Hoxa10 Maintains the Murine and Human Skeletal Stem Cell Pool

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INTRODUCTION: Skeletal stem and progenitor cells (SSPCs) are pivotal to the growth and lifelong turnover of bone, as well as to its regenerative capacity. Following their role in patterning the embryonic skeleton, *Hox* genes remain regionally expressed in SSPCs of the adult skeleton. We have previously shown that *Hoxa10* is associated with stemness using mainly in vitro assays (1). Here, we aim to understand the *in vivo* role *Hox* genes play in the SSPC populations of the tibia and its conversion in humans. We hypothesize that *Hox* maintains mouse and human skeletal stem cells in an uncommitted, multipotential state, thereby inhibiting their differentiation into bone and cartilage. To explore this hypothesis, we generated and validated an inducible *Hoxa10creERTGFP* knock-in, knockout mouse and used it in conjunction with a $HoxA^{flox}$ allele, in which the entire *HoxA* cluster is removed upon recombination, along with $Rosa^{Tomato}$ to track the descendants of control and mutant cells. We also used siRNAs against posterior *Hox* genes in order to reduce *Hox* expression in primary human SSPCs *in vitro*. We found that the reduction of *Hox* expression in mouse SSPCs *in vivo* or human SSPCs *in vitro* led to a loss of "stemness" profile of these cells, taking on more differentiated characteristics, and led to compromised fracture healing. Similarly, Hox-deficient human SSPCs exhibited a dysregulation of stem cell characteristics and greater osteogenic potential, demonstrating the therapeutic potential of targeting *Hoxa10* and its effectors in remedying skeletal ailments.

METHODS: Tibial cells from 8 to 16-week-old were isolated for experiments. For micro-computed tomography (microCT) analysis, bones were with intact muscle were incubated in 4% paraformaldehyde at 4°C for 2-3 days. Bones were then submitted to the microCT scanning core at NYU Langone to determine fracture healing capacity. For immunofluorescence, after fixation the bones were incubated EDTA for 3 weeks, then with 30% sucrose overnight and embedded with OCT compound. Bones were then cryosectioned at 40-micron thickness, and the sections permeabilized (0.25% Triton-X-100 in PBS; PBST; 30 min), blocked (5% donkey serum in PBST; 30 min), stained with primary antibodies (1:500 Aves chicken anti-GFP; 1:200 SantaCruz rabbit anti-Osterix; overnight at 4°C), washed with PBS, and stained with secondary antibody (Thermofisher Donkey anti-Chicken 488, Donkey anti-Rrabbit 647; 1:2000). For *in vitro* siRNA experiments, human siRNAs against Hoxa10, a11, c10, d10, and d11 (HoxMix) were administered to 50,000 human BMSCs at 10nM each; nontargeting control siRNA was administered at 50nM. For flow cytometry, after careful dissection, bones with intact periosteum were first cut into small pieces with fine scissors and then submitted to three serial collagenase digestions in 0.2% collagenase type 2 (Thermo Fisher Scientific) in DMEM at 37 °C for 20 minutes with gentle rocking. Cells were collected through a 70-micron filter after each collagenase step. After the last digestion, the cells were centrifuged and incubated with red blood cell lysis (NH_4Cl; StemCell Technologies) solution for 10 min on ice. Cells were centrifuged and filtered again and incubated with relevant antibodies for flow cytometry. Student's t-test and Anova used for statistical analysis.

