Fibroblast positional memory

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Summary
During embryogenesis in humans, 39 Hox genes in four different clusters are regulated in a temporal and spatial manner by epigenetics, giving rise to segmentation and specific cell differentiation. Studying gene expression, it was found that many different gene expression profiles exist, both in mesenchymal stem cells (MSC) and fibroblasts. These gene expression differences may lead to fibroblast plasticity, and ultimately gave rise to many different fibroblast subtypes. Furthermore, fibroblast Hox gene expression differences seems to be based on the location of the cell along the anatomic axes of the body, instead of being based on local interactions. Fibroblasts that are spatially close are not necessarily closely related, while distal fibroblasts gene expression profiles revealed striking similar 'signatures'. This same 'signature' could also be found for MSC, showing similarities between bone marrow MSC (BM-MSC) and cord blood MSC (CB-MSC). Regulation of Hox genes seems to be complex, but might be partially regulated by several mechanisms via miR-21 in both MSC and fibroblasts. Other gene expression differences were found, showing a diverse heterogeneity in both fibroblasts and MSC populations.

Keywords: Fibroblast, MSC, Hox, positional memory, anatomic axes, miR-21

Embryogenesis

Demystifying embryogenesis

For a long time the proliferation, differentiation and spatial organization of cells during embryogenesis have intrigued developmental biologists. How could a single-cell zygote give rise to this many different cell types, and even more striking, how do these cells know where to go? Why is there hair above the eyes, but not on the nose? How do these cells know how to specialize into so many different types of tissue? These questions were partly answered a little over 30 years ago, with the discovery of the homeotic genes [1-3]. It was found that homeotic genes were expressed in a gradient, giving rise to segmentation and spatial organization of different anatomic sites, while giving cells positional identity along the anterior-posterior anatomic axis[4,5]. Homeotic genes were sequenced in the early 80's, and were found to share a short 180 base-pair region, termed *homeobox* [3,6-8]. Homeobox containing genes are in literature also referred to as *Hox* genes. The homeobox in Hox genes encodes a DNA-binding domain, called the homeodomain[9,10]. Hox encoding proteins are able to bind specific DNA sequences in *cis*-regulatory regions in several genes, and in this way function as important transcription factors during embryogenesis[11-14]. More strikingly, Hox genes seemed to be conserved in many different animals, ranging from fruit flies [15] and mice [16] to higher order mammals including humans. These findings show the importance of Hox genes throughout evolution, and possibly depict a common ancestor[17]. Figure 1 shows the hypothetical locus of this common ancestor. In humans, 39 Hox genes are known, located on four different clusters named HoxA till HoxD. These four different clusters are on chromosome 7, 17, 12 and 2 respectively. Hox genes have temporal and spatial colinearity, meaning 3' genes are expressed in the anterior part of the body, while 5' Hox genes are expressed at the posterior part of the anatomic axis during embryogenesis (figure 1)[18,19].Thirty-five years ago cells were characterized mainly on morphology and cellular markers. However, today cells can be further subcategorized based on gene expression profiles[20]. Since the discovery of the importance of Hox genes during embryogenesis, it was found that many cell types in adults still express Hox genes, and in this way determine the positional identity along the anterior-posterior body axes. Rinn and colloagues found that differential expression of the HoxA and HoxD genes reflected the anatomic location of the fibroblast on the proximal–distal axis along the upper and lower limbs. On the other hand, differential expression of HoxC genes correlated with

the anatomic location in the torso along the anterior–posterior axis, and HoxA/HoxD genes were not expressed in the torso. Fibroblasts from internal organs showed increased HoxB expression when compared to dermal fibroblasts[20]. This overview will focus on fibroblasts from different anatomic locations, and try to compare them. We will see that cells from the same type but from different anatomic locations in the body can on one hand have entirely different gene expression profiles, while some may share a gene expression signature. However, first, to fully appreciate anatomic spatial organization, a brief background in embryonic development is necessary.

