Recent in vivo evidence suggests that Wnt signaling plays a central role in determining the fate of stem cells in the ectoderm and in the neural crest by modulating bone morphogenetic protein (BMP) levels, which, in turn, influence Msx gene expression. However, the molecular mechanism regulating the expression of the Msx genes as key regulators of cell fate has not been elucidated. Here we show in murine embryonic stem cells that BMP-dependent activation of Msx2 is mediated via the cooperative binding of Smad4 at two Smad binding elements and of lymphoid enhancing factor (Lef1) at two Lef1/TCF binding sites. Lef1 can synergize with Smad4 and Smad1 to activate Msx2 promoter, and this transcriptional complex is assembled on the endogenous promoter in response to BMP2. The Wnt/β-catenin signaling pathway can activate Msx2 via the binding of Lef1 to its promoter and synergizes with BMP2 to activate Msx2 expression, possibly via enhanced recruitment of the p300/cAMP-response element-binding protein-binding protein-co-factor. Interestingly, the Wnt/β-catenin-dependent activation of Msx2 was defective in Smad4-deficient embryonic stem cells or when Smad binding elements were mutated but persisted in the presence of various BMP antagonists, indicating that Smad4 was involved in transducing the Wnt/β-catenin signals in the absence of a BMP autocrine loop. A chromatin immunoprecipitation analysis revealed that endogenous Smad4, but not Smad1, was part of the Lef1 transcriptional complex in response to β-catenin activation, dismissing any implication of BMP signaling in this response. We propose that Wnt signaling pathway could dictate cell fate not only by modulating BMP levels but also by directly regulating cooperatively BMP-target genes.

The bone morphogenetic protein 2/4 (BMP2/4) signaling pathway is activated by the binding of the ligand to a family of type I and type II serine-threonine kinase receptors (1, 2). This activated receptor complex recruits and phosphorylates receptor Smads (Smad1, Smad5, or Smad8), which, in turn, associate with Smad4. This complex then translocates to the nucleus where it participates in transcriptional regulation. The N-terminal, Mad homology-1 domain of Smad4 binds DNA via a Smad binding element (SBE) identified as AGAC using a random pool of oligonucleotides (3) or as CAGAC based on the crystal structure of the Mad homology-1 domain bound to the SBE (4). The Smad complex can act either as a co-repressor or co-activator, depending on the co-factor with which it associates. For example, it can recruit the p300/CBP histone acetyltransferase (5–7) or the histone deacetylase HDAC-1 via its interaction with the homeodomain protein TGIF (8) to either induce or repress, respectively, transcriptional activity. The C-terminal Mad homology-2 domain of the Smad protein can transactivate transcription and form functional complexes with members of various classes of transcription factors (9, 10). Of interest, Smad4 was shown to functionally interact with the high mobility group member, lymphoid enhancing factor (Lef1), to regulate the expression of Xltn in Xenopus (11, 12). Lef1 is a transcriptional mediator of the Wnt signaling pathway activated by the binding of β-catenin (13). In unstimulated cells, β-catenin is constantly degraded by ubiquitination via phosphorylation by glucose synthase kinase-3β. Upon Wnt signaling, the constitutive kinase activity of glucose synthase kinase-3β is inhibited and allows the accumulation of β-catenin in the nucleus (14). β-Catenin binds and activates Lef1 by recruiting p300/CBP protein to the complex (15, 16).

Some of the genes known to be induced by BMP2/4 include the homeobox containing genes Msx-1 and -2 (17). In chick embryos, BMP2/4 is secreted from the neuroepithelium along the roof of the neural tube (18). Concomitant to the Bmp expression, Msx genes are expressed in the neural crest cells of the dorsal region of the neural tube to the hindbrain (17, 19). Recent studies in the avian system indicate that Msx2 expression inhibits chordogenic differentiation of the migratory cranial neural crest cells (20). The role of Msx genes in maintaining undifferentiated cells has also been well established in myoblasts (21, 22), and overexpression of Msx-1 in myotube cells triggers their de-differentiation into multipotent stem cells (23). Consistent with their role in modulating cell fate, BMP2/4 are potent neural inhibitors. In Xenopus, the default fate of the ectodermal cells is to acquire a neural phenotype. Secretion of BMP4 from the dorsal neural tube prevents neural determination and allow the cells to take on an epidermal fate (24). The Msx genes appear to mediate this response, because overexpression of Msx1 in the ectoderm of Xenopus embryos inhibits neural and promotes epidermal differentiation. Similar default mechanisms for neural differentiation is inhibited by BMP/Smad4 signaling in mouse embryonic stem (ES) cells (25). An additional role of the Msx genes is to mediate BMP-induced apoptosis. In the avian system, increased expression of
BMP2/4 in rhombomeres (r) 3 and 5 leads to increased Msx2 expression and to the apoptotic elimination of their neural crest cells (28). Consequently, misexpression of Msx2 in r2 and r4 leads to apoptosis of its neural crest cells, which would otherwise migrate and contribute to the formation of craniofacial structures (27). Taken together, these results indicate that graded expression of Msx2 can result in different cell fate, emphasizing the importance of tight regulatory mechanisms.

Although the Msx genes are sufficient to induce these biological responses, recent studies in avian embryos suggest that it is the Wnt signaling pathway that dictates these BMP-dependent responses. The Wnt-induced neural inhibition is achieved by interfering with fibroblast growth factor-induced inhibition of Bmp4 expression (28). The ectodermal cells close to the region expressing Wnt (lateral zone) take on an epidermal fate whereas the marginal ectoderm, with low Wnt expression, acquire a neural fate. Similarly, exogenous Wnt, but not BMP4, can provoke induction of neural crest cells from the neural plate (29), and increased levels of Wnt are required for the apoptotic elimination of neural crest cells in r3 and r5 (30). In this latter system, Wnt activity is enhanced by decreasing the levels of the Wnt antagonist, cSFRP2, in r3 and r5 relative to the others. Thus, it can be deduced from these studies that the levels of Wnt signaling determines cell fate, in part, by modulating BMP production, which, in turn, could influence the activation of Msx gene expression. However, the molecular mechanisms regulating the graded expression of Msx2 are unclear, because BMP-induced activation of Msx genes appears insufficient to induce most of these biological responses (29, 30).

