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FGF8 Regulates Myogenesis and Induces Runx2 Expression and Osteoblast Differentiation in Cultured Cells

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ABSTRACT

In the current study, treatment of the rat osteogenic cell line ROB-C26 cells with fibroblast growth factor 8 (FGF8) stimulated alkaline phosphatase (ALP) activity, and also induced the expression of the Runx2 transcription factor, and increased the activity of a luciferase reporter gene containing the osteocalcin (OCN) promoter and six copies of the osteoblast specific *cis*-acting element 2 (OSE2) response element. In contrast, FGF8 treatment of the mouse myoblast cell line C2C12 inhibited the expression of desmin and the synthesis of myotubes. The expression of MyoD, Myogenin, Foxc2, and Hand1 was also decreased by FGF8. Transient expression of Foxc2 in C2C12 cells induced the expression of Hand1, and chromatin immunoprecipitation (ChIP) analysis indicated that Foxc2 binds to the promoter region of the Hand1 gene. These results indicated that Foxc2 is directly involved in the regulation of Hand1 expression. The results of the current study indicate that FGF8 regulates myoblast differentiation through the regulation of MyoD expression, and that this regulation is independent of Hand1 in cultured cells. Conversely, FGF8 supports bone development and cell differentiation though the induction of Runx2 expression. J. Cell. Biochem. 106: 546–552, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: FGF8; MYOD; MYOGENIN; RUNX2; FOXC2; HAND1; DIFFERENTIATION

Fibroblast growth factor 8 (FGF8) is required for a variety of patterning events in the developing embryo. FGF8-deficient mouse embryos arrest at gastrulation with a failure of the mesoderm to migrate out of the primitive streak [Sun et al., 1999], and exhibit defects in the apical ectodermal ridge of the developing limb bud [Moon and Capecchi, 2000] and abnormal axis specification and cardiac looping reversals [Abu-Issa et al., 2002]. Limb bud initiation, growth, and patterning have been shown to be regulated by a spectrum of ligands, including FGFs, Wnt proteins, sonic hedgehog (Shh), and bone morphogenetic protein (BMPs). In particular, FGF8 is expressed in the limb ectoderm as buds begin to emerge, suggesting a possible role in very early stages of limb budding. Several reports have implicated FGF8 as a transmitter of signals for limb induction [Crossley and Martin, 1995; Moon and Capecchi, 2000]. FGF8 expression commences in anterior/posterior bands in the flank ectoderm within the limb fields from which buds will soon emerge, and expression persists and becomes localized to the newly formed apical ectodermal ridge of the early buds. FGF8 has limb bud inducing activity and presumably acts directly upon the mesenchyme [Crossley et al., 1996; Vogel et al., 1996]. Furthermore, a recent report has demonstrated that FGF8 regulates limb bud interdigital programmed cell death [Pajni-Underwood et al., 2007]. The deletion of chromosome 22q11.21 results in the disruption of pharyngeal and cardiac development, and is associated with DiGeorge and other related syndromes in humans. While the human FGF8 gene does not localize to chromosome 22q11, FGF8 deficiency in mice results in a phenotype that is similar to that of 22q11-deletion [Moon et al., 2006].

Mice that are deficient in Foxc2, a member of the forkhead family of transcription factors, die in utero, and exhibit an interrupted aortic arch and skeletal defects in the neurocranium and the vertebral column [Iida et al., 1997]. The interruption of the aortic arch that is seen in Foxc2-deficient mice is similar to that observed in some human congenital abnormalities, including DiGeorge syndrome. Foxc2 is expressed in the mesenchymal condensation around the optic vesicle, and in the mesenchyme underlying the midbrain and hindbrain in the prechordal region of early embryos. Subsequently, strong expression is localized in developing

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cartilaginous tissues, kidney, and dorsal aortas [Iida et al., 1997; Winnier et al., 1997]. Thus, it appears that Foxc2 is involved in the proliferation, aggregation, and differentiation of neural crest- and cephalic mesoderm-derived mesenchymal cells and sclerotome cells [Winnier et al., 1997], and it has been suggested to be one of the candidate genes for DiGeorge syndrome.

