was not possible. However, retrospective analysis of the data (see Supplemental data, Table S2) suggested that on the whole, expression levels in individual colonies were well above the limit of detection, which is corroborated by the reproducibility of the P1 and P2 PCRs (see Materials and Methods). This analysis also suggested that expression profiles of pooled cDNAs represent a

weighted average of the expression in individual colonies. We conclude that the heterogeneity observed is likely to be biological in origin, which is in line with the known functional heterogeneity among CFU-F colonies. Importantly, all expression profiles P1 and P2 were located within their corresponding clusters, indicating that they are representative of the single-colony profiles in their clusters.
Within their cluster, CFU-F profiles from lung and thymus formed clearly distinct groups, in contrast to those from tibia and femur, which were completely dispersed within a common hindlimb cluster. By cutting the linkage dendrogram at a different level, lung and thymus CFU-F profiles could be segregated into two distinct and coherent clusters; however, this also caused other clusters to break up into subclusters (data not shown). This indicates that *Hox* expression profiles of lung and thymus CFU-F colonies represent distinct identities, but that the difference between them is small compared to the degree of heterogeneity existing within other clusters. Tibia and femur profiles, in contrast, did not partition into two homogeneous clusters at any level of the linkage tree (data not shown). Surprisingly, CFU-F colonies from the bone marrow of sternum, forelimb and hindlimb yielded distinct *Hox* signatures, demonstrating that the type of tissue is not the only determinant of the expression profile, and suggesting that topographical location is an additional, possibly the primary factor underlying these differences in expression.

***Influence of hematopoietic cells on Hox gene expression***

In agreement with the literature, most CFU-F colonies contained hematopoietic cells, as revealed by expression of the pan-hematopoietic marker CD45 (Ptprc; data not shown). Proliferation of CFU-F fibroblasts in vitro is known to be dependent on the presence of hematopoietic cells in the culture (18,97,170). The presence of hematopoietic cells might affect CFU-F *Hox* gene expression profiles in different ways. Firstly, signals from hematopoietic cells might influence transcriptional regulation of *Hox* genes in the fibroblasts. Secondly, since hematopoietic cells also express *Hox* genes, albeit at low levels (supplementary material of (30); our unpublished observations), they could contribute to the expression profiles in a direct manner. To evaluate the contribution of hematopoietic cells, fully formed CFU-F colonies from sternum and forelimb were depleted of hematopoietic cells using ATP-mediated cyanide poisoning (211). This effectively removed all hematopoietic cells from the cultures as determined by microscopic observation (see Fig. 3A), by FACS analysis using antibodies against CD45 (data not shown), and by RT-PCR detection of Ptprc mRNA (Fig. 3B). In agreement with previous reports (18,170,211), depleted colonies maintained in culture stopped expanding and eventually detached from the substrate after several days. Also, depleted fibroblasts could not be replated upon trypsinization, whereas untreated cells could be passaged repeatedly (data not shown). At the time of harvesting however, viability of the fibroblasts was not significantly compromised, as determined by FACS analysis of cells stained with 7-amino-actinomycin D (data not shown).

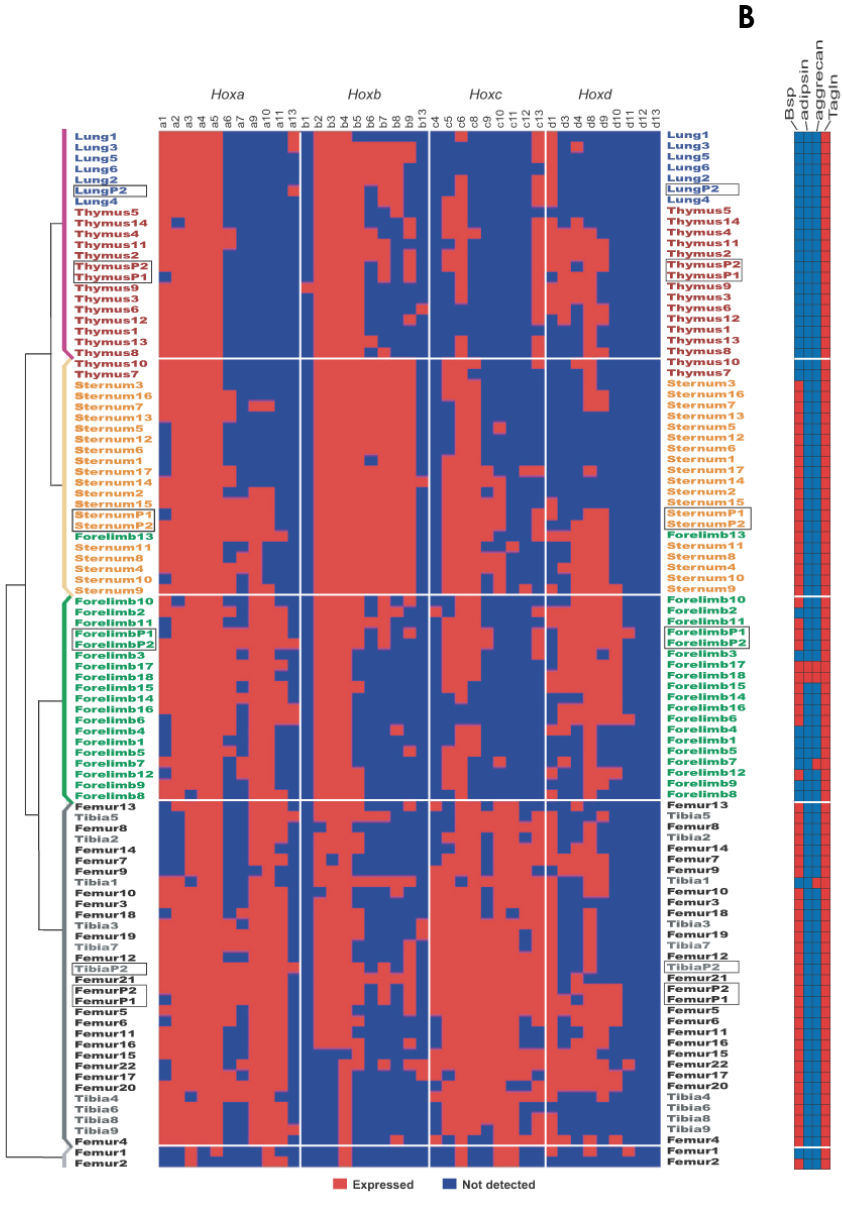



**Figure 2. Hierarchical clustering of *Hox* gene expression profiles.**
(**A**) Cluster analysis of *Hox* gene expression profiles of individual colonies and pooled cDNAs (black boxes). Profile names and colors indicate the tissue of origin, and gene expression was scored as expressed (red) or not detected (blue). The linkage dendrogram shown on the left was cut at a level yielding five clusters, indicated by brackets. (**B**) Expression of differentiation markers in the samples. These expression data were not used in the cluster analysis.

Expression of a subset of *Hox* genes, including genes differentially expressed in CFU-F colonies from these two tissues, genes expressed in both, and one gene expressed in neither, was analyzed in cDNA pooled from six colonies per tissue. All of these genes were expressed in qualitatively identical patterns in depleted and non-depleted CFU-F colonies (Fig. 3C). These data strongly suggest that the *Hox* profiles obtained faithfully reflect expression in CFU-F fibroblasts, and are independent of the presence of hematopoietic cells.

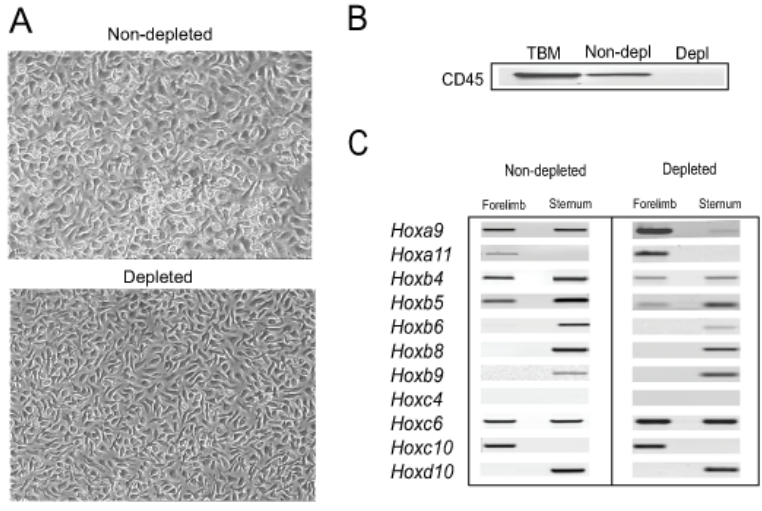



**Figure 3. Contribution of hematopoietic cells to *Hox* gene expression profiles.**
CFU-F colonies from forelimb and sternum bone marrow were depleted of hematopoietic cells, and gene expression was compared in pools of six cDNAs of depleted and non-depleted colonies from each location. (**A**), microscopic phase contrast images showing part of a non-depleted and a depleted colony. Note absence of birefringent hematopoietic cells on top of the fibroblast monolayer after depletion. (**B**), RT-PCR detection of *Ptprc* (CD45) mRNA in depleted and non-depleted colonies, compared with expression in a cDNA preparation of total bone marrow (TBM). (**C**), expression of selected *Hox* genes in depleted and non-depleted colonies of forelimb and sternum.

### Hox gene expression and differentiation

A certain amount of spontaneous differentiation is known to occur during culture of CFU-F, and it has been shown that single, clonal CFU-F colonies can simultaneously express markers for several different lineages (320,338). The smooth muscle marker Tagln (SM22α) was expressed in all CFU-F colonies, whereas expression of the chondrogenic and adipogenic lineage markers aggrecan and adipsin, respectively, was rarely observed (see Fig. 2B). Expression of the

osteogenic lineage marker bone sialoprotein (Bsp) on the other hand was detected in most bone marrow-derived CFU-F colonies but in none of the lung or thymus colonies (Fig. 2B). This suggests that CFU-F from bone marrow and non-bone marrow tissues have different propensities for differentiation along the osteogenic lineage, which agrees with previous in vitro and in vivo data (92,96).

Some *Hox* genes are differentially regulated during osteogenic (20,104,123,257,287) or adipogenic (60) differentiation. Therefore, we asked whether *Hox* profiles depend on the differentiation status of the cells, and in particular whether differences between bone-marrow and non-bone marrow *Hox* profiles could be attributed to the osteogenic differentiation that occurs in bone marrow CFU-F colonies. High-density CFU-F cultures were subjected to osteogenic and adipogenic differentiation conditions. As has been observed by others (65,136,273), cultures from different organs varied markedly with respect to the degree of terminal differentiation as determined by histochemical staining (see Fig. 4A). Interestingly, this was also true for bone marrow from different bones: CFU-F cultures from forelimb consistently differentiated poorly towards bone as well as fat. Not all of these differences were reflected in the levels of marker gene expression (Fig. 4B). Undifferentiated cells from all five sources expressed the early adipogenic marker PPARγ2, while expression of the late adipogenic marker adipsin showed the same tissue distribution as was observed for individual CFU-F colonies (Fig. 2). In contrast, the early and late osteogenic markers Runx2 and osteocalcin, respectively, were already expressed prior to induction of differentiation, in all of the cultures. This is most likely due to the higher cell densities in these cultures, since osteogenesis as detected by alkaline phosphatase staining is commonly observed in the dense center of CFU-F colonies (231) where proliferation ceases (231).

*Hox* expression profiles of undifferentiated cells resembled those of the corresponding P1 and P2 pools of CFU-F colonies (Fig. 4C, cf. Fig. 1; and data not shown). However, under these culture conditions expression of some genes, namely *Hox*a6, *Hox*a7, *Hox*c8 and *Hox*c9, and to a minor extent also *Hox*a10, was no longer bone marrow-specific, as they were also detected in lung and thymus cultures (Fig. 4, cf. Fig. 1; data not shown). This correlated with the expression of osteogenic marker genes in the latter cultures as described above. Nevertheless, during the subsequent three weeks of culture under differentiation-inducing conditions, the *Hox* codes for all five tissues remained unchanged compared to those of the undifferentiated cells (Fig. 4C, and data not shown), showing that topographic distinctions are maintained during terminal differentiation.

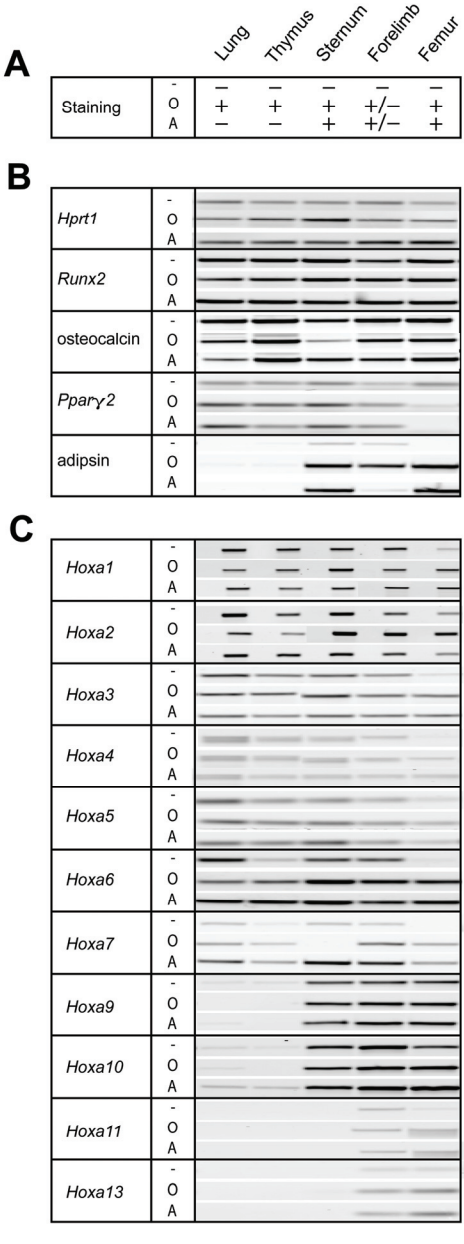


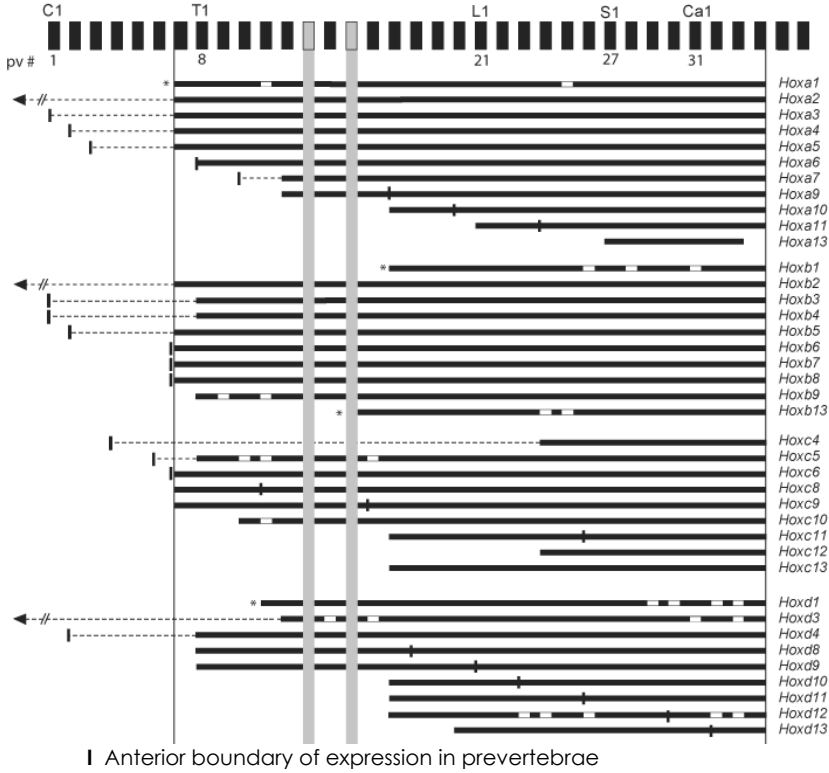

**Figure 4. Regulation of *Hox* genes during differentiation.**
CFU-F cultures from different tissues were analyzed before (-) and after 21 days of culture under osteogenic (O) or adipogenic (A) conditions. (**A**) Results of histochemical staining with Alizarin Red (for cultures in osteogenic differentiation medium) or Oil red O (for cultures in adipogenic differentiation medium). (+/- indicates that very few cells were stained.) (**B**) Expression of differentiation markers. (**C**) Expression of the *Hoxa* cluster.

***Do CFU-F Hox codes reflect embryonic patterning of the body plan?***

As *Hox* gene expression in the adult frequently follows expression patterns established in the embryo (216), we wondered whether the *Hox* codes observed in CFU-F colonies reflect the embryonic expression patterns of *Hox* genes in the corresponding tissue primordia. Making meaningful comparisons is hampered by the diverse and complex origins of the different organs under investigation, the often dynamic expression of *Hox* genes therein, and our lack of knowledge of the temporal appearance and lineal origin of CFU-F. To obtain a more clear-cut picture, we turned to the vertebrae, which are serial homologues with straightforward topographical relationships. They are formed from somitic mesoderm in a well-defined manner (reviewed by Christ et al. (55)), and are individualized at an early stage as prevertebral condensations of mesenchyme. Furthermore, expression of *Hox* genes in prevertebrae has been extensively documented. Hence, we asked whether *Hox* profiles of CFU-F from vertebral bone marrow correspond to the *Hox* codes observed in the embryonic prevertebrae (157).

Many vertebrae turned out to contain sufficient bone marrow for small-scale CFU-F cultures to be established. *Hox* profiles derived from such cultures displayed a remarkable degree of spatial colinearity, although with several exceptions to the rule that successively more 5' genes within a cluster are expressed increasingly less anteriorly, (Fig. 5A, grey bars). When these expression patterns are compared to the anterior boundaries of expression in the prevertebrae reported in the literature (vertical tacks in Fig. 5A), many discrepancies are apparent. For twenty genes it was possible to compare the expression boundary in prevertebrae with that in CFU-F cultures. Interestingly, within this group of genes, those from the 3' half of the clusters exhibited more posterior expression boundaries in vertebral CFU-F cultures than in the corresponding prevertebrae whereas, in contrast, more 5' paralog groups tended to show increasingly more anterior expression in CFU-F cultures than in the prevertebrae (see Fig. 5B). Clearly, these data are not consistent with a straightforward derivation of CFU-F *Hox* profiles from prevertebral *Hox* codes.

**A**




**I** Anterior boundary of expression in prevertebrae

**B**

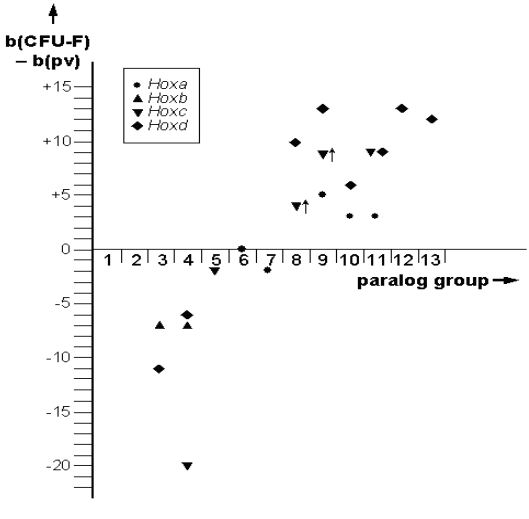


**Figure 5. *Hox* gene expression in CFU-F from vertebral bone marrow.**
(**A**) Expression domains of *Hox* genes in CFU-F. The vertebral column is represented schematically at the top of the panel, with the first cervical, thoracic, lumbar, sacral and caudal vertebrae (C1, T1, L1, S1 and Ca1, respectively) indicated above and the corresponding prevertebral numbers (pv #) below. Color-coding of vertebrae: green, CFU-F *Hox* gene expression profile successfully derived; white, insufficient bone marrow to establish a culture; grey, quality of cDNA was insufficient for expression profiling. The colored horizontal bars represent the anteroposterior domains of *Hox* gene expression in CFU-F as determined from the *Hox* profiles of cultures from individual vertebrae. Black vertical tacks indicate the anterior limits of gene expression in embryonic prevertebrae at E11.5 – E12.5, as reported in the literature, determined by in situ hybridization on sections using radioactively labeled probes. Arrowheads indicate that prevertebral expression boundaries lie more anteriorly than pv1. *, not expressed in prevertebrae at these stages. (**B**) Difference between the anterior boundaries of the expression domains in vertebral CFU-F [b(CFU-F)] and in the prevertebrae [b(pv)]. Circles indicate a defined difference, arrowheads indicate a minimum difference, as deduced from the data in panel A.

