Research Paper

Differentiation defect in neural crest-derived smooth muscle cells in patients with aortopathy associated with bicuspid aortic valves

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1. Introduction

Bicuspid aortic valves (BAV) are the most common congenital cardiovascular malformation affecting 0.9–2% of the general population (Michelea et al., 2014; Prakash et al., 2014). BAV account for more morbidity and mortality than all other cardiovascular congenital malformations combined. Thoracic aortic aneurysm (TAA) occurs in approximately 50–70% of patients with BAV which is significantly higher than the rate of TAA formation in patients with tricuspid aortic valves (TAV) (Fedak et al., 2005). Furthermore, patients with BAV have an eight-fold higher risk of aortic dissection, which carries very high mortality (Micheleena et al., 2011). There are no therapeutic agents to prevent TAA in BAV.

Whether the aneurysms in BAV patients arise due to altered hemodynamic forces from the abnormal valve or from an underlying genetic defect leading to both BAV and TAA is currently unknown (Prakash et al., 2014). The assessment of hemodynamic flow in BAV patients shows distinct differences of the flow patterns compared to TAV patients (Keane et al., 2000). However, even after aortic valve replacement to correct the hemodynamics, some BAV patients still develop an ascending aortic aneurysm (Itagaki et al., 2015). Studies using aortic tissue from BAV patients have shown potential evidence of aortopathy of the aortic wall, including immature smooth muscle cells (SMCs) (Gregwet al., 2014b; Nkomo et al., 2003). However, these results failed to delineate between the two possible causes of TAA mentioned above.

Accumulating evidence suggests an inherited component to the etiology of BAV complicated by TAA (BAV/TAA), but the responsible locus or loci are still largely unknown (Cripe et al., 2004; Horne et al., 2004). Rare variants of NOTCH1, GATAS and FBN1 were correlated with 4–10%, 2.6% and one in eight BAV patients, respectively (Bonachea et al., 2014; Garg et al., 2005; Padang et al., 2013; Pepe et al., 2014). Common variants in FBN1 were also found to be associated with BAV/TAA (LeMaire et al., 2011). Lack of knowledge of gene alterations predisposing to majority of BAV/TAA patients has prevented the development of mouse models. Due to the inaccessibility of appropriate cellular or mouse model, so far, the mechanism of TAA in BAV patients remains largely unknown.

Interestingly, the aortic root, ascending aorta, and aortic arch are commonly involved in aneurysms, whereas the descending aorta is rarely impacted in BAV/TAA (Fazel et al., 2008). Embryonic fate-mapping studies have demonstrated that the aortic root, ascending aorta

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and aortic arch are populated by SMCs arising from neural crest (NC), while the descending aorta is populated by SMCs from the paraxial mesoderm (PM) (Cheung et al., 2012; Majesky, 2007). Based on these facts, we hypothesize that the aortopathy in BAV patients is due to a defective differentiation of neural crest stem cells (NCSCs) to SMCs that spares the paraxial mesoderm cells (PMCs)-derived SMCs. Advancement of induced pluripotent stem cells (iPSCs) and lineage-specific SMCs differentiation technology now provides unique methods to test this hypothesis (Cheung et al., 2014).

2. Materials and Methods

2.1. Human Subjects

Clinical information about the subjects for iPSCs generation was summarized in Fig. S1A. 5 to 10 ml of fresh blood was drawn from each patient after written consents were obtained. PBMCs were harvested for reprogramming. This study was approved by the Institutional Review Board (IRB) at the University of Michigan Health System (Ann Arbor, MI) in compliance with Health Insurance Portability and Accountability Act (HIPAA) regulations and the Declaration of Helsinki. All our study procedures were consistent with the IRB Guide for the human subject studies.

2.2. iPSC Cells Generation and Culture

The plasmids preparation and the procedure of non-integrated iPSC derivation were performed according to the methods described previously (Su et al., 2013). In brief, PBMCs were separated from human peripheral blood, cultured and electrottransfected with non-integrating DNA plasmids containing OCT4, SOX2, KLF4, and C-MYC. After the colonies became compact at around day 30 post-infection, the colonies were mechanically picked up from the culture dishes and cultured with TesRE8 medium (Stemcells Inc.) on Matrigel (BD Corp.) coated dishes with Accutase (Life technologies Corp.) and seeded onto Matrigel (BD Corp.) coated dishes at a density of 4 × 10^6 cells/cm² with medium containing DMEM/F12, N2, Pen/Strep (Life technologies Corp.), EGF 10 ng/ml, FGF2 10 ng/ml (R&D systems Corp.) and Rocki (Y27632, Stemgent Inc.). 1 μM, Sigma Corp.), BMP4 (10 ng/ml, R&D Systems Corp.) for 1.5 days then cultured in the above medium excluding BMP4 for 3.5 days (Cheung et al., 2014).

