Dlx3 (Distal-less 3) is a homeobox-containing transcription factor required for normal placental development in mice. Here we demonstrate that Dlx3 interacts with Smad6, a member of a larger family of transcriptional regulators generally thought to regulate transforming growth factor β/bone morphogenetic protein signaling. Immunocytochemical and immunoprecipitation studies demonstrate overlapping nuclear localization and physical interaction between Dlx3 and Smad6 in human chorionic carcinoma cells and in differentiated trophoblasts from human placenta. In vitro protein interaction studies mapped the Smad6 interaction domain within Dlx3 to residues 80–163, a region of Dlx3 that includes a portion of the homeodomain. Dlx3 and Dlx4 share homology within this region, and Dlx4 was also found to bind Smad6. Using the Esx1 gene promoter as a model for a Dlx3-responsive gene, studies demonstrate two near consensus Dlx3 binding sites within the proximal 2.3 kb of the transcription start site. Interestingly, binding of Dlx3 to one of these two sites was inhibited by interaction with Smad6. Consistent with this result, expression of an Esx1 promoter luciferase reporter was increased by overexpression of Dlx3; this effect was reversed with co-expression of Smad6. Further, small interference RNA-mediated knockdown of endogenous Smad6 increased Dlx3-dependent expression of the Esx1 gene promoter. Thus, Smad6 appears to functionally interact with Dlx3, altering the ability of Dlx3 to bind target gene promoters. Smad6 appears to play a modular role in the regulation of Dlx3-dependent gene transcription within placental trophoblasts.

The Distal-less family of transcriptional regulators includes six members in mammals, arrayed in pairs and aligned with the hox gene clusters along different chromosomes (1, 2). Dlx3 is tandemly arrayed with Dlx4 on human chromosome 17 and is involved in developmental determination of multiple tissues, including the first and second branchial arches, teeth, bone, and multiple epithelia, including the skin, mammary gland primordia, and the placenta (3). The relationship between convergently transcribed pairs of Dlx family members and specific hox gene clusters has suggested that, although independent of hox gene expression patterns, these homeodomain-containing transcription factors are clearly involved in important aspects of developmental morphogenesis (reviewed in Ref. 2). The importance of Dlx3 during development and in the adult arises from several different observations of disease states. Mutations in Dlx3 are believed to be causally related to tricho-dento osseous syndrome, a genetic disorder manifested by taurodontism, hair abnormalities, and increased bone density in the cranium (4–6). The defect in Dlx3 leading to tricho-dento osseous syndrome appears to be associated with a four-nucleotide deletion just downstream of the homeodomain, resulting in a premature truncation of the protein. Amelogenesis imperfecta with taurodontism has similar characteristics as tricho-dento osseous syndrome and has also been associated with mutations with Dlx3 in some families investigated, albeit distinct from the four-nucleotide deletion/mutation described above (7). Amelogenesis imperfecta is an autosomal dominant trait leading to dental enamel defects and enlarged pulp chambers and has been associated with a two-nucleotide deletion within the homeodomain of Dlx3. This deletion again results in a frameshift and premature truncation of Dlx3 in the carboxyl terminus, primarily downstream of the homeobox. In addition to tricho-dento osseous syndrome and amelogenesis imperfecta, Dlx3/Dlx4 have been identified in the gene interval thought to be involved in some forms of craniofacial abnormalities, including cleft palate (8). The putative involvement of Dlx3 in the occurrence of cleft palate is also supported by the murine model deficient in endothelin-A receptor (the endothelin-A receptor), which results in cleft palate and hypoplasia of the mandible (9–11). In this model, Dlx3 expression is thought to be dependent upon endothelin-1 through a Gαq/Gα13-dependent mechanism. In the Gαq/Gα13-deficient mouse, Dlx3, among other factors, is specifically down-regulated, supporting the speculation of the importance of Dlx3 in cranio-facial morphogenesis (12). Thus, the role and importance of Dlx3 in morphogenetic aspects of development and in epithelial differentiated function is rather far reaching.

The Dlx3 null mouse dies in utero by embryonic day 10 due to putative placental failure (13). This was associated with a failure in the development/morphogenesis of the placental labyrinth compartment of the murine placenta. Further, genetic loss of Dlx3 was correlated with reduced expression of an additional homeobox factor, Esx1, suggesting that Dlx3 may be an important transcriptional regulator of Esx1 promoter activity. Studies from our laboratory identified Dlx3 as a cell type-specific transcriptional activator in placental trophoblasts. Dlx3 binds to
and transactivates the promoter for the glycoprotein hormone α subunit gene via a cis-acting element required for full basal activity of this gene (14). The glycoprotein hormone α subunit is a subunit of the heterodimeric glycoprotein hormone, chorionic gonadotropin (CG).3 Trophoblast-derived CG has been identified in primate and equine and appears to play a critical role in the maintenance of early pregnancy in women, providing early gonadotropic support to the corpus luteum and maintenance of progesterone production (15–17). Both for the case of the α subunit promoter and regulation of Esx1 in the Dlx3 null mouse, Dlx3 appears to function as a putative transcriptional activator. However, it has also been proposed that Dlx3 can serve as a negative regulator of gene transcription in amphibian models (2). This apparent activation/repression capability may be due to variable heterodimeric partners of Dlx3 (as proposed in Ref. 2), dependent upon cell context and physiological state. This observation was the impetus for us to examine potential binding partners of Dlx3 in the context of the human placenta. The present studies identify Smad6 as a binding partner for Dlx3 using a yeast two-hybrid screen of a human term placental cDNA library. Dlx3 and Smad6 are co-localized in the nucleus of cells of trophoblast origin, including cytotrophoblasts and syncytial trophoblasts from fully differentiated human term placenta. Interaction between Dlx3 and Smad6 alters the DNA binding properties of Dlx3 such that Smad6 serves as a negative regulator of Dlx3-dependent gene transcription of the Esx1 promoter.

