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Expression and Function of Dlx Genes in the Osteoblast Lineage

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Abstract

Our laboratory and others have shown that overexpression of Dlx5 stimulates osteoblast differentiation. $Dlx5^{-/-}/Dlx6^{-/-}$ mice have more severe craniofacial and limb defects than $Dlx5^{-/-}$, some of which are potentially due to defects in osteoblast maturation. We wished to investigate the degree to which other Dlx genes compensate for the lack of Dlx5, thus allowing normal development of the majority of skeletal elements in $Dlx5^{-/-}$ mice. Dlx gene expression in cells from different stages of the osteoblast lineage isolated by FACS sorting showed that Dlx2, Dlx5 and Dlx6 are expressed most strongly in less mature osteoblasts, whereas Dlx3 is very highly expressed in differentiated osteoblasts and osteocytes. In situ hybridization and Northern blot analysis demonstrated the presence of endogenous Dlx3 mRNA within osteoblasts and osteocytes. Dlx3 strongly upregulates osteoblastic markers with a potency comparable to Dlx5. Cloned chick or mouse Dlx6 showed stimulatory effects on osteoblast differentiation. Our results suggest that Dlx2 and Dlx6 have the potential to stimulate osteoblastic differentiation and may compensate for the absence of Dlx5 to produce relatively normal osteoblastic differentiation in Dlx5 knockout mice, while Dlx3 may play a distinct role in late stage osteoblast differentiation and osteocyte function.

Keywords

osteoblast differentiation; osteoblast lineage; Dlx2; Dlx5; Dlx3; Dlx6; GFP; bone; FACS

INTRODUCTION

Extant vertebrates consist of gnathostomes (jawed vertebrates) and cyclostomes (jawless vertebrates), and the divergence of these two lineages is to a large degree characterized by the gnathostome's elaboration of the body plan to include bone, teeth, paired appendages and jaws (Neidert et al., 2001). There is a correlation between some of those morphological innovations

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and Dlx gene family expansion and expression modification (Depew et al., 2002). The vertebrate Dlx genes, which encode a family of homeobox-containing transcription factors related in sequence to the Drosophila Distal-less (Dll) gene product, constitute one example of functional diversification of paralogs (Ghanem et al., 2003). Gnathostoma vertebrates have six Dlx genes organized into pairs of closely linked, convergently transcribed loci, Dlx1-Dlx2, Dlx3-Dlx4 (originally called Dlx7), and Dlx5-Dlx6, each located in close proximity to one of four Hox clusters in the mouse genome. Because the original duplication event that created the first linked pair of genes happened a considerable evolutionary time before the subsequent replication events that produced the three pairs that exist in mammals and birds, Dlx2, 3 and 5 are more closely related to each other than they are to Dlx1, 4 and 6 (Neidert et al., 2001; Stock, 2005; Stock et al., 1996). They are expressed in distinct but overlapping domains, primarily in the forebrain, branchial arches and tissues derived from epithelial-mesenchymal interactions (Bendall and Abate-Shen, 2000; Qiu et al., 1997; Robinson and Mahon, 1994; Bryan and Morasso, 2000). Overlapping patterns of expression of members of a gene pair can be conferred by cis-acting regulatory sequences located in the regions between gene pairs (Ghanem et al., 2003).

Dlx5 and Dlx6 are expressed in a generally similar pattern, and are expressed in almost every skeletal element, including endochondral and membranous bone (Chen et al., 1996; Simeone et al., 1994; Zhao et al., 1994). The impression from these studies was that, in general, Dlx5 is expressed at higher levels than Dlx6, although the techniques used were not quantitative. Dlx5 overexpression accelerates osteoblast differentiation of primary osteoblast cultures derived from chick calvariae (Tadic et al., 2002), and it can induce expression of the Col1a1 promoter (Tadic et al., 2001). Dlx5 knock-out mice display significant craniofacial and sensory capsule skeletal defects as well as delayed calvarial ossification and less well organized diaphyseal cortical bone (Depew et al., 1999; Acampora et al., 1999). All these data suggest that Dlx5 plays an important role in osteoblast differentiation; however the limbs and axial skeleton of Dlx5 knock-out mice are relatively normal. This raises the possibility that other Dlx genes compensate for the absence of Dlx5 in these mice. Interestingly, Dlx5/6 knock-out mice have a more severe bone phenotype than the Dlx5 single knockout, with craniofacial, axial, and appendicular skeletal abnormalities (Robledo et al., 2002); in addition, this knockout produced a jaw phenotype that was interpreted as being a transformation of the maxilla into a mandible-like structure (Koentges and Matsuoka, 2002; Robledo et al., 2002). Although many of these defects are thought to reflect aberrant pattern formation, it was also suggested that there may be delayed ossification in the mutant. Inactivation of Dlx3 in mice resulted in placental failure, so that the embryonic phenotype could not be investigated (Morasso et al., 1999), however a frameshift deletion in the human DLX3 gene causes a hereditary disease, tricho-dento-osseous (TDO) syndrome, which is characterized by increased bone density (Haldeman et al., 2004; Price et al., 1998; Price et al., 1999). This suggests the potential involvement of Dlx3 in bone development.

Because of the above considerations, we investigated the possibility that other Dlx genes expressed in bone have the ability to stimulate osteoblast differentiation. We examined expression of all six Dlx genes during mouse osteoblast differentiation by quantitative real-time PCR. Dlx3 is the only family member that is highly upregulated during differentiation, while Dlx2, Dlx6 and Dlx5 are expressed in intermediate levels and Dlx4 and Dlx1 expressed at levels that are difficult to distinguish from background. To assess the ability of these genes to stimulate osteoblast differentiation, chick and mouse Dlx6 and chick Dlx3 cDNAs were cloned into RCASBP(A), an avian replication-competent retroviral vector. We cloned murine Dlx6 mRNA that contains a long poly-glutamine/poly-proline tract that is not present in the chick, Xenopus or zebrafish proteins. We also cloned an N-terminal truncated form of the mouse mRNA that lacked the poly-glutamine/poly-proline tract, in which translation is initiated at an internal AUG. Primary chick and mouse calvarial cells and mouse marrow

stromal cells (MSC) were infected with the chick Dlx3, chick Dlx6 or mouse Dlx6 vector respectively. Our results show that Dlx3 induces osteoblastic differentiation in all of these cell types with an apparent efficiency comparable to that of Dlx5. Both forms of Dlx6 also stimulated osteoblastic differentiation. These studies suggest that Dlx3 may be a critical Dlx gene for inducing the later stages of osteoblastic lineage progression and possible osteocyte function, and that Dlx6, which is in a different class of Dlx genes from that of Dlx2, 3 and 5, is also capable of stimulating osteoblast differentiation. Our results provide important clarification of the expression levels and possible functions of the Dlx genes during osteoblast differentiation.

MATERIALS AND METHODS

Preparation of calvarial osteoblast cell culture and viral transduction

Calvarial cells were isolated from 7- day-old CD1 neonatal mice or 15 day old chick embryos (Charles River SPAFAS, North Franklin, CT, USA) using a modification of the method described by Wong and Cohn (Kalajzic et al., 2002; Tadic et al., 2002; Wong and Cohn, 1975). Calvariae were subjected to four sequential 15 min digestions in an enzyme mixture containing 0.05% trypsin and 1.5 units/ml collagenase P at 37°C. Cell fractions 2–4 were pooled and enzyme activity was terminated by addition of media containing FBS. Cells were plated at a density of 1.5×10^5 cells/well in 6-well culture dish in DMEM with 10% FBS and switched to differentiation medium (αMEM containing 10% FBS, 50 μg/ml ascorbic acid, 4 mM β-glycerophosphate) when they reached confluence. Cells were infected once a day for three days with 0.5 ml conditioned media containing RCASBP(A), RCASBP(A)Dlx3, RCASBP(A)Dlx5 or RCASBP(A)Dlx6 mixed with 1.5 ml fresh media per well beginning on the day after plating. Cells were harvested for analysis of bone markers at different stages of differentiation.

Preparation of mouse marrow stromal cell (MSC) cultures and viral transduction

Two-month-old βAKE transgenic mice were sacrificed by CO_2 asphyxiation. Marrow stromal cells were prepared using a previously described procedure (Kalajzic et al., 2002). Briefly, the epiphyseal growth plates of femurs and tibias were removed and the marrow was collected by flushing with αMEM with 10% FBS. Cells were plated at a density 5×10^6 cells/well in 6-well culture plates. On day 4, the media, along with the nonadherent cells, was replaced with fresh αMEM plus 10% FBS; on day 7, total medium was changed into differentiation medium (αMEM/10% FBS supplemented with 50μg/ml ascorbic acid, 10^{-8} M dexamethasone and 8 mM β-glycerophosphate). Afterwards, differentiation medium was changed every other day for the duration of the experiment. Cells were transduced with conditioned media containing virus particles beginning on day 4. Two transductions were performed each day for three days until the cultures were switched into differentiation medium. On each day of transduction, the cells were exposed to 0.5 ml virus mixed with 1.5 ml fresh media in the morning, this media was replaced with fresh media containing virus after 8 hr, and this media was left on the cells overnight.

