

Both the Smad and p38 MAPK pathways play a crucial role in *Runx2* expression following induction by transforming growth factor- β and bone morphogenetic protein

Kyeong-Sook Lee¹, Seung-Hyun Hong^{1,2} and Suk-Chul Bae^{*1,2}

¹Department of Biochemistry, School of Medicine, Chungbuk National University, Cheongju, 361-763, South Korea; ²Institute for Tumor Engineering, Chungbuk National University, Cheongju, 361-763, South Korea

The Runx family of transcription factors plays pivotal roles during normal development and in neoplasias. In mammals, Runx family genes are composed of *Runx1* (*Pebp2 α B/Cbfa2/Aml1*), *Runx2* (*Pebp2 α A/Cbfa1/Aml3*) and *Runx3* (*Pebp2 α C/Cbfa3/Aml2*). *Runx1* and *Runx3* are known to be involved in leukemogenesis and gastric carcinogenesis, respectively. *Runx2*, on the other hand, is a common target of transforming growth factor- β 1 (TGF- β 1) and bone morphogenetic protein-2 (BMP-2) and plays an essential role in osteoblast differentiation. *Runx2* is induced by the receptor-activated Smad; *Runx2* mediates the blockage of myogenic differentiation and induces osteoblast differentiation in C2C12 pluripotent mesenchymal precursor cells. However, Smad does not directly induce *Runx2* expression; an additional step of *de novo* protein synthesis is required. Here we report that Smad-induced *junB* functions as an upstream activator of *Runx2* expression. Furthermore, not only the Smad pathway but also the mitogen-activated protein kinase (MAPK) cascades are involved in the induction of *Runx2* by TGF- β 1 and BMP-2. Our results demonstrate that following TGF- β and BMP induction, both the Smad and p38 MAPK pathways converge at the *Runx2* gene to control mesenchymal precursor cell differentiation.

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Introduction

Members of the transforming growth factor- β (TGF- β) superfamily elicit diverse cellular responses, including inhibition of adipogenesis and myogenesis, and stimulation of chondrogenesis and osteogenesis (Hogan, 1996; Roberts *et al.*, 1988). The most important members of the TGF- β superfamily which effect on bone cell differentiation *in vivo* are TGF- β and bone morphoge-

netic proteins (BMPs). TGF- β 1 induces new bone formation when injected in close proximity to bone tissue. On the other hand, BMP-2 induces bone formation even when injected ectopically (Wozney *et al.*, 1988). In the pluripotent mesenchymal precursor cell line, C2C12, TGF- β 1 inhibits default differentiation of the cell into multinucleated myotubes without inducing osteoblast phenotypes. BMP-2 not only inhibits myogenic differentiation of C2C12 cells but also induces osteoblast phenotypes (Katagiri *et al.*, 1994).

TGF- β family members exert their cellular effects by binding to transmembrane receptors that possess serine/threonine kinase activity (Massague, 1998). Following ligand activation, the receptor kinase phosphorylates Smad proteins, which move into the nucleus to stimulate the transcription of a set of target genes. Smad2 and 3 are activated by TGF- β receptors and mediate TGF- β responses, whereas Smad1, 5 and 8 are activated by BMP receptors and transduce BMP signals (Heldin *et al.*, 1997; Massague, 1998). In addition to the Smad group of proteins, activation of mitogen-activated protein kinase (MAPK) cascades is involved in TGF- β superfamily signal transduction. Both TGF- β and BMP have been shown to activate p38, a member of the stress-activated protein kinases (SAPKs), through MAPK kinase (MKK) 6 or MKK3 (Gallea *et al.*, 2001; Hanafusa *et al.*, 1999). It has been reported that both Smad and MAPK pathways are essential components of the TGF- β superfamily signaling and affect osteoblast differentiation (Derynck *et al.*, 2001; Fujii *et al.*, 1999; Gallea *et al.*, 2001; Nishimura *et al.*, 1998; Yamamoto *et al.*, 1997). However, how these signaling pathways affect osteoblast differentiation is poorly understood.

Runx family of transcription factors encoded by three distinct genes, *Runx1* (*Pebp2 α B/Cbfa2/Aml1*), *Runx2* (*Pebp2 α A/Cbfa1/Aml3*) and *Runx3* (*Pebp2 α C/Cbfa3/Aml2*), plays pivotal roles during normal development and in neoplasias. *Runx1* plays a critical role in the formation of hematopoietic stem cells (North *et al.*, 1999; Okuda *et al.*, 1996; Yokomizo *et al.*, 2001). It is the most frequent target of chromosome translocation in leukemia and is responsible for about 30% of human acute leukemia cases (Look, 1997).

