SM22α-targeted deletion of bone morphogenetic protein receptor 1A in mice impairs cardiac and vascular development, and influences organogenesis

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Expression of bone morphogenetic protein receptor 1A (BMPR1A) is attenuated in the lung vessels of patients with pulmonary arterial hypertension, but the functional impact of this abnormality is unknown. We ablated Bmpr1a in cardiomyocytes and vascular smooth muscle cells (VSMCs) by breeding mice possessing a flox allele of Bmpr1a (Bmpr1aflox) expressing R26R with SM22α-Cre mice. SM22α-Cre;R26R;Bmpr1aαflox/mice died soon after embryonic day 11 (E11) with massive vascular and pericardial hemorrhage and impaired brain development. At E10.5, SM22α-Cre;R26R;Bmpr1aαflox embryos showed thinning of the myocardium associated with reduced cell proliferation. These embryos also had severe dilatation of the aorta and large vessels with impaired investment of SMCs that was also related to reduced proliferation. SM22α-Cre;R26R;Bmpr1aαflox mice showed collapsed telencephalon in association with impaired clearing of brain microvessels in areas where reduced apoptosis was observed.

Transcript and protein levels of matrix metalloproteinase (MMP) 2 and 9 were reduced in E9.5 and E10.5 SM22α-Cre;R26R;Bmpr1aαflox embryos, respectively. Knock-down of BMPR1A by RNA interference in human pulmonary artery SMCs reduced MMP2 and MMP9 activity, attenuated serum-induced proliferation, and impaired PDGF-BB-directed migration. RNA interference of MMP2 or MMP9 recapitulated these abnormalities, supporting a functional interaction between BMP signaling and MMP expression. In human brain microvascular pericytes, knock-down of BMPR1A reduced MMP2 activity and knock-down of either BMPR1A or MMP2 caused resistance to apoptosis. Thus, loss of Bmpr1a, by decreasing MMP2 and/or MMP9 activity, can account for vascular dilatation and persistence of brain microvessels, leading to the impaired organogenesis documented in the brain.

KEY WORDS: Bmpr1a (Alk3), Vasculogenesis, Heart development, Craniofacial development, Matrix metalloproteinase (metalloproteinase), MMP2, MMP9, Smooth muscle cell proliferation, Pericyte apoptosis, SM22α (transgelin, Tagln), Mouse

INTRODUCTION

Bone morphogenetic protein receptors (BMPRs) are members of the transforming growth factor β superfamily of receptors (de Caestecker, 2004; Mehra and Wrana, 2002). Heteromeric complexes form between BMPR1 and BMPR2 (Gilboa et al., 2000). Aberrant BMP signaling has been linked to pulmonary disease (Deng et al., 2000; Lane et al., 2000; Thomson et al., 2001). Moreover, independent of a mutation, expression of BMPR1A is known as transgelin, Tagln) (Beppu et al., 2000), Bmpr2 (Park et al., 2006), lethality occurs between E10.5 and E11.5, in association with massive abdominal hemorrhage. These mice exhibit dilatation of large vessels owing to poor recruitment of VSMCs around the EC layer, but it is not clear whether the vascular phenotype is due to Bmpr1a-deficient ECs or SMCs (Park et al., 2006). In this study, we determined whether VSMC deletion of Bmpr1a could cause abnormalities in vasculogenesis that might explain a propensity to PAH. We bred mice expressing floxed Bmpr1a and ROSA26 with SM22α-Cre mice [SM22α is also known as transgelin (Tagln) – Mouse Genome Informatics]. Progeny homozygous for deletion of Bmpr1a, SM22α-Cre;R26R;Bmpr1aαflox, died soon after E11 with massive vascular and pericardial hemorrhage. These mice had a thin ventricular wall and aneurysmal dilatation of large vessels associated with reduced myocyte proliferation related to decreased MMP9 and MMP2 activities. Defective brain development documented in the SM22α-Cre;R26R;Bmpr1aαflox mice was associated with impaired clearing of brain microvessels related to a resistance of pericytes to apoptosis and decreased levels of MMP2.

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MATERIALS AND METHODS
Experimental model: SM22α-Cre;R26R;Bmpr1a<sup>flox/flox</sup> mice

We crossed SM22α-Cre mice with mice homozygous for the floxed Bmpr1a gene (Mishina et al., 2002) and the Cre reporter gene ROSA26 (R26R) (Soriano, 1999) (Bmpr1a<sup>flox</sup>;R26R<sup>Cre</sup>). F2 breeding was then realized by backcrossing F1 mice (SM22α-Cre;R26R<sup>Cre</sup>;<Bmpr1a<sup>flox</sup>/><Bmpr1a<sup>flox</sup>/> mice) to produce mice that were SM22α-Cre;R26R;Bmpr1a<sup>flox/flox</sup>. All studies were performed under a protocol approved by the Animal Care Committee at Stanford University in accordance with the guidelines of the American Physiological Society.