2.3. Karyotype Analysis

The iPSCs were incubated in culture medium containing 0.25 μg/ml colcemid (Invitrogen) for 2 h and were harvested and incubated in hypotonic solution containing 0.4% NaCl (Sigma Corp.), at 37 °C for 20 min. And cells were fixed with a mixture of methanol:acetic acid (3:1, v/v). Slides were aged for 8–12 h at 65 °C and digested with 0.8% trypsin for 15–30 s. Slides were then stained with Giemsa (Gibco Corp.) for 10 min and air-dried.

2.4. NCSCs Differentiation From iPSCs

To differentiate iPSCs into NCSCs, iPSCs were dissociated with Versene (Life Technologies Corp.) into single cells and seeded at 2 × 10⁵ cells/cm² with TesRE8 (Stemcells Inc.) medium supplemented with Rocki (Y27632, Stemgent Inc.). When the cells reached a confluence of 40% to 60%, medium was changed into NCSC differentiation medium, which contained DMEM/F12 (Life technologies Corp.), N2 (Life technologies Corp.), Pen/Strep, FGF2 (20 ng/ml, R&D Systems Corp.), LY294002 (10 μM, Sigma Corp.), BMP4 (10 ng/ml, R&D Systems Corp.) for 9 days then changed into NCSC differentiation medium excluding BMP4 for 9 days then cultured in the above medium excluding BMP4 for 3.5 days (Cheung et al., 2014).

2.5. PMCs Differentiation From iPSCs

To differentiate iPSCs into PMCs, iPSCs at 80–90% confluence were dissociated with Versene (Life technologies Corp.) and split (diluted in fresh medium) at a ratio of 1:8 and seeded on Matrigel (BD Corp.) coated dishes with TesRE8 (Stemcells Inc.) medium supplemented with Rocki (Y27632, Stemgent Inc.). When the cells reached 40% to 60% confluence medium was changed into PMC differentiation medium, which contained DMEM/F12 (Life technologies Corp.), N2 (Life technologies Corp.), Pen/Strep, FG2 (20 ng/ml, R&D Systems Corp.), LY294002 (10 μM, Sigma Corp.), BMP4 (10 ng/ml, R&D Systems Corp.) for 1.5 days then cultured in the above medium excluding BMP4 for 3.5 days (Cheung et al., 2014).

2.6. SMCs Differentiation From NCSCs

NCSCs were dissociated into single cells with Accutase (Life technologies Corp.) and sorted on HNK1 and P75 double positive using FACS and seeded onto Matrigel (BD Corp.) coated dishes at a density of 4 × 10⁶ cells/cm² with medium containing DMEM/F12, N2, Pen/Strep (Life technologies Corp.), EGF 10 ng/ml, FGF2 10 ng/ml (R&D systems Corp.) and Rocki (Y27632, Stemgent Inc.). The medium was refreshed on the second day with the same media without Rocki. At day 3, SMC differentiation medium was applied which contained DMEM/F12, 15%KSR (Knockout Serum Replacement, Life technologies Corp.), and Pen/Strep (Life technologies Corp.). TGFβ1 was added at 2 ng/ml to the SMC differentiation medium in specific experimental groups. Medium was refreshed daily for 9 days, changed into DMEM/F12, N2, Pen/Strep (all from Life technologies). After two days, SMCs were treated with rapamycin (20 nM, Millipore Corp.) or mock treated with DMSO for two days until harvested for further analysis.

2.7. SMCs Differentiation From PMCs

PMCs were dissociated into single cells with Accutase (Life technologies Corp.) and seeded onto Matrigel (BD Corp.) coated dishes with density of 4 × 10⁶ cells/cm² with medium containing DMEM/F12, 15%KSR (Knockout Serum Replacement, Life technologies Corp.), Pen/Strep (Life technologies Corp.), TGFβ1 2 ng/ml and BMP4 (all from Life technologies Corp.). Medium was refreshed daily for 9 days then changed into DMEM/F12, N2, Pen/Strep (all from Life technologies Corp.). Medium was refreshed daily for four more days, SMCs were harvested for further analysis.

