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BMPER, a Novel Endothelial Cell Precursor-Derived Protein, Antagonizes Bone Morphogenetic Protein Signaling and Endothelial Cell Differentiation

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The development of endothelial cell precursors is essential for vasculogenesis. We screened for differentially expressed transcripts in endothelial cell precursors in developing mouse embryoid bodies. We cloned a complete cDNA encoding a protein that contains an amino-terminal signal peptide, five cysteine-rich domains, a von Willebrand D domain, and a trypsin inhibitor domain. We termed this protein BMPER (bone morphogenetic protein [BMP]-binding endothelial cell precursor-derived regulator). BMPER is specifically expressed in flk-1-positive cells and parallels the time course of flk-1 induction in these cells. In situ hybridization in mouse embryos demonstrates dorsal midline staining and staining of the aorto-gonadal-mesonephric region, which is known to host vascular precursor cells. BMPER is a secreted protein that directly interacts with BMP2, BMP4, and BMP6 and antagonizes BMP4-dependent Smad5 activation. In *Xenopus* embryos, ventral injection of BMPER mRNA results in axis duplication and downregulation of the expression of Xvent-1 (downstream target of Smad signaling). In an embryoid body differentiation assay, BMP4-dependent differentiation of endothelial cells in embryoid bodies is also antagonized by BMPER. Taken together, our data indicate that BMPER is a novel BMP-binding protein that is expressed by endothelial cell precursors, has BMP-antagonizing activity, and may play a role in endothelial cell differentiation by modulating local BMP activity.

Vasculogenesis, the formation of blood vessels de novo from precursor cells, is the first means by which the vascular system forms during embryogenesis; many of the events in these early stages of vascular development are recapitulated in adult vascular outgrowth and disease. The first discernible event in the vasculogenic process is the differentiation of endothelial cells from mesoderm-derived precursors; failure of vascular growth and developmental arrest occur in the absence of endothelial cell differentiation (57). Although poorly understood, the early cues that trigger endothelial cell differentiation from their immediate progenitors affect many subsequent morphogenic and organogenic events in the embryo.

During the earliest stages of mammalian development, endothelial cells arise from precursors located both in the extraembryonic compartment and within the embryo proper. Differentiation of endothelial cells from precursors occurs in close proximity to primitive hematopoeitic cells in structures called blood islands, and it is widely accepted that a population of bipotential cells in the early developing embryo can give rise to both hematopoietic (32) and endothelial (71) cell lineages (and possibly also some smooth muscle cell populations as well [20, 27; P. Carmeliet, Comment, Nature **408:**43, 45, 2000]). These cells are termed hemangioblasts and can be identified by the presence of fetal liver kinase 1 (flk-1, also known as vascular endothelial cell growth factor receptor 2) and other markers (46). Persistent expression of flk-1 in these cells is associated with differentiation along the endothelial cell lineage, whereas loss of flk-1 expression characterizes differentiating hematopoietic progeny (70). During mouse embryonic development, hemangioblasts are initially located in the aorto-gonadal-mesonephric (AGM) region and in the yolk sac. Yolk sac-derived hemangioblasts are the source of primitive hematopoietic and endothelial cells that express some but not all markers of mature endothelium and that form a primitive vasculature (11, 12). Precursor cells within the AGM region are also fated to give rise to hematopoietic and endothelial cell lineages (47). The AGM region hosts hemangioblasts (54); it is a source of the definitive hematopoietic system and contributes to the definitive vasculature in the mouse embryo (40). The molecular events that determine the generation and differentiation of endothelial cell precursors are not well defined at present.

A number of signaling pathways downstream of endothelial cell differentiation—notably, those activated by members of the vascular endothelial cell growth factor (VEGF) and angiopoietin family—have been implicated in vascular patterning (22). In addition, positive and negative cell surface markers are available for the characterization of embryonic endothelial cells and their precursors; among them are flk-1 (which decorates hemangioblasts and all endothelial cells) and PECAM and VE-cadherin (which identify more mature endothelial cells) (18). In contrast, upstream signaling molecules and transcription factors that trigger mesodermal differentiation toward the vascular endothelial cell lineage are less well understood. Notably, recent studies have indicated a necessary role for Indian hedgehog, a secreted ligand for the Patched recep-

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tor, in blood island formation and endothelial cell precursor specification (8, 19); however, the effects of Indian hedgehog are not specific to the vasculature, and other signals must surely cooperate with Indian hedgehog signaling to establish the cues necessary to recruit vascular progenitors from their mesodermal precursors.

Bone morphogenetic proteins (BMPs) represent another family of signaling molecules that have been implicated, albeit less directly, in hematopoietic and endothelial cell differentiation. BMPs are members of the transforming growth factor β (TGF-β) superfamily of proteins, which consists of more than 30 members classified into several subgroups according to their structural features. Signaling events mediated by these proteins are tightly regulated and play crucial roles in embryonic development. In *Drosophila*, Decapentaplegic (Dpp), the homolog of vertebrate BMP2 and BMP4, and its specific antagonist, Short gastrulation (Sog), the homolog of vertebrate Chordin (Chd), form a gradient that is necessary for dorsal-ventral patterning of the embryo (21). In vertebrates, BMPs were initially described by their ability to induce ectopic bone and cartilage formation (64). However, BMP expression studies and loss-of-function mouse models have demonstrated a broad range of activities of the different BMP family members for various cell types. BMP signaling plays an essential role in left-right asymmetry of the embryo, neurogenesis, mesoderm patterning, and organogenesis (4). BMP2 or BMP4 is required for the genesis of hematopoietic cell lineages in *Xenopus* (37, 66), and BMP4 cooperates with VEGF to specify lymphohematopoietic cell development in mouse embryonic stem (ES) cells (44). In addition, recent studies have implicated BMPs in angiogenesis through a VEGF-dependent pathway (17).

Because so little is known about the early events in endothelial cell specification, we have sought to identify their molecular determinants using an inductive approach. We have used differentiating mouse ES cell-derived embryoid bodies grown in attached cultures as a model of blood vessel development; the anatomic and molecular events in embryonic endothelial cell differentiation and blood vessel development are faithfully recapitulated in this model (65, 67). In this report, we describe the cloning and characterization of BMP-binding endothelial cell precursor-derived regulator (BMPER), which binds BMP4 and inhibits BMP-dependent developmental events in several models, including Smad activation, mesoderm specification, and endothelial cell differentiation.

MATERIALS AND METHODS

Cells and reagents. Recombinant BMP4, BMP2, and BMP6 and the BMP4-blocking antibody MAB757 were obtained from R&D Systems. The anti-BMP4 antibody used for Western blotting was obtained from Chemicon. Phycoerythrin (PE)-conjugated anti-flk-1 antibody, fluorescein isothiocyanate-conjugated anti-immunoglobulin G2 antibody, and purified anti-VE-cadherin antibody were purchased from Becton Dickinson. Anti-Myc antibody 9E10 was obtained from Santa Cruz Biotechnology.

ES cell culture. R1 mouse ES cells were grown on gelatinized tissue culture plates with maintenance medium containing conditioned medium from human 5637 bladder carcinoma cells. Differentiation was initiated by harvesting ES cell aggregates with dispase and culturing embryoid bodies in differentiation medium (Dulbecco modified Eagle medium with 10% fetal calf serum and 50 μM 2-mercaptoethanol). Cells were initially grown in bacterial dishes to allow for the formation of cystic embryoid bodies and were plated on tissue culture dishes after 60 h for attachment. Attached embryoid bodies were grown under these conditions for various times (5).

For BMP-dependent ES cell differentiation assays, R1 cells were grown under conditions in which embryoid bodies survive but significant endothelial cell differentiation occurs only after stimulation with growth factors (2, 43). After commencement of differentiation, ES cells were grown in Dulbecco modified Eagle medium supplemented with 10% knockout SR serum replacement (Gibco) in bacterial dishes. After 48 h, cells were placed in six-well tissue culture dishes with fresh medium and transfected with pSecTag2BMPER (see below) or control plasmid by using the Fugene-6 method. Recombinant BMP4 or BMP4 preincubated with neutralizing antibody MAB757 was added to the dishes 24 h after transfection. On day 5 of differentiation, the medium was replaced, and BMP4 or BMP4-MAB757 was added again. On day 7, the embryoid bodies were dissolved to single-cell suspensions by using trypsin and were analyzed by flow cytometry.

