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The LIM Homeodomain Transcription Factor LHX6

*A TRANSCRIPTIONAL REPRESSOR THAT INTERACTS WITH PITUITARY HOMEOBOX 2 (PITX2) TO REGULATE ODONTOGENESIS**

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Background: Odontogenesis is tightly controlled by the interactions of transcription factors.
Results: LHX6 acts as a transcriptional repressor and interacts with PITX2 to regulate craniofacial development.
Conclusion: LHX6 is required for mandible and dental morphogenesis through repression of gene expression.
Significance: This is the first report of LHX6 transcriptional repressor activity and its ability to inhibit the activity of other transcription factors.

LHX6 is a LIM-homeobox transcription factor expressed during embryogenesis; however, the molecular mechanisms regulating LHX6 transcriptional activities are unknown. LHX6 and the PITX2 homeodomain transcription factor have overlapping expression patterns during tooth and craniofacial development, and in this report, we demonstrate new transcriptional mechanisms for these factors. PITX2 and LHX6 are co-expressed in the oral and dental epithelium and epithelial cell lines. *Lhx6* expression is increased in *Pitx2c* transgenic mice and decreased in *Pitx2* null mice. PITX2 activates endogenous *Lhx6* expression and the *Lhx6* promoter, whereas LHX6 represses its promoter activity. Chromatin immunoprecipitation experiments reveal endogenous PITX2 binding to the *Lhx6* promoter. LHX6 directly interacts with PITX2 to inhibit PITX2 transcriptional activities and activation of multiple promoters. Bimolecular fluorescence complementation assays reveal an LHX6·PITX2 nuclear interaction in living cells. LHX6 has a dominant repressive effect on the PITX2 synergistic activation with LEF-1 and β-catenin co-factors. Thus, LHX6 acts as a transcriptional repressor and represses the expression of several genes involved in odontogenesis. We have identified specific defects in incisor, molar, mandible, bone, and root development and late stage enamel formation in *Lhx6* null mice. Amelogenin and ameloblastin expression is reduced and/or delayed in the *Lhx6* null mice, potentially resulting from defects in dentin deposition and ameloblast differentiation. Our results demonstrate that LHX6 regulates cell proliferation in the cervical loop and promotes cell differentiation in the anterior region of the incisor. We demonstrate new molecular mechanisms for LHX6 and an interaction with PITX2 for normal craniofacial and tooth development.

LIM homeodomain-containing proteins are transcriptional regulators that play major roles in pattern formation and cell type specification of various tissues (1–5). The LIM domain has been identified as a crucial zinc finger motif mediating protein-protein interactions and required for the biological functions of many LIM proteins. LIM domains do not appear to bind DNA, which is modulated by the homeodomain motif. The LIM domain confers specific protein-protein interactions (for reviews, see Refs. 2 and 6–8).

LHX6 is a LIM homeodomain protein similar in structure to ISL-1 (9). LHX6, like ISL-1, contains tandem LIM domains and a homeodomain that regulate its activity both in *cis* and through interaction with cell-specific factors (2, 6, 7, 10). LHX6 is highly expressed in the neural crest-derived mesenchyme throughout odontogenesis and down-regulated after birth (9, 11, 12). However, LHX6 is also expressed in the palate epithelium, oral epithelium, and dental epithelium during craniofacial development (12). LHX6 regulates migration and specification of neuron subtypes and marks specific neurons (13–16). LHX6 expression in the craniofacial region and during odontogenesis would suggest that *Lhx6* null mice would present with severe craniofacial anomalies. A previous report indicated that *Lhx6* null mice have no obvious craniofacial defects, and the related *Lhx7* (L3, Lhx8) null mice present with defects in palate formation (11, 17). Interestingly, *Lhx7* expression is only observed in the palate and odontogenic mesenchyme and not expressed in the epithelial tissues (11, 18). The isolated cleft palate in the *Lhx7* null mice appears due to abnormal *Lhx7* expression in the palate mesenchyme. *Lhx6/7* double homozygous mice present with cranial skeletal defects, cleft palate, molar agenesis, and supernumerary incisor-like teeth (11). These experiments demonstrate some redundancy between these two LIM domain proteins and their involvement in craniofacial development. The molecular mechanisms of LHX6 transcriptional activity are unknown, and in this report, we demonstrate new transcriptional activities of LHX6 and identify PITX2 as an interacting factor, which also activates LHX6 expression. Analyses of the $Lhx6^{-/-}$ mice reveal defects in lower incisor develop-

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ment and a role for LHX6 in regulating incisor stem cell proliferation and preameloblast differentiation.

PITX2, a member of the paired-like homeobox transcription factor family, is expressed in the brain, heart, pituitary, mandibular and maxillary regions, oral epithelium, eye, and umbilicus (19–21). PITX2 acts as a transcriptional activator but can be repressed through its interaction with other transcription factors (22, 23). Thus, the emerging theme for PITX2 is that its activity can be negatively and positively regulated through protein interactions and cell-specific factors. CLIM protein family members have been shown to interact with PITX1 (1, 24–27). Because PITX1 interacts with the LIM domain, we asked if PITX2 could also interact with other LIM domain-containing transcription factors.

Protein-protein interactions are crucial for many developmental and biological processes. Proteins may contain more than one interaction domain, which promotes the formation of multiprotein complexes that can regulate gene expression in a tissue/cell-specific pattern. PITX2 and LHX6 are two factors that contain different protein interaction domains and interact with a variety of cell-specific factors. PITX2 contains a homeodomain and C-terminal OAR domain that can each independently interact with factors to regulate its transcriptional activity (22, 23, 28–34). Because these two factors play predominant roles in craniofacial/tooth morphogenesis, we asked if they interact to regulate gene expression.

To investigate downstream target genes of PITX2 and LHX6 (initially identified through DNA microarrays), chromatin immunoprecipitation (ChIP) assays identified endogenous PITX2 binding to the *Lhx6* promoter in the LS-8 mouse oral epithelium cell line expressing PITX2 and LHX6. PITX2 and LHX6 protein-protein interactions regulate gene expression. LHX6 acts as a transcriptional repressor and interacts with PITX2 to attenuate PITX2 transcriptional activation. LHX6 represses *Lhx6*, *Pitx2c*, and *Lef-1* promoter activity, whereas PITX2 activates the *Lhx6* promoter. We demonstrate that endogenous PITX2 regulates LHX6 expression. Furthermore, LHX6 represses PITX2 activity in the presence of PITX2 cofactors. Analyses of the *Lhx6* null mice reveal subtle defects in mandible size and lower incisor development. The lower incisor is smaller than wild type littermates with a defect in ameloblast and odontoblast differentiation that is associated with decreased amelogenin and ameloblastin expression. LHX6 regulates progenitor cell proliferation in the incisor cervical loop and promotes cell differentiation in the anterior region of the incisor. LHX6 appears to regulate late stages of tooth development through its interactions with other transcription factors, including PITX2. We have uncovered a new transcriptional mechanism where PITX2 activates LHX6 expression; LHX6 represses its own expression directly or by interacting with PITX2 to attenuate PITX2 activation. This interaction reveals new transcriptional hierarchies for craniofacial/tooth development.

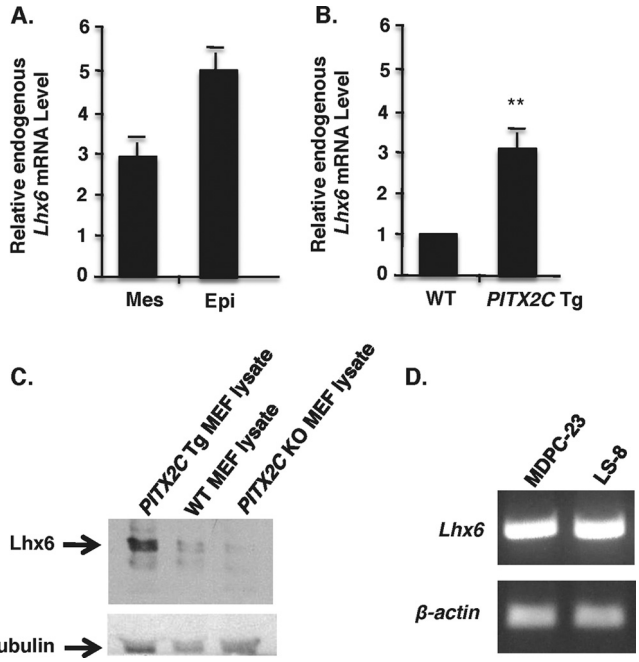

FIGURE 1. **LHX6 expression and regulation by PITX2 in odontogenic tissues and cells.** *A*, real-time PCR of *Lhx6* transcripts from excised P1 dental mesenchymal (*Mes*) and epithelial (*Epi*) total RNA. *Lhx6* transcripts were more abundant in the epithelial tissues compared with the mesenchyme tissues. *B*, real-time PCR of *Lhx6* transcripts from WT and PITX2C Tg MEF total RNA. *Lhx6* transcripts were increased 3-fold in the PITX2C Tg MEFs compared with WT. *C*, PITX2C Tg MEF lysates reveal increased endogenous LHX6 protein expression compared with WT MEFs, and little LHX6 protein is detected in PITX2 knock-out (*KO*) MEFs. *D*, RT-PCR assays detect *Lhx6* transcripts in MDPC-23 odontoblast mesenchyme cells and LS-8 oral epithelial cells. **, $p < 0.01$. *Error bars*, S.E.

EXPERIMENTAL PROCEDURES

Animals—All animals were housed at the Institute of Biosciences and Technology under the care of the Program of Animal Resources and were handled in accordance with the principles and procedure of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Texas A & M Health Science Center Institutional Animal Care and Use Committee. The *Lhx6* mutant mice were described previously, and cryopreserved embryos were obtained from the investigators (15). The embryos were injected in donor female C57BL/6 mice at the Institute of Biosciences and Technology. These mice were maintained in the C57BL/6 background and mated to wild-type C57BL/6 mice. Mice were subsequently mated to 129/sv mice to maintain them in a mixed background and facilitate survival. Embryos were collected at various time points, considering the day of observation of a vaginal plug to be embryonic day 0.5 (E0.5).[3] Genotyping PCR primers for $Lhx6^{-/-}$ and $Lhx6^{+/-}$ mice and embryos were described previously (15). Genotyping PCR primers for *Lhx6* mutant and wild type mice are as follows: knock-out forward (PLAPGllrc), 5′-TCCTCAACTGGGATGATGC-3′ and reverse (424SU), 5′- AGAGGCTTGGATTGCAAAGG-3′, 379-bp knockout (KO); wild type forward (424TUF), 5′-TTGCCAGGTCCATTTTCCTC-3′ and reverse (424TUP), 5′-GAACACTTGCCCAAAGCTGC-3′, 70-bp WT. All PCR products were sequenced to confirm their identity.

[3] The abbreviations used are: E*n*, embryonic day *n*; P*n*, postnatal day *n*; BiFC, Bimolecular Fluorescence Complementation; EYFP, enhanced yellow fluorescent protein; MEF, mouse embryo fibroblast; Tg, transgenic.

