Comparative roles of Twist-1 and Id1 in transcriptional regulation by BMP signaling

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Summary

Basic helix-loop-helix (bHLH) transcription factors are known as key regulators for mesenchymal differentiation. The present study showed that overexpression of Twist-1, a bHLH transcription factor, suppresses bone morphogenetic protein (BMP)-induced osteoblast differentiation, and downregulation of endogenous Twist-1 enhances BMP signaling. Maximal inhibition of BMP signaling was observed when Twist-1 was bound to E47, which markedly enhanced the stability of Twist-1. Co-immunoprecipitation assays revealed that Twist-1 formed a complex with Smad4 and histone deacetylase (HDAC) 1 in MC3T3-E1 cells stably expressing Twist-1. With trichostatin, an HDAC inhibitor, osteogenic factors such as alkaline phosphatase, Runx2 and osteopontin increased. Those results suggested that Twist-1 inhibited BMP signaling by recruiting HDAC1 to Smad4.

Furthermore, the inhibitory effects of Twist-1 on BMP signaling were overcome by Id1 through induction of Twist-1 degradation. These findings suggest that Twist-1 can act as an inhibitor of BMP signaling, and Id1 can regulate BMP signaling through a positive feedback loop repressing Twist-1 function. These two molecules may therefore regulate differentiation of mesenchymal cells into progeny such as osteoblasts by controlling BMP signaling.

Key words: Twist-1, Id1, BMP, Smad, HDAC

Introduction

Members of the transforming growth factor (TGF)-β superfamily regulate important biological and developmental processes, including cell proliferation, differentiation and migration (Derynck and Zhang, 2003). This is achieved through the ability to induce or repress transcription of diverse gene targets. For example, differentiation of mesenchymal cells into components of bone, cartilage or adipose tissue is regulated by bone morphogenetic proteins (BMPs), which belong to the TGF-β superfamily. BMPs induce not only new bone formation in vivo when implanted into ectopic sites (Urist, 1965), but also osteoblast differentiation of mesenchymal cells in vitro (Katagiri et al., 1994; Thies et al., 1992). TGF-β/BMP signaling is initially mediated by interactions with heterodimeric complexes of type I and type II serine/threonine kinase receptors. Activated receptor kinases phosphorylate receptor-regulated Smads (R-Smads). R-Smads then form activated complexes with common-mediator Smads (C-Smads). These complexes translocate into the nucleus to act as transcriptional regulators (Derynck and Zhang, 2003; Massague, 2000; Wrana, 2000). Studies on the mechanisms by which Smads mediate TGF-β/BMP-regulated gene transcription have led to the discovery of co-activators and co-repressors (Derynck and Zhang, 2003). However, the molecular mechanisms underlying the inhibition of BMP signaling have not been fully elucidated.

Twist-1, originally identified in *Drosophila*, is a member of the basic helix-loop-helix (bHLH) family of proteins (Leptin, 1991; Thisse et al., 1987). Twist-1 is expressed in mesodermal and cranial neural crest cells during embryogenesis (Wolf et al., 1991). In homozygous Twist-1-knockout mice the cranial neural tube fails to close and they die at embryonic day 11.5 (Chen and Behringer, 1995). Twist-1 heterozygous mice present a craniosynostotic phenotype (Bourgeois et al., 1998). Expression of Twist-1 has been implicated in the inhibition of differentiation for multiple mesenchymal cell lineages, including muscle (Hebrok et al., 1997; Spicer et al., 1996) and bone cells (Lee et al., 1999; Rice et al., 2000). The mechanisms of inhibition have been well established in muscle (Hamamori et al., 1999; Hebrok et al., 1997; Spicer et al., 1996), but little is known about the mechanisms behind inhibition of osteoblast differentiation by Twist-1. Very recently, Twist-1 has been reported to interact directly with Runx2, a key transcriptional factor regulating osteogenic gene expression. Direct interaction of Twist-1 with Runx2 causes inhibited DNA binding of Runx2 followed by gene inactivation in osteoblast precursors (Bialek et al., 2004). The fact that neurogenin, another bHLH family member, inhibits glial cell differentiation by sequestering Smad1 of the transcription complex away from glial differentiation genes (Sun et al., 2001), suggests that Twist-1 may likewise affect BMP signaling in the process of mesenchymal cell differentiation into osteoblasts.

The best-studied example of dimerization partners for known tissue-specific bHLH transcription factors involves the gene products of the *E2A* gene (Lassar et al., 1991). Through differential splicing, this gene gives rise to two different bHLH

proteins, E12 and E47, the so-called E-proteins (Murre et al., 1989; Sun and Baltimore, 1991). Id is an internal dominant negative form of HLH transcription factor, lacking a basic region. By sequestering E-proteins, Id prevents myogenic transcription factors, such as MyoD, from forming heterodimer complexes (Benezra et al., 1990; Sun et al., 1991). Recent reports have indicated that BMP induces expression of Id1, resulting in degradation of tissue-specific bHLH transcription factors (Vinals et al., 2004; Vinals and Ventura, 2004). Furthermore, Id1 is critical to BMP-induced osteoblast differentiation (Peng et al., 2004). As a result, we hypothesized that Id1 may be an antagonist of Twist-1 in osteoblast differentiation. The present study found that Twist-1 inhibits BMP-induced osteoblast differentiation. Inhibition of BMP signaling by Twist-1 is enhanced by E-protein. Moreover, the inhibitory effect of Twist-1 is overcome by Id1 through the induction of Twist-1 degradation. These findings suggest that Twist-1 and Id1 can regulate differentiation of mesenchymal cell lineages by controlling BMP signaling.

Results

Overexpression of Twist-1 inhibits BMP-induced osteoblast-specific gene expression

To examine the effect of Twist-1 on expression of BMP2-induced osteoblast differentiation marker genes, a cell line (MC3T3-E1-Tw) stably expressing Twist-1 was established. Since MC3T3-E1 cells contained less endogenous Twist-1 (Tamura and Noda, 1999) and Id1 (data not shown) than C3H10T1/2 cells, the cell line was appropriate to minimize the effect of endogenous Twist-1 and Id1. Exogenous Flag-Twist-1 was detected in MC3T3-E1-Tw1 by western blotting of immunoprecipitates using anti-Flag antibody (Fig. 1A).