2.8. Immunofluorescence Staining and Flow Cytometry

Immunofluorescence staining and flow cytometry was performed as described (Jiao et al., 2013). The following primary antibodies were used: anti-OCT4 (Santa Cruz Biotechnology Inc.), anti-SOX2 (Santa Cruz Biotechnology Inc.), anti-NANOG (Cosmo Inc.), anti-HNK1 (Sigma Corp.), anti-P75 (Advanced Targeting Systems Inc.), anti-TBX6 (Santa Cruz Biotechnology Inc.), anti-TAGLN (Abcam plc.), anti-MYH11 (Abcam plc.), anti-ACTA2 (Sigma Corp.), anti-CNN1 (Sigma Corp.). The following fluorochrome-conjugated secondary antibodies were used: Alexa Fluor 594 goat anti-rabbit (Molecular Probes), Alexa Fluor 633 goat anti-mouse IgM (Molecular Probes), Alexa Fluor 633 goat anti-mouse IgG (Molecular Probes), and Alexa Fluor 633 rabbit anti-goat IgG (Molecular Probes). Cells were applied to MoFlo Astrios or slides were mounted with anti-fade mounting media containing DAPI (Prolonggold, Life technologies Corp.). Stained cells were observed on a Nikon A1 confocal microscope.

2.9. qPCR

Total RNA was purified with TRIzol Reagent (Life technologies, Corp.). 1 μg of RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Promega Corp.) and RNasein RNase Inhibitor (Promega Corp.). QPCR was carried out with SYBR universal mix (BioRad Corp.). The primers were listed in Table S1. The expression level of genes in each sample was normalized to GAPDH.
2.10. Western Blot Analysis and ELISA

Whole cell extracts were prepared using RIPA buffer, resolved on SDS-PAGE gels, and transferred to acetate cellulose membranes. Primary antibodies used were anti-TAGLN (1:1000, Abcam plc.), anti-MYH11 (1:500, Abcam plc.), anti-ACTA2 (1:200, Sigma Corp.), anti-CNN1 (1:500, Sigma Corp.), anti-pSMAD2 (1:500, Cell Signaling Technology Inc.), anti-pS6 (1:500, Cell Signaling Technology Inc.), anti-GAPDH (1:2000, Santa Cruz Inc.). Secondary antibodies used were IRDye680RD Donkey anti-Mouse, IRDye680LT Donkey anti-Rabbit, IRDye800CW Donkey anti-Mouse, IRDye800CW Donkey anti-Rabbit (All secondary antibodies were from Licor Inc.). Licor western blot detection system was used for the dual-color imaging. ImageJ was used for the quantification of bands.

For the ELISA assay of the secreted TGF-β1, fresh medium (DMEM/F12, N2, Pen/Strep, all from Life technologies Corp.) without serum was applied to the cells and harvested after 24 h. ELISA assay was carried out using the Human TGF-beta 1 Quantikine ELISA Kit from R&D systems following the manufacturer’s guidelines.

2.11. Teratoma Formation

One million iPSCs were injected subcutaneously into each flank of the recipient nonobese diabetic/severe combined immunodeficient mice (NOD/SCID) mice (Jackson Laboratory, Bar Harbor, Maine). Paraffin sections and H&E staining of formalin-fixed teratoma specimens isolated from the mouse flanks were prepared 3–5 weeks after injection.

2.12. Collagen Gel Assay

SMCs were suspended at 1 × 10^6 cells/ml in DMEM with type I collagen (Cell Biolabs Inc.) at 2 mg/ml and NaOH (Cell Biolabs Inc.). The mixture was poured into 24-well tissue culture plates (1 ml/well) and allowed to gel in a 5% CO₂ incubator at 37 °C for 30 min, and then 1 ml of medium (DMEM/F12, N2, Pen/Strep, all from Life technologies Corp.) was added. After 3 days, gels were lifted and contraction was assessed after 1 h by scanning the plates.

