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A 4 bp deletion mutation in *DLX3* enhances osteoblastic differentiation and bone formation *in vitro*

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Abstract

A 4 base-pair deletion mutation in the Distal-Less 3 (*DLX3*) gene is etiologic for Tricho-Dento-Osseous syndrome (TDO). A cardinal feature of TDO is an increased thickness and density of bone. We tested the effects of the *DLX3* gene mutation responsible for TDO on the osteoblastic differentiation of preosteoblastic MC3T3E1 cells and multipotent mesenchymal C2C12 cells. Differential expression analysis of C2C12 cells transfected with wild type *DLX3* or mutant *DLX3* was performed and desmin gene expression, an early myoblastic differentiation marker in mesenchymal cells, was evaluated by RT-PCR, western blot analysis, and desmin promoter transcriptional activity. Transfection of wild type *DLX3* into MC3T3E1 and C2C12 cells increased alkaline phosphatase-2 activity, mineral deposition, and promoter activities of the osteocalcin and type1 collagen genes compared to empty vector transfected cells. Transfection of mutant *DLX3* into these cells further enhanced alkaline phosphatase activity, mineral deposition, and osteocalcin promoter activities, but did not further enhance type 1 collagen promoter activity. Transfection of mutant *DLX3* into C2C12 cells markedly down regulated desmin gene expression, and protein expression of desmin and MyoD, while increasing protein expression of osterix and Runx2. These results demonstrate that the *DLX3* deletion mutation associated with TDO enhances mesenchymal cell differentiation to an osteoblastic lineage rather than a myoblastic lineage by changing the fate of mesenchymal cells. This *DLX3* mutation also accelerates the differentiation of osteoprogenitor cells to osteoblasts at later stages of osteogenesis.

Keywords

Distal-Less 3; Tricho-Dento-Osseous syndrome; Osteoblast differentiation; Osteocalcin; Collagen

Introduction

Distal-less 3 (*DLX3*) is a highly conserved homeobox-containing transcription factor which is widely expressed in vertebrate tissues including terminally differentiated epidermal cells, neural crest, hair follicles, dental epithelium, dental mesenchyme, otic placodes, olfactory

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placodes, limb bud, placenta, and cement gland. [1] BMP-2 activates *DLX3* transcription in hair follicle cells [2] and in combination with Smad can induce *DLX3* transcription in keratinocytes. [3] *DLX3* expression induced by bone morphogenetic protein (BMP) regulates tissue specific gene expression in developing embryonic ectoderm, [4,5] suggesting important roles of *DLX3* in developing tissues modulated by the BMP signaling pathway. *DLX3* is also an essential factor for normal placental development in mice. Placental failure in mice lacking the homeobox *DLX3* gene results in embryonic death between E 9.5 and E 10 due to placental defects that prevent normal development of the labyrinthine layer, possibly due to an abnormality in placental growth factor (PGF) expression. [6-8]

DLX genes play important roles in skeletal patterning, and expression of *DLX3* in the mouse embryo is associated with new bone formation and regulation of osteoblast differentiation. [9-12] *DLX3* is expressed in osteoblasts, and over-expression of *DLX3* in osteoprogenitor cells promotes the induction of osteoblastic differentiation markers such as type 1 collagen, bone sialoprotein, osteocalcin, and alkaline phosphatase. [13] Chromatin immunoprecipitation assays have revealed a *DLX3* binding element in the proximal promoter region of the osteocalcin (*OC*) gene. Transcriptional repression of the *OC* gene is controlled by *MSX2* in proliferating osteoblasts. *DLX3*, *DLX5*, and *Runx2* are recruited in the differentiated osteoblast to initiate transcription of the *OC* gene, demonstrating that in addition to *DLX5* and *Runx2*, *DLX3* is also important in osteoblast proliferation and differentiation. [13]

A 4 bp deletion mutation in the *DLX3* gene is associated with Tricho-Dento-Osseous syndrome (TDO), [14-16] an autosomal dominant condition characterized by variable clinical expression of kinky/curly hair, taurodontism, thin enamel and enhanced bone thickness. Increased density and thickness of cranial bone, distal radius/ulna, femoral neck, and lumbar spine in TDO [17-19] suggest that *DLX3* is important in remodeling and homeostasis of skeletal bone and that this *DLX3* gene mutation affects both endochondral and intramembranous bone development.

In this study, we have investigated the role of the *DLX3* 4 bp deletion mutation (MT-*DLX3*) on osteoblastic differentiation of preosteoblastic MC3T3E1 cells and multipotential mesenchymal C2C12 cells.

Materials and methods

Materials

C2C12 and MC3T3E1 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and α -Minimum Essential Medium (α -MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (JRH, Lenexa, KS) and 1% antibiotics (Invitrogen). QuikChange® II XL Site-Directed Mutagenesis Kits (Cat # 200521) were obtained from Stratagene (La Jolla, CA), and restriction enzymes used were from New England Biolabs (Beverly, MA). Chemicals were purchased from Sigma (St Louis, MO) and plasmid DNA isolation kits were from Qiagen (Valencia, CA). Transfection kits (VCA-1003) were purchased from Amaxa Biosystems (Gaithersburg, MD). Mouse *DLX3* cDNA (NM_010055, catalog number; MMM1013-9201696), human *DLX3* cDNA (NM_005220, catalog number; MHS1010-7429884), and Bac clone (RP24-125D9) for the isolation of 4.5 kb mouse desmin promoter, were obtained from Open Biosystems (Huntsville, AL). NE-PER nuclear and cytoplasmic extraction reagent was obtained from Pierce chemical (Rockford, IL) and the non radioisotope EMSA kit was purchased from Roche Applied Science (Indianapolis, IN).

