INTRODUCTION

Intracranial aneurysms (IA), also known as cerebral aneurysms, are dilatations in the brain arteries. Rupture of an intracranial aneurysm leads to a subarachnoid hemorrhage (SAH), which is fatal in about 50% of the cases. Despite the high prevalence of intracranial aneurysm in the general population and the high morbidity and mortality rate of their rupture, the pathogenic mechanisms underlying intracranial aneurysm are still poorly understood (1, 2). And endovascular or intracranial surgery (coiling or clipping) is normally performed to prevent their rupture. To date, no drugs to address intracranial aneurysm have been approved (3, 4). For the further improvement of current therapies, better understanding of the pathophysiology of intracranial aneurysms is needed.

A significant proportion of patients with intracranial aneurysm are young. And there have been anecdotal reports concerning the relationship between intracranial aneurysm and other vasculopathies, such as autosomal-dominant polycystic kidney disease (ADPKD) (5, 6). Patients with autosomal-dominant polycystic kidney disease have a higher incidence of intracranial aneurysm than the general population. These intracranial aneurysms also rupture at an earlier age in patients with ADPKD and are associated with high morbidity and mortality (7, 8). ADPKD is a genetic disease caused by the mutations of components of primary cilia and intracranial aneurysm is one of the major complications of ADPKD (9–12). The primary cilia are polarized structures that are found almost ubiquitously in vertebrate cells, where they serve a diverse set of signaling functions. A multitude of human diseases have now been attributed to defects in ciliary proteins, including cardiovascular diseases (13–18).
MATERIALS AND METHODS

Chemicals

Chemicals and drugs used in this study were purchased from Sigma. Stock concentrations of reagents were made by dilution in solvents (DMSO or H2O) according to the manufacturer’s protocols and stored at −20 °C. The final concentrations of reagents were: PHA 680623 60 mg/kg, Alisthet 60 μg/kg, VX86030 μg/kg, Baraseth 30 μg/kg, Tubastat A 1 mg/kg, Tubacin 20 μg/kg, Nilotubacin 120 μg/kg, Trehalose 600 μg/kg, BECN1 240 μg/kg, Ramu- mycin 1 μg/kg, Dextrane 500 μg/kg, Forskolin 1 g/kg, 8-BromocAMP (8-Bromoadenosine-3'-5'-cyclic monophosphate) 100 mg/kg and Cilastatin 1000 mg/kg.

Mouse model of intracranial aneurysm

The angiotensin II–elastase mouse model of intracranial aneurysm was established as previously described (19, 25). Briefly, mice were inbred on a C57Bl/6 background, and Cdh5(PAC)-CreERT2 transgenic mice. To activate the Cre system, tamoxifen (25 mg/kg) was administered intraperitoneally to mice four times with intervals of 5 days. Angiotensin II (1000 mg/kg, Sigma, St. Louis, Mo) for 20 days (full the end of the experiments), and injected with 35 μl elastase (Sigma) into the cerebrospinal fluid at the right basal cistern. Systolic arterial pressure was monitored by tail cuff, and neurological function was assessed daily. Mice were considered symptomatic and euthanized if ≥2 of the following deficits were observed: decreased activity and hunched posture, leaning or circling to one side, or decreased food and water intake leading to weight loss of >20% of baseline. Mice without neurological signs were euthanized 20 days post IA induction, and the presence of IA and SAH was assessed. The brain samples were perfused with PBS followed by a gelatin (Sigma) containing bromophenol blue dye to visualize cerebral arteries as well as to assess for aneurysm formation and SAH. Two blinded observers assessed the formation of intracranial aneurysms by examining sections of Circle of Willis and its major branches under a dissecting microscope (>10). Intracranial aneurysms were operationally defined as a localized outward bulging of the vascular wall in the Circle of Willis or in its major primary branches, as previously described (19, 20). After inspecting the Circle of Willis, the arteries were frozen in optimum cutting temperature for immunohistochimical staining.

