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Hox Genes in the Adult Skeleton: Novel Functions Beyond Embryonic Development

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***Hox* genes encode evolutionarily conserved transcription factors that control skeletal patterning in the developing embryo. They are expressed in regionally restricted domains and function to regulate the morphology of specific vertebral and long bone elements. Recent work has provided evidence that *Hox* genes continue to be regionally expressed in adult tissues. Fibroblasts cultured from adult tissues show broadly maintained *Hox* gene expression patterns. In the adult skeleton, *Hox* genes are expressed in progenitor-enriched populations of mesenchymal stem/stromal cells (MSCs), and genetic loss-of-function analyses have provided evidence that *Hox* genes function during the fracture healing process. This review will highlight our current understanding of *Hox* expression in the adult animal and its function in skeletal regeneration. *Developmental Dynamics 246:310–317, 2017*. © 2016 Wiley Periodicals, Inc.**

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Introduction

Hox genes have essential functions in patterning the skeleton during embryonic development. Genetic loss-of-function experiments demonstrate severe, region specific malformations of the developing embryonic skeleton. *Hox* function in the adult skeleton has received relatively little attention, but recent studies have led to increased interest in *Hox* gene function in this tissue. Several studies report that *Hox* genes continue to be expressed in the adult bone and function during repair following fracture injury (Gersch et al., 2005; Ackema and Charite, 2008; Leucht et al., 2008; Bais et al., 2009; Liedtke et al., 2010; Rux et al., 2016). In this review, we will briefly highlight the embryonic skeletal functions of *Hox* genes and then review and discuss what has been reported on their expression and function in the adult animal. These most recent discoveries regarding *Hox* genes in the adult skeleton open unexplored avenues of research that meaningfully impact the fields of both mesenchymal stem/stromal cell biology and fracture healing.

Hox Genes During Embryonic Development

Hox genes are among the longest studied genes in developmental biology. This group of homeodomain-containing transcription factors is essential for patterning the anterior to posterior axis of the developing embryo. Spontaneous, homeotic phenotypes (segment identity transformation) were first described in *Drosophila* by William Bateson and by Calvin Bridges in the late 19th and early 20th centuries (Bateson, 1894; Bridges and Morgan, 1923; Maeda and Karch, 2009). Edward B. Lewis provided the seminal discovery that these phenotypes are attributed to a tightly linked region on a single chromosome. The *Bithorax* complex (comprised of the three *Hox* genes; *ultrabithorax, abdominalA* and *abdominalB*) is a cluster of genes that function in a segment specific manner to pattern the posterior body plan of the fly (Fig. 1) (Lewis, 1978). Loss-of-function mutations in *Bithorax* genes result in anterior homeotic transformations wherein body segments that normally express the mutated *Hox* gene acquire the identity/morphology of more anterior regions (Lewis, 1978). Complementary work showed that gain-of-function mutations in more anterior segments result in posterior homeotic transformations (Schneuwly et al., 1987; Kaufman et al., 1990). Taken together, these findings demonstrate that the *Drosophila Hox* genes are the key regulators in developing the morphology of specific body segments during embryonic development.

Importantly, *Hox* genes are deeply evolutionarily conserved and common to all bilaterian animals (Garcia-Fernandez, 2005). In *Drosophila*, eight colinear *Hox* genes are responsible for segmental patterning. During vertebrate evolution, gene amplifications and chromosomal duplications gave rise to the 39-gene, four-cluster *Hox* complex that is observed in all mammals (Scott, 1992; Krumlauf, 1994; Garcia-Fernandez, 2005) (Fig. 1). These 39 genes are further subdivided into 13 paralogous groups (*Hox1* to *Hox13*) based on sequence similarity and position within the cluster (Fig. 1). During development, the expression pattern and functional domain of each *Hox* paralogous group directly mirrors their colinear chromosomal organization. *Hox1* and *Hox2*