Genotyping
PCR was used to amplify Cre (Saam and Gordon, 1999), R26R (Soriano, 1999), the floxed Bmpr1a gene (Mishina et al., 2002), and the Cre reporter gene ROSA26 (R26R) (Soriano, 1999) (Bmpr1a<sup>flox</sup>;R26R<sup>Cre</sup>). F2 breeding was then realized by backcrossing F1 mice (SM22α-Cre;R26R<sup>Cre</sup>;<Bmpr1a<sup>flox</sup>/><Bmpr1a<sup>flox</sup>/> mice) to produce mice that were SM22α-Cre;R26R;Bmpr1a<sup>flox/flox</sup>. All studies were performed under a protocol approved by the Animal Care Committee at Stanford University in accordance with the guidelines of the American Physiological Society.

Preparation of embryos for histological analyses
Isolated E9.5-11 mouse embryos were fixed with formalin or 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), embedded in paraffin and cut transversely (7 μm).

Histology and immunostaining
Paraffin sections of brains, hearts and dorsal aortae of formalin-fixed embryos were stained with Hematoxylin and Eosin (H&E) to assess the phenotype resulting from deletion of Bmpr1a. To assess apoptosis, we performed the TUNEL assay using the ApopTag Peroxidase In Situ Ligation Apoptosis Detection Kit (Chemicon International, Temecula, CA). Sections were counterstained with Methyl Green (Vector Labs, Burlingame, CA).

To assess alpha smooth muscle actin (α-SMA-actin) or the proliferating cell nuclear antigen (PCNA), formalin-fixed tissue sections were incubated with either mouse anti-α-SMA-actin antibody (1:200, Sigma-Aldrich, St Louis, MO) or with biotinylated mouse anti-PCNA antibody (1:100, Zymed, South San Francisco, CA). For α-SMA-actin staining, sections were then incubated with goat anti-mouse-biotinylated antibody (1:500, Jackson ImmunoResearch, West Grove, PA). For both α-SMA-actin and PCNA staining, sections were incubated with streptavidin-biotin-horseradish peroxidase (HRP)-conjugated antibody (1:500, Jackson ImmunoResearch). Brown immunoreactivity was observed by subjecting the sections to diaminobenzidine substrate (DAB; Vector Labs). Sections stained with antibodies to α-SMA-actin and PCNA were counterstained with Hematoxylin and Methyl Green, respectively.

To assess apoptosis in brain pericytes, TUNEL assay using the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon) was followed by immunostaining for the pericyte marker NG2 (CSPG4 - Mouse Genome Informatics) (primary antibody, 1:100, Chemicon) on formalin-fixed head sections.

Expression of MMP2 and MMP9 in aortic walls and heart was analyzed in tissue sections of PFA-fixed embryos incubated with either an anti-MMP2 (Ab-4) mouse mAb (75-7F7) or an anti-MMP9 (Ab-3) mouse mAb (56-2A4) (1:100, Calbiochem, EMD Biosciences, San Diego, CA) followed by Alexa Fluor 488 goat anti-mouse IgG (H+L, 1:200, Molecular Probes, Invitrogen, Carlsbad, CA).

Gelatin zymography
Conditioned media collected from the upper compartments of the Boyden Chambers were kept under serum-free conditions for an additional 24 hours, after which apoptosis was assessed by measuring caspase 3 and 7 activity using the Caspase 3/Caspase 7 Luminescent Assay Kit (Caspase-Glo, Promega, Madison, WI), and by cell counts. Transfected HBVPs were kept under serum-free conditions for an additional 24 hours, after which apoptosis was assessed by measuring caspase 3 and 7 activity using the Caspase 3/Caspase 7 Luminescent Assay Kit (Caspase-Glo, Promega, Madison, WI), and by cell counts. Transfected HBVPs were kept under serum-free conditions for an additional 24 hours, after which apoptosis was assessed by measuring caspase 3 and 7 activity using the Caspase 3/Caspase 7 Luminescent Assay Kit (Caspase-Glo, Promega, Madison, WI), and by cell counts. Transfected HBVPs were kept under serum-free conditions for an additional 24 hours, after which apoptosis was assessed by measuring caspase 3 and 7 activity using the Caspase 3/Caspase 7 Luminescent Assay Kit (Caspase-Glo, Promega, Madison, WI), and by cell counts.
any mutant (f/f\textsuperscript{26R}) or WT (WT\textsuperscript{26R}) mouse expressing Cre and the R26R gene, express \(eta\)-galactosidase and can be used for lacZ staining. (B) Cre activity in SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} embryos. Whole-mount lacZ staining of SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} embryos showing blue staining (a) in the heart (asterisk) and dorsal aorta (arrow) at E9.25, and (b) in smaller vessels as well as somatic myotomes (arrows) at E10.5. (C) Phenotype of SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} (b,d,f) compared with WT (a,c,e) embryos. SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} mutants appear normal at E9.5 (b versus a) and relatively reduced in size at E10.5 (d versus c); at E11, areas of hemorrhage are noted in the heart (asterisk) and abdomen (asterisk), as well as near the mouth (arrow) and brain (arrowhead) (f versus e). (D) Percentage of total mice of SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} genotype. Numbers of genotyped mice of each age are depicted in parentheses.