Cloning of the mouse DLX3 cDNA in eukaryotic expression vector and generation of the mutated (4 bp deletion) DLX3 cDNA

Mouse and human DLX3 cDNAs were double digested with EcoRI/NotI and subcloned into the pcDNA3 eukaryotic expression vector (Invitrogen). Mutant mouse and human DLX3 cDNAs were generated with the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Briefly, 10 ng of wild type DLX3 (WT-DLX3) cDNA in pcDNA3 vector and 125 ng of sense and antisense primers encoding DLX3 sequence with a 4 bp deletion in NCBI mouse DLX3 cDNA database (NM_010055) (sense strand; 5'-GCT CTA TAA GAA TAG GTG CCG CTG G-3' and antisense strand; 5'-CCA GCG GCA CCT ATT CTT ATA GAG C-3') and human DLX3 cDNA database (NM_005220) (sense strand 5'-CTC TAC AAG AAC AGG TGC CGC TGG-3' and antisense strand 5'-CCA GCG GCA CCT GTT CTT GTA GAG-3'), were added to the mutagenesis reaction mixture containing *PfuUltra*™ High Fidelity DNA polymerase. WT-DLX3 and MT-DLX3 clones were sequence analyzed to confirm their identity using an ABI 3100 automatic DNA sequencing machine.

Generation of GFP-WT-DLX3 and GFP-MT-DLX3 fusion constructs

To investigate the cellular localization of WT-DLX3 and MT-DLX3 protein, GFP-WT-DLX3 and GFP-MT-DLX3 fusion constructs were produced. Briefly, PCR products generated using Sense Strand (5'-ACT CTC GAG ATG AGC GGC TCC TTC GAT CGC AAG-3') and Antisense Strand (5'-ACT CTC GAG TAC TCA GTA CAC AGC CCC A-3') primers with WT-DLX3 and MT-DLX3 cDNA as template, were digested with XhoI (recognition sequence underlined) and subcloned into pcDNA3-GFP vector containing an XhoI site instead of the GFP stop codon. These constructs, and pcDNA3-GFP vector as a control, were transfected into MC3T3E1 cells and the cellular localization of WT-DLX3 and MT-DLX3 was examined using an inverted fluorescence microscope.

Transfection of WT-DLX3 and MT-DLX3 cDNA into multipotent mesenchymal C2C12 cells and preosteoblastic MC3T3E1 cells and differentiation of these cells into osteoblastic cells

Exponentially growing C2C12 cells (2×10^5) were plated into six-well plates 24 hours before transfection and then transfected with 2 µg of WT-DLX3 cDNA, MT-DLX3 cDNA, or pcDNA3 empty vector (EV) using the Lipofectamine 2000 kit (Invitrogen). WT-DLX3 or MT-DLX3 cDNA were transfected into preosteoblastic MC3T3E1 cells using a nucleofactor device (Amaxa Biosystems) and the nucleofactor transfection kit (VCA-1003) following the manufacturer's protocol. Briefly 5×10^5 MC3T3E1 cells were suspended with the transfection solution containing 1 µg of WT-DLX3 cDNA, MT-DLX3 cDNA, or EV and electroporated with the nucleofactor device using the T-20 program. Green fluorescence protein (GFP) vector was used as a transfection efficiency control. Twenty-four hours after transfection, cells were trypsinized and plated on 48-well plates (5×10^4 cells/well in 1 ml of medium). 80% confluent MC3T3E1 and C2C12 cells were then treated with osteogenic differentiation media [20] containing 50 µM ascorbic acid and 10 mM beta-glycerophosphate for 3 to 21 days by exchanging half of the media with fresh media containing fresh osteogenic factors every three days. At day 3, cells were fixed with 0.2 ml/well of fixative (formalin/methanol/H₂O=1:1:1.5) for 10 min at room temperature, washed with distilled water three times and stained for alkaline phosphatase activity with FAST 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). At day 15 to 21, von Kossa staining was performed on the fixed cells using 2.5% silver nitrate solution (Sigma). Stained cells were counted in a blind manner with an inverted microscope and photographed. In selected experiments, cultured cells were stained with 1% Alizarin Red S (Sigma) to confirm calcium deposition on cultured cells. To generate stably transfected clones, MC3T3E1 and C2C12 cells transiently transfected with WT-DLX3 cDNA, MT-DLX3 cDNA, or EV were treated with 500 µg/ml of G418 for 3 weeks. Surviving

MC3T3E1 and C2C12 cells were serially diluted into 96-well plates to isolate single cell clones. Isolated clones were expanded, and the expression levels of WT-DLX3 and MT-DLX3 were determined by western blot and PCR analyses followed by sequence confirmation. Positively selected single cell clones were used for the osteoblastic differentiation assays described.

Non Radioisotope Electrophoresis Mobility Shift Assay (EMBA)

Nuclear proteins from C2C12 cells stably transfected with EV, WT-DLX3, or MT-DLX3 cDNA were extracted using an NE-PER nuclear and cytoplasmic extraction reagent according to the manufacturer's protocols. Non radioisotope EMSA was performed with digoxigenin-11-dUTP (DIG)-labeled probes and gel shift kit. Briefly, double strand oligonucleotides for the wild type DLX3 homeobox domain binding element (wTT-HDBE) consensus sequence (5'-ATG ACC CCC AAT TAG TCC TGG CAG-3') and the mutant HD binding element (mGG-HDBE) consensus sequence (5'-ATG ACC CCC AAG GAG TCC TGG CAG-3') [13,21] were labeled with DIG using a terminal transferase. wTT-HDBE or mGG-HDBE consensus sequence probes (30 fmol) and nuclear protein (5 ug) were incubated in 10 ul of binding buffer containing 0.5 µg of poly(dI-dC) and 0.05 ug of L-lysine for 25 minutes at room temperature. The DNA-nuclear protein complex was subjected into a 6% retardation gel (Invitrogen) at 60 V for 1 hr 40 min followed by electroblotting onto a positively charged nylon membrane (Roche Applied Science). The nylon membrane was then incubated with anti-DIG antibody conjugated with alkaline phosphatase and detected with CSPD (disodium 3-(4-methoxyspiro[1] decan]-4yl)phenyl phosphate) on x-ray film. In selected experiments, unlabeled wTT-HDBE or mGG-HDBE double strand oligonucleotides were added during the binding reaction to perform a competition assay.