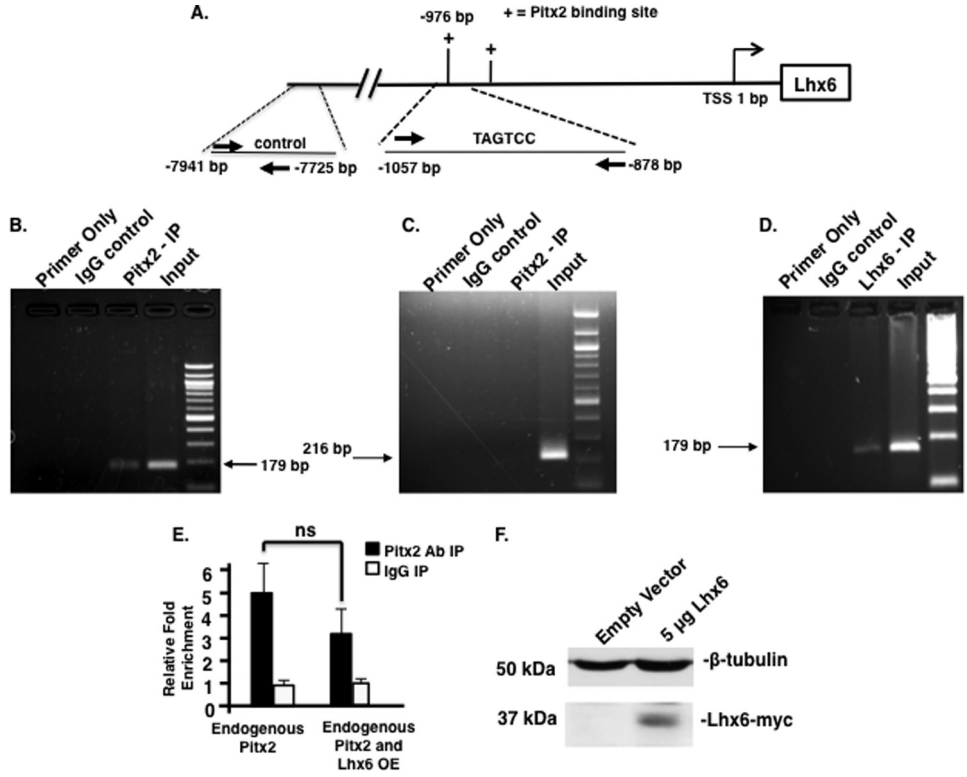

FIGURE 2. **Endogenous Pitx2 binds to the *Lhx6* promoter.** *A*, schematic of the *Lhx6* 5′-flanking sequence and location of PITX2 binding sites and the location of primers used to amplify the chromatin bound by PITX2 antibody. *B*, ChIP assay results. The chromatin input and PITX2 immunoprecipitated (*IP*) products are shown. As a control, IgG antiserum did not immunoprecipitate the chromatin. *C*, controls used primers to an upstream region of the *Lhx6* promoter that does not contain a PITX2 binding element. This region was not immunoprecipitated by IgG or PITX2 antibody; however, the primers did amplify the input chromatin. *D*, ChIP assay using LHX6 antibody and identical primers as in *B*. LHX6 was bound to the chromatin containing the PITX2 binding site. *E*, DNA fragment concentrations were analyzed by real-time PCR to calculate the -fold enrichment of the PITX2 antibody immunoprecipitation group normalized to the IgG group. No significant change was seen between the LHX6-transfected cells and control cells. *F*, Western blot shows LHX6 expression in the transfected cells. *OE*, overexpression. *ns*, not significant.

ChIP Assay—The ChIP assays were performed as described previously using the ChIP assay kit (Upstate) with the following modifications (33, 35). LS-8 cells were fed for 24 h, harvested, and plated in 60-mm dishes. Cells were cross-linked with 1% formaldehyde for 10 min at 37 °C the next day. All PCRs were done under an annealing temperature of 62 °C. The sense primer (5′-TTTGAGGGGGTGCTGTTTAC-3′) and the antisense primer (5′-CCTCAGCCTTCTTCCTC-AAA-3′) used to amplify the *Lhx6* promoter are located at −1057 and −898 bp in the distal promoter. Two control primers for amplifying a distal region of the *Lhx6* promoter without PITX2 binding elements are as follows: −7941 bp sense, 5′-AAATGTCTTTCTGCCGAGGA-3′; −7725 bp antisense, 5′-ACACCCAAAACCAAAAACCA-3′. All of the PCR products were evaluated on a 2% agarose gel in 1× TBE for appropriate size and confirmed by sequencing. As further controls, the *Lhx6* primers were used without chromatin, and normal rabbit IgG was used replacing the PITX2 antibody to reveal nonspecific immunoprecipitation of the chromatin. Quantitation of PITX2 binding to DNA fragment was performed using the same primers and conditions as above. The same amount of immunoprecipitated DNA was used in real-time PCR experiments. Each reaction was done in triplicate, and S.E. values were calculated from five independent experiments.

Cell Culture, Transient Transfection, Luciferase, and β-Galactosidase Assays—Chinese hamster ovary (CHO) cells or LS-8 (oral epithelial) cells (36) were cultured in DMEM supplemented with 5% fetal bovine serum (FBS) and penicillin/streptomycin and transfected by electroporation. Cultures were fed 24 h prior to transfection, resuspended in PBS, and mixed with 2.5 μg of expression plasmids, 5 μg of reporter plasmid, and 0.5 μg of SV40 β-galactosidase plasmid. Electroporation of CHO cells were performed at 380 V and 950 microfarads (Gene Pulser XL, Bio-Rad). Electroporation of LS-8 cells has been previously described (33). Transfected cells were incubated for 24 h in 60-mm culture dishes and fed with 5% FBS and DMEM and then lysed and assayed for reporter activities and protein content by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.). All luciferase activities were normalized to β-galactosidase activity.

Expression and Reporter Constructs—The expression plasmid containing the cytomegalovirus (CMV) promoter linked to the mouse *Lhx6* cDNA was purchased from Origene and cloned into pcDNA3.1 vector (Invitrogen). The mouse *Lhx6* 1.0-kb promoter amplified from mouse genomic DNA was constructed into the thymidine kinase-luciferase vector by replacing the minimal thymidine kinase promoter (28). The PITX2C and LEF-1 reporter constructs have been described as well as

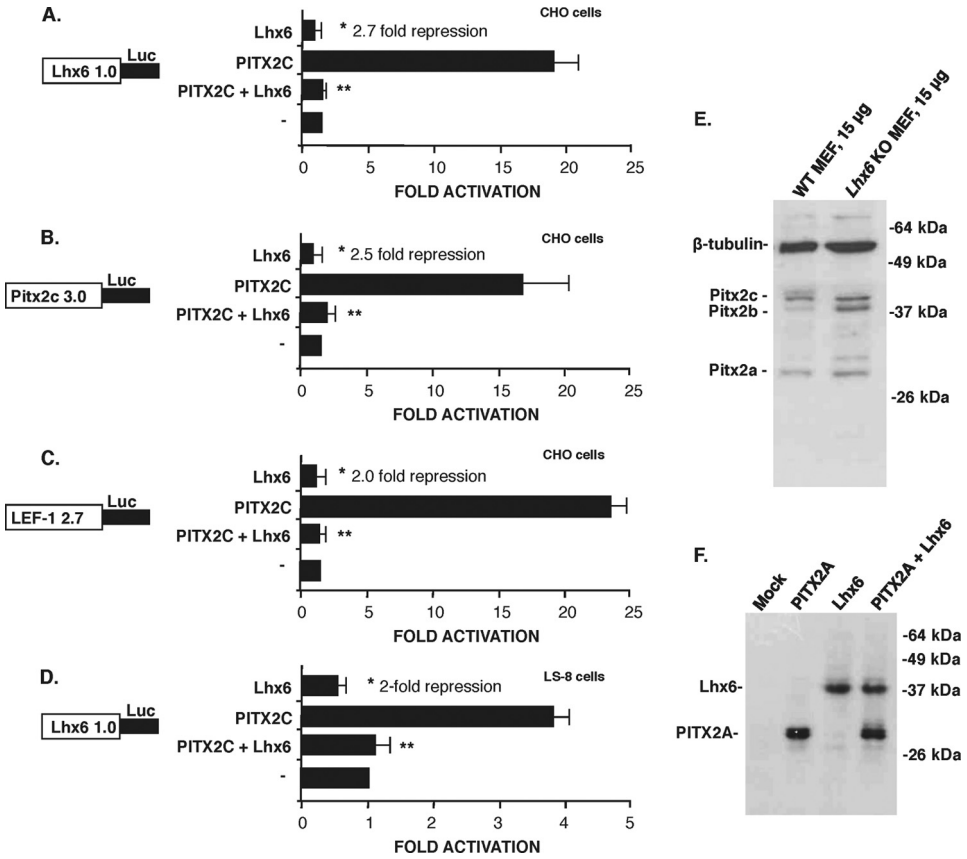

FIGURE 3. **Lhx6 acts as a transcriptional repressor and attenuates PITX2 transcriptional activity.** *A*, CHO cells were transfected with 2.5 μg of LHX6 and/or PITX2 expression plasmids and 5 μg of *Lhx6* 1.0-kb promoter plasmid. All DNAs were double CsCl-banded for purity, and cells were transfected by electroporation. To control for transfection efficiency, all transfections included the SV40 β-galactosidase reporter (0.5 μg). The activities are shown as mean -fold activation compared with the *Lhx6* promoter plasmid with empty expression plasmid and normalized to β-galactosidase activity; *error bars* indicate S.E. from at least three independent experiments. *B*, CHO cells were transfected as in *A*; however, the *Pitx2c* 3.0-kb promoter construct was used in place of the *Lhx6* promoter plasmid. *C*, CHO cells were transfected as in *A*, except the *Lhx6* promoter was replaced with the *Lef-1* 2.7-kb promoter plasmid. *D*, LS-8 oral epithelial cells were transfected as in *A*. *E*, Western blot of endogenous PITX2 expression in WT and *Lhx6*$^{-/-}$ MEFs. All three PITX2 isoforms are increased in the *Lhx6* null MEFs. *F*, the transfected proteins are shown to demonstrate expression in the cells and visualized using a Myc antibody and ECL reagents. *, $p < 0.05$; **, $p < 0.01$.

the β-catenin, PITX2, and LEF-1 expression plasmids (33, 37). Short hairpin LHX6 and short hairpin Scramble clones were constructed in the pSilencer4.1 vector containing the CMV promoter and the following short hairpin sequences targeting each transcript: Sh LHX6 forward, 5′-GATCCAGACGCAGAGGCCTTGGTTCAAGAGACCAAGGCCTCTGCGTCTAGA-3′; Sh LHX6 reverse, 5′-AGCTTCTAGACGCAGAGGCCTTGGTCTCTTGAACCAAGGCCTCTGCGTCTG-3′; Sh Scramble forward, 5′-GATCCCCGGCCTAAGGTTAAGTCGCCCTCGTTCAAGAGACGAGGGCGACTTAACCTTAGGTTTTAGA-3′; Sh Scramble reverse, 5′-AGCTTCTAAAACCTAAGGTTAAGTCGCCCTCGTCTCTTGAACGAGGGCGACTTAACCTTAGGCCGGG-3′.

