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Identification of MMP1 as a novel risk factor for intracranial aneurysms in ADPKD using iPSC models

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Cardiovascular complications are the leading cause of death in autosomal dominant polycystic kidney disease (ADPKD), and intracranial aneurysm (ICA) causing subarachnoid hemorrhage is among the most serious complications. The diagnostic and therapeutic strategies for ICAs in ADPKD have not been fully established. We here generated induced pluripotent stem cells (iPSCs) from seven ADPKD patients, including four with ICAs. The vascular cells differentiated from ADPKD-iPSCs showed altered Ca^{2+} entry and gene expression profiles compared with those of iPSCs from non-ADPKD subjects. We found that the expression level of a metalloenzyme gene, matrix metalloproteinase (MMP) 1, was specifically elevated in iPSC-derived endothelia from ADPKD patients with ICAs. Furthermore, we confirmed the correlation between the serum MMP1 levels and the development of ICAs in 354 ADPKD patients, indicating that high serum MMP1 levels may be a novel risk factor. These results suggest that cellular disease models with ADPKD-specific iPSCs can be used to study the disease mechanisms and to identify novel disease-related molecules or risk factors.

Autosomal dominant polycystic kidney disease (ADPKD) is the most prevalent, potentially lethal, monogenic disorder, and is characterized by the development of multiple renal cysts and various extrarenal manifestations^{1,2}. The disorder is caused by mutations to either of two genes, *PKD1* and *PKD2*, which encode polycystin-1 and -2, respectively^{3,4}. Polycystin-1 is a plasma membrane receptor-like protein that has functions in cell-cell or cell-extracellular matrix interactions⁵. Polycystin-2 is a nonselective cation channel protein with high permeability for Ca^{2+} and interacts with polycystin-1 to form a functional receptor-ion channel complex that regulates

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Patient	Age at skin biopsy	Sex	Clinical features	Molecular Defect (in exon)	Vascular complications	eGFR (ml/min per 1.73 m ²)	Dialysis
P1	45Y	Female	Numerous kidney cysts; Family history	ND*	HT, ICA	<15	+
P2	64Y	Female	Numerous kidney cysts; No family history	W429S, S2235L, V3008M, S3404Y, S3405Y, Missense, <i>PKD1</i> gene	HT	54	—
P3	71Y	Male	Numerous kidney cysts; No family history	A190T, Missense, <i>PKD2</i> gene	HT, ICA, temporal arteritis	<15	+
P4	49Y	Male	Numerous kidney cysts; Family history	G3818R, Missense, <i>PKD1</i> gene	HT, ICA, SAH	10	—
P5	41Y	Male	Numerous kidney cysts; Family history	E2111K, R2327W, Missense, <i>PKD1</i> gene	HT	65	—
P6	45Y	Female	Numerous kidney cysts; Family history	Q3895X, Nonsense, <i>PKD1</i> gene	—	43	—
P7	69Y	Female	Numerous kidney cysts; Family history	7024delAC, Frameshift, <i>PKD1</i> gene	HT, ICA	<15	+

Table 1. Profiles of seven ADPKD patients whose dermal fibroblasts were converted into iPSCs. ND*, not determined in the Exons. Intronic mutations 8161 + 38G > A and 2986-15C > T were found, which are described in ADPKD Mutation Database [PKDB]. (<http://pkdb.mayo.edu/>). HT, hypertension; ICA, intracranial aneurysm; SAH, subarachnoid hemorrhage.

intracellular Ca²⁺ homeostasis^{6–10}. Patients with *PKD2* mutations show milder clinical phenotypes than those with *PKD1* mutations¹¹.