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## 2.4 Discussion

***Topographic Hox codes in CFU-F colonies***

It has long been recognized that MSC from different organs are different in many respects. Differential expression of some *Hox* genes in CFU-F cultures from different tissues has been observed in one previous gene expression profiling study (108). Here, we provide here the first evidence that MSC possess, or acquire during explantation and culture, a complex "*Hox* code" that is specific to the organ of origin and/or (possibly primarily) to the topographic location of the cells. Using degenerate PCR and cDNA cloning, Phinney and coworkers have previously derived a *Hox* gene expression profile of CFU-F cultured from hindlimb bone marrow (243). This profile almost completely overlaps with the hindlimb CFU-F profiles obtained by us, but lacks about 50% of the genes expressed by our hindlimb CFU-F colonies, and did not cluster with any of the CFU-F profiles (data not shown). The discrepancy between these profiles could have been caused by differences in culture conditions, but is more likely to be due to the fact that the profile obtained by Phinney et al. is dominated by a few abundantly expressed genes.

***Are CFU-F Hox codes inherent or induced?***

In keeping with previous data showing that the "identity" of CFU-F as reflected in their in vivo differentiation properties is maintained in vitro, topographic *Hox* codes appear to be an intrinsic property of CFU-F-derived fibroblasts. We found them to be independent of influences of hematopoietic cells in the cultures, and to be largely insensitive to culture under differentiation-inducing conditions. Whether *Hox* gene expression observed in CFU-F colonies reflects *Hox* codes pre-existent in the MSC from which they are derived, is an open question. Cell fate choice of MSC can be influenced by extrinsic cues, as evidenced by the finding that soluble signals released from injured skeletal muscle can instructively drive differentiation of MSC along the myogenic lineage in vitro (275). Thus, it is possible that MSC as they reside in different tissues are all equivalent and naïve, and acquire a specific identity only upon explantation and in vitro culture, which may mimic conditions of tissue injury. In preliminary transwell experiments to address this issue, we found no evidence for transfer of tissue-specific *Hox* codes by soluble factors from a heterologous CFU-F culture (data not shown). However, it is possible that this requires cell-to-cell contact, or higher concentrations of tissue-derived factors than can be attained in transwell cultures. Further experimentation will be necessary to conclusively address whether *Hox* codes are intrinsic or acquired, and whether they are maintained when MSC colonize a heterologous tissue, which may be relevant to transplantation of MSC in a clinical setting. The results described here provide a basis for addressing these questions.

***Functions of Hox proteins in MSC***

Our findings align well with the canonical role of *Hox* proteins as master regulators of regional diversification and organ development in the embryo, and are compatible with the hypothesis that *Hox* codes are part of a "blueprint" required for MSC to carry out a program of regeneration that is appropriate for the tissue they reside in. It remains to be established which aspects of tissue regeneration are regulated by *Hox* proteins, and whether currently available assays are suitable for investigating their function. Given the circumstantial evidence linking *Hox* genes to adipogenic and osteogenic differentiation (20,60,104,123,182,257,287), an interesting question is whether differential expression of *Hox* genes underlies the different propensities for osteogenic and adipogenic differentiation exhibited by bone marrow-derived and non-bone marrow CFU-F cultures (see Figs. 2 and 4). As multiple genes may be involved, and complex interactions among *Hox* proteins may determine how the mRNA *Hox* code translates into cellular phenotypes (see refs. (158,192) for reviews), elucidating the functional importance of *Hox* genes expressed in MSC will be a difficult task, which will be the subject of future studies.

***Hox genes and the embryonic origin of MSC***

It is not known how MSC populations are established in different organs. They could originate locally, from organ primordia or in adult organs. Alternatively, they might be born elsewhere and later colonize different organs. The developmentally earliest putative precursors of MSC thus far described are the mesangioblasts, which can be isolated from the dorsal aorta of the E9 mouse embryo (209). Later in development, CFU-F can be found at sites of active hematopoiesis (206,324), suggestive of an ontogeny parallelling that of the hematopoietic stem cells. CFU-F have been detected in the embryonic circulation (38,206), supporting the idea that MSC migrate to distant sites. However, MSC might primarily colonize tissues as constituents of the wall of ingrowing blood vessels (27). Consistent with a perivascular origin, expression of smooth muscle markers is commonly detected in CFU-F cultures (see, e.g., Fig. 2), which suggests that MSC are pericyte-like cells (69).

No lineage analyses pertaining directly to MSC have been done to our knowledge. However, results of fate mapping experiments in the chick suggest that vascular pericytes have heterogeneous origins. In the head, pericytes derive from the neural crest, which seeds the vascular primordia in a spatially coordinated manner (84). For the femoral bone marrow on the other hand, available data suggest that stromal pericytes originate in the femoral primordium, specifically the perichondrium (150). This is in agreement with histological observations made by others (reviewed by Simmons et al (292) ). If these findings can be extrapolated to the vertebrae, one might expect CFU-F colonies from vertebral bone marrow to have *Hox* codes corresponding to those of the prevertebrae. Yet, our data appear to be at variance with this. Different explanations are possible. It is conceivable that at the moment MSC are born, *Hox* gene expression domains in (pre)vertebrae or their precursors, are distinct from the prevertebral domains listed in Fig. 5, which were determined in E11.5-E12.5 embryos. Alternatively, MSC may originate from a tissue that is

patterned along the anteroposterior axis independently of the prevertebrae, and colonize the vertebrae in a coordinated fashion.
Clearly, more work needs to be done to reconcile these different lines of evidence. *Hox* expression profiling of embryonic CFU-F and lineage tracing experiments in different systems will be helpful in further elucidating the relationships between patterning of the body plan and ontogeny of MSC.

## 2.5 Materials and Methods

***CFU-F assay***

In all experiments FVB/N female mice (Harlan, The Netherlands) were used, which were sacrificed at eight weeks of age by cervical dislocation according to institutional guidelines. Sternum, femur and tibia (without joints) and humerus and ulna (combined, including the joints) were dissected out and attached muscle tissue was removed. Bone marrow was collected in $Ca^{2+}$/$Mg^{2+}$-free Hanks Balanced Salt Solution (HBSS, Gibco) supplemented with 10% fetal bovine serum (FBS, Summit Biotech, Fort Collins, CO, Lot# 50E42) by flushing and scraping with a hypodermic needle. Thymus and lung were cut into small pieces. Marrow and tissue fragments were dissociated by repeated pipetting, and the cell suspensions were filtered through a 70 μm nylon mesh filter (Cell-Strainer, Becton Dickinson). Nuclei were counted on a Beckman Coulter Z2 after cell lysis using Zap-oglobin II reagent (Beckman Coulter Inc.). Cells were pelleted at 200g for five minutes at room temperature and resuspended in DMEM (Cambrex, Belgium) supplemented with 20% FBS, penicillin/streptomycin and $2\times10^{-5}$ M ß-mercaptoethanol. Cells were plated in 6 cm polystyrene Falcon tissue culture dishes (Becton Dickinson) and cultured at 37°C in a humidified incubator in 5% $CO_2$ in air. The medium was replaced after 5 days of culture. At day 13, CFU-F colonies with a diameter greater than 1 mm were counted. CFU-F colonies which after 13 days of culture had attained a diameter of approximately 3-5 mm in diameter and had a compact, circular morphology (representing the majority of colonies) were lysed with 0.8 ml of Trizol reagent (Invitrogen) using stainless steel cloning cylinders.

***CFU-F cultures from individual vertebrae***

Individual vertebrae were dissected free of surrounding tissue and crushed in HBSS with 10% FBS to collect the bone marrow. The marrow was processed as described above for the standard CFU-F assay, and all cells collected from one vertebra were plated in a 6 cm polystyrene Falcon dish. After 7 or 10 days, when colonies began to overlap, the entire dish was trypsinized using 0.25% trypsin (Gibco), and replated in a single well of a 6-well plate (Greiner). Upon reaching confluency, the hematopoietic cells were depleted as described below, and cells were lysed in 1 ml of Trizol.

***Depletion of hematopoietic cells***

Dishes containing CFU-F colonies or monolayers were depleted of hematopoietic cells as described by Modderman et al. (211). In brief, cells were washed once with $Ca^{2+}$/$Mg^{2+}$-free HBSS, and incubated in HBSS containing 2 mM ATP (Sigma) for 5 minutes at 37°C. Then potassium thiocyanate (Baker, the Netherlands) was added to a concentration of 1 mM. After a further 30 minutes at 37°C, an equal volume of cold HBSS containing 8 mM $MgCl_2$ and 1% FBS was added to the cells. All liquid was subsequently removed and replaced with culture medium. This procedure was repeated after 24 hours, and cells were harvested 24 hours after the second treatment.

***Osteogenic and adipogenic differentiation***

Cell suspensions were generated from a total of four mice as described above for standard CFU-F assays, and the equivalent of 2 thymuses, 1.5 pairs of lungs, and bone marrow from 2 forelimbs, 1.5 sternums, or 1 femur was plated per 10 cm tissue culture dish (Greiner). The medium was replaced after 4 days and at day 11 the cells were lifted from the plate using 0.25% trypsin and seeded in seven 3.5 cm Falcon dishes, which yielded a near-confluent monolayer the next day, at which time the medium was replaced with differentiation medium. Cells were maintained in either osteogenic medium (DMEM with 10% FBS, 0.2 mM ascorbic acid, 1 mM β-glycerol phosphate and $10^{-8}$ M dexamethasone, all from Sigma) or adipogenic medium (DMEM with 10% FBS, 5 μg/ml Insulin (Sigma) and $10^{-9}$ M dexamethasone) for 21 days with medium replacement three times per week. Cells were harvested before transfer to differentiation medium and after 21 days of differentiation, and duplicate plates were used for histochemical staining and RNA isolation. Intracytoplasmic lipids in adipocytes were visualized with Oil red O (259), and matrix mineralization was visualized using Alizarin Red (113).

***Analysis of gene expression***

All cell lysates were supplemented with 10 μg of bakers' yeast tRNA (Roche Diagnostics) as a carrier, and total RNA was extracted from the Trizol fraction according to the manufacturer's protocol. The RNA was treated with 1 unit of DNase I (amplification grade, Invitrogen) for 30 minutes at 37°C. The enzyme was inactivated as recommended by the supplier, and the RNA was reverse-transcribed with oligo(dT) (Invitrogen) and SuperRT reverse transcriptase (SphaeroQ, the Netherlands) according to the supplier's protocol in a reaction volume of 25 μl with RNase inhibitor (RNase-out, Invitrogen). All cDNAs were diluted with deionized water to a final volume of 200 μl, and either 0.2 μl (for the transwell experiment) or three μl (all other samples) was amplified in a 25 μl PCR reaction, using the primer pairs listed in Table S1 (supplementary data) and either SuperTaq (SphaeroQ, the Netherlands), Taq DNA Polymerase (Invitrogen) or Silverstar DNA polymerase (Eurogentec, Belgium). Reaction conditions optimized for each primer pair are available on request. Hprt1 was amplified for 30 cycles, all other targets for either 35 cycles (for the differentiation experiment) or 40 cycles (all other samples). The

entire PCR reaction was run on ethidium bromide-stained agarose gel and the products visualized on a Typhoon 9200 Imager (Amersham). The images were processed with ImageQuant 5.2 (Molecular Dynamics), adjusting gamma and brightness for optimal visualization of PCR products. Using this procedure, a minimum of 1-2 ng of product was required to generate a visible band.
Gene expression in cDNA pools consisting of equal amounts of cDNA from six to twenty-one colonies was analyzed in duplicate. Duplicate reactions yielded identical results except for *Hox*d3 and *Hox*d4 in a few cases, presumably because of the very low yields of PCR product for these two genes (data not shown). In these cases, PCR reactions were repeated in duplicate, and expression was ultimately scored as positive or negative according to the result of the majority of the reactions. The results of (-RT) control reactions were negative in all cases (data not shown).

#### *Hierarchical cluster analysis*

Gene expression data were represented in binary format based on the presence (1) or absence (0) of PCR product. The binary *Hox* gene expression profiles were subjected to agglomerative hierarchical clustering using the Treescape module of the OmniViz® software package. The similarity between the profiles was determined using the Tanimoto metric, and profiles were arranged in a dendrogram based on average linkage.

## 2.6 Acknowledgments

We are grateful to Elwin Rombouts for generously providing reagents and advice on CFU-F assays, to Peter van der Spek for introducing us to cluster analysis and for providing computing facilities, and to Marcel Reinders for helpful discussions.
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## 2.7 Supplementary data

**Table S1.** Primers for RT-PCR analysis of gene expression

| Gene | Acc. nr. | Forward primer (5'-3') | Reverse primer (5'-3') | Ref.§ |
|---|---|---|---|---|
| **Hoxa1** | NM_010449 | CCAACTTCTGAGGCATCTGAC | GATGTCGGCTGGAGAACAGT | |
| *Hoxa2* | NM_010451 | GTGACTTCTCTTCCTCGTCT | CTCCACGGGCCCTGCCTG | |
| *Hoxa3* | NM_010452 | GTGCAGGAGGCTATCTGAAT | GCAGTAAGGTCCGTGTAGGT | |
| *Hoxa4* | NM_008265 | TGGATGAAGAAGATCCACGTGAGC | TGTGAGTTTGTGCTTTCCCAGG | |
| *Hoxa5* | NM_010453 | GCTGCACATTAGTCACGACAA | GCAAAGGGCATGAGCTATTTCG | (333) |
| *Hoxa6* | AF247663 | CTGGTGCCCTTGTCTGCTCC | GTGGCTCACAGAGCGCAGAG | |
| *Hoxa7* | BC036986 | GCTGGAACTGGAGAAGGAAT | GGGTTGTGGGTATCTACTGG | |
| *Hoxa9* | BC055059 | CTTCGTGTGGCAGATCATTC | CTCCATTACAATAAGCACTCACT | |
| *Hoxa10* | NM_008263 | GAAGAAACGCTGCCCTTACACGAAG | AACCCAGCCCATTCAGGACTTGAC | |
| *Hoxa11* | NM_010450 | GTGTGGTCACTGGAGATTGG | TGATGAGCACAGAGTGCAGG | |
| *Hoxa13* | NM_008264 | ACGGCCAAATGTACTGCCC | TCCCGTTCGAGTTCTTTCAAC | (333) |
| *Hoxb1* | NM_008266 | ATCAGCCTACGACCTCCTCT | AGCGTTGGAAGCCCAGTTA | |
| *Hoxb2* | NM_134032 | TTCGCTGCAGACTCCTGTC | CTGCAAGTCGATGGCACAG | |
| *Hoxb3* | NM_010458 | GCTTCATGAACGCCTTACAC | AAAGGTGATTGAGGCCGTAG | |
| *Hoxb4* | NM_010459 | AGAACCCCCTGCATCCCA | CCGAGCGGATCTTGGTGT | |
| *Hoxb5* | NM_008268 | AGTATGAGTCTGGCTACAGC | ATTGTAACACAGGACTGGGG | |
| *Hoxb6* | NM_008269 | CGCCAGACCTACACACGCTA | AGCACCTTCACTCGGCTGGC | |
| *Hoxb7* | NM_010460 | TACAATCGCTACCTGACTCG | CATCTGGAACCACAGTTCAC | |
| *Hoxb8* | NM_010461 | AGTACGCAGACTGCAAGCTCG | GCTCCTCCTGCTCGCATTTAC | (152) |
| *Hoxb9* | NM_008270 | GGCAAAGAGTAAAGATGACCAC | GCTCGACTAGGGTCTCTTTC | |
| *Hoxb13* | NM_008267 | CCAACGCTGATGCCAACTGT | AGCAGAGAATCGTGGCGAGG | |
| *Hoxc4* | NM_013553 | CGGCTTCAAGTACGACTACA | AGAAGAAGGAGAGAGCTTGC | |
| *Hoxc5* | NM_175730 | TGACTCCAAGCCCTTCCTGA | CCAACACCTCTTTACCAAGCA | |
| *Hoxc6* | NM_010465 | CTGACCGAGCGACAGATCAA | GCAGACAAGCCAGGAAGAAG | |
| *Hoxc8* | NM_010466 | ATCCTCCGCCAACACTAACAG | CTCATTCCCTTCTTCTTCCACC | (152) |
| *Hoxc9* | AK078758 | GGCAGCAAGCACAAAGAGGAGAA | TTGCGCTGGGGAAGAGAACG | (152) |
| *Hoxc10* | NM_010462 | ACCGCAGACTCCAGTCCAGA | AGATGACGCTGGCTCAGGTG | |
| *Hoxc11* | XM_111600 | CTACGTGCCTGAGTTCTCCA | GTAGGCGTTGTCGAAGAAGC | |
| *Hoxc12* | NM_010463 | GTTGGCTCGCGTGGAGGATA | CGAATACGGCTTGCGCTTCT | |
| *Hoxc13* | NM_010464 | CGTCAGGTCACCATCTGGTT | ACAAGCTGAGGCACAGGAAG | |
| *Hoxd1* | NM_010467 | CTGGCCGGAATCTAGGAAGC | CACGAAGAGGTAGGAGCGCA | |
| *Hoxd3* | AL928733 | CTGCATTCTCCAGCCGGTCA | GCGGCGCAACTGTAGTCCAC | |
| *Hoxd4* | J03770 | CGGCCTACACCAGACAGCAA | CCGCAGCTTCGCTCAACCTA | |
| *Hoxd8* | AK016033 | CTGGTAGACGGAGAGGAAGA | TAGGACAGCTCAAGGACTGC | |
| *Hoxd9* | NM_013555 | CACTACGGGATTAAGCCTGAAACC | TTTGGGTCAAGTTGCTGCTGC | |

| | | | | |
|---|---|---|---|---|
| *Hoxd10* | NM_013554 | CCTATGGAATGCAAACCTGTGG | ATATCCAGGGACAGGAACCTCG | (34) |
| *Hoxd11* | X71422 | CAAGTACCAGATCCGCGAAC | CTCAGGCTGTAGTGGTCGCT | |
| *Hoxd12* | X58849 | GTCTGGTTCATTCGGCTCTC | CAATTCCTTACGCTTCTGCC | |
| *Hoxd13* | NM_008275 | GCCTGGCTACATCGACATGG | GCCGCCGCTTGTCCTTGTTA | |
| **Hprt1** | NM_013556 | CACAGGACTAGAACACCTGC | GCTGGTGAAAAGGACCTCTC | |
| **Tagln** | NM_011526 | GCAAATTGGTGAACAGCCTG | TGCTTCCCCTCCTGCAGTT | (262) |
| ***aggrecan*** | NM_007424 | CACGCTACACCCTGGACTTTG | CCATCTCCTCAGCGAAGCAGT | (335) |
| **Pparg2** | NM_011146 | ATGAATTCCTTAATGATGGGAGAAG | GCCTGGGCGGTCTCCACTGAGA | (332) |
| ***adipsin*** | NM_013459 | ACTCCCTGTCCGCCCCTGAACC | CGAGAGCCCCACGTAACCACACCT | (18) |
| **Runx2** | NM_009820 | GGCAGCACGCTATTAAATCCAAA | TGACTGCCCCCACCCTCTTAG | (309) |
| **Bsp** | L20232 | CAAGCGTCACTGAAGCAGGTG | CATGCCCCTTGTAGTAGCTGTATT | (18) |
| ***osteocalcin*** | NM_031368 | CAGACAAGTCCCACACAGCAGC | AGAGCAGCCAAAGCCGAGC | (18) |
| **Ptprc** | NM_011210 | ATGACTCATGTGCTCCAGC | AGGTTTAGATACAGGCTCAG | (63) |