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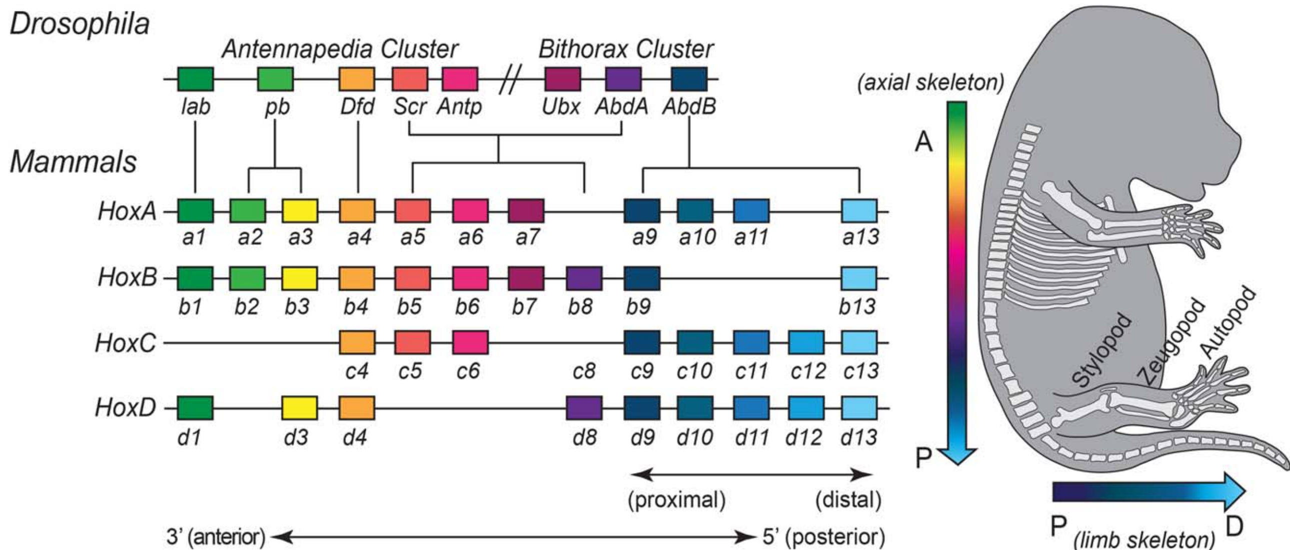

Fig. 1. Schematic of *Hox* gene clusters and regional expression in the embryo. Each line represents an area of a single chromosome. Color-coding of *Hox* genes show the conserved relationships between *Drosophila* and mammalian *Hox* genes, and the paralogous relationships within the mammalian cluster. The depiction of the mouse skeleton reflects the anterior to posterior patterning of the axial skeleton and the proximal to distal patterning of the limb skeleton.

paralogs are expressed the earliest in development and in the most anterior regions of the embryo. The remaining paralogs are expressed sequentially later and in more posterior regions (Fig. 1). Strong epigenetic regulation guides this spatiotemporal onset of expression during gastrulation and limb development (Duboule, 1994; Zakany et al., 1997; Iimura and Pourquie, 2006; Denans et al., 2015).

