Id Genes Are Direct Targets of Bone Morphogenetic Protein Induction in Embryonic Stem Cells*

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Bone morphogenetic proteins (BMPs) are morphogenetic signaling molecules essential for embryonic patterning. To obtain molecular insight into the influence of BMPs on morphogenesis, we generated new genes directly activated by BMP signaling. In vitro cultured mouse embryonic stem (ES) cells were used, cultivated in chemically defined growth medium (CDM). CDM-cultured ES cells responded very selectively to stimulation by various mesoderm inducers (BMP2/4, activin A, and basic fibroblast growth factor). BMP2/4 rapidly induced transcript levels of the homeobox genes Msx-1 and Msx-2 and the proto-oncogene JunB, whereas c-jun transcripts displayed delayed albeit prolonged increase. Using differential display and differential display of cDNA cloning, six direct BMP target genes were identified. These include Id3, which showed strong mRNA induction, and the moderately induced Cyr61, DEK, and eIF4AII genes, as well as a gene encoding a GC-binding protein. Besides Id3, also the Id1 and Id2 genes were activated by BMP4 in both ES cells and a range of different cell lines. Id genes encode negative regulators of basic helix-loop-helix transcription factors. In vivo we observed local ectopic expression of Id3 and Msx-2 mRNAs in Fli/+ embryos at overlapping regions of ectopic BMP4 misexpression. We therefore propose that the Max and Id genes are direct target genes of embryonic BMP4 signaling in vivo.

One important stage in the development of the vertebrate embryo is reached when the three germ layers are formed and the body plan gets established during the process of gastrulation (1, 2). Our understanding of the molecular mechanism mediating gastrulation is still insufficient, although over the past years many secreted growth factor-like molecules have been identified to play important roles in these early embryonic events (3). Among these signaling molecules are several members of the BMP1 family. BMP-related growth factors belong to the TGF-β-superfamily and have been identified in a wide variety of organisms, ranging from insects to mammals (4). BMPs have originally been isolated for their ability to induce ectopic bone formation when injected under the skin or into the muscle of rodents (5, 6), but meanwhile many of the BMPs have been implicated in a variety of other developmental interactions, including early embryonic inductive events. For example in Drosophila embryonic dorsal-ventral patterning is partly accomplished through the action of DPP (decapentaplegic) (7, 8), which is the BMP member most closely related to mammalian BMP2 and BMP4. At the functional level these factors can substitute for one another in vertebrate and Drosophila embryos. Human BMP4 is able to rescue the dorsal-ventral pattern defects of dpp null mutants (9), whereas Drosophila DPP protein can induce ectopic bone in mice (10). The striking evolutionary conservatism of BMP2, BMP4, and DPP suggested that these molecules play crucial roles in early vertebrate development, as is confirmed meanwhile by many studies. In Xenopus laevis BMP4 ventralizes early mesoderm (11–13) and promotes the differentiation of epidermis from ectoderm (14). Blocking BMP2/4 receptor activity in the ventral part of the embryo eliminates blood formation and dorsalizes the mesoderm (15, 16), whereas in contrast overexpression of BMP4 RNA leads to an increased expression of ventral genes and inhibits the formation of anterior structures (11, 12, 17). Use of a dominant-negative BMP2/4 receptor to block BMP signals in the ectoderm (18), or disaggregation of ectodermal cells (19), causes differentiation into neural tissues. Mutagenesis by homologous recombination of the genes encoding BMP4 (20), BMP2 (21), and the BMP2/4-RI receptor (22) have demonstrated the requirements for BMP2 and BMP4 during early mouse development. Embryos homozygous for the Bmp4 null mutation do not proceed beyond the egg cylinder stage and show little or no mesodermal differentiation (20), whereas mice deficient for BMP2 have defects in amnion/chorion and cardiac development (21). Mice lacking the BMP2/4 type I receptor die prior to gastrulation and fail to form mesoderm altogether (22). BMPs, like other members of the TGF-β superfamily, signal through heteromeric complexes of type I and type II transmembrane Ser/Thr kinase receptors (23, 24). Binding of BMP dimers to either type of receptor triggers well defined signaling steps (25, 26) that finally lead to the activation of crucial target genes. However, relatively little is known about direct target