Heart and neural crest derivatives expressed transcript 1 (Hand1/eHand) is a basic helix-loop-helix (bHLH) transcription factor that is expressed in the heart and certain neural-crest derivatives during embryogenesis. Hand1-deficient mouse embryos die between embryonic days 8.5 and 9.5, and exhibit yolk sac abnormalities due to a deficiency in extraembryonic mesoderm. Heart development is also perturbed, and does not progress beyond the cardiac-looping stage [Firulli et al., 1998]. Due to these observations, Hand1 has also been proposed to be a candidate gene for DiGeorge syndrome. During limb development, HAND1 expression is first observed in the ventral mesoderm of the emerging limb, and is then restricted to an anteroventral area of mesoderm at mid-level in the proximodistal axis. At later stages, expression is observed in the autopod encompassing the ventral tendons of the digits in chick embryos. In mouse embryos, only the anteroventral domain of expression is conserved. Early ventral expression is undetectable and the late pattern of expression differs clearly from that of the chick. A constant feature of all areas of expression is their ventral and anterior localization [Fernandez-Teran et al., 2003]. Hand1 plays an important role in the development of extraembryonic, mesodermal, and cardiac cell lineages, presumably through heterodimerization with other bHLH proteins and binding to specific DNA sites. Several reports have demonstrated that Hand1 is a potent inhibitor of gene activation by some but not all bHLH proteins in transient transfection assays. Hand1 prevents E-box DNA binding by bHLH proteins, and can strongly inhibit transactivation by a MyoD-E47 tethered dimer. Hand1 also inhibits MyoD-dependent skeletal muscle cell differentiation and expression of the muscle-specific myosin heavy chain protein [Bounpheng et al., 2000].

Taken together, these findings indicate that FGF8, Foxc2, and Hand1 are expressed in limb bud and aortic arch, and regulate bone and muscle development, as well as cardiac looping. Osteocytes and myocytes differentiate from mesenchymal stem cells. The differentiation of osteoblasts is promoted by Runx2, which belongs to the runt-domain gene family. Mice that are deficient in Runx2 die just after birth without breathing, and an examination of their skeletal systems showed a complete lack of ossification [Komori et al., 1997]. On the other hand, the differentiation of myoblasts is promoted by MyoD at early stages. At latter stages, myoblasts differentiate into cardiac-, smooth-, or skeletal muscle. In particular, the differentiation into skeletal muscle is promoted by myogenin. The evidence for an essential role for myogenin in differentiation came from examinations of muscle cells that expressed adenovirus protein Ela which suppresses the activation of the myogenin gene and concomitantly blocks differentiation, although the cells continued to express Myf-5 and MyoD [Enkemann et al., 1990; Braun et al., 1992]. Additionally, anti-sense oligonucleotides that inhibit myogenin expression prevented myoblast differentiation in these cells [Brunetti and Goldfine, 1990; Florini and Ewton, 1990]. The deficient mice were born immobile, died immediately after birth, and they showed severe reductions in skeletal muscle mass [Hasty et al., 1993 Nabeshima et al., 1993].

In the current study, the role of FGF8 in the differentiation of mesenchymal progenitor cells into osteoblasts and myoblasts was examined in cultured cells.

MATERIALS AND METHODS

CELL CULTURE

The rat osteogenic cell line ROB-C26 were maintained in α-modified essential medium (α-MEM, Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Japan Bioserum, Hiroshima, Japan). The mouse myoblast cell line C2C12 was maintained in Dulbecco's modified minimal essential medium (DMEM, Wako) supplemented with 15% FBS in a humidified atmosphere of 5% CO_2 at 37°C. Media contained 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium).