**Table S2.** Relationship between detection of *Hox* gene expression in P2 and the fraction of positive colonies in P2[a].

| | pool size | a1 | a2 | a3 | a4 | a5 | a6 | a7 | | a9 | a10 | a11 | | a13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lung** | 6 | 100 | 67 | 100 | 67 | 100 | 0 | 0 | | 0 | 0 | 0 | | 33 |
| **Thymus** | 14 | 100 | 93 | 100 | 50 | 100 | 14 | 0 | | 0 | 0 | 0 | | 0 |
| **Sternum** | 17 | 65 | 100 | 100 | 88 | 100 | 53 | 29 | | 47 | 24 | 0 | | 0 |
| **Forelimb** | 18 | 67 | 94 | 94 | 83 | 100 | 61 | 33 | | 100 | 100 | 50 | | 11 |
| **Femur** | 22 | 50 | 73 | 100 | 50 | 95 | 27 | 23 | | 86 | 100 | 86 | | 0 |
| **Tibia** | 9 | 78 | 87 | 100 | 44 | 100 | 44 | 22 | | 100 | 100 | 78 | | 22 |

| | pool size | b1 | b2 | b3 | b4 | b5 | b6 | b7 | b8 | b9 | | | | b13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lung** | 6 | 0 | 100 | 100 | 100 | 83 | 67 | 83 | 83 | 33 | | | | 0 |
| **Thymus** | 14 | 7 | 100 | 100 | 100 | 100 | 43 | 36 | 21 | 43 | | | | 0 |
| **Sternum** | 17 | 0 | 100 | 100 | 100 | 100 | 94 | 100 | 100 | 100 | | | | 6 |
| **Forelimb** | 18 | 0 | 100 | 100 | 100 | 44 | 11 | 22 | 11 | 17 | | | | 0 |
| **Femur** | 22 | 0 | 59 | 64 | 95 | 41 | 5 | 9 | 14 | 27 | | | | 14 |
| **Tibia** | 9 | 0 | 67 | 56 | 100 | 44 | 22 | 33 | 11 | 22 | | | | 11 |

| | pool size | | | | c4 | c5 | c6 | | c8 | c9 | c10 | c11 | c12 | c13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lung** | 6 | | | | 0 | 17 | 50 | | 0 | 0 | 0 | 0 | 0 | 83 |
| **Thymus** | 14 | | | | 0 | 43 | 79 | | 14 | 0 | 0 | 0 | 0 | 79 |
| **Sternum** | 17 | | | | 0 | 76 | 100 | | 100 | 29 | 47 | 6 | 12 | 6 |
| **Forelimb** | 18 | | | | 6 | 56 | 89 | | 89 | 6 | 6 | 0 | 0 | 17 |
| **Femur** | 22 | | | | 59 | 59 | 100 | | 86 | 55 | 100 | 91 | 64 | 68 |
| **Tibia** | 9 | | | | 44 | 89 | 89 | | 100 | 89 | 100 | 100 | 33 | 89 |

| | pool size | d1 | | d3 | d4 | | | d8 | d9 | d10 | d11 | d12 | d13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Lung** | 6 | 100 | | 0 | 17 | | | 0 | 0 | 0 | 0 | 0 | 0 |
| **Thymus** | 14 | 50 | | 50 | 50 | | | 86 | 36 | 0 | 0 | 0 | 0 |
| **Sternum** | 17 | 12 | | 6 | 29 | | | 53 | 41 | 6 | 0 | 0 | 0 |
| **Forelimb** | 18 | 44 | | 67 | 56 | | | 100 | 67 | 61 | 6 | 0 | 0 |
| **Femur** | 22 | 86 | | 36 | 54 | | | 82 | 45 | 23 | 9 | 0 | 0 |
| **Tibia** | 9 | 89 | | 0 | 0 | | | 44 | 33 | 0 | 0 | 0 | 0 |

[a], Indicated for each tissue are the number of colonies in P2 and the percentage of colonies in each pool that tested positive for expression of a given *Hox* gene. The background color of the cells reflects the outcome of the corresponding P2 PCRs Orange: P2 PCR positive, Blue: P2 PCR negative

# 3

# Transcriptional regulation of *Hox* gene expression in mesenchymal stem cells

Karin Ackema & Jeroen Charité

## 3.1 Summary

Mesenchymal Stem Cells (MSC) are multipotent cells that have been isolated from nearly every tissue or organ in the mammalian body. They can be analysed in vitro as CFU-F (colony forming unit-fibroblast), and upon expansion retain certain characteristics that reflect their tissue of origin. We have previously shown that CFU-F colonies derived from different locations in the adult mouse express characteristic combinations of *Hox* genes. However, it is unclear how these topographic expression patterns are established at the molecular level, and how these relate to the origin of MSC in the embryo. To begin to answer these questions, we have now investigated where the regulatory sequences responsible for driving *Hox* gene expression in CFU-F are located. The results of these studies suggest that most of these cis-acting elements are located within the confines of the *Hox* clusters. We also show that known region-specific enhancers can drive expression in CFU-F, but without any topographic specificity, and without any correlation to the expression in the corresponding cartilage primordia. These data corroborate the hypothesis that MSC in bone marrow do not originate locally from the same embryonic precursors as the bone they are found in, but colonize these from a distinct source.

## 3.2 Introduction

The vertebrate body is home to different kinds of multipotent stem cells that exhibit a limited differentiation potential, dedicated to a specific tissue or organ (183). Among these are the mesenchymal stem cells (MSC) that have been found in essentially all organs and tissues (65). MSC can be assayed in vitro, as colony forming units-fibroblast (CFU-F), based on their plastic adherence and clonogenicity. MSC expanded in culture can be induced to differentiate into several mesenchymal cell types including osteoblasts, chondrocytes, adipocytes and smooth muscle cells (reviewed by Prockop (253)). MSC from different organs have intrinsic differences in their proliferative capacity and differentiation efficiency (e.g., (65,94,96,98,136,273)). In asking how organ-specific properties of MSC are encoded, we have previously investigated the involvement of the *Hox* genes, which encode homeodomain transcription factors that are responsible for generating much of the regional diversity in the body. In vertebrates, 39 *Hox* genes have been identified that are organised in four paralogous clusters (A-D). During development, the clustered *Hox* genes are transcriptionally activated in a temporally and spatially coordinated manner, which correlates rather strictly with the linear order of the genes within their clusters. Genes located more 3' in the clusters, relative to the common direction of transcription, start being expressed at earlier stages, and are expressed in more anterior regions, than successively more 5' genes. These correlations have been termed temporal and spatial colinearity, respectively (reviewed by McGinnis and Krumlauf (204)).

Studies about the regulation of colinearity showed that the underlying diverse and complex mechanisms are often highly conserved. The most intensively studied regulatory elements of colinear *Hox* gene expression are region-specific enhancer

elements, many of which have been identified in the vicinity of the genes, e.g. (15,29,49,201,256,304,327,350). Enhancers can be shared among several genes (88,103,160,162,285), and can also be located outside the clusters. The latter has for instance been found for *Hox* gene activation during mouse limb development in which several *Hox*d genes are activated by an enhancer located more than 100 kb upstream of the cluster (300).
Spatial colinearity of *Hox* gene expression is preserved to some degree in the adult animal (reviewed by Morgan (216)). Gene expression analysis of whole organs (308,343) or fibroblasts derived from different parts of the body display expression profiles that resemble the fetal colinear *Hox* patterning (266). Along the same lines, we have recently shown that *Hox* genes in CFU-F colonies derived from different locations of the adult mouse are also expressed in a somewhat colinear fashion. The combinations of *Hox* gene expression products are specific for the anatomical origin of the MSC, which we referred to as the topographic *Hox* code (2), analogous in concept to the vertebral *Hox* code as formulated by Kessel and Gruss (157).

These findings raised two interconnected questions: Where do MSC originate, and how is *Hox* gene expression in these cells regulated? To begin to answer these questions, we now used transgenic mouse models to investigate where the regulatory sequences responsible for driving *Hox* gene expression in CFU-F are located, and used reporter constructs to track their tissue specificity of expression at fetal and adult stages.

The results of these studies suggest that most of these cis-regulatory elements are located within the confines of the *Hox* clusters. We also show that small constructs containing previously defined region-specific enhancers, can drive expression in CFU-F colonies, but without any topographic specificity, and without any correlation to the expression in the corresponding cartilage primordia that is the hallmark of these region-specific enhancers. These data corroborate the hypothesis that MSC in bone marrow do not originate locally from the same embryonic precursors as the bone they are found in, but colonize these from a distinct source.

## 3.3 Results

### *HOXA transgenes reproduce regionally restricted and organ-specific Hoxa gene expression*

To capture as much of the complex regulatory interactions governing expression of the *Hox* loci as possible, we initiated our search for regulatory elements responsible for expression in CFU-F colonies by generating transgenic mice with two overlapping human *HOX*A cluster constructs covering the entire *HOX*A cluster and its flanking regions (Fig. 1A).
Three independent lines were established for each construct. A rough estimate of transgene copy number was obtained for several of these, suggesting that one to three copies had integrated. In keeping with the observation that one or two copies of an intact human *HOX*D cluster are well tolerated in the mouse (301), all *HOX*A transgenic mice were viable and fertile and appeared healthy. They exhibited no

overt phenotypes except for line 170O19I-2, which occasionally developed elephant's teeth.

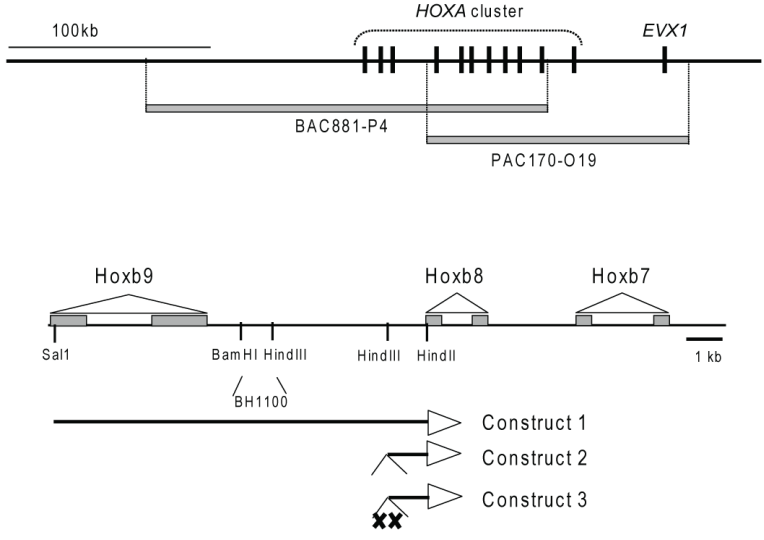



**Figure 1. Overview of the transgenic constructs used in this study.**
**A**, the human PAC/BAC inserts aligned with the *HOXA* cluster. Arrows indicate the direction of transcription. **B,** the configuration of the murine *Hoxb* cluster around *Hoxb8*. Arrows indicate the direction of transcription and grey boxes represent coding region. The *Hoxb8* reporter constructs are schematized below. The open arrowhead represents the *LacZ* gene followed by SV40 polyadenylation sequences. The position of the BH1100 regulatory region is also indicated. The mutated *Cdx* binding sites in BH1100 in construct *Hoxb8-III* are indicated with **X**. The black arrowhead indicates the integration site of *LacZ* in *Hoxb8lacZneo*$^{-}$.

We asked whether the human *HOX*A constructs were able to reproduce the colinear, regionally restricted expression patterns of *Hox*a genes in the embryo. We addressed this question by micro-dissection of embryos and RT-PCR. In order to distinguish between human and mouse *HOX*/*Hox* transcripts we designed species-specific primer sets. Human specific primer sets are shown in Table 1, mouse primer sets are shown in Table S1 from Chapter 2. Lines 881P4-1 and 170O19I-1 were analyzed in this manner. Within the limits of accuracy afforded by this method, transgenes in both lines were expressed with correct regional specificity and colinearity, and did not show any ectopic expression in the telencephalic region of the embryo (Fig. 2a and data not shown).

**Table 1. Primers used in this study**

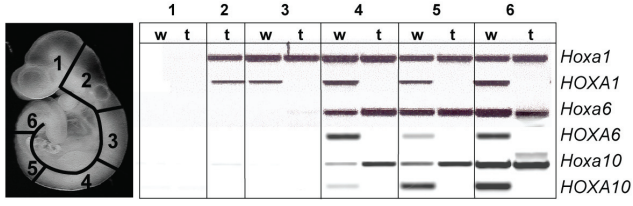
| Gene | Forward primer 5'-3' | Reverse Primer 5'-3' |
|---|---|---|
| *HOXA1* | CAGATTGGGTGCCAGCATAC | GGAAGCCGGGTTTCTGAAGT |
| *HOXA2* | CTTAGTTATTGACCTGGAGACTGG | GTGCACAGGTTCAAGCCATAG |
| *HOXA3* * | CACAGTGGCCAAACAAATCTTCC | CTGATCCTTTTTGTACTCATGCGG |
| *HOXA4* | AGCAGGTCTTGGAGCTGGAG | CACACCTGGCAGCCTTGTT |
| *HOXA6* | GGCAAAGGCGGGCGAGTAG | CGAGGTCGGGGAGCTCAG |
| *HOXA7* | CGCTGCATGGCGCGGCTGAG | GCAGCAGTGGCGGCGGCAGA |
| *HOXA9* # | CCCAGCAGCCAACTGGCTTCATGCGC | CACTCGTCTTTTGCTCGGTCTTTG |
| *HOXA10* | CTGATGAATCTCCAGGCGACG | TGACACTTAGGACAATATCTATCTC |
| *HOXA11* | CATTGGATTCTGAGAGCTGTGC | AGTCCACTCTGTGTCGAGGCTT |
| *Venus* | TGGATAACCGTATTACCGCCATGC | CGCTTACAATTTACGCCTTAAGAT |
| *LacZ* | ACTTGCTGATGCGGTGCTGA | GCGGCTGATGTTGAACTGGA |

* (134) # (280)

Adult organs and tissues have distinct *Hox* gene expression profiles when analyzed at a gross level (reveiwed by Morgan (216)). We used species-specific primers to compare expression of the human and murine *Hox*a10 and *Hox*a2 paralogs in several organs from adult mice of lines 881P4-1 and 170O19I-1. Expression of the transgenes agreed rather well with that of their murine paralogs, (Fig. 2b), suggesting that both constructs can also reproduce expression at adult stages with reasonable fidelity.

We also investigated whether the human transgenes are expressed in various hematopoietic cell populations sorted from the bone marrow, including the $CD45^{+}Lin^{-}$ population that is enriched in progenitor cells and was previously shown to exhibit the most pronounced expression of *Hox* genes. We failed to detect expression of any *HOX*A transgene in hematopoietic cells, in contrast to some of their murine paralogs (see Chapter 4). This suggests that the enhancers responsible for the hematopoietic lineage are located outside the genomic region covered by RP11-881P4 and RP1-170O19

**A**


1
2
3
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6
w t
t
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Hoxa1
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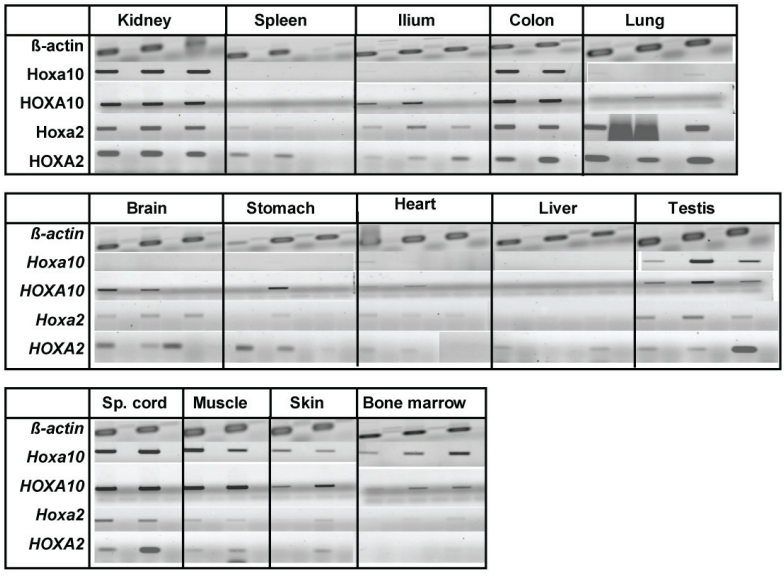
HOXA6
Hoxa10
HOXA10

**B**


Kidney
Spleen
Ilium
Colon
Lung
ß-actin
Hoxa10
HOXA10
Hoxa2
HOXA2
Brain
Stomach
Heart
Liver
Testis
ß-actin
Hoxa10
HOXA10
Hoxa2
HOXA2
Sp. cord
Muscle
Skin
Bone marrow
ß-actin
Hoxa10
HOXA10
Hoxa2
HOXA2

**C**

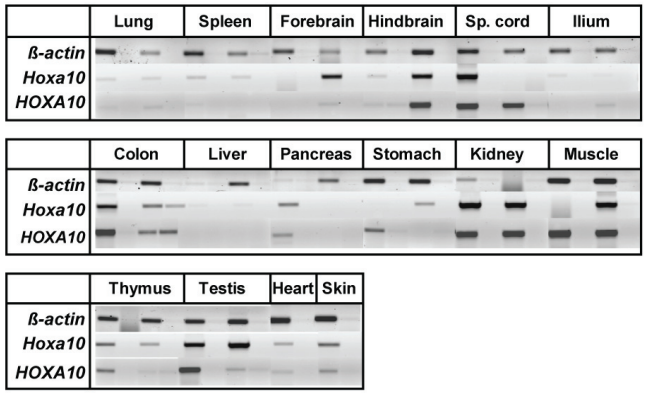

Lung
Spleen
Forebrain
Hindbrain
Sp. cord
Ilium
ß-actin
Hoxa10
HOXA10
Colon
Liver
Pancreas
Stomach
Kidney
Muscle
ß-actin
Hoxa10
HOXA10
Thymus
Testis
Heart
Skin
ß-actin
Hoxa10
HOXA10

**Figure 2. Region- and organ-specific expression of *HOXA* transgenes.**
(**A**), expression of selected *Hox* genes in axial structures of E9.5 embryos. The panel on the left shows regions 1-6 in which the anteroposterior axis of the embryo was subdivide; region 2 extends down to the first somit, regions 3-5 are sections of the axis comprising 7 somites each. The panel on the right shows expression of the *Hoxa* paralogue group 1, 6, and 10 genes in regions 1-6 of a line 881P4-1 transgenic embryo (t) and wild type littermate (w). (**B**), expression of *Hoxa2* and *Hoxa10* and their human paralogues in various organs of line 881P4-1 adult mice. Expression of the endogenous *β-actin* gene is shown as internal control for cDNA quality. Sp.cord, spinal cord. (**C**), expression of *Hoxa10* and *HOXA10* in organs of line 170O19I-1.

