Hox gene expression determines cell fate of adult periosteal stem/progenitor cells

Vivian Bradaschia-Correa1, Kevin Leclerc1, Anne M. Josephson1, Sooyeon Lee1, Laura Palma1, Hannah P. Litwa1, Shane S. Neibart1, Jason C. Huo1 & Philipp Leucht1,2

Hox genes are evolutionarily conserved transcription factors that during embryonic development function as master regulators of positional identity. In postnatal life, the function of Hox proteins is less clear: Hox genes are expressed during tissue repair, but in this context their function(s) are largely unknown. Here we show that Hox genes are expressed in periosteal stem/progenitor cells in a distribution similar to that during embryonic development. Using unbiased sequencing, we established that periosteal stem/progenitor cells from distinct anatomic sites within the skeleton significantly differ in their transcriptome, and that Hox expression status best defines these differences. Lastly, we provide evidence that Hox gene expression is one potential mechanism that maintains periosteal stem/progenitor cells in a more primitive, tripotent state, while suppression of Hox genes leads to fate changes with loss of tripotency. Together, our data describe an adult role of Hox genes other than positional identity, and the modulatory role of Hox genes in fate decisions may offer potential druggable targets for the treatment of fractures, non-unions and bone defects.

During embryonic development, homeobox (Hox) genes establish the cranial-caudal ‘Bauplan’ of the body (reviewed in1). In the caudal region, Hox genes are expressed in a nested pattern that terminates in the cranial region in the expression of a single Hox gene (reviewed in1). Anterior to the second branchial arch, from which the mandible and hyoid bones form, skeletal tissues are Hox-negative3. This Hox-positive and Hox-negative status of the skeletal elements is maintained into adulthood4–7, but why these genes remain transcriptionally active throughout the entire lifespan is not known. In adult animals and humans, the skeleton loses its potential to regenerate missing segments, and therefore a ‘Bauplan’ for restoration is not essential. Yet the continued presence of Hox expression in the mature skeleton argues for an alternate function; here, we tested the hypothesis that Hox status regulates the fate of periosteal stem/progenitor cells, which are ultimately responsible for healing skeletal injuries.

Periosteal stem/progenitor cells, irrespective of their anatomical origin, are thought to be one cell population, equal in function and character; and thus far, studies have not revealed significant differences in the properties of periosteal stem/progenitor cells from different skeletal elements. If Hox genes in fact regulate periosteal stem/progenitor cells function, then this would add another layer of complexity to this sparsely characterized stem/progenitor cell8; and our research thus aims at investigating whether the presence or absence of Hox expression imparts differential functional information that influences regenerative behavior of the periosteal stem/progenitor cell.

While most musculoskeletal research over the last few decades has focused on bone marrow-derived stromal cells, more recent scientific advances have focused on the periosteal stem/progenitor cell niche. In particular, the periosteal stem/progenitor cell pool demonstrates greater self-renewal, more regenerative potential, and superior in vitro proliferative capacity9. This heightened interest has resulted in the identification of a unique surface marker profile describing the periosteal stem/progenitor cell9–11.

In this study, we establish that Hox expression status regulates adult periosteal stem/progenitor cell lineage commitment. We observe a more osteogenic phenotype in Hox-negative periosteal stem/progenitor cells, while Hox-positive periosteal stem/progenitor cells are more chondrogenic and adipogenic. Gene silencing approaches,

1Department of Orthopaedic Surgery, New York University School of Medicine, New York, NY, USA. 2Department of Cell Biology, New York University School of Medicine, New York, NY, USA. Vivian Bradaschia-Correa and Kevin Leclerc contributed equally. Correspondence and requests for materials should be addressed to P.L. (email: Philipp.leucht@nyulangone.org)
using siRNA and antisense oligonucleotides (ASOs) against the long noncoding RNAs Hotairm1 and Hottip, suppress Hox expression in Hox-positive periosteal stem/progenitor cell populations, and this Hox-suppression led to a transcriptional and phenotypic change suggestive of a reversal of lineage commitment. These data demonstrate that Hox proteins are developmentally conserved master regulators of stem/progenitor cell fate during wound healing.

Results
Embryonic Hox gene signature is maintained in adult skeletal stem cells. “General Purpose” control genes, such as the Hox gene cluster, control the body plan of the embryo along the anterior-posterior axis (reviewed in12). During this process, patterns of Hox gene activity assign each anatomic body part a segmental identity, which culminates in the creation of a complex tissue, organ or organism. While such “Bauplan” is essential during development, it becomes less clear why these control genes would be necessary during adulthood. The most likely function may be found during regeneration of an injured tissue. Here, stem cells, once activated, organize within the regenerate to restore form and function of the injured body part, and it is in this scenario that a body plan gene cluster may provide vital regulatory function. We hypothesized that Hox genes continue to function as “general purpose” genes far into adulthood, and in order to test this conserved function of the Hox gene cluster, we made use of the skeleton, a contiguous organ, spanning the entire body from cranial to caudal. The skeleton is one of the few adult tissues that regenerates instead of repair/scar13 and contains skeletal progenitor cells that are located within distinct anatomic sites of the skeleton, such as the periosteum10,14–16. First, we had to confirm that indeed Hox gene expression is conserved and present in adulthood. Periosteal stem/progenitor cells were harvested from four anatomic locations 4, spanning the entire body, and were subjected to transcriptional profiling. RNAseq analysis revealed embryonic Hox-negative periosteal stem/progenitor cells maintained their Hox-negative status into adulthood (Fig. 1A), while embryonically Hox-positive periosteal stem/progenitor cells continued to express Hox genes (Fig. 1A). qRT-PCR analysis confirmed that anterior Hox genes continued to be expressed in the hyoid, while posterior Hox genes, such as Hoxa11 and Hoxa13, were expressed in periosteal stem/progenitor cells originating from the tibia (Fig. 1B). These findings were confirmed using in situ hybridization, demonstrating expression of the anterior Hox gene, Hoxa2, in the cambial layer of the hyoid periosteum, while the posterior Hox gene, Hoxa11 and Hoxa13, were expressed within the tibial periosteum (Fig. 1C).