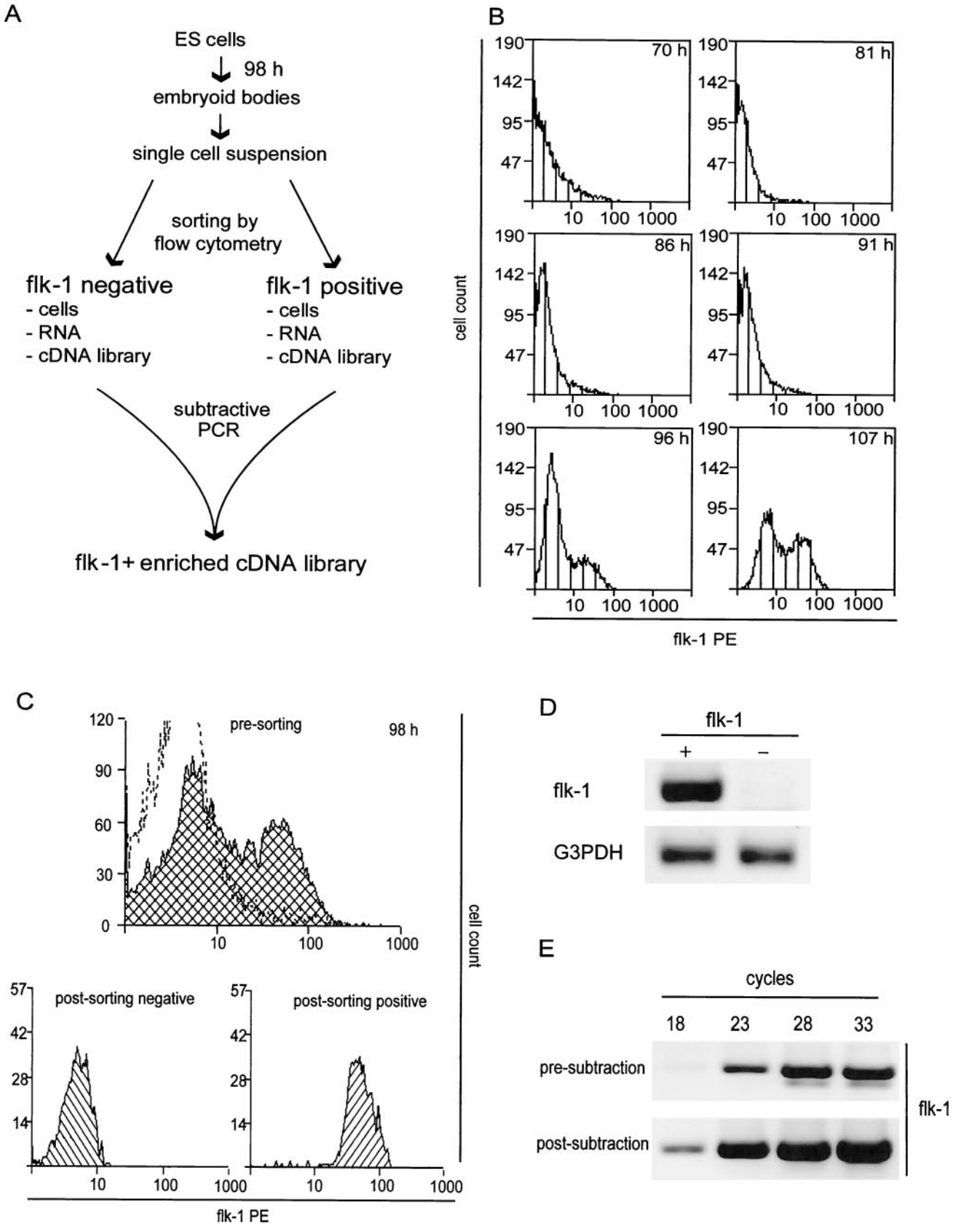
Flow cytometry and cell sorting. ES cells were harvested with trypsin, washed in phosphate-buffered saline (PBS)–0.1% bovine serum albumin (BSA), and passed through a 40-μm cell strainer. Cells were stained with the appropriate antibody for 30 min, washed twice in PBS–0.1% BSA, and analyzed by using a Becton Dickinson FACScan with Cytomation Summit software. For cell sorting experiments, 10^7 cells were harvested in a similar manner, stained with PE-conjugated anti-flk-1 antibody, washed, and sorted into the respective positive or negative population by using a MoFlo instrument (Cytomation).

RNA preparation, Northern blotting, and PCR. Total RNA from cells in culture or sorted cell suspensions was prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride (53).

The quality of the RNA was analyzed by agarose-formaldehyde gel electrophoresis, and quantification was performed spectrophotometrically. For Northern blotting, total RNA was separated by agarose-formaldehyde gel electrophoresis and transferred to a nitrocellulose membrane by capillary action (53). The membrane was subjected to hybridization in hybridization buffer (Quickhyb; Stratagene) with [^{32}P]dCTP randomly labeled cDNA fragments and was exposed to film. An adult mouse multiorgan blot was purchased from OriGene Technologies. Semiquantitative PCR was performed with the following primers: flk-1—forward, 5′-GGACTTCCTGACCTTGGAGC-3′, and reverse, 5′-GGGGTAGT GTAGTCAGGAGC-3′; glyceraldehyde-3-phosphate dehydrogenase (G3PDH)—forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACC CTGTTGCTGTA-3′; and BMPER—forward, 5′-CCCGGCTGAGCCATGTG TCC-3′, and reverse, 5′-CAGCTCCACAGACTTGGTCC-3′.

cDNA library cloning and subtractive PCR. flk-1-positive and flk-1-negative cDNA libraries were cloned by using a modification of Clontech SMART technology. Reverse transcription of total RNA from sorted ES cells was performed with a modified oligo-dT primer containing an internal priming site, and an asymmetrical primer site was attached at the 5′ end of the reverse transcription product. The cDNA libraries were amplified by PCR with the internal priming site. An flk-1-enriched cDNA library was created by subtracting the flk-1-negative library from the flk-1-positive library by using subtractive PCR technology (PCR-SELECT; Clontech). The resulting cDNA library was cloned into vector pCR2.1 (Invitrogen), transformed into *Escherichia coli*, plated on Luria-Bertani–ampicillin agar, and screened for inserts with blue-white color selection. White colonies were grown in 96-well plates and used as a template for insert amplification by PCR. PCR products were spotted to create two identical nylon membranes and were hybridized separately with [^{32}P]dCTP randomly labeled probes derived from the flk-1-positive and flk-1-negative sorted cDNA libraries. Differentially expressed clones were sequenced. The full-length BMPER cDNA fragment was cloned by 3′ and 5′ rapid amplification of cDNA ends with adult mouse lung RNA as a template. For mammalian expression, the coding sequence, without the signal peptide, was cloned into pSecTag2 (Invitrogen) to create pSecTag2BMPER, which contains a carboxy-terminal Myc tag for immunodetection and the mouse kappa-chain immunoglobulin signal sequence at the amino terminus for efficient secretion (35).

Western blotting, protein purification, and coimmunoprecipitation. COS-7 cells were transfected with pSecTag2BMPER by using the Fugene-6 method to obtain Myc-tagged protein. At 24 h after transfection, the medium was changed to OptiMEM-1, and cell supernatants were collected. To obtain cell lysates, cells were washed with PBS and harvested in radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EGTA, 1% Igepal, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate). Cell supernatants were concentrated with Amicon Centriplus columns and analyzed by gel electrophoresis. Empty vector transfections were used as controls. For BMP4 coimmunoprecipitation, samples were immunoprecipitated with an anti-Myc–agarose conjugate for 2 h at room temperature in immunoprecipitation (IP) binding buffer {150 mM NaCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 0.1% deoxycholate, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5% glycerol, 0.1% BSA}. Samples were washed three times in IP binding buffer, pellets were resuspended in IP binding buffer, and BMP4 (5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nM) and/or competing proteins (125 nM) were added for 2 h of incubation. Samples were washed again three times in IP binding buffer without BSA and an additional three times in radioimmunoprecipitation buffer for the competition experiment and resuspended in 6× loading buffer (0.1 M Tris-HCl, 30% glycerol, 4% SDS, 0.05%, bromophenol blue, 1 M β-mercaptoethanol), and Western blotting was performed.

Reporter gene assay. A pGL2-based reporter construct containing an Smad5-binding sequence (3GC2) upstream of the luciferase reporter gene was kindly provided by M. Kato (26). 293T cells grown in six-well plates were transfected with the reporter gene and pSecTag2BMPER or empty vector (control). BMP4 (final concentration, 0.5 nM) was added at 18 h after transfection. At 38 h after transfection, cells were harvested in reporter lysis buffer (Promega), and luciferase activity was analyzed by luminometry with a luciferase assay system (Promega). Each condition was analyzed with at least six independent samples, and each sample was analyzed in triplicate.

In situ hybridization. Embryos were collected at different times from pregnant CD-1 mice. After fixation in 4% paraformaldehyde, embryos were stained with a digoxigenin-labeled antisense RNA probe or a respective sense control. Digoxigenin was detected with an alkaline phosphatase-conjugated antibody (Roche), and the reaction was developed by adding nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphate toluidine salt (Promega). For in situ analysis of sections, whole embryos were fixed as described above, embedded in paraffin, sectioned at 10 μm, and placed on frosted slides before staining was performed (7).

***Xenopus laevis* embryos and microinjections.** *X. laevis* embryos were obtained by in vitro fertilization (59) and dejellied by cysteine treatment (0.1× MBS [8.8 mM NaCl, 0.1 mM KCl, 0.07 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.5 mM HEPES, 0.25 mM $NaHCO_3$, pH 7.5] –2% L-cysteine hydrochloride [pH 8.1] for 2 min; then 10 rinses with 0.1× MBS) about 1 h after cortical rotation. Embryos for microinjection were transferred to 1× MBS containing 4% Ficoll type 400 (Sigma) (10). Full-length capped sense mRNA of BMPER and *X. laevis* BMP4 (pSP64) were synthesized by using T7 and SP6 RNA polymerases, respectively (mMESSAGE mMACHINE; Ambion). For misexpression studies, 420 or 840 pg of the BMPER transcript and 50 pg of the BMP4 transcript were injected into the marginal zone of either two ventral or two dorsal cells at the four-cell stage close to the first cleavage furrow, either alone or in combination, by using a nitrogen pressure-driven system (Narishige IM-300). The embryos were transferred to 0.1× MBS 2 h after injection, allowed to develop to the desired stages, and scored for secondary axis formation or the development of dorsoanterior structures (dorsoanterior index [33]) at stage 14 or 32. The embryos were staged as described by Nieuwkoop and Faber (45). The results (see Fig. 8A) are combined totals from two independent experiments.