Functional redundancy among the members of each paralogous group is a critical feature of the vertebrate *Hox* complex. Each set of paralogs (color coordinated in Fig. 1) establishes a similar anterior limit of expression along the AP axis that correlates with the region in which they function (Dressler and Gruss, 1989; Duboule and Dolle, 1989; Graham et al., 1989; Gaunt, 1991; Izpisua-Belmonte et al., 1991; Gaunt and Strachan, 1996). Minor skeletal malformations are common in single *Hox* mutant mice, while the characteristic homeotic transformation phenotypes require the loss of more than one member of a paralogous group (Condie and Capecchi, 1994; Kostic and Capecchi, 1994; Horan et al., 1995; Fromental-Ramain et al., 1996a; van den Akker et al., 2001; Wellik and Capecchi, 2003; McIntyre et al., 2007; Wellik, 2009; Mallo et al., 2010). For example, the anterior limit of expression for the *Hox10* genes is at the thoracolumbar transition of the axial skeleton. Loss-of-function mutations in all three of the *Hox10* paralogous genes results in a morphologic transformation of the lumbar and sacral vertebrae to rib-bearing, thoracic-like vertebrae (Wellik and Capecchi, 2003). The result is an animal with a dramatic extension of floating ribs through the lumbar and sacral elements. Similarly, *Hox11* genes are expressed beginning at the lumbosacral transition and loss of *Hox11* paralogous group function results in transformation of this region to a lumbar morphology (Wellik and Capecchi, 2003).

Hox Genes in Limb Development

The posterior *Hox* genes (*Hox9* to *Hox13*) are additionally required for establishing the morphology of the skeletal elements of the limb. The limb is subdivided into three segments patterned from proximal to distal: the stylopod (humerus and femur), the zeugopod (radius/ulna and tibia/fibula), and the autopod (the wrist/forepaw, ankle/hindpaw) (Fig. 1). *Hox9* and *Hox10* function in the stylopod region (Fromental-Ramain et al., 1996a; Wellik and Capecchi, 2003; Raines et al., 2015), *Hox11* in the zeugopod region (Davis et al., 1995; Wellik and Capecchi, 2003) and *Hox13* in the autopod region (Fromental-Ramain et al., 1996b). In contrast to the homeotic transformation phenotypes observed with loss of *Hox* gene function in the axial skeleton, loss of function in the limbs result in dramatic, region-specific malformations of the skeletal elements.

Many attempts have been made to understand the mechanism of *Hox* gene function in limb development. A collective function of all posterior genes has been demonstrated at the earliest stages of limb bud formation (Kmita et al., 2005; Zakany et al., 2007; Sheth et al., 2013). However, once the three limb segments are established, surprisingly little is known regarding the region-specific mechanism of *Hox* gene function. Work on the *Hox11* group of genes shows abrogation of *Ihh* expression in the growth plates of the zeugopod elements when *Hox11* function is lost (Boulet and Capecchi, 2004). This result suggests that the *Pthrp-Ihh* feedback loop required for endochondral ossification is disrupted but a specific mechanism for *Hox* gene function in this loop has not been established.

Defining the cell type(s) in which *Hox* genes are expressed is critical to understanding their function. The *Hoxa11eGFP* mouse model was generated to carefully characterize expression during limb development. At the earliest stages, Hoxa11eGFP is observed broadly throughout the limb bud mesenchyme, but is quickly restricted to the zeugopod region by E12.5, the region that *Hox11* paralogs pattern. As skeletal development proceeds, a layer of fibroblasts surrounds the element (called the perichondrium) and eventually organizes into an outer mesenchymal cell layer of and an inner osteoblast layer (Fig. 2, E14.5) (Hall and Miyake, 2000). Consistent with other reports of in situ expression, *Hoxa11eGFP* is not expressed in the differentiating cartilage elements that will form the zeugopod bones, but is instead expressed in the