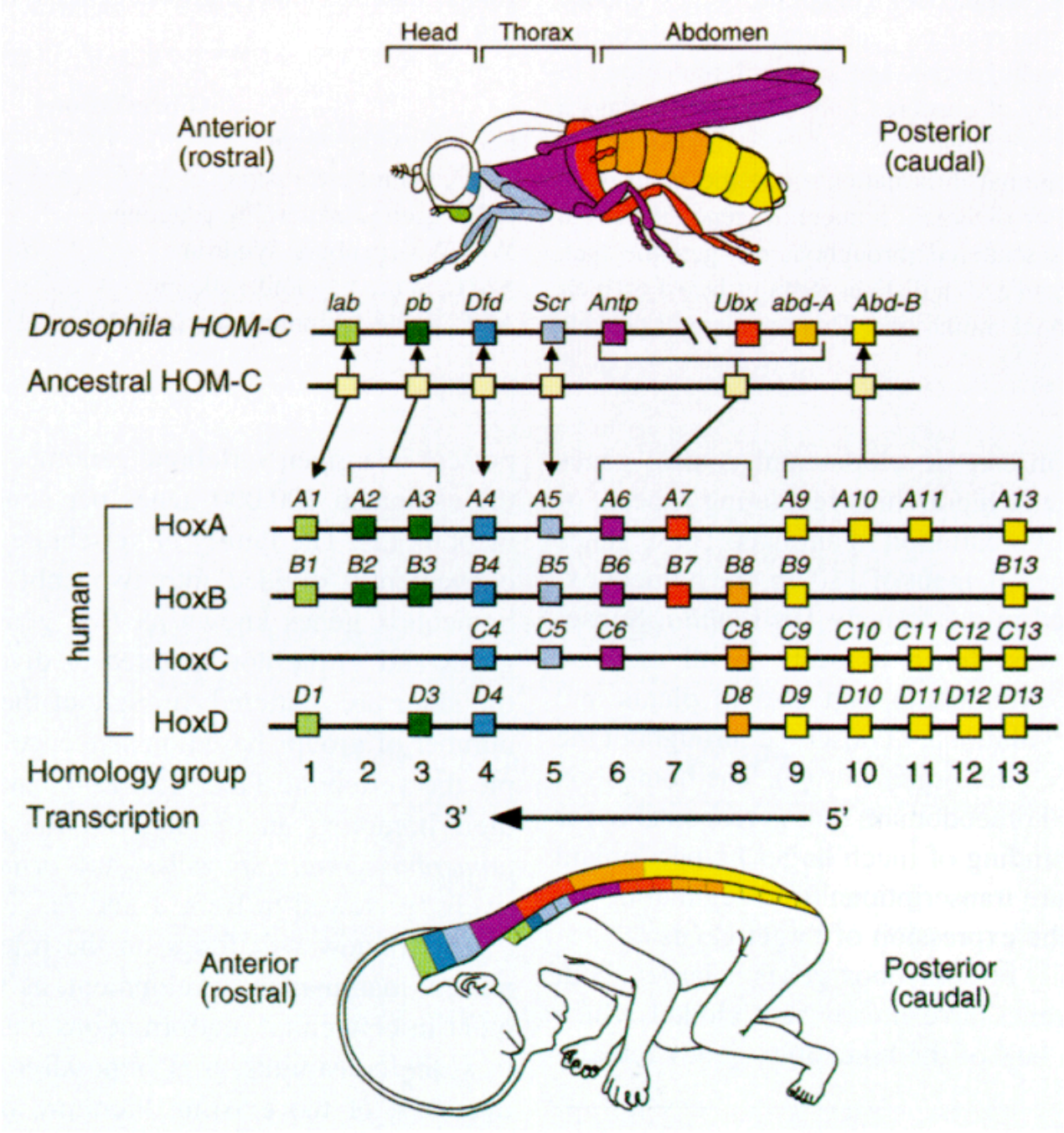

Figure 1: Temporal and spatial colinearity patterns of *Drosophila HOM* genes and mammalian *Hox* genes. Schematic representation of the *Drosophila* homeotic complex (*HOM-C*), the four human *Hox* complexes and a hypothetical ancestral homeotic complex. Figure taken from Manuel Mark, Nature, 1997{Mark:1997kl}.

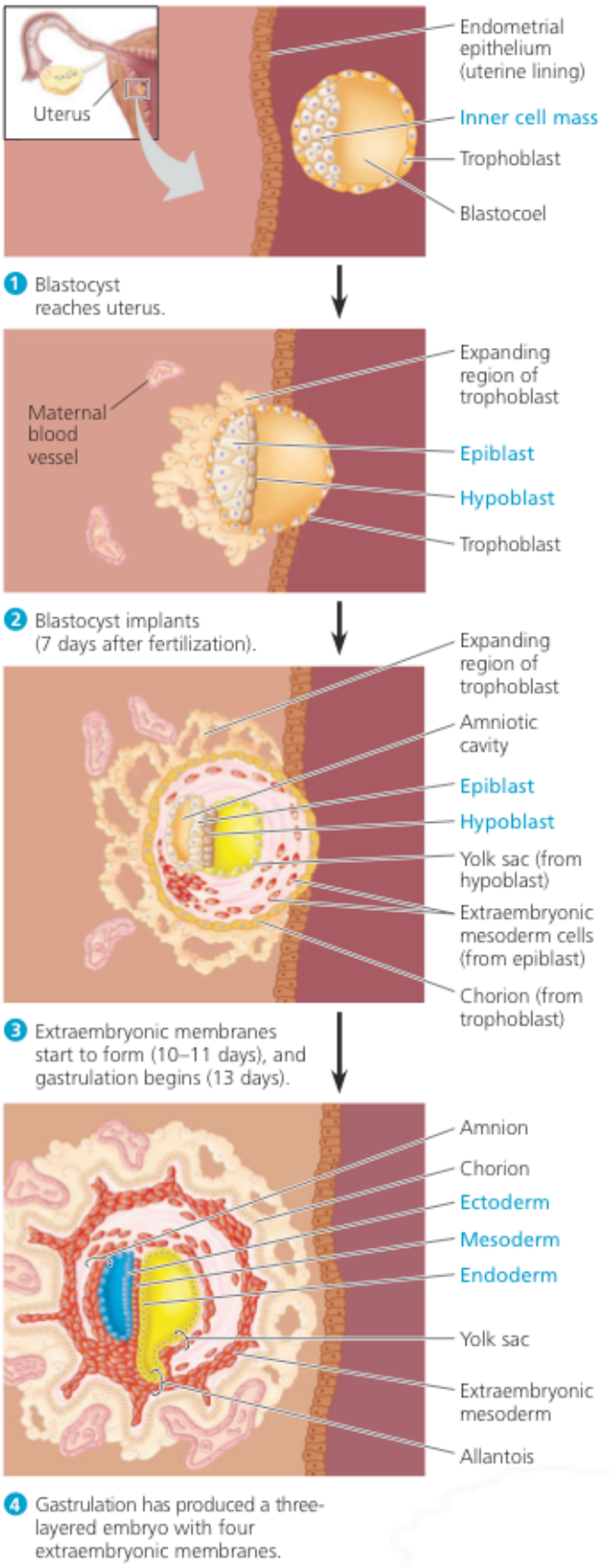

Figure 2: The four stages in the early human embryonic development. *Source: Campbell Biology 9th ed. J. Reece (Pearson).*

Embryonic development

In humans, fertilization takes place in the oviduct, and the earliest stages of development occur while the zygote completes it's journey down the oviduct to the uterus. At this stage the embryo is called the blastocyst, and constitutes of the inner mass cells, which forms the embryo, and specialized cells called trophoblasts (figure 2.1). The trophoblast do not contribute to the embryo but instead support implantation of the blastocyst in to the endometrium(figure 2.2), and later form the major part of the placenta. As implantation in to the endometrium is completed, gastrulation begins(figure 2.3). During gastrulation the inner mass cells or embryoblast, move inward from the epiblast (figure 2.3), forming ectodermal, mesodermal and endodermal germ layers(figure 2.4). Each germ layers specializes in different tissues, as will be described next paragraph. It is important to understand that every cell in the body, despite it's morphological and gene expression differences, inherits the same genetic information in the form of DNA, obtained from both parents. So how do cells 'know' which cell type to differentiate into? How do these cells know where to go? The answer lays in gene regulation. Epigenetic modifications are a form of gene regulation, and paly a pivotal role during embryogenesis. Epigenetic modifications do not change the DNA sequence, but change expression of genes by modifying the DNA's tertiary structure using methylation, alkylation, glycosylation and histone modifications. It is now widely accepted that epigenetics, particulary methylation of Hox associated CPG sites regulate cellular differentiation fate during embryogenesis. Furthermore, epigenetic inheritance from parent to offspring leads to an alternative way of vertical inheritance of information, without changing the DNA sequence[21].