GROWTH FACTOR STIMULATION

For 24 h stimulation, confluent cells were deprived of serum for 12 h by incubation in medium that did not contain FBS (starvation medium), and then the cells were cultured with the indicated concentrations of FGF8 (PeproTech, Hamburg, Germany) in starvation medium to avoid the influence of growth factors that are present in FBS. For 12- and 14-day cultures, confluent cells were cultured with the indicated concentration of FGF8 in media that contained 3% FBS (low serum medium). The culture medium was changed every 2 days.

HISTOCHEMICAL ANALYSIS

Cells were plated in a 24-well tissue culture dish at a density of 5×10^4 cells/well in complete medium until they were confluent. Confluent cells were then cultured in the presence of the indicated concentrations of FGF8 in low serum medium. For alkaline phosphatase (ALP) staining, cells were cultured for 14 days in the presence of FGF8, and then fixed. Cells were then incubated in a solution of NBT/BCIP (Roche, Mannheim, Germany). For immunohistochemistry, cells were cultured for 12 days in the presence of FGF8, and then fixed. Cells were incubated with an anti-desmin antibody (diluted 1:100, Abcam, Cambridge, UK), washed, and then immunoreactive proteins were visualized using DakoCytimation LSAB + System-HRP (Dako, Kyoto, Japan).

WESTERN BLOT ANALYSIS

Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with one of the following primary antibodies: rabbit anti-Runx2 (diluted 1:3,000, Abcam), rabbit anti-Myogenin (diluted 1:3,000, Abcam), goat anti-MyoD (diluted 1:3,000, Santa Cruz, CA), goat anti-Foxc2 (diluted 1:3,000, Santa Cruz), rabbit anti-Hand1 (diluted 1:3,000, Abcam), or goat anti-Actin (diluted 1:5,000, Santa Cruz), as indicated. After washing, the membrane was incubated with peroxidase-conjugated secondary anti-goat IgG (diluted 1:10,000, Santa Cruz) or anti-rabbit IgG (diluted 1:10,000, Amersham

Pharmacia Biotech, Little Chalfont, UK) for 1 h. Immunoreactive proteins were detected using an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

CONSTRUCTION OF PLASMIDS AND TRANSIENT TRANSFECTION ASSAY

The luciferase reporter plasmid containing six copies of osteoblast specific *cis*-acting element 2 [(OSE2)x6-Luc] and the expression vector for Foxc2 were described previously [Mikami et al., 2007; Omoteyama et al., 2007]. Transient transfections were performed using Lipofectamine™ LTX Reagent and PLUS™ Reagent, according to the manufacture's instructions (Invitrogen, Carlsbad, CA). For gene reporter assays, 24 h before transfection, cells were plated in a 24-well tissue culture dish at a density of 1×10^4 cells/well. Cells were co-transfected with a total of 250 ng DNA, which included 225 ng of reporter plasmid and 25 ng of phRG (Promega, Madison, WI) as an internal control. Twenty-four hours after transfection, the medium was replaced with low serum medium containing the indicated concentrations of FGF8. Cells were harvested after 24 h of FGF8 treatment and assayed for luciferase activity using a Dual Luciferase Assay kit (Promega), according to the manufacturer's instructions. Luciferase activity was normalized to *Renilla* luciferase activity. Data represents the average of at least three experiments. For all other transfection assays, 24 h before transfection, cells were plated at a density of 1×10^5 cells/35 mm plate. Cells were transfected with 2.5 μg of the indicated expression vector in 3 ml of medium containing 6.25 μl LTX Reagent, 2.5 μl PLUS Reagent for 48 h. Cells were harvested and then subjected to Western blot analysis using the indicated antibodies.

