Developmental Biology 319 (2008) 258–266

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

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ARTICLE INFO

Article history:
Received for publication 15 August 2007
Revised 21 March 2008
Accepted 8 April 2008
Available online 24 April 2008

Keywords:
BAF180
SWI/SNF
PBAF
Chromatin remodeling
Epicardium
Coronary development
EMT
Angiogenesis
Vasculogenesis
Surface nodule

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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doi:10.1016/j.ydbio.2008.04.020

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).