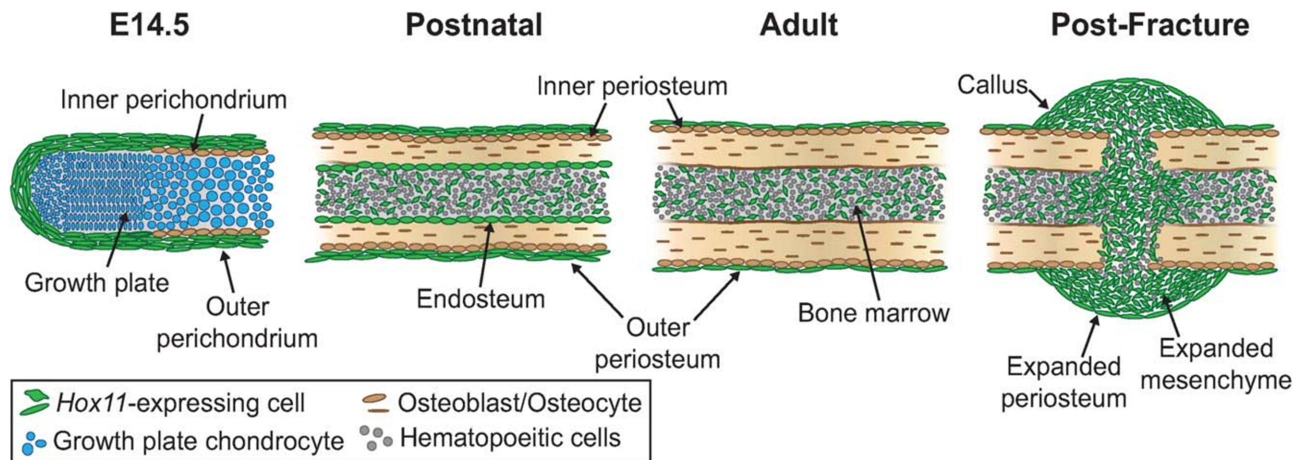

Fig. 2. Expression of *Hox11* during development and fracture repair. *Hox11*-expressing cells are expressed through embryonic development, postnatal, and adult stages, as well as during fracture healing. In the embryonic skeleton, *Hox11*-expressing cells are observed in the outer perichondrium surrounding the cartilage anlagen of the skeleton. During postnatal stages and adult stages, *Hox11* continues to be expressed in the outer periosteum surrounding the limb skeleton. It is additionally expressed in the bone marrow at both of these stages, and very highly in the endosteum during postnatal growth. Following fracture injury, *Hox11*-expressing cells expand with the forming callus.

perichondrium immediately surrounding these elements (Suzuki and Kuroiwa, 2002; Nelson et al., 2008; Swinehart et al., 2013; Neufeld et al., 2014). As the cartilage element matures, *Hoxa11eGFP* is visualized only in the outer perichondrium immediately adjacent to the layer of osteoblasts that surround the cartilage element (Swinehart et al., 2013) (Fig. 2). Swinehart et al. also reported the interesting observation that *Hoxa11eGFP* remains highly expressed in zeugopod regions through newborn stages; a result that suggested continued *Hox* gene function beyond initial skeletal patterning (Swinehart et al., 2013).

Hox Genes Beyond Embryonic Skeletal Patterning

Continued regional expression of *Hox* genes in adult tissues has been suggested by several independent studies, largely by the characterization of cells in culture. Human skin fibroblasts dissected from different anatomical locations were cultured and subjected to unbiased, whole transcriptome analyses. Results reveal broadly restricted *Hox* gene expression profiles that have some predictive values regarding the anatomical origin of the cultured population (Chang et al., 2002; Rinn et al., 2006, 2008). Similar studies of fibroblasts from adult human organs also show differential *Hox* gene expression profiles (Yamamoto et al., 2003; Takahashi et al., 2004), demonstrating that *Hox* gene expression is maintained more generally in adult tissues and organs. Another in vitro study provided evidence to support that *Hox* genes are expressed in cells that display progenitor behaviors in vitro, at least in the skeletal system. In these analyses, bone marrow cells were isolated, plated at low density, and depleted of hematopoietic cells. Colonies of fibroblasts that formed from single cells (CFU-Fs) were subjected to unbiased expression analyses and results indicate that CFU-Fs derived from different anatomical locations display region-specific *Hox* gene expression profiles (Ackema and Charite, 2008). Related to this, cord blood MSCs and bone marrow MSCs, populations that are used widely for tissue engineering and regenerative medicine also display differential *Hox* gene expression signatures in vitro (Liedtke et al., 2010; Bosch et al., 2012). The collective evidence from in vitro studies support the idea that regional *Hox* gene expression is maintained in adults and continues to function.