***X. laevis* animal cap assay and RT-PCR.** For the animal cap assay, 840 pg of in vitro-transcribed BMPER capped mRNA was injected into each of the four animal blastomeres of eight-cell-stage embryos. Animal caps of injected and noninjected embryos were explanted at the blastula stage (stage 8), cultured in 1× MBS with activin A (5 U/ml), and harvested at stage 10 (51). Total RNA from *Xenopus* embryo caps was prepared by using a High Pure RNA kit (Roche). Residual DNA was digested with DNase I, and RNA was quantified. For first-strand synthesis, equal amounts of RNA were reverse transcribed with SuperScript reverse transcriptase (RT) (Invitrogen) according to the manufacturer's instructions and with a random octamer primer mixture (Sigma). The PCR was performed with *Taq* polymerase (Clontech Titanium) and with primers for *X. laevis* transcripts for histone H4, Brachyury, Xvent-1, Chordin, and BMP4 (primer sequences were from http://www.hhmi.ucla.edu/derobertis). Histone H4 was included in the RT-PCR as a loading control.

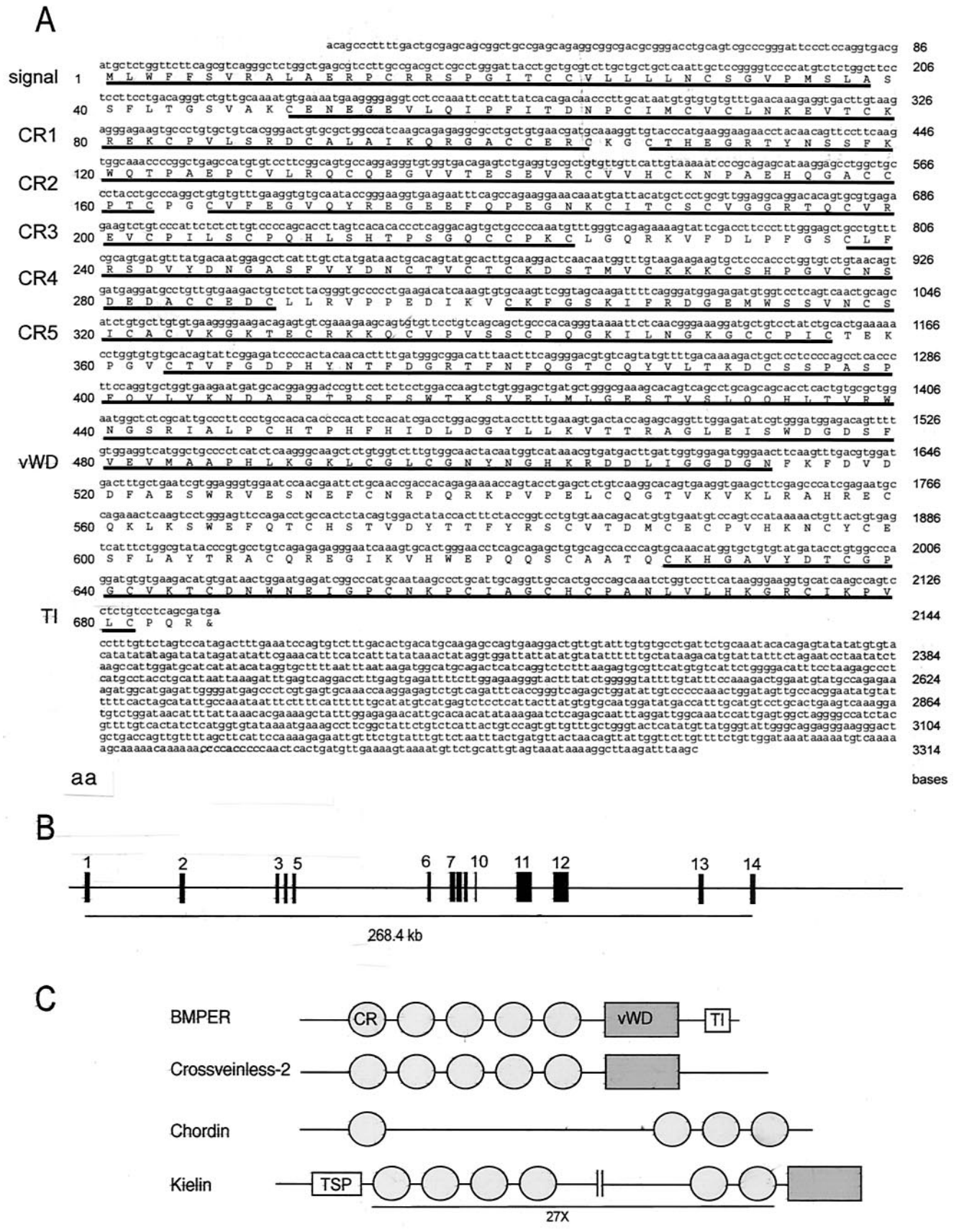
Nucleotide sequence accession number. The BMPER full-length cDNA sequence has been deposited in GenBank under accession number AY263358.

RESULTS

We used an unbiased screen to identify genes potentially involved in the earliest stages of endothelial cell differentiation. This screen relied on the differentiation of ES cells, the enrichment of endothelial cell precursor-specific cDNAs, and molecular cloning (summarized in Fig. 1A). R1 mouse ES cells were differentiated under conditions optimized for the development of vascular precursor cells. Similar to previous reports (32), we found with this model system that endothelial cell progenitors identified by flk-1 expression were not detected in appreciable quantities until 96 h after the initiation of differentiation; also, their abundance as a percentage of total cells peaked at 40% after 107 h (Fig. 1B), indicating rapid and coordinated differentiation of endothelial cell precursors. At the time of early—but significant—flk-1 expression (98 h), ES cells were sorted into flk-1-positive and flk-1-negative fractions (Fig. 1C); cDNA libraries were prepared from the respective sorted cell populations. The population of flk-1-negative cells could be separated from the population of flk-1-positive cells with only minimal amounts of cross-contamination, confirming successful cell sorting according to flk-1 status (Fig. 1D).

To further enrich for differentially expressed transcripts in the flk-1-positive population, we performed a subtractive PCR procedure that resulted in over 30-fold enrichment of flk-1 transcripts (Fig. 1E). Approximately 600 differentially expressed clones were analyzed, and among them we identified a sequence for a previously undescribed cDNA (other transcripts identified in this screen will be reported elsewhere). An initial 1.2-kb *Rsa*I-*Rsa*I fragment of this sequence obtained from the library was extended by successive rounds of 5′ and 3′ rapid amplification of cDNA ends to complete a cDNA of 3,314 bp (Fig. 2A). The cDNA contains a single open reading frame that initiates at a consensus Kozak sequence and that encodes a protein of 685 amino acids and with a predicted molecular mass of 76.1 kDa. Analysis of the predicted protein by comparison with the Pfam database (http://www.sanger.ac.uk/Software/Pfam/) and the CBS signalP database (http://www.cbs.dtu.dk/services/SignalP/) indicated the presence of a 39-amino-acid signal peptide at the amino terminus, five tandem cysteine-rich (CR) von Willebrand C-like domains, a single von Willebrand D domain, and a sequence with homology to the family of trypsin inhibitors at the carboxyl terminus. These domains are all characteristic of secreted proteins; in particular, the presence of tandem CR domains is typical of BMP-binding proteins, such as Chordin. The gene is located on mouse chromosome 9A4 and contains 14 exons spanning 268.4 kb (Fig. 2B). A BLAST search indicated that the protein most closely related to this sequence is encoded by the *Drosophila*

FIG. 1. Screening for differentially expressed cDNAs in endothelial cell precursors. (A) Outline of the screening strategy. (B) Time course of flk-1 expression in embryoid body cultures. Hours are given to indicate the time after the initiation of differentiation. Single-cell solutions of embryoid bodies were stained with PE-conjugated anti-flk-1 antibody. Subsequent studies were performed to coincide with the initial wave of flk-1 cell surface expression. (C) Separation of flk-1-positive endothelial cell precursors from flk-1-negative cells by sorting of a mixed single-cell solution of embryoid body cultures at 98 h. Reanalysis demonstrated near homogeneity of the postsorting populations. (D) PCR (25 cycles) for flk-1 with cloned cDNA libraries from flk-1-positive or -negative cells as a template. Highly efficient enrichment of flk-1 was seen. G3PDH was amplified as a control. (E) PCR for flk-1 with the flk-1-positive cDNA library before (upper panel) or after (lower panel) subtraction of the flk-1-negative library as a template. After subtraction, flk-1 was enriched approximately 32-fold (2^5).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gene *crossveinless-2* (GenBank accession number AF288223) (14). Crossveinless-2 interacts genetically with the BMP signaling pathway during fly wing development and contains both CR and von Willebrand D domains but lacks the trypsin inhibitor domain. Based on our analysis described below, we have called this mouse sequence BMPER.

RT-PCR confirmed strong preferential expression of this cDNA in flk-1-positive cells derived from embryoid bodies; expression was below the lower limits of detection in cells not expressing flk-1 (Fig. 3A). The time course of BMPER expression during ES cell differentiation was examined by Northern blot hybridization of a BMPER-specific probe to RNA obtained at different times during ES cell differentiation (Fig. 3B). A single BMPER transcript of 3.5 kb was detected at low levels in undifferentiated ES cells but was strongly downregulated coincident with the initiation of differentiation. The highest levels of BMPER mRNA were detected at day 5 of embryoid body development, and expression slowly decreased through day 9. Although flk-1 is not expressed in undifferentiated R1 ES cells, the pattern of flk-1 induction during differentiation mirrored that of BMPER, consistent with the preferential expression of BMPER in flk-1-expressing cells (Fig. 3B).