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1 The abbreviations used are: BMP(s), bone morphogenetic protein(s); CDM, chemically defined medium; FCS, fetal calf serum; ES cells, embryonic stem cells; CHX, cycloheximide; TGF, transforming growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; dd, differential display; FCS, fetal calf serum; bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline.

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genes of BMP signaling during early mammalian cell fate determination. Therefore, the identification of BMP-induced immediate early genes appeared of great interest. Toward this aim we analyzed BMP-mediated gene activation in murine ES cells. The in vitro differentiation process of ES cells can serve as a model for early mammalian development, permitting the expression of a variety of embryonic markers to be monitored (27, 28). When ES cells are cultivated in a chemically defined medium (CDM), they exhibit specific responses to defined growth factors. These cellular responses are influenced by the nature as well as the concentration of the factors. For example, BMP4 treatment induces expression of ventral mesoderm markers (29), and, at higher BMP concentrations, hematopoietic cell development is induced (30). We used this system to investigate BMP2 and BMP4 effects on the expression of early embryonic marker genes by using the differential display (dd) RT-PCR technique (31). We report the identification of several transcription units directly regulated by BMPs, including members of the Id gene family.

EXPERIMENTAL PROCEDURES

Cells—Cells used in this study were CCE (29, 32), E14.1 (33), C3H/10T1/2 (ATCC, CCL-228), C2C12 (ATCC, CRL-1772), PC-12 (ATCC, CRL-17211), Saos-2 (ATCC, HTB-85), U-2 OS (ATCC, HTB-96), 3T3-L1 (ATCC, CCL-92), Raji (ATCC, CCL-86), MOLT-4 (ATCC, CRL-1582), HeLa (ATCC, CCL-2), HL-60 (ATCC, CCL-240), and RK1 (ATCC, CCL-37).

ES Cell Culture and Stimulation Conditions—The used ES cell line CCE (129/Sv/Ev-derived) was already adapted to grow in the absence of feeder cells. The cells were routinely grown on gelatinized flasks in Dulbecco's modified Eagle's medium supplemented with 1000 units/ml leukemia inhibitory factor, 15% heat-inactivated fetal calf serum, 150 μM α-monothioglycerol, 0.2 mM L-glutamine, and antibiotics (50 μunits/ml penicillin, 50 μg/ml streptomycin). The medium was changed every day, and passaging was required every 2nd to 3rd day. The ES cell line E14.1 (129/Ola-derived) had been adapted to grow in the absence of feeder cells (27, 34) and was maintained under the same conditions as the CCE cells.

For stimulation studies, ES cells were trypanosized, washed free of leukemia inhibitory factor and FCS, and seeded in 10 ml of CDM medium at a density of 5 × 10⁶ cells per 9-cm diameter gelatinized tissue culture dish. CDM was prepared as described (29). The medium was changed every 2nd day. If not stated otherwise, cells were stimulated on day 5 of cultivation for the indicated times using BMP2 (5 ng/ml), BMP4 (5 ng/ml), activin A (20 ng/ml), bFGF (20 ng/ml), FCS (10%), or 12-O-tetradecanoylphorbol-13-acetate (1 μM). Cycloheximide (100 μg/ml) treatment started 30 min and actinomycin D (5 μg/ml) treatment 15 min prior to the addition of the inducers. We used CHX at concentrations that are known to inhibit completely cellular protein synthesis of embryonal cells (35, 36).

Cloned cDNA fragments were cloned into the pGEM®-T vector using the TA cloning system (Promega). Cloned cDNAs were sequenced with the Cyclist™ Taq DNA Sequencing Kit (Stratagene) using either T7 or Sp6 primers. DNA data bank searches were performed using the NCBI nr database.