RUNX3 has been shown to be a tumor suppressor of gastric cancer (Li *et al.*, 2002). *RUNX3* was frequently

*Correspondence: S-C Bae, E-mail: scbae@med.chungbuk.ac.kr
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inactivated in human gastric cancer tissues and tumorigenicity of gastric cancer cell lines in nude mice was inversely related to their level of *RUNX3* expression. Furthermore, a mutation identified from a gastric cancer patient abolished the tumor suppressive effect of *RUNX3*.

Runx2, on the other hand, is an essential transcription factor required for osteogenesis (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997). Haploinsufficiency of the *Runx2* gene was shown to be the cause of the human disease cleidocranial dysplasia (CCD), an autosomal dominant bone disorder (Lee et al., 1997; Mundlos et al., 1997). The oncogenic properties of *Runx2* were demonstrated in transgenic mice where *Runx2* overexpression perturbs T cell development and synergizes strongly with *c-myc* in lymphomagenesis (Vaillant et al., 1999).

Runx2 expression is transiently up regulated by TGF- β 1 and BMP-2-activated Smads and mediates the blockage of myogenic differentiation of C2C12 cells. *Runx2* is essential for the common activities of by TGF- β 1 and BMP-2 and cooperation between *Runx2* and receptor activated Smads is required for the ligand specific gene expression (Lee et al., 2000; Zhang et al., 2000). However, Smad does not directly induce *Runx2* expression; an additional step of *de novo* protein synthesis is required (Lee et al., 2000). Our results suggest that *junB* is the newly synthesized protein required for the induction of *Runx2* by TGF- β 1 and BMP-2. Furthermore, we show that activation of p38 MAPK is also involved in *Runx2* induction. These results provide important insights into how the TGF- β 1 and BMP-2 signals are transmitted to their major target gene, *Runx2*.

Results

Involvement of *junB* in *Runx2* induction by TGF- β 1 and BMP-2

Previously we reported that activation of Smads by TGF- β 1 and BMP-2 resulted in the induction of *Runx2* and that *Runx2* played an important role in blocking myogenic differentiation in C2C12 cells. However, in addition to the activation of Smad, induction of *Runx2* requires *de novo* protein synthesis (Lee et al., 2000). Since *Runx2* induction is observed as early as 2 h after stimulation, we assumed that an immediate early gene product might be involved in the *Runx2* induction. Among the immediate early genes, *junB* is known to be induced by TGF- β 1 and BMP-2 in C2C12 cells, while expression of *c-jun*, *junD* and *c-fos* is not changed (Chaloux et al., 1998; Jonk et al., 1998). Since *junB* also mediates blockage of myogenic differentiation of C2C12 cells (Chaloux et al., 1998), we asked whether *de novo* synthesis of *junB* is required prior to the induction of *Runx2*. Firstly, we examined the time course of *junB* and *Runx2* induction in response to TGF- β 1 stimulation. As shown in Figure 1a, the maximum level of *junB* mRNA was detected 1 h after TGF- β 1 stimulation, which is 1 h earlier than *Runx2*, implying that *junB* could be an upstream activator of *Runx2*. In contrast,

