BAF200 Is Required for Heart Morphogenesis and Coronary Artery Development

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Abstract

ATP-dependent SWI/SNF chromatin remodeling complexes utilize ATP hydrolysis to non-covalently change nucleosome-DNA interactions and are essential in stem cell development, organogenesis, and tumorigenesis. Biochemical studies show that SWI/SNF in mammalian cells can be divided into two subcomplexes BAF and PBAF based on the subunit composition. ARID2 or BAF200 has been defined as an intrinsic subunit of PBAF complex. However, the function of BAF200 in vivo is not clear. To dissect the possible role of BAF200 in regulating embryogenesis and organ development, we generated BAF200 mutant mice and found they were embryonic lethal. BAF200 mutant embryos exhibited multiple cardiac defects including thin myocardium, ventricular septum defect, common atrioventricular valve, and double outlet right ventricle around E14.5. Moreover, we also detected reduced intramyocardial coronary arteries in BAF200 mutants, suggesting that BAF200 is required for proper migration and differentiation of subepicardial venous cells into arterial endothelial cells. Our work revealed that PBAF complex plays a critical role in heart morphogenesis and coronary artery angiogenesis.

Introduction

Epigenetic regulation of embryonic development involves DNA methylation, histone modifications and ATP-dependent chromatin remodeling [1]. ATP-dependent chromatin remodeling complexes are specialized multi-protein machines that utilize ATP to non-covalently restructure, mobilize, or eject nucleosomes to regulate access to the DNA. One key group of this superfamily is the SWI/SNF subfamily, consisting of two closely related SWI/SNF remodeling complexes BAF (SWI/SNF-A) and PBAF (SWI/SNF-B) in mammalian cells. SWI/SNF family, as one of the most-studied chromatin remodeling complexes, plays critical roles in embryogenesis, cancer, and stem cell specification and differentiation [1–3].

The differential usage of the greater diversity of mammalian SWI/SNF subunits is essential for the development of specific cell fates and lineage conversion, including the progression from pluripotency to multipotency to committed cardiomyocytes and neurons [4]. For example, BAF60C is expressed specifically in the heart and somites in early mouse embryo and is essential for normal heart development by recruiting BAF chromatin remodeling complexes to heart-specific enhancers [5]. BAF53 has been implied for neuronal stem cell proliferation in mice [6], while BAF57 was reported to have important role in T-cell development in mice [7]. ATPase subunit Brg1 play an important role in heart muscle development and diseases [8], trabeculation [9] and cardiac morphogenesis and hair follicle regeneration and repair [10]. Our previous studies have shown that BAF-specific BAF250 knockout mouse embryos die early around implantation and are defective in mesodermal differentiation [11], whereas PBAF-specific BAF180 deficiency in mouse embryos leads to severe hypoplastic ventricle development and trophoblast placental defects [12], and defect in coronary vessel formation [13]. Our studies suggest that BAF and PBAF play distinct roles in vivo.

Arid2 or BAF200 has been defined as an intrinsic subunit of PBAF complex [14]. Recent study using exome sequencing of hepatocellular carcinomas revealed novel inactivating mutations of BAF200 in the liver cancer [15]. This study suggested that BAF200 could be a tumor suppressor gene that is mutated in HCV-associated hepatocellular carcinomas. In vitro functional studies showed that suppression of BAF200 by interfering RNA reduced interferon-responsive gene expression [14]. Moreover, BAF200 was required for the stability of chromatin remodeling complex. Recent work suggested BAF200 is essential for osteoblast differentiation, suggesting its role in maintaining cellular identity and activating tissue-specific gene expression [16]. However, the in vivo role of BAF200 was not known. Here we generated...
BAF200 knockout mice and aim to characterize its in vivo function during embryonic development.

**Materials and Methods**

**Animal study**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Approved protocol number 2011-AN-2). All efforts were made to minimize suffering. BAF200-LacZ mouse line was generated by knock-out-first strategy by EUCOMM/Sanger Institute [17]. Mice were bred on C57BL6/J background and heterozygous male and females were mated to generate BAF200−/− mutants. For proliferation study, BrdU (100 μg/g body weight) was dissolved in PBS and injected intraperitoneally into pregnant mice 4 hours before embryos harvest.

