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BMPER-induced BMP signaling promotes coronary artery remodeling

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ABSTRACT

The connection of the coronary vasculature to the aorta is one of the last essential steps of cardiac development. However, little is known about the signaling events that promote normal coronary artery formation. The bone morphogenetic protein (BMP) signaling pathway regulates multiple aspects of endothelial cell biology but has not been specifically implicated in coronary vascular development. BMP signaling is tightly regulated by numerous factors, including BMP-binding endothelial cell precursor-derived regulator (BMPER), which can both promote and repress BMP signaling activity. In the embryonic heart, BMPER expression is limited to the endothelial cells and the endothelial-derived cushions, suggesting that BMPER may play a role in coronary vascular development. Histological analysis of BMPER$^{-/-}$ embryos at early embryonic stages demonstrates that commencement of coronary plexus differentiation is normal and that endothelial apoptosis and cell proliferation are unaffected in BMPER$^{-/-}$ embryos compared with wild-type embryos. However, analysis between embryonic days 15.5–17.5 reveals that, in BMPER$^{-/-}$ embryos, coronary arteries are either atretic or connected distal to the semilunar valves. In vitro tubulogenesis assays indicate that isolated BMPER$^{-/-}$ endothelial cells have impaired tube formation and migratory ability compared with wild-type endothelial cells, suggesting that these defects may lead to the observed coronary artery anomalies seen in BMPER$^{-/-}$ embryos. Additionally, recombinant BMPER promotes wild-type ventricular endothelial migration in a dose-dependent manner, with a low concentration promoting and high concentrations inhibiting migration. Together, these results indicate that BMPER-regulated BMP signaling is critical for coronary plexus remodeling and normal coronary artery development.

Introduction

During early heart development, simple diffusion of blood is sufficient to supply the heart with nutrients and oxygen. As the heart remodels, however, the coronary vasculature must develop to support the oxygen and nutrient needs of a larger, more muscular heart. A wide range of coronary anomalies have been documented, consisting of both severe (a failure of the coronary vasculature to connect to the aorta) and less severe (abnormal locations and numbers of coronary stems and irregular branching patterns) anomalies (Angelini, 2002). It comes as no surprise that the absence of both coronary arteries has clearly deleterious effects on the developing organism; however, even less severe anomalies can lead to sudden death (Maron, 2003; Turkmen et al., 2007), illustrating the need for a comprehensive understanding of the mechanisms and pathways involved in the development of the coronary vasculature.

The coronary vasculature begins forming before the heart has fully septated and connects to the aorta long after the heart has begun beating (Bernanke and Velkey, 2002). The coronary vasculature begins as a plexus of endothelial cells that spread from the sinus venosus to encompass the ventricles (Lavine et al., 2006; Red-Horse et al., 2010). These endothelial cells derive from the proepicardium and traverse through the sinus venosus to yield the subepicardial venous plexus and the intramyocardial arterial plexus (Red-Horse et al., 2010; Katz et al., 2012; Wu et al., 2012; Tian et al., 2013). This initial network remodels to form a more mature pattern comprising arteries and veins, with a number of small vessels initially connecting to the aorta and coalescing to form two distinct ostia or openings (Ando et al., 2004; Pérez-Pomares and de la Pompa, 2011). In addition to the endothelial cells that form the coronary plexus, smooth muscle cells derived from the proepicardium surround and support the portion of the coronary artery that connects with the aorta, known as the coronary stem (Gittenberger-de Groot et al., 1999; von Kodolitsch et al., 2004; Grieskamp et al., 2011). Even though the overall progression of coronary vascular development has been well documented (Riley and Smart, 2011), key gaps remain in our understanding of this process, particularly with respect to the signaling cues that guide endothelial cell migration.

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One signaling pathway that is important in endothelial cell biology is the bone morphogenic protein (BMP) pathway. Endothelial cells express a range of BMPs, including BMPs 2, 4, 6, and 7, as well as type 1 and 2 BMP receptors (Bobik, 2006). The effects of the BMPs on endothelial cell function are highly context dependent, with a single BMP being capable of signaling through multiple intracellular pathways. BMPs can stimulate cell migration, angiogenesis, apoptosis, and proliferation in some settings yet inhibit proliferation and apoptosis in others (Bobik, 2006; Wiley and Jin, 2011). The BMP signaling pathway is complex and regulated at many different levels. One level of regulation is afforded by extracellular BMP regulators such as chordin, noggin and BMPER (BMP-binding endothelial cell precursor-derived regulator). Whereas most of these regulators behave as either a repressor or inducer of BMP signaling, BMPER can act as both (Kelley et al., 2009). In vivo, BMPER has pro-BMP roles in the mouse eye, kidney, and skeleton (Ikeya et al., 2006); zebrafish gastrulation (Rentzsch et al., 2006) and axis formation (Moser et al., 2007); and drosophila crossvein formation (Conley et al., 2000). However, BMPER has also been attributed anti-BMP roles in mouse retinal vascularization (Conley et al., 2000; Moreno-Miralles et al., 2011) and Xenopus axis formation (Moser et al., 2003). In vitro, BMPER has been ascribed a pro-BMP role and promotes sprouting and migration in human umbilical vein endothelial cells (HUVECs) (Heinke et al., 2008).

Despite the well-documented effects of the BMP pathway on endothelial cell biology, its role in coronary vascular development has yet to be demonstrated. Therefore, this study was undertaken to explore the role that BMP and its extracellular regulator BMPER has on the development of the coronary vasculature. We hypothesized that BMPER would regulate BMP signaling to promote coronary vascular development and tested this theory using the BMPER$^{-/-}$ embryo as our model system. Embryonic BMPER$^{-/-}$ hearts present with a range of coronary anomalies, despite a normal initiation of coronary plexus formation. Further, endothelial apoptosis and subsequent contributions from the proepicardium appear normal in the BMPER$^{-/-}$ hearts when compared with wild-type hearts. Analysis of ventricular endothelial cells isolated during coronary vascular remodeling reveal that BMPER$^{-/-}$ endothelial cells have impaired migration and remodeling abilities compared with their wild-type counterparts. In addition, recombinant BMPER promotes wild-type ventricular endothelial migration in a dose-dependent manner, with a low concentration promoting and high concentrations inhibiting migration. Together, these results indicate that BMPER-induced BMP signaling promotes normal coronary vascular development and that BMP signaling is critical for normal coronary stem formation.

Materials and methods

Mice

The creation of the BMPER$^{-/-}$ mouse was previously described (Kelley et al., 2009). Mice were maintained on a C57BL/6N/129 background. Because the BMPER$^{-/-}$ embryo exhibits perinatal lethality (Kelley et al., 2009), timed matings were performed using BMPER$^{+/-}$ mice. All experiments were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Based on the frequency of the coronary defects, eight BMPER$^{-/-}$ embryos were required for each experiment that compared wild-type and BMPER$^{-/-}$ embryos to have sufficient statistical power (power calculations: $\alpha=0.05$, 95% confidence, size effect=0.67 based on coronary defects). Thus, the in vivo quantitative analyses have a minimum of eight embryos per group.