Next, ALP activity stimulated by BMP2 was examined. BMP2-induced ALP activity was elevated in parental cells (MC3T3-E1-WT; Fig. 1B). By contrast, Twist-1 overexpression significantly suppressed BMP2-enhanced ALP activity. Basal levels of ALP activity in untreated MC3T3-E1-WT and MC3T3-E1-Tw clone 1 (MC3T3-E1-Tw1) cells were low. We analyzed ALP activity under BMP stimulation in other stable transformant clones. ALP activity in all the clones (data not shown) was much lower than in the parental MC3T3-E1-WT. We further examined whether Twist-1 overexpression also suppressed expression of other osteoblast marker genes, such as osteopontin (Hullinger et al., 2001; Shi et al., 1999; Yang et al., 2000) and osteocalcin (Katagiri et al., 1994; Thies et al., 1992). The level of BMP2-induced osteopontin expression in MC3T3-E1-Tw1 cells was lower than in MC3T3-E1-WT cells. BMP2-induced osteocalcin expression also decreased in MC3T3-E1-Tw1 cells (Fig. 1C). These results indicate that Twist-1 could affect BMP2-induced osteoblast differentiation.

Downregulation of endogenous Twist-1 enhances transcriptional activity mediated by BMP signaling

To examine the function of Twist-1 in BMP signaling, we attempted to downregulate endogenous Twist-1 expression in C3H10T1/2, a mesenchymal progenitor cell line with high levels of Twist-1 expression, using RNA interference (RNAi) technology. We selected the most effective Twist-1-specific siRNA (siTwist-691). Transient transfection of siTwist-691 resulted in a 50-60% decreases in mRNA levels (Fig. 2A). Next, we examined the direct effects of downregulating

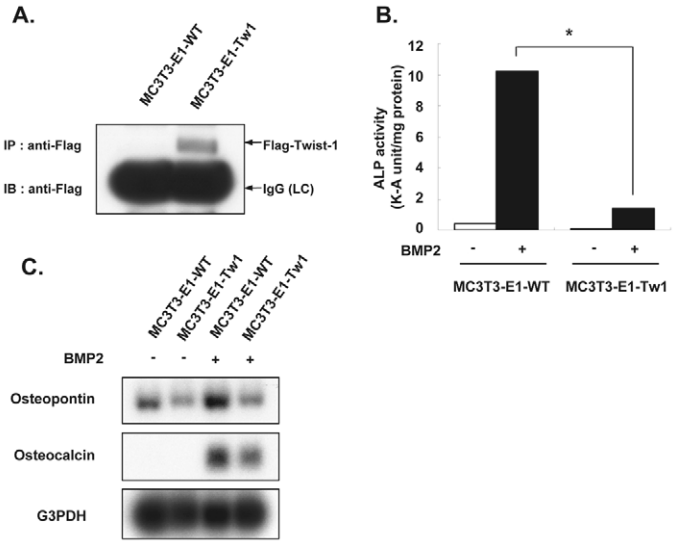


Fig. 1. Twist-1 overexpression suppresses BMP2-induced osteoblast differentiation. (A) Immunoprecipitation of overexpressed Flag-tagged Twist-1 in the nuclear extracts of MC3T3-E1-Tw1 and MC3T3-E1-WT cells. (B) MC3T3-E1-Tw1 and MC3T3-E1-WT cells were grown in the presence or absence of rhBMP2 (300 ng/ml) for 6 days. ALP activity was measured as described in the Materials and Methods (*P<0.01). (C) MC3T3-E1-Tw1 and MC3T3-E1-WT cells were grown in the presence or absence of rhBMP-2 (300 ng/ml) and total RNA was isolated on day 6. Northern blot analysis was performed using osteopontin and osteocalcin cDNA probes.

endogenous Twist-1 on transcriptional activity mediated by Smads using 3GC2-Lux, which contains three tandem repeats of a Smad-binding GC-rich sequence linked with the collagen X core promoter inserted into pGL2-Basic (Ishida et al., 2000). 24 hours after transfection of C3H10T1/2 cells with Twist-1-specific siRNA, cells were transfected with 3GC2-Lux and TK-*Renilla* luciferase and fed with or without rhBMP2 treatment. Suppression of endogenous Twist-1 expression by Twist-1-specific siRNA resulted in increased BMP-dependent Smad transcriptional activity (Fig. 2B). Additionally, when the siTwist-691 was co-transferred to C3H10T1/2 cells with Twist-1 expression plasmid by lipofection, real-time PCR analysis indicated that endogenous Twist-1 expression was partially recovered (at most 32% compared with Twist-1 level in the cells received the siRNA alone). However, no significant increase of endogenous Twist-1 expression was obtained using control plasmid, pCAGIP, without Twist-1 cDNA (data not shown).

Next, we overexpressed Smad1, Smad4 and BMPR-IB(QD) in C3H10T1/2. Downregulation of endogenous Twist-1 using Twist-1-specific siRNA also enhanced transcriptional activity mediated by overexpressed Smads (Fig. 2C). These results indicate that Twist-1 could inhibit BMP/Smad signaling.

Twist-1 inhibits BMP signaling cooperatively with E-protein

To examine whether overexpressed Twist-1 could inhibit BMP-induced transcriptional activity, co-transfection studies were performed. P19 cells that respond to BMPs and express some of the BMP target genes were transfected using Twist-1,