RNA extraction from cell cultures and Northern blot analysis

RNA extraction and Northern blotting were performed as detailed in (Kalajzic et al., 2002). Briefly, total RNA was extracted from cultures using TRI Reagent (Invitrogen) according to the manufacturer's instructions. RNA pellets were redissolved in GTC buffer and further precipitated in isopropanol. 15μg of RNA was separated on a 2.2 M formaldehyde/1% agarose gel and transferred onto a nylon membrane (Nytran, Schleicher and Schuell). Membranes were probed with (^{32}P)dCTP labeled rat Col1a1, mouse OC, and mouse BSP for mouse osteoblast cultures and chick BSP, OC, Dlx3 and Dlx6 for chicken cultures.

Histochemical analysis of cell cultures

ALP activity staining was performed by using a commercially available kit (86-R Alkaline Phosphatase, Sigma Diagnostics, Inc. St. Louis, MO, U.S.A.) according to the manufacturer's instructions. Von Kossa staining was utilized to assess mineralization after ALP staining by adding 5% silver nitrate solution at 1ml/well into 6 well culture plates, which were then irradiated using two Auto Cross Link cycles in a UV Stratalinker 1800 (Stratagene), washed with water and air dried.

Real-time PCR

Calvarial cell cultures were prepared from 7-day-old CD1 neonatal mice and RNA was extracted using methods described above. cDNA was synthesized using an Invitrogen Superscript First-strand Synthesis System for RT-PCR. TaqMan® Gene Expression Assays specific for Dlx genes and marker genes for osteoblast differentiation were purchased from ABI and performed on the 7500 Real-Time PCR System (assay ID: Dlx1, Mm00438424_m1; Dlx2, Mm00438427_m1; Dlx3, Mm00438428_m1; Dlx4, Mm0043842858_m1; Dlx5, Mm00438430_m1; Dlx6, Mm01166201_m1, 18s, 4319413E; DMP1, Mm01208365_m1; BSP, Mm00492555_m1; OC, Mm00649782_m1). The TaqMan assays are designed so that they do not detect the antisense Dlx1 and 6 transcripts that are in the mouse (Liu et al., 1997). 18S ribosomal RNA was used as internal control. Before using the ΔΔCT method for quantification, validation experiments were performed to demonstrate that the amplification efficiencies of target genes and the reference gene were approximately equal. Q-PCR was performed using two sets of independently transcribed cDNAs from each experiment. Gene expression levels were averaged from two biological replicates.

Mouse Dlx6 cDNA Cloning

Tissue RNA was extracted from C57BL/6 mouse femurs and treated with DNase to degrade the remaining genomic DNA. The cDNA array was generated by reverse transcription using SuperScript II and oligo dT (Invitrogen) according to the manufacturer's instructions with slight modification. One to 0.1 μl of the final reaction was used for the PCR reaction. A series of primers were designed according to the mouse genomic sequence (Accession number: **AC122240**, GI: **50839090**) to amplify targeted regions as shown in Fig. 7A. Primers were the following: sense primer P1: 5′-TTTATCGATGTGAAAGAAACCCGGGAGA-3′; antisense primer P2: sequence 5′-TTTATCGATGCCTCCTTCAGAAGCTCCGTA-3′; Sense primer P3: 5′-CTCGCAGCACAGCCCTTACCTCCAGTCC-3′. The PCR fragment amplified by P1 and P2 was sub-cloned into a TOPO TA vector (Invitrogen, Carlsbad, California) and further cloned into an RCAS BP(A) virus vector. PCR conditions for Dlx6 cDNA amplification using primers P1 and P2 are: 2.5 unites Invitrogen Pfx Taq polymerase, 5mM Mg^{2+}, 0.3μM primers of each, 100ng of cDNA; 94°C, 4′, 1 cycle; 94°C, 60″, 55°C, 60″, 68°C,2′, 32 cycles.

Retroviral vectors and retrovirus production

The coding sequence of chicken Dlx3 gene was amplified by PCR from plasmids kindly supplied by M. Kessel (Max-Planck Institute, Göttingen, Germany) in order to remove 5′ and 3′ untranslated regions. Pfu polymerase (Stratagene, La Jolla, CA, USA) was used and the blunt-end product was cloned into Zero-Blunt vector (Invitrogen, Carlsbad, CA, USA) and then placed into RCASBP(A), a helper-independent avian retroviral vector using the Gateway cloning system (Life Technologies, Rockville, MD, USA). Dlx6 cDNA was amplified by RT-PCR from total RNA extracted from chicken long bone, using primers designed from the sequence of a cosmid clone that contains the chicken Dlx6 gene. Superscript II (Invitrogen, Carlsbad, CA, USA) was used for reverse transcription and Taq polymerase for PCR Invitrogen, Carlsbad, CA, USA). The amplified fragment was cloned into a modified Bluescript vector and sequenced. The sequence of this clone was identical to the sequence submitted to

Genbank by S. Brown and A. Groves, accession number **AY640308**, except for a base variation at nucleotide 813 from a C to a T which converts a proline to a leucine. This amino acid is downstream of the homeodomain, and its functional significance is not known. We confirmed the sequence of our clone by PCR amplifying and sequencing the Dlx6 cDNA from chick osteoblast RNA and found the sequence identical to our cDNA clone. We did not detect any sequences that matched **AY640308** at nucleotide 813. The **AY640308** sequence may represent a polymorphism, or a sequencing error. Because we confirmed that the clone we had isolated was a true wild-type sequence, we chose to use it for our functional studies. Our cDNA was cloned into the Cla1 site of the RCASBP(A) retroviral vector. Control virus was RCASBP(A) vector without inserted cDNA. Vectors were transfected (Lipofectamine 2000, Life Technologies, Rockville, MD, USA) into the DF1 producer cell line (chicken embryonal fibroblasts). Cells were expanded by splitting multiple times. Conditioned media from transfected cells was collected when cells became superconfluent and stored at −70°C. Virus production was confirmed by Reverse Transcriptase Assay (Roche). Virus titer was acquired by performing immunohistochemistry using antibody against pGAG (AMV-3C2, Developmental Studies Hybridoma Bank). In general, virus titer was between 6×10^7 to 6×10^8.

Preparation of Nuclear Extracts and Western Immunobloting

Nuclear extracts of day 7 calvarial cultures were produced based on the procedures of Shapiro et al. (Shapiro et al., 1988). 25μg of protein were separated on SDS-PAGE gels, transferred to nitrocellulose membranes, and blots were probed with a polyclonal rabbit anti-human Dlx6 antibody (Aviva Systems Biology, San Diego, CA) under manufacturer's instructions. Antibody binding was visualized using the ECL Plus system (GE Healthcare).

In situ hybridization

The 600-bp antisense Dlx3 riboprobe was prepared by in vitro transcription of linearized chick Dlx3 cDNA using T7 RNA polymerase (Life Technologies, Rockville, MD, USA). Nonspecific hybridization signals and emulsion background were controlled for by hybridization of adjacent tissue sections with nonspecific riboprobe or with no probe at all. Whole calvaria from 15-day and mandibles from 10-day old chick embryos were isolated, fixed in 4% paraformaldehyde at 4°C overnight, washed and dehydrated using methanol. Tissues were embedded in paraffin, sectioned at 7-μm thickness and processed for in situ hybridization using [32P] UTP-labeled riboprobes. The sections were stained with hematoxylin, mounted, examined and photographed using an E600 Nikon microscope and a Spot RT TM camera. In the studies of Dlx3 expression in 18 day mouse mandible, paraffin sections were prepared as described above. Dlx3 antisense and sense RNA probes used for hybridization were prepared from an Xho1 and Not1 linearized pBluescript II SK Dlx3 subclone and transcribed in vitro in the presence of digoxigenin-U-NTP mixture (Roche # 11277073910) with T3 and T7 polymerase, separately.

Preparation of Cells for Sorting

Cells were prepared for sorting as previously described (Kalajzic et al., 2005). Briefly, 7-day-old Col3.6(GFP) calvarial osteoblast cultures were digested in 0.25% trypsin, 1 mM EDTA for 5 minutes. 17-day-old Col2.3(GFP) culture or 19-day-old DMP1(GFP) cultures were digested in 0.2% collagenase A (Roche), 0.2% hyaluronidase and 2.5% trypsin for 10–15 minutes. Cell sorting was done using a FACS Vantage (BD Biosciences) with 488 nm excitation and 530/30 emission filters. Cells were separated using a 100-μm nozzle and collected into DMEM/30% FBS media. Prior, during, and following sorting the cell suspensions were kept cold to minimize changes in gene expression.

