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Coronary development is regulated by ATP-dependent SWI/SNF chromatin remodeling component BAF180

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ABSTRACT

Dissecting the molecular mechanisms that guide the proper development of epicardial cell lineages is critical for understanding the etiology of both congenital and adult forms of human cardiovascular disease. In this study, we describe the function of BAF180, a polybromo protein in ATP-dependent SWI/SNF chromatin remodeling complexes, in coronary development. Ablation of BAF180 leads to impaired epithelial-to-mesenchymal-transition (EMT) and arrested maturation of epicardium around E11.5. Three-dimensional collagen gel assays revealed that the BAF180 mutant epicardial cells indeed possess significantly compromised migrating and EMT potentials. Consequently, the mutant hearts form abnormal surface nodules and fail to develop the fine and continuous plexus of coronary vessels that cover the entire ventricle around E14. PECAM and α-SMA staining assays indicate that these nodules are defective structures resulting from the failure of endothelial and smooth muscle cells within them to form coronary vessels. PECAM staining also reveal that there are very few coronary vessels inside the myocardium of mutant hearts. Consistent with this, quantitative RT-PCR analysis indicate that the expression of genes involved in FGF, TGF, and VEGF pathways essential for coronary development are down-regulated in mutant hearts. Together, these data reveal for the first time that BAF180 is critical for coronary vessel formation.

Introduction

Coronary vessel development consists of a series of dynamic events (Mu et al., 2005; Reese et al., 2002; Tomanek 2005). Although the origin of certain cell types is not entirely clear (Jiang et al., 2000; Mikawa and Gourdie, 1996; Moretti et al., 2006; Perez-Pomares et al., 2002; Poelmann et al., 1993), it is well established that the precursors of the coronary vessel and epicardium arise mostly from the proepicardium (PE). Cells from the PE migrate to and then envelope the heart to form the primitive epicardium around E9.5 in mice. Shortly afterwards, some epicardial cells undergo an EMT to become migratory mesenchymal cells (E11.5–E12.5), and give rise to vascular smooth muscle, perivascular fibroblasts, and also perhaps endothelial cells of the coronary vessels. Primary vascular plexus is formed by vasculogenesis (*de* novo generation of blood vessels), and then the major coronary vessels, smaller-caliber arteries, veins, and capillaries are formed by angiogenesis. Finally, after joining the geneal circulatory system by E13, the discontinuous and randomly oriented coronary vessels connect, expand, and orient to form the functional coronary network.

Tissue differentiation and organ formation such as coronary vessel development require signal-dependent and tissue- and stage-specific gene expression. The specific gene expression program is ensured by chromatin modifying enzymes to make gene-specific DNA accessible to the transcription machinery in response to developmental signals. Among them, ATP-dependent chromatin remodeling complexes and histone modifying complexes are two well-defined classes (Narlikar et al., 2002). ATP-dependent chromatin remodeling complexes are specialized multi-protein machines that utilize ATP to modify nucleosomes or higher order chromatin structures to regulate access to the DNA. The chromatin remodeling factors are themselves often classified as transcription cofactors: chromatin remodeling is intimately linked to transcription, and the remodeling factors often physically interact with transcription factors or cofactors and can potentiate transcriptional activity of certain activators (Levine and Tjian, 2003).

The delicate and dynamic nature of tissue differentiation and organ development dictates that the chromatin remodeling activity must be highly specific for the tissue-, stage-, or gene cluster. This is indicated by the large number of different chromatin remodeling complexes and the tissue- or developmental stage-specific composition of the subunits within a particular complex. There are five families of chromatin remodeling complexes identified so far: SWI/SNF, ISWI, NURD/Mi-2/CHD, INO80, and SWR1, and numerous complexes have been discovered within each family (Saha et al., 2006). In addition, the composition of a particular complex can be also tissue- or stage-specific. For example, heart- and neuron-specific subunits have been identified

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in the mammalian SWI/SNF complexes (Lessard et al., 2007; Lickert et al., 2004; Wu et al., 2007). And indeed, the heart-specific BAF60c in SWI/SNF complexes is required for early heart formation (Lickert et al., 2004), and the neuron-specific BAF53b is essential for neuron dendritic pattern formation (Wu et al., 2007). Furthermore, neuron-specific subunit composition switch is required for the transition from neural stem/progenitors to postmitotic neurons (Lessard et al., 2007). Despite the important biochemical functions and the identified roles at various stages of development, the role for chromatin remodeling and the specific chromatin remodeling factors essential for coronary development have not been explored.

SWI/SNF complexes has been further characterized to include two major subclasses: BAF and PBAF (Mohrmann and Verrijzer, 2005; Moshkin et al., 2007; Wang, 2003). These complexes appear to be evolutionarily conserved from yeast, to fly, and to humans. Components of BAF complexes can associate with Rb or HDACs for transcriptional repression (Zhang et al., 2000), or associate with CARM1 or other factors for transcriptional activation (Xu et al., 2004). The two complexes share eight common subunits but are distinguished by four polypeptide components: BAF180 and BAF200 (unique to PBAF), and BAF250a and BAF250b (both unique to BAF) (Wang, 2003; Yan et al., 2005). Interestingly, a recent study identified another signature subunit SAYP, a Trithorax group protein, in *Drosophila* PBAF complex (Chalkley et al., 2008). These unique subunits may represent key regulatory components that functionally distinguish PBAF from BAF, as suggested by numerous studies (Chalkley et al., 2008; Lemon et al., 2001; Moshkin et al., 2007; Yan et al., 2005).

In this study, we identified that BAF180, a polybromo protein uniquely present in PBAF complex (Moshkin et al., 2007; Xue et al., 2000), is critical for coronary development. We observed a significant reduction of epicardial cells in BAF180 mutant hearts that underwent EMT and migrated into the subepicardial space at E11.5. Three-dimensional collagen gel assays indicated that epicardial cells lacking BAF180 are severely compromised in migration and EMT. Consequently, the mutant hearts failed to develop the fine and continuous plexus that covered the entire ventricle at later stages. Numerous nodules were formed within the epicardium as a result of a failure of epithelial and smooth muscle cells within these structures to branch out to form vessels, and the coronary vessels penetrated only slightly into the myocardium. Finally, we observed that BAF180 regulates the expression of genes involved in FGF, TGF, and VEGF signaling pathways, which are essential for coronary development.