Histology

For immunohistochemical analyses, E12.5–E16.5 embryos were harvested in phosphate-buffered saline (PBS), fixed in 4% formaldehyde overnight at 4 °C, washed in PBS, and processed for paraffin or frozen sectioning, performed by the Histology Research Core Facility at UNC. Additional hearts were harvested and utilized for whole-mount immunohistochemistry. Unless indicated, immunohistochemistry was performed as previously described (Waldo et al., 1996). Primary antibodies included MF-20 (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), PECAM (Becton-Dickinson, San Jose, CA, USA), phospho-Smad1, 5, 8 (pSmad; Cell Signaling Technology, Danvers, MA, USA), phospho-Erk1/2 (Cell Signaling Technology), anti-Crossveinless-2 (also known as BMPER, R&D Systems, Minneapolis, MN, USA), and α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO, USA). Primary antibodies were detected using species-specific Alexa Fluor antibodies (Invitrogen, Grand Island, NY, USA) or ABC Elite kits and diaminobenzidine (Vector Labs, Burlingame, CA, USA). pSmad and pERK immunohistochemistry was performed as described in Dyer et al. (2010) with the addition of 1% BSA to the block solution for pERK, and BMPER immunohistochemistry was performed as previously described (Zakin et al., 2008). For whole-mount confocal microscopy, hearts were dehydrated and cleared with methyl salicylate prior to visualization.

In situ hybridization

The Tbx18 probe was kindly provided by Dr. Andreas Kispert (Medizinische Hochschule Hannover, Germany). In brief, embryos were collected and processed for frozen sectioning under RNase-free conditions. In situ hybridization was performed on 5-μm sections by the In Situ Hybridization Core Facility at UNC.

Microscopy and image processing

All microscopy was performed in conjunction with the Microscope Services Laboratory at UNC. Sections were imaged using an Olympus BX61 upright fluorescence microscope, and whole hearts were imaged using a Zeiss LSM5 Pascal confocal laser scanning microscope for fluorescently labeled hearts or a Leica-Wild M420 Macroscope for diaminobenzidine-labeled hearts. Live cell imaging was performed using an Olympus IX70 inverted fluorescence microscope that was fitted with a humidified, temperature-controlled chamber that was maintained at 5% CO_2. Volocity software was used to obtain all images. Static images were processed using Adobe Photoshop, and confocal image stacks were processed with Imaris. To compare phosphorylated protein expression, all acquisition parameters were identical when photographing the slides and processing the images to ensure that any differences in expression levels were real. Fluorescence intensity was assessed using the histogram tool, and the intensity in the BMPER$^{-/-}$ embryos was assessed as the fold change with respect to the average intensity of the wild-type embryos in each individual litter to account for inter-litter variability and to minimize other processing-related variables, such as time between immunohistochemistry and visualization.

Immunoblots

The aortic valves of E13.5 and E14.5 wild-type and BMPER$^{-/-}$ embryos were excised, harvested in ice-cold lysis buffer containing phosphatase inhibitors, and homogenized using TissueLyser LT (Qiagen, Valencia, CA) (Meng et al., 2008). Each sample comprises

four individual valves. Membranes were blotted for pSmad and β-actin as a loading control. Band density was quantified in ImageJ (www.nih.gov) and is presented as the percentage of the wild-type pSmad/β-actin ratio.

Endothelial cell isolation

Endothelial cells were isolated from the ventricles of embryonic day 14.5–16.5 mice as recently described (Dyer and Patterson, 2013a). In brief, ventricles were isolated from the embryos, minced gently, and digested in collagenase type I for 45 min at 37 °C with gentle agitation. The resulting cell suspension was washed twice in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Sigma-Aldrich) prior to an incubation with anti-PECAM-conjugated Dynabeads (Invitrogen), prepared according to the manufacturer's recommendations. PECAM is expressed by both coronary endothelial cells and endocardial cells. However, contributions from the ventricular endocardium to the coronary endothelium primarily occur at E11.5, with endocardial migration decreasing dramatically thereafter (Zhang and Zhou, 2013); thus, all migratory cells at the assessed time points are considered coronary endothelial cells, and all samples should be similarly affected by non-migratory endocardial cells. After a 30 min incubation at 4 °C, the bead–cell solution was washed five times with 10% FBS–DMEM and once with DMEM prior to dissociating the cells from the beads using trypsin–EDTA (Gibco). The resulting cells were pelleted and resuspended as follows. For live cell imaging of the tube formation assay, cells were resuspended in endothelial cell growth medium (ECGM) (DMEM containing 20% FBS, 5 μM β-mercaptoethanol, 50 μg/ml ECG (Biomedical Technologies, Inc., Stoughton, MA, USA), and penicillin/streptomycin) and plated on collagen-coated plates. Plates were prepared as described in Runyan and Markwald (1983); all endothelial cells remained on the surface of this collagen gel and preferred migrating on this collagen gel compared with a simple coat of collagen (Dyer and Patterson, 2013a). The number of cells per well was counted after plating to ensure that similar numbers of cells were analyzed. For transwell assays, endothelial cells were resuspended in DMEM and placed in the upper chamber of a transwell (Corning, 8.0 μm pore size, polycarbonate membrane).

Endothelial cell analysis – tube formation assay

Approximately 24 h after plating, isolated endothelial cells were incubated with the fluorescent marker Syto-16 (Invitrogen), which is highly enriched in endothelial cells (Arima et al., 2011). After 1 h, the medium was replaced with fresh ECGM, and time-lapse photography was performed. Isolated cells were maintained in a humidified chamber at 37 °C with 5% CO_2 and were imaged every 15 min for 22 h. The resulting movies were analyzed with the MTrack plugin (Meijering, Dzyubachyk (Smal)) for ImageJ (rsbweb.nih.gov/ij/). The following parameters were analyzed: migration distance, cell–cell interactions, chain length, and chain branching. For migration distance, the five most motile cells per sample were tracked. Embryos were not genotyped until the analyses were complete to prevent biasing the results.

Endothelial cell analysis – transwell migration assay

For transwell migration analysis, wild-type isolated endothelial cells were resuspended in DMEM (approximately 2,000 cells per 75 μl DMEM). DMEM (500 μl) was added to the lower chamber, and the cells were given one hour at 37 °C to settle. Vehicle control or recombinant BMPER (10–75 ng/ml) was added to the lower chamber, and endothelial cells were allowed to migrate for four hours at 37 °C. VEGF was used as a positive control. After 4 h, the upper chamber side of the membrane was swabbed gently with a cotton-tipped applicator, and the membranes were incubated in 0.1% crystal violet for 1 h (Mandelboim, 2006). The membranes were thoroughly washed with distilled water, and four 10× images were obtained for each membrane. For each experiment, equal cell numbers were plated in controls and treated wells, and the results are presented as the fold change compared with the control to account for slight variability between experiments.

