Research Paper

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

Jiao Jiao [a], Wei Xiong [a], Lunchang Wang [a], Jiong Yang [b], Ping Qiu [a], Hiroyuki Hirai [a], Lina Shao [b], Dianna Milewicz [c], Y. Eugene Chen [a], Bo Yang [a,*]

[a] Department of Cardiac Surgery, Frankel Cardiovascular Center, University of Michigan, Ann Arbor, MI 48109, USA
[b] Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA
[c] Department of Internal Medicine, University of Texas, Houston, TX 77030, USA

ARTICLE INFO

ABSTRACT

Individuals with bicuspid aortic valves (BAV) are at a higher risk of developing thoracic aortic aneurysms (TAA) than patients with trileaflet aortic valves (TAV). The aneurysms associated with BAV most commonly involve the ascending aorta and spare the descending aorta. Smooth muscle cells (SMCs) in the ascending and descending aorta arise from neural crest (NC) and paraxial mesoderm (PM), respectively. We hypothesized defective differentiation of the neural crest stem cells (NCSCs)-derived SMCs but not paraxial mesoderm cells (PMCs)-derived SMCs contributes to the aortopathy associated with BAV. When induced pluripotent stem cells (iPSCs) from BAV/TAA patients were differentiated into NCSC-derived SMCs, these cells demonstrated significantly decreased expression of marker of SMC differentiation (*MYH11*) and impaired contraction compared to normal control. In contrast, the PMC-derived SMCs were similar to control cells in these aspects. The NCSC-SMCs from the BAV/TAA also showed decreased TGF-β signaling based on phosphorylation of SMAD2, and increased mTOR signaling. Inhibition of mTOR pathway using rapamycin rescued the aberrant differentiation. Our data demonstrates that decreased differentiation and contraction of patient's NCSC-derived SMCs may contribute to that aortopathy associated with BAV.

1. Introduction

Bicuspid aortic valves (BAV) are the most common congenital cardiovascular malformation affecting 0.9–2% of the general population (Michelena 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 (Michelena 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) (Grewal et 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*, *GATA5* 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

* Corresponding author.
  E-mail address: boya@med.umich.edu (B. Yang).

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. iPS 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 electrotransfected 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. iPSCs were passaged every 4 to 6 days with Versene (Life Technologies Corp.). IPSCs at passages 25–35 were used for the experiments.

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 2×10^4 cells/cm^2 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 (Life technologies Corp.), Noggin (R&D systems Corp.) at 200 ng/ml and SB431542 (Stemgent Inc.) at 10 μM. NCSC differentiation medium was refreshed daily for 10 days.

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, FGF2 (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 4×10^4 cells/cm^2 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 4×10^4 cells/cm^2 with medium containing DMEM/F12, 15%KSR (Knockout Serum Replacement, Life technologies Corp.), Pen/Strep (Life technologies Corp.), TGF-β1 2 ng/ml (R&D systems 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-TCF15 (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 manufacture'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% CO_2 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 SMC 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. And 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, S1A). 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, S1C), expressed pluripotent markers, including OCT4, NANOG, SOX2, SSEA4, TRA-1-60 and TRA-1-81 (Figs. 1d, S1B), and formed teratomas in NOD/SCID mice (Figs. 1e, S1D). 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, S1E). The NCSCs from BAV/TAA and control iPSCs expressed the NCSC marker genes SOX9, PAX3 and SLUG at similar levels (Figs. 1g, S1F–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. 1h). >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. 1i).