Transcriptome analysis reveals difference between Hox-negative and Hox-positive periosteal stem/progenitor cells. Besides Hox gene cluster expression, periosteal stem/progenitor cells present another unique identifying signature: distinctive embryonic origins4. Most of the craniofacial skeleton, except for the parietal bone15, are derived from the neural crest18, while the entire axial and appendicular skeleton are derived from the mesoderm19 (Fig. 2A). These different embryonic origins, superimposed with a distinct Hox gene expression pattern, allowed us to define four unique periosteal stem/progenitor cell populations, different
in embryonic origin and Hox expression (Fig. 2A). If in fact Hox gene expression imprints a certain positional identity to these periosteal stem/progenitor cells, then transcriptional profiling using RNAseq should be able to identify which distinct feature (embryonic origin or Hox expression) better describes these stem/progenitor cell populations. Hierarchical cluster analysis of the transcriptome of the frontal, parietal, hyoid and tibial periosteal stem/progenitor cells was carried out. Clustering revealed that transcriptional similarities assigned the periosteal cells into two clusters defined by their Hox expression status: Periosteal stem/progenitor cells without Hox gene expression (frontal and parietal) homogenously clustered together and separately from periosteal...
stem/progenitor cells with Hox gene expression (hyoid and tibia)(Fig. 2B). To further dissect the discrepancies/ similarities/differences either between periosteal stem/progenitor cells from neural crest- (frontal and hyoid) and mesoderm-derived (parietal and tibia) skeletal elements (Fig. 2D) or between periosteal stem/progenitor cells with or without endogenous Hox gene expression (Fig. 2E). The MA plot distinguishing periosteal stem/ progenitor cells according to their embryonic origin revealed that periosteal stem/progenitor cells (SSCs) from NC-derived and MD-derived bones demonstrated significant differences in FPKM reads of 216 genes (Fig. 2D). However, when plotted as Hox-expressing and Hox-negative SSCs, 5,390 out of 17,569 genes measured by RNAseq revealed statistically different expression levels (Fig. 2E, red dots), indicating that if there is a functional difference between periosteal stem/progenitor cells from the craniofacial and appendicular skeleton, then this difference can be best described by the cells’ Hox gene expression status. To further understand this difference in transcriptional signature, we performed ATACseq to identify genes that were not only differentially expressed but also differ in their chromatin accessibility. Integration of the RNAseq and ATACseq data allowed us to identify genes that are differentially regulated between the comparisons. In line with the RNAseq data, the integrated analysis revealed that only 79 genes were differently expressed in cells from the neural crest versus mesoderm (Fig. 2F). However, in the comparison of Hox-positive and Hox-negative SSCs, 1,135 genes exhibited differential regulation (Fig. 2G), again supporting our hypothesis that the Hox gene expression status best differentiates SSCs from distinct anatomic skeletal regions.

Hox-negative and Hox-positive periosteal stem/progenitor cells respond differently to injury. We have previously shown that skeletal stem/progenitor cells reside within the periosteum of the tibia (appendicular skeleton) and the mandible (craniofacial skeleton), and contribute to repair and regeneration of each specific skeletal element after injury. RNAseq data have now revealed that Hox-expressing periosteal stem/progenitor cells are transcriptionally different than Hox-negative periosteal stem/progenitor cells. We next sought to examine these cells in vivo and investigate whether the transcriptional difference results in a morphological change in an uninjured and injured state. Histologic evaluation of uninjured periostea from Hox-negative and Hox-positive bones revealed an identical anatomic composition with its characteristic two-layer structure (Fig. 3A–H). In response to a scratch injury, Hox-negative periostea responded with a pure osteogenic reaction within the layer immediately adjacent to the cortical bone (Fig. 3I,J,M,N). In stark contrast, Hox-positive periostea exhibited a mixed response with both cartilaginous and osseous components (Fig. 3K,L,O,P). Immunofluorescence for Osx and Sox9 demonstrated a prevalence of Osx-positive cells in the periostea of Hox-negative bones after injury, further confirming the more osteogenic phenotype of this periostea (Fig. 3M,N). In contrast, periostea from Hox-positive bones showed a mixture of Osx-positive and Sox9-positive cells, confirming the histological appearance of a mixed chondrogenic and osteogenic injury response (Fig. 3O,P).