Generation of polyclonal anti DLX3 antibody and Western immunoblot analysis

Mouse DLX3 cDNA generated by PCR (sense strand 5'-GCC ATA TGA GCG GCT CCT TCG ATC GC-3' and antisense strand 5' TGC TCG AGG GTGG GTA CTC AGT AC-3') was double digested with *NdeI* (recognition sequence underlined) and *XhoI* (recognition sequence bolded) and ligated into the PET 14b vector. The recombinant protein was expressed in BL-21 *Escherichia coli* and purified on His-Bind resin (Novagen Co., Madison, WI) as described previously. [22] The recombinant protein was used as an antigen to raise a rabbit anti-mouse DLX3 polyclonal antibody according to the standard protocol. The specific reactivity of the DLX3 antiserum was determined by testing its capacity to detect the mouse DLX3 protein in western blot analyses. Cell lysates from MC3T3E1 and C2C12 cells transiently and stably transfected with mouse and human WT-DLX3, MT-DLX3, or EV as described above, were separated using 10% SDS PAGE. Electrophoretic transfer of proteins from the polyacrylamide gel to nitrocellulose (Schleicher & Schuell) was performed using a semi-dry blotting unit (BioRad) at 20 V for 60 min. After transfer, the nitrocellulose membrane was blocked with 5% skim milk and hybridized with the anti-mouse DLX3 polyclonal antibody followed by horseradish peroxidase (HRP)-labeled anti-rabbit IgG (A9169, 1:10,000, Sigma-Aldrich, MO). The expression levels of desmin, MyoD, Runx2, and osterix protein were examined using desmin specific antibody (ab15200, 1:500, Abcam, Cambridge, MA), MyoD specific antibody (sc-304, 1:2000, Santa Cruz Biotechnology, CA), Runx2 specific antibody (sc-10758, 1:1000, Santa Cruz Biotechnology, CA), and osterix specific antibody (ab22552, 1:500, Abcam, Cambridge, MA) followed by anti-rabbit HRP. HRP conjugated β-actin antibody (A3854, 1:5,000, Sigma-Aldrich, MO) was used as a loading control. The nitrocellulose membrane was then washed and visualized with the ECL system (Amersham Pharmacia Biotech) on the Kodak X-AR5.

Measurement of rat osteocalcin, mouse Col1A1, and mouse desmin promoter activities modulated by WT-DLX3 or MT-DLX3

The 0.7 kb rat osteocalcin promoter, 2.4 kb Col1A1 promoter, and 4.5 kb mouse desmin promoter DNA were cloned into the pGL2Enhancer reporter vector (Promega, Madison, WI). 1 ug of promoter luciferase reporter constructs were co-transfected with 0.5 ug of Renilla luciferase (as an internal transfection control) in the presence or absence of 0.5 ug of mouse MSX2 cDNA in pcDNA3 into the MC3T3E1 and C2C12 cells stably transfected with WT-DLX3, MT-DLX3, or EV. Following incubation for 48 - 72 hours in 5% CO₂ at 37°C, the Firefly and Renilla luciferase activities were measured with a luminometer (Promega). At the end of the culture period, cells were washed with PBS, suspended in passive lysis buffer and centrifuged. Firefly and Renilla luciferase activities from cleared cell lysates were measured using a dual luciferase assay system (Promega) and the relative activity (a ratio between Firefly and Renilla luciferase activity) was calculated.

Two dimensional-differential in gel electrophoresis (2D-DIGE) and protein identification with MALDI-Time of Flight-Mass Spectrometry (MALDI-TOF-MS)

To identify proteins differentially expressed between C2C12 cells stably transfected with WT-DLX3 or MT-DLX3 cDNA, 2D-DIGE (Amersham, Piscataway, NJ) was performed according to the manufacturer's instruction. Briefly, equal amounts of cell lysates from C2C12 cells stably transfected with WT-DLX3 or MT-DLX3 cDNA were minimally labeled with Cy3 and Cy5 dye in the dark on ice and reactions were quenched by adding lysine. Equal amounts of Cy dye labeled cell lysates were mixed together and loaded onto an immobilized pH 3-10 nonlinear gradient (IPG) strip, and then electrophoresed using an Ettan IPGphor IEFsystem for isoelectrofocusing (IEF). Equilibrated strips with 6 M urea, 30% glycerol, 2% SDS, 100 mM Tris (pH 8.0), trace amounts of bromophenol blue, and 10 mg/ml DTT were applied to a 12% NuPAGE gel and electrophoresed at 120V. Cy dye images were collected using a 9400 Typhoon scanner (Amersham, Piscataway, NJ) in a fluorescence mode at a pixel size of 100 microns. Cy3 and Cy5 images were scanned using 532 and 633 nm lasers, respectively, and an emission filter of 580 and 670 nm band pass filters, respectively.