Many mechanisms of adult fracture healing are recapitulated events from embryonic skeletal development (Vortkamp et al., 1998; Ferguson et al., 1999; Gerstenfeld et al., 2003). Recently, *Hox* gene expression, and possible function, has been reported in mouse models of fracture injury. The first of these studies showed that at least some homeodomain-containing genes (*Msx-1, Msx-2, Prx1, Hoxa2*, and *Hoxd9*) are reactivated during repair of femur fracture by section in situ hybridization and by quantitative polymerase chain reaction (Gersch et al., 2005). In a similar study, calluses from femur fractures were subjected to microarray analysis at several stages following injury. It was reported that *Hox* genes are expressed in the fracture callus following injury of the skeleton and remain expressed throughout the repair process (Bais et al., 2009). A possible function for *Hox* genes during fracture repair was supported using a fracture transplant model. Mesenchymal cells were dissected from the periosteum of either the tibia or the mandible and were transplanted into the fracture site of either the same or the opposite bone. Results of the study show formation of cartilage in the mandibular injury when tibial cells were transplanted there. Mandibular injuries heal by intramembranous ossification (direct bone formation by osteoblasts); the presence of cartilage in these injuries suggests that the transplanted tibial cells (and, therefore, potentially the differential *Hox* function in these cells) induced the endochondral ossification process resulting in ectopic cartilage formation (Leucht et al., 2008).

These studies highlight exciting new potential functions for *Hox* genes in the adult skeleton. However, the extent to which the region-specificity more closely mirrors embryonic expression patterns was not carefully examined. In a new body of work, the adult region specificity of *Hox* genes was examined further using previously generated mouse genetic models that informed embryonic expression patterns and functions.

Hox Gene Expression in Adult MSCs

The expression of *Hox11* genes through postnatal and adult stages was examined using the *Hoxa11eGFP* mouse model.

Results reveal that Hoxa11eGFP + cells continue to be expressed through postnatal development of the skeleton and into adulthood (Pineault et al., 2015; Rux et al., 2016). *Hox11*-expressing cells are observed on the outer periosteal surface of the zeugopod long bones and they are a rare population of cells within adult bone marrow, consistent with expression expected for a mesenchymal stem/progenitor population (Fig. 2).

MSCs of the bone marrow (also called skeletal stem cells) are defined by several in vitro and in vivo characteristics (reviewed extensively by others; Schipani and Kronenberg, 2008; Bianco et al., 2013; Bianco and Robey, 2015). In vitro, they adhere to plastic, form CFU-Fs (an assay of self-renewal) and differentiate to multiple mesenchymal lineages (e.g., chondrocytes, osteoblasts, and adipocytes). In vivo, MSCs are a rare population of nonhematopoietic, nonendothelial stromal cells (CD45-, TER119-, CD31/PECAM-) and they have essential functions in bone maintenance and repair. Recent studies and have definitively shown that they contribute to the mature cell types required for both processes (Morikawa et al., 2009; Mendez-Ferrer et al., 2010; Park et al., 2012; Liu et al., 2013; Mizoguchi et al., 2014; Ono et al., 2014; Zhou et al., 2014; Worthley et al., 2015; Yue et al., 2016).