**MATERIALS AND METHODS**

**Plasmids and cDNAs** — All plasmids used in these studies were prepared by two cycles through cesium chloride using standard protocols. Expression vector for human Dlx3 was generously provided by Dr. Maria Morasso (National Institutes of Health, Bethesda, MD). A series of deletion mutants of the Dlx3 cDNA were constructed by PCR. To facilitate cloning into the pKH3 vector (generously provided by Dr. Jun-Lin Guan, Cornell University, Ithaca, NY), EcoRI and Clai restriction sites were added to the forward and reverse primers, respectively. The forward primers used in these reactions were as follows: forward 1, 5′-TCAGGAATTCAAATGACCTCTTTACCCTGATGC-3′, forward 20, 5′-TCAGGAATTCAAATGGTGAACGGCAAGCCCAAAA-TCAGGAATTCAACTGGGCTA-3′, forward 121, 5′-TCAGGAATTCAAATGGTGAACGGCAAGCCCAAAA-TCAGGAATTCAACTGGGCTA-3′, and forward 195, 5′-TCAGGAATTCAAATGGTGAACGGCAAGCCCAAAA-TCAGGAATTCAAATGGTGAACGGCAAGCCCAAAA-TCAGGAATTCAACTGGGCTA-3′. The reverse primers used in these reactions were as follows: reverse 128, 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′; reverse 135, 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′; reverse 163, 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′; reverse 202, 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′; reverse 287, 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′. PCR products were cloned initially into the pGEM T Easy vector (Promega Corp., Madison, WI). Once verified by nucleotide sequence analysis, fragments were subcloned into the pKH3 vector for use in studies. Smad6 expression plasmid was a gift from Dr. Ali H. Brivanlou (The Rockefeller University, New York, NY). Smad4 expression vector was a gift from Dr. Colin Clay (Colorado State University, Fort Collins, CO). The human Dlx4 cDNA was obtained by PCR from RNA isolated from JEG3 cells using the following primers: 5′-TCAGGAATTCAAATGACCTCTTTACCCTGATGC-3′ and 5′-GTACATCGTACGACGGCTTTTCGCATGCCTC-3′. The resulting Dlx4 cDNA was cloned into pKH3 and verified by nucleotide sequence analysis. The Esx1-2.3kb promoter was obtained by PCR using mouse genomic DNA and the following primers: 5′ primer (5′-GGTACACGACCGGCTCATCACAACCATCA-3′) and 3′ primer (5′-GCTAGCTACGACGGCTTTCCCGTT3′). To facilitate cloning, KpnI and NheI restriction enzyme sites were engineered at the end of the 5′ primer and 3′ primer, respectively. The PCR products were cloned into pGEM T-Easy vector. After KpnI and NheI digestion, the promoter fragment was subcloned into a luciferase reporter vector. The fidelity of the construct was confirmed by nucleotide sequence analysis. PCR-based site-directed mutagenesis was used to disrupt the distal Dlx3 binding site within the Esx1 luciferase reporter. This mutation substituted a Not-1 restriction site for the consensus Dlx3 binding site. The mutation was confirmed using nucleotide sequence analysis. The human α subunit gene luciferase reporter has been reported previously (14).

**Yeast Two-hybrid Screen** — To investigate novel protein–protein interactions, full-length Dlx3 served as the bait protein with a human term placental cDNA library serving as the target. The bait, human Dlx3 cDNA, was cloned into the vector pGBK7 and transformed in the yeast strain AH109. A pre-transformed human term placental Matchmaker cDNA library was in yeast strain Y187 (BD Biosciences/Clontech, Palo Alto CA). The bait and library plasmids were expressed as GAL4 fusion proteins. 3-Amino-1,2,4-triazole was titrated (5–35 mM) using the bait strain to control background yeast growth. A concentration of 12 mM 3-aminio-1,2,4-triazole was used in the library screen. The bait and library strains were mated with an efficiency of ~4%. The bait strain required Leu+ synthetic dropout (SD) minimal medium, and the library strain required Trp+ SD minimal medium. Mating was carried out in YPDA media containing 0.003% adenine hemisulfate. Following mating of the bait strain with the human placental library, yeasts were initially plated on intermediate stringency SD media (His-/Leu+/Trp+ plates). When colonies were of sufficient size, colonies were replica-plated on high stringency SD medium (Ade-/His-/Leu-/Trp+/X-α-gal) plates to verify that they maintained the correct phenotype. A colony filter lift assay was performed to access β-galactosidase activity to identify and rank the strength of potential interactions. Once identified, yeast plasmids were isolated using disruption with glass beads, and plasmids were rescued/purified using the Qiagen Miniprep reagents and a spin column (Qiagen Inc., Valencia, CA). Identity of the rescued plasmids was verified by nucleotide sequence analysis. The interaction between Dlx3 and target genes was examined using a reconstitution assay, where both plasmids

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3 The abbreviations used are: CG, chorionic gonadotropin; PBS, phosphate-buffered saline; KBPS, potassium phosphate-buffered saline; siRNA, small interference RNA; EMSA, electrophoretic mobility shift assay; TKD, trophoblast Kunitz domain protein; TGFβ, transforming growth factor β; BMP, bone morphogenetic protein; JRE, junctional regulatory element; E3, ubiquitin-protein isopeptide ligase; X-α-gal, X-α-galactosidase.
were co-transformed into the AH109 yeast strain and plated on high stringency SD medium.

Preparation of JEG3 Cell Nuclear Extracts—Subconfluent JEG3 cells were used for the preparation of nuclear extracts. Cells were washed twice with ice-cold Dulbecco’s phosphate-buffered saline (PBS; Invitrogen). Cells were collected by scraping in ice-cold PBS supplemented with a 1:1000 dilution of protease inhibitor mixture (referred to as protease inhibitor mixture; Sigma), 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride. Cells were pelleted by centrifugation and resuspended in a hypotonic buffer consisting of 120 mM potassium chloride, 30 mM sodium chloride, 30 mM Heps (pH 8.0), 0.3 M sucrose, protease inhibitor mixture, 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride and allowed to swell for 15 min on ice. Cells were lysed by Dounce homogenizing, and nuclei were isolated by layering the broken cell lysate over a sucrose cushion (0.9 M sucrose) followed by centrifugation at 4 °C for 15 min on ice. Cells were lysed by Dounce homogenizing, and nuclei were isolated by layering the broken cell lysate over a sucrose cushion (0.9 M sucrose) followed by centrifugation at 2000 × g for 30 min at 4 °C. The nuclear pellet was resuspended in a buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM EDTA, protease inhibitor mixture, 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride. Additional sodium chloride was added to a final concentration of 450 mM, and nuclear proteins were extracted with constant rocking at 4 °C for 30 min. Nuclear extracts were clarified by centrifugation (85,000 × g for 60 min), and the nuclear extract was stored in aliquots at −80 °C until later use. Protein concentrations of the nuclear extracts were determined by Bradford assay.