In this study we show that BMP-induced activation of Msx2 is regulated by two canonical SBEs and two Lef1/TCF binding sites. The Smad4/1 co-activators functionally cooperate with the transcription factor Lef1 and together form a complex on the endogenous Msx2 promoter in response to BMP2. Activation of the Wnt pathway induces Msx2 expression via these same Lef1/TCF binding sites, and this response is partially dependent on the presence of Smad4 protein and its ability to form a complex with Lef1, indicating that Smad4 can act as a transcriptional co-activator for Wnt signaling. Thus, Smad4 integrates signals from the Wnt or the BMP pathways either by respectively interacting with the Lef/β-catenin complex or by recruiting Smad1 to the DNA-bound Lef1. We proposed that in the presence of both signals, the levels or affinity of p300/CBP is enhanced via its independent interaction with β-catenin or Smad1 (7, 16, 31) and 17) and facilitate the recruitment of transcriptional machinery to the Msx2 promoter. These findings reveal a molecular mechanism explaining how Wnt signaling can act as a determinant factor influencing cell fate by directly modulating the expression of BMP-target genes.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The wild-type ES cells and Smad4−/− ES cells (clone F9/−2A2) (32) were maintained on 0.1% gelatin-treated tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum (Hyclone), 0.1 μg/ml leucomy inhibitor factor, 1 mM sodium pyruvate, 2 mM L-glutamine, and β-mercaptoethanol (10−4 M). All transfections were performed using wild-type ES cells (unless otherwise specified). They were prepared in triplicates in 96-well dishes containing 5000 to 8000 cells seeded the previous day, and LipofectAMINE plus transfection reagent (Invitrogen) was used as described by the manufacturer. Equal amounts of DNA (total of 110 ng/well) were co-transfected for all experimental constructs except for Smad1-expressing construct, which was transfected at 1/3 of the total amount of DNA to reduce background activity. All transfections were normalized to Renilla Luciferase (pRL-CMV) expression vector (10 ng) or to protein content measured at A280. The normalized luciferase values were expressed relative to the specified reporter. BMP2 was added at 25 ng/ml the following day of transfection for 10 to 24 h depending on cell density of seeding. All transfections were performed in triplicates and repeated several times. Luciferase assays were carried out using the Dual-Luciferase Reporter Assay System (Promega).

Plasmid Construction—Msx2-lux was derived by cloning a 1.7-kb BamHI genomic fragment –3 kb upstream of the transcriptional start site of Msx2, into pGL3-promoter vector (Promega) (33). Mutations of the Smad and Lef/TCF binding elements were done by a multi-step PCR system using Msx2-lux as a template. The first PCR reaction generated Fragment A using a common forward primer, 5′-GGAGGG-GCCGCAATAAAAAA-3′, in combination with one of the following reverse primers: 5′-ACCTCAACGTGCGGCTAATUCCGAGG-3′, 5′-ATCAACGAGGGGCGGCTAAATGGGGCCAGCA-3′, 5′-CCTGTCACGGCCGCGGCA ACCGTCCCCGCGCTGACTAATTGAGG-3′, or 5′-ACCTCAACGTGCGGCTAATUCCGAGG-3′ containing mutations (lowercase) in potential Smad and Lef1/TCF consensus binding sites at −766, −600, −1210, and −1060, respectively. In the second PCR, a common reverse primer, 5′-AAAAATTACCGACAGTGCGGGGCCC-3′, was used in conjunction with the reverse complement of the above mentioned primers containing mutations in the SBEs and Lef1/TCF consensus sites to obtain Fragment B.

Reverse Transcription (RT)-PCR Analysis—ES cells were plated on 10-cm tissue culture dishes (BD Biociences) at 5 × 105 cells/dish for 24 h. Cells were then starved in serum-free medium for 3–4 h, and RNA was extracted at different time points after addition of BMP2 (25 ng/ml) and 1% fetal bovine serum. Extracted RNA was reverse-transcribed with the Advantage RT-PCR kit (Clontech) and amplified for 30 cycles at an annealing temperature of 58 °C using the following primers (sense/antisense): Msx2, 5′-GGAGCGGCTGGTTGCTGACTAATTGAG-3′ and 5′-GCGGAGCTCTGGTTGCTGACTAATTGAG-3′, to amplify 1.7 kb fragment with a terminal BamHI site (underlined) at the 3′ end. The amplified fragment was then subcloned in the MBal and BlII sites of pGL3 promoter and sequenced. The same approach was used to construct constructs with double mutations using the specified single mutation constructs as templates. cDNA of full-length Smad4 protein was cloned in BamHI sites of pGex-4T2 (Amersham Biosciences).

Total Cell Extracts and GST Fusion Proteins—Wild-type and Smad4−/− ES cells were plated on 15-cm tissue culture dishes (BD Biociences) at 2.5 × 106 cells/dish for 24 h. Cells were then starved in serum-free medium for 3–4 h, and RNA was extracted at different time points after addition of BMP2 (25 ng/ml) and 1% fetal bovine serum. Extracted RNA was reverse-transcribed with the Advantage RT-PCR kit (Clontech) and amplified for 30 cycles at an annealing temperature of 58 °C using the following primers (sense/antisense): Msx2, 5′-GGAGCGGCTGGTTGCTGACTAATTGAG-3′, or 5′-GCGGAGCTCTGGTTGCTGACTAATTGAG-3′, containing mutations (lowercase) in potential Smad and Lef1/TCF consensus binding sites at −766, −600, −1210, and −1060, respectively. In the second PCR, a common reverse primer, 5′-AAAAATTACCGACAGTGCGGGGCCC-3′, was used in conjunction with the reverse complement of the above mentioned primers containing mutations in the SBEs and Lef1/TCF consensus sites to obtain Fragment B.