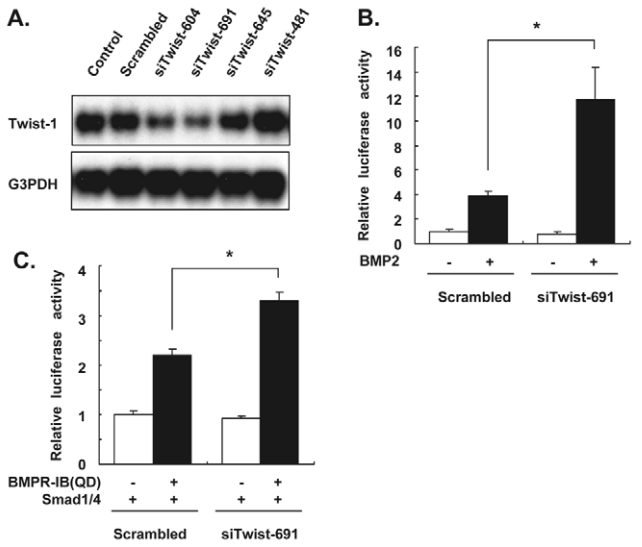


Fig. 2. Downregulation of endogenous Twist-1 using RNAi methods enhances BMP-induced transcriptional activity mediated by Smads. (A) C3H10T1/2 cells were transfected with either Twist-1-specific siRNA (siTwist-604, siTwist-691, siTwist-645, siTwist-481) or control siRNA (Scrambled) as described in the Materials and Methods. Total RNA was extracted 24 hours later, and northern blot analysis was performed using a Twist-1 cDNA probe. (B) C3H10T1/2 cells were transfected with either siTwist-691 or control siRNA (Scrambled). Cells were transfected with 3GC2-Lux and TK-*Renilla* luciferase 24 hours after siRNA transfection. At 12 hours after second transfection, cells were treated, with BMP (300 ng/ml) or left untreated, for 12 hours. Cells were lysed and luciferase activity was assayed (*P<0.01). The mean value of firefly luciferase and *Renilla* luciferase activity in the scrambled sample without BMP was approximately 2,000 and 34,000 RLU (relative light unit), respectively. (C) C3H10T1/2 cells were transiently transfected with either 90 pmol of siTwist-691 or Scrambled in combination with 100 ng of 3GC2-Lux luciferase construct, Smad1, Smad4 and BMPR-IB(QD). Cells were lysed and luciferase activity was assayed 24 hours after transfection (*P<0.01). The mean value of firefly luciferase and *Renilla* luciferase activity in the scrambled sample without BMPR-1B(QD) was approximately 411,000 and 30,000 RLU, respectively.

Smad1 and Smad4 expression constructs and 3GC2-Lux containing a Smad-binding sequence. Co-transfection of BMPR-IB(QD) with Smad1 and Smad4 enhanced transcription of 3GC2-Lux (Fig. 3A). Exogenous Twist-1 inhibited this activity, but inhibitory effects were very weak. Since Twist-1 reportedly inhibits MyoD trans-activation by E-protein sequestration, and the Twist-1-E-protein heterodimer inhibits myocyte enhancer-binding factor 2 (MEF2) trans-activation by direct interaction (Spicer et al., 1996), we hypothesized that the Twist-1-E-protein heterodimer could also act as a repressor in BMP signaling. We examined the effects of E47, an alternatively spliced product of the *E2A* gene (Murre et al., 1989; Sun and Baltimore, 1991), on suppression of Smad signaling by Twist-1. Twist-1 bound to E47 further increased the inhibition of BMP-induced transcription of 3GC2-Lux in a dose-dependent manner (Fig. 3A).

Next, the effect of Twist-1 and E47 on TGF-β signaling was analyzed using 3TP-Lux, which was empirically designed to have maximal responsiveness to TGF-β (Wrana et al., 1992). However, TGF-β signaling was not inhibited by Twist-1 and E47. Id proteins are dominant-negative-type HLH proteins that lack the basic DNA-binding domain. In muscle development, Id1 forms heterodimers with E-protein and prevents myogenic bHLH proteins from forming complexes with E-protein (Benezra et al., 1990; Sun et al., 1991). BMP stimulation also reportedly induces Id1 expression (Katagiri et al., 1994; Nakashima et al., 2001; Ogata et al., 1993), an adverse pattern as compared to Twist-1 expression (Tamura and Noda, 1999). We therefore investigated whether Id1 also cooperated with E-protein to inhibit BMP signaling. In contrast to Twist-1, however, Id1 failed to repress BMP signaling in the presence or absence of E47 (Fig. 3B), suggesting distinctly different roles for Id1 and E47 in BMP signaling. Moreover E47 also failed to repress Smad signaling without Twist-1 (Fig. 3B). To assess whether formation of heterodimer complex with E-protein is critical for the inhibitory effect of Twist-1, we overexpressed a Twist-1 mutant (Twist-NBCT), which lacks the HLH domain (El Ghouzzi et al., 2000; Hamamori et al., 1997; Hebrok et al., 1997; Spicer et al., 1996). This Twist-1 mutant did not interact with E-protein (Fig. 3C). In contrast to with wild-type Twist-1, Twist-NBCT failed to suppress transcriptional activity in the presence or absence of E47 (Fig. 3D). These findings suggest that maximal inhibition of BMP signaling by Twist-1 requires heterodimer formation with E-protein. Additionally, by over expression of Twist-1 and E47, the inhibition of luciferase gene expression by siRNA was abolished (Fig. 3E).

Twist-1 inhibited BMP signaling by recruiting HDAC1 to the Smad1-Smad4 complex

Histone deacetylases (HDACs) are involved in the repression of MyoD and MEF2 by Twist-2, which shares high homology with Twist-1 (Gong and Li, 2002; Li et al., 1995; Tamura and Noda, 1999). To analyze the involvement of HDAC in the inhibition of BMP signaling by Twist-1, Flag-tagged Twist-1 was immunoprecipitated using anti-Flag antibody in MC3T3-E1-Tw1 and the presence of HDAC and Smad was investigated in the precipitate. As shown in Fig. 4A, HDAC1 and Smad4 were detected in the precipitate, but not Smad1. In MC3T3-E1-WT, neither HDAC1 nor Smad4 was precipitated. Next, trichostatin (TSA), an HDAC inhibitor, was used to rescue the inhibition of osteogenic gene expression mediated by Twist-1. As shown in Fig. 4B, TSA treatment significantly increased the expression of ALP, Runx2 and osteopontin in the MC3T3-E1-Tw1 under BMP2 stimulation. These results suggested that Twist-1could inhibit BMP signaling by recruiting HDAC1 to Smad complex via Smad4.