Immunoprecipitation from JEG3 Nuclear Extracts and Western Blotting Analysis—JEG3 cell nuclear extracts (200 μg) were suspended in 1 ml of 0.1% Triton X buffer (50 mM Tris (pH 7.6), 50 mM sodium chloride, 0.1% Triton X, protease inhibitor mixture, 5 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride). To preclar the nuclear extracts, protein A/G-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to each suspension and allowed to mix for 1 h at 4 °C with gentle rocking. Following centrifugation (1200 × g for 1 min) to remove protein A/G-agarose, antibodies were added at the following dilutions: normal rabbit serum at 1:1000; Dlx3 antibody (Santa Cruz Biotechnology) at 1:1000; Smad6 antibody (Santa Cruz Biotechnology) at 1:100. Following 2 h of gentle rocking at 4 °C, protein A/G-agarose (Santa Cruz Biotechnology) was added and allowed to mix for an additional 2 h. Complexes were then washed four times with 0.1% Triton X buffer. Samples were then suspended in an equal volume of 2× SDS loading buffer (100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, and 200 mM dithiothreitol). Protein samples were boiled for 3 min and chilled for 5 min on ice. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes by electroblooming. Membranes were blocked with nonfat dried milk (5%) in Tris-buffered saline (10 mM Tris (pH 7.6), 150 mM sodium chloride) containing 0.1% Tween 20 (TBST). For Western blots, the Dlx3 antibody was used at 1:5000 in TBST, 5% nonfat dried milk. The reciprocal Western blot from immunoprecipitations (IPs) using the Smad6 antibody was not possible, since the IP heavy chain IgG blocked visualization of Smad6 on Western blot due to similar molecular size. In other Western blot studies, the Smad6 antibody was used at 1:500, and the actin antibody (Santa Cruz Biotechnology) was used at 1:1000 dilution. Proteins bands were visualized by chemiluminescence reagents (PerkinElmer Life Sciences).

Immunocytochemistry—JEG3 cells were cultured on glass slides or coverslips for 24 h, rinsed one time with potassium phosphate-buffered saline (KPBS), and then fixed for 20 min with 4% paraformaldehyde. Slides or coverslips were then stored in 70% ethanol until used. Prior to use, slides were rinsed in KPBS seven times over 1 h. JEG3 cells were incubated with primary antibody (Smad6 at 1:100; Dlx3 at 1:500) overnight at 4 °C. Slides were again rinsed with KPBS, followed by incubation with a fluorescence-conjugated secondary antibody (Alexa 594, Molecular Probes, Inc., Eugene OR; and Cy2, Jackson Immunoresearch Laboratories, Westgrove, PA) in KPBS-Triton X at 37 °C for 2 h. Cells were rinsed in KPBS, dehydrated through a graded series of ethanol, and cleared with xylene, and coverslips were attached with Krystalon (EM Science, Gibbstown, NJ).

Samples of human term placenta (derived from elective caesarian section) were obtained from Cayuga Medical Center (Ithaca, NY) under the guidelines and approval of the Cornell University and the Cayuga Medical Center Committees on the Use of Human Subjects in Research. Samples were collected, fixed with 4% paraformaldehyde for 48 h, and transferred to 70% ethanol until processing. Tissues were paraffin-embedded, and 5-μm sections were obtained. Immunocytochemistry was performed as previously described (14), except that fluorescence-conjugated secondary antibodies were used as described above.

Recombinant Proteins and Immunoprecipitation Analysis—Recombinant Smad6, Smad4, Dlx4, Dlx3, and deletions of Dlx3 were prepared using a coupled transcription and translation Wheat Germ Extract System (Promega Corp., Madison, WI) following the prescribed protocol. Proteins were radioactively labeled using [35S]methionine (1000 Ci/mmole at 10 mCi/ml; Amersham Biosciences). A portion (10%) of each recombinant protein was saved for input analysis. Protein combinations were added at 1:1 (by volume) mixture to a 0.1% Triton X buffer along with appropriate antibody at specified concentrations (Dlx3 at 1:1000; Smad6 (Santa Cruz Biotechnology) at 1:100; and Smad4 (Santa Cruz Biotechnology) at 1:500). Following 2 h of gentle rocking at 4 °C, protein A/G-agarose (Santa Cruz Biotechnology) was added and allowed to mix for an additional 2 h. Complexes were then washed four times with 0.1% Triton X buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the gel was fixed in 25% methanol and 15% glacial acetic acid for 1 h with gentle rocking at room temperature. The gel was then washed three times in 40% isopropyl alcohol solution and dried, and bands were visualized by autoradiography.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assays (EMSAs) were carried out as described previously (14, 18) using the indicated antibodies. Reactions (without probe) were maintained at room temperature for 30 min followed by the addition of 32P-labeled oligonucleotide Dlx3 binding site probes (the junctional regulatory element (JRE) from the glycoprotein hormone α subunit promoter (14) and two putative Dlx3 binding sites identified within the Esx1 promoter). The binding reactions were maintained an additional 30 min and then resolved on native poly-
**Dlx3 and Smad6 in Placenta**

**A.**

- **Smad6 + Dlx3**
- **Dlx3**
- **Smad6**
- **Merge**

**B.**

- **Dlx3**
- **Smad6**
- **Term Placenta**

**FIGURE 1. Dlx3 and Smad6 interact in yeast two hybrid and co-localize in placental trophoblasts.** A yeast two-hybrid screen of a human term placental library was carried out using full-length Dlx3 as bait. Following plasmid rescue, Gal4 DNA binding domain-Dlx3 bait was co-transformed along with Gal4 activation domain-Smad6 into the AH109 yeast strain and plated on (His−, Leu−, Trp−) media as described under "Materials and Methods." B, JEG3 cells were plated on glass slides and fixed as described under "Materials and Methods." Double-labeled immunocytochemistry was used to determine localization of Dlx3 (green) or Smad6 (red). The merged images demonstrate overlapping expression patterns. Human term placenta was obtained, fixed, and sectioned as described under "Materials and Methods." Similar double-labeled immunocytochemistry was used to localize Dlx3 and Smad6. The white arrowhead identifies nuclei in syncytiotrophoblasts, whereas the black arrowhead identifies nuclei in cytotrophoblasts. Bar, 20 μm.

**FIGURE 2. Dlx3 and Smad6 interact in the JEG3 choriocarcinoma cell model.** A, JEG3 cell nuclear extracts were used in IP studies. IPs included the use of normal rabbit serum (NRS) or antisera (ab) directed against Dlx3 or Smad6. Recombinant Dlx3 (rDlx3; without hemagglutinin epitope tag) and rDlx3 were resolved by SDS-PAGE, and Western blots for Dlx3 were carried out (IB Dlx3). Molecular size standards (MW) are depicted to the left. B, recombinant Dlx3, Smad6, and Smad4 were produced using wheat germ lysates in a coupled transcription/translation reaction containing [35S]methionine (Input). Dlx3 and either Smad6 or Smad4 were combined, followed by IP with the Dlx3 antibody (Dlx3 IP). The input and IPs were resolved by SDS-PAGE, the gels were fixed and dried, and autoradiography was used to visualize bands.