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Fig. 1. BMP-induced expression of Msx2 is an immediate early response that functionally requires a bipartite SBE. A, endogenous Msx2 is induced in response to BMP2 in murine ES cells. RNA was extracted from ES cells at different time points after BMP2 (25 ng/ml) stimulation. RT-PCR reactions were performed with oligonucleotides specific for Msx2 and the housekeeping gene G6PDH. Maximal stimulation was observed after 1 h of treatment. B, BMP-induced expression of Msx2 does not require de novo protein synthesis. ES cells were treated with cyclohexamide (CHX) (10 μM) 10 min prior to BMP2 treatment, and RNA was extracted 1 and 4 h later. RT-PCR analysis for Msx2 revealed that its expression was not affected in the presence of the protein synthesis inhibitor. C, genomic organization of Msx2 locus and subcloning of a distant control region into the pGL3prom luciferase vector. This region contains two SBEs (−600 and −766) that were mutated alone or in combination as indicated by the underlined nucleotides. D, both SBEs contribute to the activation of Msx2' promoter by BMP2. Wild-type (WT) ES cells were transfected with reporter constructs containing wild-type or mutated SBEs as indicated and the next day incubated with increasing amounts of BMP2 (2.5, 5, 10, 25, and 50 ng/ml) for 12 h. The experiments were done in triplicate, and the values were normalized with a Renilla luciferase vector.

well for 24 h and then transfected with equal amounts of either an empty vector (pCDNA3.0) (Invitrogen) or Msx2-lux and AN89-β-catenin constructs to give a total of 1 μg of DNA/well. The next day, the cells were treated with BMP2 (25 ng/ml) for 10 h. The cells were then cross-linked with 1% formaldehyde for 25 min at room temperature, and glycerine (125 mM) was added for 10 min at room temperature to quench the formaldehyde. The cells were washed with cold phosphate-buffered saline and lysed in 1 ml of lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitors) for 30 min on ice. Nuclei were centrifuged (5000 rpm) for 10 min at 4 °C and resuspended in 0.4 ml of nuclear lysis buffer (50 mM Tris, pH 8.0, 1% SDS, protease inhibitors). 0.1 g of glass beads (32–300 μm; Sigma) was added, and samples were vortexed for 30 min at 4 °C and sonicated four times for 10 s using a Branson 450 Sonifier (output control 5 and 60% duty cycle). The average DNA fragments ranged between 300 and 1000 bp. The lysates were then centrifuged (13,000 rpm) for 10 min at 4 °C and diluted 5-fold in ChIP dilution buffer (15 mM Tris, pH 8.0, 1% Triton X-100, 0.01% SDS, 1 mM EDTA, 150 mM NaCl, protease inhibitors). The sonicated samples were precleared using Protein G-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C, and 1% of the lysate was used for Input control. Samples were incubated with 5 μg of anti-Lef1 (Santa Cruz Biotechnology), anti-Smad4 (Santa Cruz Biotechnology), or anti-Smad1 (Upstate Biotechnology) or no antibody at 4 °C for 1 h. Immunoprecipitation was carried out using Protein G-Sepharose beads for 1 h at 4 °C. Immune complexes were washed consecutively for 5 min with each of the following solutions: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl), high salt wash buffer (same as low salt wash solution but 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0) and twice with TE. Complexes were then eluted twice at 65 °C for 10 min in elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS). Immunoprecipitated DNA was reverse cross-linked at 65 °C overnight and purified using a PCR purification kit (Qiagen). 2 μl of purified DNA from cells transfected with the reporter construct were used for PCR amplification (30 cycles), and 15 μl of purified DNA from non-transfected cells was used and amplified for 55 cycles. All reactions were done under an annealing temperature of 63 °C. The set of primers for amplifying the Lef1/TCF binding sites (292 bp) are as follows: forward, 5′-GAAAAACGAGAAGGCACGACGACGTCG-3′; reverse, 5′-CAGGAGGATGGAAGGGGGAGGCAAGGG-3′; and for the SBEs (301 bp) they are as follows: forward, 5′-CCGGATCTCTCTTAACTTCCCTGTG-3′, reverse, 5′-TTTCCTTTAATCGGCGCTTGGTT-3′. A sonication control PCR (734 bp) was done using the forward Lef1/TCF primer and the reverse SBE primer.

