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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 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*, *Dlx3* and *Dlx5* are more closely related to each other than they are to *Dlx1*, *Dlx4* and *Dlx6* (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-

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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* knockout 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* knockout mice are relatively normal. This raises the possibility that other *Dlx* genes compensate for the absence of *Dlx5* in these mice. Interestingly, *Dlx5*/6 knockout 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, 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*, *Dlx3* and *Dlx5*, 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 (1975) (Kalajzic et al., 2002; Tadic et al., 2002). Calvariae were subjected to four sequential 15-min digestions in an enzyme mixture containing 0.05% trypsin and 1.5 U/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 3 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 of 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, were replaced with fresh αMEM plus 10% FBS; on day 7, total medium was changed into differentiation medium (αMEM/10% FBS supplemented with 50 μg/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 3 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, these media were replaced with fresh media containing virus after 8 h, and these media were 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. Fifteen micrograms 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, USA) according to the manufacturer's instructions. von Kossa staining was utilized to assess mineralization after ALP staining by adding 5% silver nitrate solution at 1 ml/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 *Dlx6* 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. The final reaction (1–0.1 μl) 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 subcloned 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 as follows: 2.5 U of Invitrogen Pfx Taq polymerase; 5 mM of Mg^{2+}; 0.3 μM primers of each; 100 ng 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 *Cla*I 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 were 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 and 6×10^8.

Preparation of nuclear extracts and Western immunoblotting

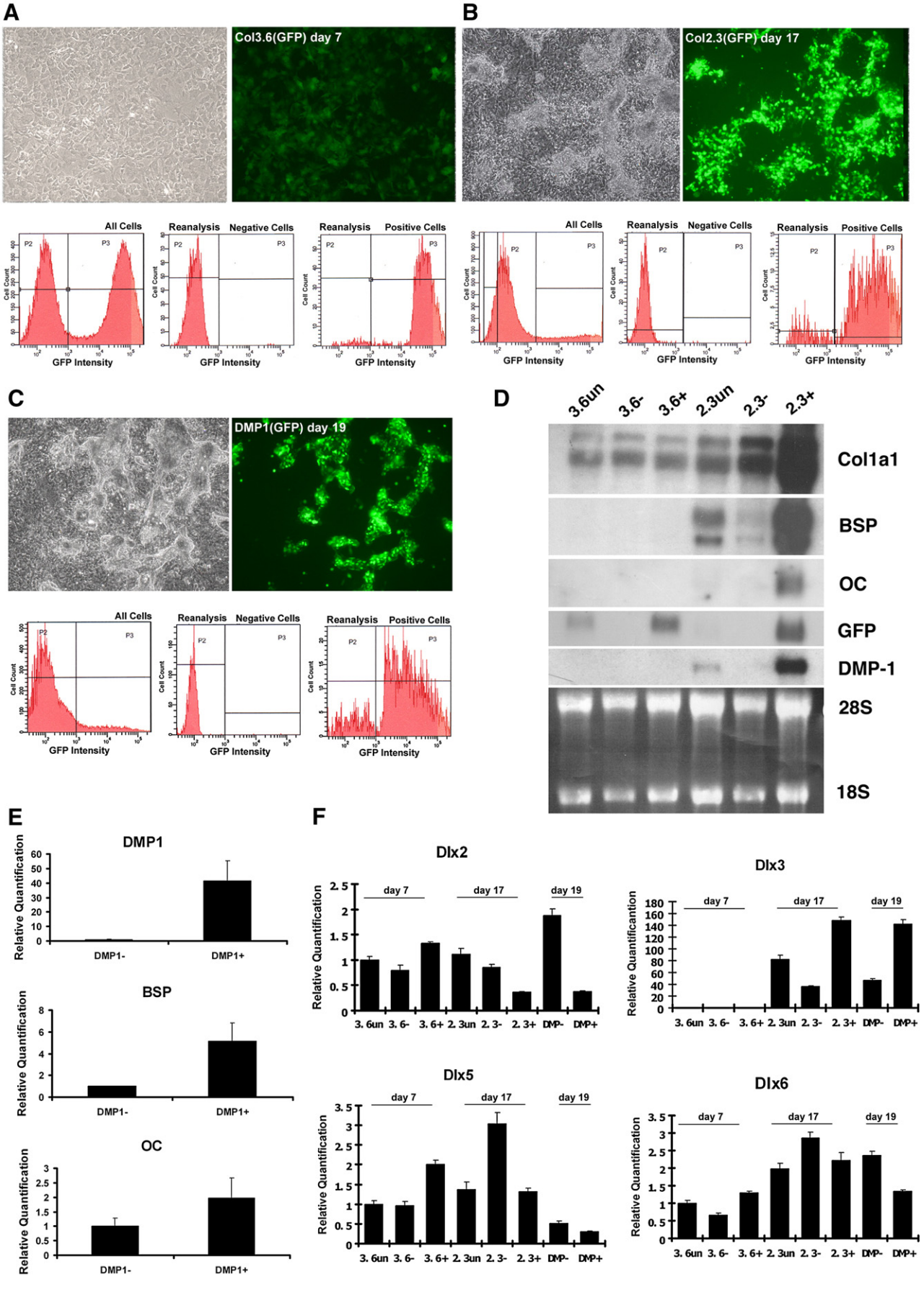
Nuclear extracts of day 7 calvarial cultures were produced based on the procedures of Shapiro et al. (1988). Twenty-five micrograms of protein was separated on SDS–PAGE gels, transferred to nitrocellulose membranes, and blots were probed with a polyclonal rabbit anti-human *Dlx6* antibody (Aviva Systems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