Bimolecular Fluorescence Complementation (BiFC) Assay—The BiFC assay was performed as described previously (38, 39). The EYFP N-terminal fragment (amino acids 1–155) was inserted between the HindIII and NotI sites in pFLAG-CMV-2 plasmid (Sigma). PITX2C cDNA was then inserted in the modified plasmid between the XbaI and BamHI sites to build the YN-PITX2C construct. The EYFP C-terminal fragment (amino acids 156 –240, with stop codon) was inserted between the XbaI and BamHI sites in pFLAG-CMV-2 plasmid to build the FLAG-YC construct. LHX6 cDNA was then inserted between HindIII and NotI sites in the FLAG-YC plasmid to build a LHX6-YC construct. The EYFP N-terminal fragment (amino acids 1–155, with stop codon) was inserted between the XbaI and BamHI sites in pFLAG-CMV-2 plasmid to build the FLAG-YN construct. The constructs are shown in Fig. 5. The complementation of the YN and YC fusion proteins produce a fluorescence complex. The constructs were transfected into HEK 293 cells, and the fluorescence complementation in living cells was observed after 24 h using a Zeiss LSM510 confocal microscope equipped with a ×63 plan-apochromat oil objective and low noise fluorescence imaging capability.

Immunoprecipitation Assay—Approximately 24 h after cell transfection with LHX6 and PITX2, CHO cells were rinsed with 1 ml of PBS and then incubated with 1 ml of ice-cold radioimmune precipitation assay buffer for 15 min at 4 °C. Cells were harvested and disrupted by repeated aspiration through a 25-gauge needle attached to a 1-ml syringe. Cellular debris was pelleted by centrifugation at 10,000 × *g* for 10 min at 4 °C. An aliquot of lysate was saved for analysis as input control. Supernatant was transferred to a fresh 1.5-ml microcentrifuge tube on ice and precleared using the mouse IgG. Precleared lysate was incubated with protein A/G-agarose beads for 1–2 h at 4 °C. After a brief centrifugation, supernatant was transferred

to a new tube, and immunoprecipitation was performed with anti-PITX2 antibody (Capra Sciences). The supernatant was incubated with protein A/G-agarose beads at 4 °C overnight. Immunoprecipitates were collected by brief centrifugation, washed two times with PBS, and resuspended in 15 μl of double-distilled H_2O and 3 μl of 6× SDS loading dye. Samples were boiled for 5 min and resolved on a 10% polyacrylamide gel. Western blotting was performed with anti-LHX6 (Cell Science) antibody and HRP-conjugated reagent to detect immunoprecipitated proteins.

Histology and Immunohistochemistry—Embryos were treated with 4% paraformaldehyde and dehydrated with sequential concentration of alcohol and finally with xylene. The embryos were embedded in paraffin, and sections were made at 7-μm thickness. To examine tissue morphology, standard hematoxylin and eosin staining was used. For immunohistochemistry, sections were deparaffinized and treated with 0.1 M sodium citrate buffer for 12 min at 100 °C. The slides were incubated with 10% goat serum for 30 min, followed by overnight incubation with amelogenin antibody at a 1:500 dilution (sc-32892, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), Ki67 antibody at a 1:500 dilution (ab15580, Abcam), and ameloblastin antibody at 1:500 dilution (sc-50534, Santa Cruz Biotechnology, Inc.). Secondary antibodies (Alexa Fluor 488 goat anti-rabbit HCA) from Invitrogen (A11034) were used at a 1:500 dilution at room temperature for 30 min. The slides were then mounted with DAPI and analyzed using a Nikon 80i fluorescence microscope.

Real-time PCR—LHX6 primers were as follows: forward, 5′-TCGTTGAGGAGAAGGTGCTT-3′; reverse, 5′-CGTCATG-TCCGCTAGCTTCT-3′. β-Actin primers have been reported previously (33). All PCR products were sequenced to confirm their identity. Incisor and molar tooth germs were dissected from E14.5 mice using a dissection microscope. To separate epithelium and mesenchyme, the tooth germs were treated with dispase II and collagenase I (Worthington) for 30 min at 37 °C. This procedure separates the epithelium from the mesenchyme and allows for specific RNA extraction of the two tissue types. RNA extraction was performed using an RNeasy minikit from Qiagen. RT-PCR was performed using the iScript Select cDNA synthesis kit form Bio-Rad. Real-time PCR was performed using the iQ SYBR Green Supermix kit, and all *Ct* values were normalized by β-actin level.

Western Blot—CHO, LS-8, MDPC-23, and MEF cells were cultured and harvested, and lysates were analyzed for endogenous and transfected protein expression. Approximately 20 μg of lysate was resolved on 10% SDS denaturing gels. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted with LHX6 antibody (1:3000; ab57064, Abcam), β-tubulin antibody (1:500; sc-9104, Santa Cruz Biotechnology, Inc.), Myc antibody (1:500), or PITX2 antibody (1:500; PA-1020-100, CAPRA Science) and detected using ECL Plus reagents from GE Healthcare.

Scanning Electron Microscopy Imaging—Hemimandibles of *Lhx6*$^{-/-}$ and matched wild type littermate mice (E14 and E16) were dissected and stored in 70% ethanol. Images were taken with a Zeiss Evo LS 10 scanning electron microscope (Carl Zeiss, Peabody, MA) in secondary electron mode of uncoated specimen at 7 kV, 3 pA under high vacuum to assess gross morphology. For further analysis of tooth morphology and enamel microstructure, the incisors and molars were fractured and mounted on stubs using adhesive copper tape. Samples were then gold-coated (Denton V Sputter coater, Denton (Moorestown, NJ)) using 30 mA for 30 s. Scanning electron microscopy images were collected in secondary electron mode at 15–20 kV and 5–15 pA probe current in high vacuum mode at a working distance of 7–9 mm.

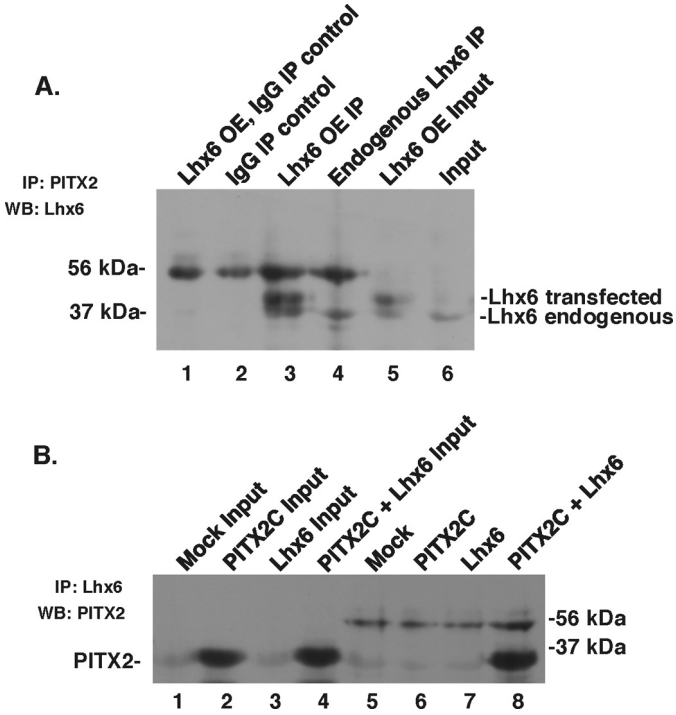

FIGURE 4. **Lhx6 and PITX2 interact.** *A*, co-imunoprecipitation assay. LHX6 expression plasmid (2.5 μg) was transfected in LS-8 cells and incubated for 24 h. Cells were harvested and lysed, and the LHX6·PITX2 protein complex was immunoprecipitated (*IP*) using the PITX2 antibody. The immunoprecipitated complex was resolved on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride filter, and Western blotting was done using the LHX6 antibody. Endogenous PITX2 complexed with endogenous and transfected LHX6 (*lane 3*) and endogenous LHX6·PITX2 complex was observed (*lane 4*). *B*, LHX6 and PITX2C were co-transfected in CHO cells, and a co-immunoprecipitation assay used the LHX6 antibody to immunoprecipitate the PITX2 protein interacting with LHX6. Western blot was done using the PITX2 antibody. Input controls are shown in *lanes 1–4*, and the LHX6·PITX2C complex is shown in *lane 8*.

Microcomputed Tomography—Hemimandibles of *Lhx6*$^{-/-}$ and matched wild type littermate mice (E14 and E16) were dissected and stored in 70% ethanol. Samples were analyzed in ethanol using a MicroCT 40 system (Scanco Medical, Brüttisellen, Switzerland) at 70 kV, 114 μA, 8 watts, and 10 μm resolution with an integration time of 300 ms. Image sequences in dicom format were processed in ImageJ (National Institutes of Health) and resliced to achieve orientation of samples for comparison in images and movies.

RESULTS

PITX2 Regulates Endogenous LHX6 Expression—A DNA microarray screen for new genes involved in craniofacial/tooth development identified *Lhx6* expression in both the dental epithelium and mesenchyme. Tooth germs from wild type E14.5

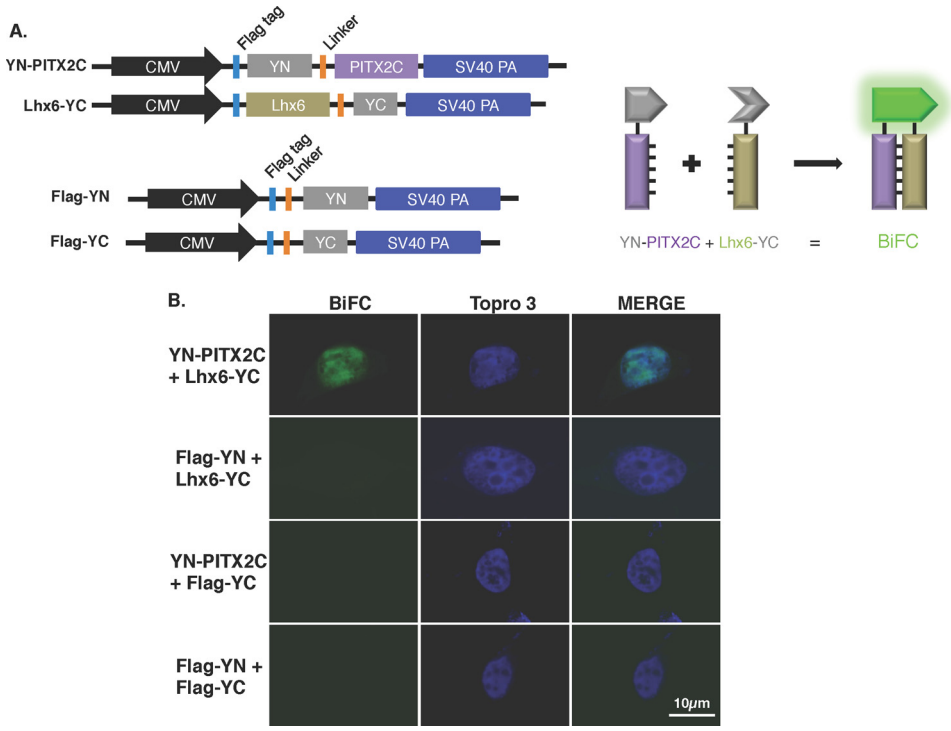

FIGURE 5. **Lhx6 and PITX2 proteins interact in the nucleus.** The BiFC assay was used to visualize protein interactions in living HEK 293 cells. *A*, schematic of the constructs used in the BiFC assay. LHX6 cDNA was linked to the EYFP C-terminal fragment, and PITX2C cDNA was linked to EYFP N-terminal fragment. Controls included the YN and YC fragment only vectors. A schematic of the complementation that produces fluorescence complex is also shown. *B*, the constructs were transfected into HEK 293 cells. Fluorescence complementation in living cells was observed after 24 h using a Zeiss LSM510 confocal microscope equipped with a ×63 plan-apochromat oil objective with low noise fluorescence imaging capability.