Intracranial aneurysms (ICAs) are among the most serious cardiovascular complications in patients with ADPKD, since their rupture carries a severe morbidity-mortality rate of ~50%¹². A prevalence of 8% for asymptomatic ICAs has been reported in patients with ADPKD, and those with *PKD1* and *PKD2* mutations appear to be at an equal risk of developing ICAs, while patients with mutations to the 5' half of *PKD1* are more likely to develop ICAs^{12–15}. Both polycystin-1 and -2 are expressed in the vascular endothelia and smooth muscle cells in humans and mice, and may play a role in the structural integrity of blood vessels^{16–21}. Several mouse models were established to study the functional roles of the polycystins in vascular cells using targeted disruption of the *Pkd1* or *Pkd2* gene. It is likely that aberrant expression or localization of polycystins affects the fluid shear stress sensing in endothelia and the stretch-activated channel activity in smooth muscle cells, which results in altered intracellular Ca²⁺ homeostasis and the vascular phenotype associated with ADPKD^{22–28}. However, little is known about the molecular mechanisms of ICA formation in ADPKD, and novel cellular assay systems are needed to study the mechanisms. In this study, we demonstrate the potential of using patient-derived iPSCs to create disease models and to identify novel risk factors for the vasculopathy associated with ADPKD.

Results

Generation of ADPKD-specific iPSCs from Patient Fibroblasts. We obtained skin samples from seven ADPKD patients (P1–7) whose characteristics are shown in Table 1. All the patients met the clinical diagnostic criteria for ADPKD²⁹. Four (P1, P3, P4 and P7) out of the seven patients had ICAs, while the remaining three patients did not. P1, P3 and P4 were diagnosed as having ICAs by magnetic resonance angiography (MRA) within three years before the skin biopsy for iPSC derivation and P7 was diagnosed as having ICAs 26 years before the biopsy by angiography. P4 also had a past history of subarachnoid hemorrhage (SAH). All three patients without ICAs (P2, P5 and P6) were examined by MRA within three years before and after the skin biopsy for P2 and P5 and six years before and five years after the biopsy for P6. All patients except P6 had hypertension, and P3 was diagnosed as having temporal arteritis at the time of the biopsy. The skin fibroblasts were converted into iPSCs after transduction either with four retroviral vectors encoding OCT4, SOX2, KLF4 and c-MYC or with three vectors encoding OCT4, SOX2 and KLF4 (Fig. 1a,b)^{30,31}. The different combinations of factors does not cause molecular or cellular differences in the resulting iPSCs³¹. Quantitative PCR with reverse transcription (qRT-PCR) was used to evaluate ADPKD-iPSC clones with repression of the exogenously introduced genes (Figure S1). Using these analyses, one iPSC clone whose repression level was highest for each of the seven patients was selected for further examination in order to minimize the effects of the exogenously introduced genes on the differentiated cells (Table S1). The genetic identity between patient fibroblasts and the corresponding iPSCs was confirmed by short tandem repeat (STR) analyses (Table S2).

All seven iPSC clones exhibited characteristic human embryonic stem cell (hESC) morphology (Fig. 1b) and were positively stained with pluripotency markers, including OCT4, NANOG, SOX2, SSEA4, TRA-1–60, TRA-1–81, and alkaline phosphatase (AP), but not a mouse ESC/iPSC marker, SSEA1 (Figs 1c and S2). These clones also exhibited mRNA expression of multiple pluripotency markers, including *NANOG*, *GDF3*, *DPPA4*, *TERT* and *REX1* (Figure S3), and normal karyotypes, except for minimal abnormality in one clone, P3-iPSC (Fig. 1d and Table S1). Microarray analysis showed that the global gene expression profiles of ADPKD-iPSCs were more similar to those of hESCs (H9) than to those of the parental fibroblasts (Figs 1e and S4). We observed an obvious decrease in the methylation level of the *OCT4* and *NANOG* promoters in ADPKD-iPSCs compared with the parental fibroblasts using bisulfite sequencing analysis (Figs 1f and S5). ADPKD-iPSCs could be differentiated into all three germ layers by embryoid body (EB) and teratoma formation following the intratesticular injection of undifferentiated ADPKD-iPSCs into non-obese diabetic/severe combined immunodeficient (NOD-SCID) mice