The germ layers

During human embryogenesis three germ layers are formed. The ectoderm, the most outer layer, forms the central nervous system, the ganglia and nerves, the epidermis, hair and mammary glands. The endoderm, being the most inner layer, forms the epithelial lining of the digestive system from the esophagus to the terminal part of the rectum. It also forms the liver, the pancreas and thyroid gland. Furthermore it forms the trachea, the bronchi, the alveoli and the urinary bladder. Between the ectoderm and endoderm, lies a third germ layer called the mesoderm. The mesoderm forms the skeletal muscle, skeleton, connective tissue, blood, the heart, part of the respiratory system, the kidney and the spleen. Connective tissue gives structure to animals, forming cartilage, bone, adipose tissue, blood, hemopoietic tissue and lymphatic tissue. Connective tissue has three main components: cells, mainly fibroblasts, fibers and extracellular matrix. Many organs in humans constitute of two germ layers; skin of both ecto- and mesoderm, the respiratory system of both the endo- and mesodermal germ layer.

Fibroblasts and the Hox 'zip code'

In this overview we will mainly focus on cells from the mesenchymal germ layer. Fibroblasts are the most abundant cell type in the mesenchymal germ layer, and are responsible for the production of many components of connective tissue. Connective tissue plays a pivotal role as it provides a structural framework for the body, connects body tissues, such as muscle and epithelium, but also provides a means of protecting internal organs while maintaining body heat. Furthermore, fibroblasts play an important role in wound healing, as fibroblasts replace damaged specialized tissue (muscle, endothelium) with collagen during wound repair. Though in general wound repair is necessary to maintain organ and tissue function, excessive scar formation by deposition of connective tissue, called fibrosis, causes serious damage to surrounding tissue in many different diseases[22]. Examples of such diseases are pulmonary fibrosis in COPD and cirrhosis due to hepatitis B or C. Because fibrosis is found in many different diseases, many studies have been performed to attenuate and normalize fibrotic scar tissue formation. Fibroblasts taken from different anatomic locations can be studied *in vitro*, and can be forced into a pro-fibrotic phenotype by the addition of Transforming Growth Factor-β (TGF-β)[23-25]. Though many studies have been performed, no cure for fibrosis is available to date.

Fibroblast and positional memory

In recent studies it was found that the anatomic location of fibroblasts in the body determines gene expression profile, and this may lead to fibroblast heterogeneity. In a study by Rinn and colleagues, it was found that gene expression between fibroblasts varied along the coordinates of the anatomic axes, and the site of fibroblast origin could be determined from gene expression profiles [20]. This coincides with early findings that fibroblasts from different anatomic locations have different morphologies[26-28], ECM deposition[26,29,30], proliferative capacities[26,29,31,32], and distinct cell- surface antigen presentation and surface receptors[33]. This means the anatomic origin of fibroblasts used in experimental designs are crucial to fully understand the studied disease. Indeed it is odd that so may differences have been found between fibroblasts over three decades ago, and only now we are starting to study the plasticity and subpopulations of fibroblasts.

Hox genes and pattern formation

The organization of specialized cell types in the body can be achieved by two different proposed mechanisms. Cells can form patterns by differentiating into specific cell (sub)types based on local interactions, and 'know' where to go by mutual attraction and repulsion. Alternatively, cells can form patterns based on their positional identity relative to reference points on the anatomic axes. The difference in both

systems is that based on local interactions, cells closely spaced from each other are more likely to have shared local interactions, and therefore may have greater similarity to each other in gene expression than cells that are more spatially distant. On the other hand, based on the coordinate system, cells that are spatially distant from each other (like hand and feet) may share a similar relative distal position along the anatomic axes, and therefore may share a similar Hox gene expression signature gained during embryogenesis. In 2006 the group of Rinn found that spatial organization is mainly based on a coordinate system along the anatomic axes, and that Hox gene regulation plays a crucial role in spatial organization. Hox gene regulation provides a 'zip *code*' for fibroblasts, homing fibroblast subsets to specific anatomic locations[20]. Equally important, this zip code is maintained throughout adulthood and passed along many cell divisions.

Hox genes in adults

As said in previous paragraph, fibroblasts from different anatomic locations have different Hox gene 'zip codes' which are gained during embryogenesis and maintained throughout adulthood. Even more striking, this 'zip code' is maintained over decades *in vivo* and over thirty cell divisions *in vitro*[34]. Furthermore, positional memory of fibroblasts is regulated by epigenetic modifications, and this epigenetic profile is maintained even after culturing cells for over 35 passages[20,34,35]. Equally important, epigenetic modification of Hox genes is maintained throughout adulthood[20,34]. A citation from the paper by Koch and colleagues; "Hierarchical clustering of CpG sites related to Hox genes revealed a separation of fibroblasts from the top half (anterior) versus the bottom half (posterior) of the human body"[35]. This means epigenetic modifications of CpG sites that regulate Hox genes are maintained throughout adulthood and are necessary to regulate and maintain positional memory along the anatomic axis of fibroblasts. The same separation between top and bottom fibroblast gene expression in the human body was seen by Rinn and colleagues[20].

Hox genes in respiratory fibroblasts

HoxA5 plays an important role in positional memory of fibroblast of the mesenchymal layer of the respiratory system. HoxA5 mutant knock-out mice are incompatible with life, and die at birth due to a defective respiratory tract[36]. Furthermore, HoxA5-/- knockout led to defective motility of alveolar myofibroblast precursors, and in turn to mis-positioning of the differentiated myofibroblast in the respiratory system. The wrong anatomic positioning of these myofibroblasts led to abnormal elastin deposition postnatally[37]. HoxA5 expression seems to be restricted to the mesenchymal germ layer, and appears to control mesenchymal-epithelial interactions during organogenesis.

