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TRANSCRIPTIONAL REGULATION OF OSTEOBLAST AND CHONDROCYTE DIFFERENTIATION: TEMPORAL RECRUITMENT OF HOMEODOMAIN PROTEINS TO CHROMATIN OF THE RUNX2 GENE

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ABSTRACT

Runx2, a transcription factor essential for osteoblast maturation, is also expressed in embryonic pre-cartilaginous mesenchymal condensations. However, regulatory controls for expression of Runx2 during skeletal formation are unknown. We hypothesize that Runx2 is regulated by both activators and repressors during the transition from mesenchyme to cartilage and bone. Our results demonstrate that Runx2 is a transcriptional target of Nkx3.2, a homeodomain regulatory factor for chondrogenesis. Runx2 repression by Nkx3.2 is necessary for activation of a chondrocyte-specific program of gene expression. Gene regulation in relation to several Homeodomain (HD) proteins during chondrocyte/osteoblast differentiation using Chromatin Immunoprecipitation assays was characterized in these studies. We find that multiple HD proteins constitute a regulatory network that mediates development through sequential association of HD proteins with promoter regulatory elements.

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1. INTRODUCTION

1.1 Skeletogenesis

Skeletogenesis is the complex developmental process by which the embryo of an organism organizes skeleton formation. The process requires coordinated differentiation of a variety of cell types, each of which play a crucial role in terms of the skeletal elements and their development. The axial skeleton is prefigured via somatic mesenchymal condensations that migrate to specific spatial locations within the embryo. The direction of migration is determined through soluble factors that are secreted by the notochord. Some of the cells commit to the osteoblast lineage and begin forming bone through a process known as *intramembraneous ossification*, which can be seen in the calvarium of developing embryos. Other components of the mesenchymal condensations take a longer route to bone formation by undergoing chondrogenesis and creating a cartilaginous framework for future bone. This cartilaginous tissue is often invaded by vasculature and replaced by bone in a process known as *endochondral ossification*. The activity of the chondrocytes in this process is found to be operating around embryonic day 13 and the osteogenic component around day 15.

1.2 Chondrogenesis

The process of chondrogenesis is initiated in unsegmented presomitic mesoderm. These cells receive soluble chemical signals that originate in the notochord and are bound to surface receptors. These receptors initiate a signal transduction cascade that activate and repress transcription of genes that are related to skeletogenesis. It has been shown that one of the notochord-derived factors, sonic hedgehog (Shh), induces the expression of a variety of transcription factors including Sox9 (a master regulator of chondrogenesis) and Nkx3.2 (Zeng et al., 2002; Lettice et al., 2001) (see figure 1.1). Nkx3.2 is a homeotic protein that belongs to

the NK gene family. The homolog of Nkx3.2 in *Drosophila*, *bagpipe* also acts on mesodermal tissue by controlling its differentiation. Shh signaling has been shown to be crucial in chondrogenesis by rendering exposed mesoderm cells competent for becoming chondrocytes. The chondrocytes require further signaling from bone morphogenetic proteins (BMPs), which belong to the transforming growth factor β (TGF- β) superfamily. The TGF-β superfamily consists of multifunctional paracrine factors that regulate cell proliferation, differentiation and death in a variety of tissues (Katagiri and Takhashi, 2002).

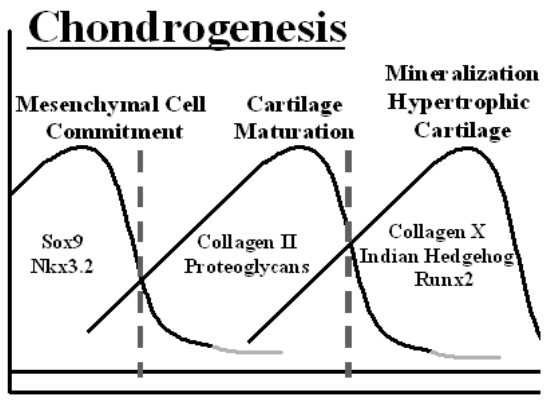

Figure 1.1 Stages of chondrogenesis and the charactiistic up regulation of phenotypic marker genese

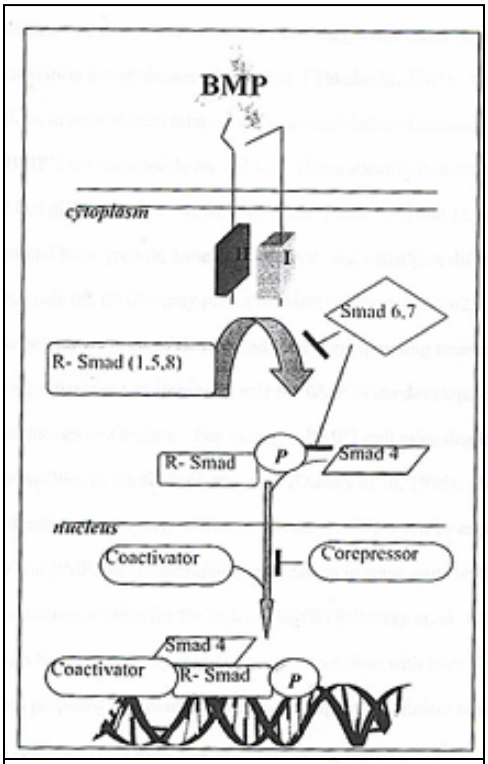

Figure 1.2 A schematic model for BMP signal transduction (Karlin 2004)

TGF-β and BMPs are homologous in amino acid sequence and are functionally similar since they both activate heterodimeric kinase receptors. They are secreted into the extracellular matrix (ECM) by osteoblast cells and initiate signaling by binding dimeric receptor complexes comprised of type I and type II serine/threonine kinase receptor combinations (Koenig et al., 1994; Liu et al., 1995). When there is binding of BMPs, the activated receptors initiate phosphorylation of the intracellular Smad proteins. Once phosphorylated, the Smads form dimers with Smad4 in the cytoplasm. This dimer can then transport the BMP signal to the nucleus where tran-

scriptional complexes can be formed (see figure 1.2). These transcriptional complexes have a variety of functions and operate by activating or repressing transcription of a number of target genes (Derynck et al., 1998). BMPs are the only growth factors that can induce ectopic bone formation in vertebrates (Katagiri and Takahashi, 2002).

According to Zeng et al., 2002, Sox-9 and Nkx3.2 are in a positive autoregulatory loop that is maintained through BMP signaling (see figure 1.3). The maintenance of this loop is necessary for constitutive expression of Sox-9, which in turn activate transcription of a number of phenotypically chondrogenic genes such as collagen type II, aggrecan, etc. In addition, Nkx3.2 knockout mice show reduced Sox9 expression and loss of Runx2 in the axial skeleton (Tribioli et al., 1997). During endochondral ossification, the chondrocytes undergo further transformation to allow ossification to occur. The mesenchymal tissue enters hypertrophy where the cells expand and begin producing collagen type X; this is also believed to signal vascular invasion. Finally the ECM is mineralized and absorbed by osteoclasts and replaced with bone matrix by osteoblasts.

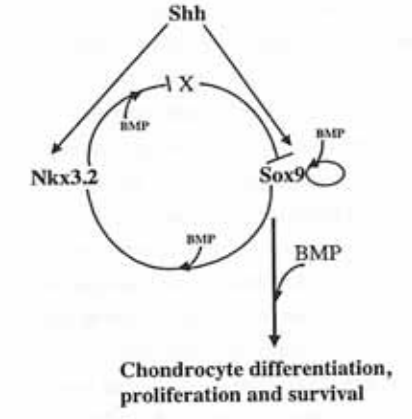

Figure 1.3 Positive autoregulatory loop between Nkx3.2 and Sox-9 that is maintained through the presence of BMP-2 (Zeng et al., 2002)

1.3 Osteogenesis

Osteoblasts, unlike chondrocytes, arrive at the site of differentiation via vascular invasion of the mesenchymal tissue. The osteoprogenitor cells undergo differentiation after receiving a BMP signal. While mesenchymal cells require Shh to become competent for chondrogenesis, osteoprogenitor cells do not. Osteoblast differentiation can be divided into three stages, proliferation, maturation and mineralization (Lian et al., 2004, see figure 1.4). The

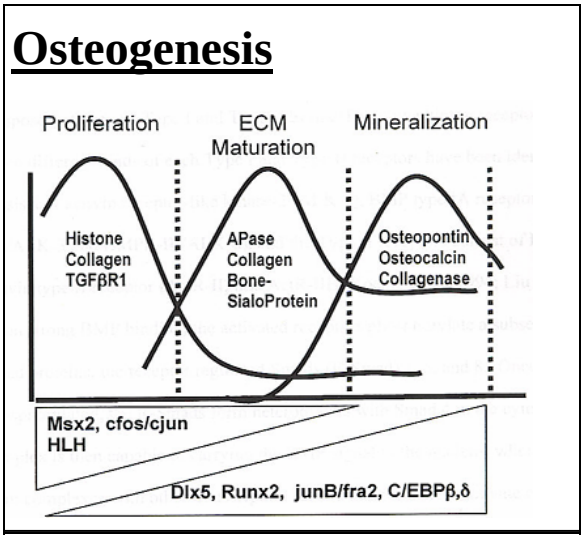

Figure 1.4 Schematic model of the major stages of osteoblast differentiation recognizable in in vitro and in vivo models beginning with the morphologically distinct osteoprogenitor near the bone-forming surface. Frequently used phenotypic markers for these osteoblast stages are indicated (Lian et al., 2004)

course of the process begins with an osteoprogenitor cell and ends with a mature osteocyte during which phenotypic genes are temporally expressed or repressed due to signaling from regulatory proteins (i.e. BMPs) and transcription factors.