Material and methods

Generation and identification of BAF180 mutant embryos

A promoterless gene targeting strategy was applied to generate BAF180 mutant mice as described (Wang et al., 2004). β-Geo, a fusion protein of β-galactosidase and neomycin phosphotransferase, is inserted and spliced in frame to exon 11 of BAF180 in the BAF180 mutants after homologous targeting, so β-galactosidase activity can represent the expression of endogenous BAF180. A three-primer PCR analysis was designed to distinguish the BAF180 wild-type (WT) from its mutants (Wang et al., 2004). All the mice in this study were bred into CD1 genetic background for at least five generations.

X-gal staining

Whole-mount X-gal staining was performed as described previously (Wang et al., 2004). E9.5 embryos and E11.5 to E14.5 dissected hearts were fixed in fixation buffer (0.2% glutaraldehyde, 0.1 M sodium phosphate, pH 7.3, 5 mM EGTA, 2 mM $MgCl_2$) for 15 to 30 min, then washed with 0.1 M phosphate, 2 mM $MgCl_2$ wash buffer for three to five times. Samples were then stained with staining buffer (1 mg/ml filtered, freshly added X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1 M phosphate, 2 mM $MgCl_2$) at room temperature overnight (O/N) or 37 °C for several hours. X-gal stock solution was prepared at 50 mg/ml in dimethylformamide. After X-gal staining, some samples were postfixed with 4% paraformaldehyde at 4 °C O/N, then dehydrated and paraffin embedded. Sections of 6–8 μm were prepared on SuperFrost slides and counter-stained with eosin.

Hematoxylin and eosin (H&E) staining

Embryos at indicated stages were collected in ice-cold PBS, fixed in 4% paraformaldehyde at 4 °C O/N, washed in PBS, and dehydrated through an ethanol series before paraffin embedding. Sections of 6 μm were prepared on SuperFrost slides and stained with H&E as described (Wang et al., 2004).

PECAM staining

The whole-mount PECAM staining was performed as previously described (Lavine et al., 2006). A 1:150 dilution of 1 μg/ml rat antibodies against mouse PECAM (Pharmingen) in PBS, 1.5% skim milk, and 0.1% triton-100 and a 1:1000 dilution of HRP-conjugated affinity purified $F(ab)_2$ fragment goat anti-rat IgG antibodies (Jackson ImmunoResearch) were used for this assay. Color reaction was revealed by peroxidase substrate DAB kit (DAKO). After PECAM staining, hearts or embryos were photographed and analyzed. Some samples were paraffin embedded and sectioned (6 μm). Sectioned samples were dewaxed, rehydrated, counterstained with nuclear fast red (Vector Laboratories), and mounted. PECAM fluorescent immunohistochemistry was performed with frozen sections as described (Lavine et al., 2006). Coronary vessel density at the ventricular surface was quantitatively measured by counting the cycles among PECAM-positive vessels as described (Huang et al., 2006).

PECAM and α-SMA double-fluorescent-immunostaining

Freshly dissected embryonic hearts were embedded in OCT and snap frozen. Cryosections were generated at 7 μm on a Leica Cryostat. The slides were heated at 50 °C for 1 h, washed with PBS, and then permeabilized with PBS/Triton X (0.25%). After PBS wash and goat serum block, mouse antibodies against α-SMA (DAKO) and rat antibodies against PECAM (Pharmingen) were incubated with the tissue. Alexa 594-conjugated goat anti-mouse (α-SMA) and Alexa 488-conjugated goat anti-rat (PECAM) were used for the double-fluorescent-immunostaining. DAPI was used to stain nucleus.

Quantitative RT-PCR

Fresh embryonic hearts at indicated stages were dissected and RNAs were extracted with the Tri-reagent (Sigma), and then purified by the Qiagen RNeasy miniprep system. Typically, RNAs from 4 wild-type or mutant E11.5 hearts were used for one set. cDNAs were prepared using Superscript III reverse transcriptase (Invitrogen) or Iscript™ cDNA synthesis kit (Biorad) with standard procedures, and at least two independent cDNA preparations were applied for the RT-PCR analysis. GAPDH was used as an internal control for quantification. PCR primers were designed to amplify 120–200-bp fragments from the coding region of candidate genes. Primer sequences are available upon request. RT-PCR was carried out with iQ™ SYBR green supermix (Biorad) in an Eppendorf Mastercycler^R ep realplex PCR machine. Fold-change in expression was determined by the $2^{-\Delta CT}$ method described in the Eppendorf User Bulletin. *P* values for two variable comparisons were calculated with Student's *t* test.

EMT evaluation: collagen gel assay

Hearts from embryonic day 12.5 were dissected and ventricular chambers were placed epicardial side down on three-dimensional gels containing 1% collagen type I (Vitrogen 100; Collagen Aesthetics, Palo Alto, CA), as described (Merki et al., 2005). After 48 h, the explanted hearts were removed and incubated for 72 h in DMEM medium containing 10% FCS and supplemented with penicillin/Streptomycin and glutamine. Transformation to mesenchymal cells was defined as the invasion of cells into the three-dimensional gels. The number of mesenchymal cells per monolayer was counted manually using an inverted microscope equipped with phase contrast optics. Statistical analysis: mesenchymal cells per monolayer were counted manually. Averages were calculated and compared using Student's *t* test for two variable comparisons at a level of significance of $p \leq 0.05$.

Results

BAF180 is abundantly expressed in PE and epicardium

To better understand the cardiac tissue-specific function of BAF180, we examined the expression pattern of BAF180 in heart tissues during embryogenesis. β-Geo, a fusion protein of β-galactosidase and neomycin phosphotransferase, is spliced in frame to a 5' exon of BAF180 in the BAF180 mutants (Wang et al., 2004), so β-galactosidase activity is a very sensitive way to show the expression of endogenous BAF180. Indeed, X-gal staining revealed broad BAF180 expression in the heart tissues with abundant expression in PE and epicardium (Fig. 1).