RESULTS

Relative quantification of Dlx gene expression during osteoblast differentiation

To assess the potential for functional redundancy of Dlx genes in bone development, we wished to study the expression and potential role of Dlx genes in bone tissues and in osteoblastic cell cultures and to have a more comprehensive understanding of the relative expression levels of the Dlx genes at different stages of osteoblastic differentiation. To address this question, we carried out an analysis of the expression of all of the Dlx genes in cultured mouse calvarial osteoblasts at several time points using quantitative real-time PCR. Because the amplification efficiency of all of the primer pairs used for the study was similar and all CT values were normalized to the same internal standard, we can also compare expression levels of different Dlx genes at the mRNA level. Dlx3 was expressed at low to moderate levels in the proliferating phase of the cultures, and demonstrated a dramatic increase as the cultures differentiated (Table 1). Somewhat surprisingly, unlike the impression from previous in situ hybridization studies (Simeone et al., 1994), Dlx6 was expressed at levels that were greater than Dlx5. Dlx2 was expressed at levels generally comparable to Dlx6, while Dlx1 and 4 were expressed at levels that were so low that they may represent background PCR signal. Dlx 2 and 6 levels increased somewhat as the cultures differentiated, and Dlx5 increased to a greater degree, but the increases in Dlx3 levels were much more extreme.

Primary osteoblast cell cultures are heterogeneous with a high proportion of cells that are not in the osteoblast lineage. In addition, osteoblast lineage cells at various levels of maturation are present at any given time of culture. Thus, amplification of gene expression in such mixed cultures represents the average gene signal derived from a very heterogeneous population and may not accurately reflect changes in expression during the maturation of the osteoblast lineage.

To further analyze Dlx gene expression in more defined osteoblastic populations, we quantified Dlx gene expression levels in FACS sorted cultured mouse calvarial osteoblasts. The use of transgenic GFP markers to label cells in different stages of osteoblast lineage has been extensively studied in our lab. It has been demonstrated that in early osteoblast cultures, the 3.6 kb type I collagen promoter directs GFP expression to cells that are at an early stage of osteoblast differentiation. In contrast, the 2.3 kb of type I collagen promoter activates when osteoblast cell cultures begin to form mature osteoblastic nodules. Isolation of Col2.3GFP positive cells by flow cytometry and Northern blot analysis has shown that these cells are highly enriched for markers of osteoblast differentiation including osteocalcin and bone sialoprotein mRNA (Kalajzic et al., 2002) indicating that they possess a mature osteoblastic phenotype. In addition, activation of dentin matrix protein 1 (Dmp1) directed GFP is closely associated with the pre-osteocyte and osteocyte stages. (Fig. 1D, E) (Kalajzic et al., 2004; Yang et al., 2005). In summary, the 3.6 kb collagen I promoter (Fig. 1A), the 2.3 kb collagen I promoter (Fig. 1B), and the DMP1 promoter (Fig. 1C) drive GFP expression in preosteoblasts, mature osteoblasts and osteocytes, respectively (Fig. 1A–E). We sorted different stages of osteoblasts from primary cavarial cell cultures derived from transgenic mice using these markers. Dlx gene's expression patterns were measured in these populations using real-time PCR (Fig. 1F). Dlx2, Dlx5 and Dlx6 were more enriched in Col3.6GFP positive cells from 7 day old cultures which represent preosteoblasts, but were more highly expressed in Col2.3GFP negative cells than in Col2.3GFP positive cells from 17 day old cultures, suggesting that these Dlx genes begin to be down regulated as osteoblasts attain the fully differentiated state. Dlx3 exhibited very high levels of expression in Col2.3GFP and DMP1GFP positive cells, which represent mature osteoblasts and osteocytes (Fig. 1D–E).

Dlx3 mRNA expression and its effects in osteoblast differentiation

Because of the distinctive expression pattern of Dlx3, we wished to explore patterns of Dlx3 expression in vivo and its possible roles in osteoblast differentiation.

RT-PCR revealed the presence of Dlx3 mRNA in total RNA of chick long bone, calvaria and skin as well as in RNA from chick calvarial osteoblast cultures at days 13 and 20, when cells are at a more differentiated stage (Fig. 2A). No expression was seen in brain, heart or liver. Northern blot analysis of mouse tissues showed that Dlx3 signal is present in bone and tail (Fig. 2); expression in tail may reflect expression in both vertebral bone and skin. In chick calvarial cultures Dlx3 expression was detected at days 13 and 20 (Fig. 2C) and in mouse calvarial and marrow stromal cultures from day 18 (Fig. 2D), a time when cultures become osteogenic and expressed markers of mature osteoblasts such as osteocalcin and BSP (data not shown). To further assess expression of Dlx3 in bone, calvaria of 15-day-old chick embryos were hybridized with a Dlx3 antisense riboprobe. Dlx3 signal was detected in the osteogenic cells in the periosteum and in the osteogenic cells lining the bone marrow spaces (Fig. 3A–E). The Dlx3 signal was also present in condensing preosteogenic mesenchyme and periosteal osteogenic cells of mandible sections from 8–10 days old chick embryos (Fig. 3F–I). We further studied Dlx3 expression in 18 day old mouse mandible by in situ hybridization (Fig. 3J and K). In agreement with a previous study (Ghoul-Mazgar et al., 2005), Dlx3 transcripts were detected in the osteoblasts and newly formed osteocytes, however, we also detected Dlx3 mRNA expression in well differentiated osteocytes (Fig. 3K).

To evaluate the ability of Dlx3 to regulate osteoblast differentiation, we tested the effect of overexpression of Dlx3 in primary chick calvarial fibroblasts (cCF) and osteoblasts (cCOB). To obtain cCF, we plated the first fraction of cells derived from sequential trypsin/collagenase digestion of calvariae from 15 day old chick embryos. When grown in differentiating conditions, cCF fail to differentiate into osteoblasts, ie. they do not express markers of late bone differentiation markers such as osteocalcin or form mineralized nodules (Tadic et al., 2002), but often peel off the dish several days after they reach confluence. In contrast, cCF cultures overexpressing Dlx3 displayed a different morphology than control cells. They appeared more cuboidal and formed mineralized nodules at day 12, while control cells infected with RCAS retained a fibroblastic spindle shape (data not shown).

After infection with RCAS(A) Dlx3, cCF cultures differentiated into osteoblasts, with increased AP staining at day 12 and increased von Kossa staining at days 12 and 14 (Figure 4C). Upregulation of type I collagen and osteocalcin was also observed at days 12 and 14 (Fig. 4A). CCOB, obtained from fractions 2–4 of these same calvarial digests, were also infected with RCAS Dlx3. More extensive von Kossa staining was observed in infected cells along with upregulation of type I collagen and osteocalcin at day 12 (Fig. 4B, C) when compared to control cultures.

In addition to our studies in chick calvarial osteoblasts, we also wished to determine whether mammalian osteoblast differentiation was regulated by Dlx proteins. We have utilized bone marrow derived stromal cells that originate from a different developmental lineage than calvarial osteoblasts. We isolated MSC from β-AKE mice, a transgenic model that expresses the receptor for the RCAS(BP)A retroviral vector. MSC cultures were transduced with vectors expressing Dlx3 or Dlx5 and RNA was harvested at various time points. Northern blot analysis was carried out to test the expression of osteocalcin and bone sialoprotein (Fig. 5). On day 11 of culture both Dlx3 and Dlx5 induced expression of both bone markers. At later time points, the levels of both markers were increased. Similar induction effects were also observed in mouse calvarial osteoblast cultures (data not shown). It is interesting that Dlx5 generally induced BSP more strongly than osteocalcin, while Dlx3 induced osteocalcin more strongly than BSP. This was observed in several experiments with both chick and mouse osteoblasts.

Cloning Dlx6 and its effects on osteoblast differentiation

We initially cloned chick Dlx6 cDNA into the RCAS retroviral vector. Calvarial cultures from 15 day old chicken embryos were infected by virus expressing Dlx6 protein. Northern blot analysis showed up-regulation of type I collagen and osteocalcin (Fig. 4D). Increased mineralization is also detected by von Kossa staining (Fig. 4C).