RESULTS

Identification of SBE on the Msx2 Promoter—The ability of BMP2/4 to induce the expression of Msx genes has been established in several biological systems, including the avian and Xenopus embryos. To determine whether BMP2 can induce endogenous expression of Msx2 in murine ES cells, kinetic analyses were performed by RT-PCR. Total RNA was obtained from wild-type ES cells at various time points after BMP2 stimulation. As early as 15 min after the addition of BMP2, expression of Msx2 was induced and reached maximal activation after 4 h (Fig. 1A). This BMP response was preserved in the presence of the protein synthesis inhibitor, cyclohexamide (Fig. 1B), indicating that Msx2 activation was an immediate early response following BMP2 stimulation. Our previous study demonstrated that a potential enhancer element present in a 1.7-kb fragment of the Msx2 locus, 3 kb upstream of the initiation start site, required Smad4 and synergistically interacted with Smad1 for its activation (33). Sequence analysis of this region revealed two potential SBEs (GTCTG), at −600 and −766 (Fig. 1C). Interestingly, the homology between these two
sites extended to seven additional nucleotides (ATTTgtctgCCC) and was similar to functional SBEs found in the several other promoters (35–37), suggesting that they could contribute to the efficient binding of Smad proteins. To determine whether these sites played a functional role in the Smad4-dependent activation of Msx2, we performed a PCR-based site-directed mutagenesis of the −600 and −766 CAGA boxes either separately or in combination (Fig. 1C). When transolated into wild-type ES cells, inactivation of either the −600 or the −766 SBE reduced the BMP-dependent activation by 2-fold relative to the wild-type Msx2-lux reporter construct (Fig. 1D). The simultaneous inactivation of both SBEs resulted in a further 2-fold reduction, bringing the luciferase expression to basal levels. These results indicate that both SBEs were required for the transcriptional activation of Msx2. The activation of the wild-type reporter remained relatively elevated at low levels of BMP2 concentrations (0.25 ng/ml) and was not significantly enhanced over a 50-fold increase in BMP2 (Fig. 1D), suggesting that increasing BMP2 levels might not be sufficient to enhance Msx2 expression.

Smad4 Specifically Binds Both SBEs—To confirm Smad4 binding to the SBE, the EMSA was performed with 100 bp end-labeled oligonucleotide probes containing either one of the two SBEs (Fig. 2A). The band shift was initially performed with recombinant GST-Smad4 fusion protein. A DNA-protein complex was obtained with the radiolabeled Probe A containing the wild-type −600 SBE, but not with the probe containing nucleotide substitutions at the core sequence of the SBE (Fig. 2B, lanes 1–4), indicating that the SBE was responsible for the binding of Smad4. Binding of Smad4 to the radiolabeled wild-type probe was competed with as little as 30-fold molar excess of unlabeled wild-type probe but not with unlabeled probe mutated at the −600 SBE (Fig. 2B, lanes 5–8). These results indicated that Smad4 binding to this SBE was specific. Similar results were obtained for the −766 SBE, whereby the radiolabeled Probe B containing the wild-type SBE, but not the one containing nucleotide substitutions at the SBE, formed a DNA-protein complex with Smad4 protein (Fig. 2B, lanes 9–12). Moreover, competition assays with the unlabeled −766 SBE revealed that only the fragment containing the wild-type SBE, but not the one containing a mutated SBE, could prevent Smad4 protein from binding to the wild-type radiolabeled probe (Fig. 2B, lanes 13–16). These results indicate that Smad4 binds to both SBEs with similar efficiency.

To examine whether Smad4 was part of an endogenous transcriptional complex binding to the SBE, nuclear extracts from wild-type and Smad4-deficient ES cells, treated or not with BMP2, were subjected to EMSA. A specific DNA-protein complex was formed with Probe C, encompassing both SBEs, with cellular extract derived from wild-type but not Smad4-deficient ES cells (lane 19), demonstrating a BMP-specific response. Addition of α-Smad4 antibody resulted in a supershift of this complex (arrowhead), confirming that Smad4 is part of the complex (lanes 23 and 24). Lanes 17 and 22 represent the free probe.
transcription factors is crucial for transcriptional activation of transforming growth factor-β-related target genes (9). To identify transcription factors involved in regulating Msx2 expression in response to BMP2, we searched for potential binding sites of known transcription factors using the TRANSFAC data base (38). We found, among other sites, two Lef1/TCF binding sites 300 bp upstream of the SBEs that matched perfectly the consensus sequence WWCAAWGG (W = T or A). Based on previous studies demonstrating functional interaction between Lef1/TCF family members and Smads (11, 12, 39), we first examined whether inactivation of the Lef1/TCF binding site would interfere with BMP-induced activation of Msx2. To this effect, the core sequence of the binding site was mutated by nucleotide substitution changing the WACAAAGG sequence to WAtgAAGG. Interestingly, the BMP-dependent activation of Msx2-lux reporter construct was completely abrogated when the Lef1/TCF binding sites were mutated either alone or in combination (Fig. 3A). These results revealed that BMP-induced activation of Msx2 requires the binding of Lef1/TCF family members and suggested that the Smad complex functionally interacted with them. To confirm the functional cooperation between Smad and Lef1/TCF family members in the activation of Msx2, wild-type ES cells were co-transfected with Smad1, Smad4, and Lef1 either alone or in combination. The Msx2 promoter was slightly induced relative to non-transfected cells when co-transfected with Smad1, Smad4, or Lef1, with or without BMP2 (Fig. 3B). When Lef1 was co-transfected with Smad1 or Smad4, the BMP response increased by 2- to 3-fold, but when all three vectors were co-expressed, Msx2-lux was activated 10-fold in response to BMP2 above non-transfected cells.