**X-gal staining**

X-gal staining was performed according to previous protocol [18]. Briefly, embryonic tissues were fixed in LacZ fix solution (0.2% glutaraldehyde, 5 mM EDTA, and 100 mM MgCl₂ in PBS) for 15–30 minutes based on the size at 4°C. After washing three times for 15 minutes in LacZ wash buffer (2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40 in 100 mM sodium phosphate buffer), embryonic tissues were stained overnight at 37°C in LacZ stain solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in LacZ wash buffer). After washing with PBS for 3 times, embryonic tissues were processed for microscopy (Leica, M165FC). For section x-gal staining, embryos and hearts were collected in PBS on ice and then fixed in 4% paraformaldehyde at 4°C for 1 hour. After washing in PBS, tissues were treated with 30% sucrose overnight. Then they were embedded in optimum cutting tissue (OCT, Sakura) and snap frozen. Cryosections of 10 μm thickness were collected on positively charged slides. Then the section was washed by PBS for 3 times, and treated with LacZ stain solution at 37°C for 3–4 hours, after PBS wash, slides were mounted with 50% glycerol. In some samples, X-gal staining was followed by immunohistochemistry staining,

**Immunohistochemistry**

Immunostaining was performed according to protocols described previously [20]. Briefly, embryos were collected in PBS on ice and then fixed in 4% paraformaldehyde at 4°C for 1 hour. After washing in PBS, tissues were treated with 30% sucrose overnight. Then they were embedded in optimum cutting tissue (OCT, Sakura) and snap frozen. Cryosections of 10 μm thickness were collected on positively charged slides. Then the section was washed by PBS for 3–4 hours, after PBS wash, slides were mounted with 50% glycerol. In some samples, X-gal staining was followed by immunohistochemistry staining, immunohistochemistry staining were performed according to standard protocols after x-gal staining finished. Images were acquired on Olympus confocal microscope FV1000, Zeiss confocal microscope LSM510 and Olympus microscope BX53. Quantification was performed by a blinded observer to experiment.

**TUNEL staining**

TUNEL staining was performed using In Situ Cell Death Detection Kit, TMR Red (Roche, 12156792910). Frozen tissue sections were fixed with 4% paraformaldehyde for 10–20 minutes at room temperature, and washed 30 minutes with PBS, then moved slides into PBS (0.1% Triton X-100 in PBS) for 2 minutes at 4°C. Slides were incubated with TUNEL reaction mixture for 1 hour at 37°C, wash with PBS 3 times. Afterwards, tissue sections were then counter-stained with cardiomyocyte marker ACTN2. Images were acquired on fluorescence microscope (Olympus, BX53).

**Whole-mount PECAM staining**

Whole-mount PECAM staining was performed as previously described [18]. Briefly, mouse embryos were collected in PBS on ice and fixed in 4% paraformaldehyde overnight at 4°C. The next day, embryos were washed in PBS three times, followed by serial dehydration by methanol. Then embryos were bleached in 5% hydrogen peroxide in 100% methanol for 2 hours at 4°C, followed by rehydration. Embryos were blocked in PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 1 hour at 4°C. Embryos were then incubated in block solution containing PECAM antibody (BD PHarmingen, 553370), AP2 (Abcam, AB13979, 1:100), LYVE1 (Abcam, AB14917, 1:100), TNNI3 (Abcam, AB36357, 1:200) was performed on cryosections. Images were acquired on Olympus confocal microscope FV1000, Zeiss confocal microscope LSM510 and Olympus microscope BX53. Quantification was performed by a blinded observer to experiment.

**Paraffin embedded and H&E staining**

Histology was performed as previously described [19]. Mouse embryos were fixed in 4% paraformaldehyde overnight at room temperature. The following day, embryos were dehydrated through 70%, 80%, 95% and 100% ethanol for 10–30 minutes each base on the embryos’ size, then embryos were moved into butyl alcohol for 2 hours and embedded with Leica parafin. Paraffin sections (10 μm) were cut through the entire heart. Hematoxylin/eosin staining was performed on the paraffin sections. Sections were Dewaxed in xylene 2 times, and rehydrated by 100%, 95%, 80%, 75% and distilled water serially, then stained by hematoxylin solution for 4 minutes, differentiated by 1% acid alcohol for additional 1 minute, bluing in 1% ammonia water for 1 minute. Afterwards, the sections were counterstained by eosin Y solution for 10 seconds, and dehydrated by serial ethanol and xylene.
from E12.5 embryos. Explants were placed with the epicardium facing downwards and allowed to attach for 5 hours at 37°C, 5%CO₂. DMEM containing 10% FBS and antibiotics was added and the cultures were incubated for up to 3 days.

Quantitative RT-PCR analysis

Mice hearts were collected from E13.5 embryos, RNA was extracted with Trizol according to manufacturer’s protocol (Invitrogen) and converted the RNA to cDNA using M-MLV reverse transcriptase (Promega, M170A). For qPCR, SYBR Green qPCR master mix (Applied Biosystems) was used and cDNA was amplified on a Applied Biosystems® 7500 Real-Time PCR System. qRT-PCR primers for BAF200 is designed to span exon 3 and exon 4 (Figure 1A): Primer1 (exon3): TGTTCCAAC-GCTGCTTTG; primer 2 (exon4): TGGCTTTGGATTG-CCTGGTG; primer 3 (exon3):AGAAGTTGTTCCAACCGT-GCC; Primer 4 (exon4): TGTGGCTTTGGATTGCCTGG; Fabp4 qRT-PCR primers: (F):CACCATCCGGTCAGAGAG-TA; (R):TGATGCTCTTCACCTTCCTG.