Proteins were visualized by Coomassie Blue staining using the SimpleBlue SafeStain kit (Invitrogen) and subsequently removed for MALDI-TOF-MS analysis. Gel plugs were washed, digested with trypsin, and mixed with α -cyano-4-hydroxycinnamic acid matrix on a matrix-assisted laser desorption ionization (MALDI) target slide. Mass fingerprint analysis was performed using a MALDI-TOF-MS (Proteomics Analyzer 4700, Applied Biosystems, Foster City, CA) as described previously. [23]

Database searches based on Mascot 2.0 (Matrix Science) individual MSMS ion scores were performed against SwissProt. Ion scores >95% CI were considered significant. Protein identification was further validated manually through BLAST using the SwissProt database at National Center for Biotechnology Information.

Statistical Analysis

Statistical significance was determined by Student t-test and P<0.05 was considered significant.

Results

Multipotent mesenchymal C2C12 cells and preosteoblastic MC3T3E1 cells transfected with MT-DLX3 cDNA and treated with osteogenic media show enhanced osteoblastic differentiation

To examine the effects of WT- and MT-DLX3 on osteoblastic differentiation and bone formation *in vitro*, we transiently transfected human and mouse WT-DLX3 and MT-DLX3 cDNA into preosteoblastic MC3T3E1 cells and treated the cells with osteogenic differentiation media [20] for 3 to 21 days. While MC3T3E1 cells transfected with WT-DLX3 cDNA show slightly enhanced AP activities at 3 days, cells transfected with MT-DLX3 cDNA show enhanced AP activities compared to those of WT-DLX3 or EV transfected cells (figure 1A and 1B upper panel). To quantify AP activity, AP positive cells were counted in a blinded manner for 10 random fields (200 X) using an inverted microscope. Cells transfected with WT-DLX3 cDNA show a significant increase in the number of AP positive cells, and transient transfection with MT-DLX3 cDNA show a significant increase in the number of AP positive cells compared to EV or WT-DLX3 cDNA transfected cells (figure 1C and 1D). MC3T3E1 cells transfected with mouse and human MT-DLX3 cDNA also show enhanced mineral deposition compared to the WT-DLX3 or EV transfected cells (von Kossa) (figure 1A and 1B middle panel). Furthermore, transfection of WT-DLX3 into MC3T3E1 cells increases the number of Alizarin Red positive colonies and transfection of MT-DLX3 further increases the number of those colonies (figure 1A and 1B lower panel). To determine the mRNA and protein expression levels of mouse and human WT-DLX3 and MT-DLX3 in MC3T3E1, Western blot and RT-PCR analyses were performed using cells transiently transfected with mouse and human WT- and MT-DLX3 cDNA. As shown in figure 1C and 1D lower panel, mouse WT-DLX3 (32kDa) and mouse MT-DLX3 (35kDa) bands are detected in cells transfected with these cDNAs and endogenous DLX3 is not detected in MC3T3E1 cells transfected with EV. Human WT-DLX3 protein (32kDa) and MT-DLX3 protein (29kDa) are also detected in cells transfected with these cDNAs. Similarly, mRNA transcripts for WT-DLX3 (320 bp) and MT-DLX3 (320 bp) are detected in these cells. Samples that are not treated with reverse transcriptase do not show transcripts (data not shown). To further analyze the role of MT-DLX3 on osteoblastic differentiation, we generated MC3T3E1 and C2C12 cells stably transfected with MT-DLX3, WT-DLX3, or EV by treating transiently transfected cells with 500 µg/ml of G418. The integrated DLX3 cDNA was amplified by PCR without reverse transcriptase and sequence analyzed to confirm whether the clones were integrated with the WT-DLX3 or MT-DLX3 cDNA. Single cell clones from MC3T3E1 and C2C12 cells stably transfected with WT-DLX3 or MT-DLX3 were then treated with osteogenic differentiation media for 3 to 21 days and assayed for their ability to induce osteoblastic differentiation. As shown in figure 2A, two independent MC3T3E1 cell clones stably transfected with MT-DLX3 show marked increases in the number of AP positive cells following the treatment of cells with osteogenic differentiation media for 3 days. MC3T3E1 clones stably transfected with WT-DLX3 cDNA also show enhanced AP activity. von Kossa staining results also show enhanced mineral deposition in MC3T3E1 stably transfected with MT-DLX3 cDNA compared to those of MC3T3E1 cells stably transfected with WT-DLX3 or EV (figure 2A). Similarly, C2C12 clones stably transfected with MT-DLX3 cDNA show markedly enhanced AP activity and mineral deposition compared to those of WT-DLX3 or EV transfected C2C12 cells. C2C12 cells transfected with WT-DLX3 also show markedly increased mineral deposition (figure 2B). Mouse WT-DLX3 (32 kDa) and MT-DLX3 (35 kDa) proteins are similarly expressed in MC3T3E1 and C2C12 cells stably transfected with these cDNA (figure 2A and 2B lower panel).

Cellular localization of WT-DLX3 and MT-DLX3

To test whether the mutated C-terminal DLX3 peptides can affect the nuclear translocation of MT-DLX3 protein, we generated GFP fused WT-DLX3 and MT-DLX3 constructs in pcDNA3 vector, transfected them into MC3T3E1 cells, and observed GFP signal using fluorescence microscopy. As shown in figure 3, both GFP-WT-DLX3 and GFP-MT-DLX3 fusion protein are translocated into nucleus while unfused GFP protein is predominantly located in the cytoplasmic fraction.