### *HOXA transgenes are expressed in CFU-F colonies*

We have shown that topographic *Hox* codes in CFU-F colonies are heterogeneous but highly characteristic for their origin. In two independent experiments, this topographic specificity was exhibited correctly by cDNA pools comprising six or more colonies (Chapter 2). Expression of *HOX*A transgenes was therefore analyzed in cDNA pools of ten CFU-F colonies derived from several tissues of RP1-170O19I and RP11-881P4 transgenic mouse lines using human-specific primer sets. Expression of *HOX*A5 and *HOX*A13 was not determined because we were unable to design human-specific primers that yielded a correct product. *HOX*A transgene expression was compared to the endogenous *Hox* profiles of CFU-F cDNA pools previously derived for wild type mice (Chapter 2). The results are summarized in Figure 3. The *HOX* expression profiles of equivalent CFU-F colonies of human origin are unknown and could conceivably be different from the murine profiles. However, despite several differences, some of which were consistently observed in independent lines, the expression of human and murine paralogs in CFU-F colonies from the transgenic lines agreed remarkably well. This indicates that the regulatory elements responsible for topographic specificity of expression in CFU-F colonies are located within the confines of the *HOX*A cluster and its immediate environs. Moreover, the fact that the genes in the region of overlap between RP11-881P4 and RP1-170O19 were expressed with largely correct specificity in both 881P4 and 170O19I lines, suggests that at least some of these regulatory elements might be relatively close to the genes they activate.

### *Is expression in CFU-F regulated through region-specific enhancers?*

Having established that cis-acting sequences responsible for expression of *Hox* genes in CFU-F colonies appear to act locally, we asked whether these elements might coincide or overlap with region-specific enhancers known to be active during development.

To answer this question, we decided to investigate the *Hox*b8 gene, since (i) it is robustly expressed in all CFU-F colonies from sternebral bone marrow but rarely in those from femoral bone marrow (see Chapter 2), and (ii) several region-specific enhancers have been identified in the vicinity of *Hox*b8, some of which have been analyzed in detail (49,50,327).

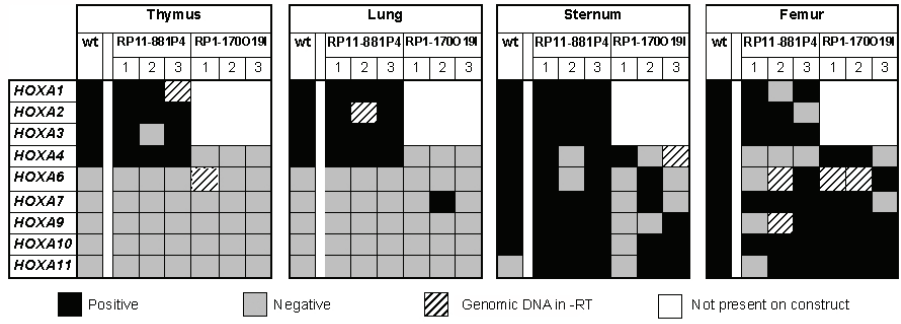



**Figure 3. *HOXA* gene expression profiles of BAC/PAC trangenic animals.**
Expression of *HOXA* genes analyzed in cDNA pools derived from several tissues of RP1-170O19I and RP11-881P4 transgenic mouse lines. *HOXA* transgene expression was compared to the endogenous *Hox* profiles of CFU-F cDNA pools previously derived for wild type mice (wt). RT-PCR results for three independent lines for each of the constructs is indicated by the shading, as explained in the legend.

We used previously described reporter constructs, in which the LacZ reporter gene is inserted as a translational fusion close to the N-terminus of *Hox*b8, and followed by SV40 polyadenylation sequences (see Fig. 1B). These fusion transcripts thus lack the 3'-untranslated region (3'-UTR) of *Hox*b8, which has been demonstrated to be a target for micro RNA-mediated mRNA degradation by miR-196 (132,344). This mechanism appears to prevent ectopic activation of *Hox*b8 by exogenous retinoic acid in the developing hindlimb (132), and conceivably could also be responsible for the lack of *Hox*b8 expression in CFU-F colonies from femoral bone marrow. Therefore, we first verified that the lacZ reporter gene in *Hox*b8lacZneo$^-$ knock in mice, which is fused slightly more downstream in exon 1 and also lacks the *Hox*b8 3'-UTR ((323); see fig. 1B), correctly reproduces expression of *Hox*b8 in CFU-F colonies. This appeared to be the case (Fig. 4A). However, none of the colonies showed ß-galactosidase expression detectable by Xgal staining (data not shown).

Next, we investigated a transgenic mouse line, L048, that harbours a randomly integrated *Hox*b8 reporter construct containing 11 Kb of genomic sequence upstream of the gene (construct 1 of ref. (50), here called *Hox*b8-I, see Fig. 1B). Several region-specific regulatory elements have been mapped within this 11 Kb of upstream sequence (50). CFU-F colonies from sternebral bone marrow of L048 mice all expressed lacZ, including one colony that did not express *Hox*b8 (Fig. 4A). In addition, lacZ was expressed in a significant fraction of CFU-F colonies from femoral bone marrow that did not express *Hox*b8 (Fig. 4A). To verify that this ectopic expression in femoral bone marrow CFU-F was not due to influences from the integration site in this particular transgenic line, we generated more transgenic mice with construct *Hox*b8-I.

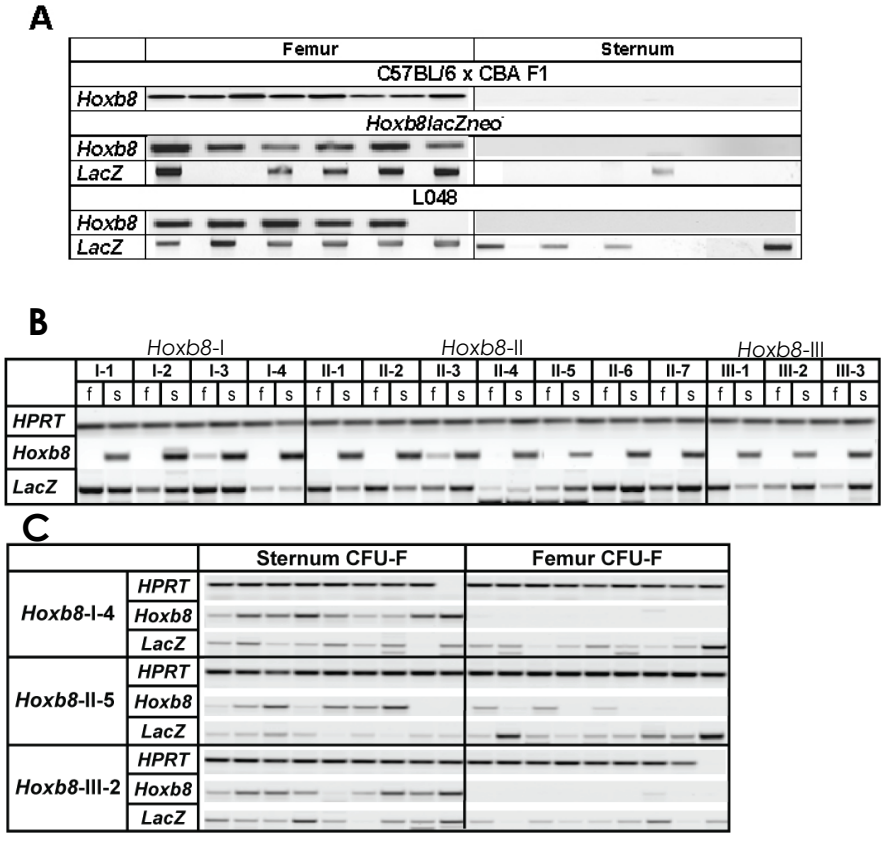



**Figure 4. Expression of *Hoxb8* and *lacZ* in CFU-F colonies from *Hoxb8* reporter mice.** (**A**), expression in individual CFU-F colonies from sternebral and femoral bone marrow of wild type, *Hoxb8lacZneo*$^{-}$, and line L048 mice. (**B**), expression of *Hoxb8* and *lacZ* in pools of 10 colonies from femoral (f) and sternebral (s) bone marrow of F0 animals carrying *Hoxb8* reporter constructs (see also Table 2). (**C**), expression of *Hoxb8* and *lacZ* in individual CFU-F colonies for three of the F0 mice.

The F0 animals (see Table 2) were sacrificed for analysis after siring a litter of embryos to determine the pattern of expression in the embryo. F1 embryos from one of these founders, *Hox*b8-I-4, showed ß-galactosidase activity in a regionally restricted pattern previously determined to be characteristic of construct *Hox*b8-I (Fig. 5C; see ref. (50)). Analysis of the founder animal also revealed expression of lacZ in CFU-F colonies from both sternebral and femoral bone marrow (Fig. 4B), suggesting that this is an intrinsic property of construct *Hox*b8-I. The embryonic expression pattern of founder *Hox*b8-I-1 could not be determined, and founders *Hox*b8-I-2 and *Hox*b8-I-3 yielded embryos with expression patterns that would be classified as "ectopic" (50), and presumably reflect the influence of enhancers located close to the integration site. Surprisingly, all three of these founders robustly expressed lacZ in CFU-F from both sternum and femur.

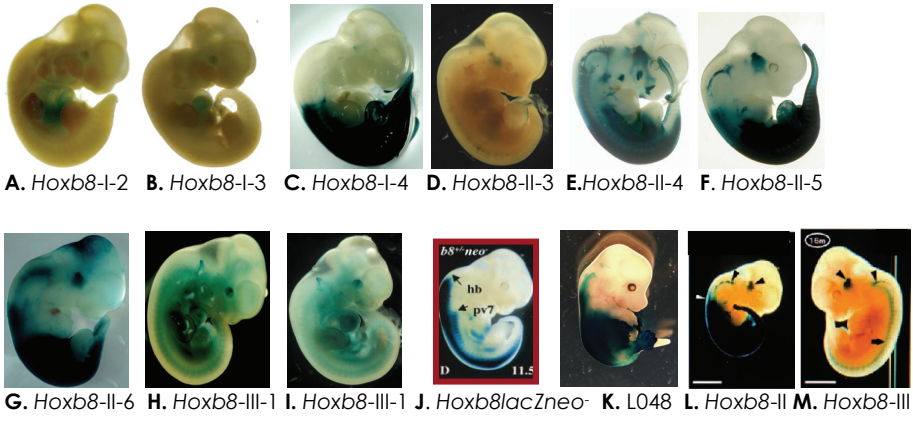
**Table 2. *Hoxb8* transgenic founders and their progeny**

| Founder | Sex | # of embryos | age (E) | # transgenic | # of Xgal(+) | panel in Fig. 5 |
|---|---|---|---|---|---|---|
| Hoxb8-*I-1* | F | 12 | 11.5 | 0 | 0 | |
| *Hoxb8*-I-2 | F | 3 | 11.5 | 1 | 1 | A |
| *Hoxb8*-I-3 | F | 8 | 11.5 | 4 | 4 | B |
| *Hoxb8*-I-4 | F | 9 | 11.5 | 3 | 3 | C |
| *Hoxb8*-II-1 | F | 11 | 11.5 | 0 | 0 | |
| *Hoxb8*-II-2 | F | 11 | 11.5 | 0 | 0 | |
| *Hoxb8*-II-3 | F | 12 | 12.5 | 9 | 9 | D |
| *Hoxb8*-II-4 | M | 10 | 12.5 | 8 | 8 | E |
| | | 9 | 11.5 | 4 | 4 | |
| *Hoxb8*-II-5 | M | 7 | 11.5 | 3 | 3 | F |
| | | 9 | 11.5 | 3 | 3 | |
| *Hoxb8*-II-6 | F | 8 | 11.5 | 5 | 5 | G |
| *Hoxb8*-II-7 | F | 7 | 11.5 | 4 | 0 | |
| *Hoxb8*-III-1 | F | 10 | 11.5 | 9 | 9 | H |
| *Hoxb8*-III-2 | F | 9 | 11.5 | 7 | 7 | I |
| *Hoxb8*-III-3 | F | 8 | 11.5 | 5 | 0 | |

Overview of F0 founder animals carrying constructs *Hoxb8-I*, *Hoxb8-II*, and *Hoxb8-III*, and the F1 embryos obtained from them in one or two litters. Listed are the total number of embryos in each litter, and the number of transgenic and Xgal-positive embryos among these. Representative expression patterns are shown in Fig. 5.

To further narrow down the sequences responsible for expression in CFU-F, we then tested construct *Hox*b8-II (construct 7 of ref. (50)), containing a 1.1 Kb region-specific enhancer element, BH1100, fused to the *Hox*b8 promoter region.
This BH1100 element is also present in construct 1, and is responsible for establishment of the anterior expression boundary. The *Hox*b8 promoter region alone is unable to direct regionally restricted expression in the embryo (50). Founder

*Hox*b8-II-5 yielded embryos that showed a ß-galactosidase expression pattern characteristic of this construct (50), including a *Hox*-like expression domain in neurectodem and mesoderm, and ectopic expression in peripheral cranial ganglia (Fig. 5F). From *Hox*b8-II-5 a transgenic mouse line was established (L-II-5). Founder *Hox*b8-II-4 yielded embryos that in addition to the pattern seen for *Hox*b8-II-5, showed more extensive ectopic expression in cranial ganglia and nerves as well as the spinal ganglia (Fig. 5E). RT-PCR analysis of cDNA pools of ten CFU-F colonies revealed that both founder *Hox*b8-II-4 and an F1 animal from *Hox*b8-II-5 expressed lacZ in CFU-F colonies from femur as well as sternum (Fig. 4B). Analysis of individual colonies from the F1 animal from L-II-5 showed that in fact, all colonies from femur expressed lacZ, irrespective of whether they expressed *Hox*b8 (Fig. 4C). From these analyses we conclude that ectopic expression of lacZ in CFU-F colonies from femoral bone marrow is an intrinsic property of construct *Hox*b8-II. Like was the case for the *Hox*b8lacZneo$^-$ mice, for all of the transgenic lines or founders described above, expression of the lacZ reporter in CFU-F colonies at the protein level was undetectable by Xgal staining (data not shown). However, in contrast to the L-II-5 F1 animal, offspring from a later generation showed readily detectable ß-galactosidase expression in a small subset (~1-5%) of CFU-F colonies from both sternebral and femoral bone marrow (Fig. 6A-C).




**A.** *Hoxb8*-I-2 **B.** *Hoxb8*-I-3 **C.** *Hoxb8*-I-4 **D.** *Hoxb8*-II-3 **E.***Hoxb8*-II-4 **F.** *Hoxb8*-II-5

**G.** *Hoxb8*-II-6 **H.** *Hoxb8*-III-1 **I.** *Hoxb8*-III-1 **J.** *Hoxb8lacZneo*$^-$ **K.** L048 **L.** *Hoxb8*-II **M.** *Hoxb8*-III

**Figure 5. ß-galactosidase expression in embryos carrying *Hoxb8* reporter constructs.** F1 embryos from F0 founders carrying *Hoxb8*-I (A-C), *Hoxb8*-II (D-G) and *Hoxb8*-III (H, I). See table 2 for details. (J), E11.5 *Hoxb8lacZneo*$^-$ embryo, reproduced from ref. (323), showing the endogenous *Hoxb8* expression pattern. (K), E12.5 line L048 embryo. (L), E11.5 *Hoxb8*-II embryo, reproduced from ref. (49). (M), E11.5 *Hoxb8*-III embryo, reproduced from ref. (49).

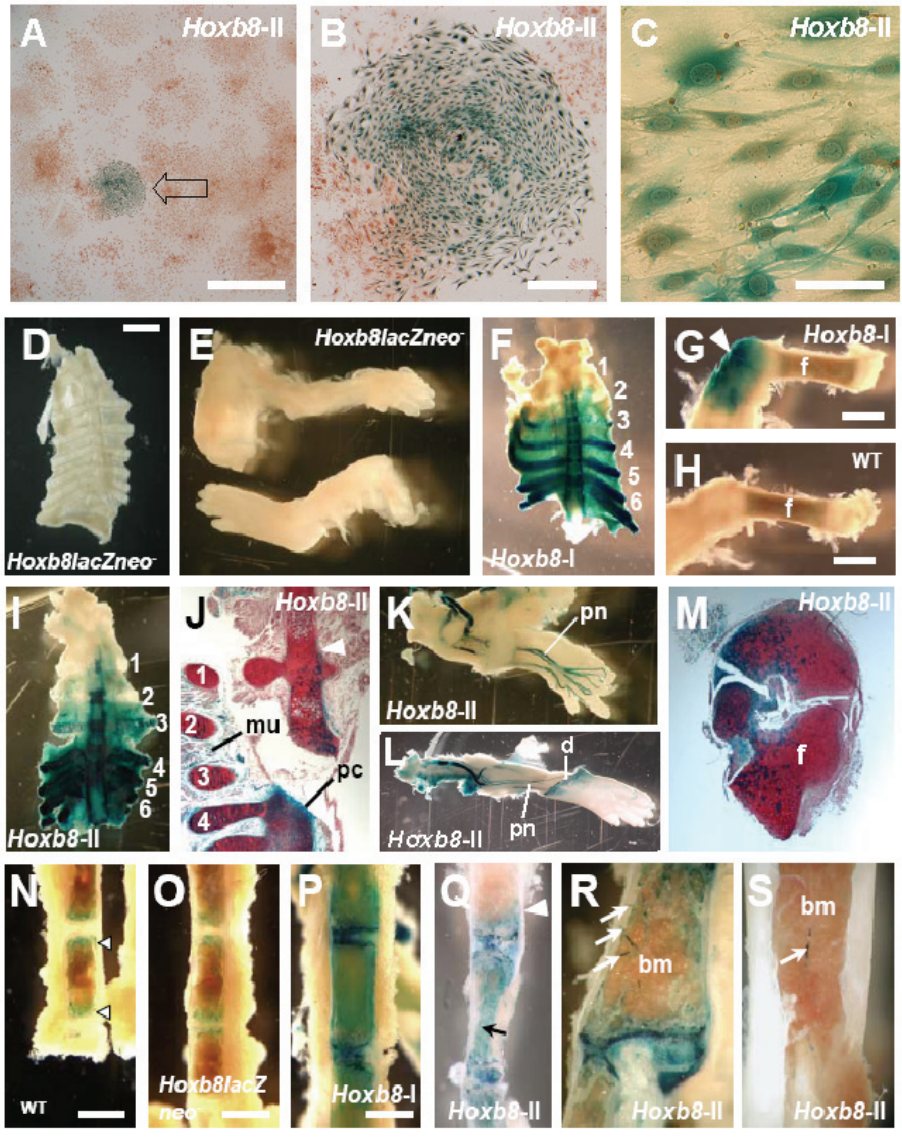
***Developmental expression***

We wondered if we could detect a relationship between *Hox*b8 expression in cartilage primordia and in CFU-F colonies. We investigated reporter gene expression at different stages of development and in the adult bone marrow of *Hox*b8lacZneo$^-$, line L048 and L-II-5 mice. Expression of ß-galactosidase in skeletal precursors in E11.5, *Hox*b8lacZneo$^-$ embryos appears to be restricted to the prevertebral condensations ((323), Fig. 5J), and this fits with the complete lack of expression in sternum and limbs at E16.5 (Fig. 6D, E). Constructs *Hox*b8-I and *Hox*b8-II on the other hand, consistently drive expression in the ventrolateral body wall and in part of the mesenchyme in the hindlimb bud at E11.5/E12.5 ((50), Fig. 5K, L). In keeping with this early expression, at late foetal stages, both lines L048 and L-II-5 show robust ß-galactosidase expression in the ventral rib cage (Fig. 6F and I, respectively), including the cartilage of the sternum and its perichondrium, the ribs, and the surrounding muscles (Fig. 6J). In the hind limbs, expression in these two lines is qualitatively different. Line L048 foetuses exhibit strong expression in the knee area but no detectable expression in the femur (Fig. 6G, compare to wild type shown in Fig. 6H). Limbs of line L-II-5 foetuses show a more elaborate pattern of expression, which includes Schwann cells of the peripheral nerves (in both fore- and hind limbs, see Fig. 6K, L; data not shown), part of the dermis (Fig. 6L), and expression in a proximal region comprising the hip joint, the head of the femur and surrounding tissues (Fig. 6M). In agreement with the expression at foetal stages, adult *Hox*b8lacZneo$^-$ mice did not show any ß-galactosidase expression in either the femur (not shown) or the sternum (Fig. 6O) that extended beyond the endogenous background activity (see wild type sternum, Fig. 6N). Line L048 adults showed expression in the cartilaginous parts of the sternebrae and in their periosteum, but not within the bone marrow cavity (Fig. 6P; data not shown). No expression was observed in the femoral bone marrow (data not shown). L-II-5 on the other hand, exhibited more pronounced expression in the sternebral bone marrow cavity (Fig. 6Q). This includes expression in blood vessels penetrating the bone (Fig. 6R). Interestingly, Xgal-stained blood vessels were also observed within the femoral bone marrow (Fig. 6S).