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...an intensifying screen. To estimate RNA loading variations further, all filters were subsequently hybridized with a glyoxaldehyde phosphate dehydrogenase (Gapdh) cDNA probe (37). Where appropriate, quantification was performed by densitometry using the MacBas software package (Fujifilm).

Differential Display (dd) RT-PCR—The RNA Image™ Kit protocol (GenHunter Corp., Nashville, TN) was largely followed. Total RNA from CCE cells, mock-treated with either vehicle (1% bovine serum albumin in PBS) or treated with BMP-2 (or BMP-4) in the presence of cycloheximide, was purified by guanidinium thiocyanate/cesium chloride step gradients (38). After treatment with RNA-free Dnase to remove RNA contamination, aliquots of each RNA were used as templates for three different first strand cDNA synthesis reactions using three different one-base anchored oligo(dT) primers, respectively (T₉, T₁₀, and T₁₁). Aliquots of these cDNAs served as templates for subsequent PCR amplifications using a combination of an arbitrary 15-mer oligonucleotide as 5’-primer and a 5’-primer identical to the one-base anchored T₁₀ oligonucleotide used in the cDNA synthesis reaction. All primers used contained additional HindIII restriction sites (5’-AAGCTT-3’) at the 5’-ends to facilitate manipulation of the amplified cDNAs after cloning. PCR was performed as follows: 94 °C, 30 s; 40 °C, 2 min; 72 °C, 30 s; for 40 cycles, with a final extension phase at 72 °C for 5 min. The PCR fragments were labeled by amplification in the presence of [γ-³²P]ATP, and aliquots of the samples were resolved on a 15% polyacrylamide sequencing gel. Aliquots of gels were autoradiographed and analyzed as described for the differential display bands. Fragments of interest were excised from the dried gel, eluted by soaking the gel slice in 100 μl of dH₂O for 10 min, followed by boiling for 15 min. The DNA was precipitated using glycyogen, sodium acetate, and ethanol, and the precipitate was dissolved in a small volume of dH₂O. A portion of this was used for the reamplification employing the same 5’- and 3’-primers used in the first PCR. The reamplified fragments were analyzed in 1.5% agarose gels, excised, purified by the Jetset extraction kit (Genomed GmbH) to remove dNTPs and protein, and aliquots were used as probes for Northern blot analysis.

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-20 °C. After reprocessing through 50% methanol, the embryos were bleached in 3% H2O2/PBS, washed 3 times for 5 min in PBS, and stored in hybridization mix until further usage. For further use, the embryos were washed once for 5 min in PBS and were hybridized at 70 °C overnight with DIG-labeled riboprobes for Bmp4, Msx-2, and Id3 in 50% formamide, 5× SSC, 1% SDS, 1% Tween 20, 50 μg/ml heparin, and 50 μg/ml Escherichia coli tRNA. Three posthybridizations were carried out in 50% formamide, 2× SSC, and 1% Tween 20 at the hybridization temperature. Embryos were then prepared for antibody incubation by processing once through PBS with 1% Tween 20 (PBST) followed by a 1-h incubation in 10% sheep serum in PBST. Antibody incubation with an anti-DIG antibody (Roche Molecular Biochemicals) was performed in 1% sheep serum in PBST (1:2000) at 4 °C overnight. After washing in PBST for at least 6 h the embryos were incubated twice (20 min each) in detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl2, 1% Tween 20) and stained in detection buffer containing 20 μl nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate per ml stock solution (Roche Molecular Biochemicals).