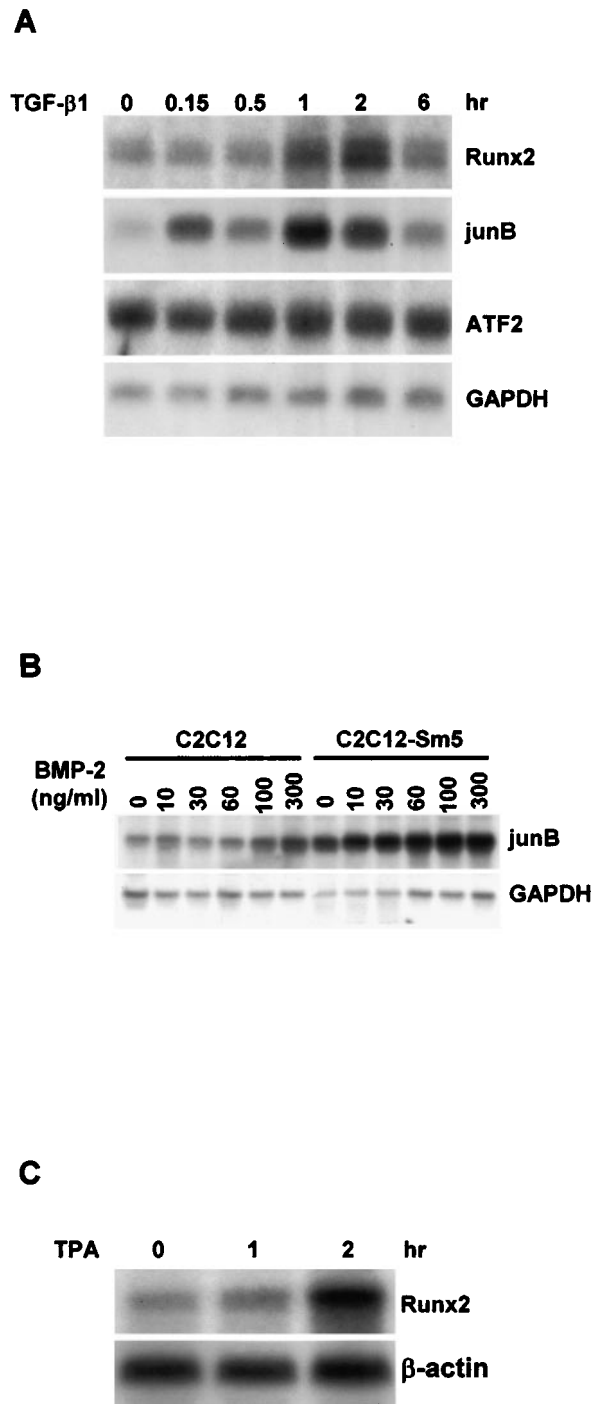


Figure 1 Induction of *Runx2* mRNA by TGF- β 1 and BMP-2. (a) C2C12 cells were treated with TGF- β 1 (5 ng/ml) for the indicated times, and total RNA was prepared. Northern blotting was performed using *Runx2*, *junB*, and *ATF-2* cDNA as probes. (b) Total RNAs were serially prepared from C2C12 cells and C2C12-Sm5 cells treated with serially diluted BMP-2 for 1 h. *junB* expression was analysed by Northern blot hybridization. (c) C2C12 cells were treated with TPA (100 ng/ml) for the indicated times, and total RNA was prepared. *Runx2* mRNA expression was measured using Northern blotting. Probes prepared from GAPDH and β -actin coding sequences were used as loading controls

the expression level of *ATF-2*, which plays an important role in osteoblast differentiation *in vivo* and makes heterodimers with *junB* (Reimold *et al.*, 1996), was not altered under the same experimental conditions.

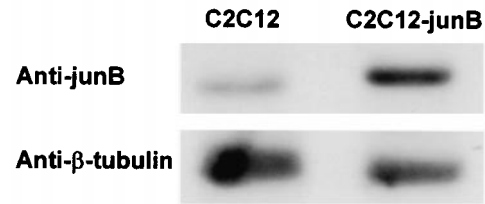
We further examined whether there is any correlation between the level of *junB* and *Runx2* expression in response to various concentrations of BMP-2. The control C2C12 and Smad5 over-expressing C2C12 cells (C2C12-Sm5) were treated with serially diluted concentrations of BMP-2 for 1 h and *junB* mRNA levels were measured using Northern blot analysis. As shown in Figure 1b, *junB* was induced by 300 ng/ml of BMP in control C2C12 cells; however, in C2C12-Sm5 cells, *junB* expression was detected in the absence of BMP-2 and increased at higher concentrations of BMP-2. The expression pattern of *junB* in C2C12 and C2C12-Sm5 cells in response to various concentrations of BMP-2 is very similar to that of *Runx2* (Lee *et al.*, 2000). These results encouraged us to examine the effect of *junB* activation on *Runx2* expression. C2C12 cells were treated with a potent *junB* activator, 12-O-tetradecanoylphorbol-13acetate (TPA) (Chiu *et al.*, 1989), and the level of *Runx2* mRNA was analysed. As shown in Figure 1c, treatment of TPA strongly induced *Runx2* gene expression (Figure 1c). Therefore, we examined whether the exogenous expression of *junB* could induce *Runx2* expression. For this purpose, C2C12 cells stably expressing *junB* (C2C12-*junB*) were obtained (Figure 2a), and the level of *Runx2* expression in response to TGF- β 1 and BMP-2 was measured by Northern blotting (Figure 2b,c). In control C2C12 cells, only very low levels of *Runx2* mRNA were detected and mRNA levels increased following TGF- β 1 and BMP-2 stimulation, as reported previously (Lee *et al.*, 2000). In contrast, C2C12-*junB* cells showed high levels of *Runx2* mRNA in the absence of stimulation, and the levels further increased following TGF- β 1 and BMP-2 treatment.