Once a BMP signal is present, the transcript levels of the transcription factor Runx2 sharply increase. Runx2 has been shown to be a master regulator of osteoblast differentiation. Runx2 belongs to the Runx transcription factor family that plays dramatically critical roles in cell fate determination in the developing embryo. The Runx family share a DNA binding domain, called the Runt homology domain, which is highly conserved across species. This domain was characterized in *Drosophila* and found to be involved in developmental body patterning and segmentation (Nusslein-Volhard and Wieschaus, 1980).

The three Runx family members provide a variety of roles within the developing mammal. Runx1 is also called AML-1 for Acute Myeloid Leukemia as well as CBFα-2 for Core Binding Factor. Runx2 is also known as AML-3 and CBFα-1 and Runx3 is AML-2 and CBFα-3. According to Levanon et al., 1994, there is a very high degree of homology within the runt domain, as one would expect; however there is also similarity within the non-DNA binding residues (around 50%).

1.4 Runx2

Runx2 knockout mice die perinatally with a complete absence of mineralized bone

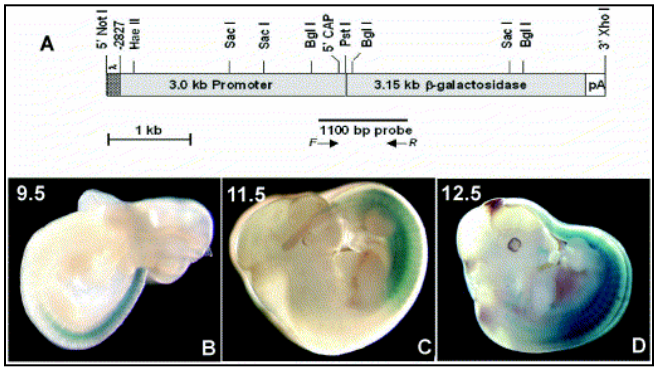

Figure 1.5 Diagram of the bone-related *Runx2* promoter construct used to generate transgenic mice. A *Lac Z/ Poly-A* cassette was cloned into the *Pst*I site in the 5□Œ untranslated region of a *Runx2* Type II genomic clone (Drissi et al., 2000). (A). (B–D) Whole-mount X-gal staining was performed on transgenic embryos from 9.5 (B), 11.5 (C), and 12.5 dpc (D) with fixation times increasing with age. Three-kilobase *Runx2* promoter activity progresses from the caudal somites (B) into developing sclerotomal mesenchyme (C,D) prior to the onset of chondrogenesis. (Lengner et al., 2002)

(Ducy et al., 1997, Komori et al. 1997, Otto et al. 1997). Therefore Runx2 is necessary for osteoblast differentiation and skeletogenesis (see figure 1.5). Studies have demonstrated that Runx2 is expressed within osteoprogenitor cells and the amount increases throughout osteoblast differentiation (Lian and Stein, 2003). As one can see from the prior references, there was a great deal of novel findings in 1997. A brief summary of these results is necessary for understanding the current research on Runx2. Runx2 was identified as a key transcription factor within the context of osteoblasts isolated from rat primary cells as well as in osteoblastic cell lines (Banerjee et al., 1997). A number of phenotypic genes are up regulated by the transcription factor Runx2 including osteocalcin, α1-collagen, osteopontin and bone sialoprotein (Ducy et al., 1997). Taken together this indicates that Runx2 is a master regulator of osteoblast differentiation.

Within the context of in vivo studies, Komori et al. and Otto et al. demonstrated the necessity of Runx2 for development and bone formation. The homozygous knockout died shortly after birth and lacked mature osteoblasts to mineralize the tissue. Interestingly, the heterozygous mice displayed a phenotype similar to cleidocranial dysplasia an autosomal dominant disease that is typified by absence of a clavicle and defects in cranial fontanelles closure (Komori et al., 1997, Otto et al., 1997)

Runx2's role in chondrogenic differentiation is apparent in the homozygous knockout mice, which displayed a lack hypertrophic chondrocytes (Kim et al., 1999). Additionally Runx2 RNA transcript was detected in prehypertrophic and hypertrophic chondrocytes by Enomoto et al. suggesting that it does in fact play a role in the maturation of the ECM and subsequent steps in osteogenesis (Enomoto et al., 2000). In the same manuscript it was shown that Runx2 antisense RNA prevented ATCD5 cells, a mouse chondrogenic cell line, from becoming hypertrophic while over expression of Runx2 increased hypertrophy.

In vitro the regulation of Runx proteins is complex due to compensatory effects between family members. Meyers et al. determined that the Runx proteins bind the same DNA motif leading one to believe that their role transcriptionally is a function of what other proteins are present (Meyers et al. 1996). The context of the cellular environment plays a significant role in the transcriptional properties of the Runx promoter and its regulation.

The Runx2 promoter contains two N-terminal isoforms, PEBP2αA1 (Type I) and *til-1* (Type II) (Geoffroy et al. 1998; Ogawa et al., 1993; Stewart et al., 1997, see figure 1.6). Type I is a 513-residue protein that initiates at exon 2 and has a downstream P2 promoter. The Type II isoform is 528 amino acid protein that is initiated at exon 1. The Type II isoform is regulated through an upstream P1 promoter and there appears to be no difference between the isoforms except their regulation through their respective promoters. It has been shown that both isoforms are capable

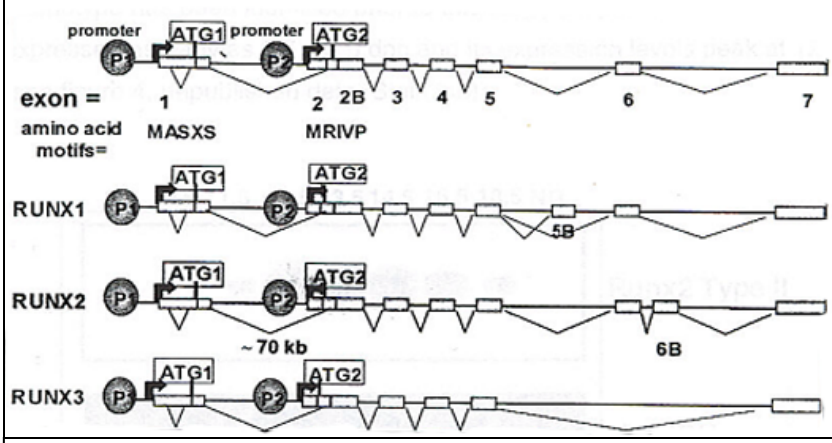

Figure 1.6 Genomic Organization of Runt-related Genes (Karlin, 2005)

of inducing osteoblast differentiation (Harada et al., 1999). There is constitutive expression of the Type I isoform in osteoprogenitor cells and nonosseous mesenchymal tissue (Banerjee et al., 2001). The Type II isoform is highly regulated and interacts with a variety of signaling molecules and proteins. BMPs and Shh have both been shown to regulate the Type II isoform during development (Banerjee et al., 2001; Yamaguchi et al., 2000).

The expression of Runx2 is tightly regulated during development, in regards to osteoblast differentiation and chondrocytes maturation. At day 12.5 in embryonic development Runx2 levels have been shown to peak which correlates with the Runx2 deficient phenotype being displayed around day 14.5 post coitum (unpublished data, Stein lab, see figure 1.7). The Type II isoform's expression during development was studied when a *lac-Z* reporter gene was fused to the Runx2-P1 promoter in a transgenic mouse (see figure 1.8). There was expression in the claudal somatic tissue as early as day 8.5. During days 9.5 through 11.5 Runx2 was present in the developing sclerotome. By day 12.5 the expression was present in the mesenchymal tissue and the prechondrocytic sclerotome (Lengner et al., 2002).

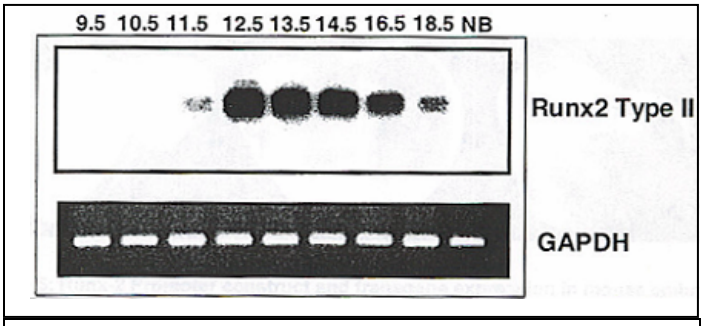

Figure 1.7 Expression of the Runx2 Type II isoform during mouse embryonic development (RT-PCR). (Stein lab, unpublished)

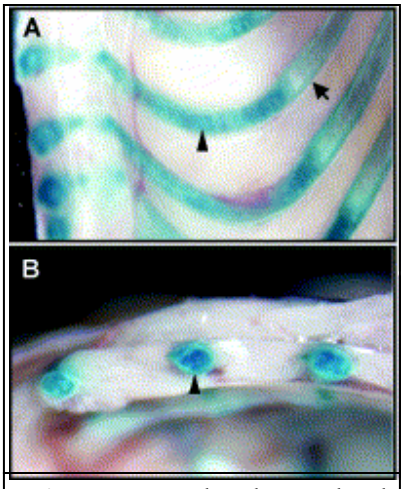

Figure 1.8 The bone-related *Runx2* promoter is active in mature chondrocytes of newborn transgenic mice. The *Runx2* promoter transgene is highly expressed in the cartilaginous portion of the rib at birth (arrowheads), but not expressed in the osseous portion of the rib (arrow). (A) Transverse section through the cartilaginous portion of the rib cage. (B) (Lengner et al., 2002)