RESULTS

BMPs and bFGF Elicit Specific and Distinct Transcriptional Responses in ES Cells Cultured in Chemically Defined Growth Media—To study patterns of gene expression induced in embryonic stem cells by stimulation with mesoderm inducing factors (BMP2, BMP4, activin A, and bFGF), ES cells (CCE) were cultivated as monolayers on gelatinized plates in the absence of feeder cells. To prevent undesired effects exerted by ill-defined components present in serum-containing growth media, we cultivated the ES cells in chemically defined medium (CDM) (29). Such cultured cells responded very sensitively and specifically to the application of different individual stimuli, as judged by transcript levels of the marker genes Msx-1, Msx-2, c-fos, and Egr-1 (Fig. 1A). Application of BMP4 led to a rapid and lasting induction of the homeobox genes Msx-1 and Msx-2, while hardly affecting c-fos and Egr-1 expression (Fig. 1A, compare lanes 1–5 with lane 11). An identical response was elicited upon induction with BMP2 (data not shown). Activin A treatment led to a transcriptional response very similar to the one induced by BMPs, except that Msx gene induction was somewhat weaker and less long-lived (not shown). In contrast, treatment with either BMP4 (5 ng/ml) (lanes 1–5) or bFGF (20 ng/ml) (lanes 6–10), harvested at the indicated time points after induction, and 10 μg of extracted total RNA was applied to Northern analyses. Gapdh rehybridizations are shown as RNA loading controls. B, expression in CCE cells of Jun genes. For control, mock treatments were for 24 h (lanes 1–6) or for 2 h (lanes 22 and 23), and BMP4 treatments (10 μg/ml) were given for the indicated times (lanes 7–21) or for 2 h (lanes 24 and 25). Additional pretreatment with cycloheximide (CHX) (30 min) was given to some cells (lanes 23 and 25). Northern blotting was performed using probes for c Jun (lanes 1–16 and 22–25) and JunB (lanes 17–21 and 22–25).

This analysis demonstrates, in agreement with Johansson and Wiles (29), that ES cells, when cultured in chemically defined medium, are able to activate distinct gene expression programs in response to specific extracellular stimuli. The Msx-1 and Msx-2 Genes Exhibit Immediate Early-type Responses to BMP4 in ES Cells—Previous studies demonstrated that BMP4 can lead to up-regulation of Msx gene expression in distinct embryonic domains, including dental mesenchyme (46), hindbrain (47), neural tube (48), limb bud (49), paraxial ectomesoderm, and facial tissues (50). It was unclear whether the Msx genes were directly activated by BMPs or indirectly. Therefore, we used our ES cell system to examine whether induction of the Msx-1 and Msx-2 genes represented a direct and possibly immediate early-type response to BMP4. CCE cells were incubated for 2 h with BMP4 in the presence or absence of cycloheximide.

In Fig. 2 we first show that induction by BMP4 of both Msx-1 and Msx-2 transcripts represents an extended response, lasting for 24 h (Fig. 2A, lanes 1–16) and even up to 3 days (not shown). The induction measured after 2 h of BMP4 treatment is unaffected in the presence of cycloheximide (Fig. 2B, lanes 1–4). Since uninduced levels of the Msx genes are not detectable at this time point (lane 1) and since CHX alone only leads to a marginal signal possibly due to message stabilization (lane 2), the revealed immediate early response of the Msx-1 and Msx-2 genes toward BMP4 suggests that these genes are direct target genes of BMP signaling. Identical results were obtained with the ES cell line E14.1 (data not shown).

By using CCE cells grown in CDM in the presence of high concentrations of BMP4 (~2 ng/ml) Johansson and Wiles (29) reported transient expression of mesoderm markers and subsequently the formation of hematopoietic precursor cells accompanied by the expression of corresponding lineage markers. Additionally, we performed dose-response experiments which...
showed that application of BMP2/4 at 0.5 ng/ml already led to a moderate enhancement of Msx-1 and Msx-2 transcripts, which increased upon using BMP concentrations up to 5 ng/ml. These responses were not enhanced further at higher BMP concentrations (data not shown). On the basis of these results, we subsequently used CCE cells treated with BMP 2/4 at 5 ng/ml, i.e. treatment conditions eliciting maximal responses regarding Msx induction.