IMMUNOPRECIPITATION

Following transfection with the indicated expression vectors, cells were washed, and then harvested in lysis buffer. Cell lysates were pre-cleared by incubation with Protein A/G PLUS-Agarose (Santa Cruz), and then incubated with an anti-Foxc2 antibody overnight at 4°C. Immune complexes were precipitated with Protein A/G PLUS-Agarose and washed. Proteins were eluted in 2× SDS buffer and then subjected to Western blot analysis.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS

ChIP analysis was carried out as described previously [Omoteyama et al., 2007]. Briefly, cells were fixed, and then the DNA of the cells was sheared by sonication. Samples were pre-cleared by incubation with Protein A/G PLUS-Agarose (Santa Cruz), and then incubated with an anti-Foxc2 antibody, or goat pre-immune serum as a negative control, for 16 h at 4°C. Immune complexes were precipitated with Protein A/G PLUS-Agarose. Complexes were eluted in elution buffer, and cross-linking was reversed by heating the sample to 65°C for 6 h. DNA was purified, and specific regions of the indicated promoters were amplified by PCR using the following

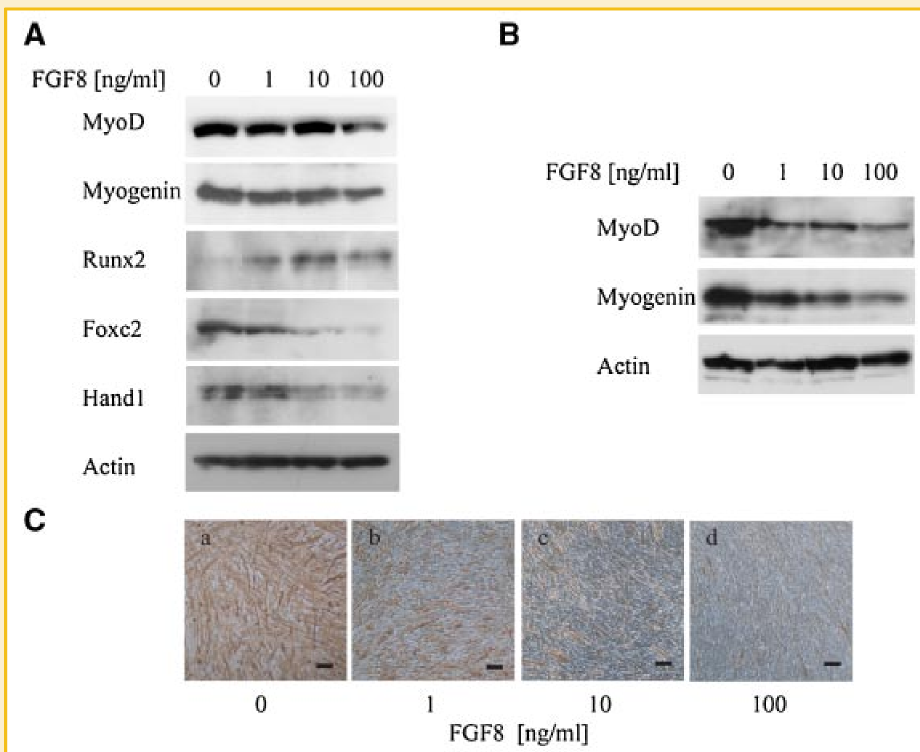

Fig. 1. FGF8 inhibits myoblast differentiation in C2C12 cells. Cells were cultured with the indicated concentrations of FGF8 for 24 h, and then analyzed by Western blot (A). C2C12 cells were cultured in the presence of FGF8 for 12 days and then the expression of myogenesis and desmin were analyzed by Western blot (B) or immunohistochemistry (C). Scale bar indicates 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

primers: for Hand1 5′-AGCTTTAGCTCCTCGCTCCT-3′ and 5′-CTCAGTGGTACAAGTAGCCT-3′; for MyoD, 5′-CGCCCCCAGCC-TCCCTTTCCAG-3′ and 5′-TGTCAGAGGTGTGGTGAAGAAA-3′; for PAI-1, 5′-CACCAGGCTTTGTAGGCTCTTG-3′ and 5′-CAGTGAC-TTGCTGGGACACGTG-3′.