For in situ hybridization in endothelial cells, PKD1+/KO, PKD2+/KO, or IFT88−/− mice, respectively, were crossed with Cdh5(PAC)-CreERT2 transgenic mice. To activate the Cre system, tamoxifen (25 mg/kg) was administrated ipronteritoneally to mice four times with intervals of 5 days. Experiments were conducted in accordance with the guidelines approved by the Animal Care and Use Committee of the Second Xiangya Hospital of Central South University (Document No. XYH20120054). All animals received routine care, including feeding standard diets, providing fresh water, and changing cages and bedding. Animal health monitoring was performed on a daily basis by animal care staff and a veterinarian. Animals showing any sign of illness, pain and weight loss more than 20% of baseline were sacrificed with ketamine (100 mg/kg) for 10 min, and washed twice in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS; the cell pellet was resuspended in 4 mL of DMEM with 10% FBS. The endothelial cells were then purified with anti-CD31 (be rank). The purified cells were cultured in 25 cm2 flasks in DMEM with 10% FBS, supplemented with 1% penicillin and 1% streptomycin. The media was changed every other day until about 80% confluence. Cells were passaged 1:3 for further use. The supernatant was collected and stored at −80 °C for further analysis.

Production and in vivo delivery of adeno-associated virus

Vector construction, production, and in vivo delivery of adeno-associated virus (AAV) were performed based on the AAV helper-free system (Agilent, San Jose, CA). The recombinant adenovaliral vector, pAAV-ITR-SmKSA, pAAV-ITR-DsRed, pAAV-ITR-Cep7, pAAV-ITR-3G, and pAAV-ITR-3G were constructed by cloning the cDNA encoding region or shRNA sequences into pAAV-ITR. The vector pAAV-ITR-GFP encoding green fluorescence protein was used as a negative control. Recombinant AAVs were produced by HEK293 cells (ATCC) transfected with pAAV-ITR vectors together with pAAV-RC and pHelper plasmids, and then purified by discontinuous iodixanol gradient centrifugation. Purified recombinant AAVs were concentrated and desalted by centrifugation through Amicon Ultra 30K filters (Millipore, Billerica, Mass). For in vivo delivery, recombinant AAVs equivalent to 1.0 × 10^12 viral genome copy were delivered though mouse tail vein.

Endothelial cell isolation and culture

Arteries were removed aseptically, rinsed in Hank’s balanced salt solution (Gibco, Worcester, Mass), minced into 1 × 1 mm squares, and digested in 20 mL of collagenase A (1 mg/mL, Boehringer, Mannheim) at 37 °C for 45 min with occasional agitation. The cellular digest was filtered through sterile 80-μm nylon mesh, centrifuged at 400 g for 10 min, and washed twice in Dulbecco’s Modified

Tissue preparation

Animals were deeply anesthetized and perfused transcardially with physiological saline followed by 4% paraformaldehyde at a speed of about 0.7 mL per hour using a peristaltic pump (RP-2000; EYELA). The intracranial arteries were isolated under the microscope and embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Fine Technical Co, Tokyo, Japan). Serial 8 μm sections were cut and mounted on silane-coated slides. For immunofluorescence staining, tissue samples were washed in PBS, blocked with 5% donkey serum in PBST (0.1% Triton X in PBS), then incubated with primary antibodies followed by the appropriate fluorescent-labeled secondary antibodies. The following primary antibodies were used: anti-VE-cadherin (BD Biosciences, Franklin Lakes, NJ; 1:100), anti-Claudin 1 (Cell Signaling, Danvers, Mass; 1:200), anti-PKD1 (Sigma, 1:200), anti-PKD2 (Sigma, 1:200), anti-acetylated α-tubulin (Sigma, 1:1,000). Secondary antibodies for immunofluorescence staining are as follows: Alexa488-, Alexa594-conjugated IgG antibodies (Invitrogen, Waltham, Mass; 1:1,000). All specimens were counterstained with DAPI (Invitrogen, 1:1,000) and finally mounted in Vectashield (Vector Laboratories, Burlingame, Calif). Immunofluorescence images were obtained using a laser scanning microscope 780 confocal microscope (Zeiss).