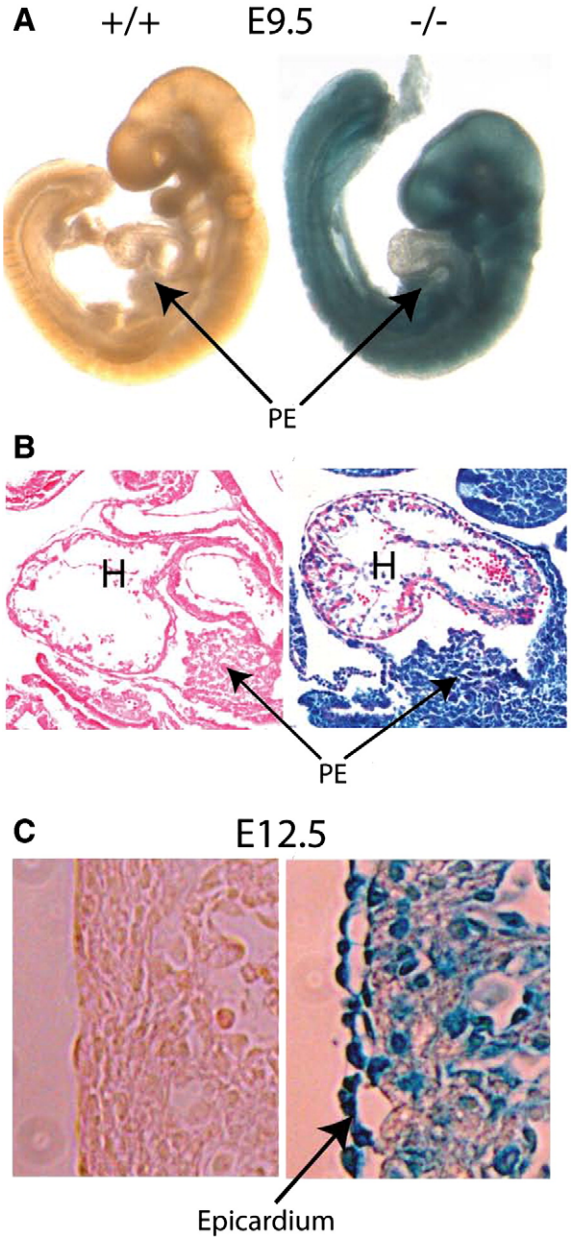

Fig. 1. BAF180 is highly expressed in PE and Epicardium. β-Geo was spliced in frame to a 5' exon of BAF180, so β-galactosidase activity reflects the expression of endogenous BAF180. (A) Whole-mount X-gal staining of E9.5 BAF180+/+ and −/− embryos. Whole-mount X-gal stained E9.5 (B) and E12.5 (C) embryonic hearts were sectioned and then counter-stained with eosin.

BAF180 ablation does not affect the formation of PE and the migration of PE cells to cover the heart tube

The high expression level of BAF180 in PE and epicardium suggest that BAF180 may be involved in the normal function of epicardial cells. We therefore examined whether global deletion of BAF180 would affect the formation of the PE and subsequent migration of cells from the PE to cover the heart. A half dozen of E9.5 BAF180 KO embryos were analyzed and it appeared that like the WT embryos (Fig. 2A, right panel), the mutant embryos develop a normal PE (Fig. 2A, left panel). Furthermore, in the mutant embryos, the PE cells form normal cysts and migrate freely to the heart tube (Fig. 2A). At E10.5, the PE-derived epicardial cells have covered most of the heart tube in both the WT and the mutant embryonic hearts (Fig. 2B). Thus, neither the formation of the PE nor the migration of the PE cells to form epicardium is affected by BAF180 ablation.

BAF180 ablation impairs the normal EMT of the epicardial cells for coronary development

We next examined whether BAF180 regulates the EMT of the epicardial cells. During embryonic development, shortly after the migration of the PE cells to form the epicardium, a subepicardial space filled with extracellular matrix appears between the epicardium and myocardium. After covering the surface of the heart, a subpopulation of the epicardial cells undergoes EMT and invades the subepicardial space to subsequently give rise to coronary vessels (Reese et al., 2002; Tomanek, 2005). The subepicardial matrix is a transient structure observed in the mouse hearts between E10.5 and E11.5 that separates the primitive epicardium from the myocardium. It is filled with proteins and growth factors critical for the EMT of epicardial cells (Mu et al., 2005), and recesses after epicardial remodeling, which renders the myocardium in direct contact to the epicardium.

H&E staining of E11.5 embryos revealed an underdeveloped subepicardial space in the mutant hearts with a reduced number of mesenchymal cells compared to their WT littermates (Fig. 3A, compare left panel to right panel), suggesting impaired EMT in the BAF180 mutant hearts. In addition, this primitive, underdeveloped epicardium is sustained in older mutant hearts, and fails to remodel and compact (compare Figs. 3B and 3C).

To further establish whether BAF180 is involved in the proper EMT of epicardial cells, we analyzed the potential of BAF180 mutant epicardial explants to convert into mesenchymal cells and migrate using the collagen gel assays. Consistent differences were observed between WT ($n=5$) and mutant explants ($n=4$), both in their ability to migrate at the gel surface as well as in their potential to invade the gel matrix. Under our experimental conditions, WT cells typically extend at the surface beyond 500 μm, and migrating cells tend to form tube like structures that detach from the main explant in the most external surface (Fig. 4A left panel). In contrast, BAF180 mutant explants do not extend beyond 250 μm and detached cells were not observed (Fig. 4A, right panel).

Differences among genotypes were also observed upon quantification of the morphologically distinguishable mesenchymal cells at different depths of the collagen gel. At the surface level mesenchymal

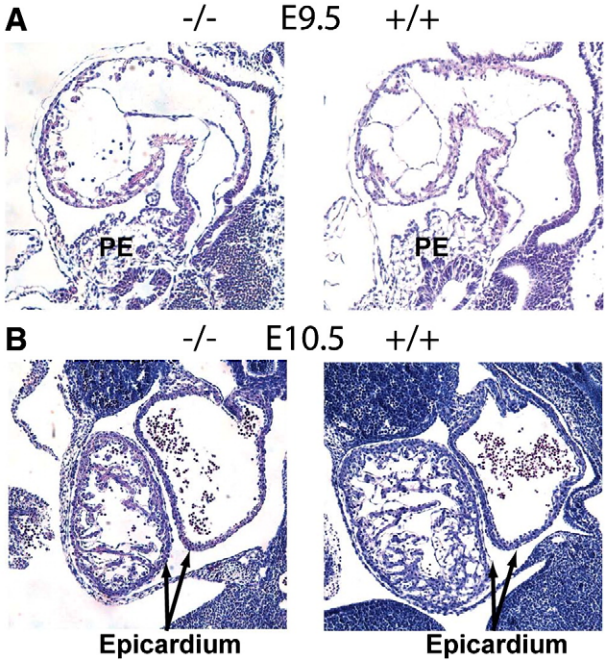

Fig. 2. BAF180 ablation does not affect formation of the PE and migration of the PE cells to cover the heart tube. (A) The PE forms normally in E9.5 BAF180 mutant embryos. (B) The KO embryos also form a near complete epicardial layer covering the heart tube at E10.5.