Hox-positive periostea contain more primitive periosteal stem/progenitor cells. Besides their transcriptional differences and their unique response to injury, periosteal cell composition demonstrated a clear distinction between Hox-positive and Hox-negative periostea. Debnath et al. recently described a periosteal stem/progenitor cell hierarchy. Using their gating strategy, we observed significantly greater numbers of periosteal stem/progenitor cells in the Hox-positive hyoid and tibia (Fig. 4A,B). In addition, we surveyed other general surface marker combinations that have been used in the past to describe stem/progenitor cells in the skeletal system. FACs analyses revealed that the Hox-positive periostea was enriched in Sca1- and CD146-expressing cells (Fig. 4C–F), while the Hox-negative periostea contained more CD166-positive cell than the Hox-positive tissue (Fig. 4G,H). Both Sca1 and CD-146 are associated with the more primitive skeletal/periosteal progenitor cell, while CD166 is labeling cells that have progressed on the lineage tree towards an osteoprogenitor cell. These data support the observation that the Hox-positive periostea is comprised of more primitive cells with both chondrogenic and osteogenic potential, while the Hox-negative calvarium is more differentiated and thus less plastic in response to injury.

Hox gene expression imparts skeletal multi-lineage differentiation potential on postnatal periosteal stem/progenitor cells. We previously demonstrated that craniofacial periosteal stem/progenitor cells exhibit superior osteogenic differentiation compared to appendicular periosteal stem/progenitor cells in an in vitro mineralization assay. Having now established that adult periosteal stem/progenitor cells come in two transcriptional flavors, Hox-positive and Hox-negative, we utilized functional in vitro differentiation assays to test whether this previously described transcriptional difference results in a measurable functional difference in osteogenic, chondrogenic and adipogenic differentiation. Periosteal stem/progenitor cells were subjected to tri-lineage differentiation. Osteogenic differentiation resulted in confluent mineralization of the frontal and parietal periosteal stem/progenitor cells assays, while hyoid and tibial periosteal stem/progenitor cells showed significantly less mineralization (Fig. 5A). Chondrogenic differentiation and adipogenic differentiation exhibited the exact opposite differentiation pattern with significantly more chondrogenic and adipogenic differentiation of periosteal stem/progenitor cells derived from the hyoid and tibia (Fig. 5B,C). These differentiation data reveal a striking similarity to the previously shown transcriptional separation of Hox-positive and Hox-negative periosteal stem/progenitor cells. Here, Hox-positive periosteal stem/progenitor cells display a more chondrogenic and adipogenic phenotype, while Hox-negative periosteal stem/progenitor cells are more osteogenic.
In order to test whether cell fate decisions of periosteal stem/progenitor cells during these differentiation assays are Hox-dependent, we utilized a knock-down approach targeting lncRNAs that regulate Hox expression of hyoid and tibial OPCs (Fig. 6A).

**Hotairm1**, a lncRNA located on the noncoding strand and situated at the 3′ end of the HoxA cluster (close to Hoxa1 and Hoxa2) (reviewed in21), has been shown to regulate Hox expression at this 3′ end (reviewed in21). We utilized an siRNA approach to silence the anterior Hoxa cluster and then assessed the effect of Hox repression (between dashed line) of the periosteum in the frontal and parietal bone, while (K,L) periostea from the hyoid and tibia responded with a mixed osteochondrogenic response (osx for osteogenesis, sox9 for chondrogenesis) (n = 5). (M,N) Immunofluorescence staining of the periostea of the hyoid bone and tibia confirmed the osteogenic response with osx-positive cells within the regenerate (between white dashed lines) and an absence of Sox9-expressing cells. (O,P) In contrast, the periosteal response to injury (between white dashed lines) of the hyoid and tibia exhibited both Osx+ and Sox9+ expressing cells, in line with a mixed osteochondrogenic healing response. Abbreviations: c, cortical bone; cl, cambial layer; fl, fibrous layer; p, periosteum.

In order to test whether cell fate decisions of periosteal stem/progenitor cells during these differentiation assays are Hox-dependent, we utilized a knock-down approach targeting lncRNAs that regulate Hox expression of hyoid and tibial OPCs (Fig. 6A). Hotairm1, a lncRNA located on the noncoding strand and situated at the 3′ end of the HoxA cluster (close to Hoxa1 and Hoxa2), has been shown to regulate Hox expression at this 3′ end (reviewed in21). We utilized an siRNA approach to silence the anterior Hoxa cluster and then assessed the effect of Hox repression on osteogenic, chondrogenic, and adipogenic differentiation. siHOTAIRM1 resulted in a significantly lower expression of Hotairm1 in hyoid periosteal stem/progenitor cells (Fig. 6B). This led to a significant reduction of Hoxa2 expression in these cells, confirming the successful knockdown (Fig. 6B). If our hypothesis that Hox expression leads to decreased osteogenic and increased chondrogenic and adipogenic differentiation, is correct, then we should observe a reversal after Hox gene knockdown. Indeed, we detected an increase in osteogenic differentiation of hyoid periosteal stem/progenitor cells after Hoxa2 knockdown, as shown by a significant increase in Collagen type 1 expression. In contrast, chondrogenic differentiation, measured by Sox9 and Collagen type 2 expression, and adipogenic differentiation, measured by Ppar-gamma and Fabp4 expression, was significantly decreased after Hoxa2 knockdown (Fig. 6B).