Immunofluorescence

Cells were cultured on poly-L-lysine-coated slides in DMEM/10% FBS (Invitrogen) to 100% confluence. Confluent cells were fixed in 4% formaldehyde, permeabilized in 0.4% Triton X-100, blocked in 1% bovine serum albumin and stained with primary antibody against acetylated α-tubulin (Sigma, 1:1,000), CD31 (1:200, Abcam, Cambridge, UK). Nuclei were labeled with DAPI (Invitrogen). Immunofluorescence samples were examined using a Nikon TE2000-E2 inverted microscope system (Nikon Instruments Inc, Tokyo, Japan) with a PlanFluo 40 × NA 1.3 objective at 1 × 1 binning. Cells were measured using Imagej (rsb.info.nih.gov/ij) software. For citra quantification, four fields in each coverslip were imaged and the number of cilia and number of endothelial cells in each field were quantified. For each sample, 10 coverslips were analyzed. The percent of ciliated cells was then averaged across the samples (n=8).

Human tissue samples

The collection and studies of human aneurysm specimens were performed under approval of our Institutional Review Board (IRB) of the Second Xiangya Hospital of Central South University (Document No. XYYH201203154) from March 2013 to December 2015. Patients signed informed IRB research consent before undergoing aneurysm surgery. Aneurysm specimens (n=9) were harvested at the time of craniotomy and aneurysm clipping surgery. Aneurysm specimens were collected from the aneurysm dome. The tissues were immediately transferred into DMEM (Invitrogen) medium on ice for cell isolation. One hundred IA patients and 100 healthy controls were used for gene mutation analysis (Supplementary Table 1, http://links.lww.com/SHK/A624). Healthy controls were recruited from the health check centre of the Second Xiangya Hospital of Central South University, who were confirmed by computed tomography and magnetic resonance imaging. All patients and healthy controls have given the informed consent. Genomic DNA was extracted from peripheral blood mononuclear cell with QiAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Exons were amplified with high fidelity DNA polymerase (New England Biolabs) and sequenced with ABI 3730. Sequence deletion, truncation and missense mutations were characterized as gene mutation in the present study.

Statistical analysis

Values are reported as the mean ± SD unless otherwise indicated. For continuous data, statistical significance was determined with the Mann–Whitney U test between two groups and the Kruskal–Wallis test followed by the Bonferroni correction for multiple-group comparison. Statistical analysis was performed with Predictive Analytics Software Statistics 18 (SPSS). Differences were considered statistically significant at p < 0.05.
RESULTS

IA region had fewer cells with primary cilia

Primary cilia projected from the apical surface of the artery into the lumen of the vessel (21). The primary cilia number was significantly decreased in the IA region and peri-IA region in the mice model of IA when compared with the healthy control mice (Fig. 1A). Furthermore, the IA region had much fewer cells with primary cilia than the peri-IA region (Fig. 1A). The endothelial cells were then isolated from the IA and peri-IA region from the mice with IA and intracranial arteries from healthy control mice. Primary cilia staining and counting also showed that IA region had fewer cells with primary cilia (Fig. 1B). These data indicate that IA regions have fewer primary cilia.

Primary cilia deficiency promoted IA development

To investigate whether genetic loss of primary cilia would increase the susceptibility of mice to IA development, the mice with endothelial cell specific knockout of PKD1 (Cdh5(PAC)-CreER\textsuperscript{T2};PKD1\textsuperscript{fl/fl}), PKD2 (Cdh5(PAC)-CreER\textsuperscript{T2};PKD2\textsuperscript{fl/fl}) and IFT88 (Cdh5(PAC)-CreER\textsuperscript{T2};IFT88\textsuperscript{fl/fl}), which are important components of primary cilia, were established by tamoxifen treatment (21, 22). Four weeks after the gene excision from endothelial cells, the IA was induced by angiotensin II and elastase treatment. All PKD1, PKD2, and IFT88 knockout mice had higher incidence of IA and rupture rate at normal IA induction condition (Fig. 2A), reduced dose of elastase (Fig. 2B), reduced dose of angiotensin II (Fig. 2C), or reduced dose of both elastase and angiotensin II (Fig. 2D). Furthermore, angiotensin II alone could induce IA in PKD1, PKD2, and IFT88 knockout mice but not in the wild type mice (Fig. 2E). These data indicate that primary cilia deficiency promotes IA development.