Ex vivo BMPER-conjugated bead treatments

Hearts were excised from E13.5 embryos and cultured as previously described (Dyer and Patterson 2013b). Agarose beads were conjugated to BMPER (72 ng/μl) or vehicle control as described in Bronner-Fraser (2008) and placed adjacent to the ventricles, tucked near the outflow tract. Hearts were cultured ex vivo with beads for 24 h. Hearts were then fixed, the position of the bead was documented, and hearts were prepared for immunohistochemistry for pSmad and PECAM to determine how BMP signaling and endothelial recruitment were affected by exogenous BMPER.

Statistics

For the migratory data and fluorescent intensity comparisons, ANOVA and unpaired Student's *t*-tests with equal variances were performed in Excel. Results are presented as the mean and s.e.m. For categorical data, comparisons were analyzed using Fisher's exact test in R. A p-value < 0.05 was considered significant.

Results

BMPER is expressed in cardiac endothelial cells and endothelial cell derivatives

Because BMPER was discovered in endothelial cell precursors (Moser et al., 2003) and BMPER mRNA is expressed in the zebrafish vasculature (Moser et al., 2007), we evaluated BMPER protein expression in the murine heart using immunohistochemistry. At E12.5–E14.5, BMPER was expressed in the outflow tract cushions and subsequent valve leaflets but was not expressed within the overlying endothelial layer (Fig. 1A, B, D–H). BMPER expression did not colocalize with the myocardium, and DAPI-positive, BMPER-negative endocardial cells are observed in the outflow tract cushions (Fig. 1A). BMPER's expression in the endocardial cells lining the trabeculae (Fig. 1A) appears to correlate with PECAM (Fig. 1B). Importantly, BMPER protein is not detected in BMPER$^{-/-}$ embryos (Fig. 1C). BMPER-positive coronary vessels were observed at the base of the outflow tract myocardium (Fig. 1D–F). Interestingly, the coronary arteries did not express BMPER during insertion into the aorta (Fig. 1G and H), suggesting that BMPER must be downregulated in the coronary endothelial cells prior to their connection with the aorta. The expression of BMPER in a subset of coronary vessels prompted us to examine whether BMPER plays a role in coronary plexus development.

Coronary plexus formation begins normally in the BMPER$^{-/-}$ embryo

Because BMPER and the BMP signaling pathway have been linked to the development of vascular networks in other models (Moser et al., 2007; Moreno-Miralles et al., 2011; Pi et al., 2012), coronary plexus development was examined in wild-type and

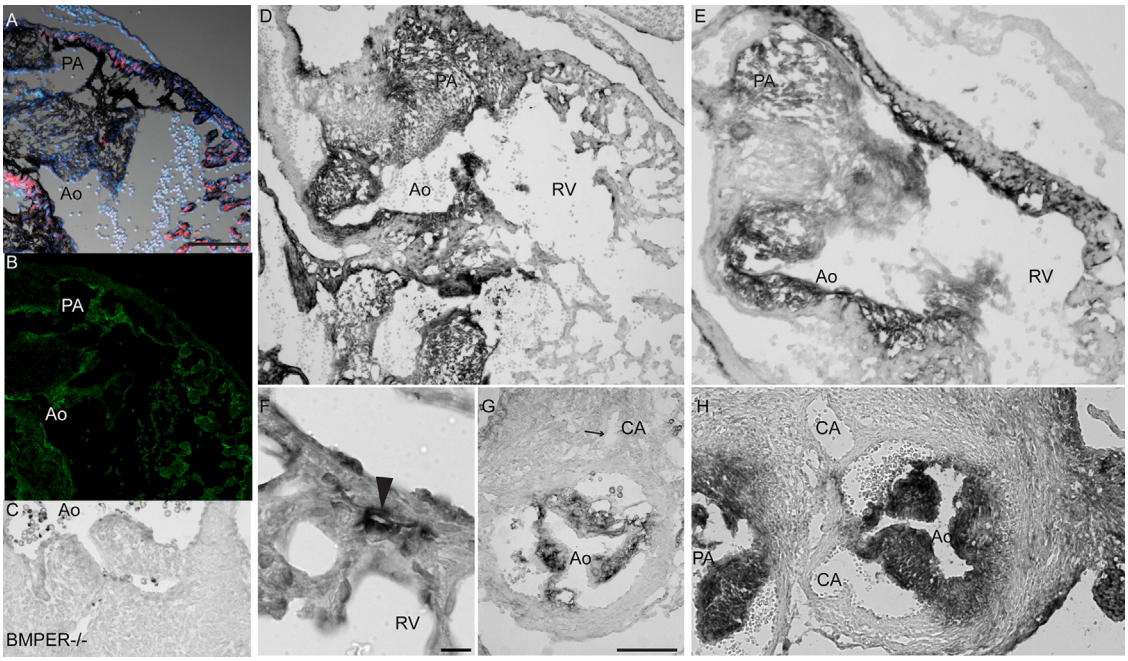

Fig. 1. BMPER is expressed in the endothelium and outflow tract cushions. Immunohistochemistry in saggital (A, B, D–F) and cross-sections (C, G, H) of E12.5 (A, B), E13.5 (D, F, G) and E14.5 (E, H) wild-type embryos and (C) an E13.5 BMPER$^{-/-}$ embryo demonstrates that BMPER expression is restricted to the outflow tract cushions and some endothelial cells during outflow tract septation and coronary artery recruitment. (A, B) BMPER (black) does not colocalize with myocardial marker MF20 (red, A), is excluded from the endocardial cells overlying the cushions (A), and shows a similar pattern in the ventricle as endothelial marker PECAM (green, B). (C) BMPER is not detected in the aortic valve of an E13.5 BMPER$^{-/-}$ embryo, highlighting the fidelity of the antibody. (D, E) Low magnification images of the outflow tract of wild-type E13.5 (D) and E14.5 (E) embryos, including aorta (Ao) and pulmonary artery. (F–H) Higher magnification images highlight a BMPER-positive coronary vessel in the compact myocardium (arrowhead, F) and BMPER expression in the aortic valve at E13.5 (G) and E14.5 (H); arrows indicate coronary stems. Ao, aorta; RV, right ventricle; PA, pulmonary artery; CA, coronary artery. Blue, DAPI. Scale bar in A–E, 100 μm; scale bar in F, 25 μm; scale bar in G, H, 50 μm.

