Oct4 and Sox2 expressing chondrocytes give rise to bone and bone lining cells during fracture healing

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INTRODUCTION: Recent genetic studies have demonstrated that chondrocytes can transdifferentiate into bone during both development and fracture repair.[1-3] This model contradicts the dogmatic view that hypertrophy is the terminal maturation state of chondrocytes and that they are subsequently destined for apoptosis. Lineage tracing experiments using multiple chondrocyte markers (collagen II, collagen X, aggrecan)[1,3] show that these cells directly contribute to the new bone in murine fracture models. However the mechanism through which the chondrocytes transdifferentiate has not been elucidated. We hypothesize that Oct4 and Sox2 programs may be activated in hypertrophic chondrocytes in the fracture callus to enable these cells to transdifferentiate into the new bone. We test this hypothesis using inducible transgenic reporter mice for Oct4 and Sox2.

METHODS: All murine studies were performed under IACUC approval. Breeding was done to create tamoxifen inducible reporter mice for the Oct4 and Sox2 genes: Oct4 (Oct4-MerCreMer, Jackson #016829) x R26R (MGI ref 11861931) and Sox2 (B6;129S-*Sox2*$^{tm1(cre/ERT2)Hoch}$, Jackson #017593) x dTomato (Green(on), Red(off): B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Lu}$, Jackson # 007676). Tamoxifen was delivered at a concentration of 75mg tamoxifen/kg body weight daily from days 6-10 post-fracture to explicitly label chondrocytes in the fracture. Tibiae were harvested 14 or 21 days post-fracture to determine the spatial temporal contribution of these genes. Oct4 and Sox2 immunohistochemistry (IHC) was performed on fractures made in WT (B6) mice to confirm the genetic patterns observed in the transgenic mice. Additionally, the soft cartilage callus from WT mice was dissected out using a microscope and cultured *in vitro* using human umbilical cord vascular endothelial cell (HUVEC) conditioned medium and analyzed by quantitative RT-PCR.

RESULTS: We have defined a histological and function region in the fracture callus called the "transition zone" (TZ) where the vasculature invades the hypertrophic cartilage callus and new bone forms (Fig 1A,E). Using a combination of IHC and the Oct4xR26R reporter mouse we see that Oct4 is not expressed in non-hypertrophic chondrocytes (Fig 1B,F), but is activated in hypertrophic chondrocytes in the TZ near the invading vasculature (Fig 1C,G). Oct4 expressing cells are found in the new bone (NB) and bone lining cells (Fig 1D,H). Similarly, Sox2 is activated in the TZ (Fig 2B,E) and can be found in the new bone (Fig 2C,F, red arrows) and bone lining cells (Fig C,F, purpose arrows) following the transition of cartilage into bone. Co-localization of Oct4 and Sox2 in the TZ was demonstrated by IHC (Fig 3A) and we find that these genes can be stimulated *in vitro* by culturing with HUVEC medium (Fig 3B).

DISCUSSION: The data presented in this abstract build on the new model of fracture healing in which chondrocytes transdifferentiate to directly contribute to the new bone. Using lineage tracing, IHC, and *in vitro* culture we investigated whether Oct4 and Sox2 play a role in this process in mice. Previously, *nanog* expression was detected during fracture healing from gene array expression, but the type of cell in which expression occurred and its role in fracture healing were not determined.[4] Here we show that Oct4 and Sox2, genes associated with pluripotency, are activated in hypertrophic chondrocytes near the invading vasculature and expression can be traced to cells both embedded in the new bone and bone lining cells. These genes were activated in chondrocytes isolated from the fracture callus *in vitro* by HUVEC conditioned medium, indicating that paracrine factors from the vasculature may coordinate this event. Taken together these data suggest that activation of Oct4 and Sox2 may enable transdifferentiation of chondrocytes into osteoblasts/cytes contributing to the new bone and the bone lining cells by inferring a stem-like capacity onto the chondrocyte that enables phenotypic transformation.