RESULTS

FGF8 INHIBITS MYOBLAST DIFFERENTIATION IN C2C12 CELLS

To determine whether FGF8 inhibits myoblast differentiation through the regulation of myogenesis expression, C2C12 cells were treated with different concentrations of FGF8 for 24 h, and then the expression of various transcription factors was analyzed by Western blot analysis. MyoD and Myogenin proteins in C2C12 were strongly expressed, and deceased at 100 ng/ml FGF8. The level of Hand1 and Foxc2 proteins, which regulate the expression of MyoD was decreased by FGF8 in a dose-dependent manner. Conversely, the levels of Runx2 protein were increased by FGF8 (Fig. 1A). It has been reported that myoblast differentiation is promoted in C2C12 cells growing in low serum medium [Davis et al., 1987]. The cells were cultured in the presence of FGF8 in low serum medium for 12 days, and then subjected to Western blot (Fig. 1B), and immunohistochemical analyses using an antibody directed against the muscle marker desmin (Fig. 1C). As seen in Figure 1B, the 12-day untreated cultured cells growing in low serum medium were more differentiated into myocytes than the 24 h cultured cells, and MyoD and Myogenin proteins were strongly expressed in these cells. FGF8 treatment strongly inhibited the differentiation of these cells into myocytes and decreased the levels of MyoD and Myogenin as compared to the untreated cells. Runx2 protein was not detected even at 100 ng/ml FGF8 (data not shown). As seen in Figure 1C, FGF8 inhibited desmin expression and myotube synthesis (panels a–d), although small myotube was observed even at 100 ng/ml FGF8 (panel d).

FGF8 PROMOTES OSTEOBLAST DIFFERENTIATION IN ROB-C26 CELLS

To determine whether FGF8 promoted osteoblast differentiation through the regulation of Runx2 expression, ROB-C26 cells were treated with different concentrations of FGF8 for 24 h, and then the expression of various transcription factors was analyzed by Western blot analysis. The levels of Runx2 protein were increased by FGF8 (Fig. 2A). The levels of Myogenin and Foxc2 protein were decreased by FGF8, although the level of MyoD protein was increased by FGF8.