Fig. 3. BMP2-induced activation of Msx2 is mediated by Lef1. A, Lef1/TCF binding sites are absolutely required for the BMP2-induced activation of Msx2. Sequence analysis of Msx2 promoter revealed two consensus Lef1/TCF binding sites (−1060 and −1210), which were mutated either alone or in combination within the Msx2-lux reporter vector. The various constructs were transfected in ES cells and stimulated with BMP2 for 12 h. The BMP-induced activation of Msx2 was reduced to basal levels when either of the Lef1/TCF binding site was mutated, suggesting that they were equally important for its activation. B, Lef1 synergistically cooperates with Smad1 and Smad4 in the activation of Msx2. ES cells were co-transfected with Smad1, Smad4, and Lef1 either alone or in combination, in the absence or presence of BMP2. Wild-type Msx2-lux reporter was induced 10-fold above non-transfected cells after BMP2 stimulation when all three expression vectors were co-transfected. C, BMP-induced activation of Msx2 via Lef-1 occurs independently of β-catenin. A mutated form of Lef1, lacking the β-catenin binding domain (LEF1Δ20), was used in the co-transfections and showed that it can still synergize with the Smad1/4 complex to induce Msx2 expression in response to BMP2. D, a ChIP analysis revealed that Lef1, Smad4, and Smad1 are recruited to the endogenous Msx2 promoter in response to BMP2 stimulation. A schematic representation of the Lef1/TCF binding sites and SBE of the endogenous Msx2 promoter is shown with the respective oligonucleotides used for the PCR (arrows). Immunoprecipitation (IP) with Lef1, Smad4, and Smad1 antibodies revealed that only nuclear extracts from BMP-treated wild-type ES cells generated an amplified product for the Lef1/TCF binding sites (left panel) and the SBEs (right panel), indicating that these transcriptional elements are brought together via the interaction of Lef1 with Smad4 and Smad1. The input control revealed that similar amounts of DNA was present in both untreated and BMP-stimulated extracts, and the no antibody (no Ab) control indicated that the amplification was specific.
cells (Fig. 3B). These results clearly demonstrated that a Smad1/4 complex could synergize with Lef1 to activate the BMP-induced expression of Msx2.

Because BMP has been reported to activate the transcription of Wnt genes in certain cells (40, 41), we wanted to rule out that the requirement for Lef1 in the BMP-induced activation of Msx2 was not mediated indirectly by the activation of the Wnt signaling pathway. To prevent LEF1 activation via Wnt/β-catenin, a deleted mutant of Lef1, LefΔ20, incapable of binding p300/CBP was co-transfected, no reduction in either the β-catenin- or BMP-induced activation of Msx2 was observed, confirming that E1A inhibition was mediated by sequestering p300/CBP.

Wnt and BMP Signaling Pathways Converge on Msx2 Promoter—The requirement for the Lef1/TCF binding site in the BMP-induced activation of Msx2 was added to the active recruitment of Lef1 to its promoter suggested that the Wnt signaling pathway could modulate Msx2 expression. To examine whether this activation was mediated by the same Lef1/TCF binding sites as those identified for the BMP response, the reporter constructs mutated at these sites were co-transfected with activated β-catenin. Mutation of the Lef1/TCF binding sites either alone or in combination completely inactivated the Msx2 promoter, suggesting that activation of the Wnt pathway can induce expression of Msx2. To confirm this activation was mediated by the same Lef1/TCF binding sites as those identified for the BMP response, the reporter constructs mutated at these sites were co-transfected with activated β-catenin. Mutation of the Lef1/TCF binding sites either alone or in combination completely inactivated the β-catenin response (Fig. 4B), indicating that binding of Lef1 to both these sites was required to induce cooperatively the transcription of the Msx2 gene in response to Wnt/β-catenin. These results suggested that Wnt and BMP signaling pathways could converge at the Lef1 transcription factor to induce Msx2 expression. When the Wnt signaling pathway was activated in combination with BMP2, the Msx2 promoter was synergistically activated, reaching levels as high as 15-fold above basal expression (Fig. 4A), confirming that these two pathways cooperate in the activation of Msx2 expression.
pate in the transcriptional activation of the target gene (7, 16, 31). To test whether p300/CBP was involved in the Wnt/β-catenin- and BMP/Smad4-induced activation of Msx2, the adeno-viral E1A protein was co-transfected with the wild-type reporter construct. The E1A oncoprotein binds p300/CBP and prevents its interaction with the transcriptional machinery and has been shown to successfully inhibit p300/CBP-dependent activation (42). Transfection of the E1A expression vector reduced both Wnt/β-catenin and BMP-induced activation of Msx2 to basal levels (Fig. 4C), suggesting that recruitment of the p300/CBP protein at the transcriptional complex by the respective co-activators is crucial for its activation. This inhibition by E1A is dependent on its ability to interact with p300/CBP, because deletion of its N-terminal region (Δ22–36 amino acids), responsible for the p300/CBP interaction (43, 44), fails to inhibit Msx2 activation in response to BMP2 or Wnt/β-catenin (Fig. 4C). Thus, the synergistic cooperation between Wnt and BMP signaling could occur via enhanced accumulation of p300/CBP on the Msx2 enhancer element.

**Smad4 Is Involved in Transducing the Wnt/β-Catenin Signal—**The requirement for a Lef1/Smad4 interaction in the BMP-induced activation of Msx2 would predict that similar associations are involved during Wnt signaling. To address whether binding of Smad4 to the Msx2 promoter region is required for β-catenin-induced activation, we examined the ability of the mutated SBE to interfere with the expression of the Msx2 reporter constructs. We found that the inactivation of either SBE, or both simultaneously, reduced by half the Wnt/β-catenin-dependent activation of Msx2 (Fig. 5A), revealing both a Smad4-dependent and independent pathway. The Smad4-independent pathway is consistent with the activation of Lef1 transcription factor in response to Wnt/β-catenin (14). However, the requirement for DNA-bound Smad4 in the Wnt/β-catenin-induced activation of Msx2 suggests that Smad4 was involved in the Wnt signaling pathway. This result prompted us to investigate Msx2 activation in Smad4-deficient ES cells in response to Wnt signaling. We found that β-catenin can partially induce Msx2 expression, and this induction was greatly enhanced when Smad4 was co-transfected back into the ES cells (Fig. 5B). Similarly, although BMP2 did not significantly induce Msx2 expression either alone or in the presence of activated β-catenin, the synergistic effect between the two pathways was restored with Smad4 co-transfection (Fig. 5B). These results confirm the cooperative interaction between Smad4 and β-catenin on LEF1 and also substantiate the requirement for Smad4 in the Wnt pathway for the activation of Msx2.