The tissue expression of BMPER in adult mice was measured by Northern blot analysis. mRNA expression was highest in the heart, lungs, and skin; lower expression was detected in the brain (Fig. 3C). Other organs (including the thymus and spleen, which are enriched in cells of hematopoietic origin) do not express BMPER at detectable levels. The anatomical distribution of BMPER mRNA expression in embryos was mapped by in situ hybridization. We observed a highly dynamic pattern of expression of BMPER mRNA, which is common during embryonic development. Analysis of mouse embryos at day 9.5 showed characteristic dorsal midline staining with a predominance in the most rostral region of the telencephalon (Fig. 4A). A substantially weaker dorsal midline signal was detected in the distal part of the tail (data not shown). Sections of the same embryo showed a sharply restricted midline signal in the cell layer adjacent to the telencephalic vesicle, corresponding to the lamina reuniens (Fig. 4B). Additionally, at the levels of somites 8 to 12, a migrating population of branchial neural crest cells ventrolateral to the neural tube and dorsal to Rathke's pouch stained positive (Fig. 4C).

At day 10.5, the midline staining was no longer detectable in whole-mount embryos. Instead, a BMPER signal could be assigned to a symmetrical region ventrolateral to the dorsal aorta by in situ hybridization of embryonic sections (Fig. 4E and G). This anatomical region has been described elsewhere as the AGM region (40, 41). A ring-shaped layer of cells around the dorsal aorta, which are presumptive endothelial cells, was also stained. Scattered adjacent mesenchymal or migrating cells also expressed BMPER. Hybridization with sense mRNA demonstrated the specificity of this expression pattern (Fig. 4F and H).

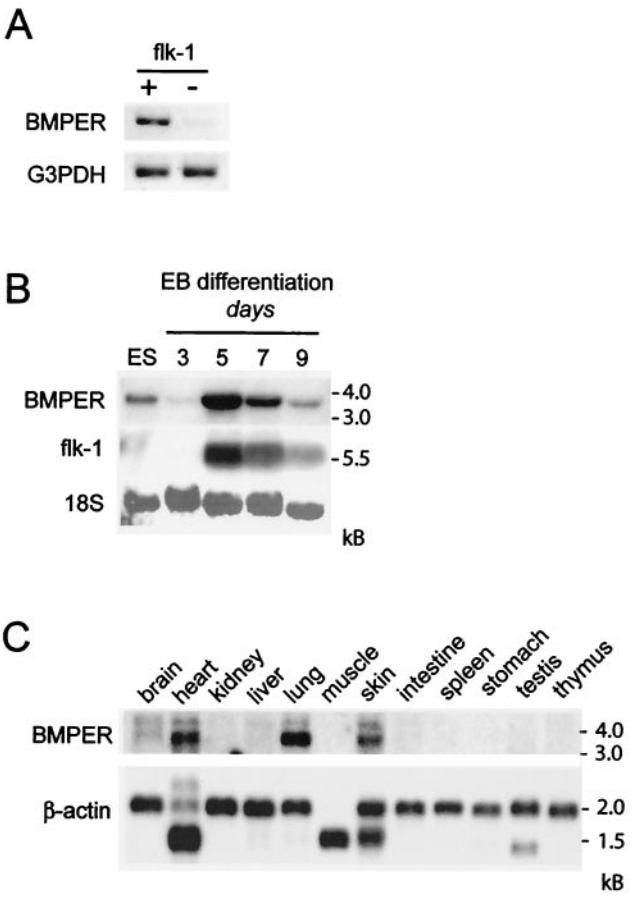
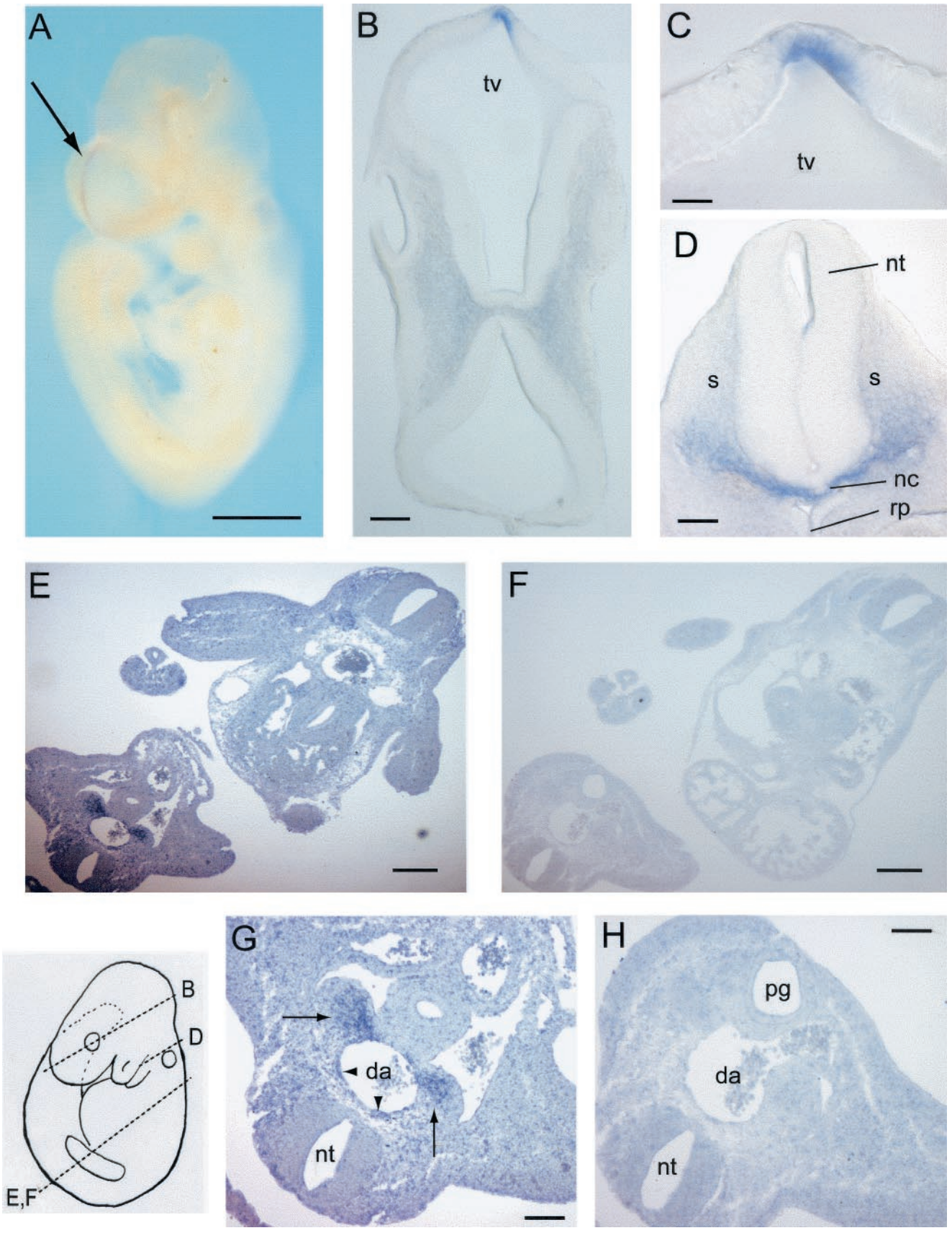


FIG. 3. BMPER mRNA expression in differentiating embryoid bodies and adult tissue. (A) Expression of BMPER in flk-1-positive and flk-1-negative cells sorted from differentiating embryoid bodies, as determined by semiquantitative PCR. (B) Northern analysis with cDNAs for BMPER, flk-1, and 18S rRNA (as an indicator of RNA loading) to probe total RNA obtained from embryoid body (EB) cultures on the indicated days of differentiation or from undifferentiated ES cells (ES). After hybridization with BMPER, a single band of 3.5 kb was visible. (C) Northern analysis with a BMPER cDNA probe of polyadenylated RNA from the indicated adult mouse tissues. Filters were probed with β-actin to visualize RNA loading. BMPER mRNA expression was highest in the heart, lungs, skin, and brain.

BMPER RNA was also detected in yolk sacs. At day 9.5, BMPER transcripts were expressed in a reticular staining pattern consistent with staining of the developing yolk sac vasculature (Fig. 5A) (48, 63). In yolk sac sections at day 10.5, BMPER was detected in endothelial cells lining the inner layer of the vascular wall (Fig. 5B). BMPER RNA was also detected

FIG. 2. Cloning and analysis of the BMPER cDNA and gene. (A) Nucleotide (upper row) and deduced amino acid (lower row) sequences. The open reading frame contains 2,055 bases coding for 685 amino acids. The predicted molecular mass is 76.1 kDa. The hydrophobic signal sequence, CR domains 1 to 5, a partial von Willebrand D (vWD) domain, and a trypsin inhibitor (TI) domain are underlined. (B) Exon-intron organization of the gene locus on mouse chromosome 9A4. (C) Structures of Crossveinless-2, Chordin, and Kielin in comparison to that of BMPER. TSP, thrombospondin domain.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in cell lines derived from yolk sac (C166) and intraembryonic (MEC) endothelial cells (Fig. 5C). Taken together, these analyses indicate that BMPER is expressed in primitive members of the endothelial cell lineage in both the embryonic and the extraembryonic compartments.

The significance of staining within the telencephalon at day 9.5 is uncertain, although BMP4 signaling has been linked to midline pattern formation in this region by others (15). Likewise, BMPs control the fates of neural crest cells, such as those expressing BMPER at day 9.5 (56). Most remarkably, the expression of BMPER in endothelial cell precursor-enriched regions of the embryo is consistent with the preferential expression of BMPER in flk-1-positive cells in embryoid bodies. The observation that BMPER is expressed at the right place and time to affect endothelial cell differentiation provided the impetus for further analysis of the cellular and developmental effects of BMPER.