The characterization of protein-protein interactions is not novel in terms of Runx2 transcriptional and post-translational

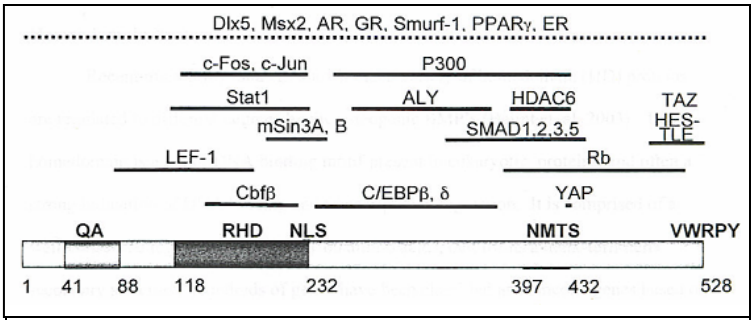

Figure 1.9 Some known Runx2 coregulatory proteins illustrated by labeled black bars positioned above the corresponding region of the Runx2 amino acid sequence with which they interact. The Runx2 protein is pictured here from N-terminus to C-terminus fashion with labeled domains referring to Glutamic Acid/Alanine (QA), Runt homology domain (RHD), Nuclear localization signal (NLS), Nuclear matrix targeting signal (NMTS), Valine/ Tryptophan/Arginine/Proline?Tyrosine (VWRPY) (Lian et al.,2004)

regulation (see figure 1.9), for instance Runx2-Smad heterodimers enhance Runx2 transcriptional regulation (Zaidi et al., 2002). In addition, Runx2 recruits Smads to nuclear domains in order to regulate Runx2 dependent genes that are osteogenic. Therefore the BMP-Smad signal is intrinsically integrated into the control of osteogenic gene expression.

It is not likely that there is a small set of factors that control differentiation, history has shown there to be many transcription factors for many stages, hinting at the complexity of life. Runx2 and Smads are only two small pieces in a myriad of osteoblastic events that are interacting with an indefinite amount of unspecified proteins.

1.5 Homeodomain Proteins

Homeodomain (HD) proteins contain a highly conserved region of amino acids in a sequence that binds DNA. A homeotic protein is one that has approximately 60 amino acid residues (see fig-

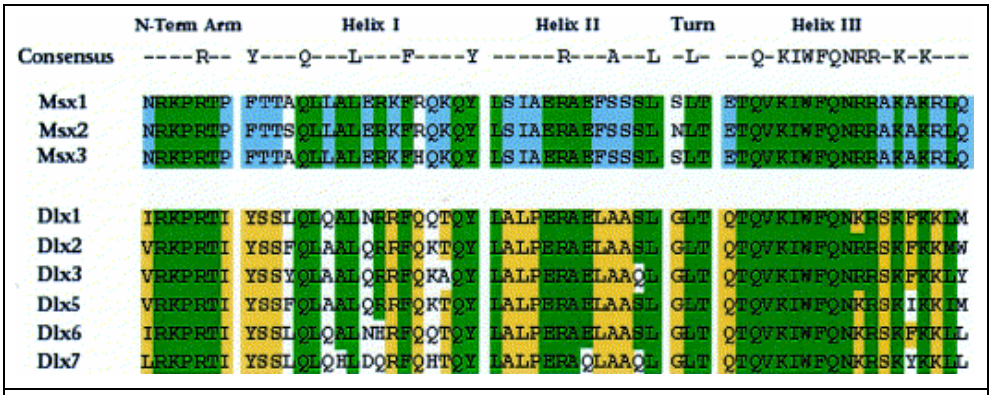

Figure 1.10 Msx and Dlx genes encode closely related homeodomains. Comparison of the murine Msx and Dlx homeodomains showing residues shared between all members (green), Msx-specific residues (blue), and Dlx-specific residues (yellow). The homeodomain consensus sequence is shown. (Bendall and Abate-Shen, 2000)

ure 1.10) that are commonly conserved between species as diverse as fruit flies and humans. The homeotic domain of these proteins binds DNA at precise sites in the promoter region. At these sites, the protein may recruit other proteins that perform transcriptional activation or repression events. These other proteins are called response elements and are part of the transcriptional complex. HD proteins are often tissue-specific and highly regulated during development and differentiation.

The conserved region of the protein takes on a characteristic structure that is directly related to its function. The HD usually has three helixes with flexible turns in between. The third helix is the primary DNA binding component that fits in the major groove of the DNA and contacts the phosphate backbone and the bases that lie within the groove. The N-terminal helix fits in the minor groove and makes other additional contacts. Transcription factors are composed of several components or modules and each has a particular role in gene regulation. The HD is just one of these elements and other examples include zinc fingers and leucine zippers.

The purpose of homeotic proteins is to bind DNA and form a protein complex in the proximity of the transcriptional start site. In this way, the transcription of the target gene is indirectly regulated by the interactions of proteins with the DNA and between recruited proteins. The genes that are controlled by HD proteins often code for other transcription factors that begin a cascade of events leading to cell differentiation. The loading and unloading of these complexes is a subject of interest in cell differentiation since the transformation of one cell type into another has many applications and implications. The growth of tissues for the replacement of damaged ones is the basis of tissue engineering and the deregulation of the cell cycle is the cause of cancer.

HD proteins have played a role in evolution and remained conserved over thousands of

years. Due to slight mutations in the sequence and structure, families of these proteins exist and redundancy in function is apparent in some cases. A particular family usually shares the same HD sequence but the functions and target tissues differ. Controlling the differentiation of cells and analyzing the chromatin is useful for characterizing the sequence of events necessary for development of particular cells and tissues.

Recent microarray studies has shown that an assortment of HD proteins are regulated by BMPs which suggests that the BMP signaling that affects Runx2 transcription may also be regulated through HD proteins. There are two classes of homeotic genes, *distaless* (Dlx) and *meshless* (Msx) that is expressed to a significant degree in mesenchymal stem cells. They have been found to be essential during cranio-facial, tooth, limb, and brain development (see figure 1.11) due to mutation and phenotype analysis in trans-genic mice. Through regulating the activity of bone morphogenetic genes, Msx and Dlx play a critical role in osteoblast differentiation and embryogenesis. The bind-ing motif of HD proteins is redundant leading one to be-lieve that protein-protein interactions with transcription factors are responsible for their regulatory activity. (Beanan and Sargent, 2000; Bendall and Abate-Shen, 2000).

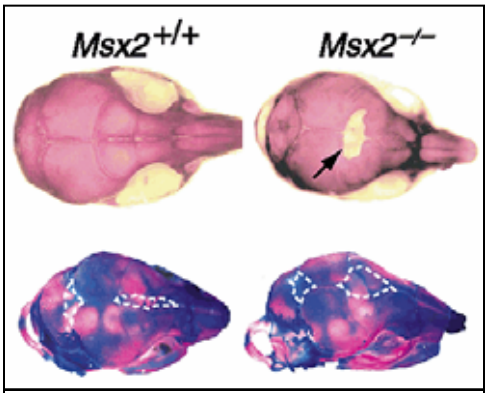

Figure 1.11 Adult *Msx2* mutants exhibit defective frontal bone ossification, re-sulting in a large foramen (arrow). At E18.5, an enlarged frontal foramen (anterior dotted line) is present in the mutant, indicating that frontal bone ossi-fication is already delayed at this stage. In contrast, the parietal foramen (posterior dotted line) is similarly sized in wild type and mutant. (Satokata et al., 2000)

Mutational analysis of Msx2, Dlx3 and Dlx5 has indicated that they lead opposing roles in embryogenesis. A mutation from proline to histidine at residue 148 in Msx2 enhances its DNA binding affinity. This causes Boston-type craniosynostosis by inducing apoptosis of the

neural crest. These cells would normally form the craniofacial features of the organism (Alappat et al., 2003). A separate mutation within Msx2 (505_508dupATTG) induces the phenotype of parietal foramina with cleidocranial dysplasia (PFMCCD), a condition similar but separate from the CCD caused by haplo-insufficiency of Runx2 (Garcia-Minaur et al., 2003). In addition, a four base pair deletion within the Dlx3 gene has resulted in the autosomal dominant condition tricho-dento-osseous (TDO) syndrome (see figure 1.12). According to Price et al., TDO is the consequence of the inability to transcribe bone specific genes and results in craniofacial abnormalities (Price et al., 1998). Dlx5 null mice exhibit a mild delay in ossification of long bones, but there is no effect on expression of Runx2. However, there is a severe phenotype with Dlx5/Dlx6 double null mice (Depew et al., 2002). Msx genes are transcriptional repressors that prevent osteoblast maturation in skull and tooth osteoblasts. Conversely, Dlx genes are transcriptional activators that are required for normal osteoblast maturation.

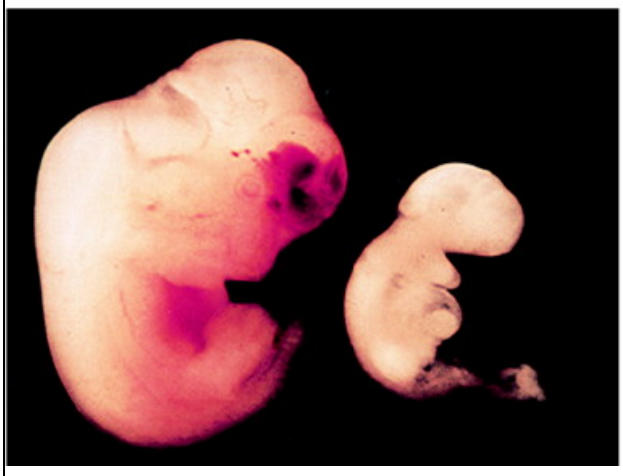

Figure 1.12 Phenotype of wild type (left) and regressed *Dlx3* / (right) E12.5 embryos genotyped by Southern blotting. (Morasso et al., 1999)

HD proteins are comprised of multiple protein and/or DNA binding modules that can bind mutually exclusively. HD motifs have been found in a variety of gene's promoters including osteogenic and chondrogenic canonical markers such as Runx2 and collagen type I (Ryoo et al., 1997). In addition heterodimers between Msx2 and Runx2 as well as Dlx3 and Runx2 have recently been discovered (Hassan et al., 2004).