Several studies, however, have reported that Wnt signaling is involved in the transcriptional activation of BMP (28, 30, 40, 45). Thus, it is possible that the Smad4-dependent induction of Msx2 in response to activated β-catenin results indirectly from the activation of the BMP pathway. To address this issue, we examined the ability of β-catenin to activate Msx2 expression in the presence of the BMP antagonist noggin. When noggin was added in concentration sufficient to inhibit both the BMP-induced activation of Msx2 and the synergistic effect of BMP2 with activated β-catenin, it did not significantly reduce β-catenin-induced activation of Msx2 (Fig. 5C). Similarly, when a dominant-negative form of the type I BMP receptor (BMPRI-K261R) was co-transfected with the reporter construct, BMP-dependent activation of Msx2 was completely abolished without affecting the β-catenin-induced response (Fig. 5C). These results indicate that the BMP pathway is not indirectly involved in β-catenin-induced activation of Msx2 and imply that the requirement for Smad4 in this response is independent of BMP signaling.

To clearly demonstrate the role of Smad4 as a co-activator involved in transducing Wnt/β-catenin responses, we performed ChIP analysis on ES cells co-transfected with the activated β-catenin(ΔN89) and the Msx2 reporter plasmid. The immunoprecipitation of the protein-DNA complex with the anti-Lef1 antibody revealed that both the Lef1/TCF binding sites and the SBEs can be amplified more efficiently in response to β-catenin activation (Fig. 5D). These results indicated, as expected, that activated β-catenin promotes the binding of Lef1 to the Lef1/TCF binding sites but also confirmed that activation of the Wnt/β-catenin pathway can recruit Smads to the Lef1 complex. The role for Smad4 in the Wnt/β-catenin signaling pathway was further substantiated with enhanced amplification of both the Lef1/TCF binding sites and the SBEs in response to β-catenin relative to unstimulated controls after immunoprecipitation with Smad4-specific antibody. These results imply that the β-catenin-induced activation of Lef1 recruits Smad4-bound SBE to the complex. This recruitment is specific to Smad4, because immunoprecipitation using anti-Smad1 antibodies does not result in enhanced amplification of these sites in response to β-catenin. However, immunoprecipitation with anti-Smad1 antibodies results in a more efficient amplification of both the Lef1/TCF binding sites and the SBEs in response to BMP2 stimulation. These results indicate that Smad1 is part of the Lef1/Smad4 complex only when activated by BMP2 and substantiate the idea that BMP signaling is not activated indirectly by Wnt/β-catenin signaling pathway. In contrast, Smad4 can form a complex with Lef1 in response to either BMP or Wnt/β-catenin signaling pathway via the recruitment of either Smad1 or β-catenin co-activators. To confirm that the Lef1/TCF and Smad binding elements were on separate fragments brought in close proximity by the formation of a Smad/Lef1 complex, we performed a sonication control PCR encompassing both Lef1/TCF binding sites and SBEs. A 735-bp fragment was detected in the input samples, but no product was amplified after immunoprecipitation, demonstrating that the elements were immunoprecipitated separately via the formation of a Smad/Lef1 complex.

Taken together, these experiments reveal a molecular mechanism of how BMP and Wnt signaling pathways cooperate to regulate graded expression of Msx2, and this can be summarized in the following model. In the presence of BMP2, activated Smad1 retains Smad4 in the nucleus and forms a protein-DNA complex with the transcription factor Lef1 on the endogenous Msx2 promoter. Although Smad4 is not absolutely required for the Wnt-induced activation of Msx2, it can form a complex with Lef1 to enhance the Wnt/β-catenin signaling. This demonstrates for the first time the molecular mechanism of how Smad4 can transduce signals other than transforming growth factor-β-related signaling pathways. Thus, Smad4 can integrate signals from both the Wnt and the BMP pathways by cooperating either with the co-activator β-catenin or Smad1, both of which can bind the histone acetyltransferase p300/CBP. The enhanced expression of Msx2 could be achieved when both β-catenin and Smad1 co-activators are translocated to the nucleus to further stabilize Smad4 to Lef1 and enhance the binding affinity, or the levels, of p300/CBP to the complex.

**DISCUSSION**

**BMP2-induced Activation of Msx2 Requires the Binding of Smad4 to the Promoter—**We had shown previously (33) that Smad4 is required for BMP-induced activation of Msx2. However, it was not clear whether this effect was because of the direct binding of Smad4 to its promoter region or involved the synthesis of intermediate proteins. In this study we showed that the expression of endogenous Msx2 occurs as early as 15 min after BMP2 stimulation, and this occurred even in the presence of a protein synthesis inhibitor, indicating that it is an
immediate early response. These results were similar to those described in a previous study (46) using murine ES cells grown in defined culture conditions and, more recently (47), using human embryonic carcinoma cells. We have now extended these studies by defining the molecular mechanism for Msx2 regulation by BMP. Smad4 can bind to the only two SBE consensus (CAGAC) found within the 1.7-kb enhancer element of the Msx2 promoter shown previously (33) to be responsive to BMP signaling. The identity between these two SBEs extended to seven additional nucleotides beyond the core (GGGCAGA-CAAT), supporting the notion that surrounding nucleotides could play a functional regulatory role. The inactivation of either one of the two SBEs reduced BMP2-dependent activation of Msx2, whereas the inactivation of both completely abrogated the response, indicating that both SBEs contribute to the regulation of Msx2 and that a bipartite Smad complex is required for its full activation. This finding is in agreement with the regulation of other Smad-dependent genes (48, 49).