**Next, we sought to suppress Hoxa cluster expression at the 5′ end, and here antisense oligonucleotides (ASO) were used to knockdown the lncRNA Hottip. Hottip is expressed in posterior anatomic locations and has been shown to be expressed in a conserved pattern from development to adulthood, where it regulates the expression**
of the distal 5′ Hox cluster. Using an ASO approach, we successfully achieved Hottip knockdown in tibial periosteal stem/progenitor cells, and in response, we observed a significant decrease in Hoxa11 and Hoxa13 expression levels (Fig. 6C). While our approach was unable to detect a significant difference in osteogenic differentiation on a transcriptional level, there was a significant increase of mineralization detected in a functional osteogenic differentiation assay. Hoxa11 and Hoxa13 suppression using Hottip ASOs also resulted in a significant reduction of Sox9 and Collagen type 2 expression, suggesting a reduction in chondrogenic potential. Finally, Ppar-gamma and Fabp4 expression was significantly decreased after Hottip knockdown (Fig. 6C), confirming our hypothesis that Hox gene expression is intimately involved in adult periosteal stem/progenitor cell differentiation.

RNAseq and ATACseq confirm stemness of Hox-positive periosteal cells. These data suggest that Hox gene expression imparts tri-lineage potential on the periosteal stem/progenitor cell, while loss/suppression of Hox expression leads to progression on the lineage tree towards a more committed osteochondroprogenitor cell, osteoprogenitor cell, or osteoblast. We returned to the RNAseq data to confirm this observation. In particular, we employed a gene ontology (GO) analysis to determine the distinct biological functions assigned to either Hox+ or Hox− skeletal cells. Gene ontology categories that were enriched in each group revealed that Hox+ cells share common GO categories such as “embryonic skeletal system morphogenesis”, “embryonic skeletal system development”, and “mesenchyme migration”, suggesting a less committed, more primitive stem-like cell population (Fig. 7A). This is in contrast to Hox− cells, which show enrichment in GO categories such as “bone morphogenesis”, “cell fate commitment”, and “cell differentiation”, indicating a more committed progenitor population (Fig. 7A). Gene set enrichment analysis demonstrates biological processes modulated by Hox gene expression. GSEA analysis of GO terms revealed that Hox gene expression regulates gene sets associated with
Figure 5. Tri-lineage differentiation potential of Hox-positive and Hox-negative periosteal cells. Osteogenic (A), chondrogenic (B) and adipogenic (C) differentiation assays of frontal, parietal, hyoid, and tibia periosteal progenitors, as revealed by alizarin red, alcian blue, and oil red O staining, respectively (n = 3). Hox-negative periosteal stem/progenitor cells are almost exclusively osteogenic, while Hox-positive periosteal stem/progenitor cells exhibit tripotency. *p < 0.05, ***p < 0.001.

Figure 6. Manipulation of Hox gene expression in periosteal stem/progenitor cells leads to cell fate changes. (A) Graphical schematic of the mammalian HoxA locus and adjacent lncRNA regulators (left) and the corresponding anatomical regions where anterior and posterior HoxA genes are preferentially expressed (right). (B, C) Using siRNA and antisense oligonucleotides (ASO) against the lncRNAs Hotairm1 and Hottip to knockdown 5′ and 3′ Hox clusters. Knockdown of Hoxa2 in hyoid periosteal stem/progenitor cells (B) and Hoxa13 and Hoxa11 in tibial periosteal stem/progenitor cells (C) using siRNA against Hotairm1 and ASOs against Hottip resulted in a change in cell fate with increased osteogenic differentiation (Osx and Collagen type I, hyoid only) and decreased chondrogenic (Sox9 and Collagen type II) and adipogenic differentiation (Ppar-gamma and FabpP4) (n = 3). (C, lower panel) Hox-deficient tibial periosteal cells, via Hottip knockdown, displayed a greater capacity to differentiate into the osteogenic lineage, as measured by alizarin red absorbance (C, lower left panel), and less adipogenic potential, as measured by Oil Red O+ cells/well (C, lower right panel) when compared with NT control. Abbreviations: NT, non-targeted control. *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 7. Gene ontology analysis of Hox⁺ vs. Hox⁻ periosteal cells. (A) Hox⁺ periosteal cells are enriched in biological processes associated with stem-like cells, such as embryonic skeletal system morphogenesis and development, and mesenchyme migration (red), while the Hox⁻ periosteal cells exhibit features characteristic of more committed progenitor cells and terminally differentiated cells (blue). GO categories ranked by significance ($p \leq 0.05$ or $-\log_{10}(p) \geq 1.3$). (B) Gene set enrichment analysis (GSEA) plots demonstrate that Hox-positive SSCs positively correlate with the gene set for stemness (RAMALHO_STEMNESS_UP). (C) Aggregated enrichment of ATAC-seq signal around all transcription start sites in Hox⁺ (red) and Hox⁻ (blue) periosteal cells.

stemness (Fig. 7B). Finally, we utilized ATACseq to quantify the accessibility of chromatin in Hox-positive and Hox-negative SSCs. Increased accessibility of chromatin is associated with stemness, and our analysis revealed that Hox-positive periosteal stem/progenitor cells exhibited a larger area of open chromatin near transcriptional start sites compared to Hox-negative periosteal stem/progenitor cells (Fig. 7C).

Taken together, the current study provides the first experimental evidence that Hox gene expression in adult periosteal stem/progenitor cells imparts lifelong genetic regulation of tri-lineage differentiation potential of these cells. Notably, we provide proof that periosteal stem/progenitor cells are not just one cell population, equal in function and character, but rather a diverse population with distinct differentiation potential, likely responsible for the unique regenerative response seen in different skeletal elements.

