Runx3/AML2/Cbfa3 Mediates Chondrocyte Differentiation.
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INTRODUCTION. The Runx related factors (Runx) are phylogenetically conserved between C. Elegans, Drosophila, zebrafish and vertebrates. The mammalian Runx family is composed of three members, Runx1, Runx2 and Runx3. Gene ablation and gain of function experiments defined multiple roles for these proteins during organogenesis. Runx1/AML1 null phenotype results in early embryonic lethality of the homozygous mice, demonstrating Runx1 requirement for definitive hematopoiesis, while Runx2 knockout results in total absence of mineralized bone. More recently, abrogation of the Runx3 gene demonstrated its critical role in the central nervous system development, in addition to its anti-oncogene function in the gastrointestinal tract. We investigated the expression patterns of Runx3 transcripts in mouse embryo mRNA and during chondrocyte differentiation using mouse embryonic limb bud-derived cells cultured in micromass. The function of Runx3 in mediating chondrogenesis was also assessed through our gain of function studies.

METHODS.
Animals: CD-1; ICR mice were used for our gene expression studies for both whole embryo and limb buds. (Charles River Laboratories Wilmington, MA). Embryos were harvested daily from timed pregnant mothers. Whole embryos were removed from the mother and prepared for RNA extraction. Adult mouse tissues were also isolated from 6 weeks old C57B6 mice.

Limb bud cell isolation: Forelimbs were dissected from E11.5 and placed in cold Puck’s Saline with glucose. The cells were pelleted and 0.5 mls of Puck’s Saline A containing 10% chick serum with 0.05 mls of Dispase (10U/ml) was added. Cells were digested for 3.5 hours at 37 degrees. Cells were resuspended in 40% DMEM/60% F-12/10%FBS and passed through a 20um Nitex nylon mesh filter to remove the ectoderm and cell clumps to generate single cells. 100,000 cells/10ul were plated in 12 well plates and incubated for 1 hour at 37 degrees to allow adherence. 2mls of fresh media was added to each well and cells were cultured and treated over a time course to collect RNA.

Adenoviral infection: Runx3 cDNA was cloned into the pTOIlox viral vector. Sf1 I linearized pTOIlox Runx3 or lacZ control plasmid was cotransfected into 292 Cre cells along with phiC31 helper virus. Infected cells were collected and a freeze thaw lysate was prepared. 1ml of lysate was used to infect 293 T cells to amplify adenovirus. A concentration of 9.5 x 10^6 particles per ml and particles per ml respectively were obtained. C3H10T1/2 cells were infected with either Runx3 adenovirus or lacZ adenovirus at a concentration of 2 x 10^5 particles per ml in a total of 5 milliliters of serum free media for 4 hours. 5 ml of growth media was added and cells were cultured for 48 hours and then RNA was collected for Real Time RT-PCR.

RNA extraction and Real-time RT-PCR: Total RNA was extracted from embryo cell cultures using the RNAeasy kit (Qiagen, Valencia, CA) following the manufacturer’s recommendations. Freshly reverse transcribed cDNA was used for Real-time PCR using the fluorescent dye SYBR Green I to monitor DNA synthesis (SYBR Green PCR Master Mix, Applied Biosystems) using specific primers designed for specific Runx components. Runx3 transcripts were highly expressed in RNA extracted from various mouse skeletal elements, including the vertebrae, the rib cage or the pharyx appendicular. Runx3 transcripts are up-regulated throughout chondrocyte differentiation along with alkaline phosphatase and osteocalcin, and are up-regulated by BMP-2 and down-regulated by TGF-β. Thus our results suggest a role for Runx3 in mediating stage-specific chondrocyte maturation. We assessed Runx3 function in mediating chondrocyte terminal differentiation using viral over expression in C3H10T1/2 cells which do not express significant endogenous levels of Runx3. Runx3 induced the expression of both early and late chondrocyte differentiation in these mesenchymal stem cells indicating that Runx3 plays a role in both early and late stages of chondrogenesis.

DISCUSSION. Overlapping expression between Runx factors in immature and mature cartilage raises the possibility that normal development of the initial skeleton in absence of Runx2, may be accounted for by other Runx family members. We have previously shown that Runx1 mediates the onset of chondrogenesis while others showed that Runx2 and Runx3 expression overlaps in prehypertrophic and hypertrophic chondrocytes suggesting a redundant or cooperative function between both factors in mediating chondrogenesis. The dual role of Runx2 and Runx3 in cartilage was also demonstrated in vivo through generation of Runx2 and Runx3 double mutant mice in which simultaneous loss of Runx2 and Runx3 functions induced a dramatic delay in cartilage formation in axial and appendicular skeleton. This reduction in cartilage maturation is attributed to the arrest of chondrocyte differentiation prior to hypertrophy. In our study we investigated the stage specific role of Runx3 in mediating chondrogenesis. Here we demonstrate that while Runx3 expression and regulation by BMP-2 and TGF-β is similar to that previously reported for Runx2, the function of Runx3 during chondrocyte maturation is not restricted to the later stages of hypertrophy and mineralization. Our study brings a novel insight into the overlapping activity between Runx2 and Runx3 in cartilage.