Cardiac defect in SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} embryos: thinning of the myocardium associated with reduced proliferation

Cre activity was confined to atrial and ventricular myocytes, with no expression in the endocardium of E10.5 \textit{fox/flox} embryos as assessed by whole-mount lacZ staining (Fig. 2A,B). Myocardial deletion by SM22\textalpha-Cre is consistent with transient expression of SM22\textalpha in the developing heart (Li et al., 1996; Umans et al., 2007).

To assess the sequelae of loss of Bmpr1\textalpha in cardiomyocytes on embryonic cardiac development, histological analysis of heart sections of viable E10.5-11 SM22\textalpha-Cre;R26R;Bmpr1\textalpha\textsuperscript{flox/flox} and age-matched littermate control embryos (WT) was carried out to show the four chambers and outflow tract anatomy at multiple levels. We noted thinning of the ventricular wall in the \textit{fox/flox} versus WT hearts (Fig. 2, compare D with C), quantified as a 35% reduction in the number of ventricular cells per heart section (P<0.05) (Fig. 2E). The cardiac phenotype was not due to enhanced apoptosis as only the occasional TUNEL-positive cell was seen (Fig. 2F,G), but rather was associated with attenuated cell proliferation. There was a reduction in the percentage of PCNA-positive cells over the total number of ventricular cells in heart sections of the \textit{fox/flox} (Fig. 2I) versus WT (Fig. 2H) (P<0.05) at E9.5 that persisted at E10.5-11 (Fig. 2J). As Bmp10-deficient embryos show thinning of the myocardium associated with decreased cell proliferation and ectopic expression of p57KIP2 (Chen et al., 2004), we assessed the expression of p57KIP2 by immunofluorescence to address the possibility that a deletion of \textit{Bmpr1\textalpha} in the heart might lead to a defect in BMP10 signaling. Our results showed no difference in p57KIP2 immunoreactivity between mutant (Fig. 2L) and WT hearts (Fig. 2K).

Vascular defect in SM22\textalpha-Cre;R26R;Bmpr1a\textsuperscript{flox/flox} embryos: dilatation of large vessels associated with reduced proliferation of vascular smooth muscle cells

To characterize the vascular defect resulting in perivascular hemorrhage and lethality in \textit{fox/flox} embryos, we performed whole-mount PECAM staining on embryos at E10.5. Gross morphological examination revealed massive dilatation of the large vessels appreciated in the dorsal aortae, mesenteric (Fig. 3A,b,d,f) and cranial vessels (not shown) of the mutants versus WT littermates.
Fig. 2. Cardiac defect in SM22α-Cre;R26R;Bmpr1aα/βflox/flox mouse embryos. (A) Blue staining (Cre activity) in the atrial (am) and ventricular (vm) myocytes but not in endocardial cells (ec) by whole-mount lacZ staining in E10.5 SM22α-Cre;R26R;Bmpr1aα/βflox/flox embryos. (B) An enlargement of A. (C,D,F,G,H,I) Consecutive transverse sections of wild-type (WT) (C,F,H) and SM22α-Cre;R26R;Bmpr1aα/βflox/flox (D,G,I) hearts taken at the same level from viable E11 embryos. H&E staining shows thinning of the ventricular wall (arrowheads in high-magnification insets) in the flox/flox mutant heart (D) versus WT (C). Apoptosis was infrequent in ventricular sections of E11 SM22α-Cre;R26R;Bmpr1aα/βflox/flox mutant (G) and WT (F) by TUNEL immunostaining. However, fewer PCNA-positive cells (brown) were observed in the mutant ventricles (I, high-magnification inset, arrows) compared with the WT (H, inset, arrows). (E) A numerical assessment of hematoxylin-stained nuclei per ventricular section of E10.5-11 WT and SM22α-Cre;R26R;Bmpr1aα/βflox/flox mutants. Bars indicate mean±s.e.m. (n=3). *P<0.05. (J) A numerical assessment of percentage of PCNA-positive cells over the total number of ventricular cells in heart sections of WT and flox/flox (ff) mutant embryos at E9.5 and E10.5-11. Bars indicate mean±s.e.m. (n=3-4). *P<0.05. (K,L) p53KIP2 immunostaining in E10.5 WT (K) and flox/flox mutant (L) heart sections. RA, right atrium; RV and LV, right and left ventricle, respectively; EC, endocardial cushions; IVS, interventricular septum. Panels depicting WT and their corresponding mutants have the same magnification. Scale bars: 100 μm.

There were more ramifications or interconnections in the interlimb vessels of the flox/flox (Fig. 3A) versus WT (Fig. 3Ae) embryos. H&E-stained transverse sections of E10.5 embryos showed dilated aortae (Fig. 3Ah) in flox/flox embryos relative to WT controls (Fig. 3Ag).