Hox genes in limb fibroblasts

HoxA13, a distal specific Hox gene, plays a pivotal role during limb development, and is continually required in humans to maintain the distal-specific gene expression profile in adult fibroblasts[34]. Furthermore, low expression levels of HoxB2, HoxB4, HoxB5, HoxB6, and HoxB7 were found in high HoxA13 expressing fibroblasts, suggesting a negative interaction between the HoxA and HoxB loci exists. In the HoxC locus of fibroblasts, a noncoding RNA (ncRNA) stretch was identified which inhibited transcription of genes on the HoxD locus[38]. The finding that several HoxB genes are negatively correlated with the expression of HoxA13 might suggest a similar regulatory mechanism for the HoxC and HoxD loci. HoxA13 expression was found in all distal parts, including hand, feet and foreskin[20].

Smad & TGF-β in Hox regulation

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily of signaling molecules that regulate cell growth, differentiation and apoptosis[39,40], and are

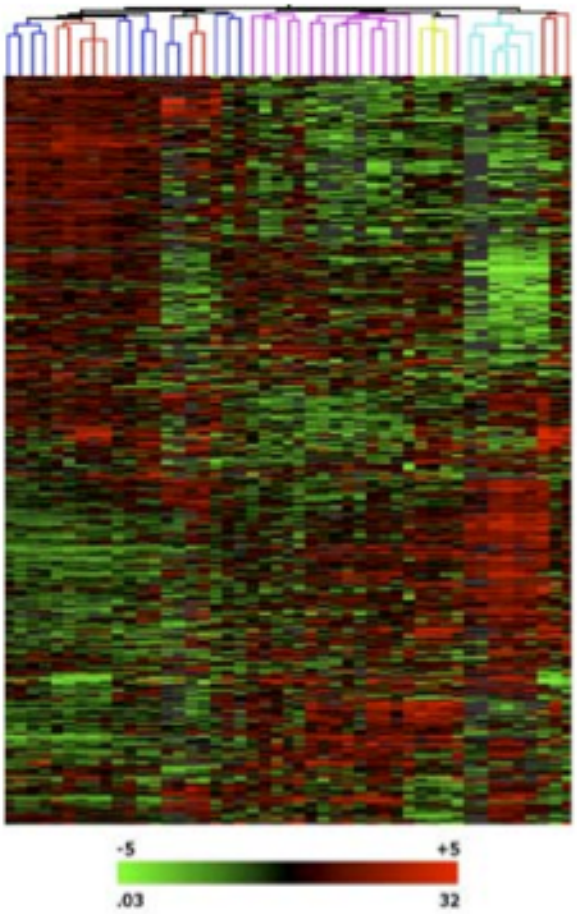

Figure 3: Diversity of gene expression programs in 47 fibroblast populations. Each row represents a gene; each column represents a fibroblast population. Figure taken from Rinn et al., 2006

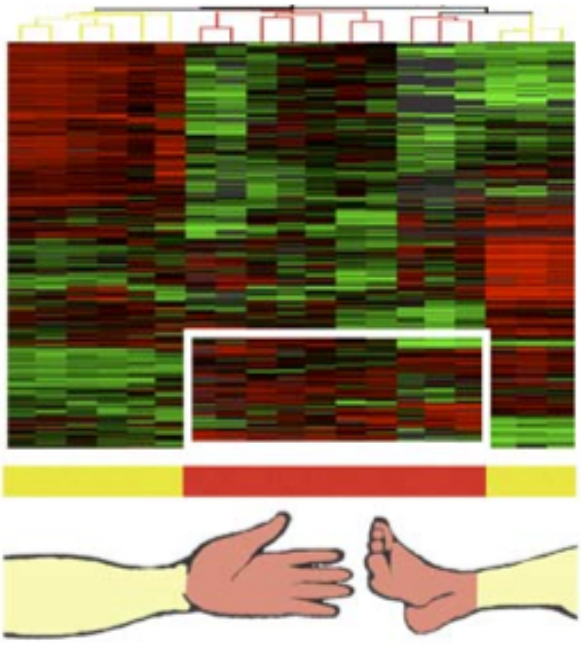

Figure 4: a gene expression signature exists between distal parts of the body, such as hand and feet. The white box highlights 98 genes that are expressed by distal fibroblasts. Figure taken from Rinn et al., 2006

important in bone and cartilage formation[39,41-43]. In previous paragraphs, we identified Hox as important proteins during embryo development by regulating many different genes, which also includes BMPs. In several papers, Smads are identified as general Hox regulators, thereby also regulating BMPs (and bone/cartilage formation) during embryogenesis[41,43-49]. For example negative feedback is acquired through the TGFβ/BMP signaling pathway, activating the common Smad (Co-smad) Smad4. Smad4 normally forms a complex with Smad2/Smad3, translocates to the nucleus and regulates gene transcription by direct DNA binding or interacting with promoter specific transcription factors[50]. However, here Smad4 interacts directly with the homeodomain of Hoxa9, and blocks the ability of Hoxa9 to bind DNA, thereby suppressing its ability to regulate downstream gene transcription[51].

Other gene expression differences

Gene expression differences between fibroblasts from different anatomic locations don't stop at Hox gene expression differences. Hundreds of different genes have been identified to be differentially expressed among fibroblasts from different anatomic locations (figure 3), and these fibroblasts may even be considered distinct cell types because their gene expression programs are as diverse as cells from different hematopoietic lineages[20,52]. We will give an overview of the many phenotypic differences of fibroblasts from different anatomic locations in the next paragraphs.