2. MATERIALS AND METHODS

Cell Culture and Transient Transfection

C3H10T1/2 cells were maintained in Dulbecco's Modified Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, GA). Transient transfections were performed in 6-well plates at 75% confluence using 5 µl of FuGENE6 transfection reagent (Roche, Indianapolis, IN) and 4 µg total DNA per well in accordance with the manufacturers protocol. For Nkx3.2 expression, 100 ng of an Nkx3.2 expression vector in a PCS2 plasmid backbone (a kind gift from Dr. Andrew Lassar at Harvard Medical School) was transfected into each well unless otherwise noted. As a control, 100 ng PCS2 expression vector (empty vector) was transfected into each well. In order to observe repression of endogenous Runx2 protein in the presence of Nkx3.2, C3H10T1/2 cells were co-transfected with either Nkx3.2 with CMV driven enhanced green fluorescence protein (EGFP), or empty vector (PCS2) and EGFP. After 24 hours, cells were trypsinized and FACs sorted to collect cells positive for EGFP fluorescence. EGFP positive cells were replated and harvested 12 hours later for western analysis. To monitor transfection efficiency, transfections included 0.5 µg of a CMV driven *LacZ* expression vector per well.

Chromatin Immunoprecipitation Assays (ChIP)

To cross-link proteins to DNA, C3H10T1/2 cells were incubated for 10 minutes at room temperature in 1X PBS (3 ml/plate) containing 1% formaldehyde, 25 uM MG-132 (Calbiochem/Sigma), and 1X protease inhibitor (Roche Molecular Biochemicals, Indianapolis, IN). A final concentration of 0.125 M glycine was added to the 1% formaldehyde-PBS solution for neutralization. Cells were collected in PBS after plates were washed twice with ice cold PBS. The cells were then lysed in lysis buffer containing 25 mM HEPES/NaOH (pH

7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 25 μM MG-132 and 1X complete protease inhibitor. The cells were pelleted and resuspended in 300 μl (300 μl/100 mm plate) sonication buffer (50 μM HEPES/NaOH (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton x-100, 0.1% Na-deoxycholate, 0.1% SDS, 25 μM MG132, 1X complete protease inhibitor). Samples were sonicated to shear the DNA into 0.2-0.6 kb fragments. Cellular debris was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C and resulting chromatin-containing solutions were distributed into multiple 1 ml aliquots that were used as the starting material of all subsequent steps.

Chromatin aliquots were precleared with 100 μl of a 25% (v/v) suspension of 2 μg single stranded DNA coated protein A/G and 1 mg/ml BSA. Samples were used directly for immunoprecipitation reaction with 2 μg of α-HA epitope, α-Dlx3 (affinity purified), α-Dlx5 (Y-20) α-Msx2 (H-70) (Covance Inc.), α-Hox a10 (N-20), or α-Runx2 (M-70, Santa Cruz Biotechnology, Santa Cruz, CA) antibody and normal rabbit/mouse IgG as a control. Chromatin immunoprecipitation reactions were allowed to proceed for 2-4 hours at 4°C on a rotating wheel. Immune-complexes were mixed with 100 μl of 25% (v/v) pre-coated protein A/G agarose suspension followed by incubation for 1 h at 4°C on a rotating wheel. Beads were collected by brief centrifugation and the immunocomplexes were eluted twice by adding 150 μl of freshly prepared elution buffer (100 mM $NaHCO_3$, 1% SDS). After reversal of crosslinks at 68°C overnight, the eluate was treated with 100 μg/ml proteinase K followed by phenol-chloroform extraction and ethanol precipitation using 5 μg glycogen as carrier. An aliquot (2-3 μl) of each sample was assayed using quantitative PCR for the presence of specific DNA fragments using primers in the Runx2 P1 promoter. The proximal region where the Nkx3.2binding motif and Runx2 autoregulatory motif is located the primers are: Forward 5'-ctc cag taa tag tgc ttg caa aaa

at-3' and Reverse 5'-gcg aat gaa gca ttc aca caa-3'. The middle region of the promoter where multiple Hox a10 sites and a Runx2 consensus site are located the primers are: Forward 5'-gca ttt gtg ttc tag cca aat cc-3' and Reverse 5'-tgg cat tca gaa ggt tat agc ttt t-3'. The distal region of the promoter where the overlapping homeodomain and Hox a10 sites are located the primers are: Forward 5'-ttg ctc aga acg cca cac a-3' and Reverse 5'-cct tca tta tta tgt cta tgg aaa agt ga-3'. Quantitative real-time PCR was carried out using 2X SYBR Green mix (Eurogentec, Belgium) and a 2-stage cycling protocol (60^{o}C annealing and extension, 94^{o}C denaturation, 40 cycles). Amplicon specificity was verified by analysis of melting temperature. All data was collected during the linear phase of amplification.

Chondrogenic Induction of C3H10T1/2 Cells

Induction of chondrogenesis was carried out by plating C3H10T1/2 cells (between passages 19 and 25) in high-density micromass cultures (10^5 cells in a 10 µl drop of media) (Ahrens et al., 1977, Mello and Tuan, 1999, Lengner et al., 2004); followed by a three-hour incubation period in which the cells were allowed to adhere. Following adhesion, micromass cultures were fed with F12 media containing 5% Fetal Bovine Serum and 100 ng/mL recombinant hBMP-2 (kindly provided by Dr. John Wozney at Wyeth-Ayerst, MA). Cultures were harvested 24 hours after induction of chondrogenesis for analysis of gene expression**.**

Osteogenic Induction of Primary Bone Marrow Stem Cells

Induction of osteogenesis in 1^{o} bone marrow stem cells was carried out in MEM media containing 0.1% L-Glutamine, 0.1% Penicillin-Streptomycin, 10% Fetal Bovine Serum, and 100 ng/mL recombinant hBMP-2. At first confluency (day 3), cells were fed 250 µL ascorbic acid/100 mLs media. The second feeding (day 5) of cultures contained 500 µL ascorbic acid/100 mLs media, while the last feeding (day 7) contained 1 mL/100 mLs (10 mM) media of Beta-glycero-phosphate (B-GPO_4). Cells were harvested 48 hours after osteogenesis was ob-

served for gene expression analysis.

RNA Isolation and Analysis

RNA was isolated from cultures of C3H10T1/2 cells using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. After purification, 5 µg of total RNA was DNAse treated using a DNA-free RNA column purification kit (Zymo Research, Orange, CA). RNA (1 µg) was then reverse transcribed using Oligo-dT primers and the Superscript 1st Strand Synthesis kit (Invitrogen) according to the manufacturer's protocol. Gene expression was assessed by quantitative real-time PCR (Runx2, Type II Collagen, Osteocalcin, Sox9, Alkaline Phosphatase, Msx2, Dlx3, Dlx5, and Hox a10). Quantitative PCR was performed using Fam-conjugated Taqman probes and Taqman 2x Master Mix (Applied Biosciences, Foster City, CA) and a 2 step cycling protocol (anneal and elongate at 60°C, denature at 94°C). Specificity of primers was verified by dissociation of amplicons when using SYBR green as a detector. Primers used for PCR reactions are found in Table 2.1.

Table 2.1: PCR Primers

Primer Name/Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Runx2	cgg ccc tcc ctg aac tct	tgc ctg cct ggg atc tgt a
Nkx3.2	aga tgt cag cca gcg ttt c	agg cgt aac gct atc ct
Sox9	gag gcc acg gaa cag act ca	cag cgc ctt gaa gat agc att
Collagen II	ctg gaa tgt cct ctg cga	tga ggc agt ctg ggt ctt cac
Alkaline Phosphatase	ttg tgc cag aga aag aga gag a	gtt tca ggg cat ttt tca agg t
Osteocalcin	ctg aca aag cct tca tgt cca a	gcg ggc gag tct gtt cac ta
Msx2	caa gag gcg gaa ctg gaa aa	gaa gcc tga ggg cag cat ag
Dlx3	tat agg cag tac gga gcg tac c	tag atc gtt cgc ggc ttt c
Dlx5	acc tcg ccc tgc cag aac	ttt cac ctg tgt ttg cgt cag t
Hox a10	ttc ttt tgc gca gaa cat caa	cat ttg tcc gca gca tcg ta
GAPDH	Applied Biosystems #4308313	Applied Biosystems #4308313

Western Blotting

For the detection of Nkx3.2, Runx2, and Actin proteins, each well of a 6 well plate was lysed in 400 µl lysis buffer containing 2% SDS, 10 mM DTT, 10% glycerol, 12% urea, 10 mM

Tris/HCl (pH 7.5), 1 mM PMSF, 1X Protease inhibitor cocktail (Roche), 25 uM MG132 proteosome inhibitor, and boiled for 5 minutes. Proteins were then quantified using Bradford reagent (Pierce, Rockford, IL) and taking spectrophotometric readings at 590 nm. Concentrations were estimated against a standard curve generated against BSA.