Twist-1 protein stability is increased by the formation of heterodimer complex with E-protein

Stability of bHLH proteins is reportedly increased by the formation of heterodimer complexes with other bHLH proteins (Deed et al., 1996; Vinals et al., 2004; Vinals and Ventura, 2004). We therefore examined whether co-expression of E47 increases the level of Twist-1 protein. Myc-tagged Twist-1 was expressed with or without Myc-tagged E47 in COS-7 cells, and the levels of both were immunodetected by western blotting. Co-expression of E47 greatly increased Twist-1 protein levels (Fig. 5A). Next, to determine whether enhancement of Twist-1 protein expression is caused by

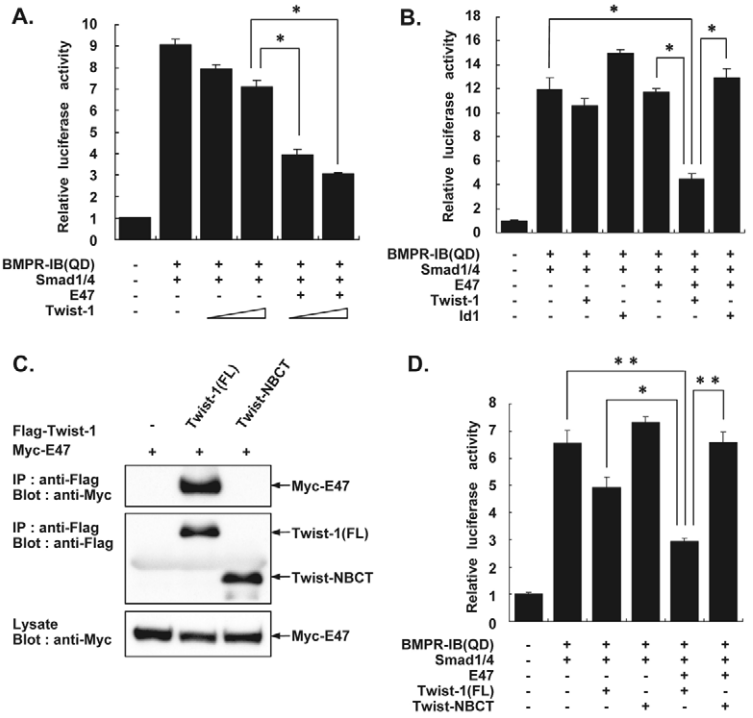
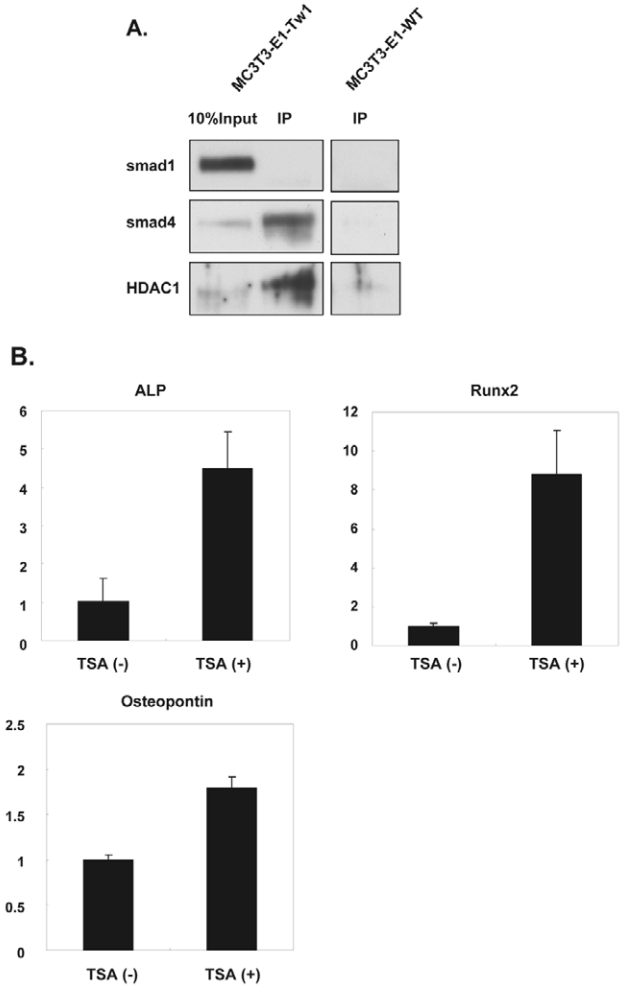


Fig. 3. Twist-1 inhibits BMP-induced Smad transcriptional activity with E-protein. (A) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1, Smad4, E47 and increasing doses (12.5 or 50 ng) of Twist-1 expression construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection (**P*<0.01). The mean value of firefly luciferase and *Renilla* luciferase activity in cells transfected with luciferase expression plasmids was approximately 109,000 and 681,000 RLU, respectively. (B) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1, Smad4, E47, Twist-1 and Id1 construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection (*P<0.01). The mean value of firefly luciferase and *Renilla* luciferase activity in cells transfected with luciferase expression plasmids was approximately 51,000 and 199,000 RLU, respectively. (C) Flag-tagged full-length (FL) Twist-1 or deletion Twist-1 mutant (Twist-NBCT) and Myc-tagged E47 constructs were transfected into COS-7 cells. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Myc antibody. (D) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1 and Smad4, E47, full-length Twist-1 and deletion Twist-1 mutant (Twist-NBCT) expression construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection (***P*<0.01, **P*<0.05).



avoidance of degradation, we examined Twist-1 protein turnover using a pulse-chase assay. Twist-1 was rapidly degraded in cells, with protein levels at 180 minutes reduced to 17% compared with the baseline at 0 minutes. By contrast, Twist-1 protein stability was greatly enhanced by co-expression with E47 (Fig. 5B). Recent reports have shown that Id genes are rapidly upregulated by BMP stimulation (Nakashima et al., 2001; Ogata et al., 1993; Peng et al., 2004). BMP2 decreases myogenin and Mash1 protein stability through induction of Id1 (Vinals et al., 2004; Vinals and Ventura, 2004). In addition, our previous data showed that Twist-1 could inhibit BMP signaling (Fig. 3A,B,D). These results prompted us to investigate whether Id1 would induce Twist-1 degradation and inhibit Twist-1 function. Twist-1 was degraded by co-transfection of Id1 in the presence of E47 (Fig. 5B).