Whole-mount staging revealed poor investment of SM22α-Cre-expressing lacZ-positive cells in the dilated aortic wall of the SM22α-Cre;R26R;Bmpr1aα/βflox/flox mutants (Fig. 3Aj) as compared with WT (Fig. 3Ai), where strong lacZ staining was evident. The lacZ-positive cells were identified as being of smooth muscle lineage by immunoperoxidase staining using an antibody for αSM-actin. There were also fewer surrounding mesenchymal cells expressing αSM-actin in SM22α-Cre;R26R;Bmpr1aα/βflox/flox (Fig. 3Bb) versus WT (Fig. 3Ba) embryos. TUNEL staining on sections of aorta revealed only occasional positive mesenchymal cells (Fig. 3Bc,d, arrows). Instead, the decreased number of αSM-actin-positive perivascular cells was consistent with reduced proliferation as assessed by PCNA staining (Fig. 3B, compare f with e, arrows). Quantitative analysis revealed a ~53% reduction in the percentage of PCNA-positive SMCs forming the vessel wall (Fig. 3Bg) (P<0.05). PECAM staining of sections did not reveal a difference in the number of ECs surrounding the dilated vessels, but the cells appeared ‘stretched’ (data not shown).

Defective brain development of SM22α-Cre;R26R;Bmpr1αα/βflox/flox embryos associated with impaired clearing of small vessels

Cre activity was seen in the forebrain of an E10.5 WT embryo by whole-mount lacZ staining (Fig. 4A). To characterize and better visualize any brain development abnormality, we examined heads of embryos incubated with ethidium bromide under UV light. Compared with WT (Fig. 4Ba,c), flox/flox mutant embryos (Fig. 4Bb,d) showed brain compression and collapse of telencephalic vesicles. These defects were apparent in H&E-stained transverse sections of the heads at multiple levels (Fig. 4Bf,h,j,l). To determine how loss of Bmpr1a in SM22α-expressing cells could impair brain development, we performed whole-mount PECAM staining on embryos at E9.5 and E10.5. We observed similar brain vessel distribution in the WT and flox/flox mutants at E9.5 (data not shown); however, at E10.5, we noted evidence of clearing of telencephalic vessels in the WT (Fig. 4Ca,c) but not the mutants (Fig. 4Cb,d).

Transverse sections of the brains stained for PECAM at the level of the nasal-mandibular processes showed histologic evidence of clearing of vessels in the WT heads (Fig. 4Ce), whereas flox/flox mutant heads (Fig. 4Ci) showed persistent vessels (brown). To determine whether the clearing of vessels is related to apoptosis, the TUNEL assay was performed on brain sections. TUNEL-positive cells were plentiful in the WT (Fig. 4Cg) but were almost absent from the flox/flox mutant heads (Fig. 4Ch). Quantitative analysis showed a ~62% reduction in the percentage of TUNEL-positive cells over the total number of cells in the flox/flox group (Fig. 4Ck) (P<0.05). Since pericytes express SM22α (Ding et al., 2004), we speculated that loss of Bmpr1a in these cells led to resistance to apoptosis and reduced clearing of brain microvessels. We therefore performed a fluorescent TUNEL assay followed by fluorescent immunostaining for NG2, a pericyte marker. Reduced apoptosis was associated with an increased number of pericytes in the mutant (Fig. 4Cj) versus WT (Fig. 4Ci) brains. Because co-localization of the TUNEL and NG2.
staining was not observed in the WT brain (Fig. 4Ci), we could not confirm ongoing apoptosis of pericytes, suggesting that this occurred before E10.5. Persistence of brain microvessels was not due to enhanced cell growth, as PCNA immunoreactivity showed no difference between E10.5 WT and flox/flox mutants (Fig. 4Cl).

Reduced MMP9 and MMP2 in embryos with SM22α-targeted deletion of Bmpr1a

We investigated the level of expression of candidate genes dysregulated by loss of Bmpr1a that could account both for resistance to apoptosis in pericytes and repression of proliferation in VSMCs. For example, BMPs increase MMP activity and mRNA expression (Mishina et al., 2004; Palosaari et al., 2003) and MMPs can regulate cell survival (Jones et al., 1997) and induce proliferation of VSMCs (Zempo et al., 1994). Quantitative RT-PCR was applied to embryonic extracts to assess differential expression of MMPs and other extracellular matrix genes that could be modulated by loss of Bmp1a and might account for these altered vascular cell phenotypes. Many of the genes that we assessed are modified in other embryonic mouse models of vascular dilatation (Oh et al., 2000). We also assessed the transcript levels of genes implicated in vasculo/angiogenesis – vascular endothelial growth factor (Vegf; also known as Vegfa) and the angiopoietins (Angpt1, Angpt2) – and of the phosphatase and tensin homolog gene (Pten), a known gene downstream of BMP signaling implicated in juvenile polyposis (He et al., 2004) that might also impact cell growth (Beck and Carethers, 2007). RNA was extracted from E9.5 embryos, preceding the appearance of the phenotype in SM22α-Cre;R26R;Bmpr1a flox/flox mice. We found a significant decrease in the mRNA expression of Mmp9 (P<0.05) and Mmp2 (P<0.05), and trends toward reduced expression of tenascin C (Tnc), fibronectin, connective tissue growth factor (Ctgf) and urokinase plasminogen activator (uPA; Plau – Mouse Genome Informatics) were observed (Fig. 5A). No differences in tissue plasminogen activator (tPA; Plat) (Fig. 5A), Angpt1, Angpt2, Vegf (data not shown) and Pten (Fig. 5A) mRNA levels were noted between the WT and mutants.