MT-DLX3 protein binds DLX3 homeobox domain binding element (HDBE)

To test whether MT-DLX3 protein translocated into the nucleus can bind the DLX3 homeobox domain binding element (HDBE), (13, 21) we performed electrophoresis mobility shift assays (EMSA) using non radioisotope labeled probe with the HDBE consensus sequence and nuclear extracts from C2C12 cells stably transfected with EV, WT-DLX3 or MT-DLX3 cDNA. As shown in figure 4, nuclear extracts from C2C12 cells transfected with WT-DLX3 forms wTT-HDBE and WT-DLX3 complex (lane 6) and the forming wTT-HDBE and WT-DLX3 complex is markedly reduced in the presence of unlabeled wTT-HDBE oligonucleotide (lane 7). In contrast, unlabeled mGG-HDBE oligonucleotide does not inhibit the formation of wTT-HDBE-WT-DLX3 complex (lane 8). Similarly, MT-DLX3 also forms a wTT-HDBE-MT-DLX3 complex (lane 3) which is inhibited in the presence of unlabeled wTT-HDBE oligonucleotide (lane 4) but not mGG-HDBE oligonucleotide (lane 5). Nuclear extract from C2C12 cells stably transfected with EV does not show a clear band compared to those of WT-DLX3 or MT-DLX3 cDNA (lanes 2). However, labeled mGG-HDBE does not form mGG-HDBE-WT-DLX3 or mGG-HDBE-MT-DLX3 complexes (lanes 10-13).

MT-DLX3 enhances the osteocalcin promoter activities in MC3T3E1 and C2C12 cells and the up-regulated osteocalcin promoter activities are reversed by MSX2 co-transfection

To test whether WT-DLX3 and MT-DLX3 can differentially modulate osteocalcin gene expression we examined the effects of WT-and MT-DLX3 on osteocalcin (OC) promoter activity,. As shown in figure 5A, the OC promoter activities are significantly enhanced in MC3T3E1 cells transfected with WT-DLX3 and are further significantly enhanced in MC3T3E1 cells transfected with MT-DLX3 compared to those of EV or WT-DLX3 transfected samples. These up-regulated OC promoter activities in MC3T3E1 cells transfected with WT-DLX3 or MT-DLX3 are down regulated by MSX2 co-transfection. Similarly, OC promoter activities are moderately but not significantly enhanced in C2C12 cells transfected with WT-DLX3. However, OC promoter activities are significantly increased in C2C12 cells transfected with MT-DLX3 compared to those of EV or WT-DLX3 transfected cells. Co-transfection of MSX2 with WT-DLX3 or MT-DLX3 into C2C12 cells reverses the up-regulated OC promoter activities to basal levels (figure 5B).

Effect of MT-DLX3 on mouse col1A1 promoter activities

Since MT-DLX3 upregulates OC promoter activity, a late marker of osteoblast differentiation, we investigated the effects of WT-DLX3 and MT-DLX3 on type 1 collagen (Col1A1) promoter activity, which is an early osteoblastic differentiation marker. [24] 2.4 kb Col1A1 promoter activities are significantly enhanced in MC3T3E1 (figure 6A) and C2C12 (figure 6B) cells stably transfected with WT-DLX3 and MT-DLX3 compared to those of EV transfected cells. The increased Col1A1 promoter activities are not significantly different in MC3T3E1 and C2C12 cells stably transfected with WT-DLX3 or MT-DLX3.

Down-regulation of desmin and MyoD gene expression in C2C12 cells transfected with MT-DLX3

To investigate differences in the protein expression profiles that are modulated by MT-DLX3 and WT-DLX3, we performed proteomic analyses using Cy-3 and Cy-5 labeled cell lysates from C2C12 cells stably transfected with WT-DLX3 or MT-DLX3. Differential expression of multiple proteins was found in C2C12 cells stably transfected with MT-DLX3 cDNA compared to those of WT-DLX3. Expression levels of desmin, vimentin, vinculin, and tubulin alpha2 are down-regulated in C2C12 cells stably transfected with MT-DLX3 and those of calumenin, NCC27, VDAC-6, and 14-3-3 eta are up-regulated (Table 1). Since desmin is an early myoblastic differentiation marker, and multipotent mesenchymal C2C12 cells can differentiate into myoblastic cells as well as osteoblastic cells depending on culture conditions, we investigated desmin gene expression levels in C2C12 cells stably transfected with WT-DLX3, MT-DLX3, or EV. As shown in figure 7A, mRNA expression of mouse desmin is markedly down-regulated in C2C12 cells transfected with MT-DLX3 compared to those of WT-DLX3 or EV transfected C2C12 cells. In contrast, mRNA expression of desmin in C2C12 cells transfected with WT-DLX3 does not significantly differ from those of EV transfected C2C12 cells. To examine desmin protein expression levels, western blot analysis was performed using a desmin specific antibody and lysates from C2C12 cells transfected with WT-DLX3, MT-DLX3, or EV. As shown in figure 7B, desmin protein expression is completely ablated in cell lysates from C2C12 cells stably transfected with MT-DLX3. Desmin protein expression levels are similar in parent, EV, and WT-DLX3 transfected C2C12 cells. Similarly, the expression level of MyoD protein, a myoblast differentiation marker, is markedly reduced in C2C12 cells stably transfected with MT-DLX3 cDNA. In contrast, expression levels of Runx2 and osterix, two major osteoblastic differentiation markers, are markedly increased in C2C12 cells stably transfected with MT-DLX3 cDNA and treated with osteogenic media for 6 days, while those expression levels are moderately increased in C2C12 cells transfected with WT-DLX3 cDNA (figure 7B). To test whether the down-regulation of desmin gene expression in C2C12 cells transfected with MT-DLX3, is due to transcriptional modulation, we evaluated the effects of WT-DLX3 and MT-DLX3 on desmin gene promoter activities using a 4.5 kb mouse desmin promoter luciferase reporter construct. As shown in figure 7C, desmin promoter activities are significantly down-regulated in C2C12 cells stably transfected with MT-DLX3 while promoter activities in C2C12 cells transfected with WT-DLX3 do not differ from those of EV transfected C2C12 cells.