SIGNIFICANCE:

(1) Yang, L., *et al*. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proc Natl Acad Sci U S A, (2014).
(2) Bahney, C. S. et al. Stem cell-derived endochondral cartilage stimulates bone healing by tissue transformation. J Bone Miner Res 29, 1269-1282 (2014).
(3) Zhou, X. et al. Chondrocytes Transdifferentiate into Osteoblasts in Endochondral Bone during Development and Fracture Healing. PLoS Genet (2014).
(4) Bais, M. *et al.* Transcriptional analysis of fracture healing and the induction of embryonic stem cell-related genes. PloS one (2009).

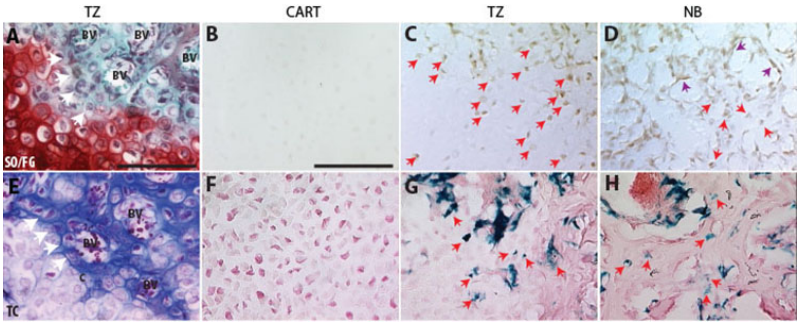

Fig 1: Oct4 expression in the day 14 fracture callus. (A) Saffranin-O staining of fracture "transition zone" (TZ). (B-D) Immunohistochemistry to Oct4. (E) Trichrome staining of TZ (F-H) x-gal staining to detect β-gal expressing cells from Oct4 x R26R mouse. Red arrows = positive chondrocytes, Purple arrows = bone lining cells

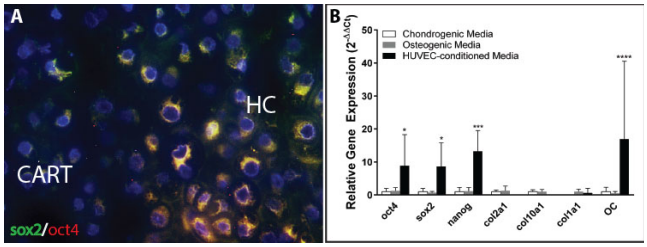

Fig 3: Colocalization of Oct4/Sox and activation by HUVEC conditione medium. (A) IHC double staining for Oct4 and Sox2. (B) RT-PCR for gene expression from fracture callus cartilage explants cultured in HUVEC conditioned medium.

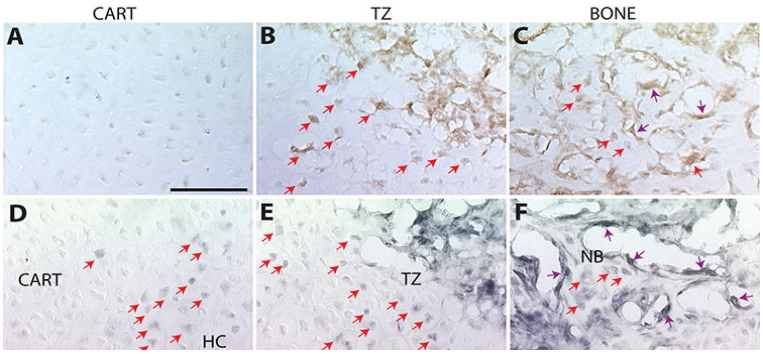

Fig 2: Sox2 expression in the day 14 fracture callus. (A-C) Immunohistochemistry to Sox 2. (E-F) eGFP IHC to detect eGFP expressing cells from Sox2 x dTomamto (Green On/Red Off) mouse. Red arrows = positive chondrocytes, Purple arrows = bone lining cells