Statistics

Data were analyzed by unpaired student’s-t-tests for two groups. Significance was accepted when P<0.05. All data are presented as mean ±SEM.

Results and Discussion

BAF200-LacZ knockin mouse line was generated by knockout-first strategy [17]. LacZ cDNA was targeted into BAF200 gene locus by homologous recombination, generating early termination of BAF200 transcription. 1–4 indicates four different primers that span exon 3 and exon 4. (B) Quantitative RT-PCR shows significantly reduced Baf200 transcripts in BAF200LacZ/LacZ mutants compared with littermate control. n = 8. *P<0.05. (C) Whole mount x-gal staining of BAF200LacZ/+ at E8.0 to E9.5. White Representative of 3 for each time point. (D) X-gal staining of E9.5 and E13.5 heart section. h, heart; red arrowheads point to endocardial cells; black arrowheads indicate cardiomyocytes in compact myocardium; green arrowheads indicate epicardial cells. Bar = 200 μm.

doi:10.1371/journal.pone.0109493.g001

Figure 1. Generation of BAF200 knockout mouse. (A) LacZ cDNA was targeted into BAF200 gene locus by homologous recombination, generating early termination of BAF200 transcription. 1–4 indicates four different primers that span exon 3 and exon 4. (B) Quantitative RT-PCR shows significantly reduced Baf200 transcripts in BAF200LacZ/LacZ mutants compared with littermate control. n = 8. *P<0.05. (C) Whole mount x-gal staining of BAF200LacZ/+ at E8.0 to E9.5. White Representative of 3 for each time point. (D) X-gal staining of E9.5 and E13.5 heart section. h, heart; red arrowheads point to endocardial cells; black arrowheads indicate cardiomyocytes in compact myocardium; green arrowheads indicate epicardial cells. Bar = 200 μm.

doi:10.1371/journal.pone.0109493.g001

BAF200 Function in Heart Development

Figure 2. Survival of offspring and heart defects in BAF200 mutant. (A) The graph displays the frequency of offspring of BAF200 mutants. The number of offspring genotyped for each time point is indicated. (B) Whole mount view of mutant and littermate control embryos. White bar = 1 mm. (C) H.E. staining of E12.5 and E14.5 BAF200 mutant and littermate control embryos show thin compact myocardium (green bar), common atrioventricular valve (asterisks), ventricular septum defect (arrows) and double outlet right ventricle (DORV, double arrows). Black bar = 0.5 mm.

doi:10.1371/journal.pone.0109493.g002
controls (Fig. 3A, B). Cell proliferation defect was confirmed by another marker phospho-histone H3 staining (Fig. 3C). Examination of a cyclin-dependent kinase inhibitor, p57kip2, was dramatically increased in mutant (Fig. 3D). However, we did not observe significant increase of cell death in mutants compared with control littermates (Fig. 3E). Cardiac differentiation markers MYL2 and MYL7 were not significantly changed in mutant, suggesting normal differentiation of cardiac chamber in BAF200 mutant hearts (Fig. 3F, G). Furthermore, GATA4 and NKX2-5, two key cardiac transcription factors [21,22], were normally expressed in BAF200 mutant hearts (Fig. 3H, I). Altogether, these data suggest that BAF200 is required for normal heart development, and regulates cardiomyocyte proliferation.

We next attempted to dissect the possible function of BAF200 in epicardium development and coronary formation, as BAF180 is involved in these processes [13]. The heart is avascular at E10.5, and coronary vessels begin to form afterwards when compact myocardium become thicker. Normal formation of epicardium is crucial for early coronary vascular development [23,24]. Staining of epicardial marker RALDH2 showed that epicardium was formed normally in mutant hearts (Fig. 4A). In addition, Ex vivo explant assays showed no difference in migrating epicardial cells number and epithelial-to-mesenchymal transition (EMT) between BAF200 mutants and littermate controls (Fig. 4B). We next examined the coronary vasculature in BAF200 mutant hearts. By whole mount PECAM staining, we found that subepicardial coronary vasculature was in similar pattern to littermate controls (Fig. 4C). However, sectional staining of PECAM revealed that intramyocardial coronary vascular endothelial cells (ECs) were significantly reduced in BAF200 mutants (Fig. 4D). To identify the vascular defects more specifically, we applied immunostaining of the coronary vascular specific marker AP2 [25]. Intramyocardial coronary arteries were significantly reduced in BAF200 mutants (Fig. 4E, F). This was also confirmed by quantitative RT-PCR (Fig. 4G). These coronary arteries were proposed to be formed by reprogramming of subepicardial coronary venous cells [26,27]. Since intramyocardial coronary vessels contain most coronary artery endothelial cells while subepicardial endothelial cells will become coronary veins, specific reduction in coronary arterial endothelial cells instead of venous population suggested that BAF200 regulated coronary artery differentiation and formation. Further study of BAF200 in regulation of coronary vessel development is warranted.