mice were isolated, the mesenchyme was separated from the epithelial component, total RNA was extracted for the DNA microarray (data not shown), and expression was confirmed by real-time PCR. The real-time PCR experiments demonstrate a slightly higher level of *Lhx6* transcripts in the dental epithelium than in the dental mesenchyme (Fig. 1*A*). Sequence analysis of the 5′-flanking region of the *Lhx6* gene revealed several PITX2 binding elements, and we asked if PITX2 regulated LHX6 expression. Furthermore, a previous report demonstrated reduced *Lhx6* expression in the *Pitx2* null mice (40). Mouse embryo fibroblasts (MEFs) were isolated from E12.5 wild type mice and PITX2C transgenic (Tg) mice and analyzed for *Lhx6* expression. Real-time PCR demonstrated increased *Lhx6* transcripts (3-fold) in the PITX2C Tg MEFs compared with wild type MEFs (Fig. 1*B*). To demonstrate increased LHX6 protein in PITX2C Tg MEFs, lysates were probed for LHX6 protein. LHX6 protein was observed in the PITX2C Tg MEFs with low levels in wild type MEFs and PITX2 KO MEFs (Fig. 1*C*). We further demonstrate *Lhx6* transcripts in MDPC-23 (dental mesenchymal cell line) cells and LS-8 cells by RT-PCR (Fig. 1*D*). Previous reports demonstrated *Lhx6* expression in the first branchial arch of E9.5 embryos and during early tooth developmental stages (E11.5 to P2) in the neural crest-derived mesenchyme (9) and dental epithelium by *in situ* hybridization experiments (12).

PITX2 is highly expressed in the dental epithelium throughout tooth development and is the first transcription marker for tooth development (21, 41). PITX2 and LHX6 are co-expressed in the dental and oral epithelium during early developmental stages of craniofacial and tooth morphogenesis (12, 21). Furthermore, because *Lhx6* is down-regulated in the *Pitx2* null mice, we asked if PITX2 directly regulated *Lhx6*.

Endogenous PITX2 Binds to the Lhx6 Promoter—Because LHX6 expression was increased in the PITX2C Tg MEFs and transfected CHO cells, we used the ChIP assay to identify endogenous PITX2 binding to the *Lhx6* chromatin. There are several PITX2 binding sites in the *Lhx6* promoter, and primers for the ChIP assay were designed to a distal PITX2 element (Fig. 2*A*). ChIP experiments demonstrate endogenous PITX2 binding to the *Lhx6* chromatin using a PITX2 antibody; however, as a control, IgG only did not precipitate the *Lhx6* chromatin (Fig. 2*B*). Other controls included primers to a more distal region of the *Lhx6* promoter with the PITX2 antibody and an IgG antibody, and these did not immunoprecipitate the *Lhx6* chromatin, demonstrating the specificity of the reaction (Fig. 2*C*). To determine if endogenous LHX6 interacted with PITX2 bound to DNA, we used the LHX6 antibody to immunoprecipitate the PITX2 binding element in the LHX6 chromatin. The identical primer set was used to amplify the PITX2 binding element, and there are no LHX6 binding sites in this region of the promoter. The ChIP assay identified an LHX6 interaction with this region of the chromatin (Fig. 2*D*). Quantitation of endogenous PITX2 binding revealed a 5-fold enrichment of bound PITX2 to the *Lhx6* promoter (Fig. 2*E*). LHX6 overexpression reduced the amount of endogenous PITX2 bound; however, the difference was not significant (Fig. 2*E*). A Western blot demonstrates the overexpression of LHX6 in the transfected cells (Fig. 2*F*). The slight reduction is due to the decrease in endogenous PITX2 expression by LHX6. These data corroborate LHX6 direct physical interactions with PITX2 and the regulation of

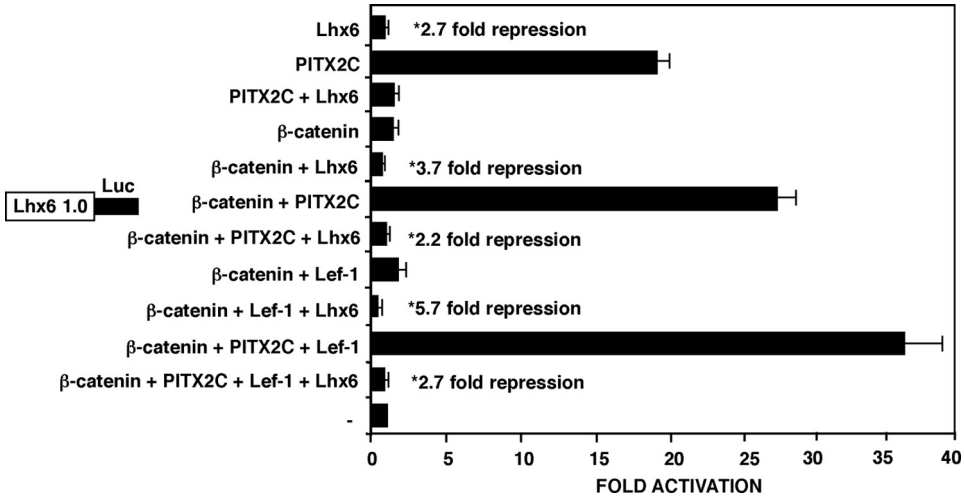

FIGURE 6. **PITX2 co-factors cannot override the Lhx6 repressive effect.** PITX2 interacting co-factors β-catenin and LEF-1 were co-transfected in CHO cells with PITX2 and/or LHX6 and the *Lhx6* 1.0-kb reporter plasmid as in Fig. 3. Equal amounts of plasmid DNA were transfected, including empty vector. DNAs were double CsCl-banded for purity, and cells were transfected by electroporation. To control for transfection efficiency, all transfections included the SV40 β-galactosidase reporter (0.5 μg). The activities are shown as mean -fold activation compared with the *Lhx6* promoter plasmid with empty expression plasmids and normalized to β-galactosidase activity; *error bars* indicate S.E. from at least three independent experiments.

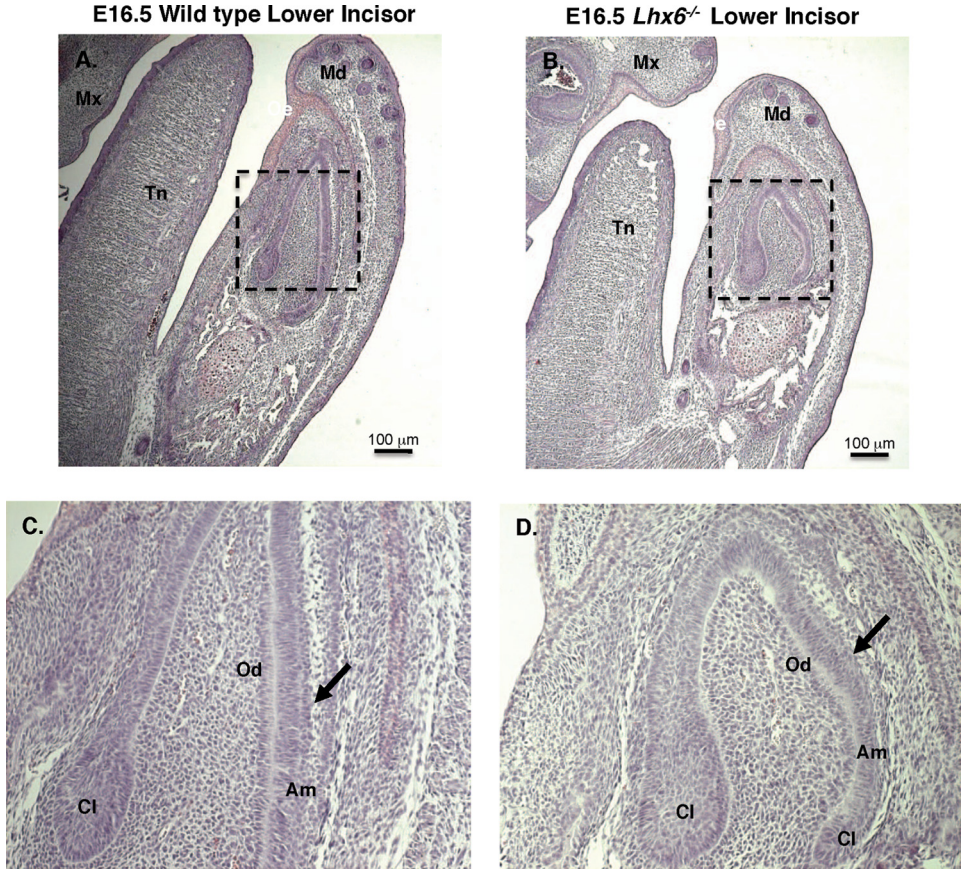

FIGURE 7. **Lhx6 null mice have a small mandible and lower incisors.** The *Lhx6*$^{-/-}$ mice have a smaller mandible and lower incisors compared with their littermates at E16. 5. WT and *Lhx6*$^{-/-}$ embryos were stained with hematoxylin and eosin. *A*, E16.5 WT mandible and lower incisor. The lower incisor tooth germ is framed by the *dotted box* to enhance the size of the incisor. *B*, E16.5 *Lhx6* null mandible and incisor. The quantitative *dotted box* frames the lower incisor to demonstrate the differences in incisor tooth germ size. The *dotted box* was copied and used in all images to compare the size differences between incisors of WT and mutant mice. *C* and *D*, higher magnification of the *boxed region* of the WT and *Lhx6*$^{-/-}$ mandible, respectively. The *arrow* points to the dental epithelium, which appears to be less well developed. *Tn*, tongue; *Mx*, maxilla; *Md*, mandible; *Cl*, cervical loop; *Am*, ameloblasts; *Od*, odontoblasts.

PITX2 expression we show in later figures using multiple experimental techniques. We next asked if transfected PITX2 activated the *Lhx6* promoter.