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. S2A). 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. 2a, 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. S2C, 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).
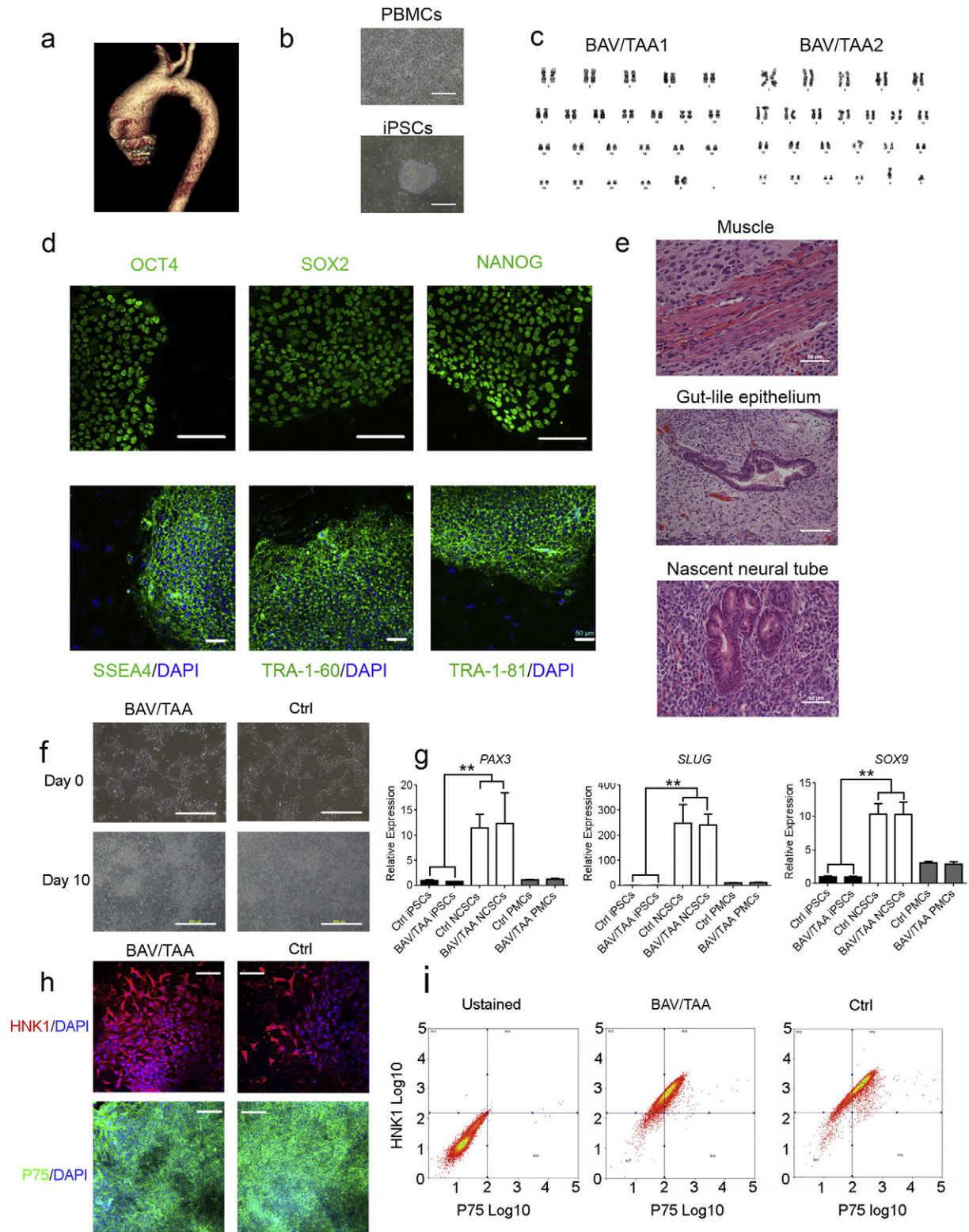
Fig. 1. iPSCs from BAV/TAA patient are pluripotent and can differentiate to NCSCs. (a) 3-D reconstruction of a Computed Tomography (CT) scan of the aorta in a BAV/TAA patient. (b) Morphology of PBMCs and iPSCs from BAV/TAA. (c) Karyotyping of iPSCs from BAV/TAA. (d) Immunostaining of OCT4, SOX2, NANOG, 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.
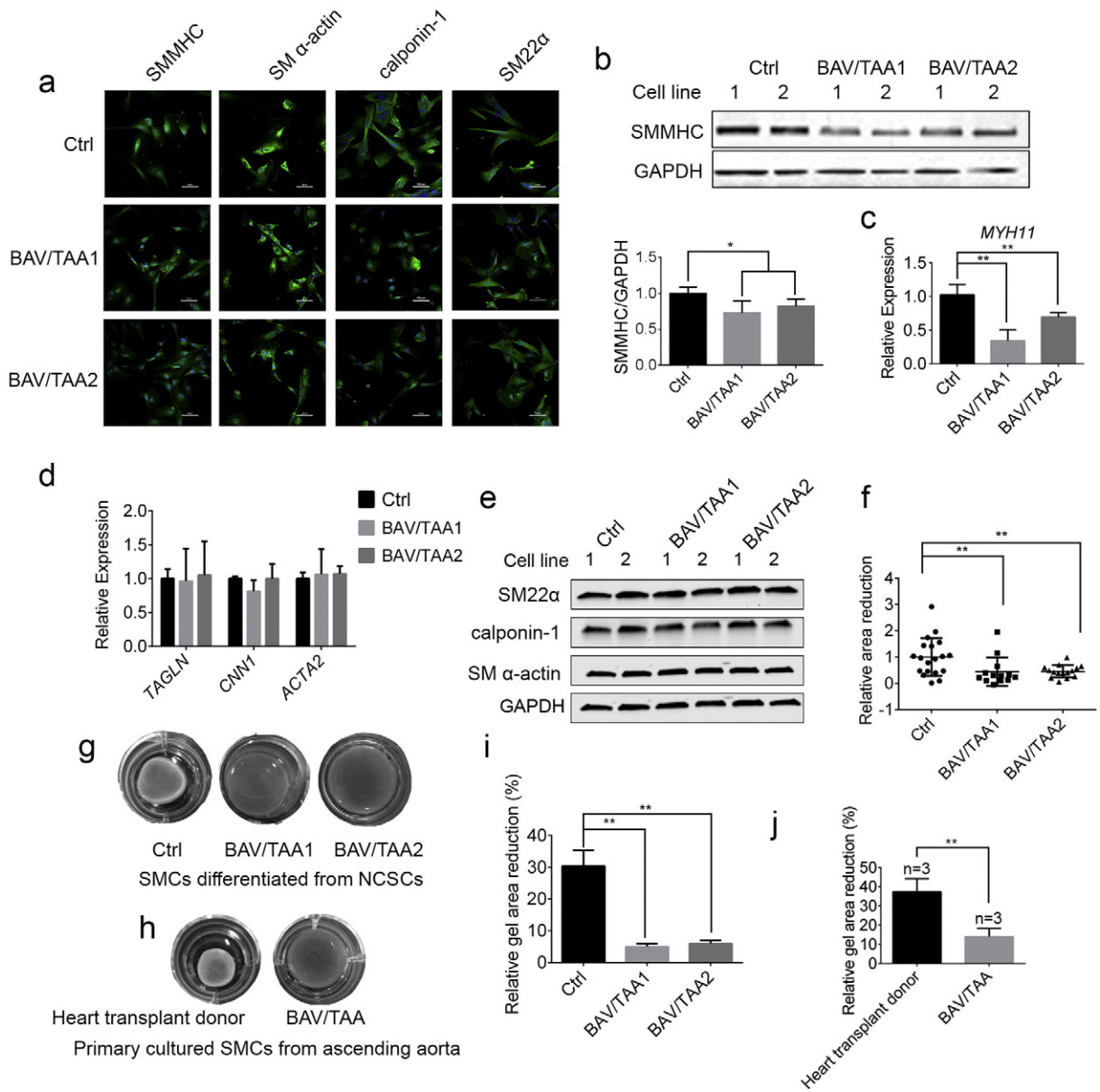
Fig. 2. BAV/TAA SMCs derived from NCSCs have decreased expression of *MYH11* and impaired contractile activity. (a) Immunofluorescence staining cells for SMMHC, SM α-actin, calponin-1 and SM22α. The scale bars represent 100 μm. (b) Western blot analysis of SMMHC in SMCs derived from BAV/TAA and control NCSCs. Lower panel: quantification of western blot data. (c) Relative expression of *MYH11* in SMCs by qPCR. (d) Relative gene expression of the indicated SMC makers. (e) Western blot of SM α-actin, SM22α, calponin-1 and GAPDH. (f) Quantification of reduction of cell surface areas after treatment with carbachol. (g–h) Representative collagen gel assay. (i–j) Quantification of gel area reduction in the collagen gel assay in (G) and (H), respectively. n = 3 in each group. Ctrl: control. Data is represented as mean ± SEM. Results were normalized to control. In (c), (d), (f), and (i), each group contains two cell lines. Experiments were repeated three times.*: P < 0.05. **: P < 0.01. See also Fig. S2.