**Fig. 5.** Smad4-dependent and -independent activation of Msx2 in response to Wnt signaling. A, activation of Msx2 by the Wnt canonical pathway requires functional SBEs. Wild-type ES cells were co-transfected with the reporter constructs containing mutated SBEs and the ΔN89-β-catenin expression vector. The luciferase expression was reduced by half when either one of the two or both SBEs were mutated, revealing a partial dependence for Smad4. B, Smad4 protein is required for the complete β-catenin-induced activation of Msx2. Smad4-deficient ES cells (clone F9–2A2) were transfected with wild-type Msx2-lux reporter construct and stimulated to activate the BMP and Wnt signaling pathway as indicated. The Wnt/β-catenin signaling pathway induced Msx2 expression by 3-fold whereas BMP2 had no effect either alone or in combination with activated β-catenin, revealing the Smad4-independent response. Co-transfection of Smad4 increased β-catenin-induced activation of Msx2-lux by a further 2.5-fold and restored the ability of BMP signaling to synergize with β-catenin, suggesting that Smad4 is central to the cooperation between the two pathways. C, the role of Smad4 in the β-catenin-induced activation of Msx2 is independent of BMP signaling. Wild-type ES cells were co-transfected with Msx2-lux reporter construct and ΔN89-β-catenin where indicated. The addition of norgin (2 μg/ml) or co-transfection with a dominant-negative form of the type I BMP receptor (DN-BMPRI) abolished the BMP-dependent response but not the β-catenin-dependent activation of Msx2, implying that β-catenin interacts with Smad4 in the absence of BMP signaling. D, ChIP analysis indicated that Smad4 but not Smad1 is assembled on the Msx2 promoter with Lef1 in response to β-catenin activation. Nuclear extracts from wild-type ES cells transfected with Msx2-lux reporter construct and either co-transfected with ΔN89-β-catenin or treated with BMP2 were subjected to immunoprecipitation (IP) with α-Lef1, α-Smad4, and α-Smad1 antibodies. PCR analysis revealed that the Lef1/TCF binding sites (left panel) and the SBEs (middle panel) were part of the Lef1 and Smad4 immuno-complex in response to β-catenin or BMP2. These results confirm the recruitment of DNA-bound Smad4 to the Lef1 DNA complex in response to β-catenin activation. However, in the Smad1 immuno-complex both of these binding sites were absent in response to β-catenin but were present in BMP-stimulated extracts, indicating that β-catenin activation does not activate the BMP-dependent recruitment of Smad1. The sonication control PCR (right panel), which encompasses both the Lef1/TCF binding sites and SBEs, did not amplify any DNA from the immunoprecipitations, confirming that the Lef1/TCF and Smad binding sites were on separate DNA fragments.
Smad4 directly mediates the BMP-induced activation of the Msx2 promoter.

**LEF1 Constitutes an Integral Part of the BMP-induced Transcriptional Complex**—Effective activation of gene expression by Smad proteins requires cooperative interaction with a transcription factor (9). We have identified two consensus Lef1/TCF binding sites, 300 bp upstream from the SBEs, that completely abrogate BMP-induced activation of Msx2 when mutated either alone or in combination. Moreover, Lef1 can cooperate with Smad4 and Smad1 to synergistically enhance Msx2 expression in response to BMP2. Significantly, ChIP analysis revealed that Lef1 binds the Lef1/TCF binding sites of the endogenous Msx2 promoter and forms a complex with DNA-bound Smad4/Smad1 in response to BMP2. These results indicate that Lef1 constitutes an integral part of the transcriptional complex regulating Msx2 expression in response to BMP signaling. These findings are consistent with recent studies (11, 12) demonstrating that Smad4 physically interacts with Lef1 and cooperates in the activation of the homeobox gene Xtnn. However, undifferentiated ES cells express several Wnt ligands, Frizzle receptors, and Lef1/TCF family members (50, 51), raising the possibility that β-catenin could be indirectly stabilized in the Smad4/Lef1 complex by autocrine mechanisms. However, several lines of evidence argue against this possibility. For instance, the Wnt1 and Wnt3a ligands capable of activating the β-catenin canonical pathway, and that are potential candidates in cooperating with BMP-dependent developmental processes (30, 52), are not transcriptionally induced in ES cells in response to BMP2 (data not shown). Moreover, co-transfection of a Lef1 mutant, incapable of binding β-catenin (12), can cooperate as effectively as wild-type Lef1 with the Smad4/1 complex in the BMP-induced activation of Msx2 (Fig. 3C). Thus, we have concluded from these experiments that Lef1 could be activated by the BMP/Smad signaling pathway independent from the Wnt/β-catenin pathway. This would be consistent with a previous study (12) showing that the same Lef1 mutant incapable of binding β-catenin can still activate, albeit at much lower efficiency, the expression of Xtnn in response to transforming growth factor-β.

**Role of Smad4 in Mediating Wnt-induced Activation of Msx2**—Previous studies have shown that the Wnt signaling pathway can induce Msx2 expression in mouse EC cells and synergize with the BMP signaling pathway for its activation (47). We have extended these studies by delineating the molecular mechanism by which this cooperative activation is achieved in mouse ES cells. We found that Wnt/β-catenin signaling can induce Msx2 expression via the same Lef1/TCF binding sites required for BMP-induced activation. The inactivation of either of the two Lef1/TCF binding site completely abolished the BMP- and the Wnt/β-catenin-induced activation of Msx2, confirming that both pathways converge on a Lef1/TCF family member. Thus, during embryogenesis, areas where Wnt levels are high, and BMP levels are low or absent, Msx2 can still be expressed. This is consistent with the recent observation that Wnt, but not BMP protein, displays the proper distribution as an ectodermal inducer of neural crest cells in chick embryos (29) and with the discordant spatial and temporal expression of Bmp4 and Msx2 during early development of the neural tube in mouse (53).