Table1
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.

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 2.3-kb 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 preosteoblasts. 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 preosteocytes and osteocytes. (D) Northern blot analysis of RNA extracted from GFP-positive, GFP-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 panel 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.


A
Col3.6(GFP) day 7
All Cells
Reanalysis Negative Cells
Reanalysis Positive Cells
GFP Intensity
B
Col2.3(GFP) day 17
C
DMP1(GFP) day 19
D
3.6un
3.6-
3.6+
2.3un
2.3-
2.3+
Col1a1
BSP
OC
GFP
DMP-1
28S
18S
E
DMP1
BSP
OC
Relative Quantification
DMP1-
DMP1+
F
Dlx2
Dlx3
Dlx5
Dlx6
day 7
day 17
day 19
Relative Quantification
Relative Quantificantion
3. 6un 3. 6- 3. 6+ 2. 3un 2. 3- 2. 3+ DMP- DMP+

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-old 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 [^{32}P] 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-old mouse mandible, paraffin sections were prepared as described above. *Dlx3* antisense and sense RNA probes used for hybridization were prepared from an *Xho*I and *Not*I 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 min. Seventeen-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 min. 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 *Dlx4* were expressed at levels that were so low that they may represent background PCR signal. *Dlx2* 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 preosteocyte and osteocyte stages (Figs. 1D and 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 (Figs. 1A–E). We sorted different stages of osteoblasts from primary calvarial 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 downregulated 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 (Figs. 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 the brain, the heart or the liver. Northern blot analysis of mouse tissues showed that *Dlx3* signal is present in bone and tail (Fig. 2B); expression in tail may reflect expression in both vertebral bone and skin. In chick calvarial cultures *Dlx3*

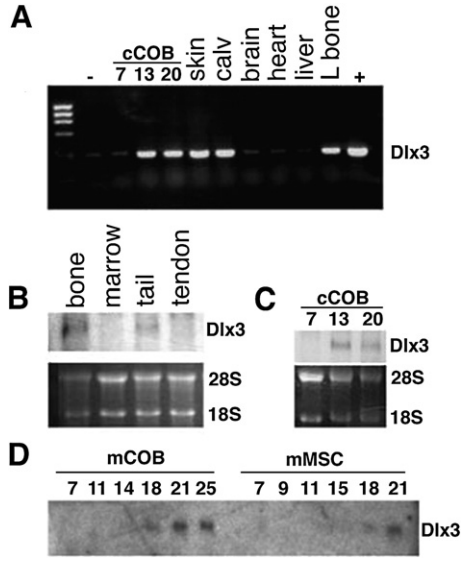


Fig. 2. *Dlx3* mRNA presence in bone and osteoblast culture. RT-PCR was performed on total RNA extracts from different tissues and cultured chicken primary calvariae for detection of *Dlx3* mRNA, as well as Northern blot analysis of cultured primary cells from chicken and mouse calvariae, mouse bone 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 the brain, the heart or the 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.