Discussion

Bone remodeling and homeostasis is maintained through a balance of bone resorption by osteoclasts and bone formation by osteoblasts. [25-27] Osteoblasts play a central role in this bone remodeling process by synthesizing multiple bone matrix proteins. Osteoblasts also regulate osteoclast maturation and osteoclast activity by modulating soluble regulatory factors and cell-cell interactions.[28,29] Imbalances between osteoclastic bone resorption and osteoblastic bone formation can result in pathologic bone phenotypes such as osteoporosis and osteopetrosis. *DLX3* gene expression is associated with osteoblast differentiation as well as bone formation.[1,9,13,14,18] The enhanced bone thickness and density found in TDO patients may be a consequence of enhanced bone formation as well as decreased bone resorption. The mechanism by which the 4 bp deletion mutation in *DLX3* (MT-DLX3) functions in osteoblast differentiation and osteoblastic bone formation is not clearly understood. When teeth are removed from the maxilla or mandible, the alveolar bone typically resorbs, ultimately resulting in hypoplastic ridges. The thin enamel and taurodontism characteristic of the TDO phenotype frequently contributes to tooth abscess and subsequent extraction. However, in TDO, after tooth extraction, the alveolar bone appears to resist the levels of resorption typically seen in edentulous individuals, [19] suggesting that osteoclast activity may be altered in TDO.

Our *in vitro* findings demonstrate that transient transfection of mouse and human WT-DLX3 cDNA into MC3T3E1 cells significantly enhanced AP activity and mineral deposition (figure 1). To overcome limitations inherent to transiently transfected cells (transfection efficiency and plasmid stability), we generated stably transfected MC3T3E1 and C2C12 cells with mouse WT-DLX3 and MT-DLX3. Using these stably transfected clones, we reconfirmed that transfection of WT-DLX3 into MC3T3E1 and C2C12 cells induced osteoblastic phenotypes (increased AP activity and mineral deposition) similar to those seen in osteoblastic differentiation induced by BMP-2 treatment.[4] Transfection of MT-DLX3 into these cells markedly enhanced the osteoblastic phenotype (figure 2). Additionally, while transfection of WT-DLX3 into C2C12 or MC3T3E1 cells increased osteocalcin promoter activity, the transfection of MT-DLX3 further increased these activities compared to those of WT-DLX3 transfected cells (3 to 7 fold increases). Up-regulation of osteocalcin promoter activity by WT-DLX3 and by MT-DLX3 was significantly reduced by MSX2 co-transfection (figure 5). These findings are consistent with previous reports that DLX3 mRNA and protein were mainly expressed at the mature stage of osteoblast differentiation in an *in vitro* culture model of preosteoblastic cells [9]. Temporal overexpression of WT-DLX3 in preosteoblastic MC3T3E1 cells enhances osteocalcin expression (~ 2 to 3 fold) and this enhanced osteocalcin expression is down-regulated by MSX2.[13] These findings demonstrate that DLX3 is expressed in the osteogenic lineage, possibly by osteoprogenitors, and in active osteoblasts on the bone surface, suggesting that DLX3 is important in osteoblast differentiation and bone formation. We found stable transfection of WT-DLX3 and MT-DLX3 into myoblastic C2C12 cells and preosteoblastic MC3T3E1 cells also enhanced mouse Col1A1 promoter activity (figure 6), consistent with results of Hassan et al. [13] who reported that WT-DLX3 overexpression in MC3T3E1 cells enhanced Col1A1 expression. However, MT-DLX3 did not further enhance the 2.4 kb Col1A1 promoter activity compared to those of WT-DLX3 transfected cells.

Bone marrow contains multipotential stromal stem cells which can differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts depending on their microenvironment. [30] During long-term culture with stimuli, multipotential bone marrow stromal cells express tissue-specific differentiation markers such as lipoprotein lipase for adipocytes, type I collagen and osteocalcin for osteoblasts, type II and type X collagen for chondrocytes, and desmin for muscle cells.[31] Multipotent mesenchymal C2C12 cells can be stimulated to differentiate into muscle (desmin positive) or osteoblast (desmin negative) lineages by BMP-2, BMP-4, or BMP-7 treatment *in vitro*. [32,33] Desmin, a member of the intermediate filament (IF) family, is the earliest myogenic differentiation marker in skeletal and cardiac muscle development. BMP-4 stimulated C2C12 cells differentiate and lose the myoblastic phenotype and do not express desmin or MyoD.[34] Similarly, treatment of C2C12 cells with enamel matrix derivative inhibits myoblastic differentiation and enhances osteoblastic differentiation, markedly increasing AP activity, osteocalcin, and type X collagen expression, while decreasing desmin and MyoD expression.[35] We demonstrated that MT-DLX3 expression in C2C12 cells inhibits desmin gene and protein expression as well as MyoD protein expression, suggesting that MT-DLX3 changes the phenotype of the multipotent mesenchymal C2C12 cells to an osteoblastic lineage. More importantly, C2C12 cells stably transfected with MT-DLX3 cDNA increased the expression levels of Runx2 and osterix protein when these cells were cultured with osteogenic media for more than 6 days suggesting that MT-DLX3 also enhanced the osteoblastic differentiation of C2C12 cells. In addition to desmin, expression levels of vimentin and tubulin alpha2, intermediate filament components in muscle structure formation [36] and vinculin, involved in cell adhesion in the attachment of the actin-based microfilaments to the plasma membrane, [37] are also down-regulated in C2C12 cells transfected with the MT-DLX3 (Table 1). However, transient transfection of MT-DLX3 into C2C12 cells did not markedly down-regulated desmin or MyoD expression suggesting that temporal expression of MT-DLX3 in C2C12 cells for 3 days might not be enough to change the phenotype of mesenchymal C2C12 cells to osteoblastic precursors. These findings indicate