**Isolation and Identification of New BMP-regulated Transcripts from ES Cells Using Differential Display RT-PCR**—By having demonstrated that CDM-cultured ES cells respond efficiently and selectively to BMP signaling, we used this cellular system to identify by DD-RT-PCR (31) new transcripts whose expression is induced by BMP4. Specifically, we searched for genes whose induction by BMP fulfilled the criteria of an immediate early-type response, i.e. displaying rapid and transient transcriptional induction in the absence of de novo protein synthesis (51). We searched for dd-RT-PCR products differentially displayed in BMP-stimulated versus mock-treated cells. Both BMP2 and BMP4 treatments were done in the presence of cycloheximide. The presence of cycloheximide ensured the isolation of those transcripts synthesized in an immediate early-type response.

60 separate DD-RT-PCR products were scored positive on differential display gels, excised, reamplified, cloned, and retested for differential expression by Northern blotting (not shown). We derived 15 independent cDNA clones that were positive in detecting mRNAs stimulated by BMP (both BMP2 and BMP4, respectively), CHX, or combined BMP/CHX treatment. Nine of these mRNAs were induced by CHX and not by BMP, and six were stimulated by BMP alone and also by a combined BMP/CHX treatment. These latter six cDNA clones, corresponding to BMP-induced “immediate early” transcripts, were characterized further by sequencing. Five cDNAs could be identified by sequence homology, whereas one (clone A79) represented a hitherto unidentified gene (Fig. 3A). Clone A51 exhibits homology to the human DEK oncogene (52), and clone A52 represents a part of the *Cyr61* gene that was first identified as a growth factor-inducible immediate early gene in mouse fibroblasts (53). Clone A59 corresponds to a gene encoding the RNA helicase translation initiation factor eIF4AII (54), and clone C74 harbors part of a gene encoding an Sp1-like GC-binding protein (55). The transcripts for these five cDNAs were moderately induced upon BMP treatment alone (1.6–2.2-fold), whereas the mRNA corresponding to clone G9 showed a strong induction (Fig. 3). Interestingly, clone G9 was identified as part of the cDNA encoding the Id3 protein, which is a negative regulator of basic helix-loop-helix transcription factors (56). Upon incubation of the ES cells with BMP4 in the presence of the RNA synthesis inhibitor actinomycin D, the mRNAs corresponding to all of the six isolated clones were not generated (Fig. 3A). This suggests that BMP-induced generation of these mRNAs is mediated, at least in part, at the level of transcription.

**Id1, Id2, and Id3 Exhibit Immediate Early Responses to BMP4 in ES Cells**—Four members of the Id family of HLH proteins have been identified so far (Id1 to Id4) (56). We were interested to see whether, in addition to the *Id3* gene identified...
Id1, Id2, and Id3 genes, and the effects of cycloheximide thereupon, in BMP4-stimulated CCE cells. A, ES cells were mock-treated (lanes 1–6) or were incubated with BMP4 (lanes 7–16) for the indicated times. Id gene expression levels were analyzed by Northern blotting. The corresponding control hybridizations with Gapdh are shown for quantitation of RNA loading. B, BMP4-induced Id gene activation in ES cells is resistant to treatment with cycloheximide. Control (Contr.) cells (lane 1), and cells treated with CHX (lane 2), BMP4 (lane 3), and cycloheximide plus BMP4 (lane 4) are investigated by Northern blotting. C, kinetic profile of BMP4-induced Id protein expression (Western blotting).

above, any of the other Id genes also responded to BMP4 treatment in ES cells. Indeed, Id1 and Id2 transcripts were also rapidly raised upon BMP4 stimulation (Fig. 3B and Fig. 4A, lanes 1–16), whereas no expression of Id4 was observed in our experiments. The inductions of Id1, Id2, and Id3 were blocked by cotreatment with actinomycin D (Fig. 3B). Cycloheximide treatment alone enhanced mRNA levels of these genes (Fig. 4B, lanes 1 and 2), and this effect contributed to the further increased expression levels seen after cotreatment with BMP4 and CHX (Fig. 4B, lanes 3 and 4). Collectively, this argues that BMP-mediated induction of the Id1, Id2, and Id3 transcripts occurred at the level of transcription, in a fashion independent of de novo protein synthesis. Furthermore, these inductions result in increased Id protein levels (Fig. 4C).