This decrease in Mmp2 and Mmp9 transcripts was associated with a decrease, although not statistically significant, in the pro (40%) and active (30%) forms of MMP2 in SM22α-Cre;R26R;Bmpr1a flox/flox mutants versus WT, as assessed by gelatin zymography on mouse embryos (data not shown).
To determine whether the decrease in mRNA levels of \textit{Mmp9} and \textit{Mmp2} in whole E9.5 mutant mouse embryos is translated into reduced protein expression at a later age, we performed immunostaining of MMP9 and MMP2 at E10.5. We found abundant MMP9 and, to a greater extent, MMP2, in the aortic walls of WT embryos (Fig. 5Ba,c) and only weak immunoreactivity in the mutants (Fig. 5Bb,d). However, a low and diffuse immunostaining was noted in the heart and brains of WT and mutants (data not shown).

Loss of \textit{BMPR1A} attenuates proliferation and directed migration of vascular smooth muscle cells and induces pericyte resistance to apoptosis via reduced MMP9 and MMP2

Subsequent studies were carried out using cultured human pulmonary artery smooth muscle cells (HPASMCs) and human brain (micro)vascular pericytes (HBVPs) to determine (1) whether reducing levels of \textit{BMPR1A} by RNAi would result in suppression of MMP9 and/or MMP2 activities and (2) whether reducing \textit{BMPR1A}, MMP9 and/or MMP2, represses proliferation of VSMCs and induces resistance to apoptosis in pericytes. We reduced the mRNA level of \textit{BMPR1A} by 66% by transfecting HPASMCs with siRNA (SiBMPR1A), and observed, by gelatin zymography, that SiBMPR1A-transfected cells had decreased levels of the pro and active forms of MMP9 (\textit{P}<0.05 for both) and of MMP2 (\textit{P}<0.01 and \textit{P}<0.05, respectively) versus SiControl-transfected cells (Fig. 6A).

Consistent with our hypothesis and our findings in the mouse embryo, we showed that RNAi-mediated reduction in mRNA of \textit{BMPR1A} (by 66%), MMP9 (to undetectable levels) or MMP2 (by >80%), resulted in a 35-40% reduction in HPASMC proliferation in response to 10% FBS as assessed by the MTT assay (\textit{P}<0.001, Fig. 6B) and cell counts (data not shown). Since MMP9 and MMP2 levels increase in migrating SMCs (Bendeck et al., 2002; Franco et al., 2006; Kuzuya et al., 2003; Mason et al., 1999), we determined whether the chemotactic migratory behavior of SMCs was impaired by loss of \textit{BMPR1A}, in association with reduced MMP9 and/or MMP2. A deficiency in SMC migration could also account for the lack of SMC investment of the aneurysmally dilated vessels in \textit{flx/flox} embryos. We serum starved HPASMCs in 0.1% FBS for 48 hours and then assessed their response to a 6-hour treatment with PDGF-BB (20 ng/ml) using a modified Boyden Chamber assay. The MMP9 and MMP2 activities in SiControl HPASMCs, as assessed by gelatin zymography, were repressed in SiBMPR1A-treated cells (\textit{P}<0.01 for MMP9 and \textit{P}<0.001 for proMMP2 and MMP2) (Fig. 6C).
Although basal levels of migration were increased in SiBMPR1A-transfected HPASMCs ($P<0.001$), these cells did not significantly migrate in response to PDGF-BB when compared with SiControl HPASMCs ($P<0.05$) (Fig. 7B).

We then investigated whether pericytes with loss of BMPR1A would be resistant to apoptosis owing to a reduction in MMP2 activity, as MMP2 activity is proapoptotic in pericytes of diabetic patients (Yang et al., 2007). Using RNAi under conditions of serum starvation (0.1% FBS) for 48 hours, we showed a 53% reduction in BMPR1A transcript levels in HBVPs. To induce apoptosis, cells were serum deprived for an additional 24 hours, after which a reduction in both pro and active forms of MMP2 was demonstrated by gelatin zymography ($P<0.05$) (Fig. 8A). We then used RNAi to reduce mRNA levels of MMP2 in HBVPs and confirmed the decrease in pro and active forms of MMP2 by gelatin zymography ($P<0.001$) (Fig. 8B). A control experiment showing the sensitivity of apoptosis to MMP9 and MMP2 expression in SM22α-driven Bmpr1a loss in mice

![Fig. 5. Attenuated MMP9 and MMP2 expression in SM22α-Cre;R26R;Bmpr1aflox/flox mouse embryos. (A) Profiling by qR-PCR the expression of genes that might be modified by the SM22α-targeted deletion of Bmpr1a. Values are shown relative to 18S RNA. n=3 where each sample combines 3-4 embryos. *P<0.05. Eln, elastin; Colla1, collagen Ia1. (B) Reduction of MMP9 (b versus WT in a) and MMP2 (d versus WT in c) protein expression in aortic walls of flox/flox embryos as assessed by immunofluorescence. Note the absence of immunoreactivity in an aortic section of a WT incubated with only the secondary antibody as a negative control (e). Panels depict WT and mutants at the same magnification. Scale bar: 50 μm.