BMPER$^{-/-}$ hearts to determine whether BMPER is important for the development of the coronary plexus. Whole mount immunohistochemical analysis of wild-type and BMPER$^{-/-}$ hearts at E14.5 showed that PECAM-positive endothelial cells completely encompassed the ventricles from sinus venosus to apex (Fig. 2A, C, and E) and were found within the compact myocardium of both wild-type and BMPER$^{-/-}$ ventricles (Fig. 2B, D, and F). Based on the whole mount immunohistochemistry, an analysis of the percentage of the heart encompassed by the forming plexus, the number of sprouts along the leading edge of the plexus, and the branch points within the plexus showed no differences between genotypes (Fig. 2G–I; see also Supplemental Fig. 1 for additional stages and details showing the measured parameters). Thus, coronary plexus formation occurs normally in the BMPER$^{-/-}$ embryo.

Coronary stem formation is disrupted in the BMPER$^{-/-}$ embryo

By E15.5, the coronary vasculature should be connected to the aorta via two distinct coronary ostia. Whole mount confocal analysis of wild-type hearts between E15.5 and E17.5 demonstrated normal connection of the coronary arteries to the aorta, with the coronary arteries connecting to the aorta below the semilunar valve, at the level of the myocardium (Fig. 3A–E). However, analysis of BMPER$^{-/-}$ embryos at these stages of development revealed varying degrees of malformation of the coronary stems. In some BMPER$^{-/-}$ embryos ($n=5$ of 18), the coronary stems connected high above the semilunar valve, known as a "high take-off coronary" (compare Fig. 3I with B). In such cases, the high take-off coronary was observed up to 200 μm above the semilunar valve, and the other coronary stem was typically in the correct location (Fig. 3H, I). In other BMPER$^{-/-}$ embryos, vessels were observed within the myocardium and even the subepicardial space, but these vessels failed to connect to the aorta ($n=9$ of 18; Supplemental Fig. 2). These defects were consistently observed in BMPER$^{-/-}$ embryos; only rarely was a coronary anomaly observed in wild-type embryos ($n=1$ of 11 wild-type hearts; $p<0.001$).

Because the C57BL/6N mouse strain is prone to high take-off and atretic coronaries (Fernandez et al., 2008), we compared the prevalence of defects in our mice to the reported prevalence of coronary anomalies in pure C57BL/6N mice, and coronary atresia was significantly more prevalent ($p<0.01$) in our BMPER$^{-/-}$ embryos on the mixed background. Thus, the presence of the C57BL/6N background in our mixed background does not explain the BMPER$^{-/-}$ phenotype.

Coronary artery smooth muscle recruitment is not impaired in BMPER$^{-/-}$ embryos

A final step in coronary stem formation is the addition of proepicardial-derived smooth muscle cells, which stabilize the coronary stems (Gittenberger-de Groot et al., 1999). Because BMP signaling promotes proepicardial addition (Schlueter et al., 2006; Ishii et al., 2010), BMPER expression was analyzed in the proepicardium at E9.5 to determine whether BMPER also affects proepicardial derivatives. BMPER expression did not colocalize with the proepicardial marker Tbx18, indicating that BMPER cannot directly regulate proepicardial addition (Supplemental Fig. 3A and B). Further, smooth muscle cells were recruited to the coronary stems by E15.5 in BMPER$^{-/-}$ embryos, as observed in wild-type embryos, indicating that proepicardial derivatives are correctly formed in the absence of BMPER (Supplemental Fig. 3C and D). Together, these data indicate that BMPER does not affect the proepicardium or its derivatives.