To further investigate the connection between expression domains in the embryo and expression in CFU-F in the adult, we generated F0 mice with construct *Hox*b8-III, in which four Cdx binding sites within BH1100 have been inactivated. These mutations completely abolish the ability of this enhancer to drive regionally restricted expression in the embryo (49). Two founders, *Hox*b8-III-1 and *Hox*b8-III-2, yielded F1 embryos with predominantly but not exclusively neural expression extending into the brain (Fig. 5H, I). This expression pattern is similar, but not identical to what was previously determined to be characteristic of this construct ((49); see Fig. 4M). Both founders, as well as a third founder (*Hox*b8-III-3), that did not show expression in F1 embryos, expressed lacZ mRNA in CFU-F colonies from sternum and femur (Fig. 4B, C). We can, however not exclude that this expression was the result of enhancers near the integration site, activating the *Hox*b8 promoter at later stages.

Taken together, these data show that (i) expression in CFU-F colonies that is readily detectable at the mRNA level may not result in detectable levels of ß-galactosidase activity, and (ii) expression in CFU-F colonies is not correlated with expression in the primordia of the bones in which they are located. This agrees with our previous finding that *Hox* codes in CFU-F derived from the bone marrow of individual vertebrae are distinct from the vertebral *Hox* codes established during embryogenesis (see Chapter 2).

**Figure 6** (next page). **Analysis of ß-galactosidase expression in situ and in CFU-F colonies.**
(**A**), Part of a 6 cm dish with CFU-F colonies from femoral bone marrow of line L-II-5 carrying construct *Hoxb8*-II, showing one Xgal-positive colony (arrow). (**B**), close up view of Xgal-positive colony shown in A. (**C**), high magnification view of blue cells showing fibroblastoid morphology. (**D, E**), ventral rib cage (D, anterior to the top), forelimb (E, top) and hindlimb (E, bottom) dissected from an E16.5 heterozygous *Hoxb8lacZneo*$^{-}$ embryo and stained with Xgal. (**F**), ventral rib cage of an E18.5 fetus of line L048, carrying construct *Hoxb8*-I, showing regionally restricted ß-galactosidase expression in part of the ribs (numbered) and sternum (arrowheads).
(**G, H**), proximal hindlimbs of a line L048 transgenic fetus and a non-transgenic littermate, respectively, with the femur dissected free of overlying muscles. Note Xgal staining over the knee area in G, but lack of expression in the femur (f; compare to femur in H). (**I**), ventral rib cage of an E18.5 fetus of line L-II-5, anterior to top, showing regionally restricted ß-galactosidase expression in ribs (numbered) and sternum (arrowheads). (**J**), frontal section through the rib cage similar to the one shown in I, showing the anterior boundary of expression in the cartilage primordium of the sternum (arrowhead), and expression in the perichondrium (pc), the ribs (numbered), and intercostal muscles (mu). (**K, L**), dissected forelimb and hindlimb, respectively, of an E18.5 fetus of line L-II-5, showing expression in peripheral nerves (pn). In the hindlimb, there is also expression in part of the dermis (d) and in the proximal part of the limb (arrowhead). (**M**), section through the proximal hindlimb of an E18.5 fetus of line L-II-5, showing ß-galactosidase expression in the hip joint including the femoral head (f) and the overlying tissues. (**N, O, P, Q**), sternums of adult wild type (N), *Hoxb8lacZneo*$^{-}$ (O), line L048 (P), and line L-II-5 (Q) mice, cut longitudinally and stained with Xgal. Note weak endogenous galactosidase activity in cells near the anterior and posterior ends of the sternebrae in N (open arrowheads), and similar expression in O, but lack of expression in the bone marrow in N, O, and P. In P and Q, stronger expression of ß-galactosidase is detected in and along the cartilage and bone of the sternebrae, the white arrowhead in Q indicating the anterior limit of expression. In the line L-II-5 sternum (Q) there is also expression within the bone marrow cavity (black arrow). (**R, S**), higher magnification of the sternebral and femoral marrow cavities (bm), respectively, of a line L-II-5 adult, showing prominent Xgal staining along blood vessels (white arrows). The scale bar in A represents 5 mm, in C 0.1 mm, those in other panels 1 mm.


A
Hoxb8-II
B
Hoxb8-II
C
Hoxb8-II
D
Hoxb8lacZneo-
E
Hoxb8lacZneo-
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1
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Hoxb8-II

## 3.4 Discussion

***Large transgenes reproduce topographic specificity of Hox gene expression in CFU-F***

*Hox* codes in CFU-F colonies from different locations display some spatial colinearity in that those derived from more posterior locations tend to express increasingly more 5' genes than those from more anterior locations. This colinearity is more apparent in CFU-F colonies from vertebral bone marrow, although there are clearly more exceptions in the expression pattern than have been observed for the canonical regional expression domains in the embryo (Chapter 2). These observations strongly suggested that *Hox* gene expression in CFU-F is driven by region-specific regulatory elements. Here, we have demonstrated that the in vivo activity and specificity of such putative elements are contained within genomic constructs comprising parts of the *HOX*A cluster. This data also suggested that these elements might act relatively locally, on genes within their immediate environment. For each of the two genomic *HOX*A transgenes, some differences were observed between independent transgenic lines. Some of these may be accidental, because CFU-F colonies are sampled from a heterogeneous population. We have previously shown that pools of CFU-F colonies in independent experiments may occasionally show differences in expression of the endogenous *Hox* genes as well (see Chapter 2). Overall, the human transgenes reproduced expression of the endogenous *Hox*a genes rather faithfully, but relatively consistent differences were apparent for *HOX*A11 and *HOX*A4, which are located near the end of constructs RP11-881P4 and RP1-170O19, respectively. This may be due to these genes being more exposed to influences from the host genome at the site of integration. However, comparing expression of *HOX*A4 in CFU-F colonies from thymus, lung, and femoral bone marrow reveals consistent differences between RP11-881P4 and RP1-170O19I lines, which are not readily explained by integration site effects. These differences might provide clues to the location of some the regulatory elements involved. *HOX*A11 on the other hand was expressed in CFU-F from sternebral bone marrow in five of the six lines, contrasting with the consistent absence of endogenous *Hox*a11 expression in sternebral CFU-F colonies. This might reflect a genuine difference in regulation of the human and murine paralogues in CFU-F from the sternum. Expression profiling of CFU-F colonies from human sternebral bone marrow could clarify this issue.

***Discrepancy between mRNA and protein expression***

When we investigated the involvement of previously identified embryonic region-specific enhancer elements in driving expression of *Hox*b8 in CFU-F colonies, a rather different picture emerged. First of all, a lacZ reporter integrated in the endogenous *Hox*b8 coding region faithfully reproduced differential expression of *Hox*b8 in CFU-F colonies from sternebral versus femoral bone marrow at the mRNA level, but did not yield detectable levels of ß-galactosidase expression in these cells. The same observation was made for the vast majority of transgenic mice carrying randomly integrated *Hox*b8 reporter constructs. This might indicate that mRNA expression levels are too low to allow for histochemical detection of the protein, although our non-quantitative RT-PCR analyses did not suggest a difference

in expression levels in CFU-F colonies versus whole embryos. Conceivably, it could also reflect translational repression of *Hox*b8 expression in CFU-F. As an exception, some of the CFU-F colonies from L-II-5 showed robust ß-galactosidase activity, suggesting that translational repression, if it exists, is not absolute. More detailed experiments, including quantitative analysis of mRNA expression levels in CFU-F colonies from L-II-5 mice, and testing the influence of the 5'-UTR on the efficiency of mRNA translation, might help resolve this issue.

***Small region-specific enhancers do not exhibit specificity in CFU-F***

Reporter constructs containing variable parts of the *Hox*b8 upstream region showed strongly decreased specificity of expression in CFU-F colonies from sternebral versus femoral bone marrow. However, the lack of specificity of expression in CFU-F colonies derived from adult mice bearing construct *Hox*b8-I, contrasts sharply with the differential expression in sternum versus femur driven by this construct prenatally. This discongruence is in agreement with the analysis of constructs *Hox*b8-II and *Hox*b8-III that showed a predominant lack of correlation between topographic LacZ expression in the embryo and in CFU-F in the adult. This observation is consistent with our hypothesis that CFU-F do not originate locally from the prevertebral condensations, which we based on the CFU-F *Hox* codes from vertebral bone marrow (see Chapter 2). Distinct regulatory elements could be responsible for expression in CFU-F and/or their actual precursor cells.

We have observed expression in some blood vessels within the bone marrow cavities in sternum and femur of line L-II-5 adults. Mounting evidence suggests that MSC are pericyte-like cells e.g. (61,64,149,288,353). Interestingly, Le Douarin and coworkers have suggested that pericytes in the femoral bone marrow are derived from the perichondrium of the femoral primordium (150). Further developmental analyses, and prospective sorting based on ß-galactosidase expression using line L-II-5 may be helpful in elucidating the developmental origin of al least the small subset of CFU-F that detectably express ß-galactosidase. Furthermore, it will be interesting to isolate pericytes from different tissues and determine if they have *Hox* codes that resemble those of CFU-F from the same location.

***Are CFU-F Hox codes functionally relevant?***

The faithful reproduction of CFU-F *Hox* codes by large *HOX*A transgenes contrasts sharply with the lack of specificity of small *Hox*b constructs. Also we observed a lack of correlation between topographic LacZ expression in the embryo and in CFU-F in the adult. These observations may be indicative of the existence of specific regulatory elements dedicated to mesenchymal stem cells or their precursors, distinct from the embryonic region-specific enhancers present in the *Hox*b8 reporter constructs used in this study. Another explanation may be found in the fact that establishment of CFU-F cultures involves activation of previously quiescent cells (94). It is possible that *Hox* codes in CFU-F colonies reflect a non-physiological activation of transcription due to the environmental stress involved with explantation

and in vitro culture. A similar effect has been observed upon activation of NK cells and T cells in vitro, which caused coordinated transcriptional activation of the *Hox*b cluster (41,258). Induction of *Hox* gene expression in this manner could conceivably be modulated by epigenetic marks retained from an earlier phase of expression, i.e., in a CFU-F precursor cell. It is reasonable to assume that large constructs, containing multiple polycomb response elements necessary for maintenance of silencing mediated by Polycomb-group proteins (226), could better preserve repressive chromatin marks than small transgenes. This might explain the differences in specificity observed between our *HOX*A and *Hox*b constructs.
Even if non-physiological, the induction of *Hox* gene expression in CFU-F-derived cells may have phenotypic consequences. Although the lack of ß-galactosidase activity in CFU-F colonies from *Hox*b8lacZneo$^-$ mice suggests that functional protein expression of at least this one gene is quite low or absent, it is unknown whether this can be extrapolated to other *Hox* genes. Furthermore, several *Hox* genes have been shown to have redundant or overlapping functions (158,192), and expression of multiple *Hox* genes with overlapping functions might have additive effects e.g. (52,53,66,100,130). Functional assays involving deletion or over expression of *Hox* genes will be required to address this issue.

## 3.5 Materials and methods

### *DNA Constructs*

Human genomic BAC and PAC clones were obtained from the BACPAC Resources Center (http://bacpac.chori.org) at the Children's Hospital Oakland Research Institute (Oakland, CA). Clone RP1-170O19 is derived from the RPCI-1 library cloned in pCYPAC2 (138), and contains an insert of 129 Kb (acc. nr. AC004080). It extends from ~10 Kb downstream of the EVX1 coding sequence to the first exon of *HOX*A3 isoform-a transcript 2, ~2 Kb downstream of the *HOX*A4 polyA sequence.

Clones RP11-881P4 and RP11-884O23 are derived from the RPCI-11 library, cloned in pBACe3.6 (229). Both clones were identified by hybridization of RPCI-11 high-density filters with a *HOX*A4 probe (data not shown). The ends of the RP11-881P4 insert were sequenced with SP6 and T7 primers, and the sequences were aligned with the human chromosome 7 sequence BL000002.1 (NCBI) using BLAST. From this analysis the insert was inferred to be 201kb in size and to extend from BL000002.1 (GI:30089982) basepair no. 26734363 (SP6 end) to 26935695 (T7). These positions correspond to basepair 69097 of AC073472 (Sp6), 140kb downstream of *HOX*A1, and to basepair 61492 of AC004080 (T7), about 3kb upstream of the first exon of *HOX*A11 (Figure 1).

The integrity and stability of the clones were verified by PCR with *HOX*A gene-specific primers listed in Table 1, and by restriction enzyme digestion. Restriction fragments were separated with standard agarose gel electrophoresis and pulsed field gel electrophoresis (PFGE) using a Rotophor system (Biometra). For each clone, several individual colonies derived from the initial stab culture were analysed, and one that conformed to the published sequence by restriction analysis was used for

generating transgenic animals. The BamHI restriction pattern of RP1-170O19 was consistent with AC004080 (data not shown). AscI and KpnI digests of RP11-881P4 were consistent with BL000002.1 except for the absence of a KpnI site predicted to be at 20107 bp from the SP6 end, resulting in a 8.5 Kb KpnI fragment replacing the predicted fragments of 6.9 and 1.6kb. This site was also missing in a partially overlapping clone, RP11-884O23 (data not shown), and might indicate a polymorphism. Based on restriction analyses, both RP1-170O19 and RP11-881P4 were stable over multiple DNA isolations.

RP1-170O19 does not contain a unique cutting site in the vector sequence that would allow linearisation. We therefore generated the retrofitting vector pRetroES-I by inserting a NotI;I-SceI;SalI;SacI adaptor into the SacI and NotI sites of pRetroES (gift of Dr. Ursula Storb) (334) The adapter was created by annealing oligos 5'-GGCCGCTAGGGATAACAGGGTAATGTCGACGAGCT-3' and 5'-CGTCGACATTACCCTGTTATCCCTAGC-3' (gift of Dr. Ali Imam).
pRetroES-I was then retrofitted into the loxP site of RP1-170O19 using published procedures ((334); see appendix), yielding RP1-170O19I.

*Hox*b8 reporter constructs *Hox*b8-I, *Hox*b8-II and *Hox*b8-III have been previously published, and correspond to constructs 1 and 7 of ref.(50), and construct 16m of ref.(49), respectively (see also Figure 1b).

***Transgenic mouse lines***

*Hox*b8lacZneo$^-$ knock in mice (323) and the L048 transgenic mouse line carrying construct *Hox*b8-I (50) were used after at least 10 generations of backcrossing to C57BL/6 x CBA F1 mice.

For the generation of transgenic mice, BAC and PAC constructs were isolated from large-scale bacterial cultures by alkaline lysis followed by caesium chloride gradient equilibrium density centrifugation. RP11-881P4 and RP1-170O19I were linearized with PI-SceI and I-SceI (New England Biolabs), respectively, and the enzymes were inactivated according to the manufacturer's instructions. The digested DNA was separated by PFGE, and the linear form was excised from the gel, electro-eluted, dialyzed against micro injection buffer (8 mM Tris-HCl pH7.5 and 0.1 mM EDTA), and concentrated by vacuum dialysis.

The inserts of constructs *Hox*b8-I, *Hox*b8-II and *Hox*b8-III were separated from vector sequences, purified from a standard agarose gel by electro-elution, and dialyzed against micro injection buffer.

Purified DNA fragments were micro injected into the pronuclei of 1-cell embryos of the FVB/N strain (Harlan), which were transferred to C57BL/6 x CBA F1 pseudo pregnant females. Mice were routinely genotyped by PCR. Transgenic lines carrying RP11-881P4 or RP1-170O19I were maintained by brother-sister mating. Integration of the constructs was verified by PCR with human-specific *HOX*A primers (see

Table 1). A rough estimate of transgene copy numbers was obtained by Southern blot analysis, comparing the signal of a human *HOX*A2 probe to that of a probe of about equal size against the endogenous Shh gene.

***Hoxb8 founder analysis***

Transgenic founder animals were mated with wild-type mice and females sacrificed between 11.5 and 12.5 days after a vaginal plug was detected. Male founders were sacrificed after producing transgenic offspring. The animals were killed by cervical dislocation according to institutional guidelines. The embryo's were dissected from the uterus and stained for ß-galactosidase activity using standard procedures. The yolk sac was used for genotyping by PCR for the LacZ gene with primers described in Table 1.

***CFU-F***

CFU-F assays were performed as described in Chapter 2 (2), except that for the L048 and *Hox*b8lacZneo$^-$ mice DMEM was replaced by IMDM (Gibco # 041-90898M).

***RNA isolation and PCR Analysis***

Mouse organs were dissected and washed in PBS0. Trizol (Invitrogen) was added according to the weight of the tissue as described by the manufacturer. The tissue was homogenized using an ultra-turrax mixer followed by centrifugation to remove remaining tissue fragments. For RT-PCR, 1 µg of RNA was used.

Dissected embryo fragments were dissolved in Trizol by shearing through a 19G hypodermic needle and processed as described for CFU-F colonies.

CFU-F RNA processing and preparation of cDNA has been described in Chapter 2. Reverse transcription reactions were diluted to 200 µl with de-ionized water, and 2 µl was amplified in a 25 µl PCR reaction, using the primer pairs listed in Table 1 with either SuperTaq (SphaeroQ, the Netherlands) or Taq DNA Polymerase (Invitrogen). Reaction conditions optimized for each primer pair are available on request. PCR reactions were analyzed by agarose gel electrophoresis as described in Chapter 2.

***Histological analysis of reporter gene expression***

E11.5 and E12.5 day embryos were dissected from the uterus, fixed in PBS0 containing 1% formaldehyde and 0.2% glutaraldehyde for 1 hour at 4°C, and washed with PBS0.

Foetuses (E16.5-E18.5) were killed by decapitation; femur and sternum were dissected out and most of the overlying muscle was removed prior to fixation for 45 minutes as described above or for 2 hours on ice. Yolk sac or tail was used for genotyping with LacZ primers (see Table 1).

Adult mice were killed by cervical dislocation according to institutional guidelines, and femur and sternum were dissected out and largely freed of overlying muscle. These bones were split longitudinally, and fixed for 1 hour.

Embryos and bones were stained for ß-galactosidase activity for 18-24 hours using standard procedures but using Xgal at 0.4 mg/ml instead of 0.8 mg/ml. For E16.5-E18.5 foetuses and adult bones, 0.2% Nonidet NP-40 substitute and 0.1% sodium deoxycholate were added to washing and staining solutions to improve staining of deeper tissues. Tissues were embedded in paraffin and sectioned and stained as described before (327).
Dishes with CFU-F colonies were washed once with PBS0, fixed for 15 minutes at room temperature, washed with PBS0, stained as described above, and counterstained with Neutral Red.

## 3.6 Acknowledgements

The authors are grateful to Sylvia Dekker and John Kong a San for microinjections, to Laura van Vliet for technical assistance, to Jacqueline Deschamps and Wim de Graaff for *Hox*b8 constructs and mice and for their hospitality during regular visits to the Hubrecht Institute to analyse these, and to Ali Imam for the I-SceI adaptor.

# 4

# Regulation of *Hox* genes in hematopoiesis

Karin Ackema, Laura van Vliet & Jeroen Charité

## 4.1 Summary

*Hox* Proteins plays an important role in hematopoiesis, in both the hematopoietic stem cells as well as in lineage resticted cel populations. They are important for correct proliferation and differentiatio, and overexpression of *Hox* genes frequently lead to leukemias. Despite their importance in hematopoiesis, nothing is known about the regulatory mechanisms that are responsible for driving *Hox* gene expression in the hematopoietic system. We have searched for putative hematopoietic enhancer elements for the *HOX*A cluster using a transgenic approach. However, our results suggest that that the enhancer elements are located outside the region that was covered in our analysis. We therefore developed a double retrofitting strategy to generate large reporter constructs for identification of these putative distant regulatory elements.