In addition, they have important roles in maintenance of the hematopoietic stem cell niche (Calvi et al., 2003; Zhang et al., 2003; Adams et al., 2007; Mendez-Ferrer et al., 2010; Frenette et al., 2013; Kunisaki et al., 2013). Several surface markers positively identify freshly isolated, progenitor-enriched, bone marrow-MSCs: CD105, platelet derived growth factor receptor-alpha (PDGFRα), Sca1, CD51 and leptin receptor (LepR) are among the most commonly used (Chan et al., 2009, 2013; Morikawa et al., 2009; Houlihan et al., 2012; Kunisaki et al., 2013; Pinho et al., 2013; Zhou et al., 2014). Recent work has shown that LepR, and the *LepR-Cre* mouse model, identifies the most highly enriched MSC population based on current methodologies (Ding et al., 2012; Ding and Morrison, 2013; Oguro et al., 2013; Zhou et al., 2014). This population overlaps almost exactly with a combination of two cell surface markers, PDGFRα and CD51 (Pinho et al., 2013). Cells marked in this lineage are largely quiescent, a feature that is shared with other defined stem cells populations (i.e., hematopoietic stem cells) (Zhou et al., 2014).

In our recent work, we find that *Hox11*-expressing cells display all of the characteristics of a progenitor-enriched MSC. When isolated from fresh bone marrow, they co-express PDGFRα, CD51, and LepR, three markers that label progenitor-enriched MSCs within the bone marrow stroma (Kunisaki et al., 2013; Pinho et al., 2013; Zhou et al., 2014). In vitro, Hoxa11eGFP + cells are capable of multi-lineage differentiation and demonstrate enhanced CFU-F capacity. In vivo, Hoxa11eGFP + cells expand following fracture injury and continue to co-express PDGFRα, CD51, and LepR throughout the repair process (Fig. 2). A lineage trace of transplanted Hoxa11eGFP + cells shows that they are capable of differentiating to osteoblasts and chondroctyes that contribute to fracture callus formation and repair (Fig. 2) (Rux et al., 2016).

Maintained Regional Specificity of *Hox* Genes in the Adult Skeleton

The expression of Hoxa11eGFP in adult, progenitor-enriched MSCs led to the question of whether this represents the normal expression for other or all *Hox* genes in regionally restricted bone

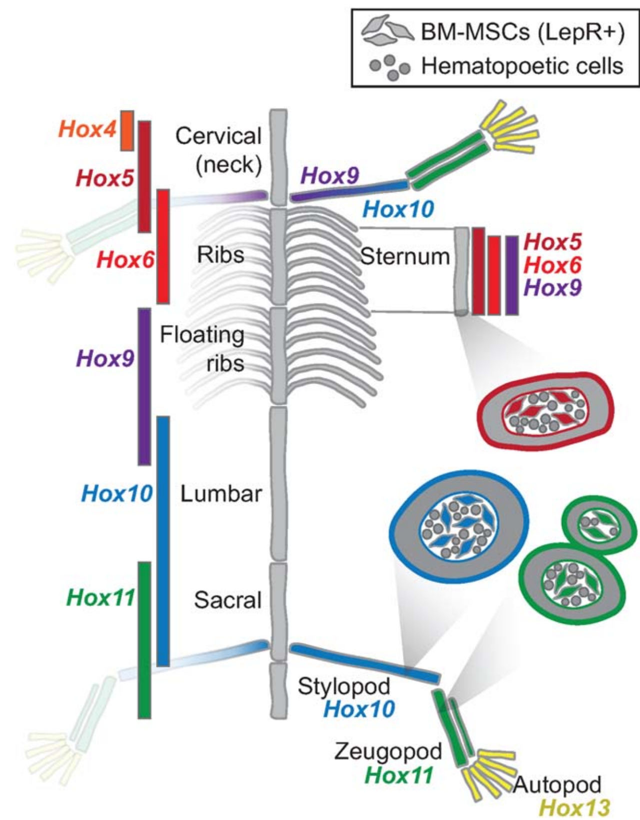

Fig. 3. Schematic of *Hox* gene expression in the adult skeleton. Recent data lead to a proposed model where *Hox* genes are expressed in adult bone marrow MSCs. The expression of *Hox* genes in these cells mirrors the regionally restricted pattern of expression and function during embryonic development.