that stable transfection of MT-DLX3 into C2C12 cells changes the phenotype of C2C12 cells to an osteoblast lineage rather than the myoblast lineage. Desmin cDNA transfection into C2C12 cells stably transfected with MT-DLX3 did not reverse AP activity or mineral deposition, osteoblastic differentiation phenotypes, suggesting that MT-DLX3 could be a dominant regulatory factor for osteoblastic differentiation of C2C12 cells. Consistent with these findings, results of our promoter studies demonstrate that desmin expression and promoter activity were down-regulated in C2C12 cells expressing MT-DLX3 compared to WT-DLX3 or EV transfected C2C12 cells.

The *DLX3* mutation identified in TDO patients introduces a frameshift that changes the last C-terminal 97 amino acids (from 191 to 287) creating a novel 65 amino acid C-terminal peptide in humans and introduces a novel 119 amino acids C-terminal peptide in the mouse. These chimeric DLX3 proteins have novel C-terminal domains, just 3' to the homeobox binding domain. The homeodomain region in both human and mouse *DLX3* genes includes a nuclear localization signal (NLS) from amino acid 124 to 189. [4] The deletion mutation does not alter the primary structure of the homeobox domain region or the NLS region and nuclear translocation of the MT-DLX3 protein is not changed (figure 4). Additionally, both WT-DLX3 and MT-DLX3 form complexes with a DLX3 homeobox domain binding element probe (figure 5). Our observation that MT-DLX3 enhances osteoblastic differentiation and osteocalcin promoter activity in C2C12 and MC3T3E1 cells could be due to the novel C-terminal amino acid peptide sequence present in the chimeric MT-DLX3 protein. The initial 65 amino acids in the chimeric mouse MT-DLX3 has 80% homology with the initial 65 amino acids of the chimeric human MT-DLX3. Although the primary structure of the homeobox binding domain is maintained, these novel 65 amino acids may have enhanced transactivation potential for osteoblastic differentiation as well as osteocalcin promoter activity. An extensive BlastP search of the NCBI database failed to identify any significant homology of these 65 amino acid peptides with the NCBI peptide database. Bryan et al. [38] reported that a derived deletion mutant that abolishes the last 41 C-terminal amino acids in DLX3 did not significantly change the transactivation potential of DLX3 using a yeast one hybrid assay suggesting that the newly introduced 65 amino acid peptide caused by the 4 bp deletion may interact with other regulatory proteins that can modulate transactivation of osteogenic factors.

These findings demonstrate that the 4 bp *DLX3* deletion mutation associated with TDO increases osteogenesis by enhancing the differentiation of mesenchymal cells to an osteoblastic lineage at both early and later stages of osteoblast differentiation. Further study using MT-DLX3 expressing cells and transgenic mice may provide an understanding for the mechanisms of the enhanced bone formation and bone density caused by this mutation.

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The abbreviations used are

DLX3	Distal-Less 3
BMP	bone morphogenetic protein
PGF	placental growth factor
TDO	Tricho-Dento-Osseous
DEXA	dual-energy x-ray absorptiometry

HDBE	homeobox domain binding element
2D-DIGE	two dimensional-differential in gel electrophoresis
EMSA	electrophoresis mobility shift assays
Col1A1	type 1 collagen
EMD	enamel matrix derivative
IF	intermediate filament
MyoD	Myogenic differentiation antigen 1
Runx2	Runt-related transcription factor 2
osx	osterix

Fig. 1. Transient transfection of mouse and human MT-DLX3 into MC3T3E1 cells enhances AP activity and mineral deposition. Preosteoblastic MC3T3E1 cells were transiently transfected with mutant type (MT)-DLX3 cDNA, wild type (WT)-DLX3 cDNA, or pcDNA3 empty vector (EV). Transfected cells were cultured with osteogenic media for 3 to 21 days. Figure 1A (mouse) and 1B (human) shows AP activity and mineral deposition (von Kossa) at 3 and 21 days. Figure 1C (mouse) and 1D (human) shows the number of AP positive cells that were counted with inverted microscope. Statistically significant differences in the number of AP positive cells were observed for the 3 differentially transfected cells (*; $P < 0.05$). Alizarin Red S staining performed on differentially transfected cells cultured for 15 days (figure 1A (mouse) and 1B (human) lower panel). Similar results were seen in three independent experiments. Protein and mRNA expression levels of WT-DLX3 and MT-DLX3 were examined by Western blot analysis and RT-PCR using mouse and human DLX3 specific sense strand primer (5'-GGC TCC TTC GAT CGC AAG CTC-3') and antisense strand primer (5'-TTC ACC GAC ACT GGG TCC TGG G-3').

Fig. 2.

Stable transfection of MT-DLX3 cDNA into MC3T3E1 and C2C12 cells enhances AP and mineral deposition. WT-DLX3, MT-DLX3, and EV were stably transfected into MC3T3E1 (A) and C2C12 cells (B) as described in methods. After single cell isolation, stable clones were assayed for their ability to induce osteogenic differentiation by assessing AP activity and mineral deposition (Von Kossa) at day 3 and day 21. Expression levels of mouse WT-DLX3 (32 kDa) and MT-DLX3 (35 kDa) protein were examined by Western blot analysis using a DLX3 antibody. Similar results were seen in three independent experiments.

Fig. 3.