Total protein (20 μg) was subjected to electrophoreses in a denaturing 10% polyacrylamide gel containing 10% SDS. Proteins were then transferred onto Immobilon-P membranes (Millipore, Billerica, MA) using a semi-dry transfer apparatus. Membranes were blocked in PBS-0.01% Tween-20 containing 2% nonfat powdered milk (Biorad, Hercules, CA). Proteins were detected by incubating with blocking solution. Antibodies used in this study are as follows: Nkx3.2, α -HA epitope mouse monoclonal antibody (Santa Cruz, Santa Cruz, CA); Runx2 mouse monoclonal antibody was a generous gift from Drs. Yoshi Ito and Kosei Ito, National University, Singapore; α -Actin goat polyclonal antibody. Primary antibodies were detected with goat a-mouse secondary antibody conjugated to HRP. Secondary antibodies were detected using Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA).

3.RESULTS

As can be seen in the Runx2 P1 promoter there are many DNA-protein interactions that are possible through a variety of consensus sequences found 600 kb upstream that play a role in regulating transcription (figure 3.1.). These interactions are context dependent within the cellular environment and are mediated through complexes of proteins that interact with the transcription factors and signaling molecules to activate or repress transcription. Competition between overlapping and nearby sites, and protein-protein recruitment are features of regulation

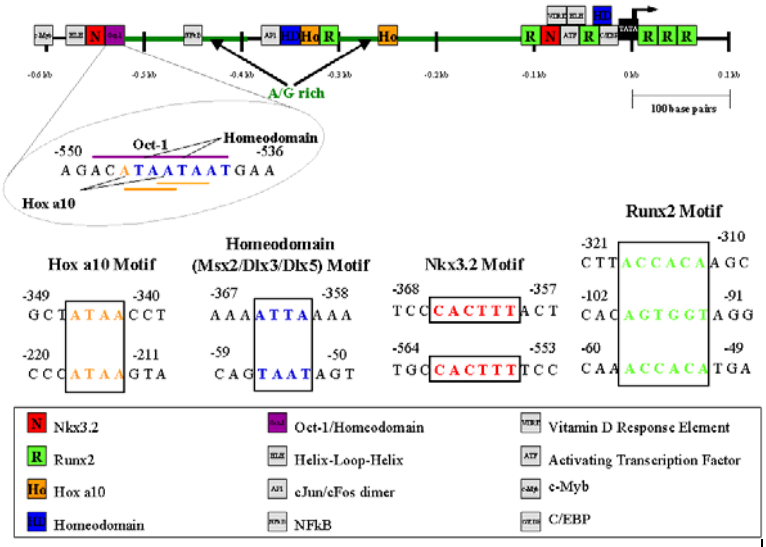

Figure 3.1 Homeodomain Protein Regulatory Elements in the Bone-Related Runx2 0.6 kb P1 Promoter

that are the determinant of what transcription factor binds when. Chromatin immunoprecipitation is a powerful technique that allows one to analyze the temporal loading of proteins that interact with DNA (figure 3.2). The DNA that is isolated is amplified and measured quantitatively using real

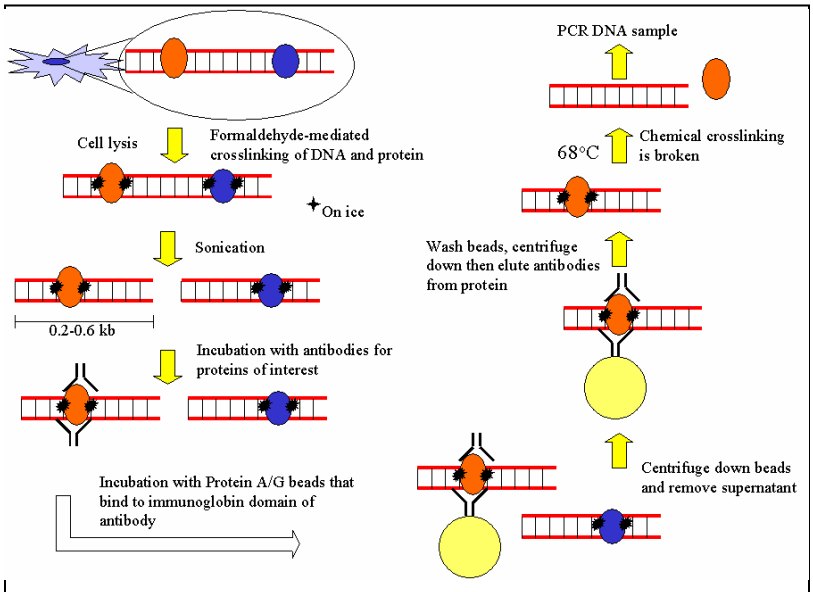

Figure 3.2 Schematic of Chromatin Immunoprecipitation

time-polymerase chain reaction. The amount of DNA that is pulled down is dependent on the protein-DNA interactions present in the cell. In this study we induced differentiation of murine primary bone marrow stem cells (BMSCs) and the murine mesenchymal cell line C3H10T1/2 to become osteoblasts and chondrocytes respectively. The primers used targeted specific regions of the Runx2 P1 promoter that contain regulatory boxes with a number of overlapping consensus sites (figure 3.3).

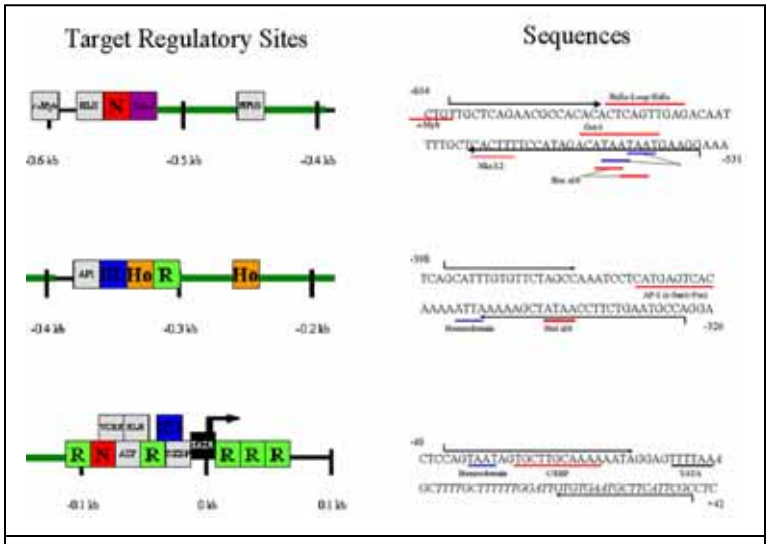

Figure 3.3 Schematic of Primer locations within the Runx2 P1 promoter and the regulatory sites that are targeted.

3.1 Nkx3.2 Repression of Runx 2

RNA was isolated from C3H10T1/2 cells induced to undergo chondrogenesis and reverse-transcribed. Gene expression was monitored over four days and using quantitative real-time PCR (see figure 3.4). Primers targeting specific cDNA sequences were used to determine relative transcript levels of phenotypic genes such as collagen type II and Sox9. Because we observed an inverse correlation between Runx2 and Nkx3.2 we hypothesized that Nkx3.2 was repressing Runx2 transcription. As early as day 7 after plating in BMP-treated media, BMSCs exhibit osteogenic nodule formation within the cell population. These nodules continue

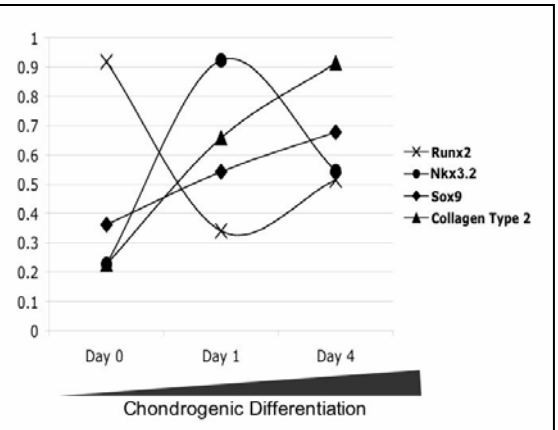

Figure 3.4 Gene expression was monitored over a 4-day period after induction of chondrogenesis. Strong expression of Nkx3.2 was observed by day one and was closely followed by activation of Sox9 and the cartilage specific ECM protein collagen type II. In contrast Runx2 expression was strongly repressed by one day. (Lengner, Serra et al., 2005)

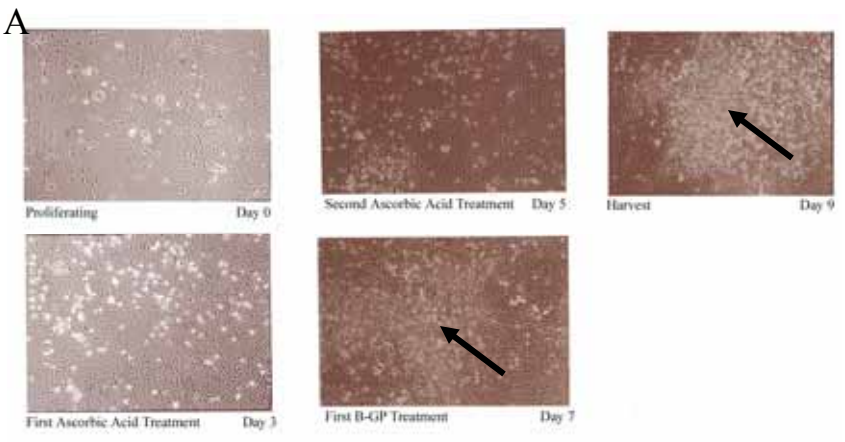

Figure 3.5 Osteogenic differentiation time course (A), Chondrogenic differentiation time course (B)

differentiation and expand in size and cell number dramatically 48 hours after β-GP treatment (arrows in figure 3.5) The osteoblast phenotypic expression of the osteoprogenitor cells indicated an opportune time to harvest for ChIP analysis. This phenotype is exaggerated within C3H10T1/2 cell cultures that have been adenovirally Runx2 infected and displayed increased osteogenesis. The osteogenic gene Alkaline Phosphatase (AP), when stained, shows prevalence in the Runx2 infected cultures. Alcian blue staining targets mucopolysaccharides and glycoaminoglycans that are associated with cartilaginous tissue. As can bee seen in figure 3.6 there is an abundance of staining in the lacZ infected culture while the Runx2 infected cultures shows dramatic loss of chondrogenic gene expression. Runx2 activity under chondrogenic conditions prevents the population from differentiating