- acrylamide gels. To determine whether Smad6 could displace pre-bound Dlx3 in EMSA, binding reactions containing Dlx3 alone were allowed to incubate with probe for 30 min to reach equilib-
out significant similarity to any mammalian gene sequence and therefore served as an appropriate negative control (OligoEngine, Seattle, WA). The forward and reverse strands of oligonucleotides containing the siRNAs and nonsense sequence also contained BglII and HindIII sites at the 5′/H11032-3′-end of the forward and reverse oligonucleotides, respectively. The oligonucleotides were annealed and inserted into the pSUPER-retro-neo vector after digestion of the vector with BglII and HindIII. These siRNAs were a self-contained hairpin loop for the double-stranded siRNA. All siRNA sequences were confirmed by nucleotide sequencing.

Cell Culture, Transfection, and Retroviral Infection of siRNAs—HEK293 Phoenix Ampho packaging cells (American Type Culture Collection; Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and were transfected with the pSUPER-retro-neo siRNA plasmids using FuGENE 6 transfection reagent (Roche Applied Science). Forty-eight hours following transfection, the culture media containing the retrovirus for individual siRNAs and control siRNA were filtered through a 0.45-μm filter, and the viral supernatant was used for infection of JEG3 cells in the presence of 8 μg/ml polybrene. JEG3 cells were exposed to the retrovirus overnight, and cells were then washed with fresh medium and allowed to recover for 24 h. Following infection, stable cell lines were selected using neomycin at 500 μg/ml for 7 days (time until untransfected cells all died), and then cultures were maintained in medium containing 500 μg/ml neomycin. Transient transfections of the siRNA cell lines were carried out as described above.

Statistical Analysis—Luciferase data were subjected to analysis of variance, and differences between treatments were determined using Tukey’s Studentized Range Test. Probability of less than 0.05 (p < 0.05) was considered statistically significant.

RESULTS

Dlx3 and Smad6 Functionally Interact in a Yeast Two-hybrid Screen—Full-length human Dlx3 was used as a bait protein in a yeast two-hybrid screen of a human term placental library. The screen included coverage of 3.4 × 10⁶ independent clones. Once colonies were identified from the original screen and library plasmids were rescued, plasmids were retransformed in a reconstitution assay with the bait vector into the AH109 yeast strain. The library plasmid resulting in the most robust interaction (as measured by β-galactosidase activity) with Dlx3 was a cDNA containing the entire coding region of Smad6. Transformation of the Smad6 library vector alone did not support yeast growth on high stringency SD medium (Ade⁻/His⁻/Leu⁻/Trp⁻/X⁻-gal) plates in the presence of 12 mM 3-amino-1,2,4-triazole. Transformation of Dlx3 bait vector minimally supported yeast growth under the same conditions. Co-transformation of Dlx3 bait and Smad6 library plasmid resulted in rescue of yeast growth (Fig. 1A).

Dlx3 and Smad6 Are Co-localized in the Nucleus in Choriocarcinoma Cells and in Human Placental Trophoblasts—Initially, studies focused on examining localization of Dlx3 and Smad6 proteins in cells of trophoblast origin. Dlx3 and Smad6 were expressed and localized primarily in the nuclear compartment in JEG3 cells (Fig. 1B), a choriocarcinoma cell line of trophoblast origin. Consistent with this observation, Dlx3 and Smad6 were nuclear localized to both cytotrophoblast and syncytiotrophoblast within microvilli of fully differentiated term human placenta. Localization of Dlx3 in term placenta pro-
**Dlx3 and Smad6 in Placenta**

![Alignment of Dlx3 and Dlx4 between residue 80 and 198](image)

**Figure 4. Dlx4 binds Smad6.** A, alignment of Dlx3 and Dlx4 between residue 80 and 198 reveals regions of conserved sequence within the Smad6 interaction domain (residues 80–163). The consensus amino acid sequence is listed in boldface type below the alignment. B, wild type Dlx3-(1–287), Dlx3-(195–287), Smad6, and Dlx4 were prepared as recombinant proteins as described in the legend to Fig. 2 (Input) and subjected to IP studies using the Smad6 antibody (Smad6 IP) and recombinant Smad6 and combinations of Dlx3 or Dlx4 as indicated. The input and IPs were resolved by SDS-PAGE, gels were fixed, and bands were visualized by autoradiography. *, bands consistent with protein-protein interactions.

vided additional insight into the expression pattern of Dlx3 during gestation in primates. We have previously shown Dlx3 in placental trophoblasts in human placenta obtained at 8 weeks gestation during peak production of human CG (14). The current studies support the conclusion that Dlx3 expression may be maintained within trophoblast cell populations until term in the human placenta.

Since both Dlx3 and Smad6 were expressed endogenously in the JEG3 choriocarcinoma cell model, we sought to use this model for subsequent analyses. We have used JEG3 cells previously for molecular analysis of gene regulatory processes related to glycoprotein hormone and Dlx3 gene promoter expression (14, 18, 19). IP studies using nuclear extracts from JEG3 cells and Smad6 antibody revealed that Dlx3 and Smad6 interact in mammalian cells (Fig. 2A). The reciprocal study using Dlx3 antibody to IP Smad6 was not possible due to the molecular size of Smad6 and interference with the IgG heavy chain used in the IP studies. However, this constraint was overcome with preparation of Dlx3 and Smad6 as recombinant proteins labeled with [35S]methionine. IP of recombinant Dlx3 and Smad6 with the Dlx3 antibody revealed specific association with Smad6 but not Smad4 (Fig. 2B). Thus, the original interaction defined in the yeast system was supported by IP studies in choriocarcinoma cells that endogenously express these two proteins and in vitro using recombinant Dlx3 and Smad6 proteins.