PITX2 Activates the Lhx6 Promoter and LHX6 Attenuates PITX2 Transcriptional Activity—The *Lhx6* promoter (1.0 kb) was cloned into the luciferase reporter and co-transfected with

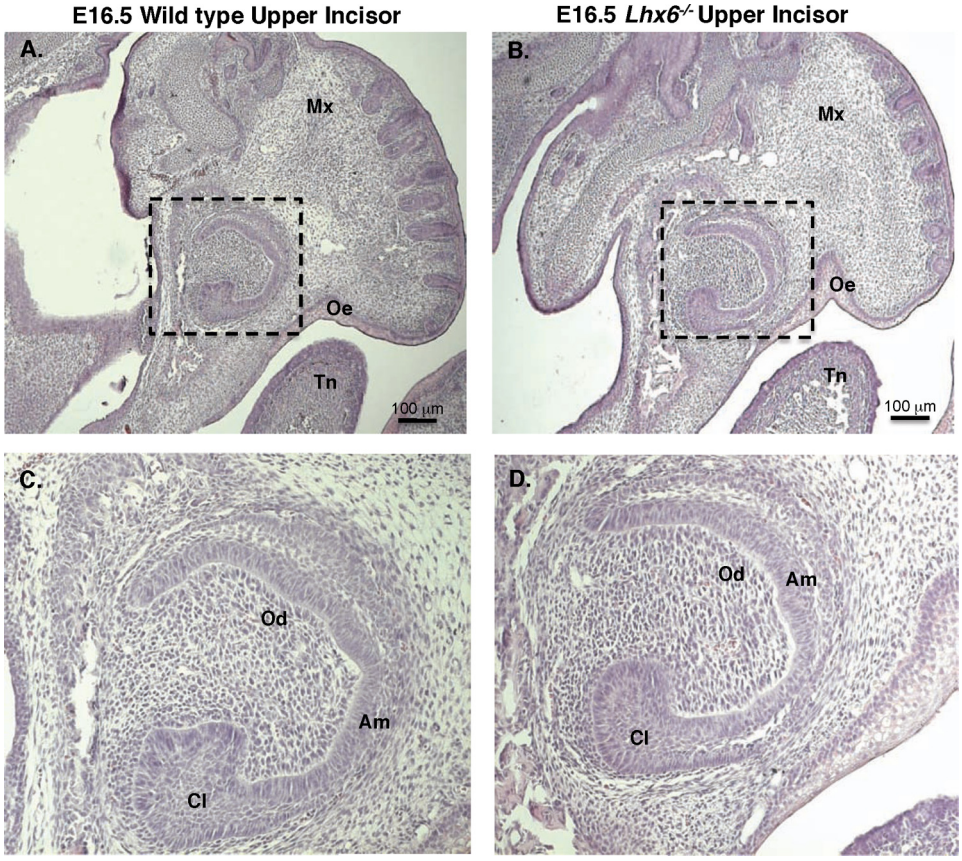

FIGURE 8. **E16.5 *Lhx6* null mice have normal upper incisors.** WT and *Lhx6*$^{-/-}$ embryos were stained with hematoxylin and eosin. *A* and *B*, WT and *Lhx6*$^{-/-}$ upper incisors (respectively) are normal in E16.5 embryos. The *dotted box* frames the upper incisor. *C* and *D*, higher magnification of the *boxed regions* in *A* and *B* of WT and *Lhx6*$^{-/-}$ incisors, respectively. *Tn*, tongue; *Mx*, maxilla; *Oe*, oral epithelium; *Cl*, cervical loop; *Am*, ameloblasts; *Od*, odontoblasts.

Lhx6 and/or PITX2 expression plasmids in CHO cells. LHX6 repressed *Lhx6* promoter activity (2.7-fold), whereas PITX2 activated the *Lhx6* promoter ~18-fold (Fig. 3*A*). However, co-transfection of both LHX6 and PITX2 resulted in complete repression of PITX2 transcriptional activity (Fig. 3*A*). To determine if LHX6 repression was promoter-specific, the *Pitx2c* and *Lef-1* promoters were co-transfected with LHX6 and/or PITX2. We have previously shown that these two promoters are activated by PITX2 (33, 37). Interestingly, LHX6 repressed the activity of both promoters in CHO cells, whereas PITX2 activated both promoters (Fig. 3, *B* and *C*). LHX6 repressed PITX2 activation of both promoters (Fig. 3, *B* and *C*). To determine if the LHX6 activity was cell-dependent, LS-8 cells (oral epithelial cells) were co-transfected with the *Lhx6* promoter and LHX6 and/or PITX2 expression plasmids. In these experiments, LHX6 repressed the *Lhx6* promoter and PITX2 activation, similar to CHO cell transfections, indicating that LHX6 repressive activity is not cell-dependent (Fig. 3*D*). These data reveal that LHX6 acts as a transcriptional repressor and that it also represses PITX2 transcriptional activation in a promoter- and cell-independent manner. Furthermore, these data suggest that LHX6 interacts with PITX2 directly to regulate PITX2 activity. To demonstrate a direct LHX6 regulation of PITX2 expression, we isolated *Lhx6* null MEFs, prepared cell lysates, and probed for endogenous PITX2 expression. Because PITX2 autoregulates its expression, we expected to observe an increase in PITX2 expression. The expression of all three PITX2 isoforms was increased in the *Lhx6* null MEFs, revealing a direct repression of PITX2 expression by LHX6 (Fig. 3*E*). The transfected PITX2 and LHX6 proteins are shown to confirm their expression using a Myc antibody (Fig. 3*F*). The PITX2 and LHX6 expression proteins have a Myc tag on the C-terminal end of the proteins.

LHX6 Physically Interacts with PITX2—LHX6- and PITX2-interacting complex was immunoprecipitated using the PITX2 antibody, and the immunoprecipitated LHX6 protein was detected using the LHX6 antibody. LS-8 cells endogenously express LHX6 and PITX2. Immunoprecipitation of LHX6- or mock-transfected cells with rabbit IgG did not immunoprecipitate LHX6 (Fig. 4*A*, *lanes 1* and *2*). Because LS-8 cells endogenously express LHX6 and PITX2, transfected LHX6 was able to form a complex with endogenous PITX2, and this complex was immunoprecipitated (Fig. 4*A*, *lane 3*). We further immunoprecipitated an endogenous PITX2·LHX6 complex, without transfected plasmids (Fig. 4*A*, *lane 4*). The difference in molecular weight of transfected LHX6, which migrates slightly slower than endogenous LHX6 due to a Myc tag on the transfected protein is compared with endogenous LHX6. The transfected LHX6 protein is shown in *lane 5*, and the endogenous LHX6 protein is shown in *lane 6*.

These experiments were repeated using LHX6 antibody to immunoprecipitate the LHX6·PITX2 complex. PITX2C protein is detected in the PITX2C-transfected and PITX2C- and LHX6-transfected input controls (Fig. 4*B*, *lanes 2* and *4*).

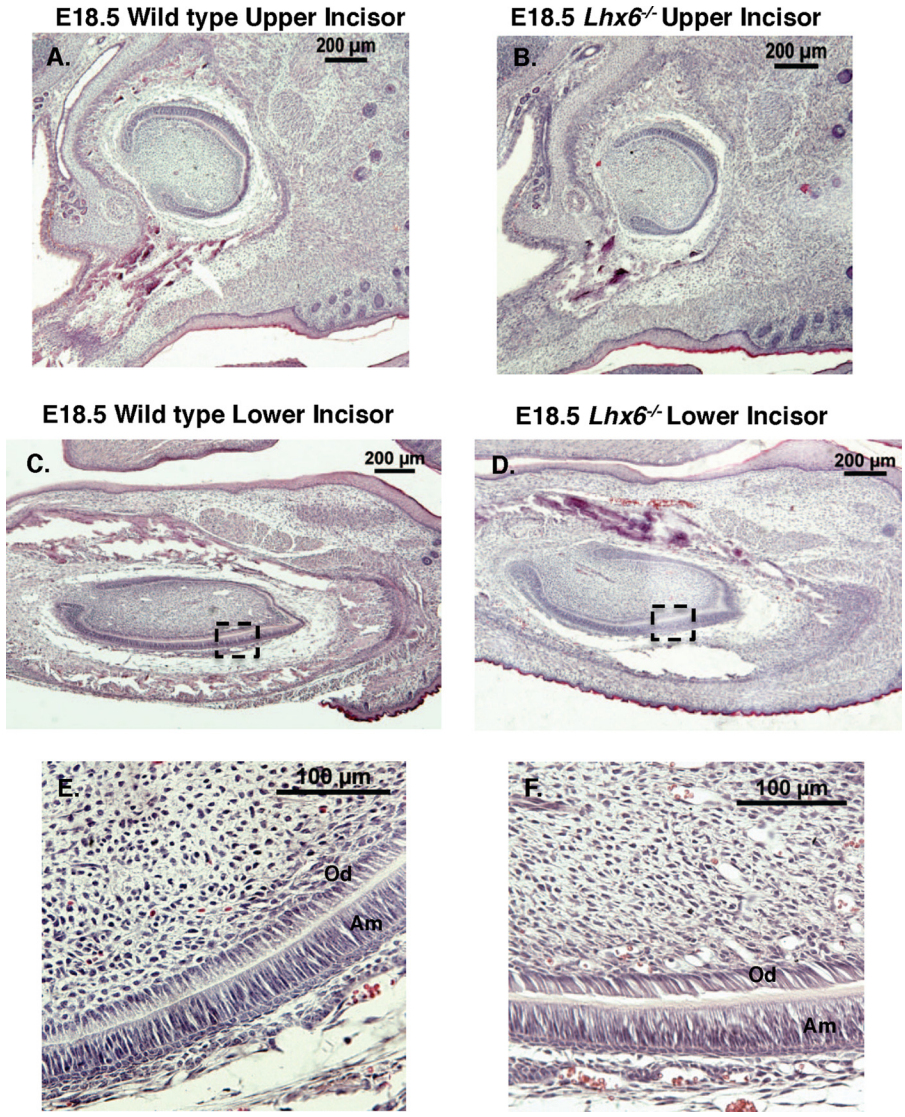

FIGURE 9. **Lower incisor ameloblast layer differentiation defects in *Lhx6*$^{-/-}$ mice.** *A* and *B*, E18.5 upper incisors are normal in *Lhx6*$^{-/-}$ embryos stained with hematoxylin and eosin; compare WT littermates (*A*) with *Lhx6*$^{-/-}$ mice (*B*). *C* and *D*, the E18.5 *Lhx6*$^{-/-}$ lower incisors (*D*) are slightly smaller and less differentiated than wild type (*C*). *E* and *F*, *higher magnification* of the *dotted box* reveals a less dense ameloblast layer in the *Lhx6*$^{-/-}$ mouse incisors (*D* and *E*). *Am*, ameloblasts; *Od*, odontoblasts.

Because PITX2 is not expressed in CHO cells, the LHX6·PITX2C complex was only detected when both proteins were co-transfected (Fig. 4*B*, *lane 8*). Thus, LHX6 directly interacts with PITX2 to repress PITX2 transcriptional activity.