The essential role of *junB* in the induction of *Runx2* was further confirmed by suppression of AP-1 activity following stable expression of a dominant negative *c-fos* (*A-fos*), which inhibits *jun* family dependent transactivation (Olive *et al.*, 1997). Two independent C2C12 cell clones that express either high level of *A-fos* (C2C12-*A-fos*-1) or low level (C2C12-*A-fos*-2) were obtained (Figure 3a), and the level of *Runx2* mRNA in the presence or absence of TGF- β 1 and BMP-2 was analysed using Northern blotting. As shown in Figure 3b, exogenous expression of *A-fos* significantly inhibited induction of *Runx2* by TGF- β 1 and BMP-2 stimulation. Notably, the inhibitory effect correlated with the level of *A-fos* protein. Collectively, these results indicate that induction of *junB* is essential for the induction of *Runx2* expression by TGF- β 1 and BMP-2.

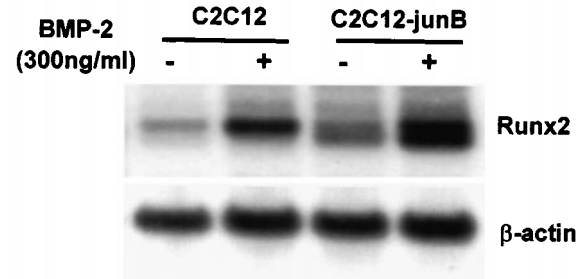
Involvement of the p38 MAPK signaling pathway in the induction of Runx2 expression

To determine whether any other kinase is also involved in the induction of *Runx2* expression following TGF- β 1 and BMP-2 stimulation, C2C12 cells were treated with various kinase inhibitors in the presence or absence of

A



B



C

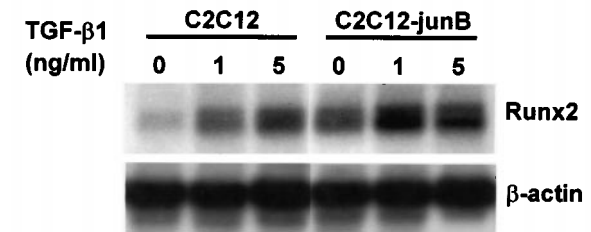


Figure 2 Induction of *Runx2* by *junB*. (a) Western blotting showing over-expressing of *junB* in C2C12-*junB* cells. Total cellular protein extracts were prepared from control C2C12 and C2C12-*junB* cells, and the *junB* protein was detected by a specific monoclonal antibody. For loading control, levels of β -tubulin were measured by anti- β -tubulin antibody. (b) Control C2C12 and C2C12-*junB* cells were treated with BMP-2 (300 ng/ml) for 2 h and *Runx2* mRNA levels were analysed using Northern blotting. (c) Control C2C12 and C2C12-*junB* cells were treated with the indicated concentrations of TGF- β 1 for 2 h. Total RNA was prepared from the cells, and *Runx2* mRNA levels were analysed using Northern blotting

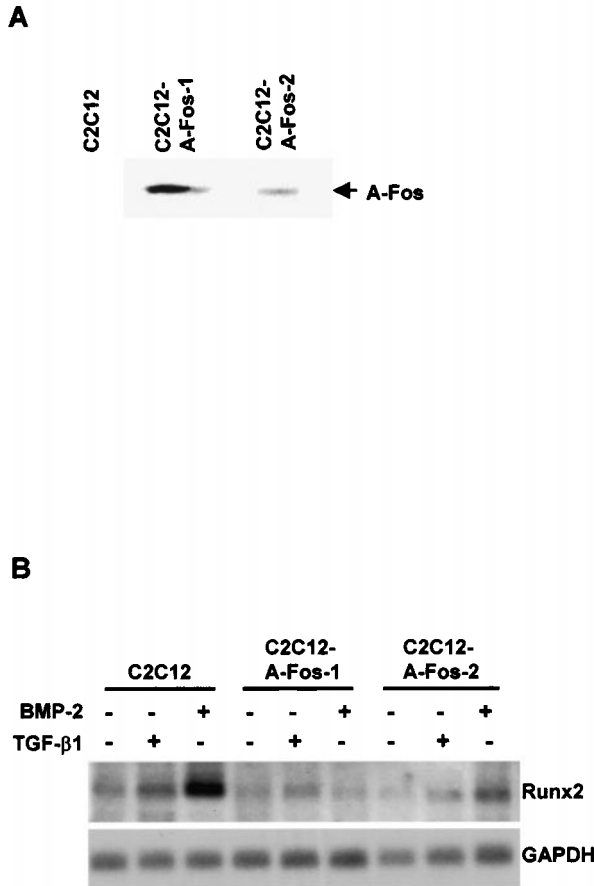


Figure 3 Inhibition of *Runx2* induction by A-fos. (a) Western blotting showing exogenous expression of A-fos in C2C12-A-fos cells. Total cellular protein extracts were prepared from control C2C12, C2C12-A-fos-1 and C2C12-A-fos-2 cells, and A-fos protein was detected by an anti-FLAG antibody. (b) C2C12, C2C12-A-fos-1 and C2C12-A-fos-2 cells were treated with TGF- β 1 (5 ng/ml) and BMP-2 (300 ng/ml) for 2 h. Total RNA was prepared from the cells, and *Runx2* mRNA levels were analysed using Northern blotting

