Hox Gene Expression Regulates the Osteogenic Maturation of Pre-Osteoblast Cells

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INTRODUCTION: Approximately 185,000 people undergo limb amputation annually in the United States, resulting in permanent physical disability [1]. Therefore, successfully restoring lost digits or limbs could improve the prognosis for amputees. Although mammals typically have limited regeneration of composite musculoskeletal tissues, adult mice exhibit the natural ability to regrow the distal digit tip following amputation, including bone and soft tissues [2,3]. Digit regeneration depends on the formation and differentiation of a blastema, a mass of mesenchymal stem/progenitor cells with characteristics of both embryonic limb development and wound healing. Previously, we discovered that *Hoxa13*, a developmental gene critical for autopod (hand/foot) morphogenesis [4], is transiently upregulated by the blastema [3]. Mice lacking *Hoxa13* exhibit disrupted skeletal outgrowth and morphology during digit regeneration [5], but how osteoblast lineage commitment and maturation are affected remains unclear. To explore how developmental transcriptional programs influence bone formation and patterning, we investigated the effect of *Hoxa13* overexpression on osteogenic gene expression and mineralization *in vitro* using murine pre-osteoblasts [6].

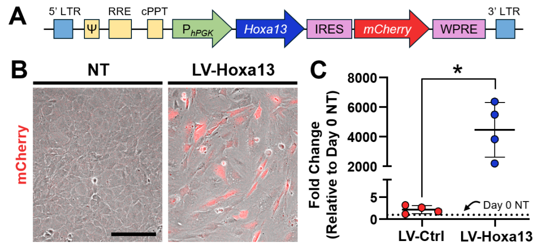

Fig. 1. (**A**) Schematic of *Hoxa13* lentiviral vector with mCherry reporter (LV-Hoxa13). (**B**) MC3T3 pre-osteoblasts express mCherry after LV-Hoxa13 transduction. Scale: 0.2 mm. (**C**) *Hoxa13* expression by transduced MC3T3s on day 0 prior to osteogenic differentiation (n=4 samples/group, mean ± SD), shown as fold change relative to NT on day 0 (dotted line). *: p<0.05 between groups.

METHODS: Lentiviral transduction: A 3rd generation lentiviral transfer plasmid for *Hoxa13* overexpression (LV-Hoxa13) was generated using PCR and DNA assembly methods. In this plasmid, the constitutive promoter from human phosphoglycerate kinase 1 drives expression of *Hoxa13*, which is transcriptionally linked to a fluorescent reporter (mCherry) (**Fig. 1A**). VSV-G-pseudotyped lentivirus was produced in HEK293T cells. A control lentivirus (LV-Ctrl) containing only mCherry was also generated. MC3T3-E1 murine pre-osteoblasts (passage 3; ATCC CRL-2593) [6] were transduced with either control virus (MOI: 2) or *Hoxa13* virus (MOI: 5) in the presence of 10 μg/mL polybrene for 2–3 days to generate LV-Ctrl and LV-Hoxa13 cells, respectively. Cells exposed only to polybrene were used as a non-transduced (NT) control. Transduced cells were evaluated for mCherry production by fluorescence microscopy. Osteogenesis: NT, LV-Ctrl, and LV-Hoxa13 cells (passage 7–10) were grown in either expansion medium (α-MEM with 10% fetal bovine serum and 1% penicillin/streptomycin) or osteogenic medium (expansion medium with 50 μg/mL ascorbate, 100 nM dexamethasone, and 10 mM β-glycerophosphate) [6]. Cells were grown in 24-well plates with media changes every 3–4 days. After 21 days, cells were fixed and stained with Alizarin Red to assess mineral deposition. Stained wells were imaged by brightfield microscopy, with the staining area fraction (%) quantified via ImageJ (n=6 samples/group). To evaluate the expression of *Hoxa13* and osteogenic genes (*Runx2, Sp7,* and *Bglap*), RNA isolation, cDNA synthesis, and quantitative real-time polymerase chain reaction were performed for cells grown in 12-well plates on day 0 prior to osteogenic induction and after 21 days in osteogenic medium (n=3–4 samples/group). Analysis was performed with the ΔΔCt method, with all samples compared to the NT group on day 0 and *Rn18s* used as the endogenous control gene. Significance was assessed by 1-way ANOVA with Tukey's post-hoc test or by a 2-tailed unpaired Student's t-test (p<0.05).