The SMCs from BAV/TAA and control PMCs had upregulated expression level of SMCs marker genes *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-SMCs in both BAV/TAA and control expressed more *MYH11* than their counterparts of NCSC-SMCs (Fig. S3F). Western blot assay also confirmed that SMMHC, SM α-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 S4, S5). These results indicated that the SMCs from BAV PMCs were as 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 were similar in BAV/TAA NCSC derived SMCs (Fig. S4B–D). TGF-β receptor 1 and 2 mRNA was found to be decreased in BAV/TAA (Fig. S4E, F). Relatedly, phosphorylated SMAD2 (pSMAD2) significantly decreased in BAV/TAA
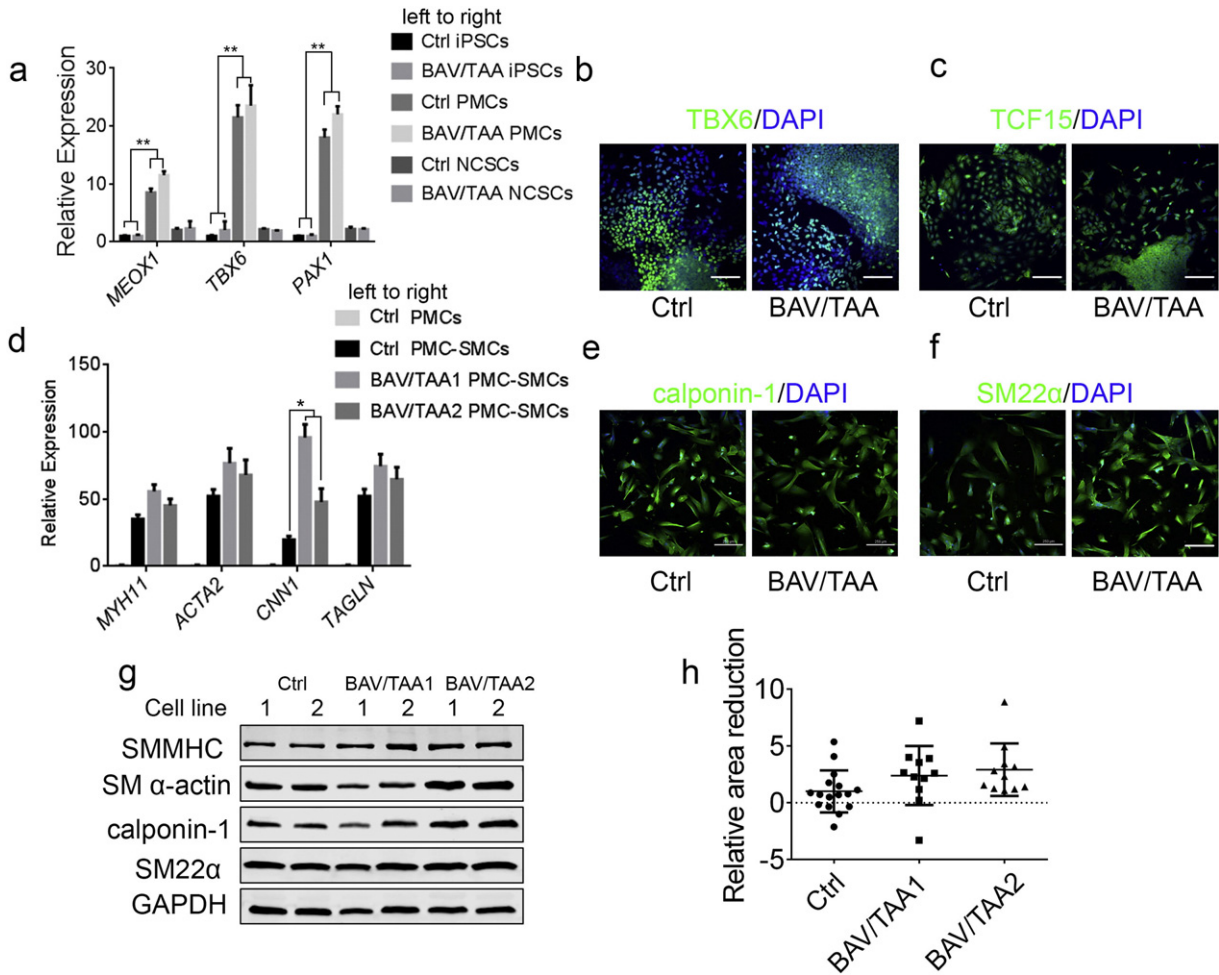
Fig. 3. Contractile function is comparable to control in SMCs derived from BAV/TAA PMCs. (a) Relative expression level of PMC marker genes *MEOX1*, *TBX6* and *PAX1* evaluated using qPCR. (b and c) Immunofluorescence staining of TBX6 and TCF15 of PMCs. The scale bars represent 50 μm. (d) Relative expression level of SMCs marker genes evaluated using qPCR. (e and f) Immunofluorescence staining of SMC markers. (g) Immunoblotting of SMMHC, SM α-actin, SM22α, calponin-1 and GAPDH. (h) Quantification of reduction of cell surface area after stimulation by carbachol. Ctrl: control. PMCs: paraxial mesoderm cells. Data is represented as mean ± SEM. Results were normalized to control. In (a), (d), and (h), each group contains two cell lines. Experiments were repeated three times. **: $P < 0.01$. *: $P < 0.05$. See also Fig. S3.