Although chick Dlx6 appears to have a relatively modest effect on osteoblast differentiation, we considered that mouse Dlx6 may be more potent, because of the strong additional effects of the Dlx6 knock out when combined with the Dlx5 knock out and due to significant sequence divergence between the mouse and chick genes. Therefore, we assessed the ability of mouse Dlx6 to stimulate osteoblast differentiation. In preparation to these studies we become aware that no definitive complete mouse Dlx6 cDNA sequence was available. GenBank annotation of the Dlx6 gene included a computer analysis prediction of a translation initiation site at 49 amino acids upstream of its homeobox domain. However, in frame genomic sequences upstream of this predicted initiation codon encode a domain containing multiple repeated glutamines and prolines which is worthy of note. The function of such poly-glutamine/poly-proline tracts in transcriptional regulation is not clear, but it may be active in protein binding and related to cell apoptosis (Perutz et al., 1994). Expansions of poly-glutamine tracts in proteins that are expressed in the central nervous system cause neurodegenerative diseases (Ferro et al., 2001). The general transcription initiator, TATA box binding protein (TBP), contains N-terminus poly-glutamine repeats which regulate C-terminus DNA binding activity (Nakamura et al., 2001). Pfeffer et al. compared the human Dlx6 genomic sequence to the Xenopus and zebrafish genomic sequences and suggested that the human Dlx6 protein included the long poly-glutamine and poly-proline tracts (Pfeffer et al., 2001). Information obtained from human mRNA and mouse genomic sequences were used to predict the mouse Dlx6 translation start site, however neither mouse mRNA nor protein studies were presented. Therefore we decided to clone the mouse Dlx6 cDNA based on this information.

Primers were designed according to the mouse genomic sequence as indicated to amplify the entire coding region (Fig. 6A). Two PCR products were obtained using primers P1 and P2, a fragment of around 1 kb (GenBank accession number **EF535989**) as predicted by Pfeffer et al. (Pfeffer et al., 2001), and a smaller fragment of around 0.8 kb (GenBank accession number **EF535990**) (Fig. 6C). Primers P3 and P2 produced only one PCR product, suggesting that the sequence variance is in the putative first exon region (Fig. 6D). Sequencing of the PCR product suggested that 146bp of the first exon was deleted and the predicted translational reading frame had a premature stop codon after amino acid 74. The shorter Dlx6 RNA was found in several RNA preps from different tissues. Although initially we were not sure of the significance of this PCR product, we later acquire evidence to demonstrate that it is likely to be a PCR artifact. We found that this band was amplified by PCR using RNA extracted from chick fibroblasts that were infected with an RCAS virus vector that expressed the 1 kb cDNA.

We wished to determine whether the size of the native form of the Dlx6 protein is consistent with translation initiation at the upstream AUG. We therefore performed Western blot on nuclear extract from mouse calvarial osteoblasts. A Dlx6 specific antibody detected a band of approximately 37 kD (Fig. 6E). This result is consistent with the predicted size of a protein initiated at 171 amino acids upstream of the homeobox domain.

To study the biological functions of Dlx6, as well as the role of the glutamine/proline repeats, we generated RCAS virus containing the 1kb and 0.8kb cDNAs; this was done before we discovered that the 0.8 kb product was a PCR artifact. We also produced a vector containing a 0.6 kb mouse Dlx6 cDNA containing only the more 3′ AUG, which produces a protein that does not contain the polyglutamine/polyproline repeat sequences. Although this form of the protein is not expressed endogenously, we used this vector to carry out an initial test of the

importance of the N-terminal region of the Dlx6 protein that contains the repeats for induction of osteoblast differentiation. Empty RCAS vector was used as a control, and the 0.8 kb expressing vector, which has not produced any biological effect in any of our experiments, in effect served as a second negative control. The phenotypes from DF1 cultures transduced with virus containing 1 kb or 0.6 kb were similar. Cells from these cultures show dramatic decreases in cell number, lose their fibroblastic phenotype and become cuboidal in shape compared to uninfected cell cultures, RCAS(A) or the 0.8 kb Dlx6 virus transduced cultures (Fig. 7A). Similar morphological changes have been observed in all DF1 cultures that have been transduced with Dlx3 or Dlx5 containing virus (data not shown).

We observed a strong induction of osteoblast differentiation when mouse marrow stromal cell cultures were transduced with 0.6 kb Dlx6 containing virus. More von Kossa positive nodules were present (Fig. 7B and C) and induction of BSP and OC expression was detected by real time PCR (Fig. 7D). Even stronger induction of osteoblast differentiation was observed from cultures transduced with the 1kb full length Dlx6 cDNA (Fig. 7D, 7E). Quantification of bone markers showed a 19 fold induction of OC and a 10 fold induction of BSP expression in day 18 cultures (Fig. 7F). In other experiments the induction of osteoblast markers by the full length sequence was not as great, while the level of induction by the 0.6 kb form was more consistent. This could be because the 1kb full length Dlx6 cDNA is not as consistently expressed from the RCAS vector as the 0.6 kb cDNA (data not shown). This could be a reason for lower induction of differentiation in some cultures transduced by full length Dlx6. Our studies indicate that the poly-glutamine/poly-proline repeat domain is not necessary for transcriptional activation of bone differentiation markers; however it may enhance the strength of induction.

DISCUSSION

Previous Dlx5 overexpression studies showed that Dlx5 can induce osteoblastic differentiation in osteoblastic cell culture (Miyama et al., 1999; Ryoo et al., 1997; Tadic et al., 2002). It has also been shown that Dlx genes are mediators of the BMP2 induction of osteoblast regulatory genes including Runx2 and Osterix (Harris et al., 2003; Lee et al., 2003a; Lee et al., 2003b).

Dlx5 knock-out mice suffer from craniofacial and sensory capsule skeletal defects (Acampora et al., 1999; Depew et al., 1999), while simultaneous disruption of Dlx5 and Dlx6 in mice results in a more severe craniofacial phenotype (Robledo et al., 2002). Knockouts of Dlx1 and Dlx2 show primarily craniofacial defects (Qiu et al., 1997; Qiu et al., 1995). Although many of the effects of Dlx gene knockouts on the skeleton are patterning defects, these studies suggest that Dlx genes also play a direct role in stimulating osteoblast differentiation. The effects of individual Dlx genes may be compensated for by expression of other Dlx genes in differentiating osteoblasts.

To evaluate this possibility, we examined the relative expression of the various Dlx genes in osteoblasts at different stages of differentiation. Our initial prediction would be that genes that are expressed at higher levels in differentiating osteoblasts are more likely to be important for differentiation. In situ hybridization, Northern blot or standard PCR cannot be used to make reliable comparisons of expression levels of different genes, because the efficiency of detection of different mRNAs is difficult to assess. Real time PCR has a greater potential to allow quantitative comparison of different mRNA levels, because the efficiency with which primer pairs amplify different mRNAs can be assessed and optimized so that they are very close to 100%. In unsorted calvarial osteoblast cultures, Dlx1 and Dlx4 mRNAs were almost non-detectable, while Dlx2, Dlx5 and Dlx6 were expressed at comparable levels to each other, and their expression in general was somewhat upregulated concomitant with the maturation of the cultures. Dlx3 showed the highest expression level in the most mature osteoblast cultures. We confirmed the presence of Dlx3 in osteoblasts using in situ hybridization, Northern blot and

PCR. High resolution in situ hybridization demonstrated expression of Dlx3 in osteobasts, pre-osteocytes, and some mature but not all osteocytes. This is in partial contrast to the studies of (Ghoul-Mazgar et al., 2005) who showed Dlx3 expression in osteoblasts, immature osteocytes but not mature osteocytes. Thus we speculate that Dlx3 may play a role in regulating osteocyte function.

Osteoblast cultures contain mixed cell types. Although late stage calvarial cultures include increasingly differentiated osteoblasts, a significant proportion of the cells are not in the osteoblastic lineage. The study of gene expression in a whole cell population can be misleading. To study Dlx genes expression in pure cell types, we analyzed FAC sorted cells.

The use of an osteoblast lineage specific promoter to drive GFP expression provides us with the ability to separate pre-osteoblasts, mature osteoblasts plus osteocytes, and osteocytes alone from cultures with mixed cell types. A model of our current belief, based on the data from FAC sorted cells concerning changes in the levels of Dlx mRNAs during osteoblast differentiation is shown in Fig. 8. Some of these conclusions are more strongly supported by the evidence than others. The most strongly supported conclusions are that Dlx3 mRNA is present at the highest levels in differentiated cultured calvarial osteoblasts and osteocytes, and shows the greatest degree of upregulation during differentiation. Our real time PCR studies are supported by microarray studies showing that Dlx3 is very highly expressed in Col2.3GFP positive cells from differentiated calvarial osteoblast cultures, but is present at much lower levels in 17 day cultures and in Col2.3GFP negative osteoblasts (Kalajzic et al., 2005). In general, expression of Dlx2, Dlx5 and Dlx6 were increased as cultures matured, peaking in the day 17 Col2.3GFP negative cells; however, their expression is decreased in more differentiated Col2.3GFP positive osteoblasts. It should be noted that the Col2.3GFP negative population contains both immature osteoblasts and cells that are not in the osteoblast lineage. We believe that it is probable that Dlx genes are primarily expressed in osteoblast lineage cells in calvarial cultures, so if anything we are probably underestimating the expression of Dlx genes in immature osteoblasts, but this has not been proven.