2.13. Carbachol Stimulated Contractility Assay

Differentiated SMCs were washed with DMEM (Life technologies Corp.) once and medium was replaced to DMEM with 1 mM carbachol (Sigma Corp.). Cells were recorded every 1 min from 0 to 30 min using a Nikon TE200 microscope with DP controller software. The pictures were condensed into a movie with DP controller software. The cells were kept in the same places and the imaging parameters were kept the same during the 30 min. Pictures at 0 and 30 min were used to calculate the cell area reduction. Single cells were identified by location and shape from pictures at 0 and 30 min. Cell surface areas were measured using ImageJ software. Change of area of each cell were normalized to its area at 0 min, and then normalized to the mean value of normalized area reduction of cells from control patient.

3. Results

3.1. iPSCs Were Used to Model TAA Associated With BAV

iPSCs were derived from peripheral blood mononuclear cells (PBMCs) using non-integrating DNA vectors with OCT4, SOX2, KLF4 and C-MYC (Okita et al., 2007; Su et al., 2013) from two patients (one male 34 years old and one female 53 years old) with BAV, aneurysmal ascending aorta and normal descending aorta, and one control male 65 years old patient with normal tricuspid aortic valve (TAV) and a normal aorta (Figs. 1a, 1A). No mutation of NOTCH1 was found in these patients by exome sequencing. The iPSC lines showed typical morphology of human embryonic stem cells, maintained a normal karyotype (Figs. 1b–c, 1C), expressed pluripotent markers, including OCT4, NANOG, SOX2, SSEA4, TRA-1-60 and TRA-1-81 (Figs. 1d, 1B), and formed teratomas in NOD/SCID mice (Figs. 1e, 1D). Based on the correct karyotype, expression of pluripotent genes, and ability to generate three germ layers in teratoma, two iPSCs lines from normal control and two iPSCs lines from each BAV/TAA patient were selected for differentiation experiments.

The iPSCs were differentiated into NCSCs by dual-SMAD inhibition (Chambers et al., 2009). Typical neural rosette morphology was detected at days 10–12 (Figs. 1f, 1E). The NCSCs from BAV/TAA and control iPSCs expressed the NCSC marker genes SOX9, PAX3 and SLUG at similar levels (Figs. 1g, 1f–h). Immunohistochemistry of the differentiated NCSCs showed that >80% of the differentiated population expressed cell membrane markers P75 and HNK1, indicating high differentiation efficiency (Fig. 1i). >60% of differentiated cells were P75 and HNK1 double positive in flow cytometry analysis, and double positive cells were purified by flow cytometry sorting for SMCs differentiation (Fig. 1j).

3.2. SMCs Derived From BAV/TAA NCSCs Express Less MYH11 and Have Impaired Contractile Function

We sought to determine if there were differences in the phenotype of NCSC-SMCs from iPSCs derived from patients with BAV/TAA when compared with control iPSCs. SMCs differentiated from the control NCSCs gradually gained an enlarged and expanded morphology (Fig. 2a), and showed increased expression level of SMCs markers (contractile protein genes), including MYH11, ACTA2, TAGLN, and CNN1 (Wang et al., 2003) (Fig. 2A). Immunofluorescence and Western blot analyses showed that >90% of the cells were SMCs based on positive staining of corresponding proteins, SMMHC, SM α-actin, SM22α and calponin-1 (Fig. 2, a, c, e). Flow cytometry showed that >70% of the differentiated cell population was positive for the SMMHC, which is the most specific marker of SMCs (Fig. S2B) (Owens et al., 2004). Functionally, the control SMCs could contract in the collagen gel assay (Fig. 2g), and upon treatment with carbachol (Fig. 2f, Movie S1). Therefore, mature contractile SMCs could be efficiently differentiated from NCSCs.

The qPCR analysis and Western blotting assay of the SMCs from two BAV/TAA-NCSCs showed significantly lower expression of SMMHC (Fig. 2b, c). In contrast SM α-actin, SM22α and calponin-1 were similarly expressed between BAV/TAA and control samples (Fig. 2d–e). The carbachol assay and collagen gel contraction assay showed that the contraction of BAV/TAA NCSC-SMCs decreased compared to control NCSC-SMCs (Fig. 2f–g, i, Fig. 5C, Movies S1, S2). Importantly, primary culture of SMCs explanted from the ascending aorta of BAV/TAA patients also showed decreased MYH11 expression in high through put sequencing of mRNA, and impaired contraction when compared with SMCs explanted from the normal aorta of heart transplant donors (Fig. 2h, j, Fig. S4A). Interestingly, the NCSC-SMCs derived from iPSCs of BAV patient with normal aorta showed levels of expression of MYH11 similar to the control (Fig. S2D). These results suggest that patients with BAV/TAA have a defect in differentiating NCSC into fully contractile SMCs.