BiFC Assay—To demonstrate an *in vivo* PITX2 and LHX6 interaction and cellular localization, we used the BiFC assay. PITX2 was cloned with the EYFP N-terminal fragment next to its N terminus, and LHX6 was cloned with the EYFP C-terminal fragment adjacent to its C terminus (Fig. 5*A*). A linker separates the YN and YC peptides from the PITX2 and LHX6 protein. If YN-PITX2 and LHX6-YC proteins interact in the living cells, the YN and YC moieties form a fluorescence protein complex (38, 39). The YN-PITX2·LHX6-YC complex is evenly distributed in small nuclear bodies (Fig. 5*B*). As controls, we demonstrate that YN-PITX2 with the FLAG-YC and LHX6-YC with FLAG-YN do not fluoresce on their own or form a complex (Fig. 5*B*). Additional controls include YC, YN, YN-PITX2, and LHX6-YC, which do not fluoresce by themselves individually or together (data not shown). These data reveal a direct PITX2·LHX6 interaction in living cells and demonstrate a nuclear interaction.

LHX6 Represses PITX2 Activation in the Presence of PITX2 Co-factors—To understand if PITX2 co-factors could negate the repressive effect of LHX6 on PITX2 transcriptional activity, these factors were co-expressed with LHX6 and PITX2 with the *Lhx6* promoter in CHO cells. We have previously shown that PITX2 interacts with β-catenin and LEF-1 to synergistically regulate promoter activity (29, 33). β-Catenin does not activate the *Lhx6* promoter, and co-transfection with LHX6 slightly increases LHX6 repressive activity (Fig. 6). PITX2 and β-catenin co-transfection increased PITX2 activation of the *Lhx6* promoter from 18-fold (PITX2 alone) to 27-fold activation (Fig. 6). Co-transfection of LHX6 with these factors repressed this increased activation from 27-fold activation to 2.2-fold repression of *Lhx6* promoter activity (Fig. 6). LEF-1 and β-catenin co-transfection did not activate the *Lhx6* promoter;

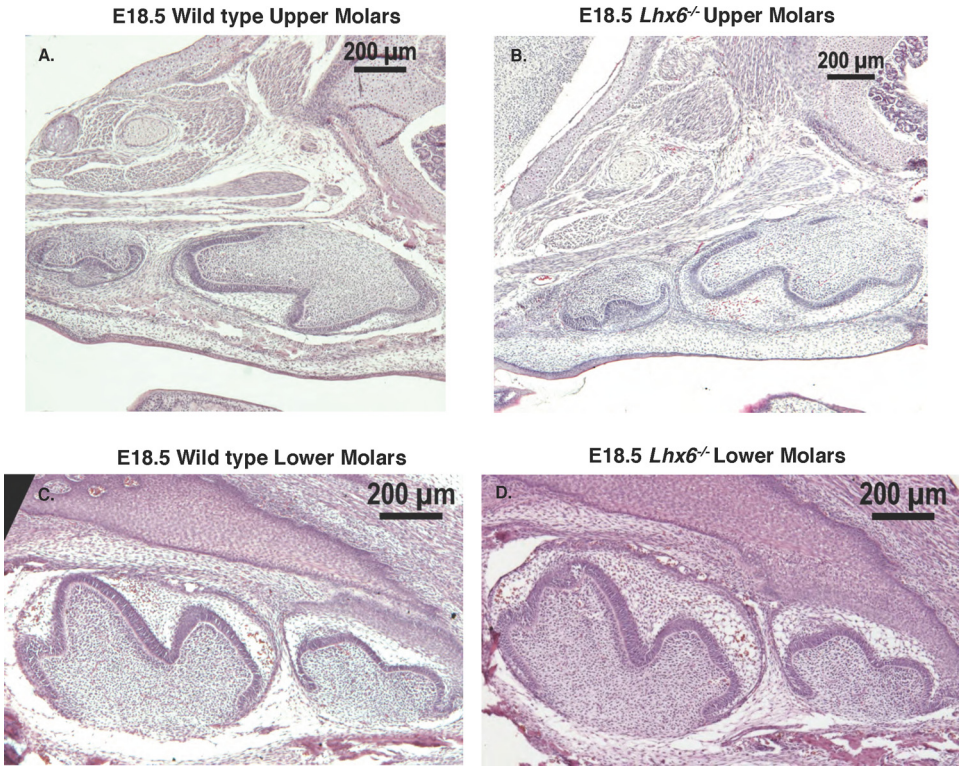

FIGURE 10. **Early stage molar development is normal in E18.5 *Lhx6*$^{-/-}$ mice.** WT and *Lhx6*$^{-/-}$ E18.5 embryos were stained with hematoxylin and eosin. *A–D*, no defects were observed in the *Lhx6*$^{-/-}$ molars compared with wild type embryos.

however, these factors enhanced the repressive activity of LHX6 to 5.7-fold repression and increased PITX2 activation to 36-fold (Fig. 6). LHX6 repressed PITX2 activation of the *Lhx6* promoter in the presence of β-catenin and LEF-1 (Fig. 6). These data indicate that LHX6 repression of PITX2 transcriptional activity appears to be dominant over β-catenin and LEF-1 co-factor interactions. These data indicate that LHX6 has strong repressive activity, and interestingly, this repressive activity is enhanced in the presence of β-catenin and LEF-1.

Lhx6 Null Mice Have a Slightly Smaller Mandible and Lower Dentition—We have shown that LHX6 acts as a transcriptional repressor and interacts with PITX2 to regulate several genes involved in odontogenesis. To understand the *in vivo* effects of *Lhx6* on tooth development, we analyzed the *Lhx6* null mice. Most of these mice are embryonic lethal; however, after back-crossing them with the 129/sv mice (C57BL/6 × 129/sv) and maintaining them on a mixed background, a few *Lhx6*$^{-/-}$ mice survived after birth. The E16.5 mouse embryos maintained on the mixed background have a smaller mandible than wild type mice and a smaller lower incisor with defects in ameloblast differentiation and polarization (*arrows* denote polarized pre-ameloblasts) compared with wild type embryos (Fig. 7). The *Lhx6*$^{-/-}$ preameloblasts (*Am* in Figs. 7–9) are polarized but are not as densely packed or as elongated as the wild type preameloblasts. The *Lhx6*$^{-/-}$ upper incisors (Fig. 8) and molars (data not shown) for E16.5 are similar at this stage compared with wild type embryos. At E18.5, the *Lhx6* null mouse lower incisors (Fig. 9*C*) are less developed than those of their wild type littermates (Fig. 9*D*), whereas the upper incisors are normal (Fig. 9, *A* and *B*). Higher magnification revealed less organized ameloblasts (*Am*) and odontoblasts (*Od*) in the *Lhx6*$^{-/-}$ mice (Fig. 9*F*) at E18.5. These defects in lower incisor development appear to affect both dental epithelial and odontoblast cells, suggesting that LHX6 plays a role in their differentiation. Interestingly, bone formation appears to be decreased in the *Lhx6* null mutant incisors compared with wild type mice (Fig. 9, *B* and *D*). Bone formation mesial of the incisor tip is the most pronounced difference; however, more experiments are required to determine the specific bone defect. Molar development in the *Lhx6*$^{-/-}$ mice appears normal at E18.5 (Fig. 10).

Late Stage Molar, Root, and Bone Development Is Affected in P14 Lhx6 Null Mice—Analyses of developed molars at P14 in *Lhx6* null mice revealed defects in enamel thickness (Fig. 11*A*, *white arrows*) compared with wild type molars. More dramatically, however, the *Lhx6* null molar root formation is severely affected with defects in root structures (Fig. 11*A*, *yellow arrows*) compared with wild type. Interestingly, bone development is defective in the mutant mice (Fig. 11*A*, *blue arrows*). Root formation can be affected by decreased alveolar bone formation, and cortical bone formation is also severely affected in the *Lhx6* null mice. Furthermore, the molars are smaller and not well developed compared with wild type molars at this stage. Analyses of the *Lhx6* null upper and lower molars show smaller cusps and smaller molars compared with wild type littermates (Fig. 11, *B* and *C*). However, the entire mandible is smaller in the *Lhx6* null mice compared with wild type mice at the same stage.

Ameloblast Differentiation and Proliferation Are Affected in the Lhx6 Null Mice—*Lhx6* null embryo lower incisors were analyzed for amelogenin and ameloblastin expression. Amelogenin and ameloblastin are well characterized differentiation markers for ameloblast cells and required for enamel formation (42–

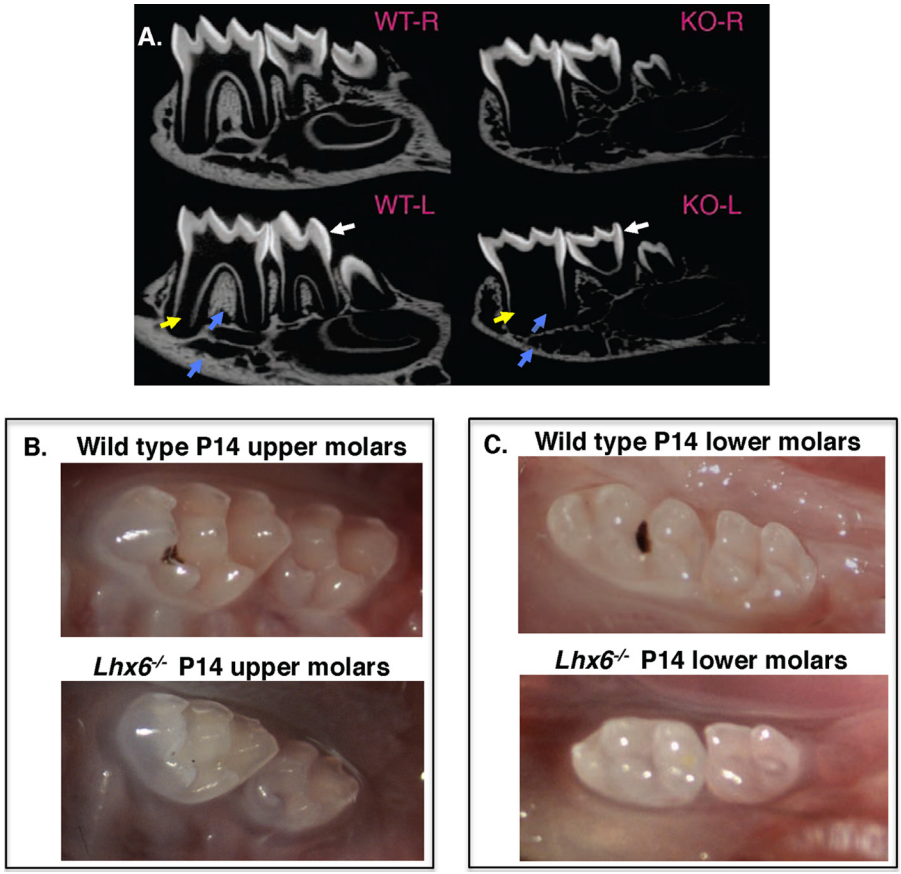

FIGURE 11. **Late stage molar, root, and bone development is affected in P14 *Lhx6* null mice.** *A*, microcomputed tomography analysis of *Lhx6* null (*KO*) P14 molars compared with WT littermates. The right side (*R*) and left side (*L*) of the mandible are shown. The *white arrows* denote defects in enamel formation, the *yellow arrows* show defects in root development, and the *blue arrows* point to bone developmental defects in the *Lhx6* null mice. *B* and *C*, analyses of the P14 molars demonstrate a decrease in size, shape, and cusp formation of both $Lhx6^{-/-}$ upper and lower molars.