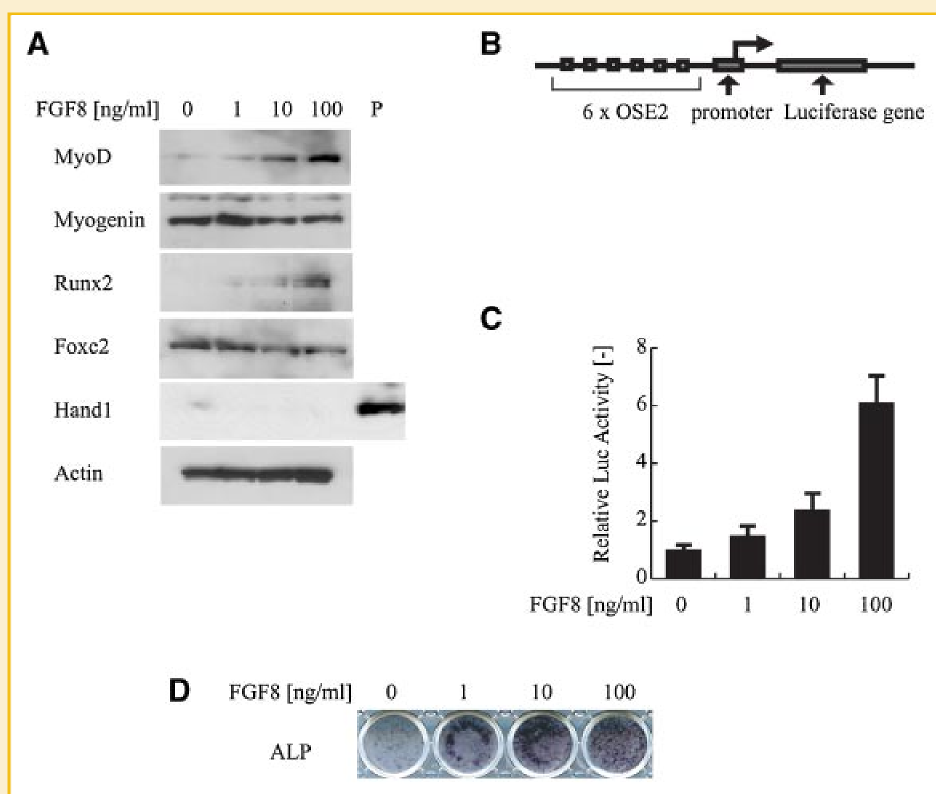

Fig. 2. FGF8 promotes osteoblast differentiation in ROB-C26 cells. Cells were cultured with the indicated concentrations of FGF8 for 24 h, and then analyzed by Western blot. The letter P indicates the positive control that is untreated C2C12 cells (A). Schematic representation of the luciferase reporter gene construct containing the OCN promoter (−34/+13 relative to the transcriptional start site at +1) and six copies of the OSE2 binding site (OSE2)x6-Luc (B). ROB-C26 cells were co-transfected with (OSE2)x6-Luc and phRG as an internal control, and then treated with FGF8 for 24 h (C). Luciferase activity was analyzed using a Dual-Luciferase reporter assay system (Promega), and is expressed relative to *Renilla* luciferase activity. Cells were cultured with the indicated concentrations of FGF8 for 14 days and then ALP activity was analyzed by NBT/BCIP (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Hand1 protein was not detected in these cells. We also examined the activity of a luciferase reporter gene that contained multiple copies of the OSE2 core sequence (5′-CCAACCAC-3′), and nucleotides −34/+13 (relative to the transcriptional start site at +1) of the mouse osteocalcin 2 (OCN2) gene promoter. OCN are extracellular matrix proteins that are enriched in bone matrix, and the gene promoters of both proteins contain Runx2 binding sites [Ducy and Karsenty, 1995]. This construct contained a TATA box as the sole *cis*-acting element (Fig. 2B). Luciferase activity was nearly undetectable in the absence of FGF8, and was strongly activated by treatment of cells with FGF8 (Fig. 2C). ROB-C26 cells that were treated with FGF8 for 14 days were subjected to histochemical analysis to examine ALP activity. FGF8 treatment resulted in an increase in ALP activity as compared to control, untreated cells (Fig. 2D).

REGULATION OF HAND1 AND FOXC2

The expression of Hand1 and Foxc2 was down-regulated by FGF8 (Figs. 1A and 2A). To determine whether Foxc2 physically interacted with Hand1, cells were transiently transfected with an expression vector for Foxc2, and then subjected to immunoprecipitation using an anti-Foxc2 antibody, followed by Western blot analysis. As seen in Figure 3A, Hand1 was undetectable in Foxc2 immune complexes.