BMP-2, and the level of *Runx2* mRNA was analysed using Northern blotting. As shown in Figure 4a, treatment with SB203580, a specific inhibitor of p38 MAPK, effectively inhibited *Runx2* induction by TGF- β 1 and BMP-2, while other kinase inhibitors had no significant effect. To examine whether the activation of p38 could induce *Runx2* expression, C2C12 cells were treated with anisomycin, a potent activator of p38, in the presence or absence of SB203580, and the level of *Runx2* mRNA was measured using Northern blotting. As shown in Figure 4b, *Runx2* expression was significantly induced by anisomycin, and induction was effectively blocked by SB203580. To further investigate the involvement of the p38 signaling pathway in the induction of *Runx2*, C2C12 cells stably expressing dominant negative p38 [p38(AGF)] (Noguchi *et al.*, 2000), were obtained [C2C12-p38(AGF)] (Figure 5a). We confirmed that p38 was activated by TGF- β 1 and over-expression of p38(AGF) effectively blocked the p38 activation (Figure 5b). Control C2C12 and

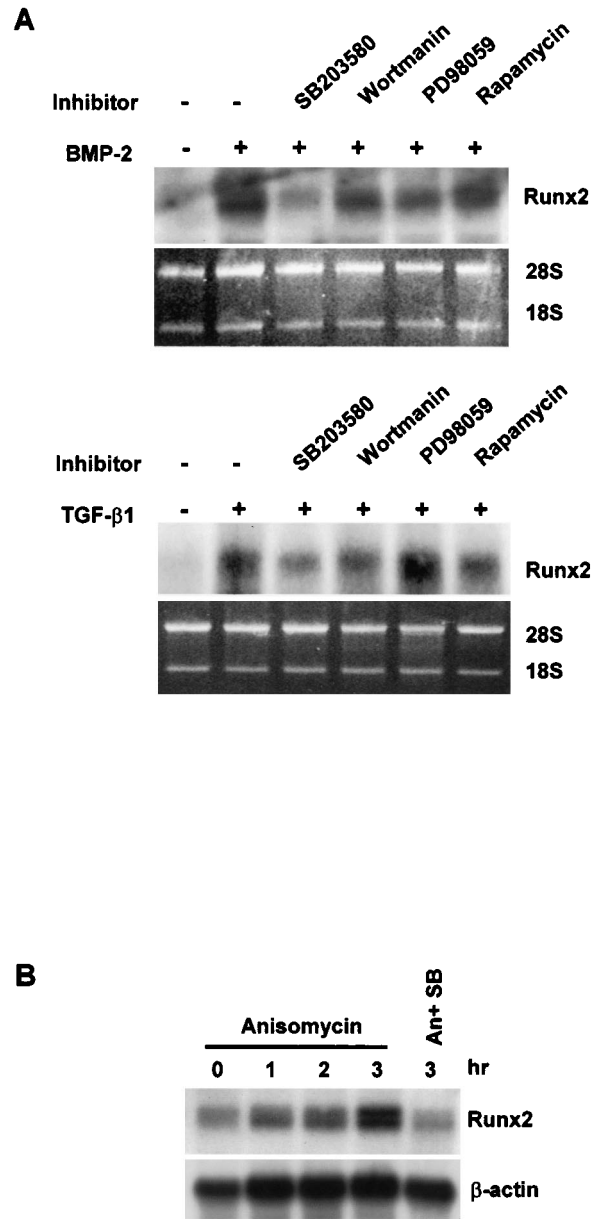
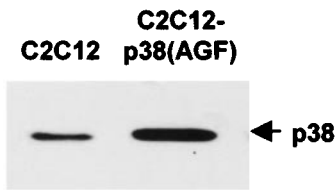