Discussion
In comparison to other tetrapod vertebrates, humans possess limited regenerative capacity. While amphibians can replace entire limb segments, including the most complex musculoskeletal structures – mammals, including humans, can only partially regenerate injured bone segments, let alone restore entire appendages. Previous work has identified maintenance of Hox gene expression in adult mouse skeletal stem cells. Picchi et al. and Ackema et al. have both reported the presence of a Hox code in bone marrow-derived stromal cells, consistent with the embryonic Hox pattern, and both have suggested a role in determining cellular identity and function in...
adulthood, but no studies have yet identified a functional role of Hox gene expression in adult skeletal stem/progenitor cells specifically in the periosteum. The current study presents first evidence that Hox gene expression provides periosteal skeletal stem/progenitor cells with an anatomic signature, and at the same time imparts differentiative cues to these stem cells, which is a prerequisite for successful skeletal element regeneration.

Our previous work has shown that Hox gene expression of adult tibial skeletal stem cells confers upon them a sense of positional identity, which is unchanged when cells are placed into a new environment, similar to experiments performed on embryonic tissues, similar to in vitro experiments performed on embryonic tissues. While this positional memory of the skeletal stem cell may be important in transplantation experiments and surgical procedures, it is not clear why skeletal stem/progenitor cells possess and maintain this identity into adulthood. Should we consider bone regeneration that occurs after fracture in a human or mouse as complex as limb regeneration in an axolotl after amputation – one that requires positional identity to undergo proper morphogenesis of specific bone or limb segments? What if organization of the early hematoma after fracture requires a “Bauplan” similar to the one established by the Hox expression patterning during embryonic development or in a blastema after amputation? Rux et al. recently published compelling data that Hox expression is limited to adult progenitor-enriched mesenchymal stem/stromal cells and is essential for proper differentiation during repair. In response to injury, it is this cell population that is essential for successful regeneration, but their initial prevalence is miniscule compared to the other cell types present in the hematoma. Communication between these few cells is likely impossible due to the physical distance between them, therefore each skeletal stem cell has to be equipped with an architectural plan outlining the final regenerative product. Similar to the developing embryo, the establishment of cellular and tissue compartments within an early regenerate occurs in an environment, where gradients of transcription factors cannot be established over many cell diameters, nor can extracellular signaling proteins/morphogens modulate positional identity of this scarce subset of cells. Activation and maintenance of Hox gene expression in the skeletal stem cell pool is auto- and cross-regulated by products of Hox genes themselves, as well as through a group of proteins that modulate transcription and chromatin conformation, the Polycomb and Trithorax group. lncRNAs, such as HOTTIP and HOTAIRM1 can bind to these complexes and can thus modulate Hox gene expression. We took advantage of this regulatory mechanism and manipulated Hox gene expression using siRNA and antisense oligonucleotides against these lncRNAs. Using this strategy, we gathered crucial information on the functional role of Hox gene expression in adult periosteal stem/progenitor cells. First, our in vitro and RNAseq data provide convincing data that the presence or absence of Hox expression delineates two very distinct populations of periosteal stem/progenitor cells, varying not only in a vast number of transcriptional products, but also demonstrating unique cell fate decisions. While Hox-negative periosteal stem/progenitor cells favor osteogenesis, Hox-positive periosteal stem/progenitor cells primarily differentiate into cartilage and fat. When Hox expression was suppressed using siRNA or ASOs, we observed a reduction of the pro-chondrogenic and pro-adipogenic phenotype in the Hox-positive periosteal stem/progenitor cell population towards a more osteogenic phenotype. While these data only provide evidence that Hox paralogs impart differential functional information on periosteal stem/progenitor cells when Hox genes are ON or OFF, it remains unknown whether different Hox genes convey specific and unique patterning, repair and morphology instructions to the regenerating skeletal element. This may add critical complexity to the scientific journey towards mammalian limb regeneration and may revolutionize the procedure of bone grafting in orthopaedic surgery and dentistry. If the positional identity of a skeletal stem cell is essential for its regenerative capacity, then bone grafting procedures should take this positional information into account when the graft donor site is chosen.

Materials and Methods

Mice. All procedures were approved by the New York University Committee on Animal Research and were performed in accordance with the institutional guidelines and regulations. The studies were conducted on 12-week-old C57BL/6J male mice purchased from Jackson Laboratories (Bar Harbor, ME).

Periosteum dissection and RNA isolation. The periosteum from four different skeletal sites (F-frontal bone, H-hyoid bone, P-parietal bone, T-tibia), each representing a unique signature of embryonic Hox code (positive/negative) and embryonic origin (neural crest (NC)/mesoderm) were analyzed. After the soft tissues were carefully removed, the periosteum was collected with a Gracey curette (Hu Friedy, Chicago, IL) and stored in RNAlater (Qiagen, Hilden, Germany). The tissues were homogenized in Qiazol (Qiagen) with a mortar and pestle complemented with QIAshredder columns (Qiagen) then purified with the miRNeasy kit (Qiagen) following the manufacturer's instructions. The RNA purity was verified with Nanodrop (Thermo Fisher Scientific, Waltham, MA) and the integrity was evaluated with Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with 260/230 ratios superior to 2.0 and RIN superior to 8 were further analyzed.