**Structure/Function Analysis of the Dlx3/Smad6 Interaction Interface**—To further understand the mechanism of the Dlx3/Smad6 interaction, we constructed a series of deletion mutants of Dlx3 (Fig. 3A). The rationale for these mutations was predicated on the existing understanding of important domains within Dlx3 defined by others (20, 21), centering upon the homeodomain (residues 130–189; Fig. 3A), the centrally located DNA binding domain. Dlx3 deletion mutants were prepared in vitro along with full-length Dlx3 and Smad6. Smad6 IP was then used to determine the domains sufficient to support interaction with Dlx3. Initially, we identified the importance of Dlx3-(1–202), which bound Smad6 at levels similar to Dlx3-(1–287) (Fig. 3B). Dlx3-(121–287) bound Smad6 as well, suggesting that Smad6 interaction domain required at least a portion of the homeodomain (Fig. 3B). Based upon these results, we predicted that Dlx3-(121–202) mutant would also bind Smad6. This was not readily interpretable in our studies, since putative degradation fragments/products of these recombinant deletion mutants were of similar molecular size as the Dlx3-(121–287) mutant. Subsequent Dlx3 deletion mutants defined a region of Dlx3-(80–163) that was sufficient to bind Smad6 in vitro (Fig. 3B). These studies again supported the conclusion that at least the amino-terminal portion of the homeodomain was sufficient for interaction with Smad6 in vitro. Alignment of Dlx3 with Dlx4 (another member of the Distal-less family expressed in placenta (22)) revealed ~37% amino acid conservation within this region (residues 80–163; Fig. 4A, with higher levels of homology particularly clustered within the homeodomain, supporting the prediction that Dlx4 may also bind Smad6. Based upon in vitro studies, recombinant full-length Dlx4 bound Smad6 similarly to Dlx3-(1–287) (Fig. 4B). Dlx3-(195–287), previously shown not to bind Smad6 (Fig. 3B), was used in these studies as a negative control.

**Dlx3 Binds to the 5′-Flanking Sequence of the Esx1 Promoter**—To begin to examine the functional significance of the Dlx3/Smad6 interaction on gene transcription, we cloned 2.3 kilobases of the 5′-flanking sequence of the Esx1 promoter. The report of the Dlx3 null mouse provided evidence that the loss of Dlx3 in vivo was correlated with a loss of Esx1 mRNA in mouse placenta (13). Taking advantage of this observation, we identified two near consensus Dlx3 binding sites within the 2.3-kb promoter fragment. The distal site was located at −2135 and the more proximal site at −585 relative to the transcription start site of the Esx1 promoter, as previously defined (23). At both of these two sites, the central core of the binding site
mune normal rabbit serum. The addition of the Smad6 or the CCAAT/enhancer-binding protein antiserum to these binding reactions did not appreciably alter complex formation in EMSA, suggesting that Smad6 may not directly participate in DNA binding with Dlx3 and that Dlx3 antibody interactions were specific. To determine the importance of this site to Dlx3-induced Esx1 gene transcription, the distal site was mutated to a NotI restriction site and examined using a luciferase reporter gene approach in transient transfection studies (Fig. 6B). The wild type Esx1 promoter was strongly induced by Dlx3. The mutation in the distal putative Dlx3 binding site reduced Dlx3-induced Esx1 expression by >50% (p < 0.05), suggesting that this site was important for promoter activity induced by Dlx3.

We then sought to determine the impact of Smad6 on Dlx3 DNA binding. Reconstitution EMSAs using recombinant Dlx3 and Smad6 demonstrated that the Dlx3/Smad6 interaction effectively reduced/blocked association of Dlx3 with the distal Dlx3 binding site within the Esx1 promoter (Fig. 7A). In this experiment, Smad6 alone did not form a complex with the distal Dlx3 binding site. When Smad6 was titrated into the binding reactions containing Dlx3, the Dlx3 binding complex was diminished in a dose-dependent manner. Using the JRE as probe, a similar titration of Smad6 protein reduced Dlx3 binding, albeit to a lesser extent compared with the distal site of the Esx1 promoter. Studies then focused on determining if pre-bound Dlx3 could be displaced by Smad6 in EMSA binding reactions (Fig. 7B). Binding reactions compared the addition of Dlx3 concurrent with Smad6 and reactions where Dlx3 binding was allowed to reach equilibrium and then Smad6 was added. These studies revealed that for the distal probe, Smad6 competed for Dlx3 binding regardless of the order of protein addition. Similar levels of competition were not observed using the JRE (stronger relative binding), suggesting that Smad6 can compete for Dlx3 binding, particularly on gene targets that have relatively weaker Dlx3 binding sites, like those characterized within the Esx1 gene promoter.

To assess the functional consequences of the Dlx3/Smad6 interaction on the Esx1 promoter, we again used the Esx1 promoter-luciferase reporter construct (Fig. 8). Based upon the binding studies described above, our prediction was that Smad6 overexpression would probably repress expression of the Esx1 promoter induced by Dlx3. Using transient transfection in JEG3 cells, overexpression of Dlx3 and Smad6 demonstrated that the Dlx3/Smad6 interaction resulted in a marked inhibition (p < 0.05) of Dlx3-induced Esx1 promoter activity. Further, Dlx3 overexpression increased Esx1 luciferase activity (p < 0.05; Fig. 8B). Titration of Smad6 into this system resulted in reduced basal activity of the Esx1 reporter gene (p < 0.05). Consistent with our prediction, co-transfection with increasing doses of Smad6 expression vector along with Dlx3 resulted in a marked inhibition (p < 0.05) of Dlx3-induced Esx1 promoter activity. Dlx3-induced activation of the α subunit gene promoter was also reduced by overexpression of Smad6 (data not shown). In an effort to determine the specificity of Smad6 action on gene transcription in general, we examined the effects of Smad6 on the trophoblast Kunitz domain protein 1 (TKDP1) (Fig. 8C) and Rous sarcoma virus promoters (data not shown). TKDP1 promoter expression (25, 26) is tropho-

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**FIGURE 5.** Two putative Dlx3 binding sites are present within the Esx1 promoter. A, the JRE (a consensus Dlx3 binding site) from the glycoprotein hormone α subunit promoter was compared with the distal (position −2135) and proximal (position −585) binding sites from the Esx1 promoter. The central core of the binding site is underlined. B, EMSAs were used to compare binding complexes formed on the JRE and the distal and proximal Dlx3 binding sites within the Esx1 promoter using JEG3 cell nuclear extract and recombinant Dlx3. Equal amounts of nuclear extracts or recombinant Dlx3 were added to each binding reaction. Recombinant Dlx3 in these studies was epitope-tagged with three copies of the hemagglutinin epitope (3×HA-Dlx3). Binding reactions were resolved on native polyacrylamide gels, the gels were dried, and bands were visualized by autoradiography. Free probe is designated by an arrow at the bottom of the gel.
FIGURE 6. The distal binding site of the Esx1 promoter binds Dlx3 and is necessary for full Dlx3-induced gene transcription. A, using only the distal binding site for Dlx3 and JEG3 cell nuclear extract (JEG3 NE), studies examined if a Dlx3 antibody would disrupt binding of endogenous proteins, including Dlx3, to this site. Binding reactions were resolved on native polyacrylamide gels, the gels were dried, and bands were visualized by autoradiography. The addition of a Dlx3 antibody (Dlx3 ab) to binding reactions resulted in the formation of a supershifted complex as indicated (supershift). Normal rabbit serum was used as a control for the Dlx3 antibody. In some binding reactions, the JEG3 NE was boiled and then added to reactions containing the Dlx3 antibody as a negative control. Free probe is designated by an arrow at the bottom of the gel. B, transient transfection studies were used to determine the relative importance of the distal Dlx3 binding site on expression of the Esx1 promoter luciferase reporter gene. JEG3 cells were transiently transfected with either wild type Esx1 luciferase reporter or an Esx1 luciferase reporter containing a mutation within the distal Dlx3 binding site. Cells were cotransfected with control plasmid or a Dlx3 expression vector (2 µg; designated − or +). Data are reported as relative luciferase activity standardized for protein for a representative study (n = 3/treatment). The designations a, b, and c indicate significant differences (p < 0.05).