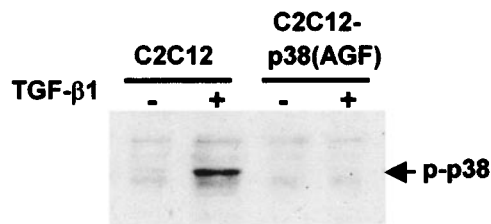
Figure 4 Involvement of p38 MAPK in the induction of *Runx2*. (a) C2C12 cells were pretreated with SB203580 (50 μ M, 1 h), Wortmanin (0.5 μ M, 1 h), PD98059 (50 μ M, 20 min) or Rapamycin (20 ng/ml, 1 h) and then treated with BMP-2 (300 ng/ml) or TGF- β 1 (5 ng/ml) for 2 h. Total RNA was prepared from the cells, and *Runx2* mRNA levels were analysed using Northern blotting. (b) C2C12 cells were treated with anisomycin (5 ng/ml) in the presence or absence of SB203580 for the indicated times, and total RNA was prepared. *Runx2* mRNA levels were analysed using Northern blotting. An: Anisomycin; SB: SB203580

C2C12-p38(AGF) cells were treated with TGF- β 1 and BMP-2, and the level of *Runx2* mRNA was measured using Northern blotting. As shown in Figure 5c, the induction of *Runx2* was significantly inhibited in the C2C12-p38(AGF) cells. These results indicate that activation of p38 is involved in the induction of *Runx2* by TGF- β 1 and BMP-2.

A



B



C

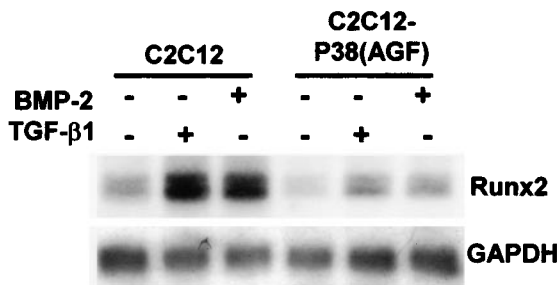


Figure 5 Inhibition of *Runx2* induction by dominant negative p38 [p38(AGF)]. (a) Western blotting showing exogenous expression of p38(AGF) in C2C12-p38(AGF) cells. The arrows indicate p38 and p38(AGF) proteins. (b) C2C12 and C2C12-p38(AGF) cells were cultured in the presence or absence of TGF- β 1 (5 ng/ml) for 2 h, and phosphorylated p38 was detected with a phospho-p38-specific antibody. Note that p38(AGF) effectively blocked TGF- β 1-dependent p38 phosphorylation. (c) C2C12, and C2C12-p38(AGF) cells were treated with TGF- β 1 (5 ng/ml) or BMP-2 (300 ng/ml) for 2 h. Total RNA was prepared from the cells, and *Runx2* mRNA levels were analysed using Northern blotting

The effect of junB and p38 on osteoblast differentiation

TGF- β and BMP are known to induce preosteoblast stage specific gene expression (collagen α (1) and fibronectin) in C2C12 cells. However, osteoblast-specific genes (for example, alkaline phosphatase and osteocalcin) are induced only by BMP. Previously, we showed that *Runx2* alone can induce preosteoblast stage specific genes, but fails to fully induce osteoblast-specific genes (Lee et al., 2000). Since we found that induction of *junB* and activation of p38 are involved in the induction of *Runx2*, we examined the effect of *junB* and p38 pathways on differentiation-specific gene expression. C2C12 cells were treated with serially diluted concentrations of BMP-2, and alkaline phosphatase activities were determined. Control C2C12 cells required 300 ng/ml of BMP-2 for the induction of alkaline phosphatase. On the other hand, in both C2C12-Rx2 and C2C12-JunB cells, alkaline phosphatase was weakly expressed even in the absence of BMP-2 and strongly expressed at very low concentrations of BMP-2 (Figure 6). The induction of alkaline phosphatase by BMP-2 was inhibited by A-fos and p38(AGF) (Figure 6). These results, together with the observation that *junB* induces *Runx2* expression, suggest that *junB* is involved in the process of osteoblast differentiation by inducing *Runx2* expression, and that the activation of p38 MAPK is an additional essential component for the TGF- β 1 and BMP-2-dependent induction of *Runx2*.