Manipulating primary cilia dynamics affects IA development

We then examined the possibility of controlling the IA development through manipulating the primary cilia dynamics. There three pathways have been demonstrated to modulate primary cilia dynamics, HEF1- Aurora Kinase A pathway, Cep97-CP110 pathway and VHL (von Hippel-Lindau) -GSK3\(\beta\) (glycogen synthase kinase 3 beta) pathway (23–25). HEF1, a prometastatic scaffolding protein, and the oncogenic AurA localize at the basal body of the primary cilia. These two proteins interact with each other and cause phosphorylation and activation of HDAC6, a tubulin deactetylase, promoting ciliary disassembly (23). Cep97 and CP110 localize at centrioles, which play a dynamic role in centrosome function and have the capacity to nucleate the assembly of primary cilia. Cep97 recruits CP110 to centrosomes and stimulate centrosome duplication and cytokinesis, resulting in ciliogenesis inhibition (24). VHL and GSK3\(\beta\) are two of key components that maintain the primary cilium structure. Their inactivation leads to loss of cilia (25). Suppressing primary cilia formation through AAV-mediated overexpressing HEF1-AurA and Cep97-CP110 or knocking down VHL-GSK3\(\beta\) and IFT88 via shRNA increased both IA incidence and rupture rate in the mice model with low dose of elastase and angiotensin II (Fig. 3A). On the other hand, stabilizing the primary cilia through AAV-mediated knocking down HEF1-AurA and Cep97-CP110 via shRNA or overexpressing VHL-GSK3\(\beta\) reduced both IA incidence and rupture rate in the mice model with high dose of elastase and angiotensin II (Fig. 3B). Overexpressing IFT88 did not affect the IA incidence and rupture rate. It might be because of the saturation of this protein in the cells.

Next, we further studied the possibility of controlling the IA development through manipulating the primary cilia dynamics by chemicals administration in the mice model of IA. Available chemicals include AurA inhibitors (PHA 680623, Alisertib, VX680, and Barasertib), HDAC6 inhibitors (Tubastatin A, Tubacin, and Niltubacinc) and other chemicals that had been demonstrated to affect the ciliogenesis process, such as autophagy enhancers (Trehalose, BECN1, and Rapamycin), calcium modulators (Gd\textsuperscript{3+}, Dantrolene), cAMP (cyclic adenosine monophosphate) modulators (Forskolin, 8-BromocAMP) and dynein inhibitor Ciliobrevins (26–28). All these chemicals could reduce the IA incidence and rupture rate in the mice model of IA with high dose of elastase and angiotensin II except dynein inhibitor Ciliobrevins (Fig. 2C). Then one chemical from each group was selected and used to further study their synergistic effects. Indeed, combing these five factors (PHA...
Fig. 2. Primary cilia deficiency promotes intracranial aneurysm development in knockout mice. A, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II and 35 mU elastase. n = 12 for each type of mice. * indicates P < 0.05. B, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II and 10 mU elastase. n = 12 for each type of mice. * indicates P < 0.05. C, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 500 ng/kg/min angiotensin-II and 35 mU elastase. n = 12 for each type of mice. * indicates P < 0.05. D, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 500 ng/kg/min angiotensin-II and 10 mU elastase. n = 12 for each type of mice. * indicates P < 0.05. E, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II. n = 12 for each type of mice. * P < 0.05. PKD1 indicates polycystin 1; PKD2, polycystin 2; IFT88, intraflagellar transport 88; KO, knockout; Ang II, Angiotensin II; IA, intracranial aneurysm.
680623, Tubastatin A, Rapamycin, Gd3⁺, and Forskolin) would further decrease the IA incidence and rupture rate significantly in the mice model of IA with high dose of elastase and angiotensin II (Fig. 2D). Thus, the IA development could be modulated through manipulating primary cilia dynamics and the primary cilia stabilizing chemicals could reduce the IA incidence and rupture rate.