FIGURE 7. Smad6 attenuates Dlx3 DNA binding. A, to examine the effects of Smad6 on Dlx3 binding, recombinant Dlx3 and Smad6 were prepared in vitro and used in EMSAs with the distal Dlx3 binding site within the Esx1 promoter and the JRE. As observed earlier, Dlx3 bound this site, whereas increasing doses of Smad6 (as represented by the gray triangle) did not bind. Binding reactions containing a constant dose of Dlx3 and increasing doses of Smad6 resulted in a dose-dependent reduction in Dlx3 binding for the distal site and to a lesser extent for the JRE. B, EMSA studies were carried out to determine if the order of protein addition had an impact on the ability of Smad6 to interfere with Dlx3 binding. Using the distal and JRE probes, binding reactions were assembled where Dlx3 was added and allowed to reach equilibrium (Dlx3), and then Smad6 was added (Dlx3 + Smad6). This was compared with binding reactions where Dlx3 and Smad6 were added concurrently (Dlx3 3 Smad6). For both A and B, free probe and Dlx3 complexes are designated by arrows.

Dlx3 and Smad6 in Placenta

The designations reported as relative luciferase activity standardized for protein for a representative study (indicated a, b, and c) did not bind. Binding reactions containing a constant dose of Dlx3 and increasing doses of Smad6 resulted in a dose-dependent reduction in Dlx3 binding for the distal site and to a lesser extent for the JRE. EMSA studies were carried out to determine if the order of protein addition had an impact on the ability of Smad6 to interfere with Dlx3 binding. Using the distal and JRE probes, binding reactions were assembled where Dlx3 was added and allowed to reach equilibrium (Dlx3), and then Smad6 was added (Dlx3 + Smad6). This was compared with binding reactions where Dlx3 and Smad6 were added concurrently (Dlx3 + Smad6). For both A and B, free probe and Dlx3 complexes are designated by arrows.

actually increased expression of the Rous sarcoma virus promoter in a dose-dependent manner. These studies support the conclusion that Smad6 does not confer global non-specific transcriptional repression, and probably the effects of Smad6 on Esx1 and α subunit promoter activity are specific.

siRNA-mediated Knockdown of Smad6 Resulted in Increased Expression of the Esx1 Gene Promoter—Whereas overexpression studies can be informative, interpretation of such studies must be cautious. To examine the effects of Smad6 on Dlx3-dependent gene expression, we developed stable cell lines expressing specific Smad6 siRNA hairpin loops (Fig. 9A). Two of the three siRNA cell lines examined were found to have specific reductions in Smad6 protein expression (Smad6 siRNA#1 and siRNA#3; 57% reduction compared with control siRNA) but not expression of Dlx3 or actin (internal controls; Fig. 9A). Transfection studies in the control siRNA and the Smad6 siRNA cell lines using the Esx1 luciferase reporter demonstrated that basal activity of this promoter was elevated (p < 0.05) in the siRNA#1 and siRNA#3 cell lines but not in the control and siRNA#2 cell lines. In response to Dlx3, Esx1 luciferase promoter activity was enhanced (p < 0.05) in Smad6 siRNA#1 and siRNA#3 cell lines but not in control or siRNA#2 cell lines (control siRNA (5.0-fold) versus siRNA#1 (7.3-fold) and siRNA#3 (8-fold)) in a manner highly correlated with the percentage loss of Smad6 in these cell lines (Fig. 9B). Thus, consistent with the overexpression studies, loss of endogenous Smad6 was highly correlated with enhanced Dlx3-dependent transcription of the Esx1 gene promoter.

DISCUSSION

The present studies provide novel evidence for the functional association between Dlx3, a Distal-less class homeobox factor required for normal placental development in the mouse, and an inhibitory Smad, Smad6. The role of Dlx3 as a transcriptional activator is clear based upon studies in mammalian systems, such as the glycoprotein hormone α subunit gene promoter (14), the

blast-specific but not dependent upon Dlx3 expression (Fig. 8C). The Rous sarcoma virus promoter is constitutively active in trophoblast cells. In contrast to the effects of Smad6 on Dlx3-induced expression of the Esx1 and α subunit promoters, Smad6 had no appreciable effect on the TKDP promoter and
expression of the homeobox factor Esx1 in the Dlx3 null mouse (13), and the role of Dlx3 in the regulation of the osteocalcin gene (27). Moreover, Dlx3 has been linked to transcriptional activation as well as transcriptional repression in Xenopus (reviewed in Ref. 2). The ability of Dlx3 to serve in several different ways as a transcriptional modulator may be best explained by the presence of variable tissue- and cell type-specific binding partners. The present study provides important evidence that Dlx3 and Smad6 form a complex in a yeast system, in mammalian cells in culture, and in vitro using recombinant proteins. Dlx3 and Smad6 also display overlapping expression in the nucleus of differentiated human term placental trophoblasts, suggesting that a functional interaction between these two proteins may be important in vivo. The finding that one Dlx3 interacting partner is an inhibitory Smad may also reflect important cross-talk between the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) signaling system and important developmental determinants that require Dlx3.