Id1, Id2, and Id3 mRNAs Are Up-regulated by BMP4 in Different Cell Types—During embryogenesis the expression patterns of Id genes overlap with the expression of BMP2 and BMP4 on many sites, for example during development of bone, cartilage, whiskers, teeth, and gut (57, 58). In addition, BMP2 has been shown to induce Id1 in osteoblast-like and myoblastic cells (59, 60) which strongly suggested the BMPs as candidates of Id gene inducing polypeptides. Based on the hypothesis that Id genes may serve as direct targets for BMP signaling in many different organs, we examined by Northern analysis a range of cell types for Id gene inducibility upon BMP4 treatment. The Id1, Id2, and Id3 genes were BMP-inducible in murine mesenchymal C3H/10T1/2 cells, murine preadipocytic 3T3-L1 cells, murine myocytic C2C12 cells, human osteogenic Saos-2 and U2-OS cells, and rat adrenergic PC-12 cells (Fig. 5). The kinetics of induction and magnitudes of response appeared highly variant between these different types of cell. Additionally, in human T-lymphocytic MOLT-4 and B-lymphoblastic Raji cells, the Id mRNA levels were increased only very moderately and transiently by BMP4 treatment. In human epithelial HeLa cells, promyelocytic HL-60 cells, and fibroblastic rabbit kidney RK13 cells, BMP did not elicit any change in Id transcripts at all (data not shown).

Ectopic BMP4 Misexpression in Vivo Correlates with Up-regulation of Mxs-1, Mxs-2, and Id3 mRNA—We next wanted to assess to what extent our results obtained in vitro using cultured cells would also relate to gene induction events in vivo. The dominant mouse mutant Fused toes (Ft) is characterized by fused toes of the forelimbs and thymus hyperplasia (41, 42). Recent experiments have shown a region of ectopic Bmp4 misexpression in the anterior distal part of the forelimbs of Ft embryos at day E12.2 Therefore, we analyzed the expression of Id3 and Mxs-2 by whole mount in situ hybridizations of limbs from heterozygous Ft/+ embryos at day E12. Fig. 6 clearly reveals at the site of strongest ectopic Bmp4 misexpression an increase in the expression of Id3 mRNA. Mxs-2 overexpression was displayed in an extended region that stretched over the entire area of Bmp4 misexpression (not shown). These embryonic expression data strongly support the notion of Mxs and Id genes being targets for BMP4 signaling in vivo.

DISCUSSION

Embryonic stem (ES) cells represent totipotent cells that can contribute to normal embryonic development when incorporated into blastocysts. In vitro, ES cells can also be stimulated to differentiate along mesodermal and neuronal pathways, which has yielded significant insight into processes of cellular differentiation during development. In the present study we confirm the ability of ES cells to respond efficiently and specifically to extracellular differentiation-inducing signals. Furthermore, we used the ES cell system to identify by dd-RT-PCR transcription units whose expression was hitherto unknown to be stimulated upon treatment with the mesoderm inducing factors BMP2 and BMP4. A similar experimental strategy led previously to the identification of Cryptic as a novel mesoderm-specific gene (61).