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 (Figs. 3A–E). The *Dlx3* signal was also present in condensing preosteogenic mesenchyme and periosteal osteogenic cells of mandible sections from 8- to 10-day-old chick embryos (Figs. 3F–I). We further studied *Dlx3* expression in 18-day-old mouse mandible by in situ hybridization (Figs. 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, i.e., 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 (Fig. 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 (Figs. 4B and 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 upregulation 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* knockout when combined with the *Dlx5* knockout 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 became 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

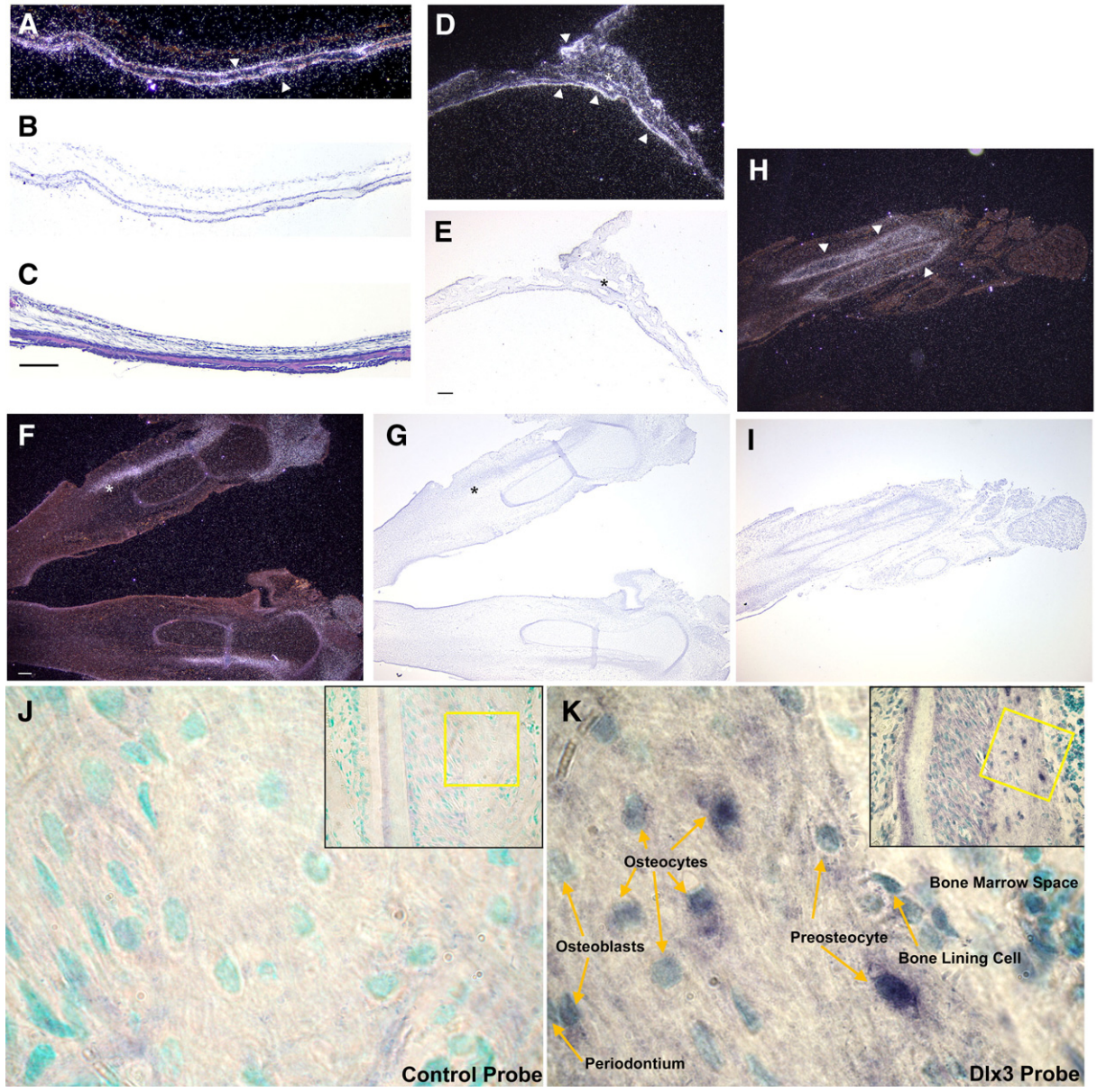


Fig. 3. Expression of *Dlx3* in developing chick calvaria and mandible and mouse mandible. Dark-field (A, D) and corresponding bright-field (B, E) images of sagittal sections through whole calvaria from 15-day-old chick embryos hybridized with the ~600-bp antisense *Dlx3* riboprobe. The hybridization 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 panel 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-day-old (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.

sequences upstream of this predicted initiation codon encode a domain containing multiple repeated glutamines and prolines that 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 that regulate C-terminus DNA binding activity (Nakamura et al., 2001). Pfeffer et al. (2001) 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. 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.