Expression of BMPER in cultured mammalian cells (as a carboxy-terminal Myc-tagged protein) resulted in the detection of a protein with the appropriate molecular mass in cell lysates (Fig. 6A). The full-length protein was also detected in cell supernatants, as was a smaller protein, of about 42 kDa, which likely represents a carboxy-terminal proteolytic fragment, as has been described for other BMP-associated secreted proteins, such as Chordin (36) and Chordin-like protein (43). The presence of this fragment only in the secreted fraction suggests that cleavage is dependent on an extracellular protease. Based on the sequence similarity between BMPER and other BMP-binding proteins, we tested the association of BMPER with BMP4 and found that recombinant BMP4 could indeed be efficiently and specifically immunoprecipitated by Myc-tagged BMPER that was immunopurified after expression in COS-7 cells (Fig. 6B). We used competition assays to determine whether the related BMPs BMP2 and BMP6, which overlap in expression with BMPER along the dorsal midline (34), could also bind BMPER (1); both of these proteins in a 25-fold excess efficiently competed for binding between BMPER and BMP4 (Fig. 6C), indicating that BMPER may also bind these proteins. Bovine fibroblast growth factor in similar concentrations did not compete for BMPER binding, demonstrating the specificity of this assay.

Based on the association of BMPER with BMP4, we used several complementary assays to determine the extent to which BMPER modulates BMP activity. As a first test, we examined the activation of a luciferase reporter gene under the control of an Smad5-binding sequence that is known to be BMP4 responsive. Stimulation of 293T cells with BMP4 resulted in approximately 3.5-fold-increased luciferase activity (Fig. 7), consistent with previous observations (26). The expression of BMPER by itself had little effect on Smad5 reporter activity. However, BMP-dependent activation of this reporter was almost completely inhibited by coexpression of BMPER. This effect occurred in a dose-dependent fashion, and BMPER had no activity for a luciferase reporter construct lacking the Smad5-binding sequence, either with or without BMP4 (data not shown). Because Smad5 binds to its DNA-binding site in response to the activation of BMP4 signaling pathways, these results provide evidence that BMPER has antagonizing effects on BMP4 activity and its downstream Smad signaling pathways.

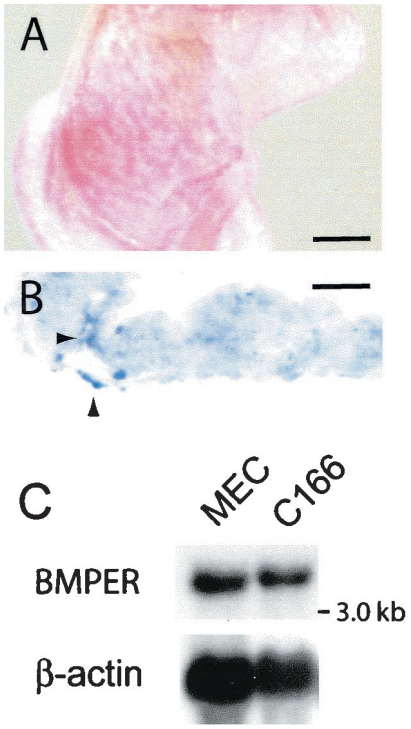

FIG. 5. BMPER expression in extraembryonic tissues and endothelial cell lines. (A) Whole-mount in situ hybridization of a yolk sac at day 9.5. Bar, 200 μm. (B) In situ hybridization of a sectioned yolk sac at day 10.5. Arrowheads indicate BMPER-positive cells. Bar, 70 μm. (C) Northern analysis of total RNA from cultured mouse intraembryonic endothelial cells (MEC) and cultured mouse yolk sac-derived embryonic endothelial cells (C166) with a BMPER cDNA probe.

We next investigated the effects of BMPER on BMP signaling in *Xenopus* based on the conservation of dorsal-ventral patterning events during early stages of development. Past studies showed that several secreted molecules expressed in the *Xenopus* organizer (such as Noggin, Chordin, and Follista-

FIG. 4. Localization of BMPER mRNA in developing mouse embryos. (A) Whole-mount in situ hybridization of a mouse day 9.5 embryo with a digoxigenin-labeled BMPER antisense cDNA probe. Note the midline staining in the rostral telencephalon (arrow). Bar, 500 μm. (B) Transverse section from the same embryo at the level indicated in the diagram at the lower left. Bar, 80 μm. tv, telencephalic vesicle. (C) Magnification of the frontal area from panel B. Bar, 20 μm. (D) Transverse section of the embryo shown in panel A at the level indicated in the diagram. Bar, 80 μm. nt, neural tube; rp, Rathke's pouch; s, somite; nc, notochord. Cells that appear to be migrating ventrally toward the branchial arch and heart region express BMPER. (E to H) In situ hybridization of sections from a day 10.5 mouse embryo. Arrows indicate the AGM region. (E and F) Overview of BMPER staining (E) versus that of the sense control (F) at the level indicated in the diagram. Bar, 400 μm. (G) Magnification of panel E. Bar, 150 μm. da, dorsal aorta. Note the staining of cells ventrolateral to the dorsal aorta and a ring-shaped layer of stained cells around the dorsal aorta (arrowheads). (H) Magnification of panel F. Bar, 50 μm. pg, primitive gut.

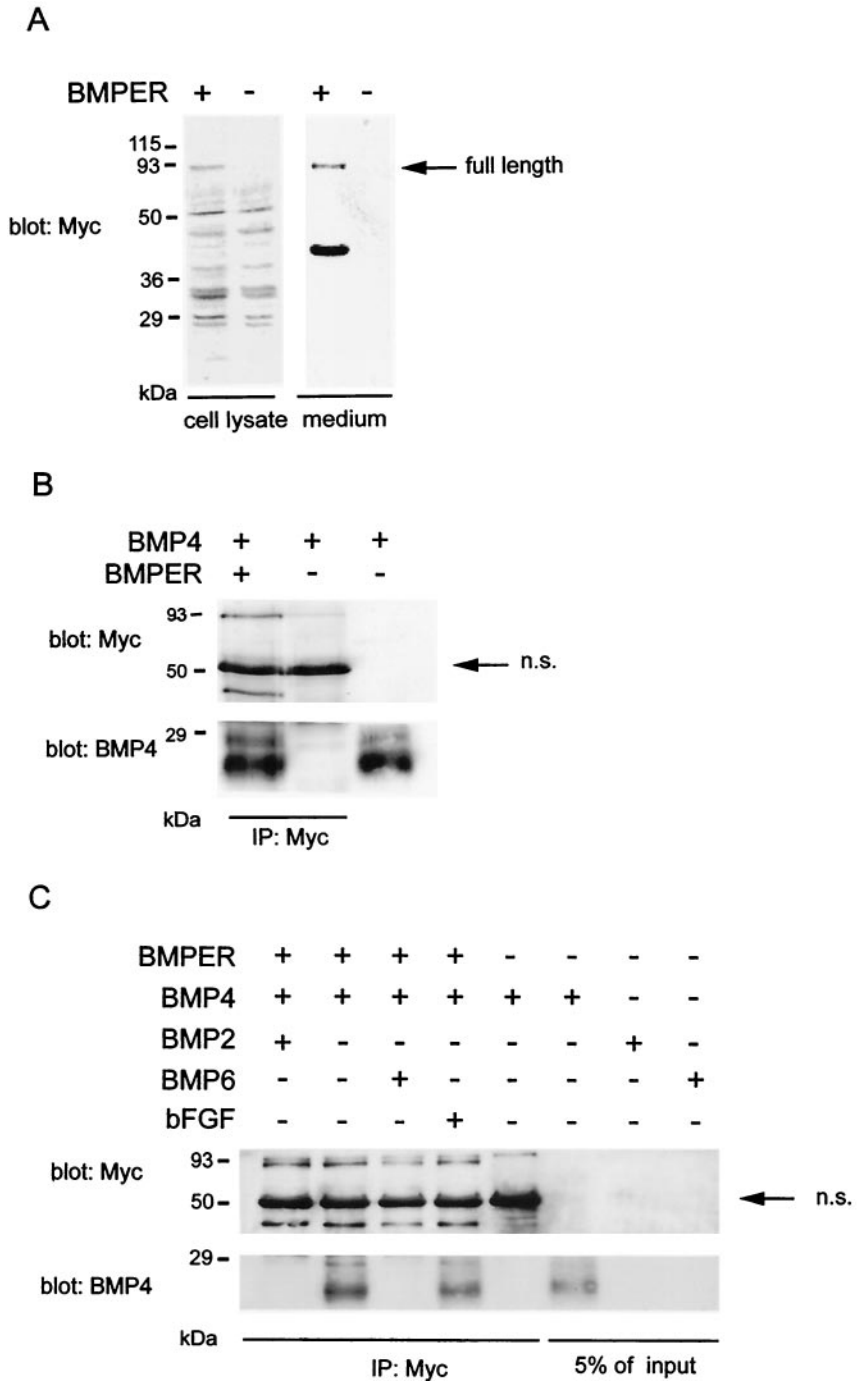

FIG. 6. Mammalian expression of BMPER and BMP binding. (A) COS-7 cells were transiently transfected with vector pSecTag2BMPER, which is tagged with a Myc epitope at the carboxyl terminus, or empty vector. Supernatant medium and whole-cell lysates were obtained and subjected to Western blot analysis with an anti-Myc antibody. The arrow indicates full-length BMPER. (B) Myc-tagged BMPER purified from cell supernatants was incubated with recombinant BMP4, as indicated, and reaction mixtures were immunoprecipitated (IP) with an anti-Myc antibody, followed by Western blotting for BMP4 (lower panel). BMP4 was immunoprecipitated only in the presence of BMPER. In the third lane, BMP4 (10 ng) was loaded directly as a control. The efficient immunoprecipitation of BMPER was confirmed by blotting for Myc (upper panel). n.s., nonspecific. (C) Competition with BMP4 binding was used to determine the promiscuity of BMPER interactions with BMPs. Competition for coimmunoprecipitation of BMP4 with BMPER was tested in the presence of a 25-fold molar excess of BMP2, BMP6, or bovine fibroblast growth factor (bFGF).