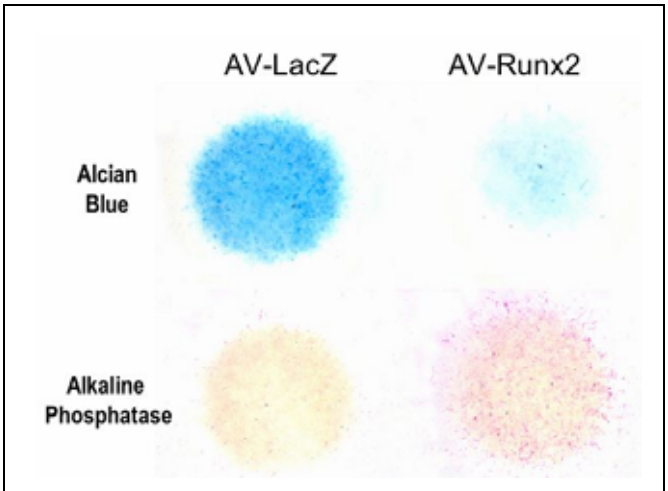

Figure 3.6 Histology of osteogenic and chondrogenic phenotypes as seen in C3H10T1/2 cell cultures with alcian blue and alkaline phosphatase staining. Notice the enhanced expression of AP in the adenovirally Runx2 infected cells that also almost completely lack a chondrogenic phenotype. (Lengner, Serra et al., 2005)

into chondrocytes, yet can induce osteogenic differentiation.

Therefore Runx2 seems to be acting as a repressor of chondrogenic genes and this repressive activity is absent during chondrogenesis. Therefore a mechanism of inhibition and derepression is hypothesized where there is a repressor (Nkx3.2) of the repressor (Runx2).

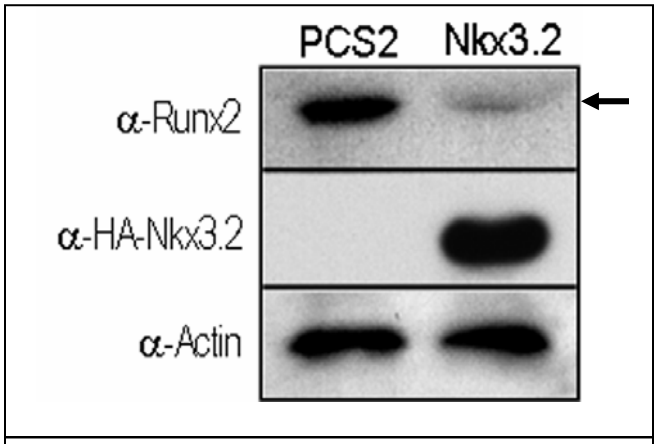

Figure 3.7 Western Blot analysis of Runx2 repression from Nkx3.2 over expression. (Lengner, Serra et al., 2005)

C3H10T1/2 cells, which have been transiently co-transfected (figure 3.7) with Nkx3.2 and GFP, were FACS analyzed for GFP-marked plasmid incorporation. The GFP positive cells were plated and grown for 48 hours, at which time they were lysed for Western analysis. As can be seen in figure 3.7, there is a significant reduction in Runx2 protein levels in the Nkx3.2 infected cells. Therefore it is believed that Nkx3.2 is in fact repressing transcription of Runx2. The mRNA transcript levels of Runx2 must be evaluated in order to determine if transcription at the Runx2 promoter is happening and the transcript is degraded before translation or if transcription is repressed at the promoter.

As can be seen in figure 3.8 the Runx2 mRNA levels are elevated during osteogenesis of BMSCs yet is repressed during chondrogenesis of C3H10T1/2 cells suggest-

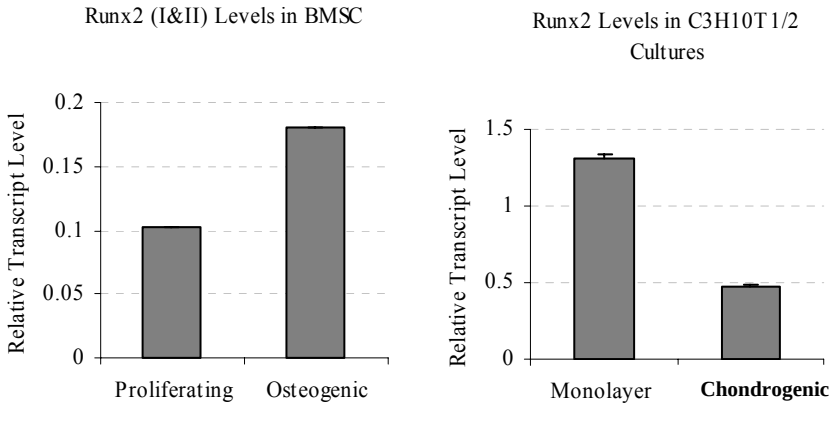

Figure 3.8 Runx2 RNA transcription levels

ing that Runx2 is in fact transcriptionally repressed during chondrogenesis. This repression is hypothesized to be due to Nkx3.2 recruitment to the promoter. When the 0.6 kb promoter sequence is examined for consensus sequences as can be found in figure 3.1, there is a highly conserved regulatory box around 100 kb upstream. Upon closer examination, one finds that there are binding sequences for a number of transcriptional regulators including HLH, ATF, Vitamin D, as well as Runx2 itself. Nkx3.2 binds to this site in a sequence specific manner as determined by EMSAs with a 24 bp oligo containing the Nkx3.2 site (data not shown). Mutation of the Nkx3.2 site within the oligo completely eliminates binding demonstrating the requirement for the intact Nkx3.2 sequence.

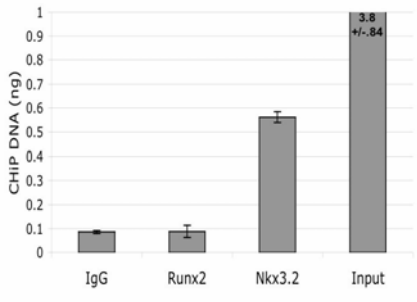

Figure 3.9 Nkx3.2 recruitment to proximal Region of Runx2 Promoter in Transfected C3H10T1/2 cells (Lengner, Serra et al., 2005)

To confirm that Nkx3.2 is occupying the regulatory site in the promoter, we performed ChIP assays 24 hours after transfection with Nkx3.2 in C3H10T1/2 cells using antibodies against endogenous Runx2 or HA-tagged Nkx3.2 (see figure 3.9). We found that the Runx2 promoter was immunoprecipitated with the αHA antibody but not the αRunx2 or non-specific IgG antibody. Therefore the transcription factor Nkx3.2 is occupying the Runx2 promoter and interacts with the consensus site that is approximately 100 bp upstream from the TATA box.

Nkx3.2 associates and regulates chondrogenic genes as can be seen in the Sox9 transcription factor promoter (data not shown.). We have recently shown that Nkx3.2 negatively regulates the Runx 2 promoter in order for the mesenchymal progenitor cell to undergo osteogenesis. During chondrogenisis, Nkx associates with the Sox9 promoter (data not shown). In addition, Nkx associates with the distal end of its own promoter perhaps auto-regulating itself in a fashion similar to Runx2 (data not shown).

3.2 Homeodomain Protein Regulatory Analysis of the Runx 2 0.6 KB P1 Promoter

As mentioned before, there are several sites on the Runx2 promoter that may influence transcription (figure 3.1). Much like the Nkx3.2 binding region approximately 100 bp upstream of transcriptional start site, there are two additional regulatory regions that have overlapping HDs. ChIP analysis of these regions using antibodies for Runx2, Dlx3, Dlx5, Msx2, and Hox a10 enables one to determine if these regions are temporally loading with one or more of these transcription factors during mesenchymal differentiation.

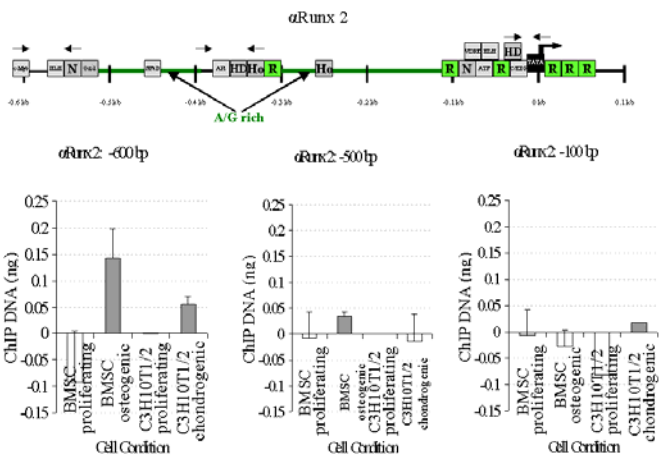

Figure 3.10 Runx2 Chromatin immunoprecipitation

Runx 2 preferentially associates with its own distal promoter under osteogenic conditions and there may be a positive effect due to this association (figure 3.10). Auto- regulation is a common mechanism for maintaining protein levels of a gene product and this may or may not be the case in this instance. The middle region of the promoter also shows some increase in the osteogenic time point; however this is believed to be an effect from the ChIP and the sonication of the chromatin fragments. The fragments are in the range of 200 to 500 kb in length, therefore the primers may be picking up a piece of DNA that was pulled down with an antibody for a protein that is recruited to a nearby region in the promoter.

