Pitx2 Prevents Osteoblastic Transdifferentiation of Myoblasts by Bone Morphogenetic Proteins*1

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Muscle cells are often exposed to bone morphogenetic proteins (BMPs) in pathological muscle and/or bone conditions. Because BMPs function as strong bone inducers as well as myogenic inhibitors, certain molecules likely prevent muscle cells from converting into pathologic bone; without these molecules, de novo bone would form as observed in myositis ossificans traumatica. When C2C12 myoblasts are exposed to BMPs, they differentiate into osteoblastic cells but cannot mature into bone cells. As the Osterix gene, a transcription factor for osteoblast differentiation, is only transiently induced upon BMP stimulation in C2C12 cells, we hypothesized that unknown transcriptional repressor(s) inhibit Osterix expression and prevent complete osteoblastic differentiation. Gene microarray analyses were performed to identify putative inhibitors for osteoblastic differentiation, and the paired-like homeodomain transcription factor Pitx2 (also termed Rieg), which plays an important regulatory role in left-right asymmetry, was identified. Pitx2 was induced 2 days after BMP stimulation in C2C12 cells in concert with Osterix down-regulation. Overexpression of Pitx2 repressed Osterix expression and subsequent osteoblastic differentiation, whereas Runx2, the most upstream regulator of osteogenesis, was unaffected. Conversely, the induction of short hairpin RNA for Pitx2 in C2C12 cells enhanced Osterix expression and osteoblastic maturation upon BMP stimulation. Moreover, mouse embryonic fibroblasts containing myoblasts from Pitx2-null embryos showed enhanced Osterix expression upon BMP stimulation. These findings suggest that Pitx2 suppresses osteogenic signals induced by BMPs in myoblasts to prevent their osteoblastic conversion.

Bone morphogenetic proteins (BMPs) are secreted signaling molecules of the transforming growth factor-β family, and they play a central role in bone formation. When artificially implanted into muscle tissues, BMPs induce ectopic bone formation (1). In myositis ossificans traumatica, a condition in which bone formation occurs in muscle after muscle trauma, BMPs are expressed at the injury site (2). However, the bone-forming effects of BMPs in vivo are self-limiting, suggesting the presence of endogenous regulatory mechanisms (3). At bone fracture sites, BMPs are released from the bone matrix or cavity (4), and muscles attached to bones should be exposed to these secreted BMPs. However, muscle cells do not form ectopic bone in normal fracture healing processes, suggesting that protective mechanisms prevent abnormal bone formation in myoblasts.

In vitro, BMPs not only inhibit myogenic differentiation of C2C12 myoblasts by inducing antagonizing factors such as Id1 (5) or Hey1 (6) that inhibit MyoD, the master regulator of myogenesis, but also induce an osteoblastic phenotype (5). However, unlike osteoprogenitor cells, C2C12 myoblasts do not undergo terminal osteoblast differentiation upon BMP treatment (7). Osterix, an indispensable transcription factor for osteoblast differentiation (8), is rapidly and strongly induced by BMP but, importantly, declines 24 h post-induction (7). In contrast, Runx2, the master regulator of osteoblast differentiation that acts upstream of Osterix (9, 10), does not show a reduction. A decrease in Osterix was not observed in the terminal differentiation of osteoblast progenitor cells, such as mouse marrow stroma cells (MSC), ST-2 cells, or mouse calvarial osteoblasts,4 suggesting that sustained Osterix expression is important for osteoblastic maturation.

We hypothesized that certain types of transcriptional repressors are induced in myoblasts after osteoblastic commitment to

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3 The abbreviations used are: BMP, bone morphogenetic protein; Pitx2, paired-like homeodomain transcription factor 2; MSC, marrow stroma cells; MEF, mouse embryonic fibroblast; ALP, alkaline phosphatase; shRNA, short hairpin RNA; RT, reverse transcription; OC, osteocalcin; KO, knockout; rh, recombinant human; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; LV, lentivivirus; m.o.i., multiplicity of infection; E14.5, embryonic day 14.5.