Expression of Transcriptional Control Genes upon BMP4 Treatment of ES Cells

Mxs Genes—We confirmed the Mxs-1 and Mxs-2 genes as target genes of BMP2/4 signaling in ES cells (Fig. 1A and Fig. 2). Since Mxs gene induction by BMPs did not require de novo protein synthesis, it is suggested that Mxs-1 and Mxs-2 represent direct target genes of BMP signaling. Mxs-1 and Mxs-2 are two members of a divergent homeobox-related gene family displaying homology to the Drosophila msh gene (muscle segment homeobox) (reviewed in Refs. 62 and 63). In the mouse embryo Mxs genes are involved in epithelial-mesenchymal interactions (49, 64–66) and are expressed in a wide range of embryonic regions, including the neural tube, the limb buds, and derivatives of the cranial neural crest (67–70). Their overlapping expression patterns and sequence similarities strongly suggest that the Mxs-1 and Mxs-2 genes function in an equivalent manner during development (71). Since expression of Mxs genes correlates with that observed of BMPs during development, BMPs have been considered likely candidates for Mxs-inducing factors (62).

2 J. Heymer and U. Rüther, manuscript in preparation.
The in vitro irradiation or heat shock (44, 45). The data presented here show that BMP4 in inhibiting the expression of myoblast differentiation markers. Additionally, Jonk et al. (74) reported the identification of a regulatory cis-element in the JunB promoter targeted by BMP signaling (74). The prolonged response of the c-jun gene correlates with similar effects elicited by TGF-β on human lung adenocarcinoma and erythroleukemia cells, as well as mouse embryo fibroblasts (75–77). The identification of c-jun and JunB as BMP response genes in ES cells implicates AP-1 as a regulator of BMP-dependent gene expression during early embryogenesis. In this respect it is interesting to note that in mice null mutations of the individual AP-1 components c-jun and JunB lead to early embryonic death (78–80).

Identification of Novel BMP-induced Immediate Early Genes by Differential Display (dd) RT-PCR

Of the six BMP4-regulated immediate early genes revealed in this study, five could be identified by their homology to known sequences. These include genes encoding one extracellular matrix signaling molecule (Cyr61/clone A52), one translational regulator (EIF4AII/clone A59), and three transcriptional regulators (DEK/clone A51, Sp-1 homologue/clone C74, and Id3/clone G9).

Cyr61—Transcriptional activation of the Cyr61 gene by FGF, PDGF, and the BMP-related molecule TGF-β has been reported previously (81, 82). The encoded Cyr61 protein is a secreted cysteine-rich heparin-binding protein that associates with the cell surface and the extracellular matrix (83). Cell-cell and cell-matrix interactions are known to exert important effects on embryonic development (84, 85). During embryogenesis cyr61 is expressed most notably in mesenchymal cells that are differentiating into chondrocytes and in vessels of the developing vascular system (86, 87). These sites overlap with the expression of BMP-related molecules (88–90).

EIF4A—The RNA helicases eIF4A1 and eIF4AII are components of the translation initiation complex eIF4A, which facilitates translation of certain mRNAs (91). Murine eIF4AII expression shows tissue-specific distribution (92). We speculate that induction of eIF4AII in response to BMP stimulation might facilitate translation of critical mRNAs present in ES cells.

DEK—DEK, a nuclear protein, has site-specific DNA binding activity that is likely involved in transcriptional regulation and...
signal transduction (92). DEK transcripts are seen at high levels from embryonic day 10 onward, and the gene is expressed ubiquitously in different adult tissues (52).

Clone C74—The BMP-inducible gene corresponding to clone C74 was previously identified as Ghp-23b, encoding a novel murine Sp1-like protein (55). Its human homologue TIEG was identified as a TGF-β- and BMP2-regulated gene in osteoblasts (2). The TIEG protein contains three zinc finger motifs homologous to the transcription factors Sp1, Sp3, WT1, and GT box-binding protein. We speculate that the clone C74 gene product may play a role as an effector of BMP-mediated differentiation.

Clone A79—The gene represented by clone A79 awaits identification.