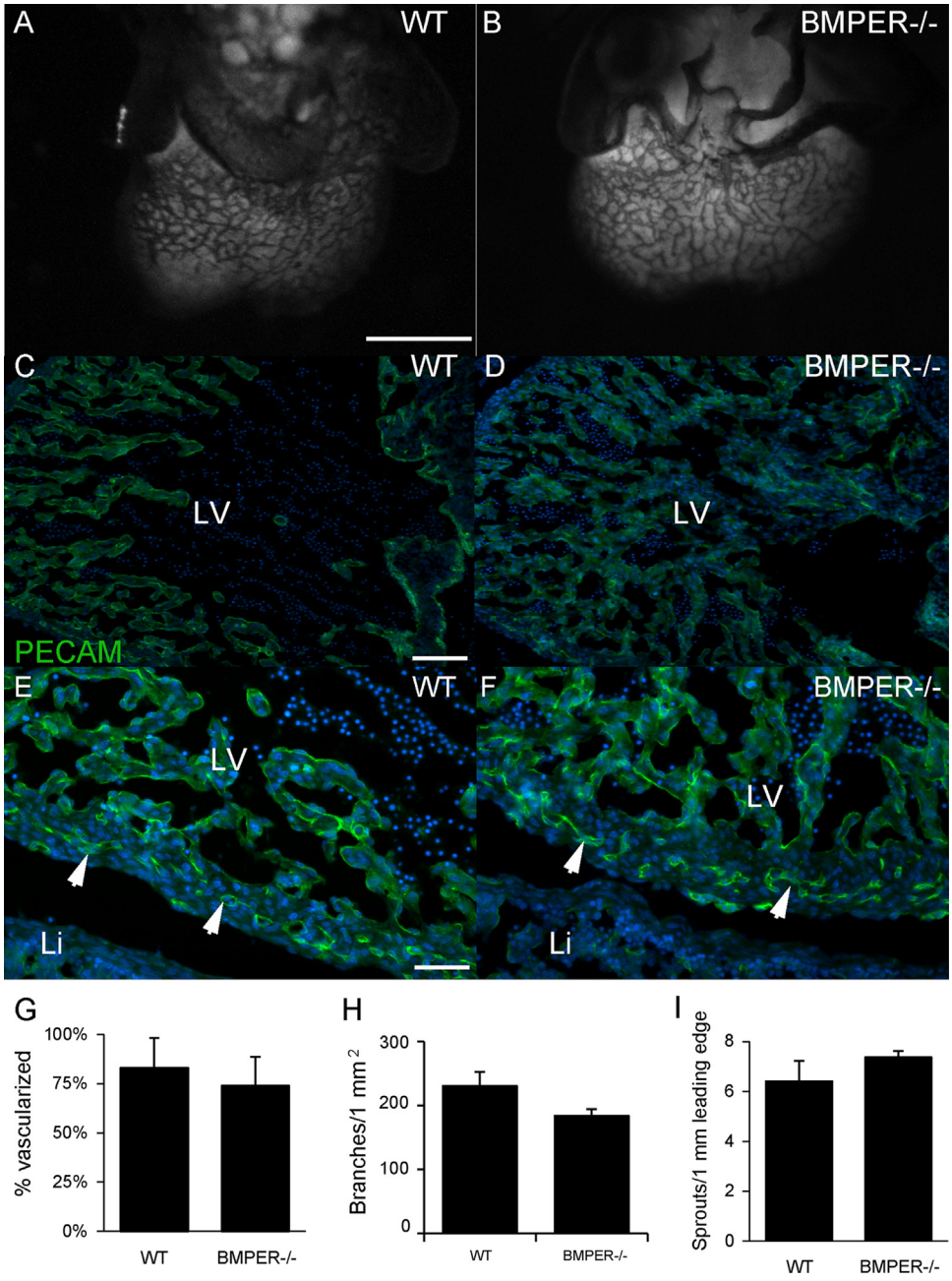

Fig. 2. The coronary plexus begins to form in the absence of BMPER. Whole mount immunohistochemistry in E14.5 wild-type (A) and BMPER$^{-/-}$ (B) hearts shows that endothelial cells (black) encompass the dorsal aspect of the ventricles and cover the ventricles by E14.5. Immunohistochemistry of sagittal sections of wild-type (C, E) and BMPER$^{-/-}$ (D, F) hearts at E14.5 shows that this vasculature permeates the compact myocardium. Shown are low magnification (C, D) and higher magnification (E, F) images of the left ventricle (LV) at the level of the mitral valve. Green, endothelium; blue, nuclei. To ensure that the vascular plexus developed normally in BMPER$^{-/-}$ embryos, the following measurements were compared in whole mount PECAM-labeled E14.5 hearts: the percentage of surface area encompassed by the plexus (G), the number of branch points (H), and the number of sprouts in the leading edge of the plexus (I). No differences were oberved between genotypes. Scale bar in A, B, 500 μm; scale bar in C, D, 100 μm; scale bar in E, F, 50 μm. Li, liver.

BMPER$^{-/-}$ coronary endothelial cells do not undergo abnormal proliferation or apoptosis

We next examined wild-type and BMPER$^{-/-}$ hearts to determine whether differences in endothelial cell proliferation or apoptosis may be a potential mechanism responsible for the coronary stem defects seen in the BMPER$^{-/-}$ hearts, given BMP's role in regulating endothelial proliferation and apoptosis. In E12.5–E16.5 wild-type and BMPER$^{-/-}$ hearts, apoptotic endothelial cells were rarely observed throughout the compact myocardium or specifically in the coronary stems, and no differences were observed between genotypes (Supplemental Fig. 4A–C). In addition, no difference in endothelial cell proliferation was observed between wild-type and BMPER$^{/}$ hearts between E13.5 and E16.5 (Supplemental Fig. 4D–F). Together, these data indicate that endothelial cell survival and proliferation are not responsible for the observed coronary defects seen in BMPER$^{-/-}$ hearts.

BMPER is required in a cell autonomous manner for coronary endothelial migration

Based on BMPER's expression pattern in the developing coronary vasculature and the outflow tract cushions, we hypothesized that BMPER may directly promote coronary endothelial migration

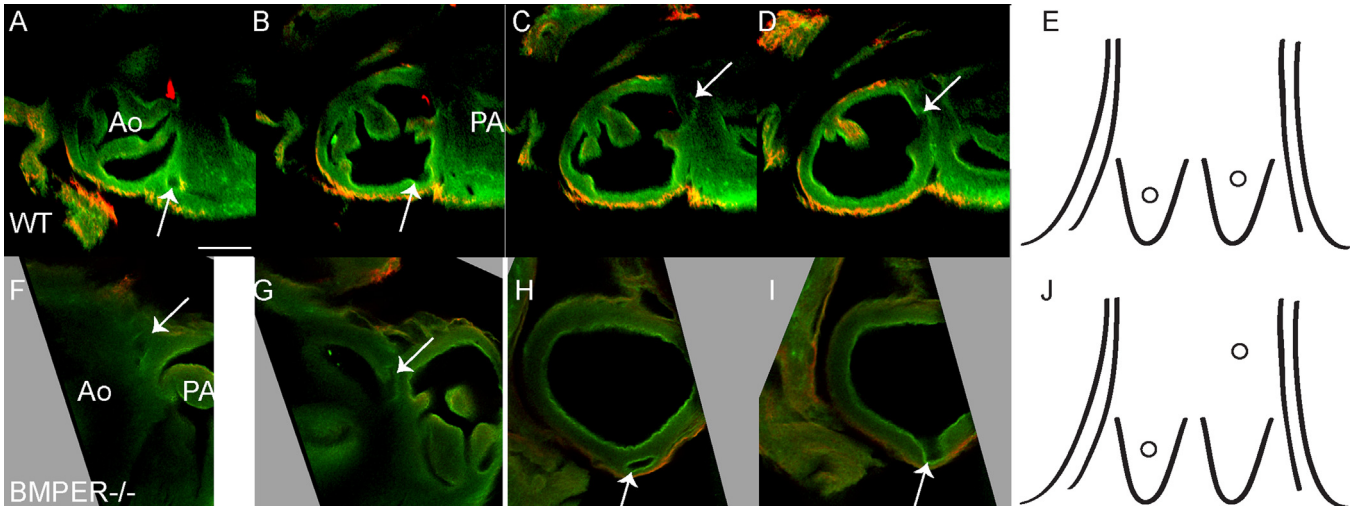

Fig. 3. BMPER$^{-/-}$ embryos exhibit coronary stem defects. Whole mount confocal imaging of E17.5 wild-type (A–D) and BMPER$^{-/-}$ (F–I) hearts through the aorta shows the connection of the coronary arteries in a wild-type heart (arrows in A–D, depicted schematically in E) and in a BMPER$^{-/-}$ heart (arrows in F–I, depicted schematically in J). In the BMPER$^{-/-}$ hearts, the coronary arteries either connect above the semilunar valve (arrow in H and I; $n=4$ of 13 embryos) or fail to form stems at all ($n=7$ of 13 embryos, Supplemental Fig. 1). These defects are rarely observed in wild-type embryos ($n=1$ of 9 embryos; $p<0.01$). (E, I) Schematics depicting the insertion of the coronary artery in the aortic sinuses; the schematic design is based on Clauss et al. (2006). Green, endothelium; red, myocardium. Ao, aorta. Scale bar, 150 μm.