## 4.2 Introduction

Throughout our entire life, blood cells of multiple lineages are produced by a small population of hematopoietic stem cells (HSC). This process involves the self-renewal of HSC, proliferative expansion of progenitor cells, and the progressive commitment of progenitors to single lineage differentiation (225).

The *Hox* family of transcription factors plays an important role in hematopoiesis. Deregulation of *Hox* gene expression effects the proliferation and differentiation of HSC and committed progenitors, as has been shown by a number of knockout and over-expression studies (reviewed in (1,180,316)). Loss-of-function studies of *Hox*a9, *Hox*b4 and *Hox*b3 have shown that these genes are involved in the regulation of stem cell self-renewal and repopulation after HSC transplantation. Genetic interaction among these genes suggests that they act in a similar pathway (32,177,194). These findings are also supported by retroviral over expression studies of the human *HOX*B4 and mouse *Hox*a9 genes that resulted in marked increase in HSC self-renewal and repopulation abilities (279,314,317). *Hox*a9-deficient mice on the other hand, also exhibit a variety of differentiation defects in the myeloid and lymphoid pathways, which suggests that this gene has multiple functions in regulation of hematopoiesis (139,178). Other *Hox* genes also appear to act at multiple levels. Overexpression of *HOX*B6 for example, leads to expansion of HSC and myeloid precursors while inhibiting erythropoiesis and lymphopoiesis (85).
Overexpression of *HOX* genes also frequently leads to the development of leukemias, as was for example shown for *HOX*A10 (315) and for *HOX*B6 (85). Aberrant activation of *HOX* genes is a common event in human myeloid leukemia and many *Hox* proteins have shown to contribute to human leukemogenesis (10,221,232). Expression of *Hox* genes in primary leukemic cells shows characteristic patterns that are distinct for specific types of leukemia (45). In addition, high-level expression of *HOX*A9 in human acute myeloid leukemia (AML) is significantly correlated with resistance to treatment (107). Mixed lineage leukemia's (MLL) caused by chromosome rearrangements involving the *HOX* maintenance factor MLL, account for the majority of infant and treatment-related

secondary leukemias, and in general carry an extremely poor prognosis (16,73). MLL can be distinguished from other leukemias by a distinct gene expression profile, which includes differential overexpression of *HOX* genes. It is assumed that this is due to inappropriate activation of *HOX* gene expression by the MLL fusion proteins (12).

The expression of *Hox* genes in hematopoietic cells has not been investigated systemically, but for a reasonable number of these genes expression in different hematopoietic lineages in human and mouse has been established. *Hox* genes were first shown to be expressed in several human and murine immortalized hematopoietic cell lines (163,286), but later also in several subsets of hematopoietic cells from the bone marrow (215,248,278). Expression of *Hox* genes is markedly higher in progenitor populations than in mature blood cells (179,248,278) and the expression appears to be mainly restricted to the *HOX*A and *HOX*B clusters (30,278). Despite the increasing knowledge about the function of *Hox* genes in normal hematopoiesis and leukemia, nothing is known about the regulatory sequences that are responsible for driving *Hox* gene expression in the hematopoietic system.
We started to search for putative hematopoietic enhancer elements for the *HOX*A cluster by testing BAC/PAC constructs covering the complete *HOX*A cluster and flanking sequences at either side in transgenic mice. These transgenes failed to drive expression in hematopoietic cells, suggesting that the enhancer elements are located outside the region covered by these constructs. We also describe the development and implementation of a novel, double retrofitting strategy to generate large reporter constructs for identification of such distant regulatory elements.

## 4.3 Materials and Methods

***BAC constructs***
RP1-170O19 and RP11-881P4 have been described in Chapter 3. Five other overlapping BAC constructs flanking both sides of the *HOX*A cluster spanning around 1Mb of genomic sequence in total, were obtained from the BACPAC Resources Center (Oakland, CA) as agar stabs. A few colonies per clone were checked for stability by comparing the restriction digestion pattern obtained with a frequently cutting restriction enzyme (data not shown). Most of the clones were sequenced with SP6 and T7 primers to map the ends of the insert. The results are shown in Table 1 and Figure 2

**Table 1. Location of the ends of genomic BAC clones**

| BAC name | NCBI ID | T7 end | SP6 end |
|---|---|---|---|
| RP11-881P4 | - | AC004080 61492 | AC073472 69097 |
| RP11-26E06 | - | AC004080 96123 | AC004009 27287 |
| RP11-749D01 | - | AC004996 99830 | AC007130 19019 |
| RP11-422K18 | - | AC007130 26155 | AC005091 86712 |
| RP11-812K17 | AC07315 | See NCBI accession number | |
| RP11-232C20 | - | Not sequenced yet | |
| RP11-627P22 | AC07374 | See NCBI accession number | |

***Retrofitting vectors***

Both pRetro-Sp/L-ΔI and pRetroES-I are modified version of pRetroES (334).
The pRetroES vector (334) was modified as follows. First, an oligonucleotide containing SalI and I-SceI sites was ligated into the SacI and NotI sites of pRetroES, creating pRetroES-I (see also Chapter 3). This vector was used to construct pRetro-Sp/L. For this, the 1.8Kb XbaI-XbaI fragment containing the GST-loxP-Cre coding region was removed from pRetroES-I. The NotI site was replaced by BglII. The PGK-neo cassette was removed as an ApaI-HindIII fragment, and replaced by an ApaI-SalI-PmeI-NheI-HindIII polylinker. The ampicillin resistance gene was removed by digesting with HpaI and SwaI, and replaced by a 2.0 Kb HindIII fragment from pNELγI (153) containing the spectinomycin resistance gene. Subsequently, a 1370 bp SphI-PshAI fragment containing the lacI gene was excised from pET28-b(+) (Novagen), fitted with SacI linkers and ligated into the SacI site of the $Sp^R$ retrofitting vector. A novel self-inactivating 6His-loxP-Cre fusion protein expression cassette was created as follows. A 2.0Kb XbaI-HindIII fragment containing the GST-loxP-Cre cassette was subcloned from pRetroES into pET28-b(+) (Novagen) digested with NheI and HindIII. Next, a ~720bp NdeI fragment encoding the GST moiety was removed from this construct, fusing the 6His tag from the pET vector to the loxP-Cre open reading frame. The XbaI site in the terminator region of the Cre cassette was destroyed by filling-in with Klenow fragment. Finally, the 6His-loxP-Cre cassette was re-inserted as an XbaI-HindIII fragment into the modified vector backbone after partial digestion with HindIII and digestion with XbaI. To generate pRetro-Sp/L-ΔI, the I-SceI site was destroyed by digestion and T4 DNA polymerase treatment.

***Hoxa7s-Venus***

The 2.7kb SacI-SacI fragment containing a *Hox*a7 enhancer/promoter region (161,235,256) was derived from RP21-500A4 (RPCI-21, 29S6/SvEvTac mouse library) (230) by restriction digestion, and cloned in the SacI site of the multiple cloning site of pVenus-N1 (219,261).

The *Hox*a7s-Venus fragment was isolated from the vector sequence and cloned into pRetro-Sp/L-ΔI. A CMV-EGFP reporter was obtained from pEGFP-N1 and cloned into pRetro-Sp/L-ΔI. For both reporters the direction of transcription is the opposite of that of the Cre gene.

***Cell sorting and cDNA preparation***

Mice were killed according to institutional guidelines. The bone marrow from adult FVB/N and transgenic mice bearing the RP1-170O19I or RP11-881P4 construct (see Chapter 3 for details), was collected from the femur and tibia in phosphate buffered saline (PBS0) (Gibco) with 10% fetal bovine serum (FBS) and 2 mM EDTA by flushing and scraping. Nuclei were counted on a Beckman Coulter Z1 after cell lysis using Zap-oglobin II reagent (Beckman Coulter Inc.). Lysis of red blood cells prior to sorting was done as follows. Cells were pelleted at 1000rpm for 5 minutes and resuspended in 1 ml of lysis buffer (150 mM $NH_4Cl$, 10 mM $NaHCO_3$, 2 mM EDTA). Lysis was allowed to proceed for 3-4 minutes at RT, then 6 ml of medium was added and gently mixed. A layer of approx 0.5 ml of FBS was placed at the bottom of the tube with a pasteur pipette. The cells were centrifuged for 5 minutes without disturbing the layers and the supernatant was removed. Cells were resuspended in PBS0 with 1% FBS and 0.1 mM EDTA. Cells were stained with directly FITC or PE-labelled antibodies for 30-40 minutes on ice with $1x10^6$ cells per 100 µl buffer. Antibodies were obtained from e-bioscience and each batch was titrated for optimal concentration before usage.

Cells were sorted on either an AutoMACS or a FASC-Vantage, both from Becton-Dickinson. For analysis of gene expression after FACS, a minimum of a 100.000 cells with a purity of >95% were collected. The cells were pelleted and lysed in Trizol (Invitrogen). RNA isolation and reverse transcription were performed as described for CFU-F in chapter 2. Depending on the cDNA quality, 1-5 µl of the RT reaction (25 µl), was used for PCR, using the *Hox*a and *HOX*A primers described in chapters 2 and 3.

***Retrofitting***

In vivo retrofitting was performed as described by as described by Wang et al (334). Retrofitting BACs in both loxP sites resulted in decreased proliferation of the host bacteria, and these constructs tended to lose the pRetro vectors in liquid cultures grown to high density. Also, double retrofitting was only successful when pRetro-Sp/L-ΔI-*Hox*a7s-Venus was retrofitted first, followed by pRetroES-I (Figure 3). After the second transformation, no colonies could be found the next day, but after at least 24 hours, clusters of satellite colonies started to appear. These satellite clusters were then streaked onto a fresh plate and if double retrofitting was successful, colonies appeared after approximately 16 hours of incubation. The colonies were screened by PCR to determine the sites of insertion of the retrofitting

constructs, using primers F1 (5'-TCAGCGTGAGACTACGATTC-3'), F2 (5'-GTTGCTACGCCTGAATAAGTG-3') and R (5'-TCGACCGGTAATGCAGGCA-3') as described by Wang et al, 2001 (334) for pRetroES. These primers also work for pRetro-Sp/L-ΔI-*Hox*a7s-Venus and pRetro-Sp/L-ΔI-CMV-EGFP. However, to obtain a specific product for these vectors, primer R was replaced by EGFP-fw (5'-TGGATAACCGTATTACCGCCATGC-3'). This was important, as during the second retrofitting the vectors can exchange their integration site.

Low DNA yields were obtained from both small and large scale plasmid preparations, as the bacteria (DH10B) tended to lose their doubly retrofitted BAC construct. For large-scale cultures, one colony was inoculated in 100 ml of LB medium containing 25 µg/ml ampicillin and 25 µg/ml spectinomycin. After over-night incubation at 37$^{o}$C with shaking, the cultures were centrifuged at 3500 rpm for 10 minutes. As a large percentage of the bacteria had died, the centrifuged culture was cloudy and only a relatively small pellet of cells was recovered. The medium was discarded and the pellet resuspended in three litres of fresh culture medium. These cultures were incubated at 37$^{o}$C while shaking for approximately 3-4 hours until they had reached a maximum optical density of 0.5 at 595 nm.
The DNA was isolated using Qiagen columns (1 Q500 column per litre of culture) according to the manufacturer's manual except that no intermediate precipitation step before loading the column was performed, and the lysate was directly loaded on the column. For elution, the elution buffer was heated to 50$^{o}$C. A three-litre culture typically yielded between 20-50µg of doubly retrofitted BAC DNA. Caesium chloride equilibrium density gradient centrifugation preparations were not successful with these cultures due to the high protein-to-plasmid DNA ratio.

#### *Nucleofection*

K562 cells were cultured in RPMI1640 with GlutaMax, collected by centrifugation, and resuspended in Cell line nucleofector solution V (Amaxa) at a density of $10^7$ cells / ml. 100 µl of this suspension was transfected with 1-3 µg of DNA in an Amaxa nucleofector device using program T-16. Cells were examined by epifluorescence microscopy 24 hours after transfection.

## 4.4 Results

#### *Analysis of transgene expression in hematopoietic cells*

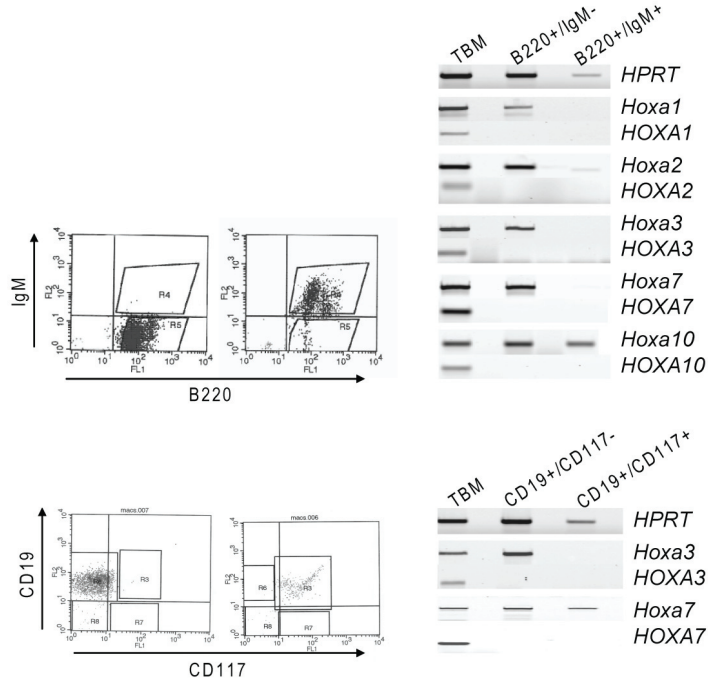
Most *HOX*A transgenes were readily detected in freshly isolated total bone marrow of RP-11881P4 and RP1-170O19I transgenic mice (Fig. 1). To establish whether the transgenes are expressed in hematopoietic cells, we analysed *HOX*A gene expression in various hematopoietic cell populations purified from transgenic mice carrying RP11-881P4 (all three lines) or RP1-170O19I (line 1 and 2; see Chapter 3). *Hox* gene expression was reported to be absent or expressed or at very low levels in terminally differentiated blood cells (179,278). We confirmed these results by RT-PCR on mature cell populations isolated by Magnetic Activated Cell Sorting (MACS) with lineage-specific (Lin) antibodies MAC-1 (macrophages), B220 (B

cells), TER119 (erythrocytes), GR-1 (granulocytes) and CD3e (T cells). In all of these populations, we found expression of the endogenous *Hox*a genes to be very weak or absent. Only B cells and macrophages showed weak expression of *Hox*a7, *Hox*a9 and *Hox*a10, which could not consistently be detected (data not shown). Separation of the B cells into a mature fraction ($B220^+/IgM^+$) and a Pre-B cell fraction ($B220^+/IgM^-$) by Fluorescent Activated Cell Sorting (FACS) confirmed that more genes could be detected in the more immature population (see Fig. 1. However, in the very early Pro-B cells ($CD19^+/ CD117^-$) a decreased number of expressed genes could be detected. This is probably in part due to the small population size that significantly increases the time required for separation, increasing cell-death and yielding lower quality cDNA. A very similar set of genes, compared to Pre-B cells, was expressed in the common progenitor population ($CD45^+Lin^-$) (see Fig. 1). In none of these populations did we detect expression of the *HOX*A transgenic constructs. This strongly suggests that the sequences responsible for expression in these cell populations are located outside RP11-881P4 and RP1-170O19.

***Long-distance enhancers***

It has been proposed that distant enhancers would control expression of *Hox* genes in tissues that represent evolutionary "novelties". These are thought to be acquired in evolution after cluster duplication, at a position that does not interfere with pre-existing *Hox* functions (70,300). Expression of *Hox* genes in the hematopoietic system might also be a function that has been acquired relatively recently in evolution. It is known that enhancers can act over very large distances up to almost 1 Mb (184). Enhancers are often able to control transcription of several genes that are unrelated in structure or function, a mechanism that underlies the method of classic enhancer trapping experiments (89). This may result in co-activation of unrelated genes that are positioned in the proximity of a long-distance enhancer, as for example was observed for the *Hox*d digit enhancer (300). We looked at expression of several genes that are positioned over 2 Mb at both sides of the cluster for their expression in hematopoietic cells. If genes at one side of the cluster would systematically be expressed in hematopoietic cells in contrast to genes on the other side of the cluster, this could potentially indicate the position of a hematopoietic enhancer. However, equal numbers of genes at both sides were either reported to be expressed in hematopoietic cDNA libraries (NCBI gene reports), or expressed in the human leukemia-derived cell lines U937, HL-60 and K562 (data not shown). These experiments therefore yielded no clues about the location of a putative enhancer for the *HOX*A cluster.

## A.




## B.

**Total bone marrow**

| *HOXA/Hoxa* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 | 11 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Endogenous | + | + | + | + | + | | + | + | + | + | - |
| Transgene | + | + | + | + | | + | + | | + | + | - |

**B220⁺/IgM⁻**

| *HOXA/Hoxa* | 1 | 2 | 3 | 7 | 9 | 10 |
|---|---|---|---|---|---|---|
| Endogenous | + | + | + | + | + | + |
| Transgene | - | - | - | - | - | - |

**B220⁺/IgM⁺**

| 1 | 2* | 3 | 7 | 9* | 10 |
|---|---|---|---|---|---|
| - | +/- | - | - | +/- | + |
| - | - | - | - | - | - |

* Inconsistent between different experiments

**CD19⁺/CD117⁻**

| *HOXA/Hoxa* | 2 | 3 | 7 | 9 |
|---|---|---|---|---|
| Endogenous | + | + | + | + |
| Transgene | - | - | - | - |

**CD19⁺/CD117⁺**

| 2 | 3 | 7 | 9 |
|---|---|---|---|
| - | - | + | + |
| - | - | - | - |

**CD45⁺/lin⁻**

| *HOXA/Hoxa* | 1 | 2 | 3 | 7 | 9 |
|---|---|---|---|---|---|
| Endogenous | + | + | + | + | + |
| Transgene | - | - | - | - | - |

**Figure 1**. **Analysis of *Hox* gene expression in transgenic mouse lines**
(**A**), analysis of *Hox* gene expression in the B-cell lineage. FACS plots of the sorted cell populations are shown on the left, and RT-PCR data in the right. The experiment shown was done with mouse line 881P4-2, and is representative for the other transgenic mouse lines. (**B**), summary of analysis of *Hox* gene expression in different FACS-sorted hematopoietic cell populations from RP11-881P4 and RP1-170O19I transgenic mouse lines. The absence or presence of a PCR product is indicated by (-) or (+) respectively. The subset of differentially expressed genes shown in this panel were selected for their reproducibility of detection in multiple FACS sorting experiments on different transgenic lines.

***Generation of a reporter construct***

We designed reporter constructs to continue a transgene-based approach to identify the *HOX*A hematopoietic enhancer,. Since *Hox*a7 was readily detectable in several of the more immature cell populations (see Figure 1, we decided to use a *Hox*a7 reporter construct, and to retrofit this onto BAC clones covering a genomic area of 1 Mb around the *HOX*A cluster (Figure 2). The 2.7 kb upstream *Hox*a7 promotor/enhancer fragment (*Hox*a7s) that we used in this study was reported to be capable of driving regionally restricted expression in mouse embryos (161,235,256). Fluorescent proteins had also been successfully used to track expression of *Hox* genes during embryogenesis (106,236,303). *Hox*a7s was cloned in front of the Venus fluorescent protein reporter gene in order to detect the expression in living cells. Venus is a modified version of EYFP that exhibits faster folding of the protein and yields brighter fluorescence (219).