tin) function to antagonize the effects of BMP signaling (25, 55, 60, 61, 69). When misexpressed, these factors either can lead to the dorsalization of embryos or, when injected into the ventral side of embryos, can cause the formation of a secondary axis. To test whether BMPER can function as a BMP antagonist in these assays, we injected BMPER mRNA into ventral or dorsal blastomeres of four-cell-stage embryos and scored the samples for the formation of a secondary axis. Consistent with the

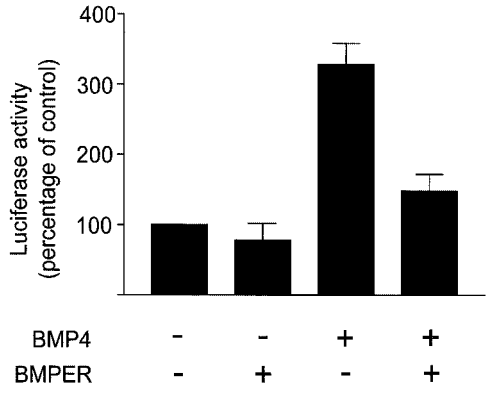

FIG. 7. Effects of BMPER expression on BMP4 activity in mammalian cells. 293T cells were transiently transfected with an Smad5-dependent luciferase reporter plasmid and pSecTag2BMPER or control plasmid, as indicated. A plasmid expressing β-galactosidase was cotransfected to control for transfection efficiency. Recombinant BMP4 or a control, as indicated, was added after 18 h and incubated for an additional 20 h. Luciferase activity was normalized for transfection efficiency and expressed as a percentage of the activity in untreated cells (mean and standard deviation).

prediction that BMPER acts as a BMP antagonist, the injection of BMPER into ventral but not dorsal blastomeres leads to the formation of a secondary axis (73 of 91 embryos had a double axis after ventral injection of 840 pg of BMPER, compared with 0 of 77 embryos after dorsal injection) (Fig.8A to F). Moreover, the effects of BMPER are dose dependent, with increasing extents of dorsalization and incidences of secondary axis formation occurring concomitantly with increasing doses of BMPER (Fig. 8A). Similar to previous descriptions of secondary axis formation in embryos injected ventrally with Chordin or Noggin, even the highest doses of BMPER rarely led to the formation of more anterior structures, such as eyes or the cement gland, in the newly formed axis (Fig. 8F) (55, 60, 61). Secondary axis formation could be blocked almost completely by coinjecting BMP4 along with BMPER mRNA into ventral blastomeres (Fig. 9A). In a complementary set of experiments, misexpression of BMP4 mRNA in dorsal blastomeres of early cleavage stages leads to a loss of dorsoanterior structures and, in more severe cases, to completely ventralized embryos (16, 28). However, coexpression of BMPER along with BMP4 mRNA in dorsal cells rescues the BMP4 phenotype by restoring dorsoanterior structures (Fig. 9B). The dorsalization of *Xenopus* embryos by BMPER is consistent with an antagonistic effect on BMP signaling, as also indicated by our experiments with the mammalian system.

To further characterize the effects of BMPER on BMP signaling in *Xenopus*, we tested the ability of BMPER to block BMP signaling in animal cap assays. In these experiments, animal caps derived from noninjected or BMPER-injected embryos were cultured in the presence of activin A, and mesoderm induction and patterning in the early gastrula (stage 10) were assessed by RT-PCR with a panel of mesoderm-specific markers (Fig. 8G). After activin A stimulation, BMPER blocked the expression of the ventral marker Xvent-1, the gene for which was previously demonstrated to be a downstream target of the BMP/Smad pathway (23). Interestingly, the BMP4 transcript itself is also partially downregulated following BMPER injection, a result which may be explained either by inhibition of ventral mesoderm formation (16, 28) or by inhibition of a positive BMP autoregulatory feedback loop, as previously described (6). However, the effect on the ventral mesoderm appears to be specific, since markers of the dorsal mesoderm, such as Chordin, are unaffected. Interestingly we do note a slight decrease in overall mesoderm formation, as indicated by a minor reduction in Xbra expression (58). Taken together, the results of our studies with *Xenopus* axis formation and cap assays confirm that BMPER can function as a BMP antagonist in a developmentally relevant context.

To examine BMPER function further in a model of mouse embryonic development, ES cells were grown under conditions that allow for cell survival but not for significant differentiation without the addition of growth factors (2, 43). Under these conditions, the stimulation of differentiating ES cells with BMP4 results in the development of an endothelial cell subpopulation after 7 days of differentiation, as determined by staining for the definitive endothelial cell marker VE-cadherin (Fig. 10). Neutralization of BMP4 by a blocking antibody inhibited the differentiation of this endothelial cell subpopulation. Comparable BMP4 inhibition was also observed in differentiating ES cells that were transfected with BMPER and subsequently stimulated with BMP4, although BMPER transfection alone had no effect on endothelial cell differentiation in the absence of stimulation. The quantitative analysis of three independent experiments indicated a significant reduction of VE-cadherin-positive cells in the MAB757- and BMP4-treated embryoid bodies as well as in the BMPER-transfected and BMP4-treated embryoid bodies compared to those treated with BMP4 alone (Fig. 10F). These results further confirm the BMP-inhibiting activity of BMPER and indicate that this activity may serve to modulate the differentiation of endothelial cells and their precursors in response to developmental cues, as suggested by the developmental expression and cellular function of this secreted protein.

DISCUSSION

Cloning of a novel BMP-binding protein. Although many of the key signaling pathways in blood vessel formation are known—including members of the VEGF and angiopoietin family—the determinants of vascular cell differentiation at the earliest stages remain poorly understood and logically require input from multiple signaling pathways. In a model that recapitulates key events in vasculogenesis under carefully controlled conditions, we performed a screen for differentially expressed genes as a means of identifying and understanding the molecular events that determine the early stages of blood vessel formation. By sorting for flk-1, a cell surface molecule that is the first marker of the endothelial cell lineage expressed during development, we defined a population of rapidly differentiating endothelial cell precursors and separated them from the surrounding cells by flow cytometry. cDNA libraries were cloned from both populations and further enriched for the flk-1-positive population by subtractive PCR. We show that the isolation of endothelial cell precursors in this fashion is efficient and that endothelial cell precursor-specific transcripts can be identified. In this population, we discovered a differen-