Discussion

JunB is an upstream regulator of *Runx2* expression following induction by TGF- β 1 and BMP-2

JunB is a direct target gene of Smads activated by TGF- β 1 and BMP-2 and mediates the blockage of

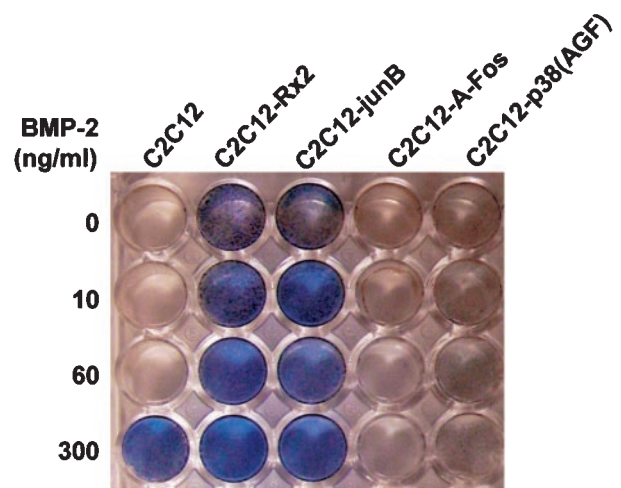


Figure 6 The effect of *Runx2* and *JunB* on osteoblast-specific gene expression. Cells were treated with the indicated concentration of BMP-2 for 2 days, and alkaline phosphatase activity was assayed as described previously (Katagiri et al., 1994)

myogenic differentiation in C2C12 cells (Chaloux *et al.*, 1998; Jonk *et al.*, 1998). We found that *junB* not only blocks myogenic differentiation of C2C12 cells but also makes the cells highly sensitive to BMP-2-induced osteoblast differentiation (Figure 6). This activity may be mediated by osteoblast-specific transcription factors induced by *junB*, since *junB* is a general transcription factor that is not specific for osteoblast differentiation. *Runx2* encodes an osteoblast-specific transcription factor and is transiently induced by TGF- β 1 and BMP-2 (Ducy, 2000; Lee *et al.*, 2000). The induction of *Runx2* is responsible for the inhibition of myogenic differentiation and over-expression of *Runx2* makes the cells highly sensitive to BMP-2-induced osteoblast differentiation (Lee *et al.*, 2000). Even though the induction of *Runx2* by TGF- β 1 or BMP-2 was observed very early after stimulation, it still required *de novo* synthesis of at least one protein. In this study, we showed that *junB* expression is induced earlier than *Runx2* following TGF- β 1 stimulation. Furthermore, activation and over-expression of *junB* resulted in the induction of *Runx2* and inhibition of AP-1 blocked TGF- β 1 and BMP-2-dependent *Runx2* induction. These results suggest that *junB* is one of the upstream regulators of *Runx2* and provide an important signal transduction pathway from receptor-activated Smads to the osteogenic master switch gene *Runx2*.

Involvement of the p38 MAPK signal pathway in the induction of Runx2

We showed that exogenous expression of *junB* by itself resulted in the induction of *Runx2*. Interestingly, the level of *Runx2* mRNA was further increased by TGF- β 1 and BMP-2 stimulation (Figure 2b,c). This result suggests that the induction of *junB* might not be the only mechanism involved in *Runx2* induction; there could be other independent pathways that function synergistically to induce *Runx2*. Our results demonstrate that the activation of p38 MAPK is also involved in the induction of *Runx2* by TGF- β 1 and BMP-2 stimulation. The activation of p38 by TGF- β 1 and BMP-2 stimulation in C2C12 cells and the crucial role of p38 in osteoblast differentiation have been reported (Gallea *et al.*, 2001; Hanafusa *et al.*, 1999). However, target transcription factors of p38 pathway responsible for the osteoblast differentiation have been poorly understood. Our results showing that the activation of p38 is involved in the induction of *Runx2* suggest that p38 pathway could contribute to the osteoblast differentiation by inducing *Runx2*.

So far, the mechanism for the induction of *Runx2* by TGF- β and BMP-activated p38 is unclear. Activation of ATF-2 could be involved in the induction of *Runx2*, since ATF-2 is known as a major target of p38 and plays an important role in bone formation *in vivo* (Reimold *et al.*, 1996). Induction of *junB* by p38 might also be responsible for the induction of *Runx2*. Further study will be required to fully understand the signaling pathway regulating *Runx2* expression through p38 MAPK.

The effect of junB and p38 on osteoblast differentiation

The sequential expression of specific genes for osteoblast differentiation has been determined (Lian and Stein, 1995). During the preosteoblast stage, several genes associated with the formation of the extracellular matrix (for example, collagen I and fibronectin) are actively expressed. Following the down-regulation of proliferation, proteins associated with the bone cell phenotype are detected (for example, alkaline phosphatase). *Runx2* is known to be an osteogenic master gene, and regulates the osteoblast specific marker genes (Lee *et al.*, 2000; Xiao *et al.*, 1999). The effect of exogenous expression of *junB* on the induction of alkaline phosphatase was almost equivalent to that of *Runx2* and inhibition of AP-1 or p38 effectively blocked BMP-induced marker gene expression (Figure 6). These results strongly support our claim that *junB* is an upstream regulator of *Runx2* and that p38 is an additional essential component for the induction of *Runx2*.