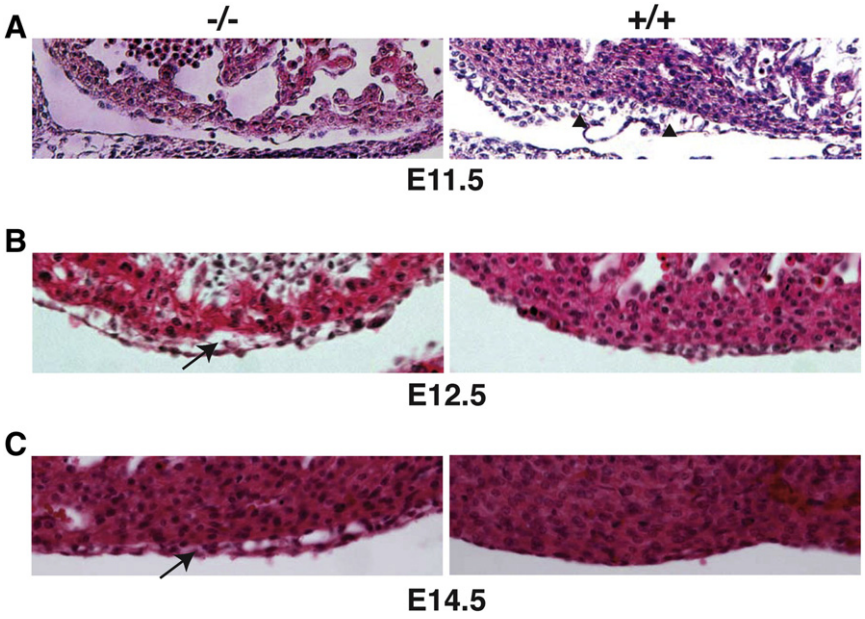

Fig. 3. Defects in the maturation of the epicardium in BAF180 null embryonic hearts. (A) At E11.5, the subepicardial space in WT is apparent and filled with cells migrated from the epicardium. In contrast, the mutant hearts generally form very limited subepicardial space, and there are far fewer cells inside the space, indicating a delayed or defective EMT of the epicardial cells in mutant hearts. At E12.5 (B) and E14.5 (C), the subepicardial space in mutant hearts is more pronounced than that in control hearts.

cells in the WT outnumbered those scored in the KO explants per area unit (41 ± 5 in WT, compared to 28 ± 7 in KO). At the level of 100 μm inside the gel 26±3 cells were observed per area unit in the WT explants compared to 5 ± 3 in the KO explants. At the 200 μm level, 3 ± 2 cells were counted in the WT cultures while no KO cells were found at this level (Fig. 4B). Therefore, the *in* vivo and *in* vitro data demonstrates that BAF180 is critical for the normal progression of epicardial cells.

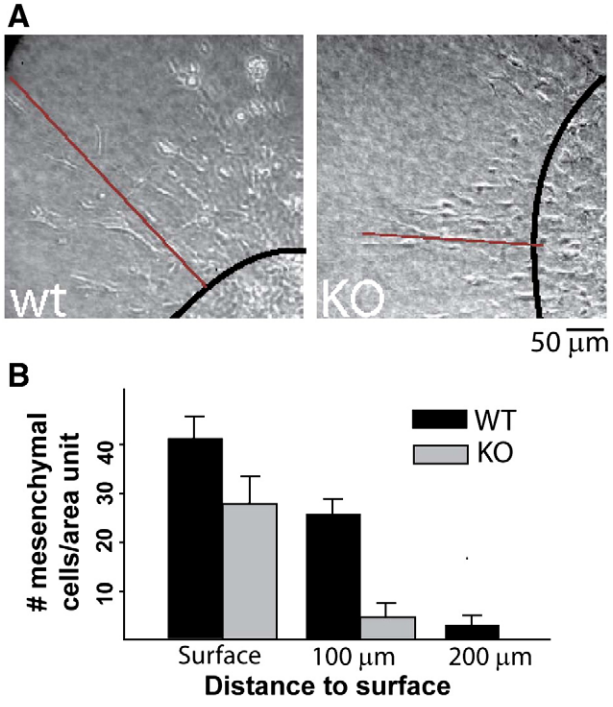

Fig. 4. Evaluation of epithelial-to-mesenchymal transformation in WT and BAF180 KO mice with the collagen gel assay. (A) Micrographs from representative fields of WT and KO explanted epicardia, showing horizontal migration of cultured cells. Observe the reduced capacity of KO explanted cells to detach from the monolayer. While WT explants typically reached 500 μm outside the continuous epithelial layer, KO cells did not extend beyond 250 μm (bar = 50 μm). (B) Quantification of invasion of collagen gel by the explanted cells, as a direct measurement of EMT. Averages were calculated and compared using Student's *t* test for two variable comparisons at a level of significance of $p \leq 0.05$.

BAF180 is essential for the normal development of the fine and continuous coronary plexus that covers the whole heart

To directly address if BAF180 ablation causes defects in the coronary vasculature, we applied antibody staining of PECAM, a marker for endothelial cells, to E12.5–E14.5 BAF180 null hearts. Indeed, we observed abnormal surface nodules in the freshly dissected E14.5 mutant hearts that are rarely seen in WT hearts (Fig. 5, compare A to B). Whole-mount PECAM staining showed that these nodules are abnormal structures resulting from the failure of PECAM-positive cells within them to branch out to form coronary vessels, similar to what has been observed in thymosin β4 mutant embryos (Smart et al., 2006). PECAM staining also indicated that, compared to WT hearts, the E14.5 BAF180 null embryonic hearts are defective in developing the fine and continuous plexus that covered the entire ventricle (Fig. 5 C, E, and G). Statistical analysis indicated that the coronary vessel density at the surface of the BAF180 knockout heart ventricles is only about 25 to 40% to that of the wild-type controls (Fig. 5I). Further, sectioned PECAM staining revealed that the coronary vessels have little penetration into the myocardium of mutant hearts (Fig. 5, compare J to K, and L to M). The aberrant nodules developed in homozygous embryos are less severe in E13.5 and E12.5 KO embryos (Fig. S1). Together, these data indicate that BAF180 regulates the proper development of the fine and continuous coronary plexus.

We also examined whether the coronary arteries are connected to the aorta in the mutant hearts, because the connection with systemic blood circulation provides nutrition and oxygen to the heart during mid-gestation, and failure to do so would severely compromise the normal development of the heart. Fig. S2 indicated that like the WT embryos, the E14.5 BAF180 KO embryos form a normal connection between the coronary vessels and the aorta.