3.3. SMCs Derived From BAV/TAA PMCs Behave Similarly to the Control SMCs

Since BAV/TAA patients rarely develop aneurysms in other segments of the aorta, we sought to determine if the PMC-derived SMCs from these patients also showed defective contractile function. QPCR analyses showed significant elevation of expression levels of the PMCs marker genes, MEOX1, TBX6 and PAX1, in the control and BAV/TAA differentiated PMCs from iPSCs (Fig. 3a). The majority (~80%) of the BAV/TAA and control differentiated PMCs were positive for paraxial mesoderm markers TCF15 and TBX6 in immunofluorescence staining assay (Fig. 3b, c).
Fig. 1. iPSCs from BAV/TAA patient are pluripotent and can differentiate to NCSCs. (a) 3-D reconstruction of a Computed Tomography (CT) scan of the aorta in a BAV/TAA patient. (b) Morphology of PBMCs and iPSCs from BAV/TAA. (c) Karyotyping of iPSCs from BAV/TAA. (d) Immunostaining of OCT4, SOX2, NANOG, SSEA4, TRA-1-60 and TRA-1-81 in the BAV/TAA iPSC colonies. The scale bars represent 50 μm. (e) H&E staining of teratomas derived from the BAV/TAA iPSCs injected into the flanks of NOD/SCID mice. The scale bars represent 50 μm. (f) Morphological change of NCSCs from day 0 to day 10 in cell culture. The scale bars represent 500 μm. (g) Relative gene expression of NCSC marker genes SLUG, SOX9, PAX3 in iPSCs, NCSCs and PMCs assessed by quantitative PCR (qPCR). Each group contains two cell lines. Experiments were repeated three times. (h) Immunofluorescence staining of NCSC markers HNK1 and P75 of the NCSCs. The scale bars represent 100 μm. (i) Flow cytometry of HNK1 and P75 of the NCSCs. PBMCs: peripheral blood mononuclear cell. iPSCs: induced pluripotent stem cells. NCSCs: neural crest stem cells. PMCs: paraxial mesoderm cells. H&E: hematoxylin and eosin. BAV/TAA: bicuspid aortic valve/thoracic aortic aneurysm. Ctrl: Control. Data is represented as mean ± SEM. See also Fig. S1.
The SMCs from BAV/TAA and control PMCs had upregulated expression level of SMC markers MYH11, ACTA2, CNN1 and TAGLN (Fig. 3d), and >90% of the SMCs were positive for calponin-1 and SM22α in the immunofluorescence staining assay (Fig. 3e, f). The expression level of CNN1 was slightly higher in the BAV/TAA SMCs derived from PMCs when compared to the control (Fig. 3d). And PMC-SCMs in both BAV/TAA and control expressed more MYH11 than their counterparts of NCSC-SCMs (Fig. S3F). Western blot assay also confirmed that SMMHC, SMα-actin, SM22α and calponin-1 were similar in protein levels in SMCs from BAV/TAA PMCs and control PMCs (Figs. 3g, S3A–D). Furthermore, with carbachol treatment, the relative change of cell surface area was comparable between SMCs derived from BAV/TAA PMCs and control PMCs (Figs. 3h, S3E, Movies 54, S5). These results indicated that the SMCs from BAV PMCs were fully differentiated and contractile as those derived from control PMCs.

3.4. SMCs Derived From BAV/TAA NCSCs Have Decreased TGF-β Pathway Signaling and Increased mTOR Signaling, and Inhibition of mTOR Signaling by Rapamycin Rescues the Impaired Contraction Phenotype

To characterize the phenotype of aortic SMCs from BAV/TAA patients, whole transcriptome sequencing of the mRNAs from SMCs explanted from the ascending aorta of one BAV/TAA and three heart transplant donors as control was performed, which showed more than one hundred differentially expressed genes. A portion of those genes were enriched in TGF-β signaling pathway, mTOR signaling pathway and PDGF signaling pathway (Fig. S4A). We found that the relative gene expression of TGF-β1 ligand and TGF-β2 ligand was decreased in BAV/TAA (Fig. S4E, F). Relatedly, phosphorylated SMAD2 (pSMAD2) significantly decreased in BAV/TAA...
SMCs derived from NCSCs (Fig. 4a, b). Also, connective tissue growth factor (CTGF) expression level in the BAV/TAA group was significantly lower than in controls (Fig. 4d). Previous studies showed that TGF-β regulates expression of Myocardin (MYOCD), an important transcription factor regulating expression of MYH11 (Davis-Dusenbery et al., 2011). Here, we found that MYOCD expression significantly decreased in the BAV/TAA patients NCSC-derived SMCs (Fig. 4e).