Id1 inhibits Twist-1 function by interfering with functional Twist-1-E47 heterodimer formation

The finding that Twist-1 stabilization by E47 was partially lost by co-transfection of Id1 suggests that Id1 may sequester E47 from Twist-1, resulting in Twist-1 degradation. We tested this

Fig. 4. Twist-1 can interact with HDAC1 and Smad4. (A) MC3T3-E1-WT and MC3T3-E1-Tw1 cells were treated with BMP2 (300 ng/ml) for 24 hours. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Smad4 and HDAC1 antibody. 10% In, 10% of input; IP, immunoprecipitated fraction. (B) MC3T3-E1-Tw1 cells were treated with BMP alone [TSA (–)] or the mixture of BMP and TSA (82.5 μM) [TSA (+)]. After 24 hours, total RNA was extracted and the expression of ALP, Runx2 and osteopontin was quantified by real-time PCR. The expression levels were normalized by GAPDH, and the ratio was shown in each sample. Data are presented as mean ± s.d. of triplicate samples (**P*<0.05).

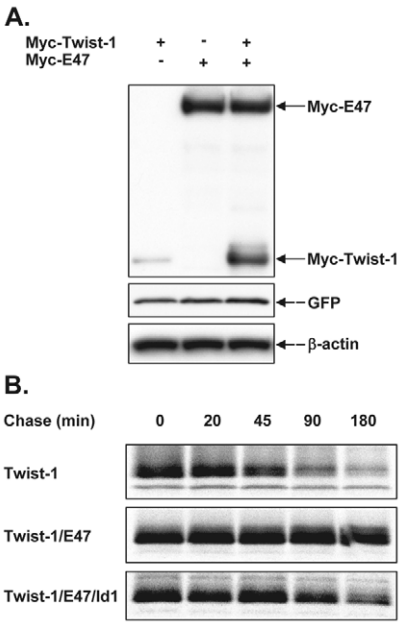


Fig. 5. Twist-1 protein stability is increased by the formation of a heterodimer complex with E47. (A) Myc-tagged Twist-1 and E47 constructs and GFP expression vector were transfected into COS-7 cells. Western blot analysis was performed with anti-Myc antibody. To show transfection efficiency, GFP protein was also detected by western blot. (B) COS-7 cells were transfected with the Flag-tagged Twist-1, Myc-tagged E47 and/or Myc-tagged Id1. At 24 hours after transfection, cells were pulsed with [^{35}S]methionine and cysteine for 3 hours, and chased with unlabeled medium for the indicated times. Labeled cell lysates were immunoprecipitated using anti-Flag antibody. Flag-tagged Twist-1 was visualized using SDS-PAGE.

possibility using immunoprecipitation assay in COS-7 cells. Increasing doses of Id1 decreased the amounts of E47 co-immunoprecipitated with Twist-1 (Fig. 6A). The amount of immunoprecipitated Twist-1 was also decreased by co-transfection of Id1, suggesting that Id1 induces Twist-1 degradation by sequestering E47 from Twist-1.

To examine whether Twist-1 also interferes with Id1-E47 heterodimer formation, we next performed the same experiment by replacing Id1 with Twist-1. In contrast to Id1, Twist-1 failed to interfere with Id1-E47 heterodimer formation (Fig. 6B). These results suggest that Id1 interacts with E47 more strongly than Twist-1 does, resulting in sequestration of E47 from Twist-1. In C3H10T1/2, endogenous Id1 was also co-immunoprecipitated with E47 using anti-E47 antibody (Fig. 6C). We examined the possibility that Id1 could rescue the inhibitory effect of Twist-1 on BMP signaling by inducing Twist-1 degradation. Twist-1-induced inhibition of BMP signaling was overcome by Id1 in a dose-dependent manner (Fig. 6D). It has been reported that differentiation of osteoblastic cells is promoted by transient expression of Id1 in early developmental stages (Peng et al., 2004). We attempted to rescue the inhibition of BMP signaling by Id1 gene transfer to MC3T3-E1-Tw1. As shown in Fig. 6E, the recovery of ALP activity in MC3T3-E1-Tw1 by Id1 gene transfer was significantly higher than by GFP gene transfer. These findings indicate that Id1 may regulate BMP signaling through a positive feedback loop that represses Twist-1 function.

Discussion

During embryonic mesoderm development, BMPs play critical roles in the commitment of mesenchymal cells into osteoblast and chondroblast lineages (Centrella et al., 1994; Hogan, 1996). Smads phosphorylated by BMP stimulation translocate into the nucleus and interact in transcription complexes with several DNA-binding transcription factors or cofactors that affect gene activation (Derynck and Zhang, 2003; Massague, 2000; Wrana, 2000). These factors have been considered critical for a variety of responses to BMP signaling in different BMP-targeted genes. Also, several inhibitors of BMP signaling have been reported. For example, Smad6 inhibits BMP/Smad1 signaling by acting as a selective Smad4 decoy (Hata et al., 1998). Tob, a member of the anti-proliferative protein family, binds Smad1, Smad5 and Smad8, and inhibits Smad-mediated transcriptional activities (Yoshida et al., 2000). Smads have also been shown to interact with bHLH transcription factors. For example, Smad3 directly interacts with MyoD and represses transcriptional activity (Liu et al., 2001). Neurogenin, another bHLH transcription factor, binds to both Smad1 and CBP (CREB-binding protein), and inhibits glial differentiation (Sun et al., 2001). The present study provides the first report that Twist-1, a DNA-binding bHLH transcription factor, inhibits transcriptional activities mediated by Smads (Fig. 3A,B,D).