Msx2 associates itself with the region near the transcriptional start site for a positive regulatory effect under osteogenic conditions. Additionally, there is a loss in binding in the distal region of the promoter during osteogenic differentiation (figure 3.11). However, Msx2 is

occupying the distal and middle region of the promoter under basal levels and is lost during chondrogenic differentiation. Additionally, when Nkx3.2 is binding during Runx2 repression, Msx2 is displaced or prevented from binding at its preferential location on the proximal region of the promoter. Therefore it appears that Msx2 is required in the distal region of the promoter in proliferating mesenchymal cells yet must leave that site and be recruited in the proximal promoter for osteogenesis. Conversely, Msx2 is sitting on the proximal promoter region during proliferation of C3H10T1/2 cells but must leave and target the distal region for Runx2 repression and subsequent chondrogenesis.

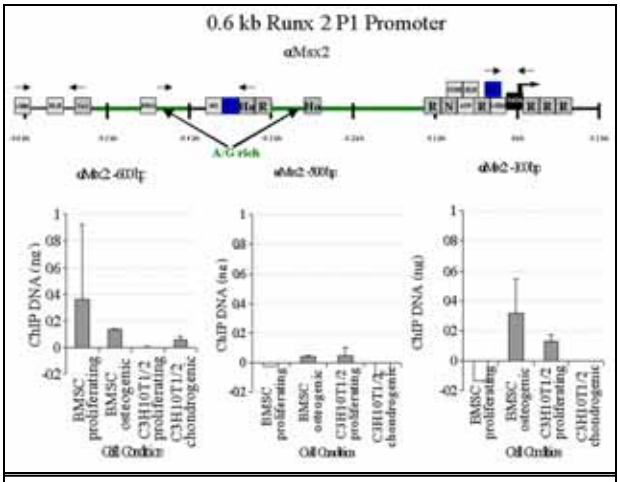

Figure 3.11 Msx2 Chromatin immunoprecipitation

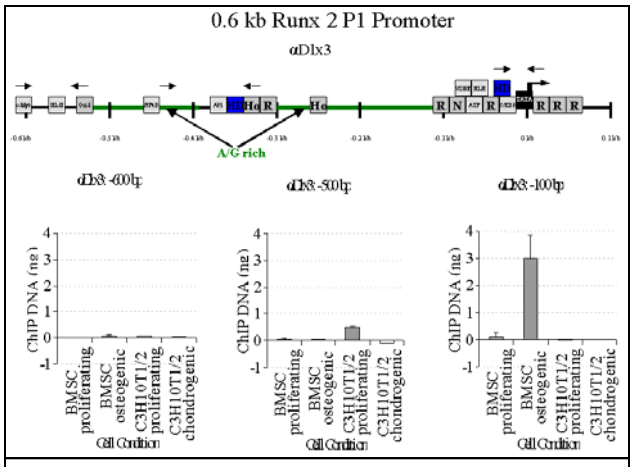

Figure 3.12 Dlx3 Chromatin immunoprecipitation

Dlx3 associates and binds in the localized region near the transcriptional start during osteogenic differentiation. There seems to be a dramatic recruitment to the proximal region that is necessary for osteogenesis (figure 3.12). This contrasts with the slight reduction in recruitment in the middle region of the promoter during Runx2 repression.

Dlx5 appears to occupy the distal promoter under basal levels yet is lost during later

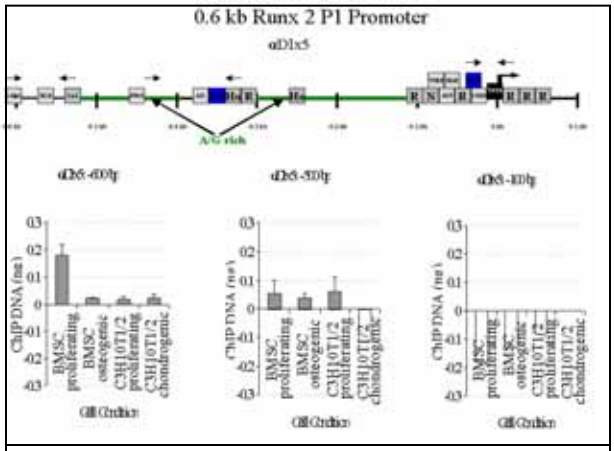

Figure 3.13 Dlx5 Chromatin immunopreciipitation

stages of osteoblast differentiation. When Runx2 is repressed there is a dramatic loss of Dlx5 from the distal region of the promoter (figure 3.13). Additionally, there is a reduction in Dlx5 binding in the central region of the promoter during chondrogenesis.

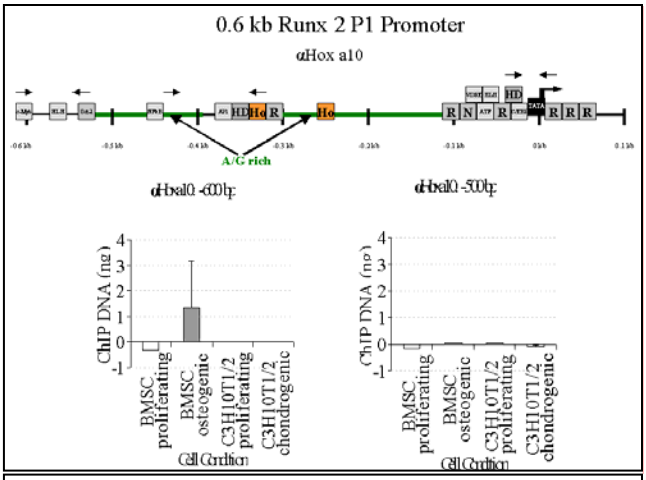

Figure 3.14 Hox a10 Chromatin immunoprecipitation

There is a recruitment of Hox A10 when the promoter is activated during osteoblast differentiation (figure 3.14). Hox A10 has recently been implicated as pro-osteogenic factor (Lian and Balint, Gene Array; Hassan personal communication). Taken together, Hox a10 may have an activating effect on the promoter through the distal region.

4. CONCLUSIONS

This project accomplished the establishment that the Runx2 gene is repressed by the homeodomain factor, Nkx3.2. The identification of the Runx2 gene being regulated by Nkx3.2 is found through the Runx2 promoter that is sensitive to coordinated signaling molecules. These findings support Runx2's critical role in chondrogenesis and provides evidence that it is also involved with BMP-2 induced chondrogenesis in pluripotent mesenchymal cells (figure 4.1). While finding the Runx2 repression at the beginning of chondrogenesis is important, the role that Runx2 plays within the cellular environment remains to be found in mesenchymal cells.

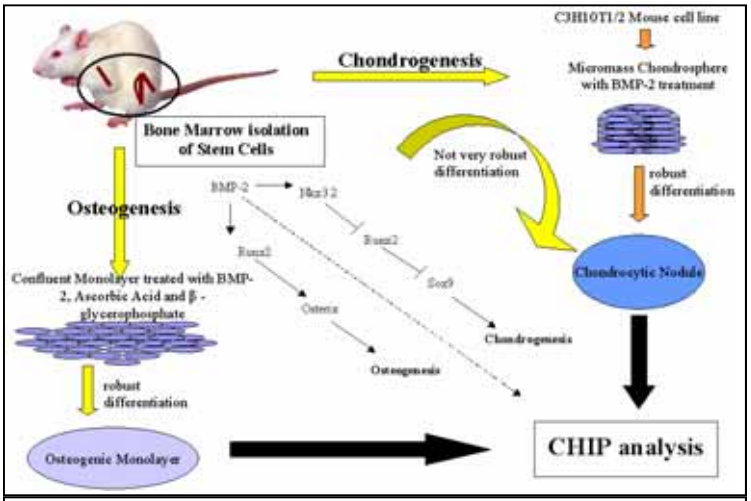

Figure 4.1 Schematic of Cell Culture and Differentiation

During osteogenic differentiation, Runx2 associates itself with the distal region of the Runx2 gene promoter. In addition, there is an induction and recruitment of Msx2, Dlx3, and Hox a10 on the proximal region of the promoter for a positive transcriptional effect. Interestingly, Dlx5 is found to bind to the distal region of the promoter under chondrogenic differentiation. This may be for a negative effect on the Runx2 promoter. Msx2 is found to increase it's binding to the proximal promoter dramatically during osteogenesis while levels decreased from

basal levels during chondrogenesis (figure 4.2).

However as differentiation progressed Dlx3, then Dlx5 displaced Msx2 and occupied the regulatory region. Additionally, the displacement found in the distal promoter during osteogenesis supports these findings. The displacement during chondrogenesis may be due to our findings of Nkx3.2 binding in the proximal regulatory element.