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, k, l). Treatment of 20 nM rapamycin for two days restored the expression of *MYH11* and SMMHC in BAV NCSC-SMCs (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-β 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. A specific altered NCSC behavior was considered to cause a specific type 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
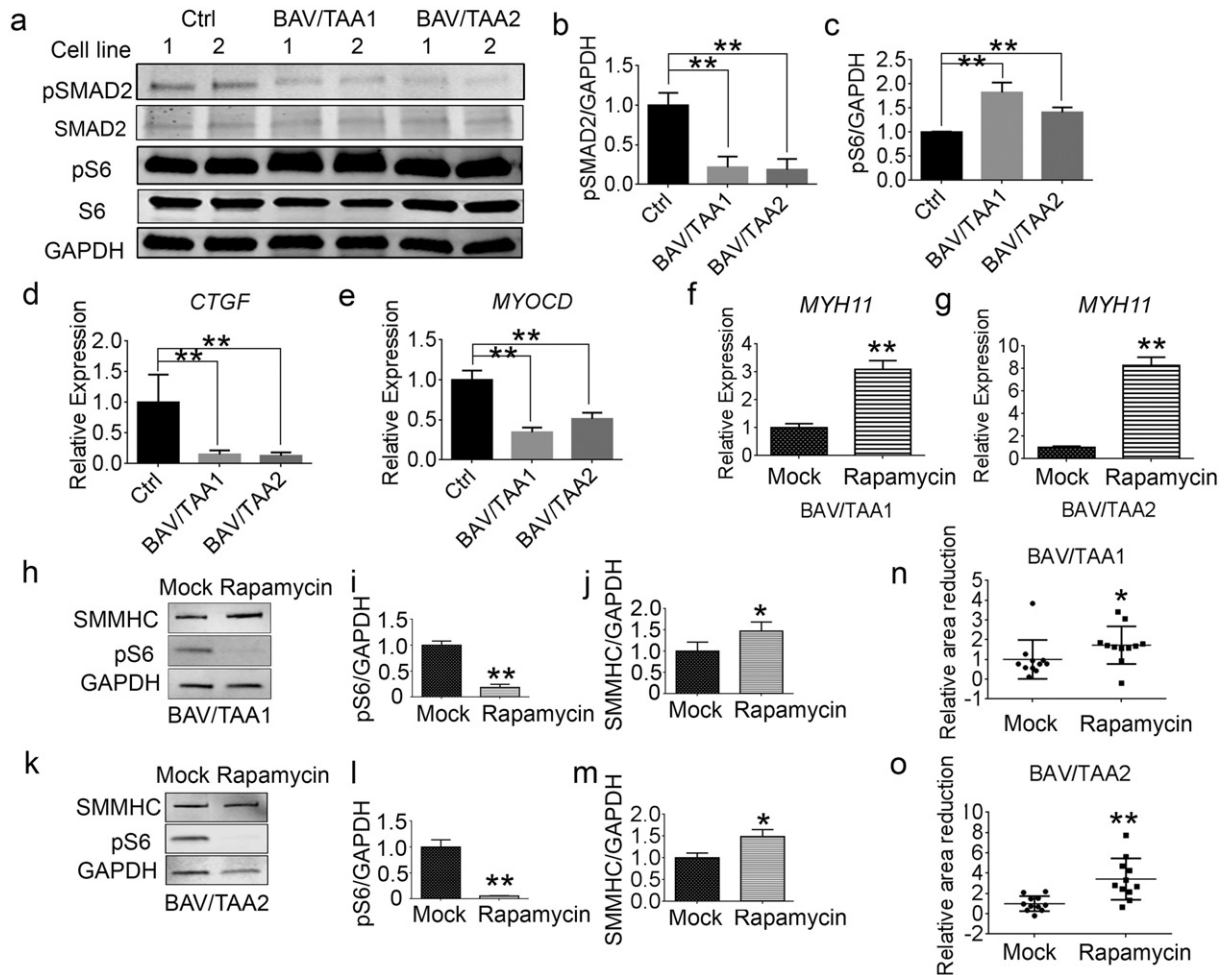
Fig. 4. SMCs derived from BAV/TAA NCSCs were impaired TGF-β signal pathway and had hyperactive mTOR pathway; inhibition of mTOR pathway by rapamycin could rescue impairment of contractile function of BAV/TAA SMCs. (a) Immunoblotting of pSMAD2, pS6, S6 and GAPDH. (b and c) Quantification of pS6 and pSMAD2 of (A) by measuring signal intensity. (d and e) Relative expression of *CTGF* and *MYOCD* in qPCR. (f and g) Relative expression of *MYH11* using qPCR in SMCs derived from BAV/TAA NCSCs treated with 20 nM rapamycin or mock treated with DMSO for 2 days. (h and k) Immunoblotting of SMMHC, pS6 and GAPDH. Mock means treated with DMSO, the solvent of rapamycin. (i, j, l, m) Quantification of pS6 and SMMHC of (h) and (k) respectively by measuring signal intensity. (n and o) Quantification of reduction of cell surface area after stimulation by carbachol. Ctrl: control. Data is represented as mean ± SEM. Results were normalized to control. In (b–o), each group contains two cell lines. Experiments were repeated three times. * indicates P < 0.05. ** indicates P < 0.01. See also Supplementary Fig. S4.

