The Dlx3 HOMEODOMAIN PROTEIN INDUCES RUNX2 EXPRESSION AND TARGET GENES TO PROMOTE OSTEObLAST DIFFERENTIATION

*Hassan, M Q; *Tare, R; *Karlin, J; *Javed, A; **Morasso, M; *van Wijnen, A; *Stein, J L; *Stein, G S; +*Lian, J B
*Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, MA
Mohammed.Hassan@umassmed.edu; Jane.Lian@umassmed.edu

INTRODUCTION:
Runx2/Cbfal is an essential transcription factor, for bone formation in the developing embryo, yet osteoblast lineage-specific activators of the Runx2 are unknown. Runx2 gene expression is observed in vivo in mesenchyme forming skeletal elements several days prior to the bone formation and induction of osteoblast genes. BMP2, an osteogenic factor during embryogenesis, induces Runx2 in non-osseous cell lines and mesenchymal stromal cells. As very few BMP target genes are known, we carried out a gene microarray profiling study (Affymetrix Mu11) using the C2C12 cell model of BMP2 induced osteoblast differentiation (1). Our experimental design focused on transcriptional regulators that would influence Runx2 expression. Several homeodomain proteins (e.g., Msx2, Dlx2, Dlx3, Dlx5) are upregulated, coincident with Runx2 induction and continued expression. Dlx3 null mice die during embryogenesis from placental failure (2). Therefore, its role in skeletal development has not been identified. Here we report characterization of the homeodomain protein Dlx3 as a strong activator of Runx2 and Runx2 target genes, and provide evidence for its role in promoting osteoprogenitor cell differentiation.

METHODS:
We used the mouse myogenic C2C12 cells (control and treated with 300ng/ml BMP2), the calvarial preosteoblast MC3T3E1 cell line and primary fetal rat calvarial osteoblasts cultured in osteogenic media containing ascorbic acid and β-glycerolphosphate to induce differentiation. RNA and nuclear extracts were prepared at stages of osteoblast maturation (days 3, 7, 14, 21) to characterize mRNA levels of homeodomain proteins by northern analysis and real-time PCR and protein levels by western blot analyses using specific antibodies. Functional assays were performed using the Runx2 and osteocalcin promoter reporter genes co-transfected into MC3T3E1 cells with plasmids expressing the HD proteins, Msx2 or Dlx3. RNA interference (RNAi) of Dlx3 was carried out with different sets of siRNA duplexes and endogenous Dlx3 knockdown and osteoblast phenotypic genes were analyzing by real-time PCR using the Syber Green dye detection method. Chromatin immunoprecipitation assays (method as described in (3)) were performed to assess the association of specific HD proteins with the Runx2 and OC genes at different stages of osteoblast differentiation.

RESULTS:
We confirmed in an independent experiment the temporal expression of four homeodomain proteins induced by BMP2 during osteoblast induction of C2C12 cells. We find Msx2, Dlx2 and Dlx3 are activated with Runx2 within 1-2 hrs after BMP2 treatment. Although Msx2 becomes downregulated by 4 hrs, Dlx3 expression is retained and Dlx5 appears highly upregulated after 12 hrs. A similar reciprocal pattern of Msx2 and Dlx3 was observed during the early stages of osteoblast differentiation in the two calvarial cell models. Increased Dlx3 expression at confluency coincided with upregulation of Runx2. By situ hybridization with a Dlx3 RNA probe, we identified Dlx3 expression in the periosteum and primary spongiosa of developing limbs in mouse (E16.5). Thus both in vivo and in vitro, Dlx3 is expressed in early osteogenic lineage cells and osteoblasts.

The proximal Runx2 gene contains several homeodomain consensus motifs. Because we have shown that the Runx2 proximal promoter is expressed in transgenic mice in prechondrogenic mesenchyme and does not contain a Smad response element (4,5), we examined Runx2 promoter activity regulated by candidate homeodomain proteins. Using functional promoter assays, we find negative regulation of Runx2 by Msx2, a well known repressor protein, but positive regulation by the Dlx3 and Dlx5 proteins. Activation of the endogenous Runx2 gene was established by expression studies. Overexpression of Dlx3 in the osteoprogenitor cell line MC3T3-E1 induced Runx2 expression from 3-5 fold. Furthermore, RNAi knockdown of Dlx3 (by 70%) almost completely inhibited Runx2 expression. These consequences of Dlx3 regulation of Runx2 gene expression were translated to Runx2 target genes (osteocalcin (OC), osteopontin and BSP). Thus Dlx3 activation of Runx2 promotes osteoblast differentiation, while RNAi of Dlx3 inhibits.

To further address the biological significance of Dlx3 and Runx2 combinatorial control of gene expression and bone formation, we examined the association of Msx2, Dlx3 and Runx2 with the osteocalcin gene at different stages of osteoblast maturation. The OC gene has a well defined single HD response element in which Msx2 replaces Dlx3 protein during proliferation, while Runx2 activates OC post-proliferatively. Harvesting primary calvarial cells, every 3 days from growth to mineralization, we find a molecular switch in association of Msx2 on the promoter when the gene is not expressed. At the point when transcription is initiated, Msx2 is replaced by Dlx3 and Runx2. In the later mineralization stage, a second molecular switch is observed when Dlx3 is replaced by Dlx5 consistent with high levels of Dlx5 expression at this stage of osteoblast differentiation. These results indicate Msx2 repression of the Runx2 and OC genes is relieved by Dlx3 for Runx2 activation Runx2 activation of OC, followed by Dlx5 recruitment to gene promoters at later stages of osteoblast maturation for further increased expression of Runx2 and OC. Therefore, our data suggest that in response to BMP2, HD proteins constitute a regulatory network which mediates development of the bone phenotype through the sequential association of distinct HD proteins with gene promoters.

DISCUSSION:
Multiple signaling pathways support skeletal development and bone formation HD proteins have been identified as key downstream targets of BMP2 induced osteogenesis through in vivo genetic approaches and by microarray gene profiling of several model systems. In these studies we identified a functional role for the homeodomain protein Dlx3 in promoting osteoblast differentiation by mediating the induction of Runx2 and their target genes. Secondly, our molecular data using the Runx2 and osteocalcin gene models suggest that during osteoblast differentiation, HD proteins appear to function in a regulatory network throughout the stages of osteoblast differentiation to control physiologic levels of target genes.

REFERENCES:

AFFILIATED INSTITUTIONS FOR CO-AUTHORS
+ Developmental Skin Biology Unit, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland

51st Annual Meeting of the Orthopaedic Research Society
Paper No: 0057