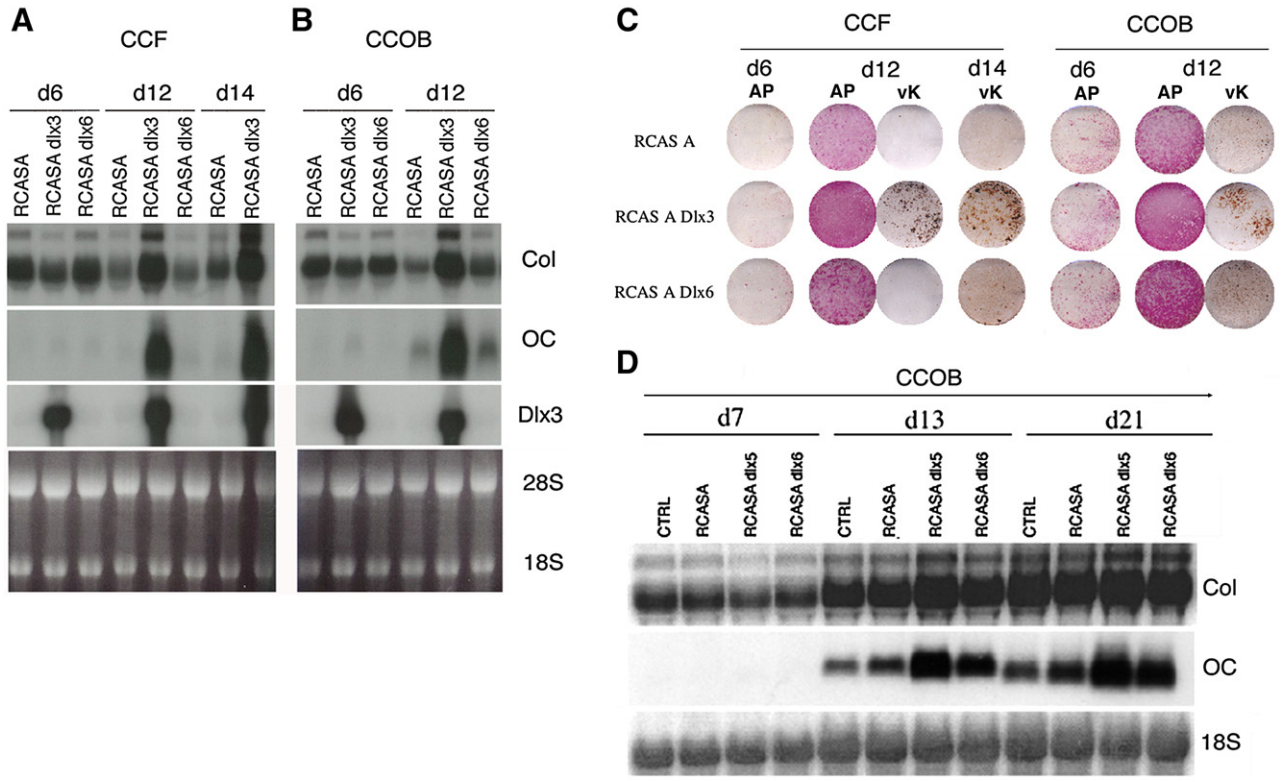


Fig. 4. Effects of retroviral overexpression of *Dlx3* and *Dlx6* on chicken calvarial fibroblast and osteoblast cultures. cCF—primary calvarial fibroblast (fraction 1) and cCOB—osteoblast cultures (fractions 2–4) were infected with RCAS Dlx3, RCAS Dlx6 and RCAS (control), and osteoblast differentiation 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 panels A and B. Both *Dlx3* and *Dlx6* induce mineralization.

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,

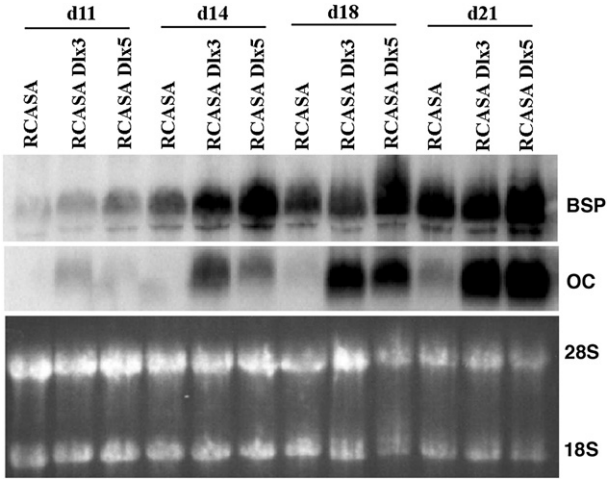


Fig. 5. *Dlx3* induces mouse marrow stromal cell differentiation. Mouse marrow stromal cell cultures were infected with virus containing empty vectors or *Dlx3* or *Dlx5* cDNAs. Cell cultures were analyzed at different time points by Northern blot to detect the expression of bone differentiation makers. 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*.