4 M. Hayashi, S. Maeda, H. Aburatani, K. Kitamura, H. Miyoshi, K. Miyazono, and T. Imamura, unpublished data.

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**A**

| Gene symbol | Gene description | Accession no. | Probe sets | Pitx2 | BMP-4 |
|-------------|------------------|---------------|------------|-------|--------|
| Adamts2     | a disintegrin- and metalloproteinase (magnesium type) with thrombospondin type 1 motif, 2 | NM_001250 | A067332_at | 242.9 | 247.1 |
| Acta2       | actin, alpha-2, smooth muscle, slow isoform | NM_000026 | A067332_at | 332.0 | 313.3 |
| AIMP2       | a disintegrin with homology to the thrombospondin motif | NM_135242 | A067332_at | 411.0 | 408.1 |
| C1r         | complement component 1, c subcomponent | NM_007109 | A067332_at | 360.1 | 356.7 |
| GA举行      | pre-B-cell factor 1 | NM_007109 | A067332_at | 720.4 | 730.0 |
| Gp130       | gp130 | NM_007109 | A067332_at | 356.2 | 360.0 |
| Igfbp7      | insulin-like growth factor binding protein 7 | NM_007109 | A067332_at | 230.5 | 230.0 |
| Itgb2       | integrin, beta 2 | NM_007109 | A067332_at | 366.8 | 360.0 |
| Lphn2       | latrophilin 2 | NM_007109 | A067332_at | 100.1 | 100.0 |
| Myo1b       | myosin IB | NM_007109 | A067332_at | 488.7 | 500.0 |
| Nov         | nephroblastoma overexpressed gene | NM_007109 | A067332_at | 356.2 | 360.0 |
| Nsg1        | neuron specific gene family member 1 | NM_007109 | A067332_at | 100.1 | 100.0 |
| Odz3        | odd Oz/ten-m homolog 3 (Drosophila) | NM_007109 | A067332_at | 488.7 | 500.0 |
| Pitx2       | paired-like homeodomain transcription factor 2 | NM_007109 | A067332_at | 356.2 | 360.0 |
| Pitx3       | paired-like homeodomain transcription factor 3 | NM_007109 | A067332_at | 100.1 | 100.0 |
| Pitx4       | paired-like homeodomain transcription factor 4 | NM_007109 | A067332_at | 488.7 | 500.0 |
| Pdcd6       | plasminogen activator inhibitor, b1 | NM_007109 | A067332_at | 356.2 | 360.0 |
| Pdcd6       | plasminogen activator inhibitor, b1 | NM_007109 | A067332_at | 356.2 | 360.0 |
| Sox2        | SRY-box containing gene 1 | NM_007109 | A067332_at | 100.1 | 100.0 |
| Tgfb2       | transforming growth factor beta 2 | NM_007109 | A067332_at | 488.7 | 500.0 |

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**B**

**EXPRESSION PROFILE**

| MEAN OF FOLD INDUCTION (n=27) | 002 24 72 (h) |
|-------------------------------|---------------|
| Pitx2 mRNA expression (relative value) | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 | 0 |

**C**

signals on microarray

**D**

Pitx2

**E**

Pitx2

**F**

| day | 0 | 1 | 2 | 4 | 7 |
|-----|---|---|---|---|---|
| BMP-2 | - | + | - | - | + |