BAF180 ablation affects the proper lining of smooth muscle and endothelial cells to form coronary vessels

The above data suggest that BAF180 is critical for the proper EMT and vasculogenesis of the coronary vasculature (Figs. 3 to 5). The DNA

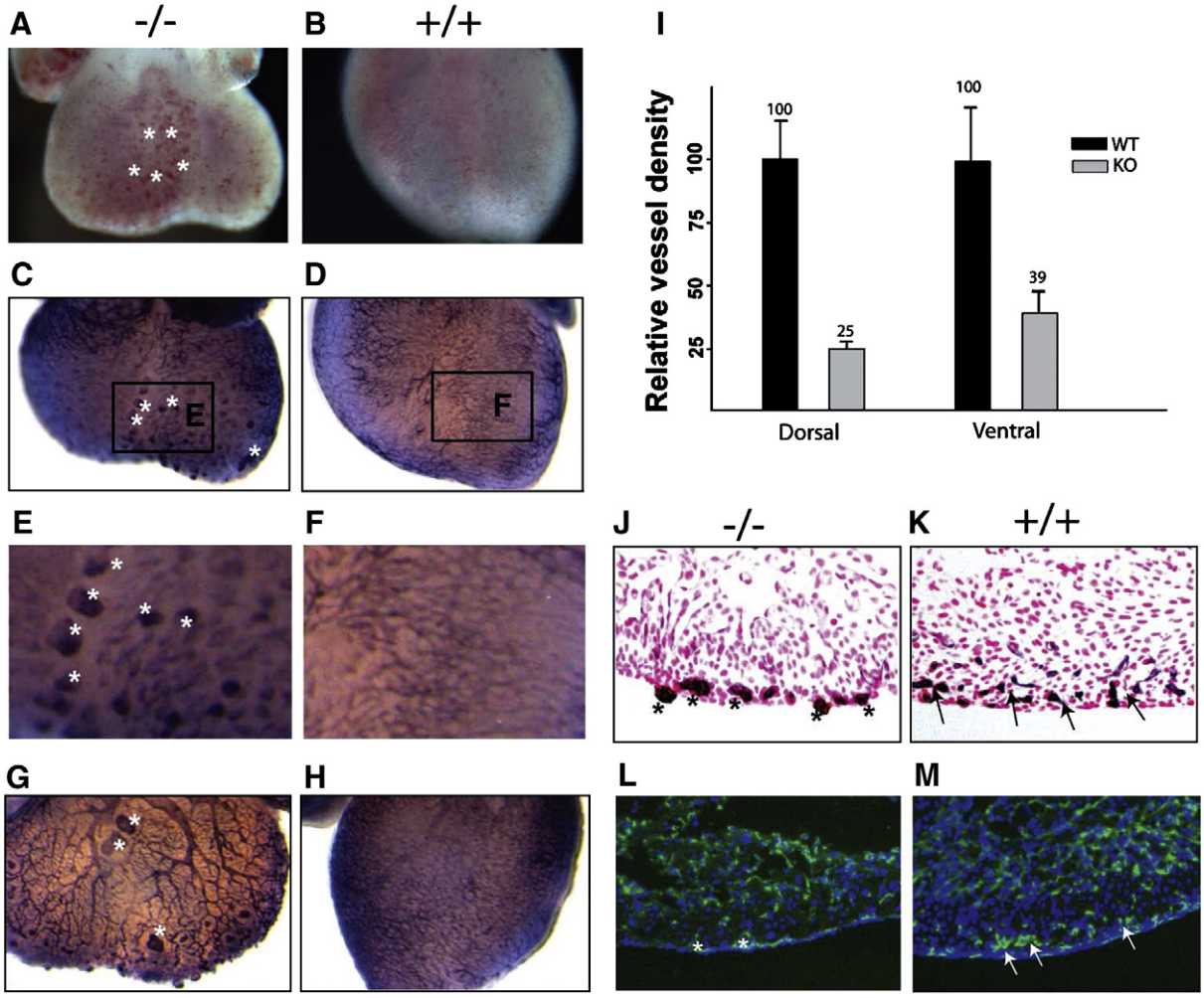

Fig. 5. Defective embryonic coronary vasculature in BAF180 KO embryonic hearts. (A, B) Ventral view of freshly dissected E14.5 BAF180 KO and WT embryos. (C–H) Whole-mount PECAM staining of BAF180-deficient (C, D, and G) and WT control (D, F, and H) E14.5 hearts. Ventral view: (C–F) Dorsal view: (G and H) Note the abnormal nodules (asterisks) and the failure of forming the fine and continuous capillary vessels in the mutant hearts (panels C, E, and G). (I) Quantitative analysis of coronary vessel density at the ventricular surface. (J, K) Histological sections of whole-mount PECAM-stained hearts counter-stained with nuclear fast red. (L, M) Sectioned fluorescent PECAM staining. Note the little penetration of coronary vessels into the compact zone of myocardium in the mutant hearts (compare panel J to K, and panel L to M). The nodules are indicated by asterisks and the coronary vessels formed within myocardium are indicated by arrows. Green: PECAM; blue: DAPI.

microarray data obtained previously and qRT-PCR data using RNA samples from BAF180 mutant hearts demonstrated that the α-SMA expression is down-regulated (Fig. 6A), pointing to the possibility that the formation of smooth muscle cells was decreased in BAF180 mutant hearts. α-SMA is mostly expressed in smooth muscle cells, although it is also expressed in early cardiomyocytes. α-SMA expression in cardiomyocytes is more prominent before E12.5, and its expression at E13.5 and E14.5 cardiomyocytes becomes weaker as observed in comparable stages of avian and rat embryonic hearts (Ruzicka and Schwartz, 1988; Woodcock-Mitchell et al., 1988). Therefore, PECAM and α-SMA double-immunostaining assays should further establish the role of BAF180 in coronary development after E13.5. Since smooth muscle cells are also present around the aorta and pulmonary trunk arteries, we first examined if the smooth muscle cells surrounding these vessels are affected.