Fibroblasts in the respiratory system

Morphological and gene expression differences between airway fibroblasts (AF) and distal lung fibroblasts (DLF) were identified by Kotaru and colleagues, and are an example of spatially nearby fibroblasts with entirely different cellular outcomes[53]. It was found that AF had a 2.3 fold greater surface area when compared to DLF, were more star-like (stellate) in appearance and had more cytoplasmic

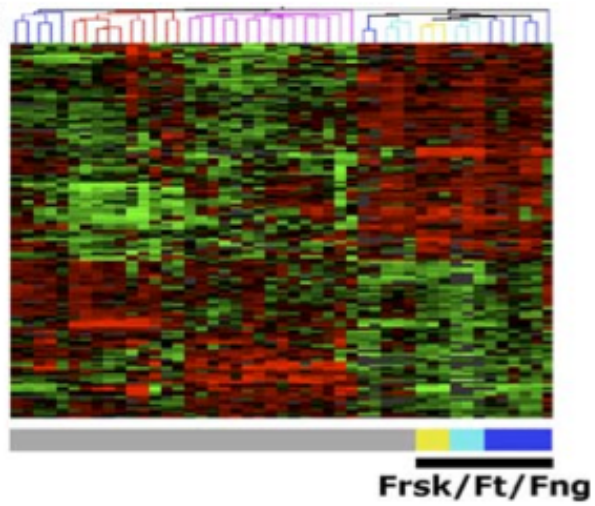

Figure 5: a clear gene expression profile (signature) can be seen between foreskin (Frsk, yellow), Feet (Ft, cyan), and Figners (blue). Figure taken from Rinn et al., 2006.

projections, while DLF had a more spindle-shaped morphology. Similarly, AFs had increased CCL11 (eotaxin-1) synthesis at baseline (2.5 fold) and after interleukin-13 stimulation (13-fold). On the other hand, AFs expressed less α-smooth muscle actin, and were less prone to proliferate after serum stimulation when compared to DLFs. Furthermore, AFs synthezised more procollagen type I than DLFs, both at baseline (unstimulated) and after stimulation with transforming growth factor β. If we look back at cartilage formation, members of the TGF-β superfamily can regulate cellular fate through Smads, by regulating Hox gene expression. Furthermore, TGF-β thrives cells into a pro-fibrotic phenotype. It is worth noting that prolonged TGF-β stimulation might force fibroblast cellular differentiation into a pro-fibrotic phenotype, thereby giving biased cellular phenotypes and distorted view on the disease. However, this finding does mean large variations can be found between spatially close fibroblasts from the lung.

Fibroblast gene signature in distal body parts

Even more striking is that fibroblasts from spatially distant anatomic locations, but with similar relative positional distance along the anatomic axis can have specific gene expression signatures, as seen by Rinn et al in hand and feet from human adults (figure 4)[20]. The white box in figure 4 depicts the specific signature of 98 genes between hand and feet. Furthermore, a similar gene expression signature can also be found on other distal body parts, such as foreskin (figure 5). This finding shows that cellular differentiation and positional identity of fibroblasts is based on a coordinate system which originates from embryonic development, and is maintained throughout adulthood.

Fibroblast wound scarring: dermal vs oral mucosal fibroblasts

The difference in fibroblast gene expression may also explain the difference in wound healing at different anatomic locations, such as wound healing on the skin (dermal) and in the mouth (oral mucosal)[54,55]. While on skin most deep wounds will scar, in the mouth almost no scarring is found. McKeown and colleagues found that oral fibroblasts, in contrast to skin fibroblasts, secrete high levels of stromelysin-1 (Matrix metalloproteinase-3, MMP-3)[55], and this may lead to increased wound contraction and less scar formation. Shannon also identified decreased expression of α-smooth muscle actin in oral fibroblasts when compared to dermal fibroblasts. Furthermore, oral fibroblasts had increased synthesis of two epithelial growth factors: keratinocyte growth factor (KGF) and hepatocyte growth factor/ scatter factor (HGF), when compared to dermal fibroblasts[54]. This data suggests a large differences exist between fibroblasts from the oral cavity and the skin. Furthermore, it reveals a complex interplay between several cytokines and matrix proteins during wound healing. Regulating several factors may in time lead to better regulation of fibrosis during wound healing and during the course of several diseases.

Heart fibroblast gene regulation

In a study by Liang and colleagues, both Transforming growth factor-β (TGF-β) and

microRNA-21 (miR-21) were upregulated while transforming growth factor-β receptorIII (TGF-βRIII) was downregulated in the border zone of mouse heart in response to myocardial infarction. Normally after myocardial infarction heart tissue is replaced by scar tissue, mainly collagen, which in time leads to decreased heart function and myocardial death. TGF-βRIII was identified as a negative regulator of TGF-β1. It was found that miR-21 decreased TGF-βRIII expression, while collagen deposition was increased. On the other hand, over-expression of TGF-βRIII reduced miR-21 expression and collagen deposition, which means a tight balance between TGF-βRIII and miR-21 exists. More important, over-expression of TGF-βRIII also gave negative feedback to TGF-β1 and pshosphorylated-Smad3 (p-Smad3). This data implies that downregulation of TGF-βRIII by miR-21 leads to overexpression of TGF-β1 and p-Smad3, which in turn leads to increased collagen deposition and scar formation[56]. Better regulation of miR-21 might lead to better control of fibrosis, and miR-21 might even be used as a scar formation marker.

Myofibroblasts in the respiratory system

Comparing this data with a study from a different group on lung fibrosis, miR-21 was found upregulated and this upregulation led to fibrogenic activation of myofibroblasts. miR-21 led to enhanced TGF-β1 production, while on the other hand, TGF-β1 led to an increase in miR-21, causing a positive feedback loop, ultimately leading to uncontrolled fibrosis[56,57]. Furthermore, knocking down miR-21 with antisense probes diminished the severity of the experimental lung fibrosis. On the other hand, increasing miR-21 promoted pro-fibrogenic activity of TGF-β1 in fibroblasts. The writers suggest a possible mechanism for miR-21 is by regulating inhibitory Smad7. Smad7 is a TGFβ type 1 receptor antagonist, and miR-21 targets Smad7, leading to a loss in TGFβ type 1 receptor inhibition. This in turn leads to increased TGFβ1 synthesis[57].

Endothelial-to-Mesechymal transition

Furthermore, miR-21 was found to be involved in the Endothelial-to-Mesechymal transition (EndMT) in the heart, where endothelial cells lose their endothelial markers and start to express certain fibroblast markers. Stimulation with TGFβ led to an increase in miR-21 expression. Induced miR-21 silenced the phosphatase and tensin homolog (PTEN), which resulted in activation of the Akt pathway, leading to further loss of endothelial markers and a gain in fibroblast markers. This process could be partly reversed by Akt inhibition[58]. In a recent publication from a different group, it was found that Akt1 might regulate Hox gene associated CpG methylation, thereby regulating Hox gene expression[59]. To summarize: TGFβ can regulate a pro-fibrotic phentotype in fibroblasts by increasing miR-21 expression. miR-21 expression can in turn regulate Smads (p-Smad3, Smad7) and this leads to Akt activation, which can regulate Hox gene expression. This in turn can change the cell's phenotype, increasing ECM deposition and stimulating an endothelial-to-mesenchymal transition in heart endothelial cells. Via this pathway, TGF-β might lead to a pro-fibrotic phenotype.