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-Dusenbery 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 downregulated. 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 *Tgfbr2* knockout mouse model, and rapamycin administration prevented aortic dilation (Li et al., 2014). In a *Tsc2* knockout mouse model, elevated mTOR signaling decreased expression of SMCs 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/TAA 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/TAA 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/TAA patients and one control with two different iPS cell lines for each subjects. However, these studies provide unique insights of aortopathy in BAV/TAA 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 identified in NCSCs-derived SMCs may contribute to the aortopathy and development of ascending aortic aneurysms. These studies demonstrate the utility of iPSCs in defining the molecular basis of a disease process without knowing the underlying genetic defect responsible for observed abnormality. Using this system, we could demonstrate a clear contractile defect in the NCSC-derived SMCs that was not present in the PM-derived SMCs. Finally, our results add to the accumulating data that decreased SMC contractile function predisposes to thoracic aortic disease.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.06.045.

Conflict of Interest Statement

None.

Funding Sources

Author Contributions

J.J. conceived the research plan and performed experimental design, did majority of experiments and data analysis. W.X. and L.W. helped with a portion of experiments and data analyses. P.Q. performed data analysis and experimental design. H.H. performed data analyses. J.Y. and L.S. did the Giemsa banding experiments. Y.C. and D.M. participated in research plan, experimental design, data analysis and manuscript writing. B.Y. conceived the research plan and performed experimental design, data analysis and manuscript writing.

Acknowledgements

References

Bonachea, E.M., Chang, S.W., Zender, G., LaHaye, S., Fitzgerald-Butt, S., McBride, K.L., Garg, V., 2014. Rare GATA5 sequence variants identified in individuals with bicuspid aortic valve. Pediatr. Res. 76, 211–216.

Cao, J., Gong, L., Guo, D.C., Mietzsch, U., Kuang, S.Q., Kwartler, C.S., Safi, H., Estrera, A., Gambello, M.J., Milewicz, D.M., 2010. Thoracic aortic disease in tuberous sclerosis complex: molecular pathogenesis and potential therapies in Tsc2 +/− mice. Hum. Mol. Genet. 19, 1908–1920.

Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., Studer, L., 2009. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 27, 275–280.

Cheung, C., Bernardo, A.S., Trotter, M.W., Pedersen, R.A., Sinha, S., 2012. Generation of human vascular smooth muscle subtypes provides insight into embryological origin-dependent disease susceptibility. Nat. Biotechnol. 30, 165–173.

Cheung, C., Bernardo, A.S., Pedersen, R.A., Sinha, S., 2014. Directed differentiation of embryonic origin-specific vascular smooth muscle subtypes from human pluripotent stem cells. Nat. Protoc. 9, 929–938.

Cripe, L., Andelfinger, G., Martin, L.J., Shooner, K., Benson, D.W., 2004. Bicuspid aortic valve is heritable. J. Am. Coll. Cardiol. 44, 138–143.

Davis-Dusenbery, B.N., Chan, M.C., Reno, K.E., Weisman, A.S., Layne, M.D., Lagna, G., Hata, A., 2011. Down-regulation of Kruppel-like factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-beta and bone morphogenetic protein 4. J. Biol. Chem. 286, 28097–28110.

Fazel, S.S., Mallidi, H.R., Lee, R.S., Sheehan, M.P., Liang, D., Fleischman, D., Herfkens, R., Mitchell, R.S., Miller, D.C., 2008. The aortopathy of bicuspid aortic valve disease has distinctive patterns and usually involves the transverse aortic arch. J. Thorac. Cardiovasc. Surg. 135 (901–907, 907 e901–902).

Fedak, P.W., David, T.E., Borger, M., Verma, S., Butany, J., Weisel, R.D., 2005. Bicuspid aortic valve disease: recent insights in pathophysiology and treatment. Expert. Rev. Cardiovasc. Ther. 3, 295–308.

Fernandez, B., Duran, A.C., Fernandez-Gallego, T., Fernandez, M.C., Such, M., Arque, J.M., Sans-Coma, V., 2009. Bicuspid aortic valves with different spatial orientations of the leaflets are distinct etiological entities. J. Am. Coll. Cardiol. 54, 2312–2318.

Forte, A., Della Corte, A., Grossi, M., Bancone, C., Provenzano, P., Finicelli, M., De Feo, M., De Santo, L.S., Nappi, G., Cotrufo, M., et al., 2013. Early cell changes and TGFbeta pathway alterations in the aortopathy associated with bicuspid aortic valve stenosis. Clin. Sci. 124, 97–108.

Garg, V., Muth, A.N., Ransom, J.F., Schluterman, M.K., Barnes, R., King, I.N., Grossfeld, P.D., Srivastava, D., 2005. Mutations in NOTCH1 cause aortic valve disease. Nature 437, 270–274.