We also found more phosphorylated S6 (pS6) protein levels in patients’ SMCs when compared with the control, indicating hyperactive mTOR signaling pathway (Fig. 4a, c). Rapamycin inhibits mTOR signaling, thus promotes SMC differentiation through Akt2 activation (Martin et al., 2007). Levels of phosphorylated S6 decreased with rapamycin treatment the patients’ cells (Fig. 4h, i, j, k). Treatment of 20 nM rapamycin for two days restored the expression of MYH11 and SMMHC in BAV NCSC-SCMs (Fig. 4F–h, k, j, m) and rescued the impaired contractile function of the BAV/TAA NCSC derived SMCs (Fig. 4n, o, Movies S2, S3). Interestingly, TGF-β3 signaling related genes and MYOCD expression level did not decrease in BAV/TAA PMC-SMCs (Fig. S4G).

4. Discussion

The thoracic aortic aneurysm in BAV patients frequently involves ascending aorta, where the SMCs are derived from the neural crest. In contrast, patients with BAV rarely have aneurysms involving the descending thoracic aorta, and the SMCs in this region of the aorta are derived from paraxial mesoderm (Cheung et al., 2014; Cheung et al., 2012; Fazel et al., 2008; Gillis et al., 2013). In this study, we found decreased MYH11 expression of NCSCs to SMCs in patients with BAV/TAA using an iPSC modeling system. Notably, PMCs derived SMCs from the same patients’ iPSCs did not demonstrate these differences, suggesting that the underlying defect leading to decreased expression of SMMHC and contractile function is specific for NCSC-derived SMCs.

As specific altered NCSC behavior was considered to cause a specific subtype of BAV, namely, Sievers type 1 (Sievers and Schmidtke, 2007) with left coronary cusp (LCC) and right coronary cusp (RCC) fusion (Fazel et al., 2008). We hypothesized that in this specific subtype of BAV, NCSCs play an important role in the pathogenesis of the ascending aortic aneurysm. We tested this with iPSCs from two unrelated patients with BAV (Sievers type 1, LCC–RCC fusion) and ascending aortic aneurysm and differentiated them into NCSCs (Fig. 1), then into SMCs (Fig. 2) and studied the phenotype of these SMCs.

This unique approach using patients’ iPSCs as a model system is a unique approach to study BAV/TAA from stem cells to somatic cells without directly involving human tissue. These results cannot be obtained from studying the aortic aneurysmal tissue harvested during surgery from BAV/TAA patients for multiple reasons. First, the aortic SMCs would be explanted from the end stage aortic aneurysm, and it has been previously shown that the disease process leads to epigenetic changes in the SMCs (Prakash et al., 2014). Second, it would not be ethical to...
sample the normal descending thoracic aorta in BAV patients as control tissue solely for research purposes. We can differentiate iPSCs into different lineages to model ascending aorta with NCSCs and descending thoracic aorta with PM, and study the difference of the SMCs from those two different lineages. Differentiation efficiency of iPSCs to NCSCs was above 60%. Flow cytometry was applied to purify the NCSCs population due to relatively low efficiency. More investigation is needed to improve the NCSCs differentiation method. Finally, this iPSC approach also avoids other confounding factors that may cloud the mechanistic study of aortopathy in BAV patients when one uses aortic tissue, such as the function of aortic valve, hemodynamics, sites of sampling, mixture of the subtypes of BAV, and size of the aorta.