a fragment of around 1 kb (GenBank accession number EF535989) as predicted by 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 146 bp 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 kDa (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 1-kb and 0.8-kb cDNAs; this was done before we discovered that the 0.8-kb product was a PCR artifact. We also

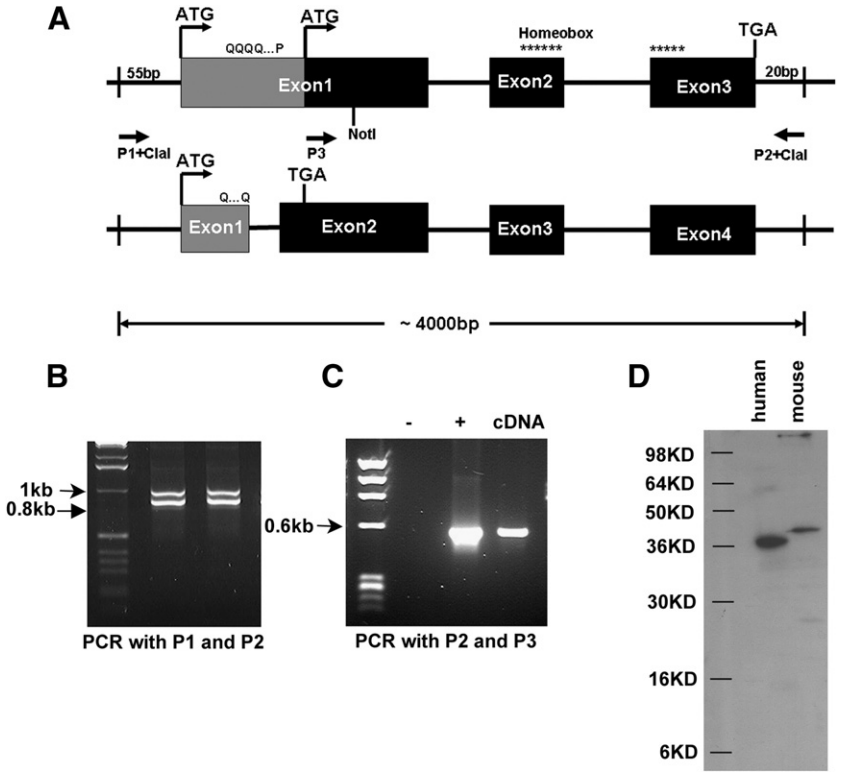


Fig. 6. Cloning mouse *Dlx6*. (A) Schematic of mouse *Dlx6* gene and primers for cloning the cDNA. A *Cla*I site was added in the 3′ end for both primers P1 and P2 for cloning. Lower panel shows the 0.8-kb cDNA amplified from mouse femur RNA. (B) Two bands were amplified by RT-PCR for *Dlx6* using primer P1 and P2. (C) Only one band was detected by RT-PCR using primer P3 and P2. (D) Western blots 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.

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 poly-glutamine/poly-proline 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 (Figs. 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 1-kb full-length *Dlx6* cDNA (Figs. 7D and E). 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 1-kb 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,b).