Cellular localization of WT-DLX3 and MT-DLX3 protein in MC3T3E1 cells. GFP fused WT-DLX3 and MT-DLX3 constructs were transfected into MC3T3E1 cells and cellular localization was examined by observing GFP signal. WT-DLX3 and MT-DLX3 protein are equally translocated into the nucleus while control GFP is located in cytoplasmic fraction.

Fig. 4. Both WT-DLX3 and MT-DLX3 protein bind the wild type DLX3 homeobox domain binding element. (wTT-HDBE; wild type DLX3 homeobox domain binding element, mGG-HDBE; mutant DLX3 homeobox domain binding element) Nuclear extract from C2C12 cells stably transfected with WT-DLX3 cDNA forms a wTT-HDBE probe-WT-DLX3 protein complex (arrow, lane 6). Formation of this complex is markedly reduced in the presence of unlabeled wTT-HDBE oligonucleotide (50X). Addition of the mGG-HDBE oligonucleotide does not reduce those complex formation. Similarly, MT-DLX3 also binds wTT-HDBE consensus sequence probe (arrow, lane 3) and the formed complex is markedly reduced in the presence of unlabeled wTT-HDBE oligonucleotide. Nuclear extract from C2C12 cells stably transfected with EV does not form a detectable complex. The mGG-HDBE consensus sequence probe does not bind either WT-DLX3 or MT-DLX3 protein. (N.S.: Non Specific band)

Fig. 5.

Effects of WT-DLX3 and MT-DLX3 on osteocalcin promoter activities in the presence and absence of MSX2. Rat osteocalcin (OC) luciferase reporter constructs were transfected into MC3T3E1 (A) and C2C12 (B) cells stably transfected with WT-DLX3, MT-DLX3, or EV in the presence or absence of MSX2. After 72 hrs incubation, Firefly and Renilla luciferase activities in cell lysates were measured and relative promoter activities were calculated. Panel A: significant statistical differences in OC promoter activities for the 3 differentially transfected MC3T3E1 cells were observed (*; $P < 0.05$). MSX2 resulted in a statistically significant down regulation of the OC promoter activities in each of the 3 differentially transfected MC3T3E1 cells (*; $P < 0.05$). Panel B: significant statistical differences in OC promoter activities were observed for the MT-DLX3 transfected C2C12 cells (*; $P < 0.05$). MSX2 resulted in a statistically significant down regulation of the OC promoter activities in each of the 3 differentially transfected C2C12 cells. Similar results were seen in three independent experiments.

Fig. 6.

Effects of WT-DLX3 and MT-DLX3 on the 2.4 kb Col1A1 promoter activities. 2.4 kb mouse Col1A1 promoter luciferase reporter construct were transfected into MC3T3E1(A) and C2C12 (B) cells. After 48hours incubation, Firefly and Renilla luciferase activities in cell lysates were measured and relative promoter activities were calculated. A significant statistical increase in Col1A1 promoter activity was observed for MC3T3E1 (Panel A) and C2C12 (Panel B) cells stably transfected with WT-DLX3 and MT-DLX3 (*; $P < 0.05$). Similar results were seen in three independent experiments.

Fig. 7.

MT-DLX3 changes the phenotype of C2C12 cells to osteoblastic lineage rather than myoblastic lineage. Total RNA from C2C12 cells stably transfected with WT-DLX3, MT-DLX3, or EV was isolated and RT-PCR was performed using mouse desmin specific primers (sense strand; 5'-GTG AAG ATG GCC TTG GAT GT-3' and antisense strand; 5'-CCC ACT CTC CAT CTC AGC TC-3'). Desmin mRNA expression level is completely ablated in C2C12 cells stably transfected with MT-DLX3. GAPDH mRNA expression was included as a loading control (A). Protein expression of desmin and MyoD in C2C12 cells stably transfected with WT-DLX3, MT-DLX3, or EV and parent C2C12 cells were examined by Western blot analyses using a rabbit anti-mouse desmin and MyoD specific antibodies followed by anti-rabbit HRP. Expression levels of desmin and MyoD protein are dramatically down-regulated in C2C12 cells stably transfected with MT-DLX3. In contrast, Expression levels of Runx2 and osterix protein are markedly up-regulated in C2C12 cells stably transfected with MT-DLX3 cDNA and cultured with osteogenic media for 6 days. β-Actin protein expression is included as a loading control for each culture condition (B). Mouse 4.5 kb desmin promoter luciferase reporter construct was transfected into C2C12 cells stably transfected with EV, WT-DLX3, or MT-DLX3 and relative promoter activities were examined. A significant reduction in desmin promoter activities were observed in C2C12 cells transfected with MT-DLX3 (*; $P < 0.05$) (C). Similar results were seen in three independent experiments.

Table 1

Proteins differentially expressed in C2C12 cells stably transfected with MT-DLX3 relative to cells stably transfected with WT-DLX3.

Locus	Name	Function	Fold change
P31001	Desmin	Early myoblastic differentiation marker	↓ 6.09
Q64727	Vinculin	Cell adhesion molecule on actin	↓ 4.26
P20152	Vimentin	Class-III intermediate filaments in mesenchyme	↓ 3.24
P05213	Tubulin α2	Tubulin alpha-2 chain	↓ 2.17
Locus	Name	Function	Fold change
Q35887	Calumenin	Vitamin K-dependent carboxylase inhibitor	↑ 5.25
Q9Z1Q5	NCC27	Chloride intracellular channel protein	↑ 5.13
Q60930	VDAC-6	Voltage-dependent anion channel protein	↑ 2.76
P68510	14-3-3 eta	Cofactor for homeodomain transcription factors	↑ 1.83