As we hypothesized, the SMCs derived from BAV/TAA NCSCs were less mature with lower levels of SMMHC and decreased contractile function when compared to control NCSC-SMCs. In contrast, the BAV/TAA SMCs derived from PM showed preserved differentiation, maturation and contractile function. This was consistent with the patients’ CT findings of ascending aortic aneurysm but normal descending thoracic aorta. The decreased contractile function in the BAV/TAA NCSC-SMCs was also consistent with the poor contractile function in primary cultured SMCs from the aneurysmal ascending aorta from the BAV/TAA patients. With the decreased expression of MYH11 and abnormal contractile properties, one can speculate that the ascending aorta consisted of poorly contractile NCSC-derived SMCs gradually develops into an aneurysm under either normal blood pressure or hypertension, and abnormal hemodynamics due to BAV. Indeed, immature SMCs and a loss of their contractile-phenotype were also found in the dilated ascending aorta in BAV patients (Fernandez et al., 2009; Grewal et al., 2014b). SMMHC is necessary to maintain SMC contractile function (Kuang et al., 2012). Patients with MYH11 mutations are also predisposed to TAA and dissection (Forte et al., 2013; Kuang et al., 2011; Kuang et al., 2012; Zhu et al., 2006). It was not surprising to see that SMCs with decreased expression of MYH11 had poor contractile function and this phenotype ultimately contributes to the aortopathy of ascending aorta, and development of aortic aneurysm. A series of genes have been identified, MYH11, ACTA2, MYLK, that when mutated cause thoracic aortic aneurysms and dissections (Gillis et al., 2013). The mutations in these genes have been shown or are predicted to lead to decrease SMC contraction (Humphrey et al., 2015). Additionally, when the genes disrupted by unique copy number variants in patients with thoracic aortic disease were analyzed at to what cellular process they were part of, these genes were predicted to disrupt smooth muscle adhesion or contraction (Prakash et al., 2014).

TGF-β signaling is critical for differentiation and survival of SMCs from NCSCs and for maintaining the contractile phenotype late in life (Humphrey et al., 2014; Mao et al., 2012; Xie et al., 2013). In...
NCSCs, p-Smad2 interacts with the myocardin-related transcription factor to regulate SMC marker gene expression. Deletion of Smad2 results in defective differentiation of NC cells into vascular SMCs in aortic arch arteries (Xie et al., 2013). TGF-β upregulates MYOCD expression, which is a master regulator of the expression of smooth muscle marker genes, including MYH11 (Davis-Dusenberg et al., 2011; Wang et al., 2003). In the SMCs derived from BAV-NCSCs, the signaling of the TGF-β canonical pathway significantly decreased as manifested by decreased pSMAD2 and CTGF. MYOCD expression levels were also down-regulated. The decreased TGF-β signaling could impair the expression of MYOCD and thus down regulate SMC marker gene expression, including MYH11. Interestingly, two groups have reported separately that impaired canonical TGF-β signaling was also found in the ascending aortic tissue in BAV patients, and might potentiate the development of aneurysms in BAV (Grewal et al., 2014a; Paloschi et al., 2015). Our in vitro model provided another piece of evidence that defective TGF-β signaling might be the cause of the defective differentiation of NCSCs and the observed aortopathy in the BAV patients with ascending aortic aneurysm.

On the other hand, impairment of the TGF-β pathway in SMCs resulted in hyperactive mTOR pathway and caused aneurysms in a SMCs conditional Tgfb2 knockout mouse model, and rapamycin administration prevented aortic dilation (Li et al., 2014). In a Tgfb2 knockout mouse model, elevated mTOR signaling decreased expression of SMC contractile genes, and rapamycin promoted SMC contractile gene expression and prevented aneurysms (Cao et al., 2010). SMC differentiation is promoted by activating phosphatidylinositol 3-kinase (PI3K) and Akt which are negatively regulated by mTOR and S6 kinase 1 (S6K1). Rapamycin promotes SMC differentiation by inhibiting mTOR, S6K1 and activating Akt2 (Martin et al., 2007). In the BAV/TAAD NCSC SMCs, we also found upregulation of phosphorylated S6, indicating upregulation of the mTOR pathway, which may also contribute to the downregulation of the contractile gene SMMHC expression. Also, the finding that rapamycin promotes MYH11 expression and contractile function in the BAV/TAAD patient derived NCSC-derived SMCs raises the possibility that rapamycin might be a potent therapeutic drug for BAV associated aneurysms.

There is limitation of this study. We only used two BAV/TAAD patients and one control with two different iPSC cell lines for each subjects. However, these studies provide unique insights of aortopathy in BAV/TAAD patients, which cannot be obtained from any other models. Future studies can validate and expand these results.

In conclusion, in the patients with BAV (Sievers type 1, LCC-RCC fusion) and TAA, the decreased contractile function in the BAV/TAAD patient derived NCSC-derived SMCs raises the possibility that rapamycin might be a potent therapeutic drug for BAV associated aneurysms.

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Conflict of Interest Statement

None.

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