Smad proteins are transcriptional regulators that can be subdivided into essentially three classes (reviewed in Refs. 28–30). Regulated Smads, or R-Smads (Smad1, -2, -3, -5, and -8), are substrates of the serine/threonine kinase catalytic activity of TGFβ/BMP type I receptors. Phosphorylation of R-Smads promotes the association with a common Smad, or C-Smad (Smad4), and subsequent nuclear translocation of the R- and C-Smad complex to affect TGFβ/BMP-specific target genes. Others have demonstrated an interaction between Smad4 and Dlx1 in the context of cytokine regulation of hematopoietic cells (31). A third unique subclass of this family is the inhibitory Smads, or I-Smads (Smad6 and -7). In the context of TGFβ/BMP signaling, I-Smads essentially oppose the activity of the stimulatory R- and C-Smads at several levels. I-Smads have been shown to compete for R-Smad binding to the type I TGFβ/BMP receptor, providing an inhibitory mechanism for R-Smad activity (32). In addition, Smad6 has been shown to interfere with the ability of Smad1 to form a complex with Smad4, independent of Smad1 phosphorylation state (33). I-Smads also form complexes with Smurfs, E3 ubiquitin ligases that appear to be involved in I-Smad nuclear export and marking target proteins (e.g. the TGFβ/BMP receptors and I-Smads themselves) for degradation by the proteosome, again contributing to an inhibitory action (28–30).

FIGURE 8. Smad6 attenuates Dlx3-dependent promoter activation in JEG3 cells. A, to determine the levels of ectopic expression of Dlx3 and Smad6 in overexpression studies, JEG3 cells were transiently transfected with expression vectors for Dlx3 (+ Dlx3; 2 μg) or Smad6 (+ Smad6; 2 μg). Whole cell lysates were prepared, and Western blot analyses were carried out using the Dlx3 and Smad6 antibodies. B, transient transfection studies in JEG3 cells were used to investigate the functional relevance of Smad6 on Dlx3-dependent gene expression. Co-transfection of the Esx1 gene promoter-luciferase reporter with Dlx3 (constant dose, 2 μg) and increasing doses of Smad6 expression vector (0, 0.5, 1.0, and 2.0 μg; represented by the gray triangle) resulted in a dose-dependent decrease in expression of the Esx1 luciferase reporter. C, similar studies were carried out using the TKDP1 gene promoter luciferase reporter. As a control for Dlx3 action, the Esx1 promoter was also used. TKDP-1-Luc activity was unaffected by Dlx3 or increasing doses of Smad6 expression vector. Data for all panels are reported as relative luciferase activity standardized for protein from representative studies (n = 3/treatment). For B and C, the designations a, b, and c indicate significant differences (p < 0.05) within the experiment; ns, not significantly different.
In the nuclear compartment, Smad6 appears to recruit the transcriptional co-repressor CtBP to target genes related to BMP action (34). Based upon the present studies, Dlx3 and Smad6 were localized to the nucleus of placental trophoblasts independent of apparent TGFβ/BMP signaling. Smad6 has been shown to serve as a transcriptional co-repressor following interaction with another homeodomain transcription factor, Hox c-8 (35, 36). In these studies, Smad6 complexes with Hox c-8 appeared to be sufficient to recruit histone deacetylase activities to Hox c-8-dependent genes, thus repressing transcription via altered organization of chromatin structure. Our studies suggest that the interaction between Dlx3 and Smad6 may utilize an entirely different mechanism for transcriptional repression in human placental cells. Smad6 interaction clearly resulted in a reduction of Dlx3 DNA binding activity, probably via steric interference. Our results demonstrate that the Smad6 interaction interface includes the first two α helices of the homeodomain. Smad6 interaction would then potentially interfere with Dlx3 DNA binding, since these two α helices are central to DNA binding as well. This mechanism potentially alleviates a need for recruitment of transcriptional co-repressor activities, since a Dlx3-Smad6 complex association may preclude association of Dlx3 with specific target gene promoter elements. Increased Smad6 protein levels using overexpression inhibited Dlx3-dependent gene expression. Conversely, reductions in the expression of endogenous Smad6 using siRNA knockdown provides important evidence that Dlx3-dependent gene expression may be particularly sensitive to relatively modest changes in Smad6 protein levels in placental cells. These complementary approaches underscore the potential importance and sensitivity of Dlx3-dependent genes to Smad6 regulation. This may be largely true of Dlx3 target genes with binding sites that have low relative binding affinity, like Esx1.

The Dlx3 homeodomain is centrally located and serves as a DNA binding domain, one of the defining characteristics of all homeobox transcription factors (37, 38). The domain structure of Dlx3 has been examined in studies defining a bipartite nuclear localization signal (residues 124–150) in a region adjacent to and including a portion of the homeodomain; transcriptional activation domains have been ascribed to residues 1–43 and 189–220; and Ser138 within the homeodomain is an apparent substrate for protein kinase C isozymes (20, 21, 24). The deletion mutagenesis carried out in the present studies defined residues 80–163 within Dlx3 as the Smad6 interaction domain in vitro. This region of Dlx3 contains the first full α helix and approximately two-thirds of helix 2 within the homeodomain. In addition, this interaction interface contains Ser138, a substrate for protein kinase C activity. Several possibilities exist for how Dlx3-Smad6 association may alter DNA binding. Perhaps the most obvious is a steric interference model, since the interaction interface includes a portion of the homeodomain. In addition, the presence of Ser138 in this interaction domain lends itself to potentially interesting speculation. The equivalent of this serine residue is conserved in all six Dlx family members (1), consistent with the observation that, like Dlx3, Dlx4 is also a Smad6-interacting protein. Using recombinant...
Dlx3, Morasso and co-workers (21) demonstrated that phosphorylation at Ser138 by protein kinase C (most strongly by protein kinase Ca) resulted in partial inhibition of Dlx3 DNA binding, consistent with the potential effects of Smad6 on DNA binding. This supports speculation that phosphorylation of Dlx3 at Ser138 may alter or increase Smad6 binding, leading to reduced DNA binding at Dlx3 target genes. Studies are currently under way to address this possibility.

R- and C-Smads appear to be constitutively expressed in most cell types, whereas I-Smads are subject to regulation by a number of growth factors, such as epidermal growth factor, TGFβ, and BMPs. In many cases, the effects of these growth factors are mediated through R- and C-Smad-dependent transcriptional mechanisms. This has led to a hypothesis implicating an intracellular negative feedback loop, whereby positive TGFβ/BMP signals are modulated over time by accumulation of induced I-Smads (reviewed in Ref. 40). Interestingly, Dlx3 expression patterns during Xenopus development depends in part upon BMP signaling gradients (41, 42). Inhibition of these gradients using the BMP receptor antagonist chordin resulted in a dose-sensitive inhibition of Dlx3, Dlx5, and Dlx6 mRNA expression. Dlx3 expression in mouse keratinocytes was also reported to be subject to regulation by BMPs (43) in a manner potentially coordinated with I-Smad expression. This supports speculation that not only might BMP-regulated Smad6 expression serve as a negative feedback mechanism controlling the duration of Smad signaling, but increased BMP-dependent Smad6 expression may lead to important modulation of Dlx3-dependent gene expression. BMPs have been shown to regulate early embryogenesis during the preimplantation period, such that these types of ligands are probably present and important during placental morphogenesis (39).