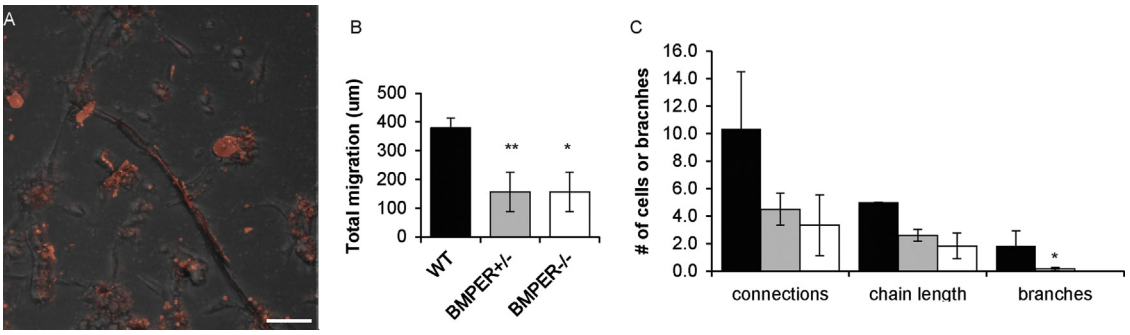

Fig. 4. Migration is impaired in isolated BMPER$^{-/-}$ ventricular endothelial cells. (A) Endothelial cells were isolated from E14.5-E16.5 wild-type (WT, $n=3$), BMPER+/− ($n=10$), and BMPER$^{-/-}$ ($n=3$) ventricles with PECAM-conjugated beads. Wild-type isolated endothelial cells were positively labeled with lectin (red). Scale bar, 50 μm. After 24 h of culture, isolated endothelial cells were labeled with Syto-16 and photographed over a 20-h period. Compared with wild-type endothelial cells (black bars), BMPER+/− (gray bars) and BMPER$^{-/-}$ (white bars) endothelial cells migrated less (B) and tended to create fewer cell–cell connections ($p=0.1$, ANOVA) and branches ($p<0.05$, ANOVA) and shorter chains (measured as the number of cells per chain, $p<0.1$, ANOVA) (C). $^{*}p<0.05$, $^{**}p<0.01$; Student's t-test.

or that BMPER may serve as a guidance cue to recruit coronary endothelial cells to the aorta at the level of the aortic valve. Thus, we first performed in vitro assays to determine the effect of BMPER specifically on coronary endothelial migration and tubulogenesis. Although siRNA is commonly used to assess the effects of gene knockdown in cell culture, siRNA knockdown techniques are typically not 100% efficient (Dennstedt and Bryan, 2011). BMPER's effects on BMP signaling appear to be concentration-dependent (Kelley et al., 2009), so a partial siRNA knockdown may have unintended consequences. Thus, to truly assess the effects of the absence of BMPER on endothelial cells, wild-type and BMPER$^{-/-}$ endothelial cells were isolated from the ventricles of E14.5–16.5 embryos. These isolated cells labeled positively with the endothelial markers isolectin, PECAM, Flk-1, and Syto-16 (Fig. 5A and Dyer and Patterson), confirming the identity of the isolated cells as endothelial cells and not supporting mural cells (Arima et al., 2011). Isolated endothelial cells were plated on a collagen gel and allowed to adhere for 24 h prior to live-cell imaging. Due to the limited number of primary cells obtained, traditional tubulogenesis assays were not possible. Instead, a 22-h live-cell imaging period was used to document the cells' behavior. Wild-type endothelial cells migrated robustly, forming cell–cell contacts that often led to chains with some branching (Fig. 4B and C). In contrast, BMPER$^{-/-}$ endothelial cells migrated shorter distances and tended to make fewer cell–cell contacts, which led to shorter chains with little to no branching (Fig. 4B and C). Further, BMPER$^{+/-}$ endothelial cells also display significantly reduced migration and fewer branches compared with wild-type endothelial cells (Fig. 4B and C). These experiments demonstrate that a specific dose of BMPER is required by ventricular endothelial cells for migration and remodeling.

BMPER promotes canonical BMP signaling in the aortic valve

BMPER's robust expression in the developing outflow tract cushions during coronary artery recruitment suggested that BMPER-mediated BMP signaling may affect coronary artery recruitment by recruiting the coronary arteries to the aortic valve. Thus, pSmad expression levels were examined in the valves at E13.5–E14.5. These specific stages were examined because the coronary stems are present by E14.0 (Compton et al., 2007). At E13.5, Smad was phosphorylated in the valves of wild-type embryos (Fig. 5A), whereas BMPER$^{-/-}$ embryos appeared reduced Smad phosphorylation levels (Fig. 5B). At E14.5, Smad phosphorylation remained high in the wild-type valves (Fig. 5C) and reduced in the BMPER$^{-/-}$ valves (Fig. 5D). These differences were confirmed by performing immunoblot. The

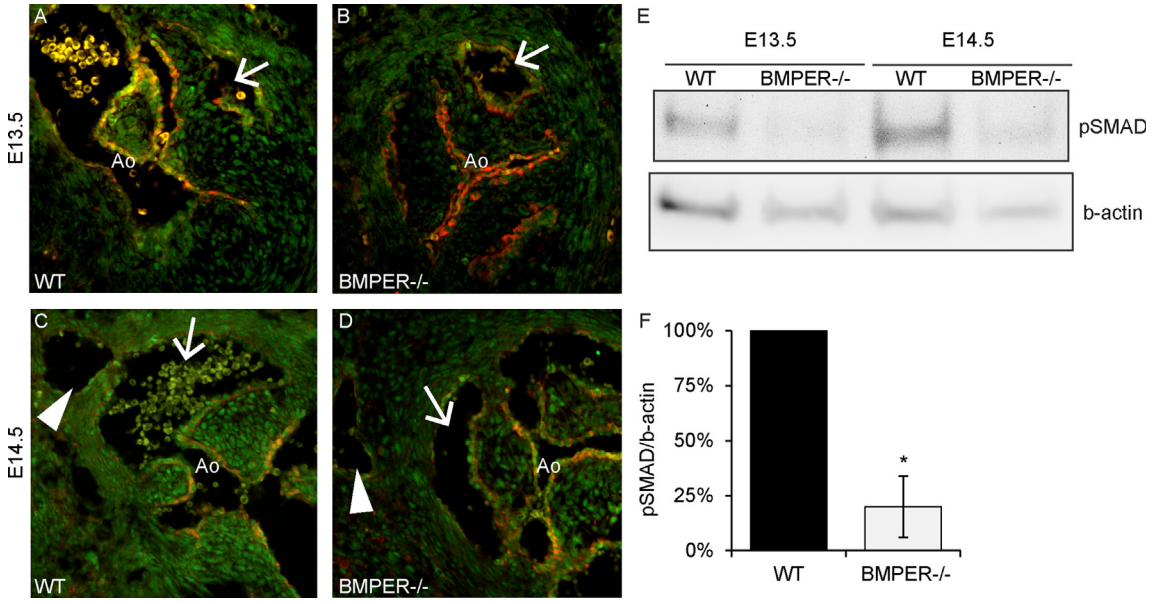

Fig. 5. Canonical BMP activity is downregulated in BMPER$^{-/-}$ aortic valves. Compared with wild-type aortic valves (A, C), BMP activity, as indicated by phosphorylated Smad1, 5, 8 expression (pSmad, green), is downregulated in BMPER$^{-/-}$ aortic valves at E13.5 (B) and E14.5 (D). (E) The pSmad levels were quantified in aortic valves dissected from E13.5 and E14.5 wild-type and BMPER$^{-/-}$ embryos. Four valves were pooled in each lane, and pSmad band density, normalized to β-actin, is quantified in (F) as the % of the age-matched wild-type sample. Red, endothelium, labeled with lectin. Arrows, aortic sinuses; arrowheads, coronary stems. Ao, aorta. Scale bar, 70 μm.

aortic valve, together with the surrounding aortic wall, was dissected from E13.5 and E14.5 wild-type and BMPER$^{-/-}$ embryos, and four embryos were pooled per sample. Compared with the wild-type valves, Smad phosphorylation levels were down-regulated in the BMPER$^{-/-}$ valves at both time points (Fig. 5E and F). By comparison, Smad phosphorylation in the valve sinus endothelium was slightly reduced in the BMPER$^{-/-}$ embryos at both E13.5 and E14.5 compared with wild-type embryos, but this difference was not significant. Thus, the specific downregulation of Smad phosphorylation specifically within the BMPER$^{-/-}$ aortic valve may be associated with the coronary artery insertion defects.