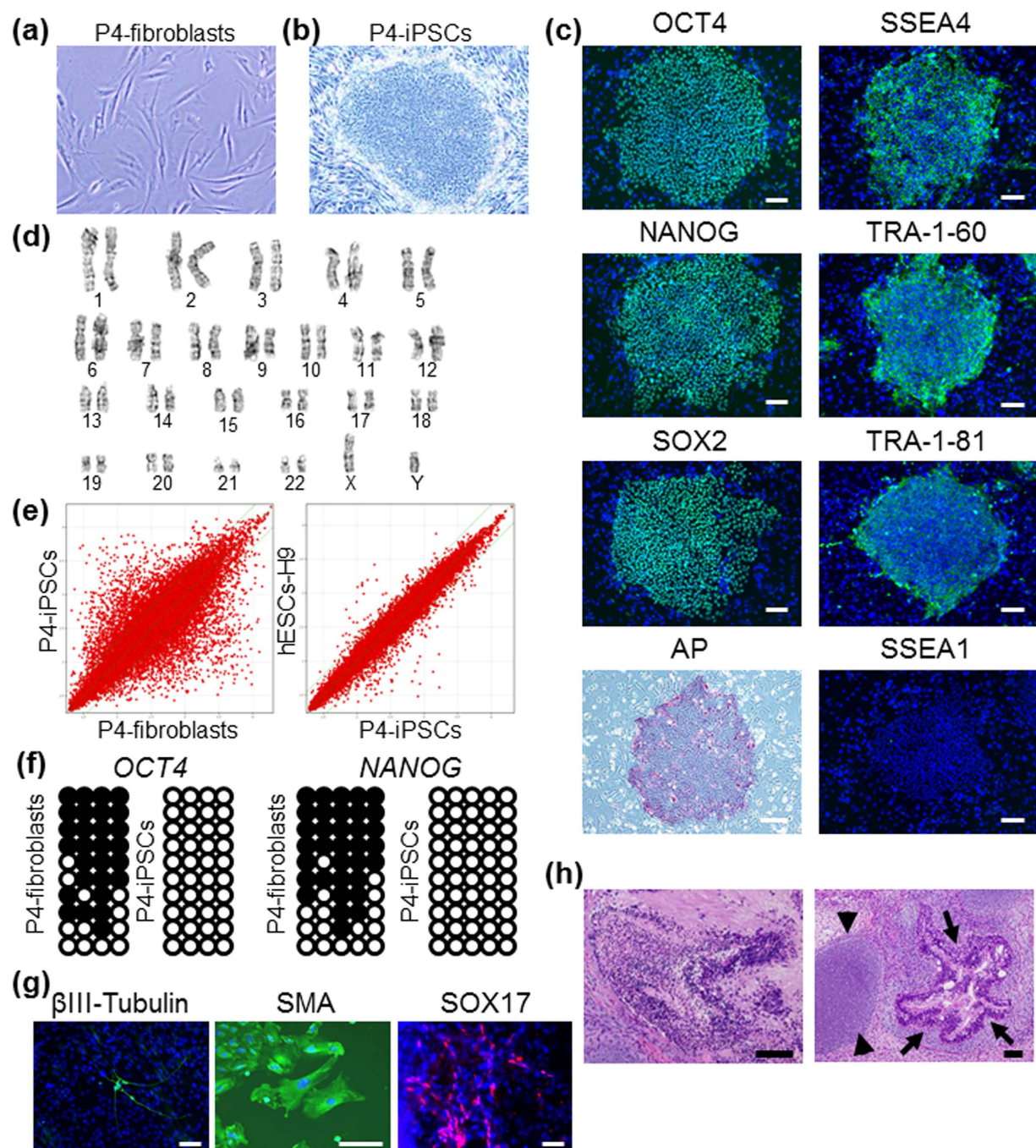


Figure 1. Derivation of Patient-specific iPSCs from ADPKD Patients. ADPKD patient's (P4) fibroblasts (a) were converted into ADPKD-iPSCs (b) after retroviral transduction with OCT4, SOX2, KLF4 and c-MYC. (c) The expression of pluripotency markers, such as OCT4, NANOG, SOX2, SSEA4, TRA-1-60 and TRA-1-81, and alkaline phosphatase (AP) enzymatic activities in P4-iPSCs. Note that a mouse pluripotency marker SSEA1 is negative. (d) The normal karyotype of P4-iPSCs (46, XY [20]). (e) The global gene expression patterns were compared between P4-iPSCs and P4-fibroblasts, and between P4-iPSCs and human ESCs (H9) using microarray analyses. (f) A bisulfite sequencing analysis of the *OCT4* and *NANOG* promoters in P4-fibroblasts and P4-iPSCs. (g) After 16 days of differentiation with embryoid body (EB) formation, cells were stained with anti- β III-tubulin (ectoderm), SMA (mesoderm) and SOX17 (endoderm) antibodies. (h) Teratomas were generated by injecting P4-iPSCs into the testes of immunocompromised NOD-SCID mice. Neural tissue (ectoderm; left panel), cartilage tissue (mesoderm; right, arrowheads) and intestinal epithelia (endoderm; right, arrows) were observed. Scale bars, 100 μ m. See also Figures S1–S6, Tables 1, S1 and S2.

(Fig. 1g,h). These results suggest that ADPKD-iPSCs have similar pluripotent properties to previously reported human ESC/iPSC lines^{30,32}.

The mutational analyses of three patients (P4, P6 and P7) and their families were performed with multipoint linkage analyses, which demonstrated that all three had a mutation in the *PKD1* gene (Table 1 and Figure S6)³³. The genomic analysis showed that the ADPKD-fibroblasts and -iPSCs possessed both wild-type and mutant *PKD1* alleles in all three patients (Figure S6). We examined the mutation status of the iPSC lines and the parental fibroblasts from the remaining four patients (P1, P2, P3 and P5) by exome