As illustrated in Fig. 6B, there is no difference of α-SMA-positive cells in number and density between the mutant and the WT surrounding aorta and pulmonary trunk arteries, indicating that the formation of these big vessels is largely unaffected. This is consistent with the idea that the smooth muscle of the outflow tract does not originate from the epicardium. In contrast, when applying this assay to examine the coronary vessels, we observed that the mutant hearts contain a significant number of individual α-SMA-positive cells within the subepicardial space in the mutant hearts (Fig. 6C, left panels). The mutant hearts form larger than normal coronary vessels that remain in the surface and aberrant nodules within the epicardium region (Fig. 6C, left panels), while the control hearts (Fig. 6C, right panels) form a fine continuous α-SMA-positive line at certain areas of the epicardium region, and within the cardiomyocyte compact zone. The PECAM/α-SMA double staining assay also revealed that the endothelial and smooth muscle cells inside the nodules are disorganized (Fig. 6C, lower left panel), and in E14.5 embryonic hearts, the nodules grow bigger with a large number of endothelial and smooth muscle cells trapped inside (Fig. 6D, left panel). The lack of fine coronary vessels at the surface, devoid of coronary vessels inside cardiomyocyte layer, and the formation of the aberrant nodules demonstrate that BAF180 is required for epicardial maturation.

BAF180 regulates various genes and signaling pathways involved in coronary development

To understand the molecular mechanism BAF180 regulated coronary development, we next systemically examined the expression of more than 30 genes (Mu et al., 2005) involved in EMT, vasculogenesis, and angiogenesis in BAF180 mutant hearts. We focused on the gene expression profile at E11.5 when epicardial defects were first observed. Our quantitative RT-PCR data indicated that the expression

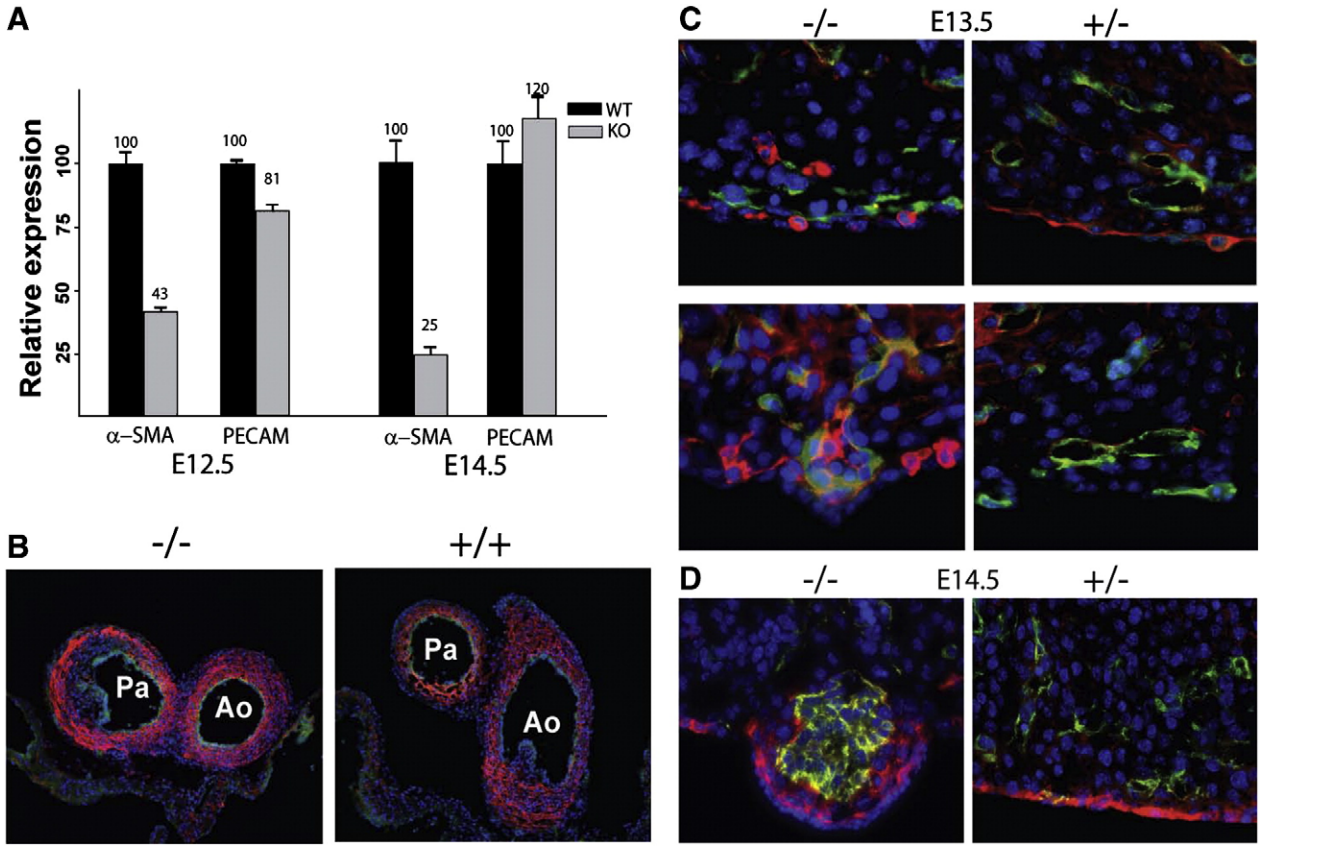

Fig. 6. BAF180 regulates the appropriate lining of endothelial and smooth muscle cells in the coronary vessels and subsequent angiogenesis. (A) qRT-PCR analyses of the relative expression of α-SMA and PECAM in BAF180 KO hearts. GAPDH was used as an internal control for quantification. (B) PECAM/α-SMA double-immunostaining of E13.5 aorta and pulmonary trunk. No obvious decrease of α-SMA is observed in these vessels. (C) PECAM/α-SMA double-immunostaining of sectioned E13.5 embryonic hearts. The control hearts (right panels) generally form a fine continuous α-SMA-positive line at a certain area of the epicardium region, and coronary vessels are frequently found in the cardiomyocyte compact zone region. In contrast, mutant hearts only form larger than normal vessels and aberrant nodules within the epicardium region (left panels). Note the disorganized endothelial and smooth muscle cells inside the nodules, and also the individual α-SMA-positive cells within the subepicardial space in the mutant hearts. (D) PECAM/α-SMA double-immunostaining of sectioned E14.5 embryonic hearts. Note the large number of endothelial and smooth muscle cells trapped in the nodule (left panel). Green, PECAM; red, α-SMA; blue, DAPI.

of pro-angiogenic genes VEGF, VEGFB, and angiopoietin-2 (Ang-2) in the heart is down-regulated, whereas the expression of Ang-1, Flt1, Flk1, VEGFC, VEGFR3, Tie1, and Tie2 remains relatively unchanged (Fig. 7A). Genes involved in TGF, FGF, PDGF pathways are selectively down-regulated, including TGFβ2, TGFR2, FGF9, PDGFB, whereas genes such as Wnt9b and β-catenin–also involved in epicardial development (Merki et al., 2005; Zamora et al., 2007) – are largely unaffected (Fig. 7B). These studies identified a number of important genes involved in coronary development that are regulated by BAF180, and provided a framework for establishing the BAF180-mediated transcriptional network during coronary development.