Twist-1 reportedly acts as an inhibitor of muscle differentiation by sequestering E-protein from MyoD and blocking DNA binding, and by inhibiting trans-activation by MEF2 (Spicer et al., 1996). Twist-2 also requires heterodimerization with E-protein for inhibition of MyoD and MEF2 (Gong and Li, 2002), as seen with Twist-1. In addition, HDACs are involved in the repression of MyoD by Twist-1 and -2. In our results, Twist-1 also required heterodimerization with E47 for both maximal inhibition of Smad-mediated transcriptional activity (Fig. 3A,B,D) and increasing Twist-1 protein stability (Fig. 5B). BMPs activate transcription through physical interaction and functional cooperation of R-Smads and coactivators CBP and/or p300 (Derynck and Zhang, 2003). Our result (Fig. 4A,B) supports the possibility that the inhibition of BMP signaling by Twist-1 and E47 was mediated by direct recruitment of HDAC1 to Smad complexes via Smad4. The repression of HDAC by TSA increased the expression of osteogenic factors probably by the activation of BMP signaling. However, gel-shift assay of Smads revealed that Twist-1 failed in the inhibition of DNA binding of Smads (data not shown). This result was not contradictory to the involvement of HDAC in inhibitory mechanism by Twist-1.

We also showed that the effect of Twist-1 in repressing BMP signaling was abrogated by Id1 (Fig. 6D,E). Id1 expression is induced by BMP stimulation in mesenchymal and neuroepithelial cells (Katagiri et al., 1994; Nakashima et al., 2001; Ogata et al., 1993). Id1 lacks the basic region necessary for binding to the E-box and acts as a dominant negative regulator by sequestering E-protein (Benezra et al., 1990). Furthermore, Id1 sequesters E-proteins away from myogenin and inhibits myogenesis by accelerating myogenin degradation (Vinals and Ventura, 2004). In neural development, transient induction of Id1 by BMP2 decreases Mash1 stability and restricts neuronal differentiation by the same mechanism (Vinals et al., 2004). These findings support the possibility that Id1 may positively regulate BMP signaling

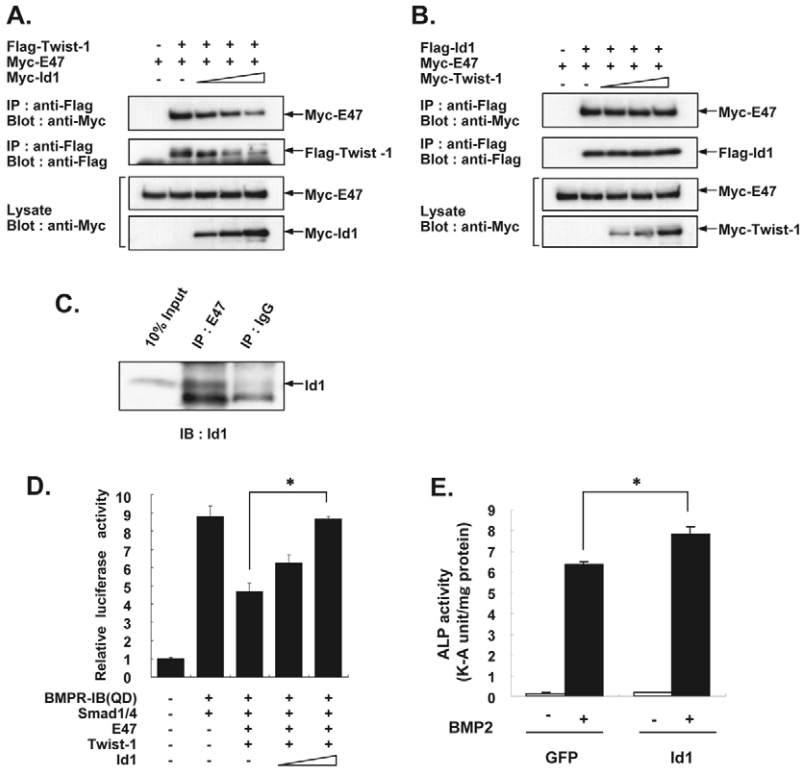


Fig. 6. Id1 inhibits Twist-1 function by sequestering E47 from Twist-1. (A) COS-7 cells were transiently transfected with Flag-Twist-1, Myc-tagged E47 and increasing doses (0.5, 1 or 2 μg) of Myc-tagged Id1 construct. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Flag and anti-Myc-antibody. (B) COS-7 cells were transiently transfected with Flag-Id1, Myc-tagged E47 and increasing doses (0.5, 1 or 2 μg) of Myc-tagged Twist-1 construct. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Flag and anti-Myc-antibody. (C) Endogenous E47 in C3H10T1/2 cells was precipitated using anti-E47 antibody. Then, Id1 was detected in the immunoprecipitates by western blot. (D) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1 and Smad4, 25 ng of Twist-1 and E47, and increasing doses (25 or 100 ng) of Id1 expression construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection (*P<0.01). (E) MC3T3-E1-Tw1 cells were transiently transfected with either Id1 or GFP expression construct by electroporation (Amaxa). The cells were grown in the presence or absence of rhBMP2 (300 ng/ml) for 6 days. ALP activity was measured as described in the Materials and Methods. Data are presented as mean ± s.d. of triplicate samples (*P<0.05).

by sequestering E-protein from Twist-1 to accelerate degradation. As shown in Fig. 6E, the recovery of ALP activity in MC3T3-E1-Tw1 by Id1 gene transfer was significantly higher than by GFP gene transfer, but the effect was not as much as expected from co-transfection experiment (Fig. 6D). We estimated that transient expression of Id1 was not sufficient to completely overcome the effect of stably expressing Twist-1, because transfection efficiency was not as high (at most 20%) in MC3T3-E1 cells.

In response to BMP stimulation, C3H10T1/2 embryonic mesenchymal cells express bone markers including collagen type I, ALP, osteopontin and osteocalcin (Ju et al., 2000). The osteopontin gene is reportedly a target of the BMP signaling pathway. Smad1 activates the osteopontin promoter by preventing Hoxc-8 (which negatively regulates osteopontin expression) from binding to this promoter (Shi et al., 1999; Yang et al., 2000). In addition, BMP stimulates direct binding of Smad proteins to the targeting sequence of the osteopontin promoter and activates transcription (Hullinger et al., 2001). In this study, we found that overexpression of Twist-1 repressed BMP2-induced expression of osteopontin and osteocalcin, and ALP activity (Fig. 1B,C). It is also known that Runx2 activates the expression of ALP, osteopontin and osteocalcin (Ducy et al., 1997; Harada et al., 1999). Furthermore, Twist-1 directly inhibits Runx2 (Bialek et al., 2004). From these reports, there is a possibility that the inhibition of BMP signaling in our experiments might result from an indirect effect mediated by the inhibition of Runx2. However, by direct binding with Runx2, Smads activate the transcription of Runx2 (Lee et al., 2000; Zhang et al., 2000). Moreover, BMP signaling was suppressed in co-transfection experiments using a reporter gene without the Runx2 recognition DNA sequence, as shown in Fig. 3A,B. Therefore, in addition to an indirect effect, through the inhibition of Runx2, it is likely that Twist-1 may have a direct inhibitory effect on BMP signaling.