Although BMP signaling is most often associated with activation of Smad, BMPs can also lead to ERK1/2 phosphorylation (Derynck and Zhang, 2003). Therefore, ERK1/2 phosphorylation in the cardiac valves was also compared between wild-type and BMPER$^{-/-}$ hearts at E13.5–E14.5. However, at these stages of development, ERK1/2 phosphorylation was limited to the mesenchymal cells underlying the tips of the valves in both wild-type and BMPER$^{-/-}$ embryonic hearts (Supplemental Fig. 5). Thus, based on the discrete localization of ERK1/2 phosphorylation, these results suggest that ERK1/2 signaling does not affect coronary stem location.

BMPER may directly recruit coronary endothelial cells

Because BMPER is expressed in the aortic valve, at the level where the coronary arteries are recruited to the aorta (Fig. 1), where it promotes canonical BMP signaling (Fig. 5), we evaluated whether BMPER could recruit coronary vessels in an ex vivo culture system. Wild-type and BMPER$^{-/-}$ E13.5 hearts were excised and cultured in the presence of BMPER- or vehicle control-conjugated agarose beads. Smad phosphorylation appeared to be upregulated in cells adjacent to BMPER-conjugated beads in the BMPER$^{-/-}$ hearts compared with control beads (Fig. 6A). Further, the control beads appeared to have no effect on Smad phosphorylation in both genotypes, and Smad phosphorylation appeared downregulated in the BMPER$^{-/-}$ hearts with control beads compared with the wild-type counterparts, consistent with Fig. 5. Consistent with a pro-angiogenic role, BMPER-conjugated beads recruited PECAM-positive endothelial cells in both wild-type and BMPER$^{-/-}$ hearts (Fig. 6B).

To determine whether BMPER directly promotes endothelial migration, wild-type endothelial cells from E13.5 to 16.5 hearts were used for transwell assays. BMPER doses were based on previous work showing that BMPER can promote HUVEC migration (Heinke et al., 2008), and VEGF was used as a positive control (Fig. 6C). Compared with vehicle control, the lowest dose of BMPER (10 ng/ml) significantly increased endothelial migration 1.6-fold over a 4-h period (Fig. 6D). Higher doses of BMPER (20–75 ng/ml) decreased endothelial migration by one-third compared with the vehicle control. This dose response suggests that a low dose of BMPER can directly promote endothelial migration whereas higher doses halt migration.

Discussion

The coronary vasculature is essential for late embryonic development and post-natal life. However, whereas early stages of coronary vascular development have been well described (Riley and Smart, 2011), the crucial connection of the coronary plexus to the aorta remains poorly understood. In the mouse in particular, the process through which the stems are formed within the aorta has not been clearly documented. This study takes advantage of the BMPER$^{-/-}$ mouse model by beginning to unravel the distinct steps required for normal coronary stem development. The BMPER$^{-/-}$ mouse, which does not display early coronary plexus defects, provides a unique opportunity to address the signaling events required to recruit the coronary arteries to the aorta.

The BMP pathway has many known roles in endothelial cells, where it can promote endothelial cell migration, proliferation, and apoptosis (Wiley and Jin, 2011). Some of these specific roles have been attributed to individual BMPs and particular intracellular pathways. Specifically examining endothelial migration, BMP2 can induce endothelial remodeling via p38/MAPK, through which it modulates transcription factor Id1 (Raida et al., 2005), and possibly via ERK1/2, though current studies are contradictory (Langenfeld and Langenfeld, 2004; Raida et al., 2005). BMP4 can promote endothelial migration via both the Smad and ERK intracellular pathways (Heinke et al., 2008) and apoptosis through the Smad