Discussion

In this study, we identified the polybromo protein BAF180 as an important regulator for embryonic coronary development. Lack of BAF180 leads to impaired EMT of the epicardial cells and arrested epicardial remodeling at E11.5 hearts. *In vivo* analysis and *in vitro* collagen gel assays revealed that BAF180 mutant epicardial cells are severely compromised in migration and EMT. Subsequently, BAF180 ablation also results in failure of the development of the fine and continuous plexus that covers the entire ventricle at later stages. Furthermore, we observed aberrant nodules at the surface of the mutant hearts with accumulation of epithelial and smooth muscle cells within these structures. We also observed little penetration of the coronary vessels into the myocardium, strongly suggesting defective migration of the already reduced number of mesenchymal cells in the BAF180 mutant hearts. In contrast, the formation of the PE, migration of the PE cells to cover the heart tube, and the connection of coronary vessels to aorta are all relatively normal in the BAF180 mutant embryos. Thus, it appears that the major function of BAF180 during coronary arteriogenesis is to mediate epicardial development.

The observed coronary defects and thin myocardial wall syndrome in BAF180 mutant hearts appear not due to a gross developmental delay in embryogenesis. Instead, the modest developmental delay of E14.5 KO embryos may be secondary to their heart defects. There is essentially no difference in somite number between WT and mutant embryos at E11.5 and E12.5 stages (Table S1) when epicardial defects are already observed (Figs. 3 and 4). The E14.5 KO embryos show slightly delayed digit segregation and are smaller. The KO embryos often show characteristic edema along their backs indicating circulatory defects. We further revealed that the defects are primarily within the heart (Wang et al., 2004). Mouse embryonic heart ordinarily develops into its mature form at E14.5 in order to provide sufficient blood flow for the rapid growth of the fetus. Thus, it is likely that the defective heart of BAF180 KO embryos would have difficulty sustaining normal circulation, leading to the observed smaller KO embryos at E14.5, a modest developmental delay around that stage, and embryonic lethality after that.

The functions of BAF180 in embryonic coronary development (this study) and cardiac chamber maturation (Wang et al., 2004) are distinct from those of several core subunits present in both PBAF and BAF complexes, such as Brg-1, BAF155, and INI1/SNF5 (Bultman et al., 2000, 2006; Guidi et al., 2001; Kim et al., 2001; Roberts et al., 2000).

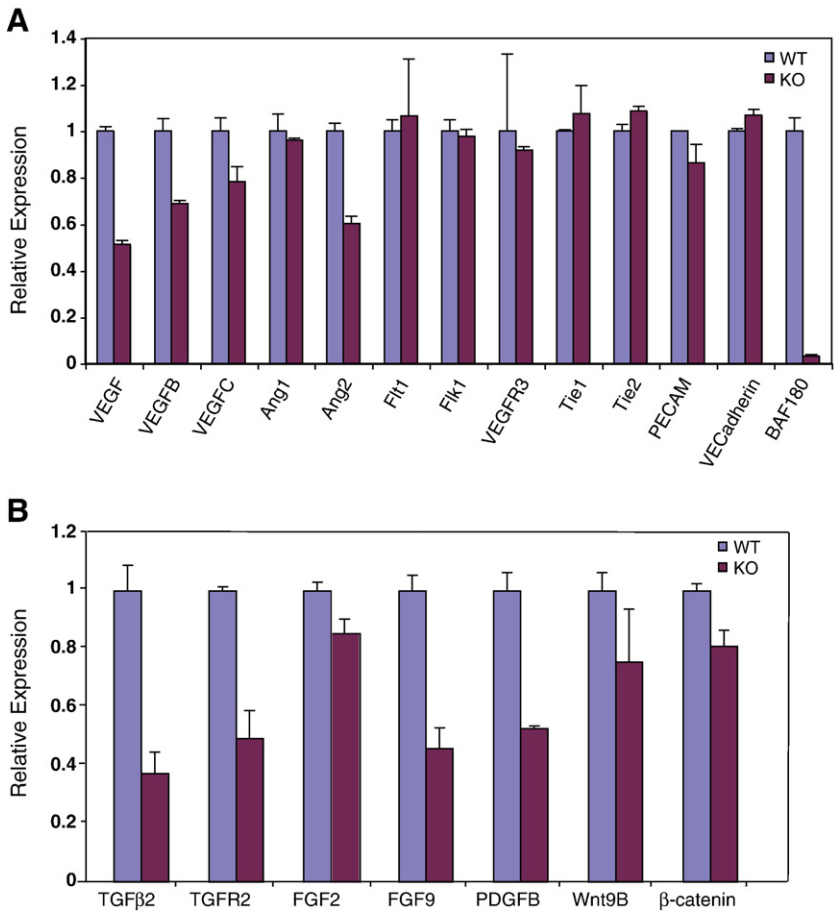

Fig. 7. Identification of genes involved in EMT, angiogenesis, and vasculogenesis in E11.5 BAF180 knockout embryos. (A) Relative expression of pro-angiogenic genes in BAF180 WT and mutant hearts. BAF180 expression was used to validate the cDNA sources. (B) Relative expression of genes involved in TGF, FGF, PDGF, Ephrin, and Wnt pathways. *P* values of the expression difference between WT and KO hearts for VEGF, Ang-2, FGF9, and PDGFB are less than 0.01, and for VEGFB, TGFβ2, and TGFR2 are less than 0.02.

The null embryos of these core subunits typically die around peri-implantation stage, and maternal Brg-1 is essential for zygotic genome activation after fertilization (Bultman et al., 2006). Interestingly, a recent study indicates that endocardial Brg-1 regulates the trabeculation of cardiomyocytes (Stankunas et al., 2008). The function of PBAF-specific BAF180 is also distinct from the BAF-specific subunits BAF250a and BAF250b. We discovered specific functions of BAF250a and BAF250b in ES cell pluripotency and BAF250a in early embryogenesis (Gao et al., 2008; Yan et al., 2008), as well as in the proper lineage development from the primary and secondary heart fields (data not shown). These data also suggest that the role in early embryogenesis found in various core subunits can be largely attributed to the function of BAF, rather than PBAF.