There appear to be subtle differences in the expression of Dlx2, 5 and 6. Dlx2 appeared to be expressed slightly earlier than 5 and 6, and to be downregulated more strongly at later stages. Dlx6 expression seemed to be maintained at higher levels at more mature differentiation stages.

RCAS retroviral expression in chick and mouse calvarial osteoblasts and in mouse MSC cultures showed that chick Dlx3 appears to be a potent inducer of osteoblast differentiation, with activity comparable to Dlx5. A definitive comparison of the potency of Dlx3 versus Dlx5 cannot be made, because we do not have antibodies to chick Dlx3 or 5 to assess levels of protein expression. We chose not to fuse an epitope tag to our expressed proteins because of concern that the tag might affect the function of the protein. It is interesting that Dlx3 seemed to consistently activate expression of osteocalcin more strongly than BSP, while Dlx5 more strongly induces BSP. This is consistent with our observation that Dlx3 is highly upregulated in differentiated cells, while Dlx5 is present at earlier stages of differentiation and is downregulated during terminal osteogenic differentiation.

While this manuscript was in preparation, a study was published which also suggests that Dlx3 plays an important role in inducing osteoblast differentiation (Hassan et al., 2004). These studies showed that Dlx3 is present in osteoblasts, stimulates osteoblastic differentiation, and binds to the osteocalcin promoter. Their chromatin immunoprecipitation studies indicate that Dlx5 displaces Dlx3 from the OC promoter in late stages of differentiation. This contrasts with our results indicating that Dlx3 mRNA is most strongly induced at later stages of differentiation, and our observation that Dlx3 appears to stimulate OC expression more strongly than BSP. At this point the reasons for this discrepancy are not clear; possibilities

include that Dlx3 and Dlx5 protein levels are post-transcriptionally regulated in an inverse manner during late differentiation, or that other transcriptional factors induced late in osteoblast differentiation favor binding of Dlx5 but not Dlx3 to the osteocalcin promoter.

Another important conclusion of these studies is that Dlx6 mRNA is expressed at relatively high levels in osteoblasts, and can stimulate oseoblastic differentiation. This is significant because Dlx6 is in a different class of Dlx genes from the Dlx genes that have been previously shown to be capable of inducing osteoblast differentiation, and thus the protein sequence outside of the homeodomain show much less sequence conservation than exists between Dlx3 and Dlx5. In addition, mouse Dlx6 contains a fairly long gluamine-proline repeat; the chick protein contains glutamine and proline repeats, but they are much shorter. Variations in repeat length have been detected in the analogous sequence in the human gene, and it has been suggested to have the potential for CAG repeat mediated expansion (Ferro et al., 2001). We speculate that, since stretches of glutamines and prolines have been shown to have transcriptional activation activity, the presence of this domain may contribute to the possible greater ability of mouse Dlx6 to activate osteoblast differentiation than chick Dlx6. Our initial studies on primary mouse marrow stromal cultures transduced by Dlx6 expressing virus indicated that, while the poly-glutamine/proline repeats are not necessary for the proteins transcriptional regulation of bone differentiation markers, they may enhance the strength of induction of these markers. A more in depth series of studies will be required to assess the role of these repeats in the ability of Dlx6 to promote osteogenesis. It is possible that the repetitive RNA sequence that encodes the amino acid repeats may not be stable during the retroviral life cycle of the RCAS vectors, so a different expression system may be needed to assess Dlx6 function. In any case, our results showing the osteogenic ability of Dlx6, coupled with our observation that Dlx6 is expressed at similar or greater levels than Dlx5 in osteoblasts, may help explain the observation that the Dlx5/Dlx6 double knockout has greater defects in skeletal development than the single Dlx5 knockout.

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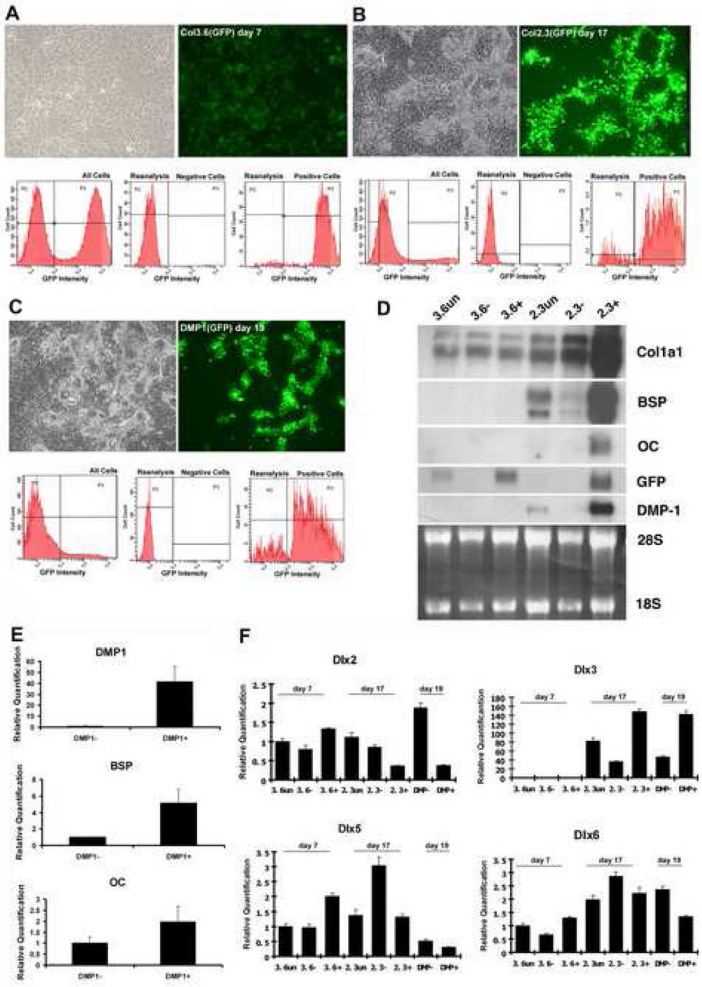

Fig. 1. Dlx gene expression in lineage marker FACS sorted cells

Calvarial osteoblast cultures were derived from transgenic mice containing GFP driven by the 3.6 kb rat Col1a1 promoter (Col3.6 GFP), the 2.3kb Col1a1 promoter (Col2.3 GFP) or the DMP1 promoter (DMPGFP). Calvarial osteoblasts were sorted according to GFP markers at day 7 for pOBCol3.6GFP cultures (A), at day 17 for pOBCol2.3GFP cultures (B) or at day 19 for DMP1GFP cultures (C).

(A) Upper panel shows phase contrast and florescent image of day 7 calvarial culture from Col3.6GFP transgenic mice. Col3.6 GFP positive cells (3.6+) represent pre-osteoblasts. Lower panel shows re-analysis of sorted populations. (B) Day 17 calvarial osteoblast culture from Col2.3GFP mice and re-analysis of cells after sorting. Col2.3 GFP positive cells (2.3+) represent mature osteoblasts. (C) Day 19 calvarial osteoblast culture from DMP1GFP mice and re-analysis of sorted cells. DMP1 GFP positive cells (DMP1+) represent pre-osteocytes and osteocytes. (D) Northern blot analysis of RNA extracted from GFP positive, negative populations or unsorted cells generated from (A and B) to detect osteoblast markers, showing enrichment in GFP+ cells. Col1a1, αI type I collagen; BSP, bone sialoprotein; OC, osteocalcin; DMP-1, dentin matrix protein 1. (E) Real time PCR analysis of RNA extracted from GFP positive and negative populations in DMP1 cultures showed in C. DMP1-GFP positive cells have 40 times enrichment of DMP1 gene expression, around five times more BSP expression and two times the amount of OC expression compared to the negative cells. (F) Quantitative real time RT-PCR was performed to study Dlx gene expression in the sorted cells. For each

gene, expression levels were normalized to the expression levels of unsorted day 7 populations. Error bars are the standard deviation of triplicate assays from the same RNA sample.