![Fig. 6. RNAi-induced loss of BMPR1A, by reducing MMP9 and MMP2 expression, attenuates proliferation of human vascular smooth muscle cells (HPASMCs). (A) Gelatin zymography using conditioned media of HPASMCs. Gelatin zymograms performed on conditioned media (left) and densitometric analysis (right) show MMP9 and MMP2 activities in SiBMPR1A-treated (SiBR1A) versus SiControl-treated (SiC) cells in response to serum (10% FBS) for 6 hours. Bars indicate mean±s.e.m. of densitometric values of SIBR1A proMMP9, MMP9, proMMP2 and MMP2 normalized to their corresponding SiC values (n=3-4). *P<0.05 and **P<0.01 between SiBR1A and SiC. (B) Proliferation of HPASMCs in response to serum using the MTT assay. HPASMCs transfected with SiC (white bars), SiBR1A, SiMMP9, SiMMP2 and combined SiMMP9 and SiMMP2 were subjected to 0.1% FBS (all white bars) or stimulated with 10% FBS (all black bars) for 72 hours, after which proliferation was assessed by the MTT assay. Bars indicate mean±s.e.m. of arbitrary OD570 values normalized to values of SiC under 0.1% FBS. n=12 for siMMP2 and/or siMMP9 and n=26 for SiC and SiBMPR1A from three independent experiments. †††P<0.001 for serum-stimulated versus unstimulated comparisons, and ***P<0.001 for comparisons with SiC at 10% FBS.
of gel zymography to detect gelatinase activity in a dose-dependent manner is provided in Fig. S1 (see supplementary material). Transfecting HBVPs with SiBMPR1A, or with SiMMP2, induced resistance to apoptosis when compared with SiControl HBVPs (P<0.001), as assessed by caspase 3 and 7 activities (Fig. 8C), without affecting cell proliferation as assessed by the MTT assay (data not shown). Therefore, lack of MMP2 in pericytes could account for the resistance to apoptosis seen in highly vascularized areas of flox/flox mutant brains.

**DISCUSSION**

**Bmprr1a in cardiac development**

The cardiac phenotype in E10.5 SM22α-Cre;R26R;Bmpr1a flox/flox mouse embryos was characterized by thinning of the ventricular wall and was attributed to reduced cell proliferation evident at E9.5. Bmp10-null mice also develop hearts with hypoplastic walls owing to reduced proliferation of cardiac myocytes at E9.0-9.5 (Chen et al., 2004). However, our results showed that BMP10 signaling in E10.5 SM22α-Cre;R26R;Bmpr1a flox/flox heart sections was not affected by Bmpr1a deletion.

It is interesting that in the mouse in which Bmpr1a was deleted following activation of the cardiac myocyte-specific promoter alpha myosin heavy chain (αMHC-Cre;Bmpr1a flox/flox) (Gaussin et al., 2002), the ventricular thinning that took place at a later time point (E11.5-12.5) was attributed to enhanced apoptosis. Ventricular thinning was also seen at E11.5-12.5 in mice lacking Bmpr1a in cardiac progenitors (Isl1-Cre;Bmpr1a nulls) (Yang et al., 2006), or at E11.5 with cardiac-specific ablation of Smad4 (Song et al., 2007), and both were associated with attenuated proliferation and enhanced apoptosis of the ventricular septal myocytes. This suggests that differences in the timing of promoter activation and Bmpr1a deletion in cardiomyocytes might dictate whether the thinning of the ventricular wall will be the result of apoptosis and/or reduced proliferation.

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**Fig. 7. RNAi-induced loss of BMPR1A, by reducing MMP9 and MMP2 expression, impairs directed migration of human vascular smooth muscle cells.** (A) Gelatin zymography in response to PDGF-BB. Gelatin zymography performed on conditioned media (left) and densitometric analysis (right) show MMP9 and pro and active MMP2 activities in SiBMPR1A-treated (SiBR1A) versus SiControl-treated (SiC) cells in response to PDGF-BB (20 ng/ml) stimulation for 6 hours. Bars indicate mean±s.e.m. of densitometric values of SiBR1A MMP forms normalized to the corresponding SiC values. (n=4). **P<0.01, ***P<0.001. (B) Migration in response to PDGF-BB as assessed by modified Boyden Chamber assay. SiC- or SiBR1A-transfected HPASMCs were stimulated with 20 ng/ml of PDGF-BB for 6 hours. Bars represent mean±s.e.m. of migrating cells in 5-6 different microscopic fields. (n=4). †P<0.05 for stimulated versus unstimulated comparisons for each Si; ***P<0.001 for comparisons between SiBR1A and SiC. Representative micrographs show the number of migrating cells under each condition. Scale bar: 100 μm.