RNA sequencing. RNA sequencing was performed utilizing the high output, paired-end reads with the Illumina HiSeq 2500 System. Bioinformatic analyses were performed with the Tophat (version 2.0.9) alignment program for reads mapping with two mismatches allowed. Cufflinks (version 2.2.0) was used to calculate FPKM values, and Htsseq (version 0.6.1.p1) was used to find the read counts for annotated genomic features. For the differential gene statistical analysis, DESeq 2 R/Bioconductor package in the R statistical programming environment was used. The contrast groups for the comparison were: Frontal (F) (2 replicates) versus hyoid (H) (2 replicates), parietal (P) (2 replicates) versus tibia (T) (2 replicates), neural crest (ND) (F + H, 4 replicates) versus mesoderm (MD) (P + T, 4 replicates) and Hox-negative (F + P, 4 replicates) versus Hox-positive (H + T, 4 replicates). Gene set enrichment analysis was performed using the GSEA software (http://www.broadinstitute.org/gsea/index.jsp) on log2 expression data of periosteal cells from the four bones aforementioned and classified in the corresponding classes. Gene sets were taken from the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). In particular, we investigated whether periosteum from each source was...
Gene sets were permuted 1000 times, the normalized enrichment score (NES) was calculated for each gene set, the nominal P value was obtained and the false discovery rate (FDR) was calculated to correct the P value.

Gene ontology (GO) analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/). Genes that were significantly enriched ($p \leq 0.05$; ≥2.33-fold) in Hox$^+$ vs. Hox$^-$ periosteal cells (1401 genes) or Hox$^-$ vs. Hox$^+$ periosteal cells (2163 genes) were used to generate GO terms corresponding to biological processes (with GOTERM_BP_DIRECT for functional annotation). Those significantly associated ($p \leq 0.05$ or $-\log_{10}(p) \geq 1.3$) with the gene list are ranked by p-value.

**ATACseq analysis.** ATACseq differential chromatin detection analysis was performed for three lanes of a paired-end 50 Illumina HiSeq. 2500 run. Per-read per-sample FASTQ files were generated using the bcl2fastq Conversion software (v1.8.4) to convert per-cycle BCL base call files outputted by the sequencing instrument into the FASTQ format. The alignment program, Bowtie2 (v2.3.4.1), was used for mapping reads of 18 mouse samples to the mouse reference genome mm10 and the application Sambamba (v0.6.7) was utilized to remove duplicate reads. The algorithm, MACS (in Python v2.7.3), was used to call peaks of signal for annotated genomic features and, similarly, the Python package NucleoATAC was functioned to call nucleosome positions. The computeMatrix and plotProfile tools in the deepTools suite (v2.3.3) were utilized for generation of signal profile plots. For the differential peak statistical comparisons between six groups of samples with three replicates each, the DiffBind package (Bioconductor v3.3.0) in the R statistical programming environment was utilized. Venn diagrams of differentially detected annotated genomic features comparing the ATACseq sample datasets with the corresponding sample sets from a related RNAseq experiment were generated using the Venny web application (v2.1).

**Flow cytometry.** Tibial, hyoid, frontal, parietal bones were harvested and periosteal cells were isolated as previously described. Dissociated cell samples were stained with PE-conjugated antibodies against CD31, CD45, and Ter-119 (Miltenyi Biotec) and APC-conjugated antibodies against Sca1, CD146, or CD166 (Miltenyi Biotec) for purification by flow cytometry (Beckman-Coulter MoFlo XDP, Brea, CA). CD31$^-$, CD45$^-$, Ter-119$^-$, and Sca1+$^+$, CD146+$^+$, or CD166+$^+$ cells were identified separately as distinct populations of skeletal stem/progenitor cells.

**Quantitative RT-PCR.** RNA was isolated from frontal, parietal, hyoid bones and the tibia as described and then reverse transcribed into cDNA with the Omniscript RT kit (Qiagen). The cDNA was amplified for specific targets using specific primers (listed in Table 1) and RT2 SYBR Green ROX PCR Master Mix in a QuantStudio3

| Primer Name | Sequence (5′-3′) |
|-------------|-----------------|
| 18S FOR     | ACGGAGACCTCGGCATGCTAACTAGT |
| 18S REV     | CGCCACTTGTCCCTCTAAGAA |
| Beta-Actin FOR | TGGTACCAACTGGGAGGACA |
| Beta-Actin REV | CTGGGTCACTTTTCCCGGT |
| Hoxa2 FOR   | GTCGAGAGCTTGGATTGATGAACT |
| Hoxa2 REV   | GTCGAGAGCTTGGATTGATGAACT |
| Hoxa11 FOR  | CTGCCAGCTCCCCTCTCTTT |
| Hoxa11 REV  | AGTAGACGTGCGACGAGTGG |
| Hoxa13 FOR  | CTGGACAGGGCCAAATGACT |
| Hoxa13 REV  | CCTCCGTTTGTGCTCTTGG |
| Hotairm1 FOR | AATCGGGGCAACTTGCTCAC |
| Hotairm1 REV | AGCATGCTCTTGGGTCTCTA |
| Horbp FOR   | TCCAGATGACCTTTCCTCGGT |
| Horbp REV   | TGATGAGGGCTTGTCCTCTAG |
| Osterix FOR | GGAAGACCTTGCTGGATAGTTTC |
| Osterix REV | GGGATCTTAGTGACTGCTCTAC |
| Type I Collagen FOR | CAGTGCATTACACTACAGACG |
| Type I Collagen REV | GGATGAGGGAGGAGTTACAGC |
| Sox9 FOR    | TACGACTGGACGCTGTG |
| Sox9 REV    | TTCATGGGCTGCTTGG |
| Type II Collagen FOR | TCCAGATGACCTTTCCTCGGT |
| Type II Collagen REV | CAGTGAACCGGATGCTTTCCTAC |
| Ppar-γ FOR | ATAGGTGTGAATCTAATGCCG |
| Ppar-γ REV | CCAACAGCTCTCTCTCTCG |
| Fabp4 FOR  | AAGAAGTGGGAGTGGGCTT |
| Fabp4 REV  | AAATCCCATTACGGCTATG |

Table 1. PCR primers. All primers were purchased from Integrated DNA Technologies.
μm thick sections were stained with Movat’s pentachrome. The sections were examined and photographed using a Zeiss LSM 710 laser scanning confocal microscope. Fluoromount (Thermo Fisher Scientific), examined and photographed using a Zeiss LSM 710 laser scanning confocal microscope.