Dlx5 knockout 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

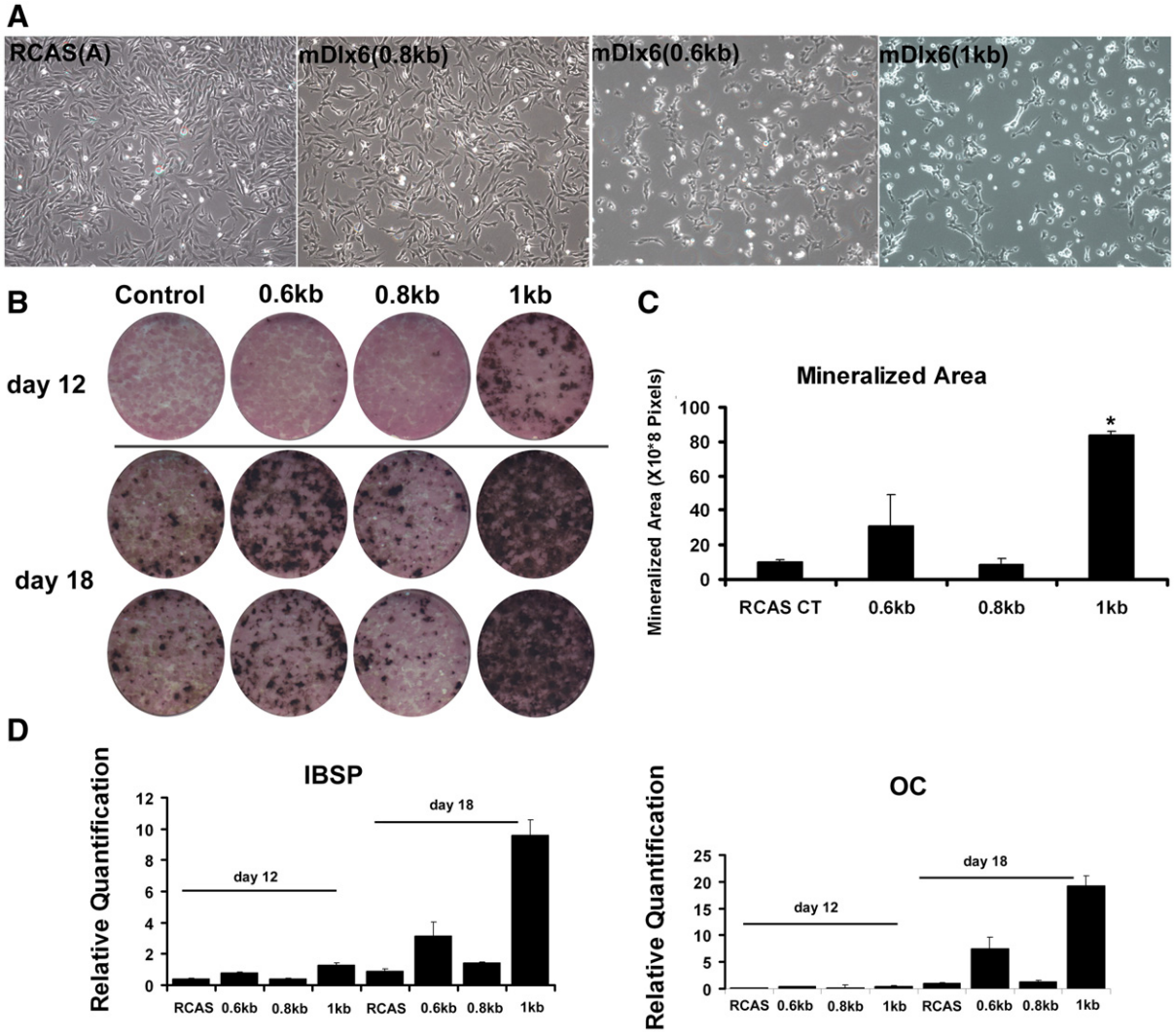


Fig. 7. *Dlx6* induces mouse marrow stromal cell differentiation. RCAS virus was generated containing mouse 0.6-kb, 0.8-kb or full-length 1-kb *Dlx6* cDNA. Virus containing empty vector was used as control. (A) Morphological changes in DF1 cultures when transduced with virus. Phenotypes of cells overexpressing 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 cuboidal. 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 panel C (*$P \leq 0.05$). (D) Real-time PCR quantification of the osteoblast differentiation makers from *Dlx6* overexpressing cultures. The 0.6-kb mouse *Dlx6* and the full-length mouse *Dlx6* dramatically induce BSP and OC expression.

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 nondetectable, 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 osteoblasts, preosteocytes 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 preosteoblasts, 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-old cultures and in Col2.3GFP-negative osteoblasts (Kalajzic et al., 2005). In general, expressions 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*, *Dlx5* and *Dlx6*. *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