analyses and long-range PCR with next-generation sequencing³⁴. The whole sequences of the *PKD1*/*PKD2* genes except for exons 8–12 of the *PKD1* gene were determined, and the genomic mutations that resulted in amino acid changes were identified in both iPSC lines and the original fibroblasts from three (P2, P3 and P5) out of the four patients (Table 1). We could not identify the genetic mutation of P1 in the exons of *PKD1*/*PKD2* genes, but found intronic mutations 8161 + 38G > A and 2986-15C > T, which are described in ADPKD Mutation Database [PKDB] (<http://pkdb.mayo.edu/>) (Table 1).

Differentiation of ADPKD-iPSCs into Vascular Cells. We used eleven iPSC clones (C1–11) established from eight non-ADPKD Japanese subjects as control-iPSCs (Table S3)³⁵. To create cellular disease models for vascular lesions, ADPKD- and control-iPSCs were differentiated into vascular endothelia and smooth muscle cells from sorted FLK1 (+) VE-cadherin (+) and FLK1 (+) VE-cadherin (–) cells on culture day 8, respectively, using our previously reported differentiation protocols (Fig. 2a)^{36–38}. Vascular endothelia and smooth muscle cells differentiated from ADPKD-iPSCs showed similar vascular marker expression to previously reported human iPSC-derived vascular cells (Figs 2b–g and S7). These results indicate that ADPKD-iPSCs can be differentiated into vascular endothelia and smooth muscle cells. Both the vascular endothelia and smooth muscle cells derived from ADPKD- and control-iPSCs showed similar morphologies (Figure S9a) and no significant differences in the expression levels of vascular marker genes, *PKD1* and *PKD2* (Figure S8). Then, we compared the proliferation and angiogenic potentials between the endothelia derived from ADPKD- and control-iPSCs using immunostaining with a proliferation marker Ki67 and tube formation assay, respectively, finding no significant differences in either assay (Figure S9b–d)³⁷.