Stem cells

Cord blood mesenchymal stem cells

For a long time, it was believed that all mesenchymal stem cells (MSCs) derived from embryonic cord blood had the same properties. Soon it was found that some populations had more proliferative capacities, or could be more easily induced to form a certain cell lineage. Today, two different CD45-, CD34– adherent stem cell populations can be isolated form cord blood; the cord blood-derived MSC (CB-MSC)[60], which has bone marrow derived MSC-like properties, and the unrestricted somatic stem cells (USSC)[61-66]. When studying gene expression profiles, remarkable expression difference between CB-MSC, BM-MSC, adipose-derived mesenchymal stem cells (AdAS) and USSC were found[67].

Characterization and gene expression profiling of USSC revealed both unique proliferation capacities and differentiation potential. Cord blood derived USSCs could be differentiated into all three germ layers; ectodermal, mesodermal and endodermal[61,68-70].

Hox code in mesenchymal stem cells

In a study by Liedtke and colleagues, a "biological fingerprint" based on Hox gene expression was identified, which also revealed a high similarity between BM-MSC and CB-MSC. Both MSC lineages were Hox positive, in contrast to the USSC lineage, which was found to be Hox negative. Furthermore, USSC were found to be more embryonic stem cell-like. Hox genes from all four different clusters (A-D) were found to be differentially expressed between MSC (both BM-MSC and CB-MSC) and USSC, and four different Hox genes were proposed as candidate markers to discriminate between MSC and USSC derived from cord blood. These four markers are: HoxA9, HoxB7, HoxC10 and HoxD8. Furthermore, Ackema and colleagues recently described mesenchymal stromal cells in mice from different organs which could be characterized by different Hox expression profiles. This difference in Hox expression profiles might be based on topographic differences[71], the same as seen in (myo)fibroblasts[20].

MSC and the Hox 'zip code'

In vitro, MSC isolated from different organs exhibit different intrinsic proliferative capacities and efficiencies with which they can be induced to differentiate towards specific mesenchymal lineages in both rat [72]and humans[71,73-75]. Tissue-specific differentiation of MSC is mainly regulated by Hox gene activity, and it seems that this Hox gene 'fingerprint' or 'signature' is maintained as MSC differentiate into different anatomic location-defined subtypes of fibroblasts[20,34,52,71].

MSC differentiation regulation

The ERK-MAPK signaling pathway plays a pivotal role during mesenchymal stem cell (MSC) differentiation. In new studies by several groups, it was found that miR-21 plays a pivotal role in regulating the ERK-MAPK pathways[76]. Furthermore, TNF-α stimulation supressed miR-21, leading to osteoporisis during estrogen deficiency[77]. Furthermore, upregulation of miR-21 promoted cellular survival of MSC exposed to hypoxia or serum deprivation. This means miR-21 plays an important role in MSC apoptosis regulation[78]. This also means miR-21 plays an important role in both MSC and fibroblasts, regulating gene expression. To date, no data is available on interaction between Hox and miR-21 in MSC, but miR-21 might play a major role in MSC differentiation.

Conclusion

Large gene expression differences exist between fibroblasts from different anatomic locations. Hox genes play an important role during embryogenesis and organogenesis, and were found to be maintained in fibroblasts during adulthood. Hox gene expression in turn seems to be regulated by epigenetics, based on a positional memory coordinate system, in which cells differentiate based on their location in respect to the anatomic axes of the body. Cell differentiation does not only seems to be regulated by local interactions, as fibroblasts from nearby anatomic locations can have completely different gene expression signatures. Furthermore, fibroblasts based distal in the body, such as hand, feet and foreskin fibroblasts share a common gene expression 'signature', based on their relative distal location along the anatomic axes. This expression difference between fibroblasts could mean fibroblasts from different anatomic locations respond different during wound repair, despite having the same membrane bound markers, and regulation seems to be delicate. In this view, fibroblasts can no longer be seen as a single cell type, but instead should be further classified in to

different subtypes based on gene expression profiles and anatomic location. Studies aimed at fibroblast progenitors, the mesenchymal stem cells, revealed gene expression differences between different MSC populations. Differential expression of Hox genes seemed to play a pivotal role in MSC differentiation and proliferation potential. Furthermore, mixed populations of MSC can be found in cord blood, revealing striking Hox gene expression differences and differentiation outcomes. This means MSC can be further categorized, and should not be treated as a single cell type, as the gene expression is as diverse as cells from completely different cell lineages. It is striking that Hox gene expression, gained during embryogenesis is maintained throughout adulthood, and can be tightly regulated by epigenetics. It is this regulation that leads to so many different cell types, each with their own form and function. Cell categorization used to be based on cellular markers and morphology. However, today cells can be further categorized based on gene expression profiles, and it is now clear that several fibroblast and MSC populations exist. We have only begun to reveal the plasticity of the many subcategories of fibroblasts and MSC, and to elucidate their extremely well coordinated role in the body.

Future

It is important to further elucidate and subcategorize the different populations of both cord-blood derived MSC and fibroblasts, to develop new therapeutic strategies for tissue generation and repair. Many diseases develop with detrimental fibrosis, leading to organ failure and eminent death. Further subcategorizing MSC and fibroblasts may lead to better understanding the role of these cells during pathogenesis and fibrosis. In time, these studies can lead to better therapeutic targeting and hopefully, a treatment against fibrosis.