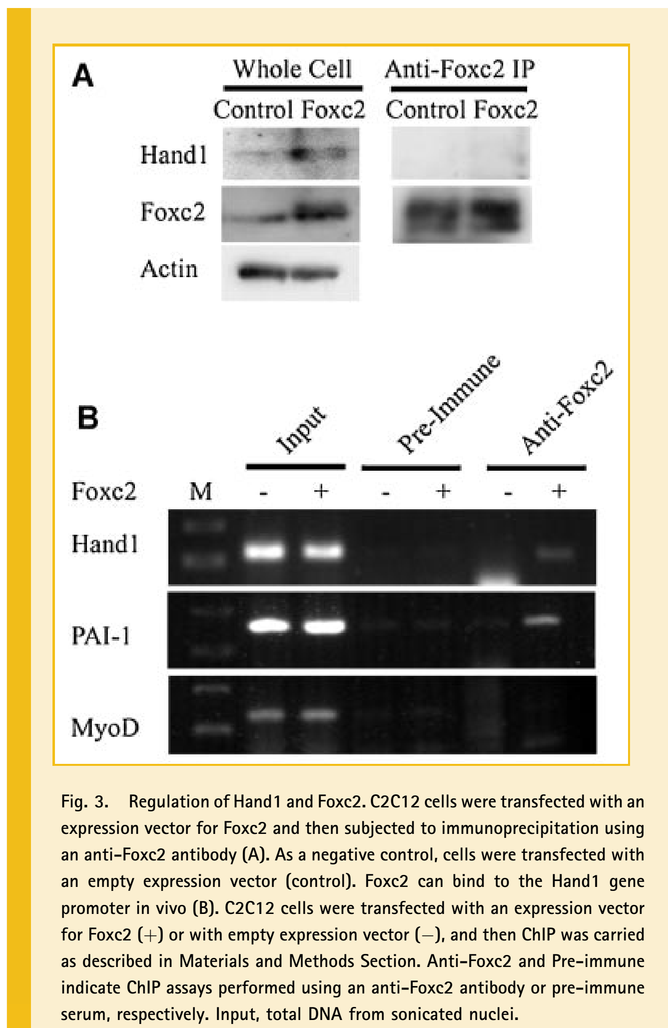

Fig. 3. Regulation of Hand1 and Foxc2. C2C12 cells were transfected with an expression vector for Foxc2 and then subjected to immunoprecipitation using an anti-Foxc2 antibody (A). As a negative control, cells were transfected with an empty expression vector (control). Foxc2 can bind to the Hand1 gene promoter in vivo (B). C2C12 cells were transfected with an expression vector for Foxc2 (+) or with empty expression vector (−), and then ChIP was carried as described in Materials and Methods Section. Anti-Foxc2 and Pre-immune indicate ChIP assays performed using an anti-Foxc2 antibody or pre-immune serum, respectively. Input, total DNA from sonicated nuclei.

However, the protein levels of Hand1 appeared to be increased in cells that overexpressed Foxc2 (Fig. 3A, whole cell). We analyzed the Hand1 gene promoter sequence (approximately 5 kb from the transcriptional initiation site; GenBank Accession No. AL732587) for putative Foxc2 binding motifs (TCTATTT) [Fujita et al., 2006], and identified several candidate Foxc2 binding sites approximately 3 kb upstream of the transcriptional initiation site in the Hand1 promoter. To determine whether the induction of Hand1 expression by Foxc2 involved a direct interaction of Foxc2 with the Hand1 promoter, we performed a ChIP assay. As a positive and negative control, we also used primers that were specific for the PAI-1 promoter [Fujita et al., 2006] and the MyoD promoter [Omoteyama et al., 2007], respectively. As seen in Figure 3B, Foxc2 associated with the Hand1 promoter in transfected C2C12 cells, but not in untransfected cells. This association appeared to be specific, since we did not detect Foxc2 in association with the MyoD gene promoter in transfected or untransfected cells. These results indicated that Foxc2 binds directly to the Hand1 gene promoter and induces Hand1 expression in C2C12 mouse myoblasts.

DISCUSSION

FGF family proteins have been implicated in bone and chondrocyte regeneration [Pitaru et al., 1993]. However, it is unclear whether FGF8 functions in bone development. In the current study, we demonstrated that FGF8 induces the activation of ALP in cultured rat ROB-C26 cells. ALP is a hallmark of osteoblast activity, and its activity increases at sites of calcification. Conversely, ALP activity is decreased under conditions in which calcification is inhibited. For example, the inhibition of BMP2-induced calcification by transforming growth factor (TGF)-β1 also results in the inhibition of Runx2 and OCN gene expression, as well as ALP activity in vitro [Spinella-Jaegle et al., 2001]. In the current study, FGF8 treatment also induced Runx2 protein expression. The differentiation of mesenchymal stem cells into immature osteoblasts that express bone matrix proteins is regulated by the action of Runx2. Runx2 triggers the expression of major bone matrix proteins at an early stage of osteoblast differentiation. Subsequently, immature osteoblasts differentiate into mature osteoblasts that express high levels of OCN. Mature osteoblasts that are embedded in the bone matrix become osteocytes [Komori, 2006]. Runx2 binding sites have been identified in the promoters of several major bone matrix protein genes, including OCN, and the expression of these genes or the activation of their promoters by Runx2 has been demonstrated in vitro [Ducy and Karsenty, 1995]. In this study, MyoD expression was increased by FGF8 in ROB-C26 cells. Recently, it has been suggested that the muscle transcription factor MyoD promotes not only myoblast differentiation but also osteoblast differentiation because MyoD stimulates Osterix promoter activity [Hewitt et al., 2008] and enhances BMP7-induced osteobast differentiation [Komaki et al., 2004]. In the current study, FGF8 induced the expression of OCN gene activity in ROB-C26 cells. Our results suggest that by increasing the levels of extracellular proteins such as OCN, which serve as protein scaffolds for calcium deposition, FGF8 creates an environment that is conducive to calcification.