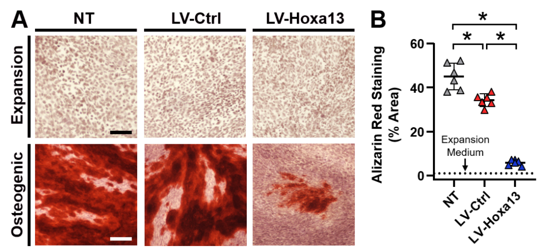

Fig. 2. (**A**) Alizarin Red staining of MC3T3s cultured in expansion or osteogenic medium for 21 days. Scale: 0.2 mm. (**B**) Alizarin Red staining (% area) of MC3T3s after 21 days in osteogenic medium (n=6 samples/group, mean ± SD). Dotted line shows mean % staining area for groups cultured in expansion medium. *: p<0.05 between groups.

RESULTS: To investigate the effect of *Hoxa13* expression on osteogenesis, we successfully constructed a lentiviral transfer plasmid for stable genomic integration of a *Hoxa13* overexpression construct coupled with an mCherry reporter (LV-Hoxa13). MC3T3 murine pre-osteoblasts transduced with LV-Hoxa13 appeared fluorescent after 48 hours of viral incubation (**Fig. 1B**). LV-Hoxa13 cells exhibited greater than a 4,000-fold increase in *Hoxa13* expression compared to non-transduced (NT) controls on day 0 prior to osteogenic induction (**Fig. 1C**, p<0.05), whereas the *Hoxa13* levels of cells transduced with control virus (LV-Ctrl) did not markedly change compared to NT cells. While all pre-osteoblast groups produced mineralized cultures when grown in osteogenic medium for 21 days, the % area of Alizarin Red staining was highest in the NT (45 ± 6%) and LV-Ctrl (34 ± 3%) groups (**Fig. 2**). In comparison, mineralization capacity was significantly attenuated in the LV-Hoxa13 group (4 ± 1% staining area) (**Fig. 2B**, p<0.05). As expected, Alizarin Red staining was absent in all groups cultured in expansion medium (**Fig. 2A**). Expression of the osteogenic gene markers *Sp7* (osterix) and *Bglap* (osteocalcin) was significantly reduced in LV-Hoxa13 cells compared to the LV-Ctrl group after 21 days in osteogenic medium (**Fig. 3**, p<0.05). However, *Runx2* expression was not different between groups after 21 days of osteogenesis (**Fig. 3**, p>0.05).

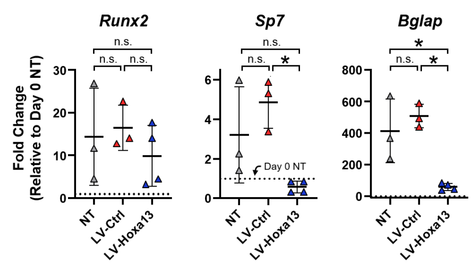

Fig. 3. Expression of *Runx2*, *Sp7*, and *Bglap* by MC3T3s after 21 days of osteogenic culture (n=3–4 samples/group, mean ± SD), shown as fold change relative to NT on day 0 (dotted line). *: p<0.05 between groups.

DISCUSSION: Successful induction of limb regeneration requires coordinated skeletal outgrowth and patterning. Here, we provide evidence that *Hoxa13* expression, which plays key roles during embryonic limb development [4] and adult digit regeneration [5], regulates the osteogenic maturation of pre-osteoblasts by reprogramming lineage-committed cells towards a more primitive, progenitor-like state. Previous studies found that *Hox* gene overexpression by periosteal cells was associated with the skeletal stem cell phenotype, whereas suppression of *Hox* genes led to loss of stem cell multipotency [7,8]. Interestingly, delivering *Hoxa10*-positive cells to the injury site after tibial fracture improved bone repair in aged subjects [8], suggesting that replenishing the stem/progenitor cell pool may augment tissue regeneration. While *Hox*-expressing cells may represent a developmentally appropriate and regeneration-competent cell source for transplantation, *Hox* gene transcription should be temporally regulated to inhibit neoplastic transformation [9]. Future work will explore the signaling pathways downstream of *Hox* gene activation and their effects on skeletal morphogenesis using murine induced pluripotent stem cells [6], with potential applications for musculoskeletal repair and regeneration.

SIGNIFICANCE: Humans have limited regenerative potential of musculoskeletal tissues following limb loss. To this end, successful attempts to regrow missing limbs could significantly improve the prognosis for amputees.

REFERENCES: [illegible] Development 2001. [5] Qu+, ORS

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