**Fig. 8. RNAi-induced loss of BMPR1A, by reducing MMP2 expression, attenuates apoptosis of human pericytes (HBVPs).** (A) Gelatin zymography using conditioned media of HBVPs in response to serum deprivation. Gelatin zymograms of conditioned media (top) with densitometric analysis (beneath) to assess MMP2 activities in SiBMPR1A-transfected (SiBR1A) as compared with SiControl-transfected (SiC) cells. Bars represent mean±s.e.m. of densitometric values of SiBR1A MMP2 forms normalized to the corresponding SiC value. n=7-8 for pro and n=3-4 for active MMP2. *P<0.05 versus SiC. (B) Reduced MMP2 activity in HBVPs transfected with MMP2 RNAi. Gelatin zymogram of conditioned media (top) and densitometric values (beneath) of MMP2 in HBVPs transfected with SiMMP2. Bars represent mean±s.e.m. of densitometric values of SiBR1A MMP2 forms normalized to SiC value (n=4). ***P<0.001. (C) Apoptosis of HBVPs in response to serum deprivation using caspase 3/7 assay. Apoptosis in HBVPs transfected with SiC, SiBR1A or SiMMP2 was induced by serum-deprivation for 24 hours and assessed by a caspase 3/7 luminescent assay. Bars represent mean±s.e.m. of arbitrary luminescent values (n=6-9). ***P<0.001 versus SiC.
**Bmp1a and vasculogenesis**

We cannot exclude the possibility that the myocardial thinning is secondary to a hemodynamic abnormality caused by the vascular phenotype observed in the *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* embryos, and characterized by aneurysmal dilatation of the dorsal aorta and other large vessels. Dilatation of the aorta was observed in embryos with Fkikl-targeted deletion of *Bmp1a* (*Fkikl-Cre;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* (Park et al., 2006) and in embryos null for *Alk1* (*Acvrl1* – Mouse Genome Informatics) (Oh et al., 2000) or *Smad5* (Yang et al., 1999). In those models, the dilatation was attributed to a paracrine effect of *Bmp1a*-deficient ECs repressing the recruitment of VSMCs or pericytes, as observed in mice lacking PDGF-BB or PDGF-Rβ (Hollstrom et al., 1999; Lindahl et al., 1997). Other possibilities suggested include poor transdifferentiation of ECs into SMCs, or a defect in SMC growth affecting vessel maturation and integrity (Park et al., 2006). The third explanation fits best with the further delineation of the phenotype of *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* embryos that we carried out.

We were able to assess the impact of *Bmp1a* deletion in reducing VSMC proliferation in the tissue as well as in cultured cells, in which we also observed impaired PDGF-BB-directed VSMC migration. No defect in the EC layer was noted in the *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* embryos by whole-mount PECA immunostaining (data not shown) that might explain the vascular defect through a non-cell-autonomous contribution. In addition, we did not observe upregulation of angiogenic factors, such as of *Angpt1* and *Angpt2* as described in *Alk1-null* embryos (Oh et al., 2000), or of VEGF as observed in both *Fkikl-Cre;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* (Park et al., 2006) and *Alk1* nulls (Oh et al., 2000). It follows that there was no concomitant angiogenic defect in *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* mutant embryos, such as the impaired yolk sac vascular remodeling seen in the *Fkikl-Cre;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* (Park et al., 2006) and the *Alk1-null* (Oh et al., 2000) embryos. Since *Fkikl* is a mesodermal marker and *Alk1* is mostly expressed in ECs, the angiogenic defect in the yolk sac is likely to be due to the loss of *Bmp1a* in ECs, a feature we reproduced by ablating *Bmp1a* using *Tie2-Cre* (our unpublished observations) (*Tie2* is also known as *Tek*). In contrast to other mice models of aneurysmal vascular dilatation, the *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* mice did not exhibit an increase in expression of proteases such as *uPA* and *tPA* (Oh et al., 2000; Park et al., 2006). In contrast to the *Smad5-null* embryos with dilated aorta, the *SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* embryos did not show apoptosis in VSMCs or in neighboring mesenchymal cells (Yang et al., 1999).

When we assessed gene expression of extracellular matrix proteins and proteases previously implicated in VSMC proliferation and migration, a consistent reduction in the expression of *Mmp9* and *Mmp2*, genes downstream of BMP signaling in other cell types (Mishina et al., 2004; Palosari et al., 2003), was observed. A direct association between reduced BMPRIA and impaired production of *Mmp9* and *Mmp2* was then demonstrated in cultured human VSMCs, in which knock-down of *BMPRIA* by RNAi attenuated *Mmp9* and *Mmp2* activities. The role of both *Mmp9* and *Mmp2* in VSMC proliferation and migration is well documented (Bendeck et al., 2002; Franco et al., 2006; Kuzuya et al., 2003; Mason et al., 1999). Expression of *Pten*, downstream of *Bmpra1a* and implicated in juvenile polyposis and affecting cell growth, was not modified in the mutants.