**α-Pitx2**

**α-tubulin**
inhibit BMP-induced Osterix expression and osteoblastic conversion. In this study, we performed microarray analyses using C2C12 cells to search for these unknown nuclear factors. We identified the paired-like homeodomain transcription factor Pitx2 (also termed Rieg), which plays a critical role in the determination of left-right asymmetry during development (16, 17) and whose mutations are responsible for Rieger syndrome (20). Here, we demonstrate that Pitx2 also inhibits BMP-simulated Osterix expression and osteoblast differentiation of myoblasts.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Osteoblastic Differentiation**—The cell culture and osteoblastic differentiation of C2C12 cells were performed as described previously (7). Recombinant human (rh) BMP-2 was provided by Astellas Pharma Inc. and used at 300 ng/ml. BMP-4 was obtained from R&D Systems (Minneapolis, MN) and used at 50 ng/ml, and BMP-6 was a kind gift from Dr. Sampath and used at 100 ng/ml. Mouse embryonic fibroblasts (MEFs) were purified from embryonic day 14.5 (E14.5) embryos. Briefly, whole embryos were digested with 0.05% collagenase at 4 °C for 48 h and seeded on dishes after homogenization by pipetting. MEFS were stimulated with rhBMP-2 at 300 ng/ml with 50 µg/ml ascorbic acid and 5 mM β-glycerophosphate. To purify primary myoblasts, the muscles were isolated from 2-day-old neonatal mice and minced into a coarse slurry using razor blades. The cells were digested with 2.4 units/ml dispase and 1% collagenase supplemented with CaCl2, to a final concentration of 2.5 mM. The slurry, maintained at 37 °C for 45 min, was triturated every 15 min with a 5-ml plastic pipette and then passed through a 70-µm nylon mesh. The filtrate was spun at 350 × g to sediment the dissociated cells, the pellet was resuspended in growth medium, and the suspension was plated on collagen-coated dishes. Primary myoblasts were maintained in growth medium consisting of Ham’s F-10 nutrient mixture containing 20% fetal bovine serum and 2.5 ng/ml basic fibroblast growth factor (Sigma) with 100 units/ml penicillin G and 100 µg/ml streptomycin. Osteoblastic differentiation of primary myoblasts was induced by 300 ng/ml rhBMP-2, 50 µg/ml ascorbic acid, and 5 mM β-glycerophosphate in medium. Osteoblastic differentiation of primary osteoblasts was induced by 300 ng/ml rhBMP-2, 50 µg/ml ascorbic acid, and 5 mM β-glycerophosphate in medium.

**Alkaline Phosphatase Assay**—Alkaline phosphatase (ALP) staining and von Kossa staining were performed as described previously (11). Quantitative analysis of ALP activity was performed using Sigma Fast p-nitrophenylphosphate tablet sets (Sigma). All samples were quantified in triplicate and normalized by protein concentration. The protein concentration in each extract was measured by DC protein assay (Bio-Rad) using bovine serum albumin as a standard.

**Pitx2 Knock-out (KO) Embryos**—Heterozygous and homozygous Pitx2 mice were generated as described previously (12). Genotyping for the targeted Pitx2 allele was performed by PCR using primers as follows: forward primer for wild-type allele, GAAAGATAAGGGCCAGCAAGG; forward primer for mutant allele, GACTCGGGCTTCCGTAGGTTG; and reverse primer, GCCCTTTGACAGGTTC. Plasmid Construction and Adenovirus Generation—Mouse Pitx2 cDNA was cloned from BMP-induced C2C12 cells into pcDEF3 using a reverse transcription (RT)-PCR-based approach. Adenoviruses carrying Pitx2 or LacZ were generated using an adenovirus expression vector kit (TaKaRa Bio, Shiga, Japan) as described previously (11).

**Generation of Lentiviruses Expressing shRNA**—Lentivirus carrying short hairpin RNA (LV-shRNA) was generated as described previously (13) using the plasmids CS-RFA-EG, pCAG-HIVgp, and pCMV-VSV-G-RSV-Rev. The shRNA sequence for mouse Pitx2 (5’-GCCTGAGACTGAAAGGTTG-3’) was designed by B-Bridge International Inc. (Mountain View, CA). shRNA was subcloned into CS-RFA-EG vector carrying a green fluorescent protein expression cassette. Lentivirus containing the control shRNA (5’-GCCTGACTGAAAGGTTG-3’) (13) was also generated. C2C12 cells were infected with LV-shRNA at a multiplicity of infection (m.o.i.) of 300, and positive cells were sorted by green fluorescent protein. Quantitative Real-time RT-PCR and Conventional RT-PCR—Total RNA was purified using TRIzol reagent (Invitrogen). cDNA was synthesized with PrimeScript reverse transcriptase (TaKaRa Bio) and oligo(dT). Quantitative real-time PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). All samples were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well as over 2-fold of the mock control were subjected to microarray analysis. Genes up-regulated over 3-fold compared with the 0 h control as well(80,696),(914,881)
were run in duplicate in each experiment, and values were normalized to glyceraldehyde-3-phosphate dehydrogenase. Primers used are listed in supplemental Table 1. Primers used to detect Pitx2 in conventional RT-PCR were GCCAGCAAG-GAAAGAATGAG (forward) and CACCATGCTGGACGA-CATAC (reverse).