We transfected pRetro-Sp/L-ΔI-*Hox*a7s-Venus (Figure 3b) in an immortalized stromal cell line derived from murine bone marrow. This resulted in fluorescent cells that were maintained upon G418 selection, confirming functionality of the reporter and the antibiotic selection cassette (data not shown). Transgenic lines carrying the *Hox*a7s-Venus construct were established to test its function in vivo. From one of these, we obtained transgenic embryos showing fluorescence in a regionally restricted pattern with an anterior limit around somite 15 (results not shown), which corresponds to the anterior boundary reported for this construct (255). However, expression was mosaic and no longer observed in later generations, probably due to position effect variegation at the integration site.

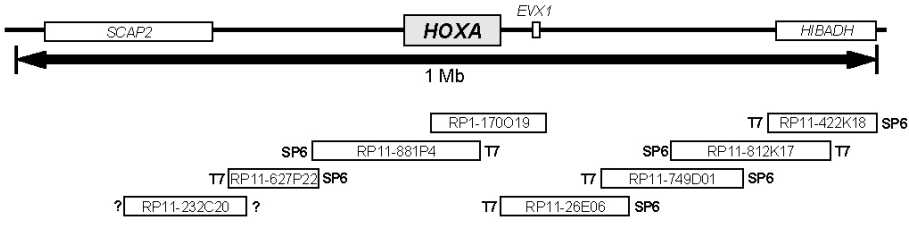



**Figure 2. Location of genomic clones**
The genomic configuration surrounding the *HOX*A cluster is shown at the top, and genomic inserts of the BAC clones used in this study, and of PAC clone RP1-170O19, are schematized below. The T7 and SP6 ends are indicated where known.

***Retrofitting of BAC clones***
Cloning of *Hox*a7s-Venus and CMV-GFP reporters, as well as other inserts into the retrofitting vector pRetroES (334) proved problematic as a high frequency of rearranged clones was observed. We therefore modified the retrofitting vector in several ways to improve its integrity and the stringency of selection. The more stringently regulated LacI promoter was used to replace the Tac promoter. Since the GST-loxP-Cre fusion protein is incompletely characterized and was shown to have decreased specificity with regard to the wild type and variant loxP sites (334), we replaced GST with a small Histidine tag. The ampicillin resistance was replaced by spectinomycin, to provide more stringent selection. The mammalian neomycin resistance cassette was removed and replaced with a multiple cloning site, to facilitate cloning of reporter cassettes.
The modified vector, pRetro-Sp/L, exhibited greatly improved stability and was successfully used to clone and retrofit *Hox*a7s-Venus and CMV-EGFP expression cassettes. However, this construct still showed a lack of specificity for the LoxP sites. The vector still integrated in both the loxP and lox511 sites with about equal frequencies (data not shown), similar to what was observed for the original pRetroES (334).

**Testing BAC constructs in vitro**
In keeping with the deregulation of *HOX* gene expression in the context of leukemias, several established hematopoietic cell lines derived from leukemia patients express *HOX* genes (163,286). It is unknown whether *HOX* gene expression in these cell lines is driven by the continued activity of hematopoietic enhancers, or whether it is due to other mechanisms, e.g., maintained by the polycomb/trithorax-group proteins after being initiated by activity of the enhancers at an earlier stage. If all factors required for enhancer activity are still present in these cells, they might allow rapid screening of reporter constructs. Knowing that RP11-881P4 is unable to express *HOX*A genes in hematopoietic cells, this construct can be used as a negative control in testing such cell lines.

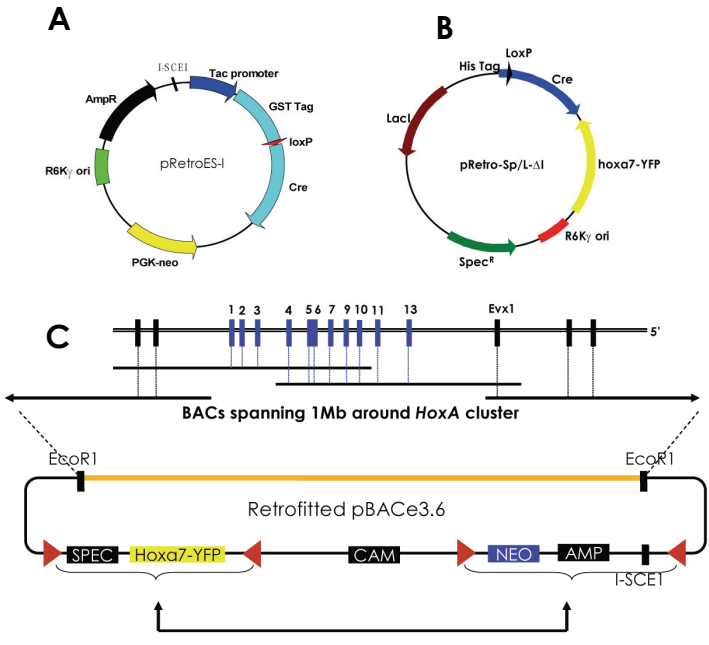



**Figure 3. Retrofitting vectors**
(**A**), scematic drawing of pRetroES-I (334). (**B**), scematic drawing of pRetro-SP/L-ΔI-Hoxa7s-Venus-. (**C**), scematic drawing of the retrofitting procedure. BACs with different genomic inserts in vector backbone pBACe3.6 are retrofitted with a retrofitting vector in both *LoxP* sites present on this vector. Shown is the integration of pRetroES-I in LoxP and pRetro-SP/L-ΔI-Hoxa7s-Venus in Lox511. All Retrofitting vectors can be integrated in either LoxP site indicated by the black arrow. SPEC, Spectinomycin resistence; Neo, Neomycin resistance; AMP, Ampicillin resistance.

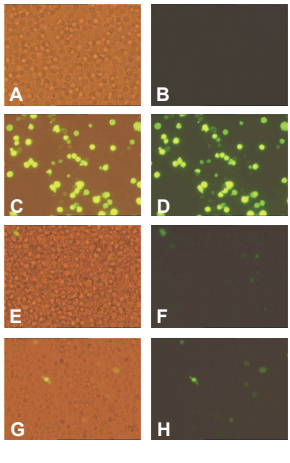



**Figure 4**
**Transfection of retrofitted BAC and PAC constructs.**
K562 cells were untransfected (**A**, **B**), or transfected with pCMV-EGFP (**C**, **D**), PAC RP1-170O19 retrofitted with pRetro-Sp/L-CMV-EGFP (**E**, **F**), or BAC RP11-881P4 retrofitted with pRetro-Sp/L-CMV-EGFP (**G**, **H**). **A**, **C**, **E** and **G**, phase contrast plus fluorescence image. **B**, **D**, **F** and **H**, fluorescence image.

To enable screening of the BACs in vitro by transfection into hematopoietic cell-lines, the loxP and lox511 sites on either side of the genomic insert were retrofitted with both pRetro-Sp/L-ΔI-*Hox*a7s-Venus and pRetroES-I, in which the first construct provides the reporter gene and the second construct a neomycin selection cassette and a unique I-SceI site for linearization. We selected clones that contained the reporter gene near the end of the insert that faces the *HOX*A cluster in the normal genomic configuration, to avoid possible complications due to presence of insulator sequences in the insert, or to the presence of vector sequences between the insert and the reporter construct. If possible, we also maintained the same orientation of the genomic insert relative to the direction of transcription of *Hox*a7. Using this approach, we retrofitted a set of six overlapping BAC clones covering the regions between the *HOX*A cluster and its neighbouring genes, SCAP2 and HIBADH (see Fig. 1 and Table 2). To establish proof of principle, we introduced BAC RP11-881P4 and PAC RP1-170O19, each retrofitted with an EGFP reporter driven by the strong CMV promoter, into the K562 erythroleukemia cell line using nucleofection. Compared to a small construct carrying the same reporter, which was very efficiently transfected (Fig. 4C, D; compare to 4A, B), the BAC and PAC constructs yielded considerably fewer cells expressing detectable levels of fluorescent protein, although some cells were fairly bright (Fig. 4E-H). This difference probably reflects at least in part the fact that the concentration of DNA molecules in the BAC/PAC transfections is at least an order of magnitude lower than for pCMV-EGFP.

**Table 2. Overview of retrofitted BAC clones**

| clone | Lox site | pRetro inserted |
|---|---|---|
| RP1-170O19 | LoxP | pRetro-Sp/L-ΔI-CMV-EGFP |
| RP11-881P4 | LoxP | pRetro-Sp/L-ΔI-CMV-EGFP |
| | Lox511 | pRetroES-I |
| RP11-26E06 | LoxP | pRetroES-I |
| | Lox511 | pRetro-Sp/L-ΔI-*Hox*a7s-Venus |
| RP11-749D01 | LoxP | pRetroES-I |
| | Lox511 | pRetro-Sp/L-ΔI-*Hox*a7s-Venus |
| RP11-422K18 | LoxP | pRetroES-I |
| | Lox511 | pRetro-Sp/L-ΔI-*Hox*a7s-Venus |
| RP11-812K17 | LoxP | pRetro-Sp/L-ΔI-*Hox*a7s-Venus |
| | Lox511 | pRetroES-I |
| RP11-232C20 | LoxP | Not determined yet |
| | Lox511 | Not determined yet |
| RP11-627P22 | LoxP | pRetro-Sp/L-ΔI-*Hox*a7s-Venus * |
| | Lox511 | pRetroES-I |

* BAC insert is in opposite orientation relative to the direction of *Hox*a7 transcription

## 4.5 Discussion

We have investigated expression of *Hox* genes in hematopoietic cells of different lineages using cell sorting and RT-PCR analysis, and found that overall, the progenitor cell populations express more *Hox* genes at detectable levels than terminally differentiated cells of various lineages. This finding is consistent with previous data obtained from mouse and human bone marrow (248,278).
Expression of several *Hox*a genes could be reproducibly detected, although fairly large numbers of cells were required for detection. We recently investigated mice carrying a green fluorescent protein reporter inserted into *Hox*a2, which is readily detectable in embryos (236). *Hox*a2 is one of the more robustly expressed genes in the cell populations we analyzed, but the fluorescence could not be detected by FACS analysis in hematopoietic cells from the bone marrow (data not shown). We assume this reflects the low expression levels of *Hox* genes in hematopoietic cells.

Using human- and mouse-specific primers for the more robustly expressed genes, we then analyzed several transgenic mouse lines carrying large transgenes covering the human *HOX*A cluster. None of these were expressed in purified hematopoietic cell fractions, strongly suggesting that enhancers for the hematopoietic system are located outside these constructs. It is possible that our failure to detect transgene expression is due to impaired functioning of the putative human enhancer in the context of murine cells. However, given the high degree of similarity between mouse and human, this seems highly unlikely. Some indirect support for this notion is provided by enhancers from avian and teleost *Hox* clusters which are functional in murine embryos e.g. (7,199), and the hematopoietic stem cell enhancer for the murine SCL/TAL-1 gene is also active in Xenopus (109).

We have developed a strategy for identifying distant enhancers using conventional transgenic analysis in vivo and in vitro, which can be easily adapted to other genes. The collection of BAC constructs described here covers the most likely region in which these enhancers may be located, and can now be tested. However, as our data suggest that expression of *Hox* genes in hematopoietic cells is below the detection limit of fluorescent proteins, it may be necessary to replace Venus with a more sensitive, enzymatic reporter such as lac*Z*.

It has been shown that regulatory DNA elements communicate with distant target genes through direct physical interactions. Several methods have recently been developed that allow identification of these physical interactions between distant DNA sequences, and are therefore applicable to identification of distant enhancers (reviewed in (295,302)). One of these methods is the Chromosome conformation Capture (3C) technology (68) with is based on the quantitation of cross-linking frequencies between two DNA fragments as a measure of their frequency of interaction in the nuclear space. This technique has succesfully been applied to analyse the conformation of a 200 kb region spanning the mouse β-globin gene cluster in its active and inactive transcriptional state (319). However, for this technique a prediction about the possible inaracting sequences is required. In our

case not only the location of the enhancer is unknown, also the responsive sequences around the genes has not been determined. Another issue in using this technique might be the large number of cells that is required for the analysis, which could be a disadvantage when working with progenitor cells. Using an established hematopoietic cell line instead might circumvent this problem.

Advanced technology that allows the identification of physically interacting loci without a-priori knowledge about the genomic organisation, based on the 3C method has also been developed. One of these methods is the open-ended Chromatin Conformation Capture approach that was used to study the chromosome dynamics of the *Hox*b1 gene during the induction of its expression in the context of retinoic acid-induced differentiation of mouse embryonic stem (ES) (339). In the standard 3C procedure the abundance of specic ligation products is determined by quantitative PCR. In the open ended 3C the technique is expanded by replacing the directed quantitative PCR with an inverse-PCR. These inverse-PCR products can then be cloned and sequenced allowing the identification of unknown sequences legated to the locus of interest. This method has, however, not shown to reveal functional interactions between genes and regulatory elements. This is most likely due to the apparent high accessibility of this locus, resulting in a very large number of potential interacting sequences over several hundreds of kilo bases.
Another technique based on the 3C method is the 4C technology, which yields a genome-wide overview of both in cis and in trans interactions of a sequence of interest (294). Like 3C, 4C technology depends on the selective ligation of cross-linked DNA fragments to a choosen target site. Then, all the crosslinked DNA fragments are amplified via inverse PCR. Tailored microarrays are used to simultaneously analyze the captured fragments. In its present implementation, it is applicable only to very long-distance interactions, as interactions in a region of several megabases surrounding the gene of interest are completely saturated (294). However, it seems possible that it could be adapted through dilution of the imput material to identify elements at intermediate distances, where we suspect the hematopoietic enhancers are located. Further investigation is needed to test the applicability for the *Hox* locus.

# 5

# Discussion

## 5.1 Introduction

During development, pluripotent embryonic stem cells are responsible for the formation of tissues and organs from all three germ layers. In post-natal life these tissues and organs are subject to cell turnover and injury. To be able to substitute for a loss of cells, adult tissues contain of tissue-specific stem or progenitor cells. The presence of these tissue-specific stem cells, has been shown for a large number of organs and include neuronal stem cells in brain (147), oval stem cells in liver (86), cardiac stem cells in heart (23,176), different types of epithelial stem cell in the skin (35), and of course the very well studied hematopoietic stem cells (HSC) in the bone marrow (223). Many studies have also been dedicated to mesenchymal stem cells (MSC) (241). Less then fifty years ago, MSC were initially discovered as osteogenic progenitor cells able to form a stroma that can support hematopoiesis (91,94). These cells were soon attributed with increasingly more functions and characteristics.

## 5.2 MSC: stem cells or progenitors

Stem cells are defined as cells that are clonogenic, self-renewing, and have a multipotent differentiation potential. One of the caveats in MSC biology is that basically every multipotent (progenitor) cell that is capable of forming CFU-F colonies and able to differentiate towards some mesenchymal cell types is defined as an MSC in the literature. Also, self-renewal of MSC has not been very well defined as in vivo demonstration of repopulation and self-renewal, analogous to the assays that exist for HSC has proven to be very challenging for MSC (133). Culture-expanded human MSC usually stop proliferating before 40 population doublings, at which time the cells become bigger and more flattened. A lot of variation has been shown between different culture conditions and the age of the donors (reviewed in (271)). In addition, MSC do not represent a homogeneous population of stem cells, as it is well established that MSC derived from different locations have distinct molecular en phenotypic characteristics (127,241,242,330). Besides the heterogeneity between MSC from different origins and preparations, the cultures are usually also composed of a mixed heterogeneous cell population. Finally, there is no standardized method for isolating and culturing MSC. All these variables make it difficult to define the exact properties of MSC and to compare the outcome of different studies. To address this issue, some criteria to define human MSC have been proposed (74), but these criteria are still too general and will most likely be applicable to most of the published studies.

In addition to MSC, several tissues were also shown to contain the totipotent Mouse Adult Progenitor Cells (MAPC). MAPC are like MSC purified through plastic adherence and are likely to be co-purified in the initial MSC cultures, but afterwards require specific culture conditions for their enrichment (144,145). Also, a population of multipotent mesodermal cells has been isolated from the bone marrow based on their SSEA-$1^+$ expression. These cells can either be directly isolated by FACS from the bone marrow or derived via CFU-F assays. They were able to contribute to most mesodermal lineages in vivo, including hematopoietic cells (8). The hierarchical

relationship between MAPC, MSC, mesodermal progenotor cells, and all other poorly defined tissue specific stem cells is unfortunately still not clear. Especially, in the present era of stem cell biology and cellular therapeutics it is extremely important to revisit the definitions and relationships of all different stem cells that have been described in literature. Also more precise molecular and cellular markers to define subsets of MSC and standardization of protocols for expansion of MSC are needed. This will hopefully allow researchers to establish a clear hierarchical description of the wide range of stem cells.

## 5.3 Trans-differentiation of MSC: fact or artefact?

A controversial issue around MSC is the matter of stem cell plasticity or trans-differentiation. This is a mechanism by which committed cells from one tissue change their fate and give rise to cells of a different type. The idea of stem cell plasticity has fascinated researchers for a long time but gained most of its popularity about a decade ago when it was shown that for example HSC could be transformed into neurons and skeletal muscle, and that the reverse was also possible (31,36,58,82,142,143,207). MSC were reported to differentiate into a variety of tissues from all germ layers (9,125,128) including cardiomyocytes (154), astrocytes (164), lung epithelium (228), endothelial cells (56) and liver cells (277).
However, it soon became apparent that some of the results could not be reproduced in other laboratories (43,155) or were shown to be caused by artefacts (141). A detailed piece of work that speaks against any form of the plasticity of HSC was done by the Weissman lab that performed single cell transplantation studies with HSC, which they tracked by a fluorescent marker in a large set of tissues. No evidence was found that these labelled hematopietic cells contributed to “foreign” tissues (329). Several trans-differentiation studies could later be explained by cell fusion (6,187,313,336,345) and it has now been shown that under some stress conditions cells of different lineages can fuse with each other leading to the hypothesys that this constitutes a quick emergency repair mechanism (37). Another explanation for apparent plasticity is that researchers may have used mixed cell isolates that contained undefined or undetectable stem cells of a different lineage that were responsible for the observed ‘plasticity’ (167). Recently it was reported that MSC could be found in virtually every tissue of the body (65). For other tissue-specific stem cells, such an analysis of anatomical distribution has not been done, but there is increasing evidence that stem cells are more widely distributed than thus far presumed (169,205,220). Because MSC in vivo are poorly characterised and usually isolated and analysed as a heterogeneous mixture of cells, this makes the transdifferentiation experiments prone to ‘contamination’ with other unexpected tissue stem cells (169).
Despite the criticisms, MSC have been used in animal experiments and clinical trials to treat a broad range of diseases affecting different tissues. In several of these studies, therapeutic transplantations with MSC were shown to be efficacious, which is remarkable, given several reports of infrequent engraftment and poor trans-differentiation efficiency. It was therefore suggested that these results were not due

to MSC plasticity but were rather induced by another characteristic of MSC, namely their ability to form a supportive stroma (reviewed in (242)). This was also one of the first properties attributed to these cells (94). It has now been suggested that MSC are not only capable to serve in hematopoietic niches, but also contribute to the repair and homeostasis of a wide range of injured tissues (242). The mechanisms that have been proposed by which MSC fulfil this task include the secretion of soluble factors that block apoptosis and inflammation, stimulation of proliferation from the injured host tissue and by stimulating angiogenesis (118,119,135,137,218,227,310,318,322,341,352).
Some of these properties (118,119,227,322,341) align well with the previously observed immunosuppressive properties in therapeutic bone marrow transplantation (17,351). A function of MSC in general tissue homeostasis also correlates very well with their widespread distribution throughout the adult body. A role in angiogenesis is strongly supported by observations, which showed that MSC may reside in the blood vessel walls as multipotent pericytes. This hypothesis was already postulated in 1988 by Owen and Friedenstein (231) and there is increasing evidence that supports this hypothesis (reviewed in (64,149,288)). In Chapter 3 we showed the presence of β-galactosidase expression in some blood vessels within the sternum and femur bone marrow cavities from a *Hox*b8-LacZ transgenic mouse line, in which *Hox*b8 is driven by a region-specific enhancer element. Isolation of these β-galactosidase expressing cells from the vasculature and comparison of their *Hox* code to the CFU-F colonies that we routinely obtain from these locations might reveal whether these cells are related to each other.