44). E18.5 *Lhx6* null mice revealed decreased amelogenin and ameloblastin expression by immunohistochemistry compared with wild type littermates (Fig. 12). The decrease in amelogenin and ameloblastin staining indicates a defect in ameloblast differentiation in the E18.5 *Lhx6* null embryos. Some $Lhx6^{-/-}$ mice survived to P14 (2 weeks old) in the mixed background, and the lower incisors are smaller and have a somewhat chalkish appearance suggestive of an enamel defect (Fig. 12*F*). The P14 wild type littermate lower incisors are opaque, and the enamel and tooth shape appear normal (Fig. 12*E*). However, amelogenin and ameloblastin may be delayed because the presence of enamel prism decussation in P14 and p16 *Lhx6* null incisors and molars indicates a delay in amelogenin secretion and enamel formation, resulting in a thinner enamel layer (data not shown). PITX2, DLX2, and LEF-1 expression are regulated by PITX2, and LHX6 represses PITX2 transcriptional activity in wild type MEFs; however, endogenous PITX2 and DLX2 expression are increased in the *Lhx6* null MEFs (Fig. 12*G*). These data further support a role for LHX6 directly repressing PITX2, DLX2, and LEF-1 expression. Furthermore, knockdown of LHX6 by shRNA in LS-8 cells shows an increase in PITX2, DLX2, and LEF-1 transcripts (Fig. 12*H*). Western blot analyses demonstrate the knockdown of LHX6 by shRNA (Fig. 12*I*).

We next performed Ki67 staining to determine if $Lhx6^{-/-}$ dental epithelial cells were defective in cell proliferation. Ki67 antibody stains actively mitotic cells and is an indicator of cell proliferation. The Ki67 staining result was unexpected and demonstrated decreased mitotic activity in the labial cervical loop and increased mitotic activity in the anterior region of the incisor tooth germ normally associated with differentiated cells, compared with wild type (Fig. 13, *D*, *E*, and *G*). The cervical loop regions contain the epithelial stem cells and transit-amplifying cells that are actively proliferating and stain for Ki67 expression in wild type tooth germs (Fig. 13*H*). However, in *Lhx6* null embryos, these cervical loop cells are not proliferating at this stage, whereas the anterior cells are mitotically active (Fig. 13*G*). These results corroborate the reduced or delayed amelogenin and ameloblastin expression and delayed cell differentiation in the $Lhx6^{-/-}$ dental epithelium.

DISCUSSION

LIM homeodomain proteins are transcriptional regulators of developmental programs and tissue morphogenetic processes, and they regulate cell specification and differentiation (7). However, the molecular mechanisms of these proteins are not well understood. It is clear that LIM homeodomain proteins can interact with other co-factors through their LIM domains, and the homeodomain motif is required to bond *cis*-elements in the genome (2). The LIM domain is thought to confer a structural basis for the formation of cell type-specific protein-protein interactions that target different genes and regulate their

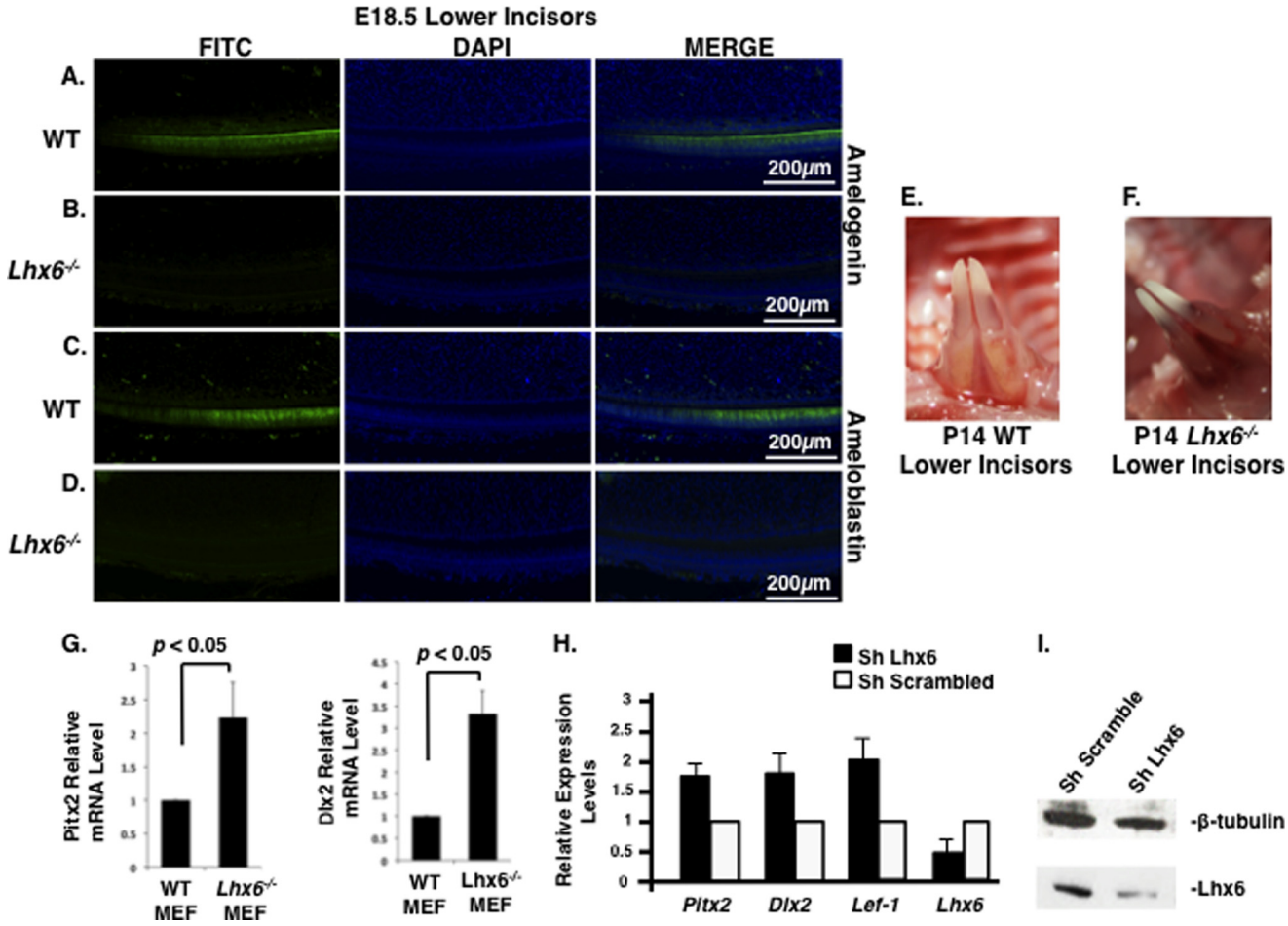

FIGURE 12. **Reduced amelogenin and ameloblastin expression in *Lhx6*$^{-/-}$ mouse lower incisors.** *A*, WT E18.5 incisor demonstrating normal amelogenin staining. *B*, loss of amelogenin expression in E18.5 *Lhx6*$^{-/-}$ lower incisors. *C*, WT E18.5 lower incisors showing normal ameloblastin expression in the ameloblast layer. *D*, loss of ameloblastin expression in the E18.5 *Lhx6*$^{-/-}$ lower incisor. Staining was performed using amelogenin and ameloblastin antibodies and secondary fluorescence antibody and analyzed using a Nikon 80i fluorescence microscope. *E* and *F*, the P14 *Lhx6* null lower incisors are smaller and appear chalky, consistent with an enamel defect. *G*, endogenous PITX2 and DLX2 transcript levels measured by real-time PCR are increased in the *Lhx6*$^{-/-}$ MEFs compared with wild type MEFs. *H*, endogenous PITX2, DLX2, and LEF-1 transcripts measured by real-time PCR are increased in the shRNA LHX6 knock-down LS-8 cells. LHX6 transcripts are knocked down ~40–50%. *I*, Western blot shows knockdown of the LHX6 protein by shRNA (5 μg). β-Tubulin was used as a loading control. All real-time PCRs were performed in triplicates, and S.E. values (*error bars*) were calculated from five independent repeats.

expression in diverse tissues (8). There are multiple examples of different LIM domain proteins interacting with diverse proteins. LHX6 is a member of the LHX7/LHX6 group of diverse LIM homeodomain proteins whose transcriptional activities and downstream targets are unknown (7). LHX6 plays an important role in the developing central nervous system, including the specification and migration of interneuron subtypes (13, 14, 16, 45, 46). In this report, we have identified a transcriptional mechanism for LHX6 during tooth development.

LHX6 Expression in Craniofacial/Tooth Epithelial and Mesenchymal Tissues and Cells—DNA microarray analyses of E16.5 and P1 excised dental epithelium and mesenchyme revealed high levels of *Lhx6* transcripts in both tissues, with slightly more in the epithelial compartment. Real-time PCR confirmed *Lhx6* expression in both tissues and also confirmed previous reports of *Lhx6* expression in these tissues by *in situ* hybridization (9, 11, 12). LHX6 expression was also observed in the dental mesenchymal (MDPC 23) cell line and oral epithelial (LS-8) cell line.

PITX2 Regulates LHX6 Expression and LHX6 Acts as a Transcriptional Repressor—Identification of LHX6 expression in the oral and dental epithelium suggests that other factors, such as PITX2, might regulate Lhx6 expression. PITX2 is the first transcriptional marker of tooth development and is specifically expressed in the oral and dental epithelium (20, 21). A previous report on the role of PITX2 in craniofacial development using targeted deletion of *Pitx2* isoforms in the mouse revealed decreased *Lhx6* expression in the maxillary primordial of the *Pitx2* null mutant mice (40). However, it was not known if the reduction in *Lhx6* expression was directly linked to PITX2 or due to defective FGF signaling (40). Analyses of PITX2C transgenic MEFs and PITX2-transfected cells demonstrated a specific activation of LHX6 expression by PITX2. Chromatin immunoprecipitation assays identified endogenous PITX2 binding to the *Lhx6* promoter, and transient transfection assays demonstrated PITX2 activation of the *Lhx6* promoter. This is the first report of a transcription factor regulating LHX6 expression in dental tissues. Interestingly, PITX2 is also