**Histology and immunofluorescence antibody staining.** Frontal, parietal, hyoid bones and the tibia were stained with alcian blue and hematoxylin. For immunofluorescence, samples were decalcified in 19% EDTA for 48 hours, then, after cryoprotection in 30% sucrose, embedded in Tissue-Tek OCT media (Sakura Finetek, Torrance, CA) and cryosectioned at 100μm thickness. Sections were incubated overnight at 4°C in OSX antibody (rabbit anti-mouse A-13, 1:100, Santa Cruz, Santa Cruz, CA) and SOX9 antibody (goat anti-human, 1:20, R&D). Next, sections were stained with DAPI (Thermo Fisher Scientific), coverslipped with Fluoromount (Thermo Fisher Scientific), examined and photographed using a Zeiss LSM 710 laser scanning confocal microscope.

**In situ hybridization.** In situ hybridization was performed using the RNAscope Probe-Mm-Hoxa2, RNAscope Probe-Mm-Hoxa11 and made-to-order RNAscope Probe-Mm-Hoxa13 (Advanced Cell Diagnostics, Newark, CA). Positive (cyclophylin B) and negative antisense controls were performed on additional samples. Hybridized probes were detected using a manual singleplex RNAscope 2.0 HD brown kit (Advanced Cell Diagnostics) following the manufacturer’s instructions. The sections were examined and photographed using a Leica digital imaging system.

**Periosteal cell isolation.** Primary SSCs were obtained from frontal, parietal, hyoid bones and the tibia. After careful dissection, bones with intact periosteum were submitted to 4 serial collagenase digestions in 0.2% collagenase type 2 (Thermo Fisher Scientific) in DMEM at 37°C for 20 minutes with gentle rocking. After each of the first three digestions, bones were subjected to light centrifugation (1000 rpm) for 5 min and then transferred to a fresh tube of collagenase. After the last digestion, bones were centrifuged at 1400 rpm for 5 min and the pelleted cells were resuspended in growth media. Selective isolation of periosteal stem/progenitor cells was confirmed using FACS analysis.

**In vitro differentiation assays.** Periosteal progenitor cells from frontal, parietal, hyoid and tibia were isolated as described above and submitted to tri-lineage differentiation. For osteogenic differentiation, cells were cultured in DMEM containing 10% FBS, 100μg/ml ascorbic acid 10 mM β-glycerophosphate and 1% penicillin/streptomycin, which was replaced every 3 days. After 21 days, cultures were stained with alizarin red and in vitro mineralization was quantified as previously described. For chondrogenic differentiation, micro masses were cultured in chondrogenic differentiation media, supplemented with TGF β-3 (Lonza, Basel, Switzerland). After 14 days, the micro masses were fixed in 4% PFA, photographed under polarized light microscope (Leica) and paraffin embedded. Ten μm-thick sections were obtained and stained with alcian blue and hematoxylin. For adipogenic differentiation, SSCs were cultured in adipogenic differentiation media (Lonza). After 14 days, cells were stained with Oil Red-O and the number of positive cells per well was counted by an examiner blinded to the groups.

**RNA interference.** Primary hyoid SSCs were transfected with commercially available Lincode Mouse Hotairm1 SMARTpool siRNAs targeting Hotairm1 with the target sequence UGGUUUACUGACUAA (GE Dharmaco, Lafayette, CO) and primary tibial SSCs were transfected with Hottip antisense oligonucleotides (ASOs). The sequence for the Hottip ASOs was TAGTGCTTCTAAAACG. A non-targeting ASO control with Dharmacon (Lafayette, CO) and primary tibial SSCs were transfected with Hottip antisense oligonucleotides (ASOs). The sequence for the Hottip ASOs was TAGTGCTTCTAAAACG. A non-targeting ASO control with Dharmacon (Lafayette, CO) was used as transfection reagent as per manufacturer’s instructions. 24 hours after the transfection, the transfection media was replaced by regular growth media, osteogenic, chondrogenic or adipogenic differentiation media as described. Total RNA was harvested 48 h later using RNeasy Mini Kit (Qiagen). The knockdown of Hotairm1 in the hyoid SSCs, Hottip in tibial SSCs, as well as the downstream effect on the expression of specific Hox genes and tri-lineage differentiation markers were assessed by qPCR as described using specific primers (listed in Table 1).

**Statistical analysis.** A priori power analysis to obtain statistical significance (p = 0.05, power 80%) resulted in an n of 5 for each group, expecting a 25% difference between the two groups. Prism 7 (GraphPad Software, Inc., La Jolla, CA) was used for statistical computations. A Student’s t test was used for all comparisons in which there were two groups; ANOVA analyses followed by the Holms-Sidak correction for post-hoc testing was applied for analyses in which there were two or more comparisons being made. Error bars represent standard deviation. An asterisk symbol (*) denotes a p value of less than 0.05.