We also showed that Smad-dependent transcriptional activity was enhanced by siRNA-mediated downregulation of endogenous Twist-1 in transient transfection analysis with a reporter construct containing BMP-responsive elements (Fig. 2B,C). Levels of Twist-1 expression gradually decrease during osteoblast differentiation (Bialek et al., 2004; Rice et al., 2000; Tamura and Noda, 1999). Taken together these results indicate that Twist-1 may maintain the population of undifferentiated mesenchymal cells by inhibiting BMP-induced osteoblast differentiation. Our data indicate a novel mechanism by which the cellular effects of BMP signals can be potentially regulated through direct competition between Twist-1 and Id1 for binding to E-protein.

Materials and Methods

Plasmid construction

Mouse Twist-1, E47, Id1 and Smad1 and Smad4 were amplified using polymerase chain reaction (PCR) from cDNA templates, which were reverse transcribed from mRNA of C3H10T1/2. To create mammalian expression vectors Myc-Twist-1, E47, Id1, Flag-Twist-1, Smad1 and Smad4, cDNA clones were introduced using Gateway technology (Invitrogen, San Diego, CA) into pCAGIP-Myc and pCAGIP-Flag vectors (Niwa et al., 2002). For Flag-Twist-1 deletion mutants, Twist-NBCT (deletion of amino acids 125-169) were created by PCR, then introduced into pCAGIP-Flag using Gateway technology. To generate mammalian expression vectors pCMV-Twist-1, pCMV-E47, pCMV-Id1 and pCMV-Smad1 and pCMV-Smad4, the corresponding cDNA clones were introduced with Gateway technology into pcDNA3.1 (Invitrogen), which was converted into the destination vector. A 3GC2-Lux luciferase construct, the constitutively active form of BMP type I receptor [BMPR-IB(QD)] (Imamura et al., 1997) and the constitutively active form of TGF-β type I receptor [TβR-I(TD)] (Wieser et al., 1995) were kindly donated by Kohei Miyazono (University of Tokyo).

Cell culture and stable transfection

The C3H10T1/2 murine mesenchymal progenitor cell line, MC3T3E-1 osteoblastic cell line and COS-7 African green monkey SV40-transformed kidney fibroblast

cells line were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. The P19 murine teratocarcinoma cell line was cultured in α-modified Eagle's medium supplemented with 10% FBS and antibiotics. Twist-1-overexpressing MC3T3-E1 (MC3T3-E1-Tw1) cells were obtained by puromycin selection of MC3T3-E1 cells transfected with pCAGIP-Flag-Twist-1. Screening of Twist-1-overexpressing clones was performed by western blotting of immunoprecipitates using anti-Flag antibody.

siRNA method

Target short interfering RNA (siRNA) was determined using the siRNA design tool (Invitrogen). The siTwist-604 sense sequence was 5′-AAGCUGAGCAAGAUUCAGACC-3′; siTwist-691 sense sequence was 5′-AAGAUGGCAAGCUGCAGCUAU-3′; siTwist-645 sense sequence was 5′-CAUCGACUUCCUGUACCAGGU-3′; and siTwist-481 sense sequence was 5′-CAGUCGUACGAGGAGCUGCAG-3′. As a control, the non-silencing siRNA sense sequence was 5′-AAGCGCGCUUUGUAGGAUUCG-3′. C3H10T1/2 cells were seeded at 70% confluence on the day before transfection. Transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen). To examine the effects of Twist-1-specific siRNA on reporter constructs, cells were transfected with 3GC2-Lux and pRL-TK vector (Promega, Madison, WI) using FuGENE6 transfection reagent (Roche, Basel, Switzerland) 24 hours after siRNA transfection. At 36 hours after siRNA transfection, cells were treated with rhBMP2 (300 ng/ml) for 12 hours. Both firefly and *Renilla* luciferase activities were measured 2 days after siRNA transfection using a dual luciferase assay system (Promega). Co-transfections of siRNA and plasmid DNAs were performed using X-treamGENE siRNA transfection reagent (Roche).

RNA extraction and northern blot analysis

Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the instructions of the manufacturer. Total RNA (15 μg) was denatured, electrophoresed in 2% agarose gels containing 18% formaldehyde, then transferred to Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ). Membranes were hybridized at 65°C for 12 hours in a hybridization buffer, PerfectHyb (Toyobo, Osaka Japan). Probes for Twist-1, osteocalcin, osteopontin and G3PDH were labeled using the RadPrime DNA labeling system (Invitrogen). After hybridization, membranes were washed four times with 2× standard sodium citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS). Blots were exposed to X-ray films using intensify screens at –80°C.

Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was assessed as previously described (Wakabayashi et al., 2002). Briefly, cell lysates were centrifuged and supernatants were used for enzyme assays. ALP activity was measured according to the methods of Kind-King, using a test kit (Wako, Osaka, Japan) with phenylphosphate as a substrate. Enzyme activity was expressed in King-Armstrong (K-A) units, normalized to protein concentration. Results are presented as mean ± standard deviation (s.d.) from a representative experiment. Statistical analysis was performed using analysis of variance (ANOVA).

Transfections and reporter assays

P19 cells were transiently transfected using 3GC2-Lux together with expression constructs of Smad1, Smad4, Twist-1, E47, Id1 and BMPR-IB(QD) using FuGENE6 transfection reagent. P19 cells were chosen because the cells responded to BMPs and expressed some of the BMP target genes. Additionally, transfection efficiency was higher in P19 cells than in other cell lines. At 24 hours after transfection, both firefly and *Renilla* luciferase activities were assayed with the dual luciferase assay system (Promega) using a Lumat LB 9507 luminometer (Berthold Technologies, Wildbad, Germany). Firefly luciferase activity was normalized with respect to *Renilla* luciferase activity. All assays were performed at least three times in duplicate or triplicate. Results are presented as mean ± s.d. from a representative experiment. Statistical analysis was performed using ANOVA.