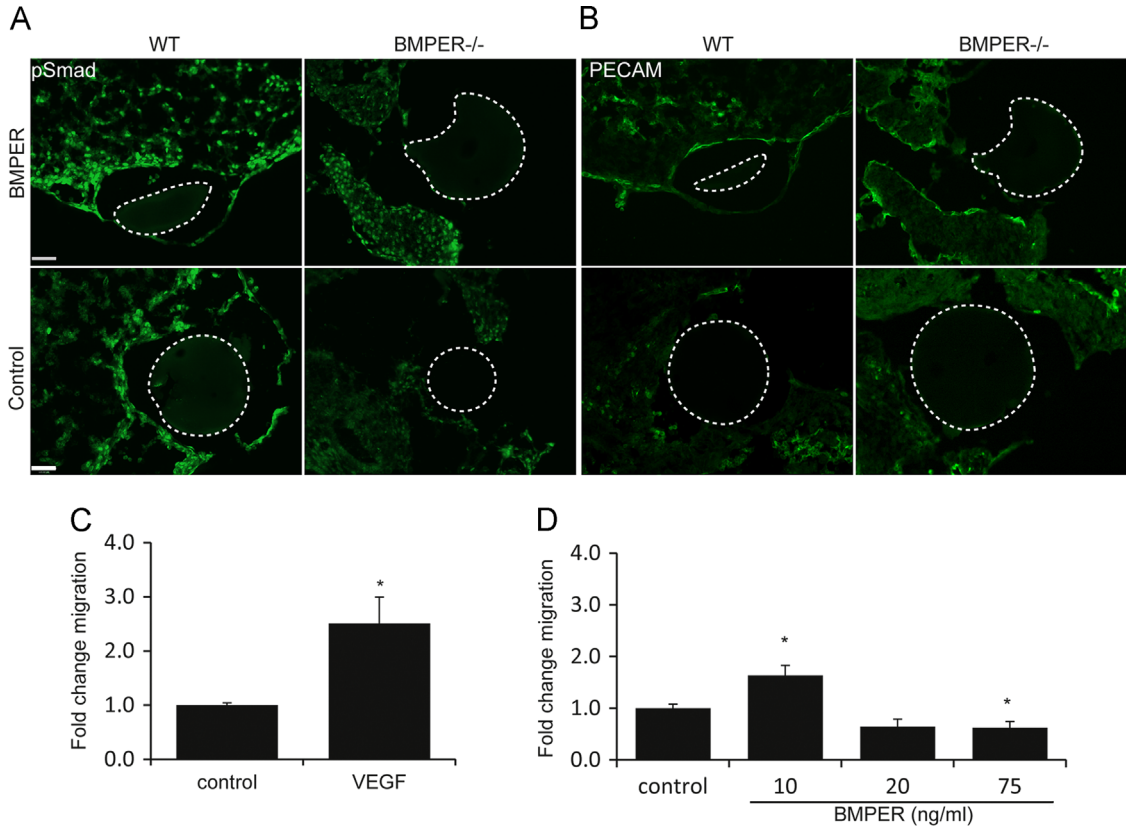

Fig. 6. BMPER indirectly promotes ventricular endothelial cell migration. (A, B) Wild-type and BMPER$^{-/-}$ E13.5 hearts were cultured ex vivo with vehichle control- or BMPER-conjugated beads. (A) Smad phosphorylation was assessed after 24 h of culture. pSmad signaling intensity was stronger in the cells adjacent to BMPER-conjugated beads compared with control beads and was similarly stronger in wild-type compared with BMPER$^{-/-}$ hearts. (B) Endothelial migration was also assessed after 24 h of culture using the endothelial marker PECAM. PECAM-positive vessels were more closely associated with BMPER-conjugated beads than with control beads. The small number of BMPER$^{-/-}$ hearts cultured with control beads precluded statistical analyses in this experiment. $n=6$ wild-type+control bead, 9 wild-type+BMPER bead, 2 BMPER$^{-/-}$+control bead, 4 BMPER$^{-/-}$+BMPER bead. Scale bars, 120 μm. (C, D) Isolated wild-type endothelial cells (E13.5–16.5) were subjected to transwell migration assays using (C) VEGF (25 ng/ml) and its vehicle control (0.1% BSA in 4 mM HCl) and (D) BMPER and its vehicle control (0.5% BSA in PBS). As expected, VEGF induced a nearly three-fold increase in migration compared with the vehicle control. In contrast, a low dose of BMPER (10 ng/ml) induced endothelial migration (1.6-fold over the control level of migration), whereas higher doses of BMPER (20–75 ng/ml) inhibited migration. *$p<0.05$ vs. control; $n=3$–7 transwells per condition.

and possibly Msx1/2 intracellular pathways (Kiyono and Shibuya, 2003). BMP6 can also promote endothelial migration via Id1 (Valdimarsdottir et al., 2002). Importantly within the context of coronary stem formation, BMPs 2, 4, and 6 are expressed either within the myocardium at the base of the aorta or in the aortic endothelium and aortic valve leaflets during the establishment of the coronary plexus and its subsequent connection with the aorta (Kim et al., 2001; Jiao et al., 2003; Keyes et al., 2003; Liu et al., 2004; Danesh et al., 2009; Jang et al., 2010). BMPER shows a similar expression pattern, with expression within the endothelial cells surrounding the outflow tract and within the endothelially derived aortic valve mesenchyme. Thus, BMPER is spatiotemporally located in a manner to regulate the BMPs that are being expressed. Further, the dose-response migration data (Fig. 6D) suggest that increasing concentrations of BMPER switch its role from acting as a pro-migratory factor to an anti-migratory factor. A quantitative assessment of the concentration of BMPER throughout the developing heart would be interesting in that it may determine whether BMPER′s expression level correlates with the dose-response migration data. However, without a reliable readout, for example, one that is comparable to using Smad phosphorylation to assess canonical BMP signaling, this experiment is not yet feasible.

Based on the expression of BMPs and BMPER and the known roles of BMP signaling in endothelial behavior, the observed coronary stem defects are not unexpected. However, coronary remodeling is poorly studied. Endothelial cells are so heterogeneous that it is difficult to translate results from one specific endothelial cell population to another (Dyer and Patterson, 2010), and embryonic coronary endothelial cells are not currently commercially available. To further complicate the study of BMPER in this population of cells, BMPER itself shows a dose-dependent biphasic effect (Fig. 6 and Kelley et al., 2009), rendering a partial siRNA knockdown model inadequate to address what happens in the absence of BMPER. To truly analyze coronary endothelial remodeling in vitro, specifically in the absence of BMPER, coronary endothelial cells from the BMPER$^{-/-}$ embryo is the ideal model.

Thus, we down-scaled well-established protocols for endothelial cell isolation and applied them to the developing heart (Dyer and Patterson). Preferably, these experiments would be performed using cells isolated just prior to connection of the coronary vasculature to the aorta; however, the yield of endothelial cells obtained from such small hearts was insufficient to perform the desired analyses, even with the down-scaled assay we employed. Instead, we utilized endothelial cells obtained from the hearts of E14.5–16.5 embryos; these stages include the coronary remodeling period (Lavine and Ornitz, 2009). Using this approach, we determined that coronary endothelial cells require BMPER to migrate. Cells isolated from BMPER$^{-/-}$ ventricles migrated less than their wild-type counterparts, and the subsequent observations of shorter chains and less branching may be directly caused by this impairment. Interestingly, though, BMPER is not expressed in the coronary endothelial cells as the coronary arteries connect with the aorta (Fig. 1G and H), suggesting that BMPER plays an earlier role in the endothelial cell that promotes migration. This early role

is supported by the in vitro coronary endothelial migration data. However, we believe that BMPER also has an indirect role in this process. The dose-dependent responses observed in the transwell migration assays suggest that wild-type coronary endothelial cells migrate in response to a low dose of BMPER but then stop migrating in response to high doses of BMPER. In the aortic valve, BMPER may affect additional signaling pathways that enhance coronary artery recruitment, leading to an even stronger effect than observed in our transwell assays. These findings may explain why coronary plexus formation can begin normally in the BMPER$^{-/-}$ ventricles but then remodels incorrectly, leading to defects in coronary stem formation. In addition, these findings may explain how the BMPER$^{-/-}$ embryo exhibits both atretic coronary stems, which may be due to a failure of the coronary endothelial cells to migrate enough to reach the aorta and/or a failure to detect the aortic valve, and high take-off coronary arteries, which may represent a simple failure of the coronary endothelial cells to detect the aortic valve. This hypothesis is further supported by the BMPER+/− embryo, which does not display coronary artery anomalies (data not shown) despite impaired endothelial cell migration (Fig. 5).

We have established that Smad-dependent BMP signaling is upregulated in the aortic valves when the coronary arteries should connect to the aorta and that BMPER is required for this activity. Further, BMPER is required specifically within the coronary endothelial cells and promotes migration and remodeling, as shown using isolated embryonic coronary endothelial cells. This study opens up an innovative tactic for examining coronary plexus formation and remodeling and the intrinsic and extrinsic factors that regulate these processes.

Author contributions

LD and CP designed the study; MM contributed reagents; LD and YW performed the experiments; LD, YW, and CP performed the data analysis; and LD and CP prepared and edited the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.12.019.

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