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REFERENCES
1. Bendall, A. J., and Abate-Shen, C. (2000) Gene (Amst.) 247, 17–31
2. Beanan, M. J., and Sargent, T. D. (2000) Dev. Dyn. 218, 545–553
3. Morasso, M. I., Mahon, K. A., and Sargent, T. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3968–3972
4. Price, J. A., Bowden, D. W., Wright, J. T., Pettenati, M. J., and Hart, T. C. (1998) Hum. Mol. Genet. 7, 563–569
5. Price, J. A., Wright, J. T., Kula, K., Bowden, D. W., and Hart, T. C. (1998) J. Med. Genet. 35, 825–828
6. Haldeman, R. J., Cooper, L. F., Hart, T. C., Phillips, C., Boyd, C., Lester, G. E., and Wright, J. T. (2004) Bone 35, 988–997
7. Dong, J., Amor, D., Aldred, M. J., Gu, T., Escamilla, M., and MacDougall, M. (2005) Am. J. Med. Genet. A 133, 138–141
8. Juriloff, D. M., Harris, M. J., and Brown, C. J. (2001) Mamm. Genome 12, 426–435
9. Clouthier, D. E., Williams, S. C., Yanagisawa, H., Wiedewilt, M., Richardson, J. A., and Yanagisawa, M. (2000) Dev. Biol. 217, 10–24
10. Clouthier, D. E., Williams, S. C., Hammer, R. E., Richardson, J. A., and Yanagisawa, M. (2003) Dev. Biol. 261, 506–519
11. Clouthier, D. E., and Schilling, T. F. (2004) Birth Defects Res. C Embryol. Today 72, 190–199
12. Ivey, K., Tyson, B., Ukidwe, P., McFadden, D. G., Levi, G., Olson, E. N., Srivastava, D., and Wilkie, T. M. (2003) Dev. Biol. 255, 230–237
13. Morasso, M. I., Grinberg, A., Robinson, G., Sargent, T. D., and Mahon, K. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 162–167
14. Roberson, M. S., Meermann, S., Morasso, M. I., Mulvaney-Musa, J. M., and Zhang, T. (2001) J. Biol. Chem. 276, 10016–10024
15. France, J. T., Keelan, J., Song, L., Liddell, H., Zanderigo, A., and Knox, B. (1996) Aust. N. Z. J. Obstet. Gynaecol. 36, 325–330
16. Acevedo, H. F. (2002) J. Exp. Ther. Oncol. 2, 133–145
17. Keay, S. D., Vatish, M., Karteris, E., Hillhouse, E. W., and Randeva, H. S. (2004) Br. J. Obstet. Gynaecol. 111, 1218–1228
18. Holland, M. P., Bliss, S. P., Berghorn, K. A., and Roberson, M. S. (2004) Endocrinology 145, 1096–1105
19. Roberson, M. S., Ban, M., Zhang, T., and Mulvaney, J. M. (2000) Mol. Cell. Biol. 20, 3331–3344
20. Bryan, J. T., and Morasso, M. I. (2000) J. Cell Sci. 113, 4013–4023
21. Park, G. T., Denning, M. F., and Morasso, M. I. (2001) FEBS Lett. 496, 60–65
22. Quinn, L. M., Johnson, B. V., Nicholl, J., Sutherland, G. R., and Kalionis, B. (1997) Gene (Amst.) 187, 55–61
23. Li, Y., Lemaire, P., and Behringer, R. R. (1997) Dev. Biol. 188, 85–95
24. Feledy, J. A., Morasso, M. I., Jang, S. I., and Sargent, T. D. (1999) Nucleic Acids Res. 27, 764–770
25. MacLean, J. A., Roberts, R. M., and Green, J. A. (2004) Biol. Reprod. 71, 455–463
26. MacLean, J. A., Chakrabarty, A., Xie, S., Bixby, J. A., Roberts, R. M., and Green, J. A. (2003) Mol. Reprod. Dev. 65, 30–40
27. Hassan, M. Q., Iaved, A., Morasso, M. I., Karlin, J., Montecino, M., van Wijnen, A. J., Stein, G. S., Stein, J. L., and Lian, J. B. (2004) Mol. Cell. Biol. 24, 9248–9261
28. Ten, D. P., Miyazono, K., and Heldin, C. H. (2000) Trends Biochem. Sci. 25, 64–70
29. Ten, D. P., and Hill, C. S. (2004) Trends Biochem. Sci. 29, 265–273
30. Chen, D., Zhao, M., and Mundy, G. R. (2004) Growth Factors 22, 233–241
31. Chiba, S., Takeshita, K., Imai, Y., Kumano, K., Kurokawa, M., Masuda, S., Shimizu, K., Nakamura, S., Ruddle, F. H., and Hirai, H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15577–15582
32. Shi, Y., and Massague, J. (2003) Cell 113, 685–700
33. Hata, A., Lagna, G., Massague, J., and Hemmatti-Brivanlou, A. (1998) Genes Dev. 12, 186–197
34. Lin, X., Liang, Y. Y., Sun, B., Liang, M., Shi, Y., Brunicardi, F. C., Shi, Y., and Feng, X. H. (2003) Mol. Cell. Biol. 23, 9081–9093
35. Bai, S., Shi, X., Yang, X., and Cao, X. (2000) J. Biol. Chem. 275, 8267–8270
36. Bai, S., and Cao, X. (2002) J. Biol. Chem. 277, 4176–4182
37. Gehring, W. J. (1993) Gene (Amst.) 133, 215–221
38. Wright, C. V., Cho, K. W., Oliver, G., and De Robertis, E. M. (1989) Trends Biochem. Sci. 14, 52–56
39. Paria, B. C., Ma, W., Tan, J., Raja, S., Das, S. K., Dey, S. K., and Hogan, B. L. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1047–1052
40. Miyazono, K. (2002) Cytokine Growth Factor Rev. 13, 7–9
41. Feledy, J. A., Beanan, M. J., Sandoval, J. J., Goodrich, J. S., Lim, J. H., Matsuo-Takasaki, M., Sato, S. M., and Sargent, T. D. (1999) Dev. Biol. 212, 455–464
42. Luo, T., Matsuo-Takasaki, M., Lim, J. H., and Sargent, T. D. (2001) Int. J. Dev. Biol. 45, 681–684
43. Park, G. T., and Morasso, M. I. (2002) Nucleic Acids Res. 30, 515–522