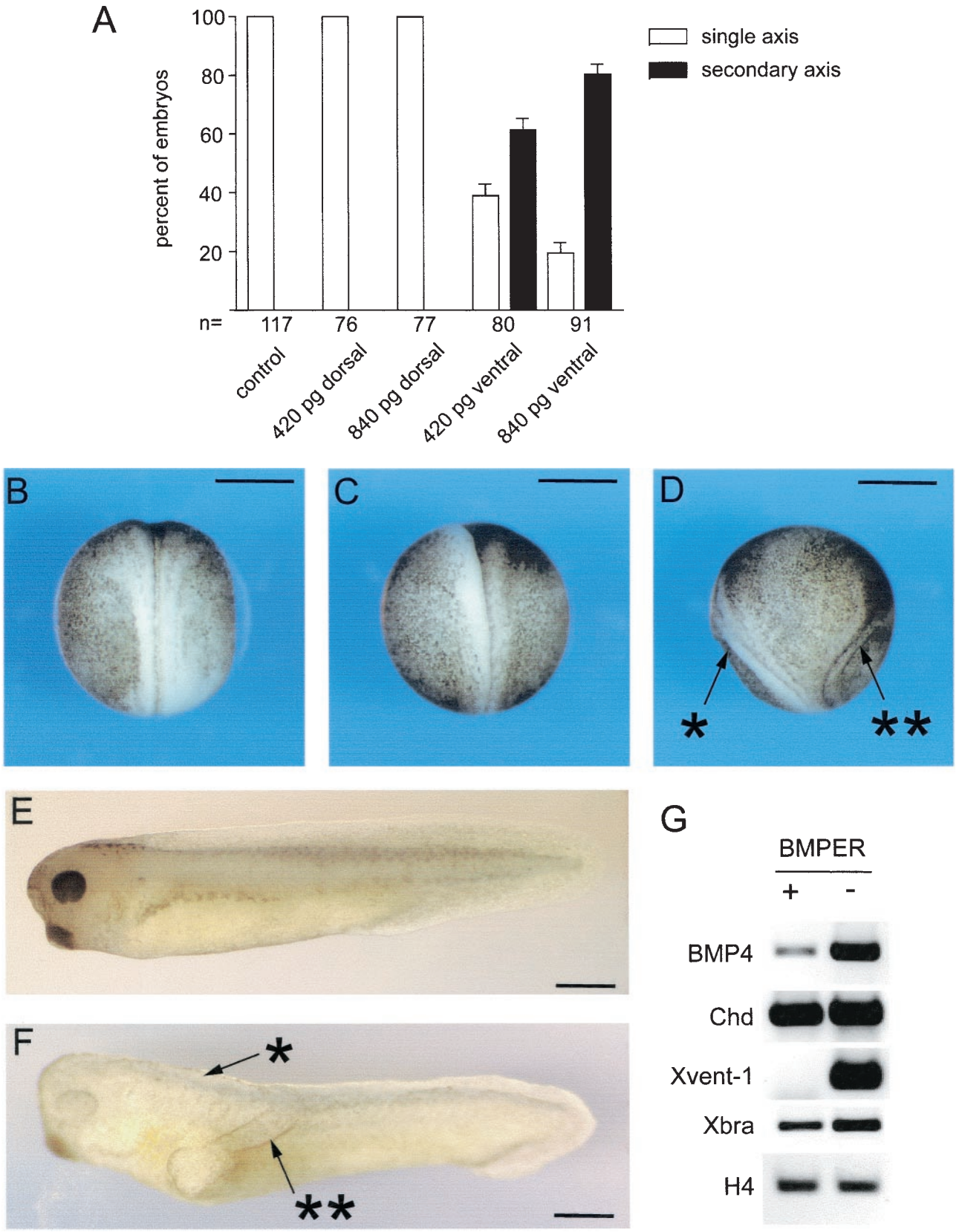

FIG. 8. Effects of BMPER on dorsal-ventral axis development in *X. laevis*. (A) Summary of the effects of BMPER injection into *Xenopus* blastomeres at the four-cell stage. n, number of embryos. Error bars indicate standard deviations. (B to D) Axis formation in *Xenopus* embryos at stage 14. Bar, 750 μm. (B) Control. (C) Dorsally injected. (D) Ventrally injected. Single asterisk, primary axis; double asterisk, secondary axis. (E and F) Representative embryos at stage 32. Bar, 1 mm. (E) Control. (F) Ventrally injected. (G) Mesoderm marker gene expression in activin A-treated animal caps, as analyzed by RT-PCR at stage 10. +, injected; −, not injected; H4, histone H4.

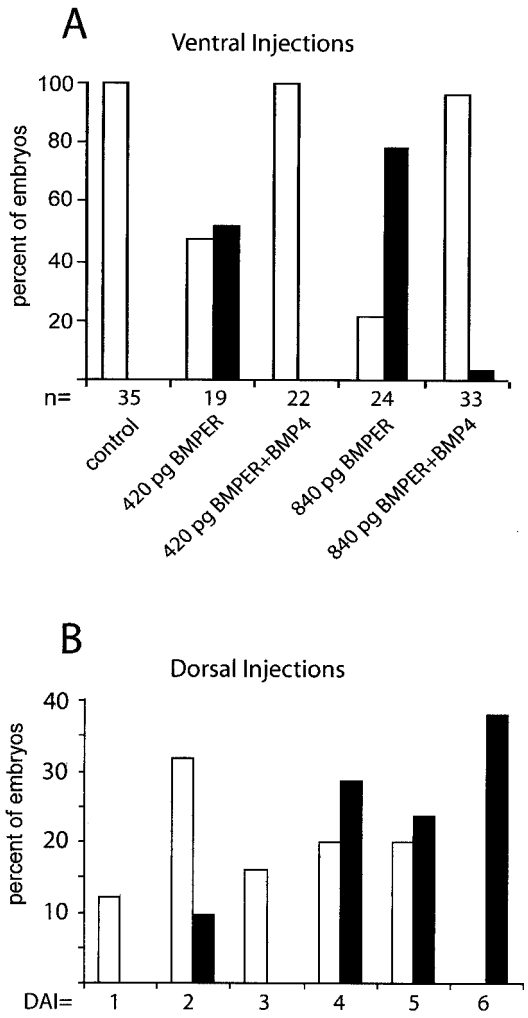

FIG. 9. Coinjection of BMPER and BMP4. (A) Effects of coinjection of BMPER and BMP4 (50 pg) into both ventral blastomeres at the four-cell stage versus injection of BMPER alone. Embryos were scored for secondary axis formation at stage 14. Symbols: □, single axis; ■, secondary axis. (B) Effects of coinjection of BMPER (840 pg) and BMP4 (50 pg) into both dorsal blastomeres versus injection of BMP4 alone. Embryos were scored for dorsoanterior structures at stage 32. DAI, dorsoanterior index. Symbols: □, BMP4, 25 embryos; ■, BMP4 plus BMPER, 21 embryos.

tially expressed cDNA, called BMPER. Its expression pattern, interactions with BMP family members, and function with respect to BMP activity and endothelial cell differentiation provide a basis for understanding how the BMP signaling pathway participates in vasculogenesis.

BMPs are a group of more than 30 proteins of the TGF-β superfamily of extracellular regulators. BMPs activate heterodimeric serine/threonine kinase receptors, which in turn phosphorylate their intracellular substrates, the transcription factors Smad1 and Smad5, to activate BMP-dependent gene regulatory events (38). Molecules within the BMP signaling pathway are critical for a number of developmental events, including left-right asymmetry, mesoderm patterning, and the development of several organs, such as the nervous system and the heart (4). The classical BMP in *Drosophila*, Decapentaplegic, and its vertebrate homologs, BMP2 and BMP4, regulate the patterning of the embryonic dorsal-ventral axis (21). The activities of BMP family members are further regulated by extracellular binding partners. Chordin and its *Drosophila* homolog, Sog, are prototypical members of one family of binding partners. Full-length Chordin binds to BMP2 and BMP4 and inhibits their binding to BMP receptors, thus antagonizing BMP activity (50). In *Xenopus*, Chordin is permissive for the development of dorsally derived tissues, including somitic muscle and neuroectoderm. Chordin may be cleaved and its BMP-binding activity may be decreased (but not abolished) by the metalloprotease Tolloid. The functional interaction of Chordin and BMPs is further regulated by Twisted gastrulation, which stabilizes the association of full-length (but not cleaved) Chordin with BMP (36). Twisted gastrulation may therefore serve as an agonist or as an antagonist of BMP activity, depending on local gradients of Chordin and Tolloid.

Chordin contains four conserved 70-amino-acid CR repeats that comprise its BMP-binding activity. It is now recognized that the presence of CR repeats defines a group of proteins that bind to and, in general, antagonize BMPs, among these being Procollagen IIA, Neuralin, and Kielin (13, 39, 73). On the basis of the five CR repeats present in BMPER, we considered the possibility that it is also a BMP-binding protein. Indeed, we found that BMPER efficiently bound BMP4 (Fig. 6B and C) and that BMPER could repress BMP-dependent Smad5 transcriptional activation (Fig. 7). Additionally, BMPER could rescue BMP4-ventralized *Xenopus* embryos (Fig. 9B). In *Xenopus* animal caps, injection of BMPER mRNA completely abolished the transcription of Xvent-1 (Fig. 8G), which is a downstream transcriptional target of BMP4 signaling via Smad5 activation (23). Interestingly BMP4 activity itself was somewhat downregulated in this assay (although not to the extent of Xvent-1), consistent with reports of BMP4 autoregulation (2, 6). In addition, BMPER resulted in axis duplication activity when injected into early *Xenopus* embryos ventrally but had no effect when injected into dorsal blastomeres. These findings are in congruence with the consequences of injection of Chordin into the same locations (55).

Coinjections of BMPER and BMP4 into ventral blastomeres rescued the dorsalizing activity of BMPER, and coinjections into dorsal bastomeres rescued the ventralizing effect of BMP4, supporting the hypothesis that BMPER acts by antagonizing BMP4 (Fig. 9). Finally, using an established ES cell assay (43), we show that BMPER inhibits BMP4-dependent endothelial cell differentiation (Fig. 10E). It has been reported, with a similar model of differentiating ES cells, that the expression of a dominant-negative BMP receptor blocks differentiation into flk-1-positive lineages (2). The effects of BMPER in our studies are similar, providing further support for the notion that BMP signaling events are important in endothelial cell differentiation, with BMPER acting as an endothelial cell precursor-derived inhibitor of this process. We have demonstrated, using multiple methods, that BMPER is a new member of the group of CR repeat-containing BMP-binding partners. Under the conditions observed here, it acts as a BMP antagonist.

Structure of BMPER. In addition to five CR repeats, BMPER also contains a von Willebrand D domain (Fig. 2C), which in some situations functions as a multimerization mod-