1. McGinnis, W., Hart, C. P., Gehring, W. J. & Ruddle, F. H. Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of Drosophila. *Cell* **38**, 675–680 (1984).
2. McGinnis, W. & Krumlauf, R. Homeobox genes and axial patterning. *Cell* **68**, 283–302 (1992).
3. McGinnis, W., Garber, R. L., Wirz, J., Kuroiwa, A. & Gehring, W. J. A homologous protein-coding sequence in Drosophila homeotic genes and its conservation in other metazoans. *Cell* **37**, 403–408 (1984).
4. Krumlauf, R. Hox genes in vertebrate development. *Cell* **78**, 191–201 (1994).
5. Keynes, R. & Krumlauf, R. Hox genes and regionalization of the nervous system. *Annu. Rev. Neurosci.* **17**, 109–132 (1994).
6. Laughon, A. & Scott, M. P. Sequence of a Drosophila segmentation gene: protein structure homology with DNA-binding proteins. *Nature* **310**, 25–31 (1984).
7. Carrasco, A. E., McGinnis, W., Gehring, W. J. & De Robertis, E. M. Cloning of an X. laevis gene expressed during early embryogenesis coding for a peptide region homologous to Drosophila homeotic genes. *Cell* **37**, 409–414 (1984).
8. Desplan, C., Theis, J. & O'Farrell, P. H. The sequence specificity of homeodomain-DNA interaction. *Cell* **54**, 1081–1090 (1988).
9. Shepherd, J. C., McGinnis, W., Carrasco, A. E., De Robertis, E. M. & Gehring, W. J. Fly and frog homoeo domains show homologies with yeast mating type regulatory proteins. *Nature* **310**, 70–71 (1984).
10. Desplan, C., Theis, J. & O'Farrell, P. H. The Drosophila developmental gene, engrailed, encodes a sequence-specific DNA binding activity. *Nature* **318**, 630–635 (1985).

11. Gehring, W. J. The homeo box: a key to the understanding of development? *Cell* **40**, 3–5 (1985).
12. Gehring, W. J. & Hiromi, Y. Homeotic genes and the homeobox. *Annu. Rev. Genet.* **20**, 147–173 (1986).
13. Gehring, W. J. *et al.* Homeodomain-DNA recognition. *Cell* **78**, 211–223 (1994).
14. McGinnis, W. A century of homeosis, a decade of homeoboxes. *Genetics* **137**, 607–611 (1994).
15. Lewis, E. B. A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565–570 (1978).
16. Hart, C. P., Awgulewitsch, A., Fainsod, A., McGinnis, W. & Ruddle, F. H. Homeo box gene complex on mouse chromosome 11: molecular cloning, expression in embryogenesis, and homology to a human homeo box locus. *Cell* **43**, 9–18 (1985).
17. Erwin, D. H. & Davidson, E. H. The last common bilaterian ancestor. *Development* **129**, 3021–3032 (2002).
18. Kmita, M. & Duboule, D. Organizing axes in time and space; 25 years of colinear tinkering. *Science* **301**, 331–333 (2003).
19. Mark, M., Rijli, F. M. & Chambon, P. Homeobox genes in embryogenesis and pathogenesis. *Pediatr. Res.* **42**, 421–429 (1997).
20. Rinn, J. L., Bondre, C., Gladstone, H. B., Brown, P. O. & Chang, H. Y. Anatomic Demarcation by Positional Variation in Fibroblast Gene Expression Programs. *PLoS Genet* **2**, e119 (2006).
21. Daxinger, L. & Whitelaw, E. Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nat. Rev. Genet.* **13**, 153–162 (2012).
22. Sappino, A. P., Schürch, W. & Gabbiani, G. Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.* **63**, 144–161 (1990).
23. Roberts, A. B. & Sporn, M. B. Transforming growth factors. *Cancer Surv.* **4**, 683–705 (1985).
24. Roberts, A. B. *et al.* Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4167–4171 (1986).
25. Roberts, A. B. & Sporn, M. B. Regulation of endothelial cell growth, architecture, and matrix synthesis by TGF-beta. *Am. Rev. Respir. Dis.* **140**, 1126–1128 (1989).
26. Conrad, G. W., Hamilton, C. & Haynes, E. Differences in glycosaminoglycans synthesized by fibroblast-like cells from chick cornea, heart, and skin. *J. Biol. Chem.* **252**, 6861–6870 (1977).
27. Pinto da Silva, P. & Gilula, N. B. Gap junctions in normal and transformed fibroblasts in culture. *Exp. Cell Res.* **71**, 393–401 (1972).
28. Ross, R. & Greenlee, T. K. Electron microscopy: attachment sites between connective tissue cells. *Science* **153**, 997–999 (1966).
29. Riederer-Henderson, M. A., Gauger, A., Olson, L., Robertson, C. & Greenlee, T. K. Attachment and extracellular matrix differences between tendon and synovial fibroblastic cells. *In Vitro* **19**, 127–133 (1983).
30. Evans, C. E. & Trail, I. A. An in vitro comparison of human flexor and extensor tendon cells. *J Hand Surg Br* **26**, 307–313 (2001).
31. Banes, A. J. *et al.* Cell populations of tendon: a simplified method for isolation of synovial cells and internal fibroblasts: confirmation of origin and biologic properties. *J. Orthop. Res.* **6**, 83–94 (1988).
32. Mackley, J. R., Ando, J., Herzyk, P. & Winder, S. J. Phenotypic responses to mechanical stress in fibroblasts from tendon, cornea and skin. *Biochem. J.* **396**, 307–316 (2006).