How BAF180-mediated chromatin remodeling regulates EMT and vasculogenesis needs further investigation. Our qRT-PCR analysis using RNA prepared from E11.5 embryonic hearts suggests that the down-regulation of VEGF, VEGFB, and Ang-2 contributes to the defects observed in BAF180 mutant hearts (Fig. 7A). In an effort to dissect whether BAF180 regulates these genes directly or regulates the possible upstream signaling pathways or transcription factors, we also examined the expression of various genes in select signaling pathways. The signaling pathways and transcription factors involved in EMT and angiogenesis during coronary development include RA and erythropoietin (epo) (Chen et al., 2002; Merki et al., 2005; Stuckmann et al., 2003; Wu et al., 1999), Fibroblast growth factors (FGFs) (Lavine et al., 2006, 2005; Morabito et al., 2001; Pennisi and Mikawa, 2005), and transforming growth factor-β isoforms (TGFβs) (Morabito et al., 2001; Olivey et al., 2006; Tavares et al., 2006). Other transcription factors include GATA4, friend of GATA2 (FOG2), Ets-1/Ets-2, Wilms tumor 1 (WT-1), serum response factor, histone acetyltransferase P300, and TBX5. BMPs, Ephrins and their receptors, platelet-derived growth factors, and Notch signaling could also be involved in coronary arteriogenesis (Mu et al., 2005). Our qRT-PCR analysis indicated that TGFβ2, TGFR2, FGF9 and PDGFB involved in TGF, FGF, PDGF pathways are selectively down-regulated, while Wnt9b and β-catenin are largely unaffected (Fig. 7B). Future studies by chromatin immunoprecipitation assays will likely reveal whether BAF180 directly regulates the expression of these genes.

Whether BAF180 mediates or works in synergy with RA signaling to regulate coronary vessel formation remains elusive. Based on previous *in vitro* and *in vivo* analyses, it seems likely that BAF180/PBAF plays a role in RA signaling during heart development, presumably by serving as a cofactor for RXRα, PPARγ, and other RA-related nuclear receptors (Lemon et al., 2001; Wang et al., 2004). Therefore, we examined the expression of RARα, RARβ, RARγ, RXRα, RXRβ, RXRγ, and PPARγ. The effect of BAF180 ablation on the expression of these genes was rather modest (data not shown). Instead of downstream targets, these receptors are likely potential partners for BAF180. Even though the six RA receptors involved in RA signaling are not affected in BAF180 mutant hearts (data not shown), our previous studies indicate that BAF180 regulates certain genes involved in RA signaling (Wang et al., 2004). Another study reveals that FGF signaling to the cardiomyoblast controls a wave of Hedgehog signaling, which in turn activates the expression of VEGF-A, -B, -C, and Ang-2 that are essential for coronary vascular development (Lavine et al., 2006). And interestingly, part of this FGF signaling mediated by FGF9 is regulated by RA signaling (Lavine et al., 2005). The down-regulation of FGF9, VEGF-A, -B, and Ang-2 in BAF180 knockout embryonic hearts suggests that BAF180 is indeed involved in RA and FGF signaling. Our further

analysis also indicates that BAF180 regulates the expression of a few early genes in RA pathway in a dynamic- and stage-specific pattern (data not shown). Future *in vivo* and *in vitro* studies will help better understand the transcriptional network and hierarchy mediated by chromatin remodeling complex PBAF during coronary development.

Even though most molecules and signaling pathways involved in coronary development appear to have a general role in vasculogenesis, the function of BAF180 is more critical in the coronary vasculature. We only observed subtle defects in the vasculature of yolk sac and embryonic bodies (Fig. S2 and data not shown), suggesting coronary development either requires specific BAF180 mediated chromatin remodeling or is more sensitive to the alteration of chromatin remodeling mediated gene network.

An intriguing question that needs further genetic investigation is whether BAF180 functions only in epicardium to regulate the proper expression of factors identified in this study. It has been well established that signals derived from the PE, epicardium, and myocardium, and maybe even from the endocardium and neural crest are important for the coronary development. For example, RA and erythropoietin (epo) signals are generated in epicardial cells to activate gene expression that promotes coronary growth and myocardium proliferation (Chen et al., 2002; Merki et al., 2005; Stuckmann et al., 2003; Wu et al., 1999). Fibroblast growth factors (FGFs) from both epicardium and myocardium are required for EMT and vasculogenesis (Lavine et al., 2006, 2005; Morabito et al., 2001; Pennisi and Mikawa, 2005). Wnt/β-catenin signaling from epicardium is essential for the coronary artery formation (Zamora et al., 2007). In addition, progenitors from the secondary heart field and neural crest can physically contribute to the formation of outflow tract and major coronary arteries (Jiang et al., 2000; Moretti et al., 2006). The normal development of the outflow tract and major coronary arteries in BAF180 knockout embryonic hearts suggest that the observed defects in coronary development are not due to BAF180's function in the tissues derived from secondary heart field or neural crest. Consistent with this, our conditional knockout studies indicated that ablation of BAF180 in the cardiac heart tube mediated by Mlc-2v-Cre and Nkx2.5-Cre does not have much effect on the coronary vessel formation (data not shown), suggesting that epicardium could be the primary target tissue for BAF180, and the thin myocardial wall syndrome reported previously could be secondary to coronary defects.

It will be critical to investigate whether a timely over-expression of BAF180 can promote the multipotency of epicardial progenitor cells. A few recent studies point to the therapeutic potential of epicardial progenitors from the PE and epicardium. Epicardial progenitor cells can maintain their multipotency even into adulthood. In zebrafish, cardiac regeneration after resection of the ventricular apex requires the activation of the entire epicardial cell layer, and a subset of these cells undergoes EMT to generate new vasculature for regenerating muscle (Lepilina et al., 2006). In both mouse and human, adult epicardial cells can undergo EMT to produce smooth muscle like cells under defined conditions (Smart et al., 2006; van Tuyn et al., 2007). The PE-derived progenitor cells might even be able to differentiate into cardiac muscle cells (Kruithof et al., 2006). These studies indicate the promising therapeutic potential of the epicardial cells. Therefore, it is of paramount importance to identify the factors or epigenetic modifications that can regulate the multipotency of these progenitor cells. The identification and characterization of BAF180 in coronary development and the elucidation of the orchestrated function of BAF180 and other transcription factors for coronary vasculogenesis and neovascularization will ultimately lead to a better understanding of the etiology of coronary related heart diseases, and may help us develop new therapeutic interventions to prevent and treat these diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.04.020.

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