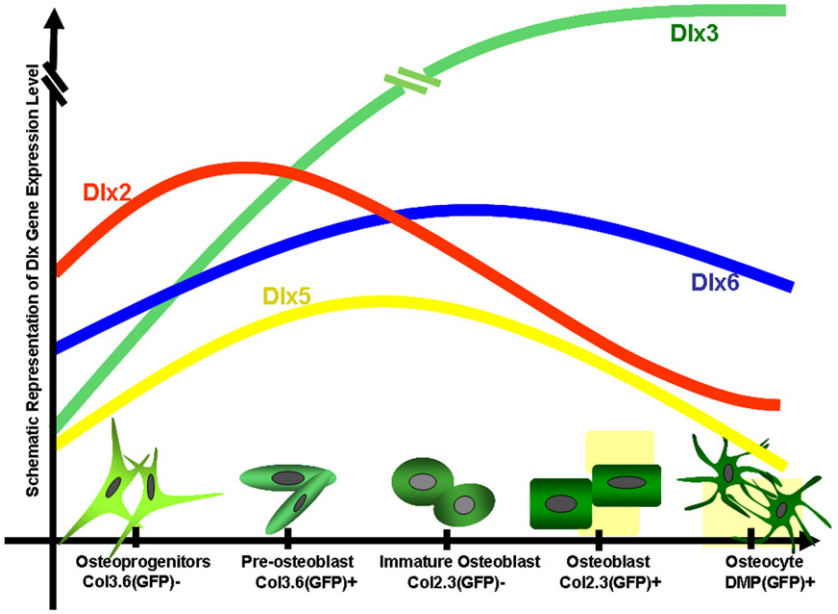


Fig. 8. Model for *Dlx* gene expression during osteoblast differentiation. We summarize *Dlx* genes mRNA expression levels during osteoblast differentiation. The horizontal axis represents the osteoblast maturation along the osteoblast lineage, which can be identified by activation of stage-specific promoters. The vertical axis schematically represents the mRNA expression level of each *Dlx* gene. *Dlx2*, *Dlx5* and *Dlx6* have relatively higher expression in the preosteoblast and immature osteoblast population, whereas *Dlx3* is dramatically enriched in the mature osteoblasts and osteocytes.

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 posttranscriptionally 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 osteoblastic 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 shows much less sequence conservation that exists between *Dlx3* and *Dlx5*. In addition, mouse *Dlx6* contains a fairly long glutamine–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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References

Acampora, D., et al., 1999. Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene *Dlx5*. Development 126, 3795–3809.

Bendall, A.J., Abate-Shen, C., 2000. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 247, 17–31.

Bryan, J.T., Morasso, M.I., 2000. The Dlx3 protein harbors basic residues required for nuclear localization, transcriptional activity and binding to Msx1. J. Cell Sci. 113 (Pt 22), 4013–4023.

Chen, X., et al., 1996. *Dlx5* and *Dlx6*: an evolutionary conserved pair of murine homeobox genes expressed in the embryonic skeleton. Ann. N. Y. Acad. Sci. 785, 38–47.

Depew, M.J., et al., 1999. *Dlx5* regulates regional development of the branchial arches and sensory capsules. Development 126, 3831–3846.

Depew, M.J., et al., 2002. Specification of jaw subdivisions by *Dlx* genes. Science 298, 381–385.

Ferro, P., et al., 2001. Are there CAG repeat expansion-related disorders outside the central nervous system? Brain Res. Bull. 56, 259–264.

Ghanem, N., et al., 2003. Regulatory roles of conserved intergenic domains in vertebrate *Dlx* bigene clusters. Genome Res. 13, 533–543.

Ghoul-Mazgar, S., et al., 2005. Expression pattern of *Dlx3* during cell differentiation in mineralized tissues. Bone 37, 799–809.

Haldeman, R.J., et al., 2004. Increased bone density associated with DLX3 mutation in the tricho-dento-osseous syndrome. Bone 35, 988–997.

Harris, S.E., et al., 2003. Transcriptional regulation of BMP-2 activated genes in osteoblasts using gene expression microarray analysis: role of *Dlx2* and *Dlx5* transcription factors. Front. Biosci. 8, s1249–s1265.

Hassan, M.Q., et al., 2004. Dlx3 transcriptional regulation of osteoblast differentiation: temporal recruitment of Msx2, Dlx3, and Dlx5 homeodomain proteins to chromatin of the osteocalcin gene. Mol. Cell. Biol. 24, 9248–9261.

Kalajzic, I., et al., 2002. Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J. Bone Miner. Res. 17, 15–25.

Kalajzic, I., et al., 2004. Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. Bone 35, 74–82.

Kalajzic, I., et al., 2005. Expression profile of osteoblast lineage at defined stages of differentiation. J. Biol. Chem. 280, 24618–24626.

Koentges, G., Matsuoka, T., 2002. Evolution. Jaws of the fates. Science 298, 371–373.