Gillis, E., Van Laer, L., Loeys, B.L., 2013. Genetics of thoracic aortic aneurysm: at the crossroad of transforming growth factor-beta signaling and vascular smooth muscle cell contractility. Circ. Res. 113, 327–340.

Grewal, N., Gittenberger-de Groot, A.C., DeRuiter, M.C., Klautz, R.J., Poelmann, R.E., Duim, S., Lindeman, J.H., Koenraadt, W.M., Jongbloed, M.R., Mohamed, S.A., et al., 2014a. Bicuspid aortic valve: phosphorylation of c-Kit and downstream targets are prognostic for future aortopathy. Eur. J. Cardiothorac. Surg. 46, 831–839.

Grewal, N., Gittenberger-de Groot, A.C., Poelmann, R.E., Klautz, R.J., Lindeman, J.H., Goumans, M.J., Palmen, M., Mohamed, S.A., Sievers, H.H., Bogers, A.J., et al., 2014b. Ascending aorta dilation in association with bicuspid aortic valve: a maturation defect of the aortic wall. J. Thorac. Cardiovasc. Surg. 148, 1583–1590.

Horne, B.D., Camp, N.J., Muhlestein, J.B., Cannon-Albright, L.A., 2004. Evidence for a heritable component in death resulting from aortic and mitral valve diseases. Circulation 110, 3143–3148.

Humphrey, J.D., Milewicz, D.M., Tellides, G., Schwartz, M.A., 2014. Cell biology. Dysfunctional mechanosensing in aneurysms. Science 344, 477–479.

Humphrey, J.D., Schwartz, M.A., Tellides, G., Milewicz, D.M., 2015. Role of mechanotransduction in vascular biology: focus on thoracic aortic aneurysms and dissections. Circ. Res. 116, 1448–1461.

Itagaki, S., Chikwe, J.P., Chiang, Y.P., Egorova, N.N., Adams, D.H., 2015. Long-term risk for aortic complications after aortic valve replacement in patients with bicuspid aortic valve versus Marfan syndrome. J. Am. Coll. Cardiol. 65, 2363–2369.

Jiao, J., Dang, Y., Yang, Y., Gao, R., Zhang, Y., Kou, Z., Sun, X.F., Gao, S., 2013. Promoting reprogramming by FGF2 reveals that the extracellular matrix is a barrier for reprogramming fibroblasts to pluripotency. Stem Cells 31, 729–740.

Keane, M.G., Wiegers, S.E., Plappert, T., Pochettino, A., Bavaria, J.E., Sutton, M.G., 2000. Bicuspid aortic valves are associated with aortic dilatation out of proportion to coexistent valvular lesions. Circulation 102, III35–III39.

Kuang, S.Q., Guo, D.C., Prakash, S.K., McDonald, M.L., Johnson, R.J., Wang, M., Regalado, E.S., Russell, L., Cao, J.M., Kwartler, C., et al., 2011. Recurrent chromosome 16p13.1 duplications are a risk factor for aortic dissections. PLoS Genet. 7, e1002118.

Kuang, S.Q., Kwartler, C.S., Byanova, K.L., Pham, J., Gong, L., Prakash, S.K., Huang, J., Kamm, K.E., Stull, J.T., Sweeney, H.L., et al., 2012. Rare, nonsynonymous variant in the smooth muscle-specific isoform of myosin heavy chain, MYH11, R247C, alters force generation in the aorta and phenotype of smooth muscle cells. Circ. Res. 110, 1411–1422.

LeMaire, S.A., McDonald, M.L., Guo, D.C., Russell, L., Miller 3rd, C.C., Johnson, R.J., Bekheirnia, M.R., Franco, L.M., Nguyen, M., Pyeritz, R.E., et al., 2011. Genome-wide association study identifies a susceptibility locus for thoracic aortic aneurysms and aortic dissections spanning FBN1 at 15q21.1. Nat. Genet. 43, 996–1000.

Li, W., Li, Q., Jiao, Y., Qin, L., Ali, R., Zhou, J., Ferruzzi, J., Kim, R.W., Geirsson, A., Dietz, H.C., et al., 2014. Tgfbr2 disruption in postnatal smooth muscle impairs aortic wall homeostasis. J. Clin. Invest. 124, 755–767.

Majesky, M.W., 2007. Developmental basis of vascular smooth muscle diversity. Arterioscler. Thromb. Vasc. Biol. 27, 1248–1258.

Mao, X., Debenedittis, P., Sun, Y., Chen, J., Yuan, K., Jiao, J., Chen, Y., 2012. Vascular smooth muscle cell Smad4 gene is important for mouse vascular development. Arterioscler. Thromb. Vasc. Biol. 32, 2171–2177.