Our observations linking reduced *Mmp9* and *Mmp2* to aneurysmal dilatation might seem at odds with clinical studies in human tissue in which increased *Mmp2* and especially *Mmp9* are observed in abdominal aortic aneurysm (Goodall et al., 2001; Thompson et al., 1995). Moreover, reduction of *Mmp9* activity by Doxycycline protects against experimentally induced aortic aneurysm (Kaito et al., 2003), as does local expression of *TIMP1*, an inhibitor of *Mmp9* activity (Allaire et al., 1998; McMillan et al., 1995). In addition, mice that are null for *Mmp9* are resistant to elastase-induced aortic aneurysms (Pyo et al., 2000). It therefore appears that during vascular development, a reduction in both *Mmp9* and *Mmp2* in SMCs is required to produce aneurysmal dilatation, as a result of reduced proliferation and perhaps migration of SMCs. It is interesting that the *Mmp2/9* double nulls (Lambert et al., 2003) do not recapitulate our phenotype. This could reflect compensatory induction of other MMPs in response to a global, rather than a tissue-specific, deletion. Alternatively, the mixed background of the *fl<sub>flox/flox</sub>* background of the *Mmp2/9* double nulls might account for the difference in the phenotype.

**Bmp1a expression in pericytes mediates vessel regression during brain development**

*SM22α-Cre;R26R;Bmp1a<sup>fl<sub>flox/flox</sub></sup>* mutants showed severe brain asymmetry and collapse of telencephalic vesicles. A vascular defect produced by impaired BMPRIA signaling that has not previously been described might explain these abnormalities.

Regression of vessels is crucial in triggering mesenchymal condensation culminating in chondrogenesis and skeletogenesis (Yin and Pacifici, 2001). As BMPRIA activity is linked to retinal pericyte apoptosis in diabetic retinopathy (Yang et al., 2007), we reasoned that suppression of *Mmp2* resulting from lack of BMPRIA signaling might make pericytes resistant to apoptosis, preventing EC apoptosis and microvessel clearing, and subsequently leading to defective brain development. Indeed, we showed that lack of BMPRIA or *Mmp2* by RNAi renders pericytes in culture resistant to apoptosis.

The same phenomenon might explain the enhanced ramification of the interlimb vessels seen in the *fl<sub>flox/flox</sub>* mutants, suggesting that vascular deletion of *Bmpra1a* might impair organogenesis of other tissues not investigated here. In the rat aortic model of angiogenesis, *Mmp9* and *Mmp2* expression and activity not only increased during the angiogenic growth phase of microvessels, but also remained elevated and were necessary for microvessel regression (Zhu et al., 2000). Consistent with this, maximal *Mmp2*...
activity is observed in the late corpus luteum concomitant with vessel regression (Duncan et al., 1998). The deletion of Bmpr1a in brain cells (Hebert et al., 2002) did not recapitulate the phenotype, further indicating the importance of the vasculature in this cell-autonomous mechanism.

The discrepancy between the phenotypes resulting from loss of BMPRIA in VSMCs and pericytes might be related to the fact that they have different basement membranes (Meyrick and Reid, 1979) and hence could exhibit different effects resulting from reduced MMPs (Fig. 9).

It is worth mentioning that our findings did not recapitulate any aspects of juvenile polyposis (JP), a condition associated with mutations in BMPRIA. However, the site of pathology in JP, the villus, forms after E15.5 (Batts et al., 2006) and mutations in MMPs (Fig. 9).

Clinical significance
Our study is the first to show that both MMP9 and MMP2 are developmentally regulated by expression of Bmpr1a and that attenuation in their levels could reduce the proliferation of SMCs leading to aneurysmal dilatation of large vessels. These observations could also explain the reduced cell proliferation that leads to thinning of the ventricular wall. Our findings linking repression of Bmpr1a-mediated MMP2 activity to reduced apoptosis of pericytes, point to a feature not only of developmental importance in clearing of microvessels, but potentially to a mechanism that might help in preserving or regenerating microvessels in disease. In our recent studies (El-Bizri et al., 2008), in which patchy deletion of Bmpr1a was induced in VSMCs, mice were actually protected against both the excessive muscularization and loss of distal vessels associated with chronic hypoxia-induced PAH.

This research is supported by the Intramural Research Program of the NIH, NIEHS to Y.M., and by NIH Grant R01 HL074186 to M.R. N.E. is supported by a fellowship from the American Heart Association (AHA)/Pulmonary Hypertension Association and M.R. by the Dunlevie Professorship. C.-P.C. is supported by NIH Grant R01 HL074186 to M.R. N.E. is supported by a fellowship from the American Heart Association (AHA)/Pulmonary Hypertension Association and M.R. by the Dunlevie Professorship. C.-P.C. is supported by NIH Grant R01 HL074186 to M.R. N.E. is supported by a fellowship from the American Heart Association (AHA)/Pulmonary Hypertension Association and M.R. by the Dunlevie Professorship.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/17/2981/DC1

References


Figure S1: *Dose-dependent gel zymogram MMP-2 activities:* Control experiment depicting ascending volumes of supernatants (from 0 to 30 μl) of centrifuged conditioned media of SiControl-transfected human brain vascular pericytes (HBVP) migrating in Boyden chambers in serum starvation (0.1% FBS) for 6 hours. Volumes of conditioned media supernatants reflect the concentrations of secreted matrix metalloproteinase (MMPs). Note the ascending concentration-dependent effect on proMMP-2 gelatinase activity as seen in the upper gel quantified by densitometry in the lower histogram. This control experiment showed the sensitivity of gel zymography to detect gelatinase activity in a dose-dependent fashion.