marrow–MSC populations. Adult Hoxa11eGFP + cells are only found in the zeugopod region of the limb, the region in which *Hox11* functions during embryonic development. Gene expression analyses comparing specific limb segments (radius/ulna to humerus) demonstrate that adult *Hox* expression patterns mirror those established in the embryo. Expression of *Hox9* and *Hox10* is found in the stylopod and *Hox11* is found in the zeugopod. Similarly, bone marrow of the sternum shows expression of *Hox5* and *Hox6* genes. Of note, *Hox* expression is only detected in LepR + bone marrow MSCs in all bones examined (Fig. 3) (Rux et al., 2016). Together, these results support that *Hox*-expressing cells maintain regionally restricted boundaries in the adult skeleton, and that expression is exclusive to LepR + MSCs in the skeleton.

These expression studies lead to questions regarding potential functions for *Hox* in the adult skeleton. To explore the function of *Hox11* at adult stages, an ulnar fracture model was used in *Hox11* compound mutant animals in which three of the four *Hox11* alleles expressed in the forelimb are mutated. The remaining wild-type allele circumvents developmental defects and embryonic lethality and allows for functional analyses in the adult. *Hox11* compound mutant animals show perturbations in fracture repair of the zeugopod skeleton that include delayed bridging of bone across the fracture gap and incomplete remodeling. Chondrogenic differentiation and soft callus formation are severely disrupted in the *Hox11* compound mutant callus. This results in reduced endochondral ossification and delayed bridging of the fracture gap. Importantly, *Hox11* mutant MSCs

demonstrate a decreased ability to differentiate to chondrocytes and to osteoblasts in vitro, revealing a function for *Hox11* in MSC differentiation. Specific to a function for *Hox* genes, these phenotypes are not observed in other regions of the limb in *Hox11* mutants. Femur (stylopod) fractures in *Hox11* compound mutant animals show no perturbations in healing of this bone (Rux et al., 2016). Combined with the demonstration that *Hox9* and *Hox10* genes are expressed in stylopod MSCs, this result suggests that these *Hox* genes function in the femur like *Hox11* genes function in the zeugopod, but this has not been directly tested. These novel findings lead to a proposed model whereby *Hox* gene expression and function in adult bone marrow MSCs mirror expression patterns from embryonic development (Fig. 3).

Perspectives and Future Directions

Knowledge of the function of *Hox* genes in the mammalian skeleton has been largely limited to the patterning information they provide during embryonic development. Results from novel work reviewed here outline two findings that critically expand knowledge about *Hox* expression and function beyond embryonic development: (1) *Hox* genes are expressed exclusively in adult, LepR + MSCs and maintain the same regional restriction established during development and (2) region-specific *Hox* function is critical for the differentiation of mesenchymal-lineage cells to chondrocytes and to osteoblasts that are required for adult fracture repair.

The discovery that *Hox* genes maintain regional restriction during adult fracture repair is a critical new finding for the field of fracture healing biology. It is now clear that the transcription factors that govern initial skeletal patterning in the embryo also function in regeneration of the adult skeleton. Importantly, the adult regional restriction exactly mirrors the region specificity of *Hox* gene function during embryonic development. An important question that remains: Do the different *Hox* paralogous groups impart different function? Elucidating these potential differences will continue to be the focus of future studies to more completely understand the role for *Hox* genes in adult skeletal repair.

Regional specificity in the context of how MSCs function in vivo is an interesting new layer of complexity for other aspects MSC biology. These findings also lead to questions about the function of *Hox* genes in contexts that are not specific to skeletal regeneration. For example, *LepR*-expressing cells perform critical roles in maintenance of the bone marrow hematopoietic stem cell niche (Ding et al., 2012; Ding and Morrison, 2013; Oguro et al., 2013; Zhou et al., 2015). Studies aimed at exploring functions for *Hox* genes in this context have the potential to advance knowledge on homing and maintenance functions of the HSC niche.