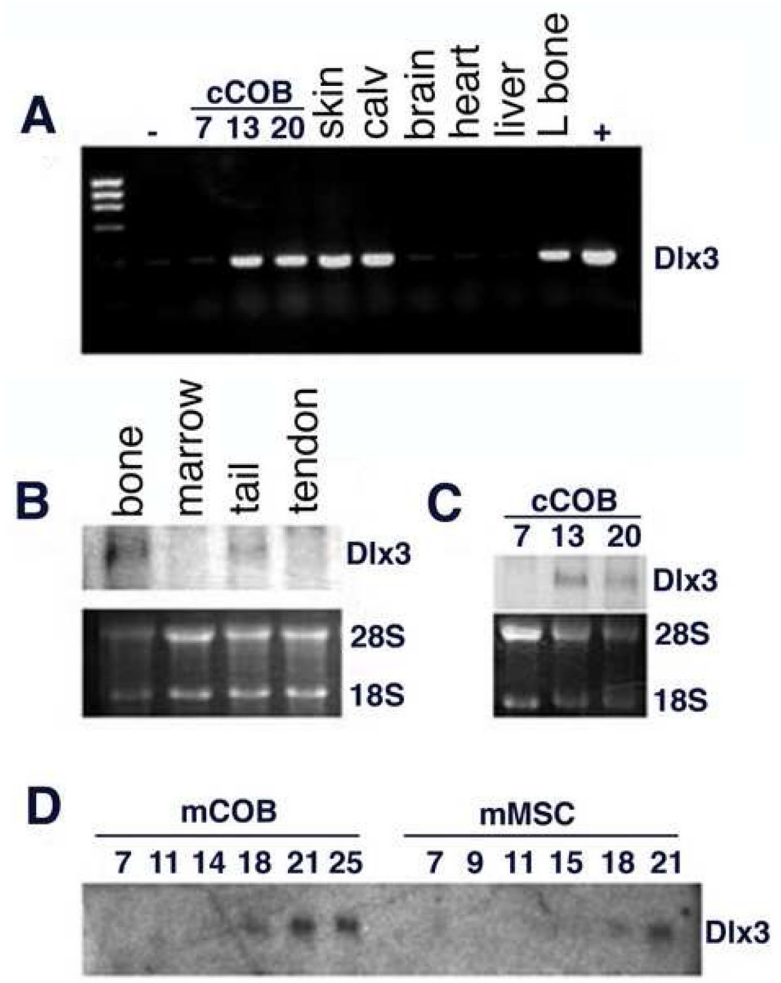

Fig. 2. Dlx3 mRNA presence in bone and osteoblast culture

RT-PCR was performed on total RNA extracts from different tissues and cultured chicken primary calvarias for detection of Dlx3 mRNA, as well as Northern blot analysis of cultured primary cells from chicken and mouse calvarias, mouse bone marrow stromal cells, long bone, bone marrow, tail and tendon.

(A) RT-PCR showing the presence of Dlx3 mRNA in chick calvaria, long bone, skin and at more differentiated stages of cultured osteoblastic cells (cCOB), but not in brain, heart or liver. (B) Northern blot analysis detected Dlx3 mRNA in mouse long bone and tail but not in bone marrow and tendon. (C) Northern blot of chicken calvarial cultures showing the presence of Dlx3 mRNA at days 13 and 20. D) Northern blot of mouse calvarial (mCOB) and marrow stromal (mMSC) cultures showing the presence of Dlx3 signal from day 18.

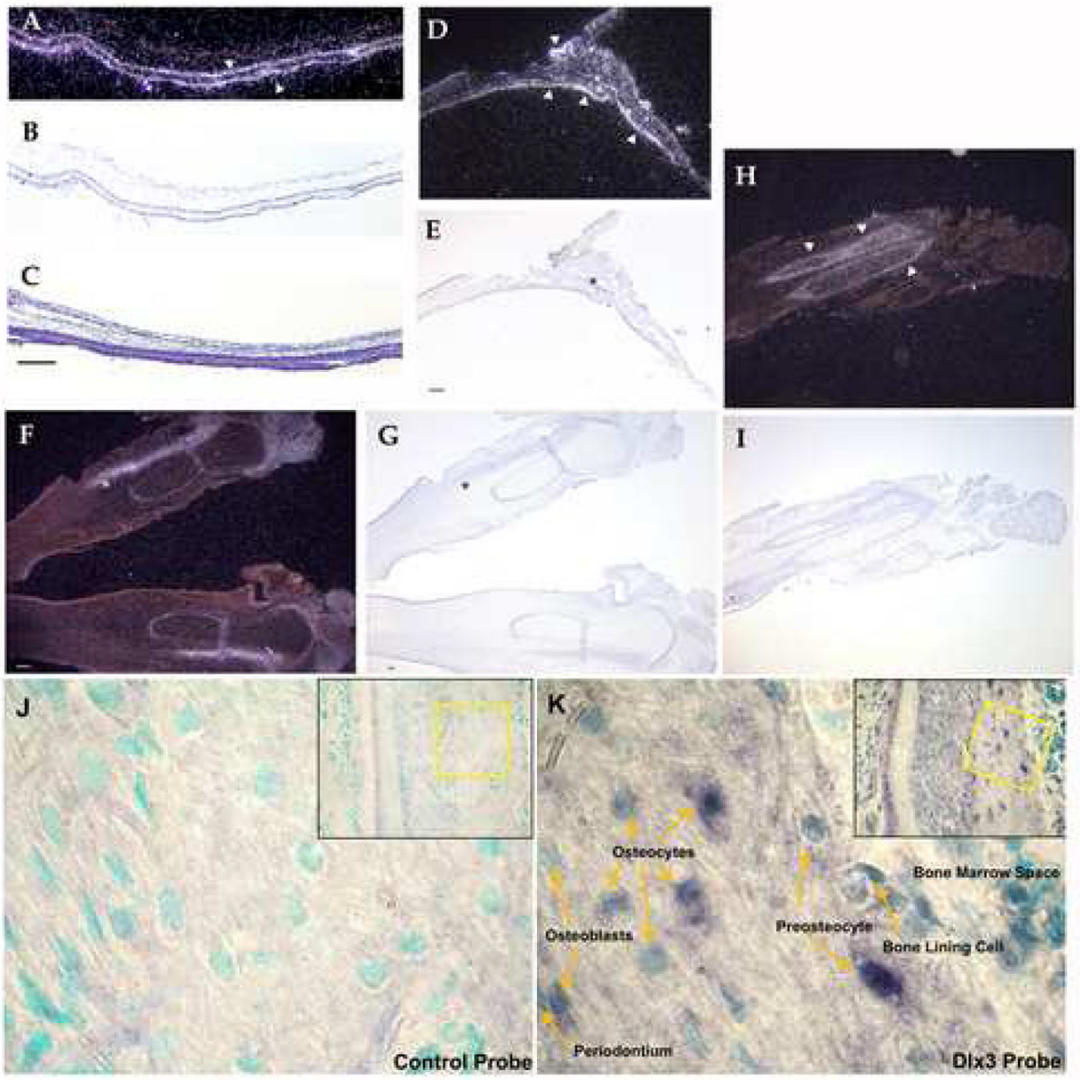

Fig. 3. Expression of Dlx3 in developing chick calvaria, and mandible and mouse mandible

Dark-field (A, D) and corresponding bright-field (B, E) images of sagittal sections through whole calvaria from 15-day-old chick embryos hybridized with the ~600 bp antisense Dlx3 riboprobe. The hybridization signal for *Dlx3* is present in the osteogenic cells in the periosteum (indicated by arrowheads) covering the newly formed bone, and in the osteogenic cells lining the bone marrow spaces (indicated by asterisk) in the more advanced stages of calvarial bone formation. (C) Adjacent section to section shown in A stained with hematoxylin and eosin. Dark-field (F, H) and corresponding bright-field (G, I) images of sagittal sections of mandibles are from 8- (F, G) and 10-day-old (H, I) chick embryo. *Dlx3* is expressed in condensing preosteogenic mesenchyme (indicated by asterisk) and osteogenic cells in the periosteum (indicated by arrowheads). Scale bars: 100 μm.

Dlx3 transcripts were detected in the sagittal section of 18 day old mouse mandible (J, control probe and K, Dlx3 probe). Mouse Dlx3 mRNA can be detected in osteoblasts, preosteocytes and osteocytes.