RESULTS SECTION: Using $Hoxa10^{creERTGFP}$ as a real-time reporter of *Hoxa10* expression and $Rosa^{Tomato}$ as a lineage reporter at post-operative day 7 of a tibia fracture, we have shown that *Hoxa10* expression is associated with the stem cell markers CD51, PDGFRα, and SCA1 as assessed by flow cytometry and is also enriched in the most primitive skeletal stem cell (SSC) and pre-Bone/Chondro/Stromal (preBCSP) populations as defined by *Chan et al., 2015* (2). The recombination of alleles in adult $Hoxa10^{creERTGFP};HoxA^{flox};Rosa^{Tomato}$ results in mice that are homozygous mutant for *Hoxa10* and heterozygous for the other genes in the *HoxA* cluster that have been shown to have sequence and functional redundancy with *Hoxa10* (3). After confirming a reduction of *Hoxa10* expression upon the administration of tamoxifen compared to control mice ($Hoxa10^{creERTGFP};Rosa^{Tomato}$), we sought to examine how *in vivo Hox* deficiency could affect the stem cell attributes and fracture healing capacity of injured mice. To assess whether *Hox* gene expression could affect the self-renewal of SSPCs, we plated SSPCs from control and mutant mice for CFU-Fs and observed a large reduction in the self-renewal capacity in mutant samples, suggesting a dysregulation of $Hoxa10^{+}$ stem cell function. To explore whether injury-induced stem cell recruitment is dependent on Hox expression and determine whether $Hoxa10^{+}$ stem cells may play a role in fracture healing capacity, control and mutant mice underwent a tibia fracture model. Mutant mice exhibited significantly lower SSC recruitment 7 days after tibial fracture when compared to control mice, as assessed by flow cytometry. Later, at POD14, mutant mice displayed significantly fewer $Hoxa10^{+}$ cells, as assessed by immunofluorescence, and displayed less bone volume per unit of callus compared to controls when analyzed by microCT. In a serial fracture model, the ability of the stem cell pool to recover from injury-induced depletion was tested. In this model, mice were allowed to recover for 6 weeks after a primary injury; after a second injury and recovery period, mutant mice exhibited fewer SSCs and had a greater propensity for non-unions when compared to control animals. To assess the differentiation capacity of control and mutant $Hoxa10^{+}$ cells, $Hoxa10GFP^{+}$ cells were sorted by flow cytometry and subjected to *in vitro* trilineage differentiation, Hox mutant cells were primed to differentiate into the adipo-, chondro-, osteo-genic lineages relative to control cells. To confirm this result i*n vivo*, we tracked control and mutant Hoxa10-lineage ($tdTomato^{+}$) cells during fracture repair. Mutant Hoxa10-lineage cells overlapped with the osteoblastic Osterix marker to a significantly greater extent than control Hoxa10-lineage cells. Altogether the *in vitro* and *in vivo* results suggest defective, premature progression along the differentiation hierarchy. To assess whether this stem cell maintenance role is conserved in humans, bone marrow samples were collected from the iliac crest of human patients (under 45 years old) undergoing orthopedic procedures. Hox expression was reduced in these cells with siRNAs against Hox genes (siHoxMix) when compared to a non-targeting control. *in vitro* assays determined that human SSPCs with diminished Hox expression exhibited fewer SSCs as defined by *Chan et al., 2018*, lower self-renewal capacity and proliferation, and greater osteogenic capacity, indicating a conserved stem cell maintenance function for Hox in humans and further cementing its potential as a therapeutic target.

DISCUSSION: Paralogous *Hox* genes are known to simultaneously display functional redundancy and distinct functions. Our transgenic mice creates a homozygous mutant for *Hoxa10* and heterozygous mutants for all other *HoxA* cluster genes except *Hoxa10*. Although *Hoxa10* is the highest expressed *Hox* gene in the tibia, it is possible that some of the phenotypes observed are due to a reduction of other *HoxA* genes that serve unique functions in tibial SSPCs.

SIGNIFICANCE/CLINICAL RELEVANCE: Many fractures can take months to heal and certain severe, traumatic injuries never properly heal. The quest for new therapies to treat these cases should be of highest priority to the research community. The adult skeleton contains tissue-specific stem cells, which are responsible for maintaining bone mass and for regenerating new bone following injury; these skeletal stem cells are thus the best target for increasing the efficacy of skeletal tissue regeneration. Approaches that focus on rapid and functional bone regeneration and repair, however, have yet to be optimized. Adjusting the potency of adult SSCs will therefore be critical to their successful application in regenerative medicine. If we can demonstrate that *Hox* genes are essential regulators of the developmental potential of SSCs, this highly translational project will have an immediate effect on patient care.

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