Immunoprecipitation and Immunoblotting—Samples for immunoprecipitation or immunoblotting were harvested and lysed with Nonidet P-40 lysis buffer as described previously (11). Anti-Pitx2 serum was generated by immunizing rabbits with a synthetic peptide (GYASVQNPASNLSC). Anti-Osterix serum was generated by immunizing rabbits with a synthetic peptide (AHGGSPEQSNLLEIC) as described previously (8). Anti-tubulin monoclonal antibody DM1A was obtained from Sigma.

Statistical Analysis—Results of the quantitative RT-PCR and ALP assays are expressed as mean ± S.D.

RESULTS AND DISCUSSION

Pitx2 Expression in C2C12 Cells Is Elevated from 1 Day after BMP Stimulation—To identify the transcriptional repressor in C2C12 cells that inhibits Osterix gene expression after BMP stimulation, we analyzed the mRNA expression profile of C2C12 cells before and 24 h after BMP stimulation. Gene expression was analyzed on an Affymetrix GeneChip Genome 430 2.0 Array. After searching for genes with delayed elevation after day 1 and excluding function-unknown expressed sequence tag genes, 27 candidate genes were identified (Fig. 1A). We confirmed that the selected genes were not up-regulated by BMP through the first 24 h and were augmented thereafter (Fig. 1B). Among these genes, only Pitx2 was a nuclear DNA-binding factor reported to act as a transcriptional repressor and activator in a context-dependent manner (14). On this microarray, genes presenting signal intensities of over 50 were designated as “Present.” Pitx2 had a relatively strong expression level (488.7) at the steady state, which was further enhanced to 1765 by treatment with BMP at 72 h (Fig. 1, A and C). The delayed induction of the Pitx2 gene after BMP simulation of C2C12 cells was confirmed by real-time RT-PCR (Fig. 1, D and E). Because Pitx2 was induced by various BMPs, including BMP-4 (Fig. 1, A and C), BMP-2 (Fig. 1, D and E), and BMP-6 (Fig. 1E), the up-regulation of Pitx2 in C2C12 was not specific to a certain BMP.

Pitx2 is expressed in the lateral plate mesoderm, and the Wnt canonical pathway is essential for Pitx2 expression during heart morphogenesis (14). Kioussi et al. (14) also demonstrated the expression of Pitx2 in C2C12 cells. Wnt/β-catenin signaling is important for C2C12 cells to maintain myogenic potential as a blockade of Wnt signaling in C2C12 cells, which leads to spontaneous adipocytic differentiation (15). Thus, Wnt signaling may be responsible for the relatively high Pitx2 expression observed in non-stimulated C2C12 cells (Fig. 1, A and C). Pitx2 is also induced by asymmetric signals encoded by Nodal, Sonic hedgehog (16), and Lefty-1 (17) and plays an important role in determining left-right asymmetry during embryonic development. However, we did not detect signals for Nodal or Sonic hedgehog in the microarray analysis (data not shown). Therefore, how BMP induces Pitx2 in C2C12 cells remains to be elucidated. In addition, Pitx2 seemed not to be a direct target of BMP signaling because the induction started 24 h after BMP stimulation.

Next, to confirm the expression pattern of Pitx2 at the protein level, we generated anti-Pitx2 serum and observed Pitx2 induction by BMP in C2C12 cells using immunoblot analysis (Fig. 1F). Protein expression level of Pitx2 was up-regulated, when compared with mock control cells, from 2 days after BMP-stimulation, reproducing the result of real-time RT-PCR.