Induced Expression of Id1, Id2, and Id3 upon BMP Treatment of ES Cells

In addition to the transcripts discussed above, our screen identified the Id3 gene to be activated by BMP2/4. We further show that its relatives, Id1 and Id2, also respond directly to treatment with BMP4. These inductions are seen in ES cells, as well as a diverse range of cell lines. Id family members encode negative regulators of the basic helix-loop-helix (bHLH) transcription factors that have been found to play a central role in the control of mammalian cell growth, differentiation, and tumorigenesis (reviewed in Ref. 56). Id proteins lack the basic region but possess the HLH motif. They can form heterodimers with the ubiquitously expressed E proteins (E2A, E2-2, and HEB) and render them inactive for DNA binding, dimerization with the tissue-restricted bHLH proteins, and, consequently, activation of their target genes. Down-regulation of Id genes is necessary for terminal differentiation in many developmental processes, including myogenesis (93, 94), myelopoiesis (95), lymphopoiesis (96), bone morphogenesis (59), glomerular mesangial cell development (97), and trophoblast development (98).

Cell cycle progression is also regulated by Id factors, in part via the induction of the p21-encoding gene, which in itself represents a BMP-regulated transcription unit (99, 100). Id proteins are viewed generally as negative regulators of differentiation and as positive regulators of proliferation. However, in our ES cell system BMP-mediated enhancement of Id expression does not coincide with a stimulation in DNA synthesis, as judged by thymidine incorporation assays (data not shown).

During organogenesis the expression patterns of Id1, Id2, and Id3 genes are highly overlapping with each other and with the expression domains of BMPs. These sites of expression are often associated with active mesenchymal-epithelial interactions (57, 58). During gastrulation Id1 and Id3 are expressed in the embryonic ectoderm, and Id2 is expressed in extraembryonic tissues, whereas Id4 expression cannot be detected at this stage of development (101). This may suggest an involvement of Id1, Id2, and Id3, but not of Id4, in gastrulation and is in line with the absence of Id4 expression in ES cells observed here. The characterization of Id1, Id2, and Id3 as BMP-induced immediate early genes in ES cells may implicate negative regulation of bHLH transcription factor activity to accompany BMP-mediated inductive events during early embryogenesis. BMP-mediated increase in Id expression may function as a molecular switch for lineage specification by functionally blocking the realization of developmental programs regulated by certain bHLH transcription factors (i.e. myogenesis). In this way, the realization of alternative programs (i.e. ventralization of mesoderm, bone formation, etc.) may be initiated.

Misexpression of Bmp4 in Embryos Induces Ectopic Induction of Msx-2 and Id3

Previous work in chicken using BMP-releasing beads implanted at selected embryonic sites has identified induced expression of Msx-1 and Msx-2 in the vicinity of the beads (46, 50). We made use of the dominant mouse mutant Fused toes (Ft) which is characterized phenotypically by fused toes of the forelimbs, coinciding with ectopic misexpression of Bmp4 in the anterior distal part of the forelimbs at E12.2 Although we found expression of Bmp4 (102) and Id3 (57) in wild-type limbs as described previously, at sites of ectopic BMP4 misexpression in Ft/+ limbs we observed the induced expression of Id3 (Fig. 6) as well as Msx-2 (not shown). This suggests that activation of these genes within the embryo is a direct consequence of local BMP expression. This in vivo correlation of BMP expression and target gene induction adds support to the experimental strategy of identifying in ES cells BMP-regulated genes whose induced expression may be relevant not only inside ES cells but also within the embryo.

In conclusion, we propose that the direct target genes of BMP signaling identified here are involved in BMP-stimulated early processes of mammalian development. It will be of special interest to compare the BMP induction patterns of Mesp-1, Mesp-2, c-fos, Id1, Id1, and Id3 in normal mice versus those deficient in BMP2, BMP4, and BMP2/4-type I receptor. The promoters of the genes identified in our study should serve as useful tools to characterize the molecular gene regulatory circuits that are governed by BMP signaling.

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