## 5.4 Embryonic origin of MSC

The complete lack of knowledge about the foetal origin of MCS is also in part responsible for its poor definition. Like in the adult, MSC can also be found in a variety of foetal tissues, but thus far it is unclear how these are related. It is for example not known whether MSC are derived from a common precursor, or whether they arise independently in different tissues with distinct characteristics. The question what the relationship is between MSC from different tissues could be addressed by clonal analysis or fate mapping (reviewed in (57,151,238)). Several different forms of fate mapping have been reviewed by Clarke and Tickle (57). Besides the invasive physical fate mapping experiments that involve the transplantation or direct labelling of a group of cells during development, genetic fate mapping has been developed. Genetic fate mapping, based on the expression of a marker gene under control of a tissue specific promoter, allows non-invasive cell labelling which makes it applicable to mouse studies. However, care should be taken to characterise the promoter activity in detail as this may otherwise result in unexpected cell labelling in different tissues. For fate mapping of MSC, a promoter with activity in the mesodermal lineage may be analysed to confirm the proposed mesodermal origin. Because of the wide-spread activity of most lineage committed genes during development, this could be combined with an inducible reporter activation that allows inductions over a short time-frame. To be able to correctly

interpret the results, the heterogeneous nature of the MSC culture must first be characterised in detail. In addition extensive analysis of background expression versus effective activation should be determined. In Chapter 2 of this thesis some classical fate mapping experiments have been discussed that showed that the bone marrow stroma is derived from the limb primordia (150,181). However this is in contradiction with the *Hox* codes that we observed in MSC we isolated from derived from individual vertebrae. These patterns did not correspond to the embryonic prevertebral *Hox* codes and were suggestive of an origin different from the vertebral primordia. Our studies described in Chapter 3 also suggest that there in no linear relationship between the sternum or femur primordia, and the MSC isolated from these locations from the adult mouse. Absence or presence of a β-galactosidase staining in fetal tissues could not be correlated with LacZ expression or β-galactosidase staining in the adult MSC. Genetic clonal analysis of MSC could be a powerful tool that might contribute to a better understanding of the diversity, distribution and potential of MSC. Especially knowledge of the origin and distribution of MSC could also lead to a better understanding of how and when cultered MSCaquire their topographic *Hox* codes.

## 5.5 Regulation of *Hox* genes in MSC

*Hox* genes are well known for their role in tissue specification and in cell identity during embryonic development. These genes may also play a role in the establishment of tissue specific variation of MSC characteristics. In Chapter 2 it was described that cultured MSC display *Hox* genes expression patterns that correlate with their anatomical origin rather than tissue type. It is unclear when the cells obtain this pattern, and how this pattern is maintained during culture. To fully understand the establishment of these patterns it is important to understand where MSC first appear in the body during development and how this correlates with the embryonic *Hox* code. Related questions to this topic are: (i) Is ectopic expression of *Hox* genes in adult MSC sufficient to reprogram positional identity. (ii) Is the MSC gene expression program instructed by the surrounding tissue? The above-mentioned clonal analysis may provide an answer to the question whether the MSC *Hox* codes are induced by cues from the surrounding tissue or are completely cell-autonomous. These issues however could perhaps more efficiently examined in transplantation studies and forced in vivo overexpression of *Hox* genes in MSC.
In addition, it is also interesting to identify the regulatory elements that are responsible for the establishment of the expression of *Hox* genes in MSC. In Chapter 3 it was described that large constructs that span most of the *HOX*A cluster produced a topographic expression pattern that largely resembled the endogenous *Hox*a expression in MSC. Furthermore, we show for at least the *Hox*b8 gene that transcription is activated through elements that are located in the proximity of the gene. It is unclear whether these elements are the same elements that are responsible for mesodermal expression in the developing embryo or that they are specialized sequences that are partially overlapping the foetal elements. Surprisingly, using these small transgenic constructs a loss of tissue-specific expression was observed,

causing 'ectopic' expression in MSC from other tissues where this gene normally is silent. This suggests that these constructs contain elements that induce gene activation, but lack those which regulate appropriate suppression.

One of the mechanisms that could play a role in tissue specific gene suppression and therefore contribute to the establishment of the MSC *Hox* code are micro-RNAs (miRNAs). miRNAs are short nucleic acid molecules with an average length of 22 nucleotides. They exert their effect by binding directly to target messenger RNAs and inhibiting mRNA stability and translation. Each miRNA can bind to multiple targets and many miRNAs can bind to the same target mRNA, allowing for a complex pattern of regulation of gene expression. At several positions in the *Hox* clusters, conserved miRNA expression sites can be found. The produced miRNAs have recognition sites in the 3'-UTRs of several *Hox* transcripts.
Some studies indicated that miRNAs help to define the regions where *Hox* genes are expressed, thereby contributing to their spatial and temporal specificity of expression during development (198,237,344). Some recent studies showed that miRNAs are able to mediate regulation of proliferation as well as differentiation in several different stem cells (51,124,311). In addition, several miRNAs have been shown to correlate with osteogenic and adipogenic MSC differentiation although for only a few of these a function has been demonstrated (83,174). To date there is no evidence in literature for miRNA involvement in the establishment of the MSC *Hox* code and future studies are required to explore this possibility. However, analysis of a *Hox*b8-LacZ knock-in mouse (323), in which the 3' UTR is replaced by a SV40 PolyA suggests that the 3' miRNA recognition site in the *Hox*b8 transcript is not involved in tissue-specific regulation of this gene (Chapter 3). It wil be interesting to extend this analysis to other *Hox* genes that are known to contain a miRNA recognition site in their 3'UTR as this could be a one of the mechanisms that contribute to establishment the topographic specific pattern.

Other key candidates for the establishment of tissue identity are the PcG and TrxG proteins. These proteins play an important role in the maintenance of *Hox* patterns by mediating mitotic inheritance of lineage-specific gene expression programs (265). Mice lacking one or more of these proteins usually display a mildly disturbed colinear *Hox* pattern in embryos together with the subsequent homeotic transformations.
A mechanism by which PcG/Trx-G proteins may influence the establishment of a *Hox* pattern can be deduced from the transcriptional repression of *Hox* genes in pluripotent stem cells like embryonic stem cells (ES cells). Although *Hox* genes are normally not expressed in ES cells, all factors required for expression, including RNA polymerase II are already present on the promotors while being epigenetically suppressed by other factors. A chromosomal region that contains both repressive and active histone modifications is called a bivalent chromatin domain (25). This is characterized by the presence of both Histone H3 lysine 4 (K4) and lysine 27 (K27) methylation within the same genomic region. K4 methylation positively regulates transcription by recruiting nucleosome remodeling enzymes and histone acetylases, while K27 methylation negatively regulates transcription by promoting a compact

chromatin structure (87,252,265,276,296,340). These histone H3 K4 and K27 methylation marks are catalyzed by trithorax and Polycomb group complexes, respectively. The K4 methylated regions of the silent *Hox* loci are cell type-specific and can be up to 60 kb in length, spanning multiple *Hox* genes (25,117). It is hypothesized that the precise locations of the epigenetic modifications of the histones are involved in controlling the colinear expression of genes from the cluster upon differentiation (13,25). It is unknown whether similar bivalent structures are present in adult stem cells like MSC as well or that these cells only display a stabilized epigenetic configuration inherited from for example its fetal precursor. However, the fact that the tissue specific *Hox* codes in MSC are maintained during culture and are insensitive to exposure to heterologous tissue or differentiation agents (Chapter 2), suggests that the epigenetic patterns in MSC are stably maintained via permanent histone marks. Interestingly, this obeservations also strongly suggest that the MSC that we obatain upon culture are a commited progenitor population rather than a true stem cell source.

## 5.6 Reporter gene expression in MSC

During our research we have used a mouse strain carrying a fluorescent reporter gene under control of the *Hox*a2 promoter. Although most of the CFU-F colonies expressed *Hox*a2 at the transcriptional level, we were unable to detect the fluorescence by microscopy and FACS analysis in MSC from bone marrow. Also, hematopoietic cells from these animals in bone marrow and fetal liver did not show a fluorescent signal that could be detected by FACS analysis.
*Hox*b8 constructs fused to a Lac reporter did in most cases also not result in any detectable signal in adult MSC. It is not yet clear whether the absence of detectable GFP or β-galactosidase expression is caused by an absence of the protein, expression levels too low for visualization, or specific down regulation of translation. Analysis of MSC cultured from C57BL/6-Tg(ACTB-EGFP)1Osb/J mice (224) that express EGFP under control of the chicken beta-actin promoter and cytomegalovirus enhancer, revealed that EGFP could only be detected in a small fraction of the MSC cultures (unpublished observation). Similar to our observations, it was recently reported that rat bone marrow derived MSC greatly repress expression of lentivirus transduced EGFP expression. EGFP in cultured rat MCS was detected in less than fifty percent of the culture. EGFP antibody staining showed that the cultures also contained cells that expressed EGFP at very low levels, undetectable by fluorescence microscopy and FACS analysis. The authors showed that this silencing of the GFP gene was caused by DNA methylation. In agreement with these results they observed that incubation of MSC with the DNA demethylation reagent 5-azacytidine increased the number of cells with detectable GFP (122).
The Escherichia coli lacZ gene, which encodes the beta-D-galactosidase enzyme, is one of the most widely used reporter genes in transgenic studies. Many spatial and temporal expression patterns of genes and their developmental profiles have been identified using lacZ reporter constructs. In postnatal studies on the other hand,

LacZ transgenes often function poorly and display variegated expression (62). It has been suggested that the GC content in the DNA that is significantly higher than encountered in most mammalian genes contributes to methylation mediated silencing. However, so far no clear evidence has been provided for this (reviewed in (213)). Analysis of our transgenic reporter construct expression should be further analysed using antibody labelling to potentially relate the lack of visibility to the expression level. Also other *Hox* genes fused to a reporter gene could be analyzed to investigate whether the same detection problems are encountered.

## 5.7 Function of *Hox* genes in MSC

After having established that cultured MSC express region specific *Hox* codes, we wondered about the function of *Hox* gene expression in MSC. As it is unknown whether the transcriptional *Hox* code is translated into functional proteins candidate *Hox* genes for functional analysis in MSC should first be analyzed for their expression of translated *Hox* proteins in MSC cultures. Multiple alternative, polycistronic and non-coding transcripts in the *Hox* clusters have been described (26,197,284), which are thought to contribute to the maintenance of an active chromatin conformation. The possibility exists that only some of the transcribed genes that we detect are actually translated into a functional *Hox* protein while the remaining neighbouring genes only become expressed due to an open chromatin state, but are repressed in their translation.

In other stem cell systems it is has been observed that *Hox* proteins serve a role in processes like proliferation and differentiation. *Hox*b1 for instance is able to mediate the maintenance and expansion of posterior neural progenitor cells. Forced expression caused progenitor amplification and reduced the number of terminally differentiated cells (110). A similar phenomenon has been observed for *HOX*B4 and *Hox*a9: upon over expression this gene is able to mediate hematopoietic stem cell expansion in vitro (279,314,317). Also, for several other *Hox* proteins a role in hematopoietic proliferation and differentiation has been described (1,193,316) as also discussed in Chaper 4. Based on the knowledge from these and other stem/progenitor cell populations it is conceivable that *Hox* genes are involved in similar processes in MSC. In fact, preliminary studies on tamoxifen inducible *Hoxa* cluster knockout mice , suggested a slight proliferative defect upon *Hoxa* cluster reduction (data not shown). These experiments have to be repeated to confirm this result. Hypotheses about a function of *Hox* proteins in differentiation are based on observations of tissue specific expression patterns in osteogenic, chondrogenic and adipocytic cell types. Expression of several *Hox* genes has been reported in the pre-adipocytic cell line 3T3-L1 and transcriptional differences were detected during differentiation. The authors also detected expression of *Hox* genes in murine retroperitoneal fat deposits (60). In agreement with this observation, most of the *HOX* genes were shown to be active in human adipose tissue in which they display varying expression patterns in different fat deposits. However, it is unclear whether these regional differences are topographically related or actually contribute to

variation in adipogenic capacities (39,328). Gersch and coworkers studied *Hox* protein expression in adult bone fracture repair, and noticed that *Hox*a2 and *Hox*d9 protein levels become upregulated in both chondrogenesis and osteogenesis during repair (104). Others have observed that during the osteoblast differentiation process of pre-myeloblastic cells *HOX*A10 is transiently expressed (20). And indeed, direct interaction of *Hox*a10 with regulatory elements of osteogenesis-specific genes has been demonstrated using chromatin immunoprecipitation (123).
In chondrogenesis, a model was proposed for the function of *Hox*c8 and *Hox*d4 in chondrocyte differentiation (347). However, the effects seem to be related to embryonic functioning and could not be confirmed in an in vitro culture system of postnatal derived chondrocytes (59). This type of experiments underscores the fact that although it is clearly demonstrated that *Hox* genes play an important role in proper skeletal development and growth during embryogenesis, the function of *Hox* proteins in adult mesenchymal tissues cannot be derived from a straightforward correlation.

One of the characteristics of MSC is the ability to form a stroma or microenvironment that is able to support HSC (48,307) and potentially also other tissue specific progenitors (242). Thus far, *Hox* genes have not been implicated to function in the formation of a stem cell supportive stroma. Also, no evidence is available that shows the interaction of *Hox* proteins with the receptors and secreted cytokine signals known to be responsible for the maintenance of the hematopoietic stem cell niche. In addition, comparative gene expression analysis between supportive and non-supportive stromal cell lines derived from the mouse fetal liver did not reveal a specific regulatory change of *Hox* transcripts that could indicate a functional role (supplementary data (120)). However, it is conceivable that the topographic *Hox* gene expression contributes to site-specific identity of MSC and serves as a blueprint of positional information for mesenchymal tissues during homeostasis and repair. This is supported by our observation that the *Hox* codes are intrinsic to the topographic origin of the MSC (Chapter 2). At this moment the question whether *Hox* genes are involved in regulating tissue homeostasis, repair and cellular identity in the adult, remains unanswered.

# 6

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## Summary

The adult mammalian body is home to several types of stem cells that are responsible for the maintanance of specific tissues or organs. In this thesis I describe one of these tissue stem cells; the Mesenchymal stem cells (MSC). MSC can be found in many locations throughout the body and are able to form different types of mesenchymal cell lineages as for example osteoblasts (bone), chondrocytes (cartilage), and adipocytes (fat). MSC can be isolated from these different locations and cultured in vitro. When placed into a tissue culture dish, MSC form clonogenic plastic adherent colonies in which the cell morphology resembles this of fibroblasts, hence the name colony forming unit-fibroblast (CFU-F). MSC isolated from different tissues show tissue specific differences reflected in for example their propensity for differentiating towards specific lineages.

In Chapter 2, we propose that *Hox* genes may be involved in determining tissue-specific properties of MSC. This was suggested as *Hox* genes are known to play an important role in regional specification and organ development in the embryo. We investigated the *Hox* gene expression profiles of CFU-F colonies derived from various locations in the body. This revealed that MSC have characteristic *Hox* expression signatures that are specific for their anatomical origin. The topographic specificity of these *Hox* expression patterns is maintained during differentiation, suggesting that they are an intrinsic property of MSC.

In Chapter 3, we examined how these topographic expression patterns are established at the molecular level, and how they relate to the origin of MSC in the embryo. We show that at the regulatory sequences responsible for driving *Hox* gene expression in CFU-F are within the confines of the *Hox* clusters. We also show that known embryonic region-specific enhancers can drive expression in CFU-F, but without any topographic specificity. We hypothesize that MSC in bone marrow do not originate locally from the same embryonic precursors as the bone they are found in, but colonize these from a distinct source.

In Chapter 4 we touch upon a different cell type in which *Hox* proteins are well known to play a role in proliferation and differentiation; the hemotopoietic cells, or blood cells. Despite a broad knowledge about the function of *Hox* genes in normal hematopoiesis and leukemia, nothing is known about the regulatory sequences that are responsible for driving *Hox* gene expression in the hematopoietic system. We started to search for hematopoietic enhancer elements in the *HOX*A cluster and its direct surroundings. The analysis suggested that the enhancer elements are located outside our analyzed region. We also describe the development and implementation of a strategy to generate large reporter constructs for identification of the putative distant enhancer.

## Samenvatting

In het lichaam van een volwassen dier bevinden zich verschillende typen stamcellen die verantwoordelijk zijn voor het onderhoud van specifieke weefsels of organen. In dit proefschrift beschrijf ik een van deze stamcellen; de mesenchymale stam cel (MSC). MSC bevinden zich op veel verschillende plaatsen in het lichaam en zijn in staat om verschillende weefsels te vormen, waaronder osteoblasten (bot), chondrocyten (kraakbeen) en adipocyten (vet). MSC kunnen uit het lichaam geisoleerd worden en gekweekt. De cellen vormen klonogene kolonies op de bodem van kweekschaaltjes. De cellen in zo'n kolonie lijken sprekend op een celtype met de naam fibroblast en daarom worden deze kolonies dan ook colony forming unit-fibroblast (CFU-F) genoemd. MSC die uit verschillende weefsels zijn geisoleerd hebben kenmerken die specifiek zijn voor de plaats in het lichaam waar ze vandaan kwamen. Deze eigenschappen uiten zich vaak in hun mogelijkheden tot het differentieren naar de verschillende mesenchymale weefsel types.

In Hoofdstuk 2 stellen we voor dat Hox eiwitten een rol kunnen spelen in het bepalen van de weefselspecifieke eigenschappen van MSC. Dit vermoeden was ontstaan omdat Hox eiwitten een belangrijke rol spelen in de regio specificatie en orgaan ontwikkeling in het embryo. We hebben het expressiepatroon van *Hox* genen in CFU-F kolonies uit verschillende plaatsen uit het lichaam bepaald, en hieruit bleek dat MSC karakteristieke expressie patronen hebben die specifiek zijn voor hun oorspronkelijke locatie in het lichaam.

In Hoofdstuk 3 onderzoeken wij hoe de locatie specifieke expressie patronen tot stand komen op moleculair niveau en of de patronen mogenlijk verwant zijn aan de plaats van oorsprong van MSC in het embryo. We laten zien dat de regulatoire elementen die verantwoordelijk zijn voor Hox gen expressie in CFU-F binnen de grenzen van de *Hox* clusters vallen. Ook laten wij zien dat reeds bekende embryonale enhancers in staat zijn om *Hox* gen expressie in CFU-F aan te zetten, maar zonder een locatie specifiek patroon. Gebaseerd op de resultaten uit Hoofdstuk 2 en 3 veronderstellen wij dat MSC uit het beenmerg niet ontstaan uit dezelfde embryonale voorlopers waaruit het bot gevormd wordt, maar dat ze deze plaatsen later koloniseren vanuit een andere locatie.

In Hoofdstuk 4 bespreken we een ander celtype waarin Hox eiwitten een balangrijke rol spelen in de groei en differentiatie; de hematopoietische cellen, of bloed cellen. Het is niet bekend welke enhancer elementen verantwoordelijk zijn voor *Hox* gen expressie in bloed cellen. Uit ons onderzoek is gebleken dat deze elementen niet in de directe omgeving van het humane *HOXA* cluster ligt, en dus waarschijnlijk een flinke afstand verwijderd zijn van dit cluster. We beschrijven ook de ontwikkeling van een methode waarmee deze enhancer elementen mogelijk opgespoord kunnen worden.

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**Afscheid betekent de geboorte van de herinnering.**

(uit het gedachtengoed van Salvador Dali)

## Curriculum Vitae

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