Lee, M.H., et al., 2003a. BMP-2-induced Runx2 expression is mediated by *Dlx5*, and TGF-beta 1 opposes the BMP-2-induced osteoblast differentiation by suppression of *Dlx5* expression. J. Biol. Chem. 278, 34387–34394.

Lee, M.H., et al., 2003b. BMP-2-induced Osterix expression is mediated by *Dlx5* but is independent of Runx2. Biochem. Biophys. Res. Commun. 309, 689–694.

Liu, J.K., et al., 1997. *Dlx* genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. Dev. Dyn. 210, 498–512.

Miyama, K., et al., 1999. A BMP-inducible gene, *dlx5*, regulates osteoblast differentiation and mesoderm induction. Dev. Biol. 208, 123–133.

Morasso, M.I., et al., 1999. Placental failure in mice lacking the homeobox gene *Dlx3*. Proc. Natl. Acad. Sci. U. S. A. 96, 162–167.

Nakamura, K., et al., 2001. SCA17, a novel autosomal dominant cerebellar ataxia caused by an expanded polyglutamine in TATA-binding protein. Hum. Mol. Genet. 10, 1441–1448.

Neidert, A.H., et al., 2001. Lamprey *Dlx* genes and early vertebrate evolution. Proc. Natl. Acad. Sci. U. S. A. 98, 1665–1670.

Perutz, M.F., et al., 1994. Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. Proc. Natl. Acad. Sci. U. S. A. 91, 5355–5358.

Pfeffer, U., et al., 2001. The coding region of the human *DLX6* gene contains a polymorphic CAG/CCG repeat. Int. J. Oncol. 18, 1293–1297.

Price, J.A., et al., 1998. Identification of a mutation in DLX3 associated with tricho-dento-osseous (TDO) syndrome. Hum. Mol. Genet. 7, 563–569.

Price, J.A., et al., 1999. Tricho-dento-osseous syndrome and amelogenesis imperfecta with taurodontism are genetically distinct conditions. Clin. Genet. 56, 35–40.

Qiu, M., et al., 1995. Null mutation of Dlx-2 results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain. Genes Dev. 9, 2523–2538.

Qiu, M., et al., 1997. Role of the *Dlx* homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and-2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches. Dev. Biol. 185, 165–184.

Robinson, G.W., Mahon, K.A., 1994. Differential and overlapping expression domains of Dlx-2 and Dlx-3 suggest distinct roles for Distal-less homeobox genes in craniofacial development. Mech. Dev. 48, 199–215.

Robledo, R.F., et al., 2002. The *Dlx5* and *Dlx6* homeobox genes are essential for craniofacial, axial, and appendicular skeletal development. Genes Dev. 16, 1089–1101.

Ryoo, H.M., et al., 1997. Stage-specific expression of Dlx-5 during osteoblast differentiation: involvement in regulation of osteocalcin gene expression. Mol. Endocrinol. 11, 1681–1694.

Shapiro, D.J., et al., 1988. A high-efficiency HeLa cell nuclear transcription extract. DNA 7, 47–55.

Simeone, A., et al., 1994. Cloning and characterization of two members of the vertebrate *Dlx* gene family. Proc. Natl. Acad. Sci. U. S. A. 91, 2250–2254.

Stock, D.W., 2005. The *Dlx* gene complement of the leopard shark, *Triakis semifasciata*, resembles that of mammals: implications for genomic and morphological evolution of jawed vertebrates. Genetics 169, 807–817.

Stock, D.W., et al., 1996. The evolution of the vertebrate *Dlx* gene family. Proc. Natl. Acad. Sci. U. S. A. 93, 10858–10863.

Tadic, T., et al., 2001. *Dlx5* induces expression of COL1A1 promoter contained in a retrovirus vector. Croat. Med. J. 42, 436–439.

Tadic, T., et al., 2002. Overexpression of *Dlx5* in chicken calvarial cells accelerates osteoblastic differentiation. J. Bone Miner. Res. 17, 1008–1014.

Wong, G.L., Cohn, D.V., 1975. Target cells in bone for parathormone and calcitonin are different: enrichment for each cell type by sequential digestion of mouse calvaria and selective adhesion to polymeric surfaces. Proc. Natl. Acad. Sci. U. S. A. 72, 3167–3171.

Yang, W., et al., 2005. Dentin matrix protein 1 gene *cis*-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. J. Biol. Chem. 280, 20680–20690.

Zhao, G.Q., et al., 1994. rDlx, a novel distal-less-like homeoprotein is expressed in developing cartilages and discrete neuronal tissues. Dev. Biol. 164, 37–51.