**IA development could be suppressed efficiently through chemical combination**

Because there might be some overlap effects among these five factors (PHA 680623, Tubastatin A, Rapamycin, Gd3⁺, and Forskolin, Fig. 3D), we further studied whether eliminating some chemicals from them would remain the efficient suppression of IA development. Eliminating Tubastatin A and Gd3⁺ showed similar efficiency on suppressing IA development when compared with five factors combination in the mice model of IA with high dose of elastase and angiotensin II (Fig. 4A). However, eliminating PHA 680623, Rapamycin, or Forskolin would affect the suppressing efficiency significantly (Fig. 4A). Therefore, the three factors (PHA 680623, Rapamycin, and Forskolin) had similar suppressing efficiency compared with five factors. And further chemical elimination from three factors would affect the suppressing efficiency significantly (data not shown). The three factor treated mice had more cells with primary cilia in the IA region (Fig. 4B). And this was also confirmed through endothelial cell isolation and primary cilia formation in vitro (Fig. 4B). Thus, the three factors could suppress the IA development through stabilizing primary cilia and maintain vascular integrity.

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**Fig. 3.** Manipulating primary cilia dynamics affects intracranial aneurysm development. A, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 500 ng/kg/min angiotensin-II and 10 mU elastase plus gene overexpression or suppression. n = 12 for each type of mice. * indicates P < 0.05 versus empty vector. B, Incidence of IA (left panel) and rupture rate (right panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II and 35 mU elastase plus gene overexpression or suppression. n = 12 for each type of mice. * indicates P < 0.05 versus empty vector. C, Incidence of IA (upper panel) and rupture rate (down panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II and 35 mU elastase plus chemicals administration. n = 12 for each type of mice. * indicates P < 0.05 versus vehicle control. D, Incidence of IA (upper panel) and rupture rate (down panel) after 20 days of IA induction with 1,000 ng/kg/min angiotensin-II and 35 mU elastase plus chemicals administration. n = 12 for each type of mice. * indicates P < 0.05 versus vehicle control. Ang II indicates angiotensin II; IA, intracranial aneurysm; sh, shRNA; Ctrl, vehicle control.
Primary cilia deficiency in human IA patients

We then explored whether primary cilia deficiency in IA development is conserved in humans. As in the mice model of IA, human endothelial cells isolated from IA region of patients showed primary cilia deficiency (Fig. 5A). To further understand the clinical relevance of primary cilia deficiency in the pathogenesis of IA, we analyzed the gene mutation frequency of primary cilia related genes in IA patients. Single-gene mutation analysis did not show any significant difference between IA patient group and healthy control group (data not shown). However, when the genes were categorized into different groups, some groups had higher gene mutation frequency than healthy control group, including BBSome group (including gene BBS1, BBS2, BBS4, BBS5, BBS7, BBS8,
Finally we demonstrated that the primary cilia deficiency in IA development is conserved in humans. And IA patients had higher frequency of gene mutations which are related to primary cilia regulation. Our data presented here implicate the important role of primary cilia in IA development and provide a promising therapeutic target for IA treatment. In the future, the therapeutic application of primary cilia stabilizing chemicals should be tested in other animal models of IA and more chemicals should be screened to find more efficient and effective primary cilia stabilizing chemicals.

CONCLUSIONS

Our study provides an important support for the role of primary cilia in the development of IA. The primary cilia stabilizing chemicals might be useful for preventing IA development.

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