Martin, K.A., Merenick, B.L., Ding, M., Fetalvero, K.M., Rzucidlo, E.M., Kozul, C.D., Brown, D.J., Chiu, H.Y., Shyu, M., Drapeau, B.L., et al., 2007. Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling. J. Biol. Chem. 282, 36112–36120.

Michelena, H.I., Khanna, A.D., Mahoney, D., Margaryan, E., Topilsky, Y., Suri, R.M., Eidem, B., Edwards, W.D., Sundt 3rd, T.M., Enriquez-Sarano, M., 2011. Incidence of aortic complications in patients with bicuspid aortic valves. JAMA 306, 1104–1112.

Michelena, H.I., Prakash, S.K., Della Corte, A., Bissell, M.M., Anavekar, N., Mathieu, P., Bosse, Y., Limongelli, G., Bossone, E., Benson, D.W., et al., 2014. Bicuspid aortic valve: identifying knowledge gaps and rising to the challenge from the International Bicuspid Aortic Valve Consortium (BAVCon). Circulation 129, 2691–2704.

Nkomo, V.T., Enriquez-Sarano, M., Ammash, N.M., Melton 3rd, L.J., Bailey, K.R., Desjardins, V., Horn, R.A., Tajik, A.J., 2003. Bicuspid aortic valve associated with aortic dilatation: a community-based study. Arterioscler. Thromb. Vasc. Biol. 23, 351–356.

Okita, K., Ichisaka, T., Yamanaka, S., 2007. Generation of germline-competent induced pluripotent stem cells. Nature 448, 313–317.

Owens, G.K., Kumar, M.S., Wamhoff, B.R., 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol. Rev. 84, 767–801.

Padang, R., Bannon, P.G., Jeremy, R., Richmond, D.R., Semsarian, C., Vallely, M., Wilson, M., Yan, T.D., 2013. The genetic and molecular basis of bicuspid aortic valve associated thoracic aortopathy: a link to phenotype heterogeneity. Annals of Cardiothoracic Surgery 2, 83–91.

Paloschi, V., Gadin, J.R., Khan, S., Bjorck, H.M., Du, L., Maleki, S., Roy, J., Lindeman, J.H., Mohamed, S.A., Tsuda, T., et al., 2015. Aneurysm development in patients with a bicuspid aortic valve is not associated with transforming growth factor-beta activation. Arterioscler. Thromb. Vasc. Biol. 35, 973–980.

Pepe, G., Nistri, S., Giusti, B., Sticchi, E., Attanasio, M., Porciani, C., Abbate, R., Bonow, R.O., Yacoub, M., Gensini, G.F., 2014. Identification of fibrillin 1 gene mutations in patients with bicuspid aortic valve (BAV) without Marfan syndrome. BMC Med. Genet. 15, 23.

Prakash, S.K., Bosse, Y., Muehlschlegel, J.D., Michelena, H.I., Limongelli, G., Della Corte, A., Pluchinotta, F.R., Russo, M.G., Evangelista, A., Benson, D.W., et al., 2014. A roadmap to investigate the genetic basis of bicuspid aortic valve and its complications: insights from the International BAVCon (Bicuspid Aortic Valve Consortium). J. Am. Coll. Cardiol. 64, 832–839.

Sievers, H.H., Schmidtke, C., 2007. A classification system for the bicuspid aortic valve from 304 surgical specimens. J. Thorac. Cardiovasc. Surg. 133, 1226–1233.

Su, R.J., Baylink, D.J., Neises, A., Kiroyan, J.B., Meng, X., Payne, K.J., Tschudy-Seney, B., Duan, Y., Appleby, N., Kearns-Jonker, M., et al., 2013. Efficient generation of integration-free IPS cells from human adult peripheral blood using BCL-XL together with Yamanaka factors. PLoS One 8, e64496.

Wang, Z., Wang, D.Z., Pipes, G.C., Olson, E.N., 2003. Myocardin is a master regulator of smooth muscle gene expression. Proc. Natl. Acad. Sci. U. S. A. 100, 7129–7134.

Xie, W.B., Li, Z., Shi, N., Guo, X., Tang, J., Ju, W., Han, J., Liu, T., Bottinger, E.P., Chai, Y., et al., 2013. Smad2 and myocardin-related transcription factor B cooperatively regulate vascular smooth muscle differentiation from neural crest cells. Circ. Res. 113, e76–e86.

Zhu, L., Vranckx, R., Khau Van Kien, P., Lalande, A., Boisset, N., Mathieu, F., Wegman, M., Glancy, L., Gasc, J.M., Brunotte, F., et al., 2006. Mutations in myosin heavy chain 11 cause a syndrome associating thoracic aortic aneurysm/aortic dissection and patent ductus arteriosus. Nat. Genet. 38, 343–349.