Immunoprecipitation and immunoblotting

COS-7 cells were transiently transfected with the expression construct using FuGENE6 transfection reagent. COS-7 cells were used because they contained no endogenous Twist-1. At 24 hours after transfection, cells were lysed in buffer containing 25 mM Hepes pH 8.0, 150 mM KCl, 2 mM EDTA, 0.1% Nonidet P-40 (NP-40) and EDTA-free complete protease inhibitor cocktail (Roche). After 20 minutes on ice, cell lysates were pelleted by centrifugation and supernatants were pre-cleared with normal mouse IgG (Santa Cruz, Santa Cruz, CA) for 30 minutes at 4°C, then incubated with anti-FLAG M2 affinity gel (Sigma, St Louis, MO) for 4 hours at 4°C. Immunoprecipitates were washed four times with the buffer used for cell solubilization. Immune complexes were eluted at 98°C for 5 minutes in Laemmli's sample buffer. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to polyvilinidendifluoride (PVDF) membrane, and immunoblotted with anti-Flag M2 antibody (Sigma) and anti-Myc antibody (MBL, Nagoya Japan). Protein bands were visualized using Chemi-Lumi One (Nacalai Tesque, Kyoto, Japan).

To detect overexpressed Smad1 and Smad4, P19 cells were lysed as described above, 24 hours after transfection. Lysates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with anti-Smad1 and -Smad4 antibody (Santa Cruz) and anti-β-actin antibody (ABcam, Cambrige, UK). Protein bands were visualized using Chemi-Lumi One (Nacalai Tesque).

Nuclear protein extracts were prepared from MC3T3-E1 cells as follows. Cells were harvested by centrifugation at 500 ***g*** for 10 minutes at 4°C. Cell pellets were washed by gentle resuspension in cold PBS-0.5 mM EDTA and nuclei isolation buffer (NIB) containing 10 mM Tris-HCl (pH 7.5), 60 mM KCl, 15 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 M sucrose, 10% glycerol, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and EDTA-free complete protease inhibitor cocktail (Roche). Cells were re-suspended with ice-cold NIB containing 0.1% NP-40 and allowed to swell for 10 minutes on ice. Swollen cells were centrifuged at 500 ***g*** for 10 minutes at 4°C. Nuclei pellets were washed in cold NIB and centrifuged at 500 ***g*** for 5 minutes at 4°C. Nuclear pellets were diluted to 1.5 mg/ml DNA with ice-cold NIB and digested using micrococcal nuclease (80 units/mg DNA; Worthington, Lakewood, NJ). Digested nuclei were rapidly cooled on ice for 10 minutes and centrifuge at 12,800 ***g*** for 10 minutes at 4°C. Supernatant (S1) was collected and pellets were re-suspended with ice-cold cell lysis buffer containing 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 10% glycerol, 300 mM NaCl, 0.1 mM PMSF and EDTA-free complete protease inhibitor cocktail (Roche), then incubated for 45 minutes on ice. Nuclear debris was spun out by centrifugation at 12,800 ***g*** for 10 minutes at 4°C, and the supernatant (S2) was collected. S1 and S2 fractions were combined, then incubated with anti-Flag M2 affinity gel (Sigma) for 4 hours at 4°C. Immunoprecipitates were washed four times with cell lysis buffer containing 0.1% NP-40. Immune complexes were eluted at 98°C for 5 minutes in Laemmli's sample buffer. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using anti-Flag M2 antibody (Sigma).

To analyze the interaction of Id1 and E47, or Smad4, HDAC1 and Flag-Twist-1, C3H10T1/2 and MC3T3-E1-Tw1 cells were lysed with RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA and EDTA-free complete protease inhibitor cocktail (Roche), and the supernatant was obtained by centrifugation of the lysates at 12,800 ***g*** for 5 minutes at 4°C. After the removal of non-specifically bound substances using non-immune IgG (Santa Cruz), the supernatant was incubated with anti-E47 (Santa Cruz) antibody for 2 hours at 4°C and precipitated with protein A beads, or anti-Flag M2 affinity gel for 4 hours at 4°C. After washing the precipitates four times with the RIPA buffer, immune complexes were eluted at 98°C for 5 minutes in Laemmli's sample buffer. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted using anti-Id1, anti-Smad4 (Santa Cruz), anti-Smad1 (Zymed, San Francisco, CA), anti-HDAC1(Upstate Temecula, CA) antibodies.

Pulse-chase assay

Pulse-chase assay was performed according to the method previously described (Deed et al., 1996), with minor modification. COS-7 cells were transfected with Flag-Twist-1, Myc-E47 and Myc-Id1 using FuGENE6 transfection reagent. At 24 hours after transfection, cells were starved in cysteine and methionine-free DMEM (Invitrogen) containing with 5% dialyzed FBS for 1 hour, then incubated for an additional 2 hours in cysteine and methionine-free DMEM containing 10% dialyzed FBS and 50 μCi/ml of Promix (Amersham). Labeled cells were then incubated in standard DMEM supplemented with 10% FBS and harvested at various time points. Immunoprecipitation was performed as described above.

Real-time quantitative PCR

MC3T3-E1-Tw1 cells (2×10^5 cells) were treated with BMP (600 ng) alone or the mixture of BMP (600 ng) and trichostatin (TSA, 330 nM; Sigma). At 24 hours after the treatment, total RNA was extracted from cells using RNeasy kits (Qiagen, Hilden, Germany) and digested with DNase I according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed into cDNA using High Capacity cDNA Archive Kits (Applied Biosystems, Foster City, CA) and amplified by real-time quantitative PCR using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Mixtures of probes and primer pairs specific for murine ALP, Runx2, osteopontin and GAPDH were purchased from Applied Biosystems (Foster City, CA). The concentration of target genes was determined using the comparative CT method (threshold cycle number at the cross-point between amplification plot and threshold) and values were normalized to an internal GAPDH control. Results are presented as mean ± s.d. from a representative experiment.

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