33. Garrett, D. M. & Conrad, G. W. Fibroblast-like cells from embryonic chick cornea, heart, and skin are antigenically distinct. *Dev. Biol.* **70**, 50–70 (1979).
34. Rinn, J., Wang, J., Allen, N., Brugmann, S. & al, E. A dermal HOX transcriptional program regulates site-specific epidermal fate. *Genes & …* (2008).
35. Koch, C. M. *et al.* Specific age-associated DNA methylation changes in human dermal fibroblasts. *PLoS ONE* **6**, e16679 (2011).
36. Boucherat, O., Guillou, F., Aubin, J. & Jeannotte, L. [Hoxa5: a master gene with multifaceted roles]. *Med Sci (Paris)* **25**, 77–82 (2009).
37. Mandeville, I. *et al.* Impact of the loss of Hoxa5 function on lung alveogenesis. *Am. J. Pathol.* **169**, 1312–1327 (2006).
38. Rinn, J. L. *et al.* Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311–1323 (2007).
39. Hogan, B. L. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10**, 1580–1594 (1996).
40. Massagué, J. TGF-beta signal transduction. *Annu. Rev. Biochem.* **67**, 753–791 (1998).
41. Duprez, D. *et al.* Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* **57**, 145–157 (1996).
42. Li, X., Nie, S., Chang, C., Qiu, T. & Cao, X. Smads oppose Hox transcriptional activities. *Exp. Cell Res.* **312**, 854–864 (2006).
43. Zou, H., Wieser, R., Massagué, J. & Niswander, L. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* **11**, 2191–2203 (1997).
44. Walsh, C. M. & Carroll, S. B. Collaboration between Smads and a Hox protein in target gene repression. *Development* **134**, 3585–3592 (2007).
45. Wang, N. *et al.* TGFbeta/BMP inhibits the bone marrow transformation capability of Hoxa9 by repressing its DNA-binding ability. *EMBO J.* **25**, 1469–1480 (2006).
46. Hullinger, T. G., Pan, Q., Viswanathan, H. L. & Somerman, M. J. TGFbeta and BMP-2 activation of the OPN promoter: roles of smad- and hox-binding elements. *Exp. Cell Res.* **262**, 69–74 (2001).
47. Wan, M., Shi, X., Feng, X. & Cao, X. Transcriptional mechanisms of bone morphogenetic protein-induced osteoprotegrin gene expression. *J. Biol. Chem.* **276**, 10119–10125 (2001).
48. Balint, E. *et al.* Phenotype discovery by gene expression profiling: mapping of biological processes linked to BMP-2-mediated osteoblast differentiation. *J. Cell. Biochem.* **89**, 401–426 (2003).
49. Li, X. & Cao, X. BMP signaling and HOX transcription factors in limb development. *Front. Biosci.* **8**, s805–12 (2003).
50. Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577–584 (2003).
51. Wang, K. C., Helms, J. A. & Chang, H. Y. Regeneration, repair and remembering identity: the three Rs of Hox gene expression. *Trends in Cell Biology* **19**, 268–275 (2009).
52. Chang, H. Y. *et al.* Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12877–12882 (2002).
53. Kotaru, C. *et al.* Regional fibroblast heterogeneity in the lung: implications for remodeling. *Am. J. Respir. Crit. Care Med.* **173**, 1208–1215 (2006).
54. Shannon, D. B., McKeown, S. T. W., Lundy, F. T. & Irwin, C. R. Phenotypic differences between oral and skin

fibroblasts in wound contraction and growth factor expression. *Wound Repair Regen* **14**, 172–178 (2006).
55. McKeown, S. T. W. *et al.* Matrix metalloproteinase-3 differences in oral and skin fibroblasts. *Journal of dental research* **86**, 457–462 (2007).
56. Liang, H. *et al.* A novel reciprocal loop between microRNA-21 and TGFβRIII is involved in cardiac fibrosis. *Int. J. Biochem. Cell Biol.* **44**, 2152–2160 (2012).
57. Liu, G. *et al.* miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J. Exp. Med.* **207**, 1589–1597 (2010).
58. Kumarswamy, R. *et al.* Transforming growth factor-β-induced endothelial-to-mesenchymal transition is partly mediated by microRNA-21. *Arterioscler. Thromb. Vasc. Biol.* **32**, 361–369 (2012).
59. Kong, K.-A., Yoon, H. & Kim, M. H. Akt1 as a putative regulator of Hox genes. *Gene* **513**, 287–291 (2013).
60. Erices, A., Conget, P. & Minguell, J. J. Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.* **109**, 235–242 (2000).
61. Kögler, G. *et al.* A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J. Exp. Med.* **200**, 123–135 (2004).
62. Chan, S. L. *et al.* Enhanced in vivo homing of uncultured and selectively amplified cord blood CD34+ cells by cotransplantation with cord blood-derived unrestricted somatic stem cells. *Stem Cells* **25**, 529–536 (2007).
63. Chang, Y.-J. *et al.* Disparate mesenchyme-lineage tendencies in mesenchymal stem cells from human bone marrow and umbilical cord blood. *Stem Cells* **24**, 679–685 (2006).
64. Kim, B.-O. *et al.* Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation* **112**, I96–104 (2005).
65. Lee, R. M. *et al.* Different signaling responses to anti-proliferative agents in human aortic and venous smooth muscle cells. *J. Cell. Biochem.* **99**, 835–844 (2006).
66. Kern, S., Eichler, H., Stoeve, J., Klüter, H. & Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**, 1294–1301 (2006).
67. Jansen, B. J. H. *et al.* Functional differences between mesenchymal stem cell populations are reflected by their transcriptome. *Stem Cells Dev.* **19**, 481–490 (2010).
68. Greschat, S. *et al.* Unrestricted somatic stem cells from human umbilical cord blood can be differentiated into neurons with a dopaminergic phenotype. *Stem Cells Dev.* **17**, 221–232 (2008).
69. Sensken, S. *et al.* In vitro differentiation of human cord blood-derived unrestricted somatic stem cells towards an endodermal pathway. *Cytotherapy* **9**, 362–378 (2007).
70. Trapp, T. *et al.* Hepatocyte growth factor/c-MET axis-mediated tropism of cord blood-derived unrestricted somatic stem cells for neuronal injury. *J. Biol. Chem.* **283**, 32244–32253 (2008).
71. Ackema, K. B. & Charité, J. Mesenchymal stem cells from different organs are characterized by distinct topographic Hox codes. *Stem Cells Dev.* **17**, 979–991 (2008).
72. Yoshimura, H. *et al.* Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res.* **327**, 449–462 (2007).
73. in 't Anker, P. S. *et al.* Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* **88**, 845–852 (2003).

74. Sakaguchi, Y., Sekiya, I., Yagishita, K. & Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum.* **52**, 2521–2529 (2005).
75. da Silva Meirelles, L., Chagastelles, P. C. & Nardi, N. B. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J. Cell. Sci.* **119**, 2204–2213 (2006).
76. Mei, Y. *et al.* miR-21 modulates the ERK-MAPK signaling pathway by regulating SPRY2 expression during human mesenchymal stem cell differentiation. *J. Cell. Biochem.* (2012).doi:10.1002/jcb.24479
77. Yang, N. *et al.* TNF-α suppresses the mesenchymal stem cell osteogenesis promoter miR-21 in estrogen deficiency-induced osteoporosis. *J. Bone Miner. Res.* (2012).doi:10.1002/jbmr.1798
78. Nie, Y. *et al.* Identification of MicroRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. *Int. J. Biol. Sci.* **7**, 762–768 (2011).