Surprisingly, however, the inactivation of the SBEs reduced by half the β-catenin-induced activation of Msx2 and reintroduction of Smad4 in Smad4-deficient ES cells doubled its response to β-catenin activation. These results revealed that Msx2 could be activated by Wnt/β-catenin pathway via both a Smad4-dependent and -independent mechanism. The Smad4-independent response is consistent with the well established functional interaction between β-catenin and Lef1/TCF family members in transcriptional activation (14) and expands the possibility to generate graded expression of Msx2. In contrast, the Smad4-dependent response suggests that Smad4 was involved in transducing part of the Wnt signal and poses an interesting mechanism of cross-talk between the two pathways whereby Smad4 would be the central determinant between BMP and Wnt signaling for some genes. A role for Smad4 in the Wnt signaling pathway has been demonstrated previously (11) for the Xtnn gene using a dominant-negative Smad4 mutant and substantiated by the in vivo interaction of Smad4 with β-catenin in various Wnt-stimulated cell lines. We have extended this result by demonstrating that this effect is not because of indirect activation of the BMP pathway, because the addition of noggin or the co-transfection of dominant-negative type I BMP receptor does not interfere with the Wnt-induced activation of Msx2 (Fig. 5C). Furthermore, the role of Smad4 in Wnt signaling was confirmed using ChIP analysis, which revealed that endogenous Smad4, but not Smad1, was part of the Lef1 transcriptional complex in response to β-catenin activation (Fig. 5D). Smad4 shuttles autonomously between the nucleus and the cytoplasm via its nuclear export and import signals (54, 55). In response to Wnt or BMP stimulation, their respective co-activators, β-catenin and Smad1, could retain independently Smad4 in the nucleus and favors its association with the transcription factor Lef1. The association of Smad4 to the R-Smads has been extensively documented (1, 56), whereas its association with β-catenin seems indirect, because they do not form a complex in vitro (11) (data not shown).

**Role of Wnt and BMP in the Graded Expression of Msx2**—Recent studies in chick embryos revealed that Wnt signaling could dictate biological fates in which BMP signaling is required but not sufficient, such as neural inhibition of the ectoderm, and neural crest induction and apoptosis (28–30). Wnt exerts its biological effect by increasing BMP expression via the modulation of other signaling pathways, such as fibroblast growth factor 2 for neural inhibition or unknown factors for neural crest cell apoptosis (28, 30). During the latter biological event, it was thought that the increase in BMP signaling resulted in enhanced expression of Msx2 leading to the apoptotic elimination of the neural crest cells. Similarly, because a role for Msx genes has been demonstrated in neural commitment of Xenopus (57), BMP-mediated dosage of Msx genes could determine neural inhibition (58). However, we have not observed a dose-dependent activation of Msx2 in response to increasing BMP2 in ES cells (Fig. 1D). Rather, we have found that the Wnt/β-catenin signaling pathway can directly activate Msx2 expression and cooperate with BMP2 in its activation. Thus, the graded expression of the dual target gene appears to be dictated by the spatial distribution of the Wnt ligand and its antagonists. For example, in the chick ectoderm, only cells that express the Wnt ligand, which alleviates the fibroblast growth factor-induced inhibition of BMP, takes on an epidermal fate, whereas the others undergo a neural fate (28). In the neural crest cells of r3 and r5, a decrease in the Wnt antagonist cSFRP2 increases Wnt activity (30) and could synergize with BMP to elevate Msx2 expression leading to their apoptotic elimination. Alternatively, enhanced BMP activity at the rostral end of the neural tube, achieved by a caudorostral decrease in noggin expression, induces migration of the rostral neural crest cells (59). Wnt signaling appears to be involved in this process, because combined Wnt1 and Wnt3a mutant results in a reduction of some neural crest cell derivatives (60). Interestingly, the neural crest cells undergo epithelial-to-mesenchymal transition as they are released from the neuroepithelium, and Msx2 expression is important to maintain these migratory
neural crest cells undifferentiated (20). Thus, it is possible that
congruence of the BMP and Wnt signaling pathways is also
required to enhance Msx2 expression to maintain neural crest
cells undifferentiated.

Wnts and BMPs are expressed in many overlapping tissues
and being morphogens, it is consistent with the idea that
formation of a gradient of ligand would result in graded expres-
sion of common target genes. It is becoming clear that gene
expression is dependent on competition over limited amount of
promiscuous co-activator such as p300/CBP (61, 62). We found
that p300/CBP is required for both the BMP- and Wnt-induced
activation of Msx2 (Fig. 4C), suggesting that the pathways
converge to increase the affinity of the level of p300/CBP to the
Lef1 transcriptional complex. This would sequester p300/CBP
from other transcriptional complexes and assure the recruit-
ment of the general transcriptional machinery to the Msx2
promoter (61, 63, 64). When the ligand concentration is lower,
the affinity or the levels of p300/CBP at the Msx2 promoter
would decrease and would become more available for other
transcriptional sites with stronger signals.

This dual regulation by Wnt and BMP appears to be frequent
in mammalian development. The homeobox gene, Emx2, has
been shown to require both Smad and lef1/TCF binding sites
for synergistic expression in the telencephalon (39). Similar to
our results with Msx2, mutating the lef1/TCF binding site has
a greater inhibitory effect on Emx2 expression than inactivat-
ing the SBE, supporting the notion that Lef1 is central to this
dual response and that Smads and β-catenin are co-activators
for the same transcription factor. Although the DNA binding
sites have not been identified, Wnt and BMP also synergisti-
cally activate the expression of Id2 and Msx1 genes (47), both
known targets for the BMP signaling pathway (46, 65). Moreover,
the expression of yet another homeobox gene, Dlx3, in the
forebrain can be activated by either Wnt or BMP signaling
pathways (66, 67). Although their synergistic activity has not
been demonstrated yet, the promoter analysis of Dlx3 will
likely uncover a similar mechanism of regulation by Smad4 and
β-catenin on Lef1/TCF family members.

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