Hox-expressing cells also represent a possible origin for adult MSCs. Reports from several groups suggest that adult MSCs arise from the perichondrium/periosteum during embryonic and postnatal stages (Maes et al., 2010; Liu et al., 2013; Mizoguchi et al., 2014; Ono et al., 2014). Of interest, Hoxa11eGFP is expressed at these times and in these regions (Fig. 2) (Swinehart et al., 2013). Moreover, the work reviewed here shows that they continue to be expressed in adult MSCs. Is it possible that *Hox*-expressing cells of the skeleton represent the progenitor population at all of these stages? Lineage-tracing studies initiated during embryonic and postnatal developmental stages will provide critical information regarding the contribution of *Hox11*-expressing cells throughout the life of the animal.

The new data that show *Hox* gene expression in adult bone marrow–MSCs also leads to the broader question: Do *Hox* genes function broadly in adult MSCs outside the skeleton? During embryonic development, *Hox* genes are also required for many other aspects of organogenesis. Similar to the pattern observed in the skeleton, the function of a paralogous group of genes is regionally restricted and is colinear with their chromosomal arrangement. *Hox3* paralogous group genes function in the thymus, *Hox5* genes in the lung, *Hox6* genes in the pancreas, and *Hox10* and *Hox11* genes in the kidney and the spleen (Roberts et al., 1994; Manley and Capecchi, 1998; Wellik et al., 2002; Yallowitz et al., 2011; Boucherat et al., 2013; Chojnowski et al., 2014; Hrycaj et al., 2015; Larsen et al., 2015). In the adult, tissue resident fibroblasts/mesenchymal cells can be isolated from all of these organs (and more), and maintained *Hox* expression has also been noted (Yamamoto et al., 2003; Takahashi et al., 2004; da Silva Meirelles et al., 2006; Crisan et al., 2008; Worthley et al., 2015). However, it is unclear whether *Hox* gene functions are also maintained in these adult organs. As a specific example, *Hox11* genes are expressed in embryonic muscle connective tissue fibroblasts and function in the muscle patterning of the zeugopod limb (Swinehart et al., 2013). Whether these cells remain in adult muscle tissue and function in maintenance and repair will be an interesting avenue to pursue.

Despite the growing body of knowledge regarding in vivo functions for adult MSCs, the major interest in these cells remains in regenerative medicine/tissue engineering applications. MSCs are capable of differentiating into bone and cartilage in vitro and this has been a major influence on tissue engineering strategies and in vivo transplantation methods (reviewed in Meijer et al., 2007). Importantly, recent studies aimed at developing novel tissue engineering protocols use strategies based on developmental signaling pathways to improve efficiency for clinical use (Lenas et al., 2009; Scotti et al., 2010). *Hox* genes have not been considered in this context, but they may be relevant to this effort. Understanding the importance of regionally restricted *Hox* gene expression and function will be critical in future studies involving MSCs for regenerative medicine. It may be important to derive MSCs from regions that are appropriate for intended use in these applications. "Matching" *Hox* gene function in vitro with the intended tissue in vivo, may prove useful for the viability of transplants. Alternatively, it may be beneficial in future tissue engineering strategies to manipulate regional *Hox* gene expression/function for specific differentiation strategies. Likewise, it will also be of interest to explore how to change *Hox* gene expression status in various in vitro organ and tissue differentiation strategies to develop desired differentiation outcomes that are relevant for transplantation in vivo.

Conclusions

Regenerative and normal physiologic processes in the adult show an increasing dependence on pathways used and established during embryogenesis. The collective results exploring *Hox* genes beyond embryonic development demonstrate that *Hox* gene expression is broadly maintained in the adult skeleton. It is also increasingly evident that the regional restriction that is established during embryonic development is maintained. This leads to a model whereby specific *Hox* genes required for patterning the morphology of skeletal elements during embryonic development may also be required for re-patterning the morphology of those

elements during maintenance, regenerative and repair processes. Continued work will provide new knowledge on this important topic.

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