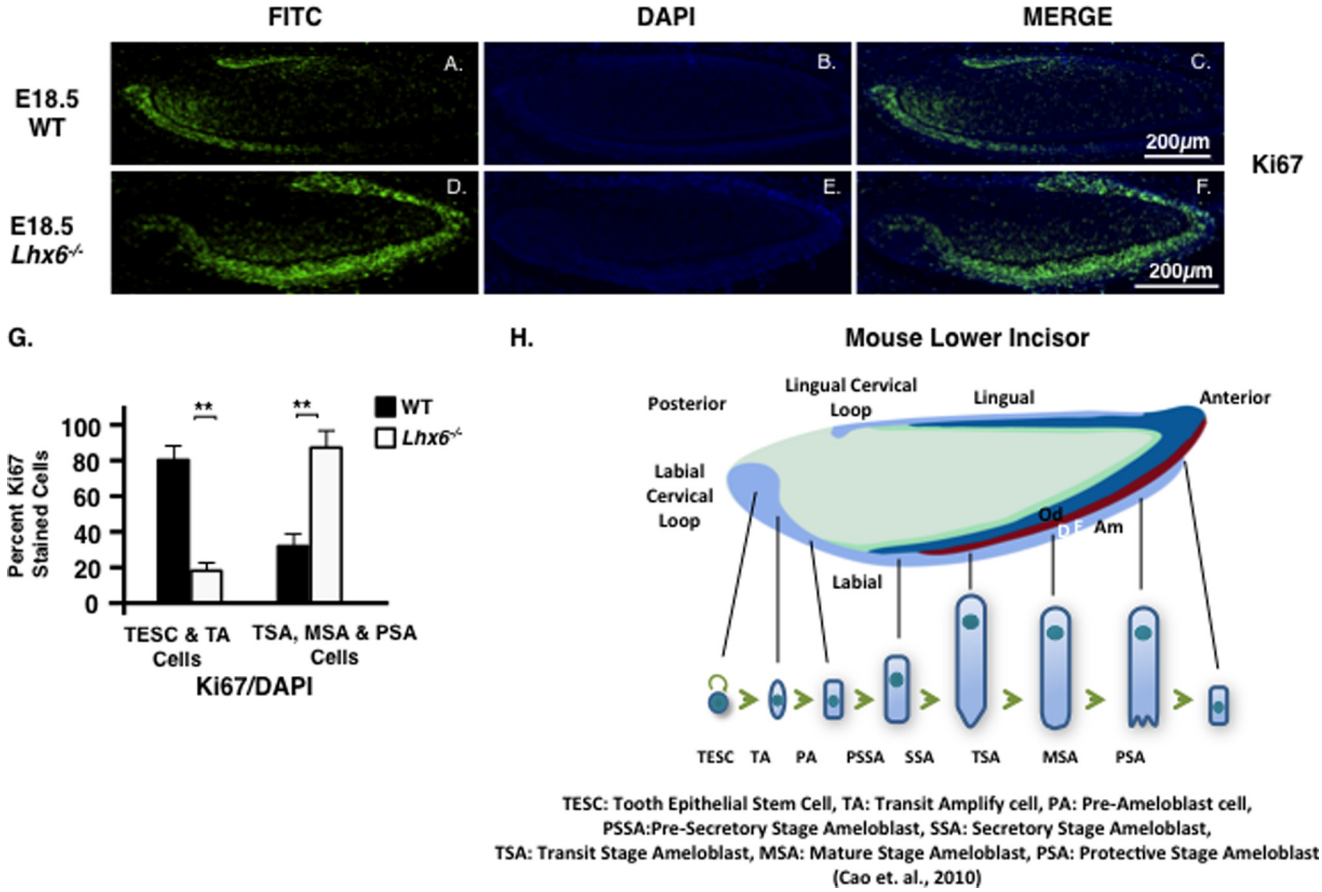

FIGURE 13. **Ameloblast cells are mitotically active in the *Lhx6*$^{-/-}$ lower incisor.** *A–C*, E18.5 WT lower incisors stained with the Ki67 antibody and a secondary fluorescence antibody. The mitotically active cells are located in the posterior cervical loop regions (*H*). In WT E18.5 incisors, Ki67 expression is localized to the posterior region and cervical loop because these are regions of cell proliferation. *D–F*, in E18.5 *Lhx6*$^{-/-}$ incisors, Ki67 stains cells predominantly in the anterior region of the incisor. This region is normally composed of preameloblasts and ameloblast cells that have migrated from the posterior region and undergone differentiation. There is a lack of Ki67 staining in the cervical loop and posterior region in these embryos. *G*, quantitation of the Ki67-positive cells demonstrates decreased cell proliferation in the posterior region and increased proliferation in the anterior region of the *Lhx6*$^{-/-}$ mice. *H*, diagram of cell morphogenesis (proliferation, migration, and differentiation) in the constantly growing mouse lower incisor. The epithelial stem cells propagate in the cervical loop regions and migrate toward the anterior region while differentiating into ameloblast stage-specific cells. **, $p < 0.01$. *Error bars*, S.E.

expressed in the brain and could potentially regulate LHX6 in specific neurons; however, it is not clear if LHX6 and PITX2 have overlapping expression patterns in these tissues (47). FGF factors can also activate LHX6 expression in de-epithelialized mandibular processes *in vitro* and are candidates to regulate LHX6 in the brain (9, 11).

A recent report demonstrated LHX6 activating an Shh enhancer element in transfected cells (45). In contrast, we demonstrate that LHX6 acts as a repressor of transcription in multiple cells with multiple promoters. However, these results are indicative of the role of LIM homeodomain proteins and their interactions with diverse cell specific proteins that can either positively or negatively regulate their transcriptional activities (2, 6, 8). In the cells and tissues tested in this report, we observe LHX6 acting as a transcriptional repressor, and LHX6 directly repressed the *Pitx2c*, *Lef-1*, and *Lhx6* promoter constructs. Interestingly, LHX6 feeds back to repress its promoter, providing a mechanism to regulate its expression (Fig. 14).

LHX6 directly interacts with PITX2, and this interaction represses the transcriptional activity of PITX2. We used the BiFC assay to demonstrate this interaction in living cells and to demonstrate a direct interaction between PITX2 and LHX6 in the nucleus, confirming the immunoprecipitation results. The specific interaction domain of PITX2 with LHX6 is not known; however, LHX6 can repress PITX2 activity in the presence of known PITX2-interacting factors. To date there are two protein interaction domains identified within the PITX2 protein

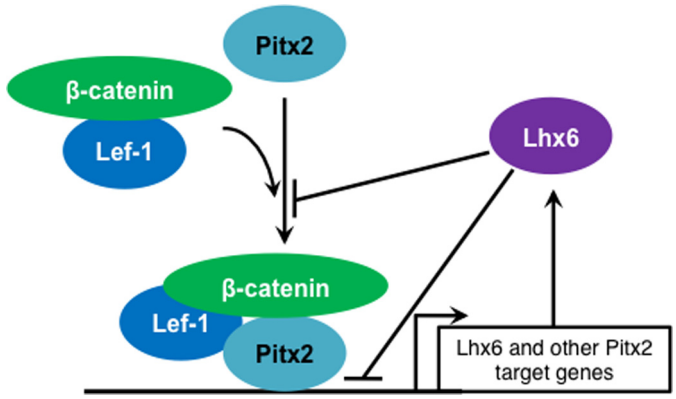

FIGURE 14. **Model for the transcriptional activity of Lhx6 and co-factors.** PITX2 activates *Lhx6* gene expression, and LHX6 represses PITX2 transcriptional activity. LHX6 attenuates PITX2 transcriptional activity in the presence of β-catenin and LEF-1 co-factors. LHX6 represses its promoter activity as well.

localized to the C-terminal tail and the homeodomain (22, 23, 28, 29). β-Catenin and LEF-1 interact with the PITX2 homeodomain and C-terminal tail, respectively, and are potent synergistic activators of PITX2 transcriptional activity (29, 33). LHX6 expression acts to repress the synergistic activation of PITX2 by these factors. These factors may form a ternary structure, or LHX6 may act to inhibit these factors from interacting with PITX2. We are currently researching the molecular mechanisms of these interactions. The increased repressive activity of LHX6 in the presence of β-catenin and LEF-1 may indicate that LHX6 directly interacts with these proteins.

LHX6 Regulation of Tooth Development—A previous report did not detect significant cranial defects in the *Lhx6*$^{-/-}$ mice (11), and in the related *Lhx7* (*Lhx8*) null mice, only an isolated cleft palate was observed (17). However, *Lhx6/7*-deficient mice present with molar agenesis, supernumerary incisor-like teeth, cranial skeletal defects, and a cleft palate (11). This study revealed redundant activities of these two co-expressed factors in craniofacial development (9, 11, 12). Our *Lhx6*-deficient mice also do not present with severe cranial defects, but we have identified both upper and lower incisor and molar developmental defects, although these defects are subtle in nature. We have observed the defect in *Lhx6* null mice at a later stage. Results from these mice were not previously reported at this late stage. The *Lhx6*$^{-/-}$ incisor is smaller than that of their wild type littermates, and the incisor has a preameloblast differentiation defect. The rodent incisor grows continuously to compensate for the destruction of the apical end, and the cells of the cervical loop (progenitor or stem cells) give rise to new epithelial cells that differentiate into ameloblast cells (Fig. 13*H*) (48–54). Notably, the cervical loop cells of the *Lhx6*$^{-/-}$ mice have reduced mitotic activity; however, the anterior cells are proliferating, which is not seen in wild type tooth germs. These mitotically active cells do not overpopulate the anterior region of the incisor or make a large tooth structure at the anterior end. They either remain in the cell cycle without mitosis or undergo apoptosis; we are currently exploring these mechanisms. From these data, LHX6 appears to control cell proliferation in the cervical loop and promotes preameloblast differentiation in the anterior region of the lower incisor.

The defective bone and root development in the *Lhx6* null mice may reflect the loss of activity of LHX6 in the dental mesenchyme. It is clear that the LHX6 is expressed in both the epithelial and mesenchymal components of the developing tooth. However, PITX2 is only expressed in the dental epithelial cells; thus, in the mesenchyme, LHX6 regulation is maintained by other factors, and its activity in regulating root and bone development is novel. The decrease in mandibular bone development may cause the small mandible observed in the *Lhx6* null mice. Furthermore, a decrease in dentin formation and bone formation may affect root and enamel formation. We are currently analyzing odontoblast differentiation as it relates to dentin and bone formation, which can modulate enamel formation.

The *K14-PITX2C* overexpression mouse has subtle craniofacial and tooth defects, including defects that are similar to those of the *Lhx6*$^{-/-}$ mice (data not shown). PITX2C overexpression affects ameloblast differentiation, and these mice have a slightly smaller mandible and lower incisors. As with the *Lhx6*$^{-/-}$ mice, which have increased PITX2 expression, the *K14-PITX2C* mice have somewhat similar phenotypes.

In summary, this report demonstrates new molecular mechanisms for LHX6 in regulating odontogenesis. PITX2 activates LHX6 expression, and LHX6 feeds back to repress PITX2 transcriptional activity through a direct protein interaction. LHX6 represses its promoter activity as well as *Pitx2c* and *Lef-1* promoters, and this repressor activity is enhanced by LEF-1 and β-catenin. PITX2 autoregulates the *Pitx2c* promoter, and LHX6 acts to attenuate PITX2 autoregulation. In all experiments, LHX6 acts as a potent transcriptional repressor, and it may interact with LEF-1 and β-catenin. We demonstrate an *in vivo* effect for LHX6 on tooth development through a unique mechanism that involves epithelial proliferation and differentiation. A lack of LHX6 protein results in decreased cell proliferation in the cervical loop region of the lower incisor and a lack of epithelial cell differentiation in the anterior region of the incisor. This is coupled with an abnormal cell proliferation of the undifferentiated cells that migrate to the anterior region of the incisor. LHX6 appears to be a major regulator of progenitor cell proliferation in the cervical loop and allows for cells to differentiate in the anterior incisor. Furthermore, LHX6 is required for normal odontoblast, bone, and root development.

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