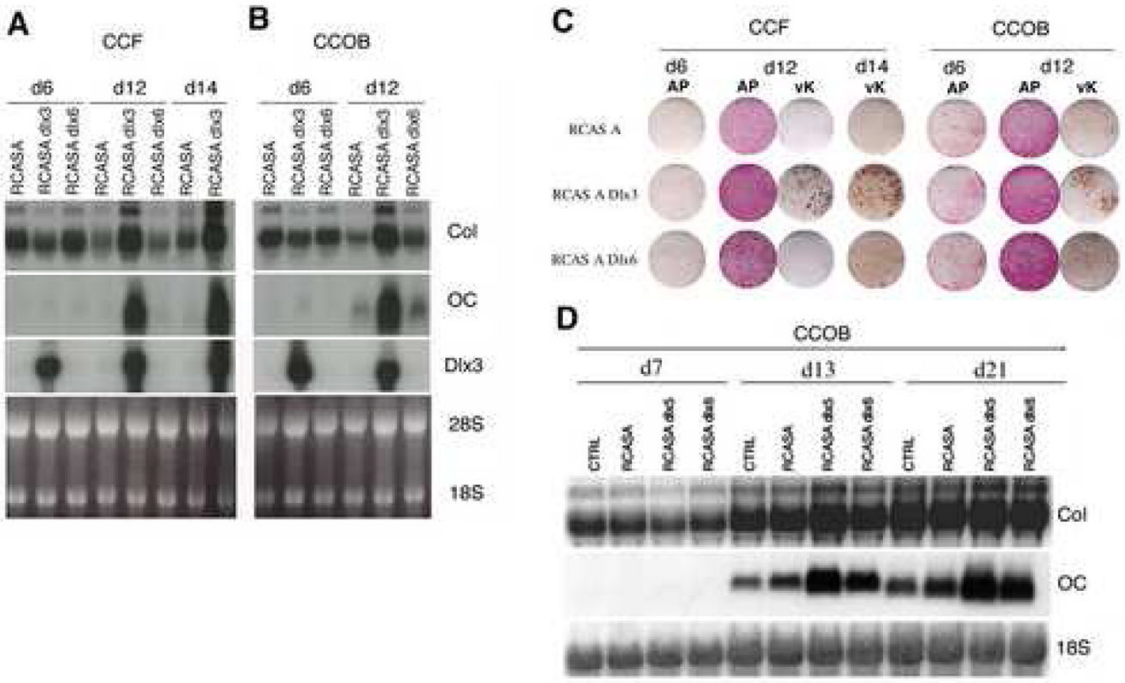

Figure 4. Effects of retroviral overexpression of Dlx3 and Dlx6 on chicken calvarial fibroblast and osteoblast cultures

cCF - Primary calvarial fibroblast (fraction 1) and cCOB - osteoblast cultures (fractions 2–4) were infected with RCAS Dlx3, RCAS Dlx6 and RCAS (control) and osteoblast differentiation was monitored by alkaline phosphatase staining, von Kossa staining and Northern blot analysis. Markers of bone differentiation, collagen and osteocalcin are strongly upregulated in cells treated with Dlx3 in both cCF (A) and cCOB cultures (B). Dlx6 also induced collagen and osteocalcin in cCOB culture at day 12 (B) and day 13, as well as day 21 (D). Alkaline phosphatase and von Kossa staining (C) reflect results of Northern blot analysis in (A) and (B). Dlx3 and both 6 induce mineralization.

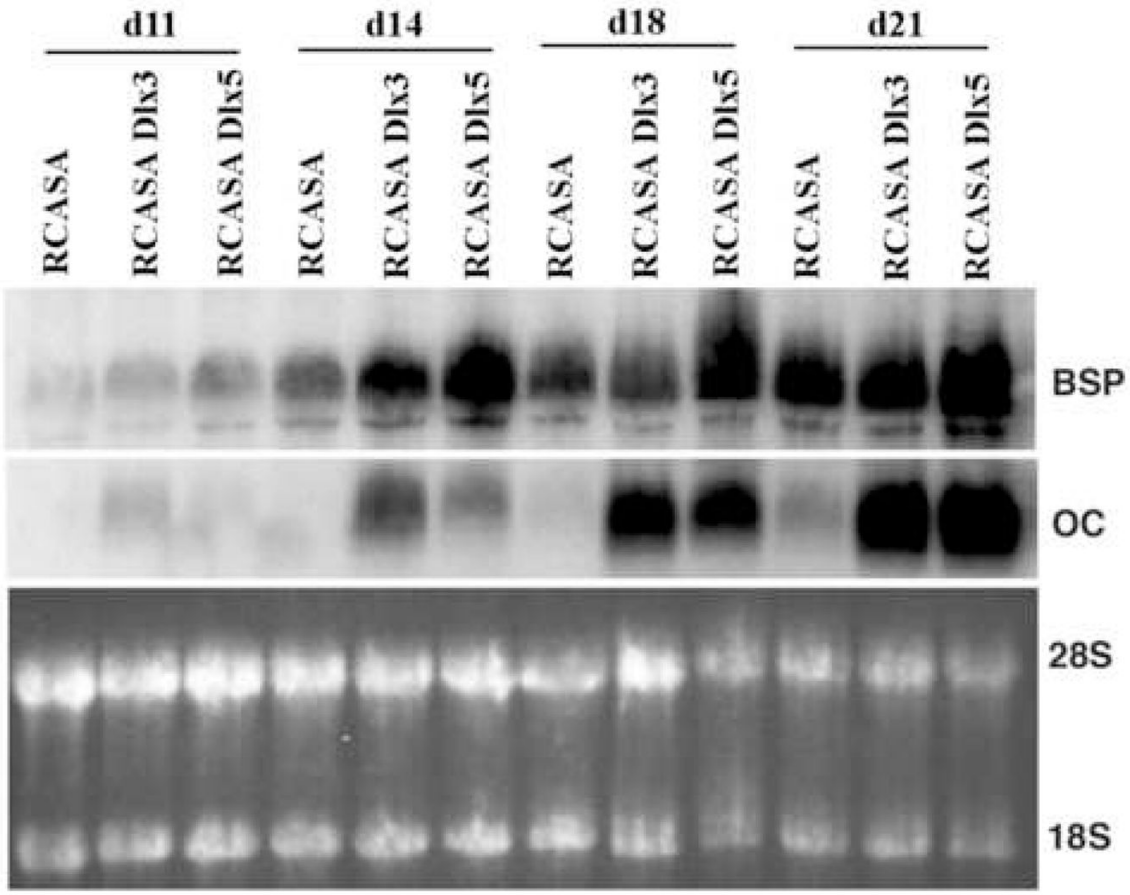

Fig. 5. Dlx3 induces mouse marrow stromal cell differentiation

Mouse marrow stromal cell cultures were infected with virus containing empty vectors or Dlx3 or Dlx5 cDNAs. Cell cultures were analyzed at different time points by Northern blot to detect the expression of bone differentiation makers. Starting from day 14, BSP and OC expression was strongly induced by Dlx3 and Dlx5. Dlx3 always induces OC expression more strongly than Dlx5, whereas Dlx5 induces BSP more strongly than Dlx3.