Figure 3. Reduced cardiomyocyte proliferation in BAF200 mutant hearts. (A, B) Staining of BRDU shows reduced cardiomyocytes (CMs) proliferation in E13.5 BAF200 mutant hearts compared with littermate controls. *P<0.05. (C) phospho-histone H3 staining shows reduced proliferation in BAF200 mutant compact myocardium. (D) Expression of p57kip2, a cyclin-dependent kinase inhibitor, is increased in mutant hearts. (E) No significant apoptosis was detected in mutant and control embryos. (F, G) Expression of chamber-specific markers MYL2 and MYL7 was unchanged in BAF200-deficient hearts. (H, I) Expression of cardiac transcription factors GATA4 and Nkx2-5 were not significantly changed in BAF200 mutants. White bar = 100 um.

doi:10.1371/journal.pone.0109493.g003
formation would aid in our understanding of reprogramming of vascular cells and may provide new avenues for vascular regeneration following injury and diseases [28].

We next determined if the defects of angiogenesis in the BAF200 mutants were cardiac coronary vessel-specific or the result of a general angiogenesis defect, giving the fact that BAF200 is widely expressed throughout the whole embryos (Fig. 1C). We performed PECAM immunostaining on E13.5 BAF200 mutants and littermate controls, and did not find any significant defect in other organs or tissues eg. brain, liver (Figure 5A and 5B). We therefore conclude that coronary vessel defect is cardiac-specific.

Figure 4. Coronary angiogenesis was impaired in BAF200 mutant hearts. (A) Epicardium marker RALDH2 indicates that epicardium integrity remains in E13.5 BAF200 mutants. (B) E12.5 heart explant assays measuring migrating epicardial cells in vitro showed no EMT defect in mutant hearts. White bar = 100 um. (C) Whole mount PECAM staining of E13.0 embryonic hearts. White bar = 0.5 mm. (D) Immunostaining of PECAM on E13.5 embryonic hearts. Dotted blue lines indicate epicardium (Epi). Endo, endocardium; LV, left ventricle; RV, right ventricle. Black bar = 0.2 mm. (E) Immunostaining of AP2 on E13.5 hearts shows vascular endothelial cell (EC) patterning in embryonic hearts. Black bar = 0.2 mm. (F) Quantification of intramyocardial ECs in BAF200 mutant and littermate control hearts. *P<0.05; n = 3. (G) Quantitative RT-PCR (qRT-PCR) of vascular endothelial cell marker AP2. *P<0.05; n = 8.

doi:10.1371/journal.pone.0109493.g004
lymphatic vessels are impaired in growth, we performed immuno-
ostaining of lymphatic vessel marker LYVE1 on BAF200 mutants. We found there was no significant defect in LYVE1+ number in BAF200 mutants compared with littermate controls (Figure 5C and 5D).

Our study provided the first in vivo evidence that BAF200 played important roles in embryonic cardiomyocyte proliferation as well as in lineage conversion of venous cells into arterial endothelial cells during coronary development. Interestingly, this data recapitulated the phenotype of BAF180 mutants [13,14], suggesting that the observed functions of BAF200 is PBAF-specific. Our studies may reveal new clues into the etiology of various congenital heart diseases. Moreover, understanding the cardiomyocyte proliferation and coronary reprogramming processes governed by SWI/SNF complexes and identifying the endogenous regulators should provide novel insights into strategies of repopulating and revascularizing the cardiac tissue after myocardial infarction or other ischemic diseases.

Acknowledgments
This work was supported by Ministry of Science and Technology (2012CB945102 and 2013CB945302), National Science Foundation of China (91339104, 31273552, 31222038, 31301189), Chinese Academy of Sciences (Hundred Talents Program and KSCX2-EW-R-09), Organization Department of the CPC Central Committee Bajian Talents Program, Shanghai Pujiang Program (11PJ1411400) and Basic Research Key Project (14JC1407400), AstraZeneca, Sanofi-Shanghai Institutes for Biological Sciences (SIBS) Fellowship, SIBS Postdoc Fund 2013KIP311, China Postdoc Fund 2013M541561, National Institutes of Health (HL109054, WZ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions
Conceived and designed the experiments: BZ. Performed the experiments: LH XT HZ. Analyzed the data: LH BZ. Wrote the paper: BZ ZW. Breed mice and did genotype: TH LZ XH.

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