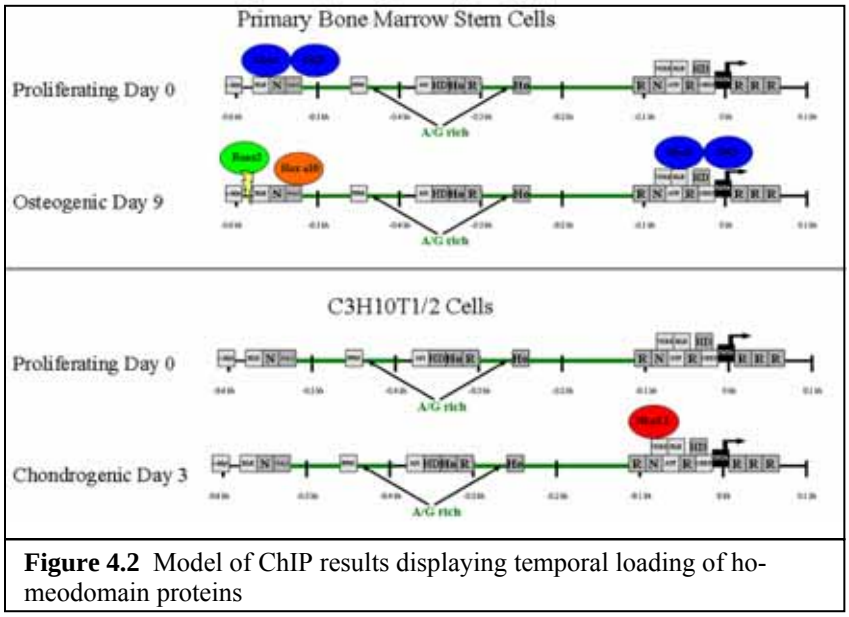

Figure 4.2 Model of ChIP results displaying temporal loading of homeodomain proteins

5. BIBLIOGRAPHY

Ahrens PB, Solursh M, Reiter RS. Stage-related capacity for limb chondrogenesis in cell culture. *Dev Biol* 1977; 60: 69-82

Alappat S, Zhang ZY, Chen YP. Msx homeobox gene family and craniofacial development. *Cell Res*. 2003 Dec; 13(6): 429-42.

Banerjee C, McCabe LR, Choi JY, Hiebert SW, , Stein JL, Stein GS, Lian JB. Runt homology domain proteins in osteoblast differentiation: AML3/Cbfa1 is a major component of a bone-specific complex. *J Cell Biochem*. 1997; 66: 1-8.

Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS, Lian JB. Differential regulation of the two principle Runx2/Cbfa1 n-terminal isoforms in response to bone morphogenetic protein –2 during development of the osteoblast phenotype. *Endocrinology*. 2001; 142 (9): 4026-39.

Beanan MJ, Sargent TD. Regulation and function of Dlx3 in vertebrate development. *Dev Dyn*. 2000 Aug; 218(4): 545-53. Review.

Bendall AJ, Abate-Shen C. Roles for Msx and Dlx homeoproteins in vertebrate development. *Gene*. 2000 Apr 18; 247(1-2): 17-31. Review.

Depew MJ, Lufkin T, Rubenstein JL. Specification of jaw subdivisions by Dlx genes. *Science*. 2002 Oct 11;298(5592):381-5.

Derynck R, Zhang Y, Feng XH. Smads: transcriptional activators of TGF-beta responses. *Cell*. 1998 Dec 11; 95(6): 737-40. Review.

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell*. 1997; 89: 747-54.

Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T, Komori T. Cbfa1 is a postive regulatory factor in chondrocyte maturation. *J Biol Chem*. 2000; 275: 8695-702.

Garcia-Minaur S, Mavrogiannis LA, Rannan-Eliya SV, Hendry MA, Liston WA, Porteus ME, Wilkie AO. Parietal foramina with cleidocranial dysplasia is caused by mutation in Msx2. *Eur J Hum Genet*. 2003 Nov; 11(11): 892-5.

Geoffroy V, Corral DA, Zhou L, Lee B, Karsenty G. Genomic organization, expression of the human Cbfa1 gene and evidence for alternative plicing event affecting protein function. *Mammalian Genome*. 1998; 9(1), 54-7.

Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M. Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol. Chem*. 1999; 274: 6972-8.

Hassan M, Javed A, Morasso M, Karlin J, Montecino M, Wijnen AJV, Stein GS, Stein JL, Lian JB. Dlx3 transcriptional regulation of osteoblast differentiation: Temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to the chromatin of the osteocalcin gene. *Mol Cell Biol*. 2004 Oct; 24(20): 9248-61.

Karlin Jeremy. Regulation of Osteoblast Differentiation. Major Qualifying Project, Worcester Polytechnic Institute. 2004.

Katagiri T, Takahashi N. Regulatory mechanisms of osteoblast and osteoclast differentiation. *Oral Dis*. 2002 May; 8(3): 147-59. Review.

Kim IS, Otto F, Zabel B, Mundlos S. Regulation of chondrocyte differentiation by Cbfa1. *Mech. Dev*. 1999; 80: 159-70.

Koenig BB, Cook JS, Wolsing DH, Ting J, Tiesman JP, Correa PE, Olson CA, Pecquet AL, Ventura F, Grant RA. Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells. *Mol Cell Biol*. 1994 Sep; 14(9): 5961-74.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell*. 1997 May 30; 89(5): 755-64.

Lengner CJ, Drissi H, Choi JY, Wijnen AJV, Stein JL, Stein GS, Lian JB. Activation of the bone-related Runx2/Cbfa1 promoter in mesenchymal condensations and developing chondrocytes of the axial skeleton. *Mech. Dev*. 2002; 114(1-2): 167-70.

Lengner CJ, Lepper C, Wijnen AJV, Stein JL, Stein GS, Lian JB. Primary mouse embryonic fibroblasts: a model of mesenchymal cartilage formation. *J Cell Physiol*. 2004; 200: 327-33.

Lengner CJ, Hassan MQ, Serra RW, Lepper C, Wijnen AJV, Stein JL, Lian JB, Stein GS. Nkx3.2 mediated repression of Runx2 promotes chondrogenic differentiation. *J Biol Chem*. 2005 (epub ahead of print).

Lettice L, Hecksher-Sorensen J, Hill R. The role of Bapx1 (Nkx3.2) in the development and evolution of the axial skeleton. *J Anat*. 2001; 199(pt 1-2): 181-7.

Levanon D, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, Broner Y. AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. *Genomics*. 1994; 23(2): 425-32.

Lian JB, Balint E, Javed A, Drissi H, Vitti R, Quinlan EJ, Zhang L, Wijnen AJV, Stein JL, Speck N, Stein GS. Runx1/AML1 Hematopoietic transcription factor contributes to skeletal development *in vivo*. *J Cell Physiol*. 2003 Aug;196(2):301-11

Lian JB, Javed A, Zaidi SK, Lengner C, Montecino M, Wijnen AJV, Stein GS, Stein JL. Regulatory controls for osteoblast growth and differentiation: Role of Runx/Cbfa/AML factors. *Critical Review in Eukaryotic Gene Expression*. 2004; 14(1&2): 1-42.

Liu F, Ventura F, Doody J, Massague J. Human type II receptor for bone morphogenetic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol Cell Biol*. 1995 Jul; 15(7): 3479-86.

Mello MA, Tuan RS. High density micromass cultures of embryonic limb bud mesenchymal cells: an in vitro model of endochondral skeletal development. *In Vitro Cell Dev Biol Anim*. 1999; 35: 262-9.

Meyers S, Lenny N, Sun W, Hiebert SW. AML2 is a potential target for transcriptional regulation by the t(8;21) and t(12;21) fusion proteins in acute leukemia. *Oncogene*. 1996; 13: 303-12.

Morasso MI, Grinberg A, Robinson G, Sargent TD, Mahon KA. Placental failure in mice lacking the homeobox gene Dlx3. *Proc Natl Acad Sci USA*. 1999 Jan 5;96(1):162-7.

Nüsslein-Volhard C, Wieschaus E. Mutants affecting segment number and polarity in Drosophila. *Nature*. 1980; 287: 795-801.

Ogawa E, Maruyama M, Kagoshima H, Inuzuka M, Lu J, Satake M, Shigesada K, Ito Y. PEBP2/PEA2 represents a new family of transcription factor homologous to the products of the Drosophila *runt* and the human *AML1* gene. *Proc Natl Acad Sci*. 1993; 90: 6859-63.

Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GWH, Beddington RSP, Mundlos S, Olsen BR, Selby PB, Owen MJ. *Cbfa*1, a candidate gene for the Cleidocranial Dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997; 89: 765-771.

Price JA, Bowden DW, Wright JT, Pettenati MJ, Hart TC. Identification of a mutation in Dlx3 associated with tricho-dento-osseous (TDO) syndrome. *Hum Mol Genet*. 1998 Mar; 7(3): 563-9.

Ryoo HM, Hoffman HM, Beumer T, Frenkel B, Towler DA, Stein GS, Stein JL, Wijnen AJV, Lian JB. Stage-specific expression of Dlx5 during osteoblast differentiation: involvement in regulation of osteocalcin gene expression. *Mol endocrinol*. 1997 Oct; 11(11): 1681-94.

Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R. Msx2 deficiency in mice causes

pleiotropic defects in bone growth and ectodermal organ formation. *Nat Genet*. 2000 Apr;24 (4):391-5.

Stewart M, Terry A, Hu M, O'Hara M, Blyth K, Baxter E, Cameron E, Onions DE, Neil JC. Proviral insertions induce the expression of bone-specific isoforms of PEBP2alpha A (CBFA1): evidence for a new myc collaborating oncogene. *Proc Natl Acad Sci*. 1997; 94(16): 8646-51.

Tribioli C, Frasch M, Lufkin T. Bapx1: an evolutionary conserved homologue of the Drosophila bagpipe homeobox gene is expressed in splanchnic mesoderm and the embryonic skeleton. *Mech Dev*. 1997; 65(1-2): 1671-83

Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. *Endocr Rev*. 2000; 21: 393-411

Zaidi SK, Sullivan AJ, Wijnen AJV, Stein JL, Stein GS, Lian JB. Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. *Proc Natl Acad Sci USA*. 2002; 99(12):8048-53.

Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP to induce somatic chondrogenesis. *Genes Dev*. 2002; 16: 1990-2005.