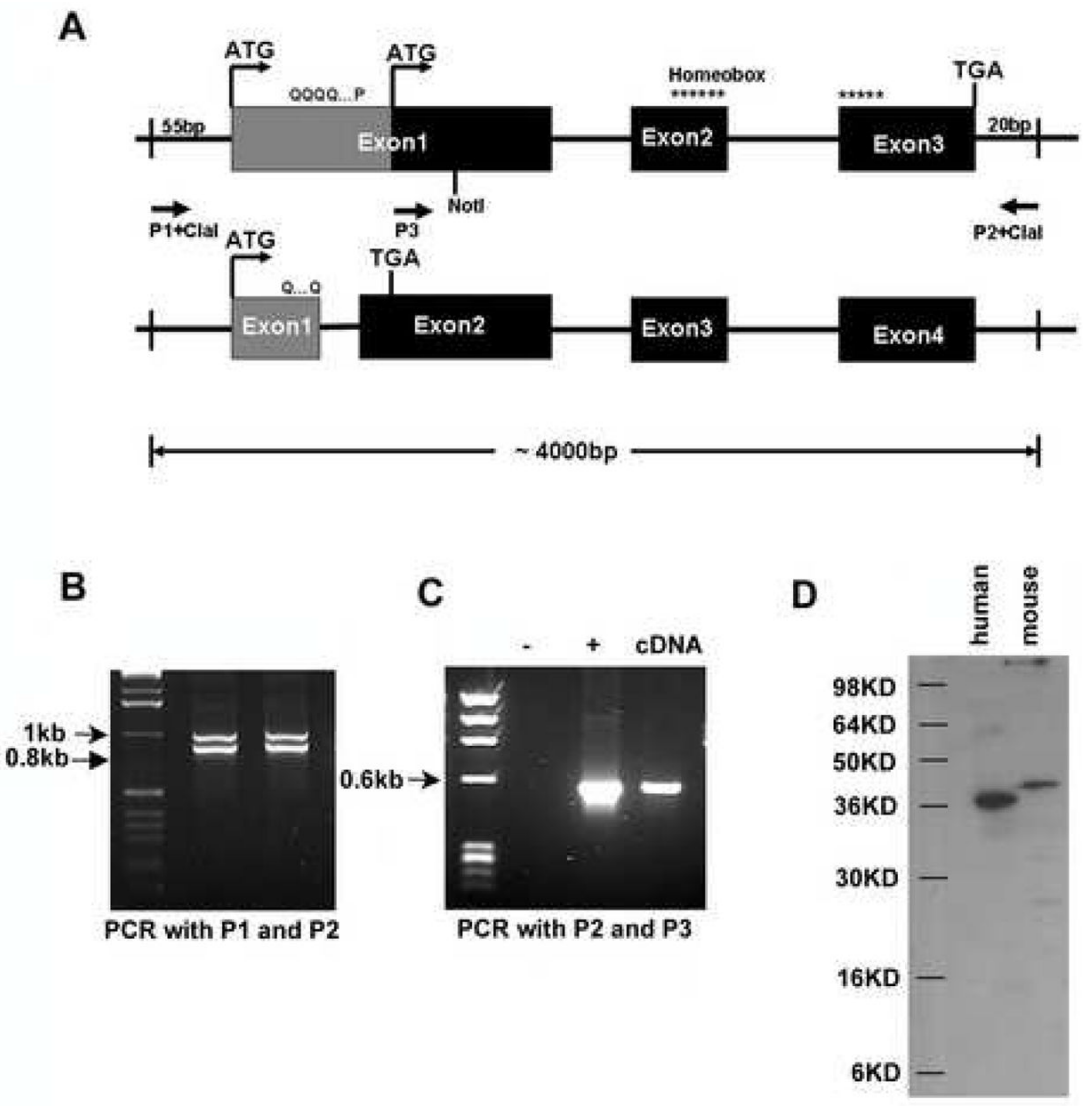

Fig. 6. Cloning mouse Dlx6

(A) Schematic of mouse Dlx6 gene and primers for cloning the cDNA. A ClaI site was added in the 3′ end for both primers P1 and P2 for cloning. Lower panel shows the 0.8 kb cDNA amplified from mouse femur RNA. (B) Two bands were amplified by RT-PCR for Dlx6 using primer P1 and P2. (C) Only one band was detected by RT-PCR using primer P3 and P2. (D) Western blot of Dlx6. Positive control, Jurkat cell lysate (human T-lymphocytes from acute T-cell leukemia, Aviva Systems Biology) and nuclear extract from day 7 mouse calvarial osteoblast cultures were blotted by Dlx6 specific polyclonal antibody. A slightly larger Dlx6 protein was detected in mouse.

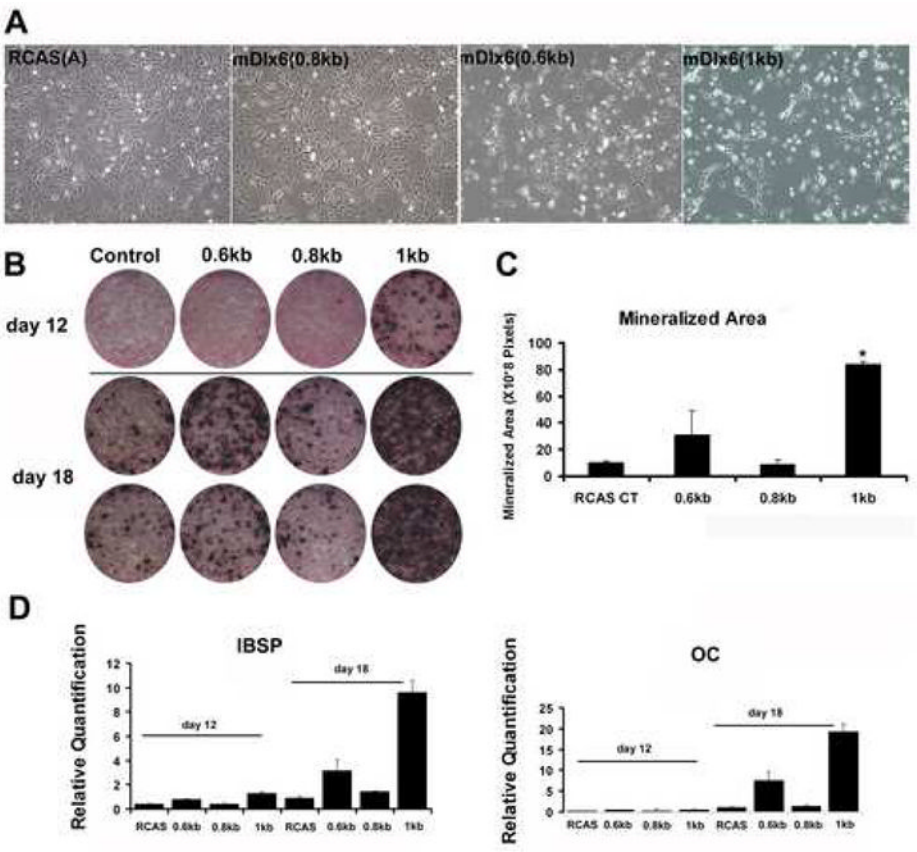

Fig. 7. Dlx6 induces mouse marrow stromal cell differentiation

RCAS virus was generated containing mouse 0.6 kb, 0.8 kb or full length 1 kb Dlx6 cDNA. Virus containing empty vector was used as control.

(A) Morphological changes in DF1 cultures when transduced with virus. Phenotypes of cells overexpressing 0.6 kb Dlx6 cDNA or 1 kb Dlx6 cDNA were similar. Cultures have decreased cell number, lose of the elongated fibroblastic phenotype, and become cubodial. Cells transduced with empty virus vector or 0.8 kb Dlx6 cDNA show no morphological changes compared with untreated cells. (B, C, D) Mouse marrow stromal cells from βAKE mice were transduced with virus containing 0.6 kb, 0.8 kb and 1 kb mouse Dlx6 cDNA. Cultures were stained for alkaline phosphatase and von Kossa at the indicated days after plating. More and stronger von Kossa staining nodules were observed in cultures overexpressing 0.6 kb and 1 kb mouse Dlx6. Mineralized areas in the day 18 cultures were quantified in figure C (* $P \leq 0.05$). (D) Real-time PCR quantification of the osteoblast differentiation makers from Dlx6 overexpressing cultures. 0.6 kb mouse Dlx6 and the full length mouse Dlx6 dramatically induce BSP and OC expression.

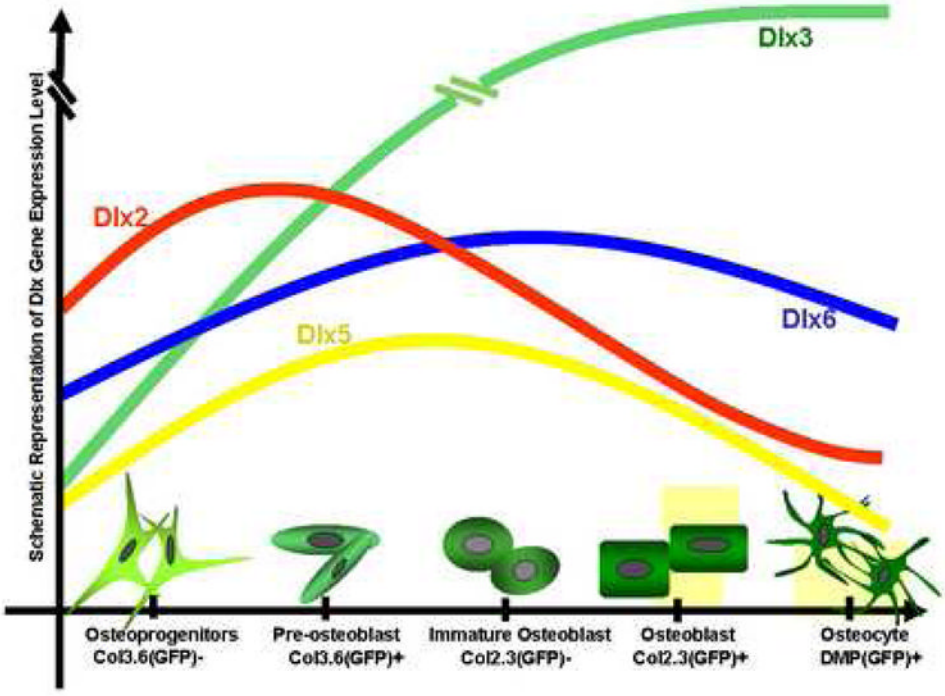

Fig. 8. Model for Dlx gene expression during osteoblast differentiation

We summarize Dlx genes mRNA expression levels during osteoblast differentiation. The horizontal axis represents the osteoblast maturation along the osteoblast lineage, which can be identified by activation of stage specific promoters. The vertical axis schematically represents the mRNA expression level of each Dlx gene. Dlx2, Dlx5 and Dlx6 have relatively higher expression in the pre-osteoblast and immature osteoblast population, whereas Dlx3 is dramatically enriched in the mature osteoblasts and osteocytes.

Table 1

mRNA Expression Pattern of Dlx Genes in mCOB

	Day 2	Day 5	Day 7	Day 14
Dlx1	1.0	0.7	0.9	1.2
Dlx2	32.1	16.2	27.8	29.5
Dlx3	12.6	4.0	28.8	980.4
Dlx4	0.1	0.2	0.7	1.5
Dlx5	3.0	3.5	6.3	19.0
Dlx6	18.6	26.1	59.2	82.6

Value was normalized to Dlx1 mRNA expression at day 2