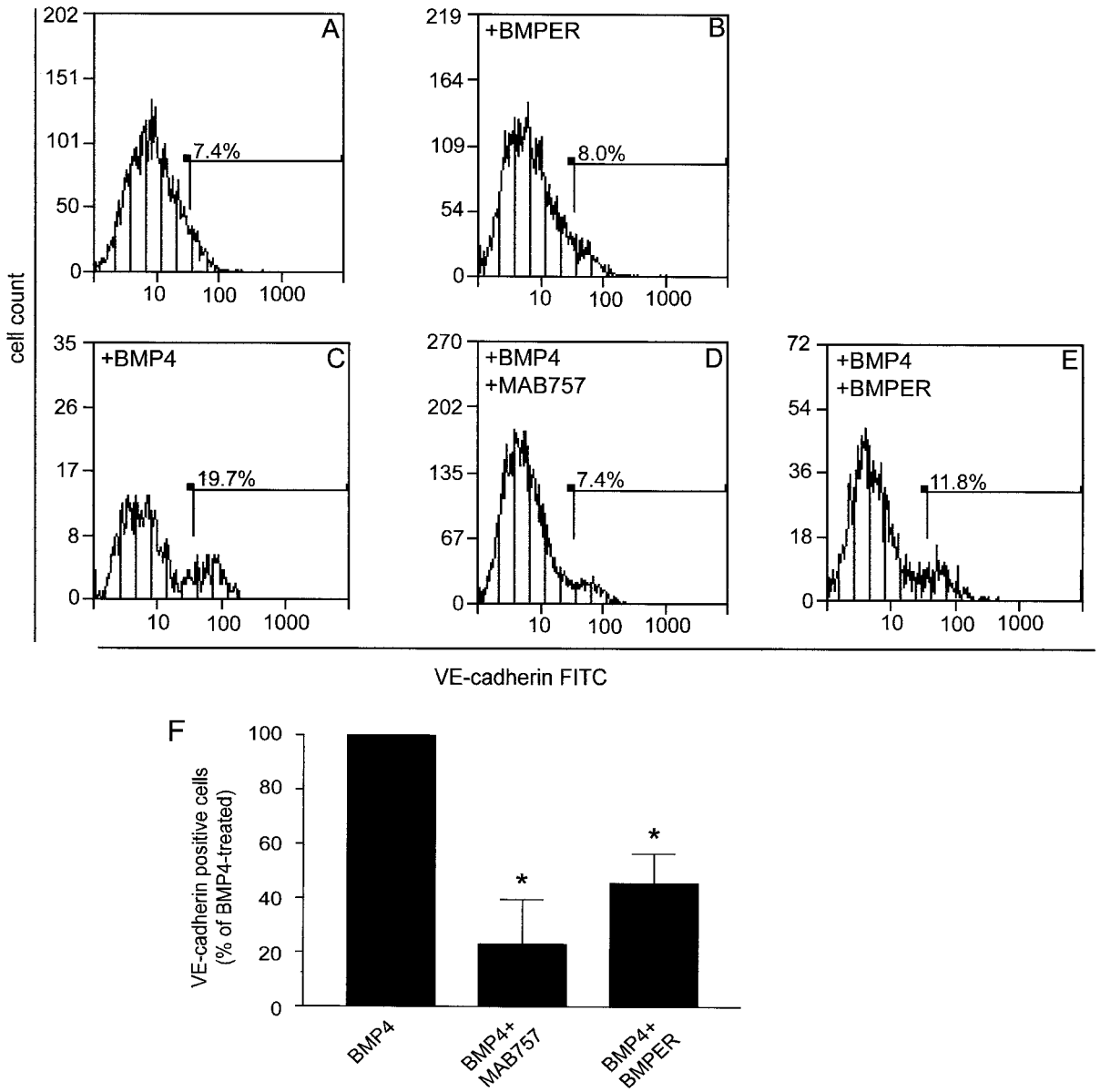

FIG. 10. Effects of BMPER on BMP-dependent differentiation of ES cells into endothelial cells. Embryoid bodies were incubated for 7 days under serum-free conditions. Definitive endothelial cells were detected by flow cytometry with an anti-VE-cadherin rat immunoglobulin G2 antibody and the respective fluorescein isothiocyanate (FITC)-conjugated secondary antibody. (A and B) VE-cadherin-positive cells were rare in the absence of BMP4 (A), and BMPER expression had a minimal effect under these conditions (B). (C and D) Expansion of the endothelial cell population increased in the presence of recombinant BMP4 (1 nM) (C), but this effect was attenuated when recombinant BMP4 was preincubated for 30 min at 37°C with a blocking antibody (MAB757) at a final concentration of 2 μg/ml (D). (E) Similarly, the expression of BMPER inhibited endothelial cell differentiation induced by BMP4. Results for panels A to E are representative of three independent experiments. (F) BMP4-dependent endothelial cell differentiation in embryoid bodies, as determined on the basis of VE-cadherin-positive cells in treated cells. Error bars indicate standard errors of the mean. An asterisk indicates a P value of <0.05 for a comparison with BMP4-treated cells.

ule (3, 30, 31). Two additional CR repeat-containing proteins also have von Willebrand D domains. Kielin (a *Xenopus* BMP repressor) has 27 CR repeats and a carboxy-terminal von Willebrand D domain; Kielin has BMP-antagonizing activity, although its ability to interact with BMPs has not been formally demonstrated (39). Crossveinless-2 is a *Drosophila* protein that contains five CR domains and a carboxy-terminal von Willebrand D domain but no trypsin inhibitor domain. The gene encoding this protein was positionally cloned based on its requisite role in wing cross vein development (14). Although the biochemical function of Crossveinless-2 (in particular, whether it directly interacts with BMPs) has not been reported, it does interact with BMP activity genetically in *Drosophila*. In particular, Crossveinless-2 potentiates Decapentaplegic signaling in the wing. This finding suggests that, unlike other reported CR repeat-containing proteins that modulate BMP activity, Cross-

veinless-2 is an agonist rather than an antagonist of BMP function.

The size and domain structure of BMPER and Crossveinless-2 are similar, and they share 30% identity and 44% similarity at the amino acid level. It is possible that BMPER and Crossveinless-2 are orthologs, although the biochemical effects of BMPER presented in this report and the genetic functions of Crossveinless-2 with respect to BMP activity are incongruent. The possibility that these two proteins are structurally similar yet have divergent functions cannot be excluded. It is possible that the presence of the trypsin inhibitor domain in BMPER confers functions different from those of Crossveinless-2. More complex models can also be developed, based on the assumption that these proteins are indeed structural and functional homologs. The precedent of Twisted gastrulation, which has both pro- and anti-BMP activities depending on the concentrations and activities of Chordin and Tolloid (36), raises the possibility that antipodal functions may be a more general property of BMP-binding proteins.

In this context, it is interesting that a second Crossveinless phenotype exists in *Drosophila*; this phenotype is due to mutation of *twisted gastrulation-2*, a second *twisted gastrulation* gene that arose spontaneously in *Drosophila* (52). The similar phenotypes suggest that Twisted gastrulation-2 and Crossveinless-2 together activate Decapentaplegic signaling in *Drosophila*. Whether the mammalian homolog of Twisted gastrulation has a similar effect on BMPER remains to be determined (in contrast to *Drosophila*, mammals contain only one *twisted gastrulation* gene), but is an interesting possibility that would reconcile these two studies. Such a model is not entirely speculative. In our studies, we noted that BMPER undergoes proteolytic cleavage in the extracellular space (Fig. 6A) to yield a carboxy-terminal fragment of approximately 42 kDa. (An amino-terminal fragment may also be present but would not be detectable in our assays because our protein was tagged at the carboxyl terminus.) Because the effects of Twisted gastrulation as an agonist or antagonist of BMPs depends in large part on Tolloid-dependent cleavage of Chordin, it is tempting to speculate that cleavage of BMPER by a protease may ultimately alter its function toward BMP4, perhaps through interactions with Twisted gastrulation or a similar protein. Although this model is attractive, we have not yet found evidence for such a dual function, and in our diverse assays, BMPER consistently behaves as a BMP antagonist.

BMPER and angioblast differentiation. The design of our initial screen, to determine genes that are preferentially coexpressed with the flk-1 gene in differentiating ES cells, raises the interesting possibility that BMPER has a role in angioblast differentiation and/or vasculogenesis. With the exception of ALK1 (49) and endoglin (24, 29, 62), which are coreceptors for TGF-β and BMP family members, a role for BMP family members in vascular formation has not been extensively studied. However, it is interesting that the deletion of several proteins involved in different steps of BMP signaling causes vascular phenotypes in mice. For example, *bmp4* mutants have compromised blood and endothelial cell development resulting from defective blood island formation (68). Not surprisingly, the phenotype that results from the deletion of BMP receptor type I is very similar to the *bmp4*$^{-/-}$ phenotype (42). Most mouse embryos lacking *bmp2* have, in addition to impaired mesoderm formation, defective heart development in the embryos that survived gastrulation (72). Targeted mutation of *smad5*, which encodes a transcription factor downstream of BMP2 or BMP4, results in the disorganization of yolk sac vasculature and heart development perturbation similar to that seen in *bmp2* mutants (9). The general conclusion of these studies is that the BMP signaling pathway has a requisite role in vascular development. However, to our knowledge, the specific expression of BMP-related proteins in the vascular compartment has not yet been described, and so it is as yet unclear how BMPs mediate specific effects on vascular progenitors.

BMPER is expressed with marked specificity in flk-1-positive ES cells (Fig. 3A), which are generally considered to represent the earliest stage of the endothelial cell lineage in this model and in mouse embryos, and the temporal expression of BMPER closely mimics that of flk-1 (Fig. 3B). In mouse embryos, BMPER is expressed in the AGM region, which is enriched in cells of the vascular lineage, as well as in endothelial cells that have been incorporated into the aorta. As an inhibitor of BMP signaling (Fig. 7 to 9), BMPER would be expected to antagonize endothelial cell differentiation, and indeed this is the effect that we observed in differentiating ES cells, which recapitulate most of the early developmental events in vascular morphology (65, 67). Although the precise role of BMPER in endothelial cell differentiation requires further elucidation, our data indicate a model in which flk-1-positive cells express BMPER in order to modulate local BMP signaling. The expression of BMPER may inhibit the recruitment of additional flk-1-positive cells to prevent vascular overgrowth, or BMPER may be secreted to encourage the differentiation of other cell lineages that can arise from the same (or spatially adjacent) precursor cells. This model would predict that multipotential precursors may be primed to assume a vascular lineage in response to stimulation by BMPs and that the vascular expression of BMPER (and perhaps other proteins that modify BMP responses) determines which cells respond to local BMPs and which do not.

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ADDENDUM IN PROOF

While this paper was in review, a gene with an identical sequence and similar embryonic expression pattern was reported as the mouse homologue of *Crossveinless-2* (C. Coffinier et al., Gene Expression Profiles **2:**189–194).

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