There is evidence that FGFs are involved in both muscle regeneration and embryonic myogenesis, and it has been suggested that FGFs play an important role in the coordinated growth and differentiation of myoblasts [Joseph-Silverstein et al., 1989]. Gene knock-out studies and mutational analysis in animals have demonstrated that the activation of MyoD by retinoic acid is mediated by FGF8-associated signaling pathways [Hamade et al., 2006]. However, FGF2 represses MyoD in cultured cells [Yoshida et al., 1996], and in the current study, FGF8 inhibited MyoD expression in cultured C2C12 cells but induced MyoD expression in cultured ROB-C26 cells. The reason for this difference between ROB-C26 cells and C2C12 cells with respect to the action of FGF8 is unknown. In this study, ROB-C26 cells were distantly related to myocytes, although the differentiated C2C12 cells were closely. One possibility is that in the early stage of muscle formation, myoblast proliferation is required even under conditions that promote differentiation, such as the presence of FGFs. In later stages, myoblasts with high differentiation potential have to respond precisely to extracellular cues for growth arrest and terminal differentiation [Yoshida et al., 1996]. Thus, the stage and site of myoblast differentiation might be important. We suggest that FGFs are required to promote myogenesis in undifferentiated cells during the initiation stage, and then down-regulate myoblast differentiation during later stages. Additional studies are required to clarify the apparent dual mechanism of action of FGFs.

Recently, it has been shown that Foxc2 induces the expression of MyoD indirectly in cultured cells [Omoteyama et al., 2007]. Our data suggests that FGF8 inhibits MyoD expression through the down-regulation of Foxc2. Although the expression of FGF8 is decreased in Foxc2 knockout mice [Seo and Kume, 2006], it is unknown whether Foxc2 is involved in the regulation of FGF8 expression, or whether Foxc2 functions upstream of FGF8 in vivo. In the current study, we demonstrated that FGF8 down-regulates Foxc2 expression. These results indicate that FGF8 and Foxc2 are regulated by each other in a reciprocal manner. The expression of Hand1, which is an inhibitor of MyoD, was also down-regulated by FGF8 treatment. Thus, FGF8-mediated inhibition of MyoD appears to be independent of Hand1 in cultured cells. Forkhead transcription factors can form heterodimers with other transcription factors, co-factors or steroid hormone receptors. For example, Foxc2 physically interacts with Smad2, Smad3, and Smad4 [Fujita et al., 2006]. Similar studies are highlighted in a recent review article [van der Vos et al., 2008]. We performed an immunoprecipitation experiment to determine whether Foxc2 physically interacted with Hand1 in cultured C2C12 cells, and found no evidence that Foxc2 forms heterodimers with Hand1. The protein level of Hand1 was increased in cells that overexpressed Foxc2, and we showed that this induction was due to a direct interaction of Foxc2 with the Hand1 promoter. Our results suggest that the Foxc2-induced expression of Hand1 functions as a negative feedback regulator of MyoD expression in cultured cells.

The genes for FGF8, Foxc2, and the Hands proteins are essential for cardiac development and the formation of the aortic arch in vivo. The results of the current study suggest a mechanism of action of these proteins in vitro, and future studies will focus on determining whether this mechanism also functions in vivo.

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