Induction of Pitx2 by BMP Is Specific to Myoblasts—Next, we examined whether the induction of Pitx2 by BMP was specific to C2C12 myoblasts or universal in other myoblasts. To resolve this issue, we first obtained Pitx2 KO embryos to monitor the expression of Pitx2 by 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) staining, in which the targeted Pitx2 allele contains a LacZ expression cassette (12). Pitx2-null embryos

![Figure 2: Induction of Pitx2 by BMP is specific in myoblasts.](image-url)

**FIGURE 2. Induction of Pitx2 by BMP is specific in myoblasts.** A, X-gal/Safranin-O staining of an E13.5 Pitx2 heterozygous embryo. Whole body (upper panel) and limb (lower panel) are shown. B, X-gal staining of wild-type and Pitx2-null MEFs. rhBMP-2 was applied at 300 ng/ml. C, expression levels of Pitx2 with or without rhBMP-2 (300 ng/ml) stimulation for 4 days were determined in C2C12 cells, primary myoblasts (myo), MEFS, primary osteoblasts (osteo), and MSCs by RT-PCR (left panel) and quantitative RT-PCR (right panel), expressed as -fold induction levels by BMP stimulation). D, osteoblast differentiation induced by rhBMP-2 (300 ng/ml) was examined by real-time RT-PCR for Osterix and ALP in C2C12 cells, primary myoblasts, MEFS, primary osteoblasts, and MSCs.
Pitx2 Protects Myoblasts from Osteoblastic Conversion

Given the role of Pitx2 in preventing BMP-induced osteoblastic conversion of myoblasts, Pitx2 should not be induced by BMP in primary osteoblasts or in osteoblastic progenitor cells. Calvarial osteoblasts and MSCs, an osteoblast progenitor, exhibited neither steady state levels of expression (Fig. 2C, left panel) nor induction (Fig. 2C, right panel) of Pitx2 gene by BMP. In contrast, BMP-induced osteoblast differentiation was not evident in C2C12 cells, myoblasts, and MEFs; the expression levels of Osterix, a pre-osteoblast marker, and maturing osteoblast marker ALP were less than those in osteoblasts and MSCs (Fig. 2D). These lines of evidence indicate that Pitx2 was induced by BMP exclusively in myoblasts.

Pitx2 Overexpression Inhibits Osterix Expression and Subsequent Osteoblastic Differentiation—We generated a N-terminal FLAG-tagged Pitx2 adenovirus and infected C2C12 cells to investigate the role of Pitx2 in osteoblastic differentiation. Pitx2 adenovirus did not affect the expression of Runx2, the most upstream regulator of osteoblast differentiation (Fig. 3A). In contrast, overexpression of Pitx2 strikingly suppressed BMP-induced Osterix mRNA expression, as well as induction of an early osteoblast maturation marker ALP and a terminal maturation marker osteocalcin.
Pitx2 Protects Myoblasts from Osteoblastic Conversion

To exclude possible off-target effects of the shRNA and to study complete loss of function of the Pitx2 gene, we examined BMP-induced osteoblast differentiation of MEFs from Pitx2 KO embryos. We purified MEFs from E14.5 embryos of littermates and determined the genotypes by PCR (Fig. 5A). Unexpectedly, mRNA expression level of Pitx2 in heterozygotes was not decreased to half of that in wild-type cells, and the induction of Pitx2 by BMP was also observed (Fig. 5B). The expression of Runx2 in KO MEFs was comparable with that in wild-type and heterozygous cells (Fig. 5C). However, Pitx2-null MEFs exhibited higher levels of Osterix expression than Pitx2 heterozygous and wild-type cells (Fig. 5D). Importantly, in Pitx2−/− MEFs, not only ALP (Fig. 5E) but also OC was strikingly up-regulated by BMP (Fig. 5F). Moreover, matrix calcification, a hallmark of mature osteoblasts, was observed upon BMP stimulation only in knock-out MEFs (Fig. 5G). The elevated expression of Osterix and its downstream genes as well as matrix mineralization after BMP application was not evident in heterozygous MEFs (Fig. 5, D–G), which was consistent with the observation that the expression level of Pitx2 in heterozygous cells is equivalent to that in wild-type MEFs (Fig. 5B). These results demonstrate that endogenous Pitx2 plays a crucial role in protecting myoblasts from BMP-induced differentiation into mature osteoblasts.