**Data Availability**
The data that support the findings of this study are available from the corresponding author upon request.
References

1. Deschamps, J. & van Nes, J. Developmental regulation of the Hox genes during axial morphogenesis in the mouse. *Development* **132**, 2931–2942 (2005).
2. Rux, D. R. & Wellik, D. M. Hox genes in the adult skeleton: Novel functions beyond embryonic development. *Dev Dyn* **246**, 310–317 (2017).
3. Grammatopoulos, G. A., Bell, E., Toole, L., Lumsden, A. & Tucker, A. S. Homeotic transformation of branchial arch identity after Hoxa2 overexpression. *Development* **127**, 5355–5365 (2000).
4. Leucht, P. et al. Embryonic origin and Hox status determine progenitor cell fate during adult bone regeneration. *Development* **135**, 2845–2854 (2008).
5. Rinn, J. L. et al. A dermal HOX transcriptional program regulates site-specific epidermal fate. *Genes Dev* **22**, 303–307 (2008).
6. Picchì, J. et al. HOX and TALE signatures specify human stromal stem cell populations from different sources. *J Cell Physiol* **228**, 879–889 (2013).
7. Ackema, K. B. & Charite, J. Mesenchymal stem cells from different organs are characterized by distinct topographic Hox codes. *Stem Cells Dev* **17**, 979–991 (2008).
8. Osorio, J. Stem cells: Back to the origins—identifying the skeletal stem cell. *Nat Rev Endocrinol* **11**, 132 (2015).
9. Duchamp de Lageneste, O. et al. Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. *Nat Commun* **9**, 773 (2018).
10. Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G. & Morrison, S. J. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* **15**, 154–168 (2014).
11. Zhang, D. R. et al. HOX Function is Required for Region-Specific Fracture Repair. *J Bone Miner Res* **32**, 1750–1760 (2017).
12. Jiang, X., Iseki, S., Maxson, R. E., Sucov, H. M. & Morriss-Kay, G. M. Tissue origins and interactions in the mammalian skull vault. *Dev Biol* **241**, 106–116 (2002).
13. Santagati, F. & Rijli, F. M. Cranial neural crest and the building of the vertebrate head. *Nat Rev Neurosci* **4**, 806–818 (2003).
14. Durland, J. L., Sferlazzo, M., Logan, M. & Burke, A. C. Visualizing the lateral somitic frontier in the Prx1Cre transgenic mouse. *J Anat* **212**, 590–602 (2008).
15. Hu, X. et al. Identification of a common mesenchymal progenitor for the adult haematopoietic niche. *Nat Commun* **7**, 13095 (2016).
16. De Kumar, B. & Krumlauf, R. HOXs and lincRNAs: Two sides of the same coin. *Sci Adv* **2**, e1301402 (2016).
17. Wang, K. C. et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature* **472**, 120–124 (2011).
18. Ugarte, F. et al. Progressive Chromatin Condensation and H3K9 Methylation Regulation the Differentiation of Embryonic and Hematopoietic Stem Cells. *Stem Cell Reports* **5**, 728–740 (2015).
19. Brookes, J. P. & Kumar, A. Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* **310**, 1919–1923 (2005).
20. Sanchez Alvarado, A. & Tsonis, P. A. Bridging the regeneration gap: genetic insights from diverse animal models. *Nat Rev Genet* **7**, 873–884 (2006).
21. Rux, D. R. et al. Regionally Restricted Hox Function in Adult Bone Marrow Multipotent Mesenchymal Stem/Stromal Cells. *Dev Cell* **39**, 653–666 (2016).
22. Le Dosarim, N. M., Creuzet, S., Couly, G. & Dupin, E. Neural crest cell plasticity and its limits. *Development* **131**, 4637–4650 (2004).
23. Marecic, O. et al. Identification and characterization of an injury-induced skeletal progenitor. *Proc Natl Acad Sci USA* **112**, 9920–9925 (2015).
24. Lee, N., Maurange, C., Ringrose, L. & Paro, R. Suppression of Polycomb group proteins by JNK signalling induces transdifferentiation in Drosophila imaginal discs. *Nature* **438**, 234–237 (2005).
25. Chinnaiya, K., Tickle, C. & Towers, M. Sonic hedgehog-expressing cells in the developing limb measure time by an intrinsic cell cycle clock. *Nat Commun* **5**, 4230 (2014).
26. 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).
27. Leucht, P. et al. Effect of mechanical stimuli on skeletal regeneration around implants. *Bone* **40**, 919–930 (2007).
28. Bradaschia-Correia, V. et al. The Selective Serotonin Reuptake Inhibitor Fluoxetine Directly Inhibits Osteoblast Differentiation and Mineralization During Fracture Healing in Mice. *J Bone Miner Res* **32**, 821–833 (2017).

Acknowledgements

RNAseq, ATACseq and analysis were performed in the NYU Genome Technology Center, supported by a P30CA016087 grant from the National Institutes of Health/National Cancer Institute. Research reported in this publication was supported by the Musculoskeletal Transplant Foundation (PL) and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number K08AR069099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions

PL conceived the project strategy; V.B.C., K.L., A.M.J., S.L., L.P., H.L., S.S.N. and J.C.H. designed, performed, analyzed and interpreted data, contributed to writing and editing of manuscript, and preparation of figures. PL analyzed data and wrote the manuscript. All authors reviewed the manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2019