Our results indicate that the independent Smad-induced *junB* pathway and the receptor-activated MAP kinase pathway both play a crucial role in the induction of *Runx2* by TGF- β 1 and BMP-2. It is worth noting that p38 is not the only MAP kinase activated by TGF- β 1 and BMP-2. Erk is also a common target of TGF- β 1 and BMP-2 and activates *Runx2*-dependent transcription without affecting the expression of *Runx2* (Xiao *et al.*, 2000). Therefore, concerted actions of the TGF- β superfamily signaling pathways converge at the *Runx2* gene for the C2C12 pluripotent mesenchymal precursor cell differentiation.

Materials and methods

Materials

Bioactive recombinant human BMP-2 was kindly provided by J Wozney (Genetics Institute, Cambridge, Massachusetts). Recombinant human TGF- β 1 was purchased from Sigma. Reagents were purchased from the following vendors: Restriction enzymes from Takara (Tokyo, Japan) or New England Biolabs; cell culture reagents, G418 and Lipofectamin from Gibco/BRL; anti-*junB* rabbit polyclonal, anti-ATF-2 rabbit polyclonal antibody from Santa Cruz; anti-p38 and anti-phospho-p38 MAPK rabbit polyclonal antibodies from New England Biolabs; monoclonal anti-FLAG antibody, alkaline phosphatase (ALP)-conjugated anti-goat IgG monoclonal antibody from Sigma; horseradish peroxidase (HRP)-conjugated anti-rabbit goat antibody from Pierce; anti- β -tubulin monoclonal antibody from Oncogene; ECL Western blotting kit including HRP-conjugated anti-mouse IgG goat antibody, Hybond-N+ nylon membrane and rediprime DNA labeling kit from Amersham; Immobilon from Milipore; SB203580, Wortmanin, PD98059, Rapamycin and Anisomycin from Sigma. All other chemicals of the purest grade available were obtained from commercial sources.

Plasmids

The plasmid expressing dominant negative Fos (A-Fos) (Olive *et al.*, 1997) was kindly provided by Dr C Vinson.

(National Institute of Health, Bethesda, MD, USA). JunB expressing plasmid (pcDNA-JunB) was kindly provided by Dr F Ventura (Universitat de Barcelona, Hospitalet de Llobregat, Spain). Dominant negative p38 MAP kinase (Noguchi *et al.*, 2000) expressing plasmid (pcDNA3-p38-AGF) was kindly provided by Dr Y Kuchino (National Cancer Center Research Institute, Tokyo, Japan).

Cell lines and cultures

The mouse pluripotent mesenchymal precursor cell line, C2C12, was purchased from the American Type Culture Collection. C2C12 cells were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 5–15% fetal bovine serum (FBS), penicillin G (100 U/ml) and streptomycin (100 μ g/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air. To avoid cell density dependent fluctuations, all the treatments were performed after cells became completely confluent.

Stable transfection

Runx2 and *Smad5* over-expressing C2C12 cells (C2C12-Rx2 and C2C12-Sm5, respectively) have been described previously (Lee *et al.*, 2000). *A-fos*, *p38(AGF)* and *junB* over-expressing cells were obtained by stable transfection of pcDNA-A-fos, pcDNA3-p38-AGF and pcDNA-JunB, respectively, into C2C12 via the Lipofectamine method according to the manufacturer's instructions (Gibco/BRL). The stably transfected cells were screened for 2 weeks in selection medium containing 600 μ g/ml G418, and viable colonies were further screened by Western blotting.

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Northern blot analysis

Northern blot analysis was performed as described previously (Sambrook *et al.*, 1988). The DNA probes were either the PCR product or cloned cDNA of mouse *Runx2*, *junB* and *ATF-2*. All probes were labeled with [α -³²P]dCTP (3000 Ci/mmol; NEN) using the rediprime DNA labeling kit.

Western blot analysis

Proteins from cell lysates were resolved by SDS-PAGE (8–10%) and transferred to Immobilon membrane (Milipore). The blots were blocked in BP solution (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat dry milk. Primary antibody (against FLAG, junB, or p38) was added to the BP solution at a 1:1000 dilution and incubated for 1 h at 25°C. The blots were washed three times with the BP solution and incubated with the secondary antibody (1:10000) conjugated with HRP for 1 h at 25°C. After three washes with the BP solution, the blots were developed with ECL and exposed on Kodak XAR-5 film.

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