Possible Roles of Pitx2 under Physiological and Pathological Conditions—Because muscles and bones are neighboring rudiments in the developing skeleton, BMPs expressed in bones may contact muscle cells. We speculate that muscle progenitor cells express Pitx2 to protect themselves from the tendency to form bone. A similar situation can be considered in adult bone fractures, where BMPs leaked from the bone cavity may stimulate residing muscles. A haploinsufficiency of the PITX2 gene in humans causes Rieger syndrome, an autosomal-dominant disorder characterized by ocular anterior chamber anomalies, dental hypoplasia, craniofacial dysmorphism, and umbilical stump anomalies (20). Pitx2 heterozygous mice exhibit phenotypes similar to Rieger syndrome (21). So far, neither muscle anomalies nor ectopic bone formation within muscles have been reported in Pitx2 heterozygous humans or mice. Our findings were based on sufficient knockdown or knock-out experiments. As the Pitx2 gene expression level seems critical (21), gene expression from a single Pitx2 allele should be sufficient to inhibit Osterix expression and osteoblastic maturation. Indeed,

(OC) (Fig. 3A). The inhibition of Osterix gene expression by Pitx2 virus was confirmed to be dependent on the expression levels of Pitx2 (Fig. 3B). Also, Pitx2-mediated suppression of Osterix expression was reproduced at the protein level by immunoprecipitation followed by immunoblotting with anti-Osterix antibody (Fig. 3C). The activity of ALP protein was also greatly reduced by the Pitx2 adenovirus (Fig. 3D). These results indicate that induction of exogenous Pitx2 gene suppressed the activity of Osterix gene promoter and its expression, resulting in elimination of subsequent osteoblast maturation.

Loss of Pitx2 Function Leads to Enhanced and Sustained Induction of Osterix Gene Expression and Osteoblast Differentiation—To study the roles of endogenous Pitx2 in BMP-induced myoblast cell fate, we used lentivirus carrying Pitx2 shRNA (LV-shRNA). Pitx2 knockdown efficiency was confirmed by immunoblotting using anti-Pitx2 serum on day 3 after BMP stimulation. B and C, C2C12 cells were infected with lentivirus carrying Pitx2 shRNA. B, knockdown efficiency was confirmed by real-time RT-PCR for Pitx2 (red lines). C, the effects of Pitx2 shRNA on expression of Runx2, Osterix, and ALP were examined by real-time RT-PCR. C2C12 cells were treated with rhBMP-2 (300 ng/ml) for indicated time periods.

FIGURE 4. shRNA for Pitx2 gene enhances BMP-induced expression of Osterix and ALP in C2C12 cells. A, C2C12 cells were infected with a lentivirus carrying Pitx2 shRNA (LV-shRNA). Pitx2 knockdown efficiency was confirmed by immunoblotting with anti-Pitx2 antibody (Fig. 4C, upper panel). Surprisingly, Pitx2 shRNA not only enhanced early induction of Osterix but also prevented its decline, and Osterix mRNA steadily increased (Fig. 4C, middle panel). As a result, the ALP expression was strongly enhanced throughout the time course (Fig. 4C, lower panel).
the expression of Osterix, ALP, and OC and matrix calcification were not altered in Pitx2 heterozygous cells, in which expression level of Pitx2 was similar to that in wild-type cells (Fig. 5). Because Pitx2 KO embryos die before early skeletal organogenesis, we were unable to examine the fate of muscular tissues in Pitx2-null mice in vivo or to generate adult bone fracture models. A myoblast-specific Pitx2 conditional KO should answer these questions.

Pathologic bone formation often occurs in muscles, including conditions such as myositis ossificans traumatica and osteogenic sarcoma. BMP expression was observed in these pathologically formed bones in muscles (2). To cure these ectopic bone formation disorders, modulating the function of endoge-

ous Pitx2 may be a novel approach. In conclusion, our results uncover a novel, indispensable role of Pitx2 in protecting myoblasts from abnormal osteoblastic transdifferentiation induced by BMP signaling.

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