Altered Calcium Handling in Vascular Cells Differentiated from ADPKD-iPSCs. Defects in either polycystin-1 or polycystin-2 expressed in primary cilia are thought to disrupt the intracellular Ca^{2+} regulation, eventually leading to renal cyst formation or vascular lesions associated with ADPKD¹⁰. Abnormal intracellular Ca^{2+} handling was detected in vascular smooth muscle cells from *Pkd1*^{+/-} and *Pkd2*^{+/-} mouse aortas, renal collecting duct cells from *Pkd1*^{+/-} mice and renal cyst cells from ADPKD patients^{24,25,27,39–41}. We first confirmed the presence of primary cilia in iPSC-derived vascular endothelia and smooth muscle cells with immunostaining for acetylated α -tubulin (Fig. 3a).

Then, in order to examine whether the vascular cells from ADPKD-iPSCs showed altered intracellular Ca^{2+} regulation, we measured the resting $[\text{Ca}^{2+}]_i$, agonist-induced Ca^{2+} release from the intracellular stores and Ca^{2+} entry using the fluorescent probe fura-2. While almost all iPSC-derived endothelia responded to ATP stimulation, only 12–38% of cells responded to carbachol (CCh) stimulation (Tables S4 and S5). We therefore used ATP as the agonist for the inositol 1,4,5-triphosphate (IP3)-IP3 receptor pathway in endothelia in subsequent analyses. In contrast, more than 50% of the iPSC-derived vascular smooth muscle cells responded to ATP or CCh stimulation, while 6–8% of cells responded to vasopressin. In addition, a small percentage of iPSC-derived endothelia or smooth muscle cells responded to caffeine stimulus. The percentage of cells responding to each of the four agonists was not significantly different between the vascular cells derived from ADPKD- and control-iPSCs (Tables S4 and S5).

The resting $[\text{Ca}^{2+}]_i$ levels and Ca^{2+} release from the intracellular stores induced by the four agonists were not significantly different between the vascular cells derived from ADPKD- and control-iPSCs (Fig. 3b–h and Table S5). In contrast, the Ca^{2+} entry under stimulation with ATP or CCh, but not vasopressin or caffeine, showed a statistically significant decrease in both endothelia and smooth muscle cells derived from ADPKD-iPSCs compared with those derived from control-iPSCs (Fig. 3c–h, and Table S5). These differences were statistically significant even after considering the effects of age, sex, original cell types from which iPSCs were generated and iPSC derivation methods ($P = 0.008$ for endothelia, $P = 0.013$ and 0.036 for smooth muscle cells induced by ATP and CCh, respectively). However, no significant differences in these parameters for intracellular Ca^{2+} handling were observed between vascular cells from ADPKD patients with ICAs (P1, P3, P4 and P7) and those from patients without ICAs (P2, P5 and P6; Table S5). These results suggest that the vascular cells derived from ADPKD-iPSCs show an altered intracellular Ca^{2+} handling.

Identification of Disease-related Molecules using iPSC-derived Vascular Cells. In an attempt to identify candidate molecules that might be involved in the pathogenesis of the vasculopathy in ADPKD, the expression profiles of vascular cells derived from ADPKD- and control-iPSCs were examined by microarray analyses. A principal component analysis (PCA) three-dimensional plot showed that there were differential gene expression patterns for the vascular cells derived from ADPKD- and control-iPSCs (Fig. 4a). Because the abnormal expression of extracellular matrix metabolism-related genes was reported in previous studies with rat and zebrafish PKD models, we focused on these genes in the analysis of vascular cells differentiated from ADPKD-iPSCs^{42,43}. Consistently, we found that the expression levels of multiple genes involved in extracellular matrix metabolism were significantly altered in vascular cells derived from ADPKD-iPSCs compared to those from control-iPSCs (Fig. 4b,c).

Vascular cells derived from the ADPKD patient group with ICAs (P1, P3, P4 and P7) and the patient group without ICAs (P2, P5 and P6) were compared in order to identify molecules that might be associated with ICA formation in ADPKD. However, there were no candidate genes whose expression levels were significantly altered between the two groups (adjusted P -value < 0.05; t-test with Holm multiple test correction). Accordingly, we

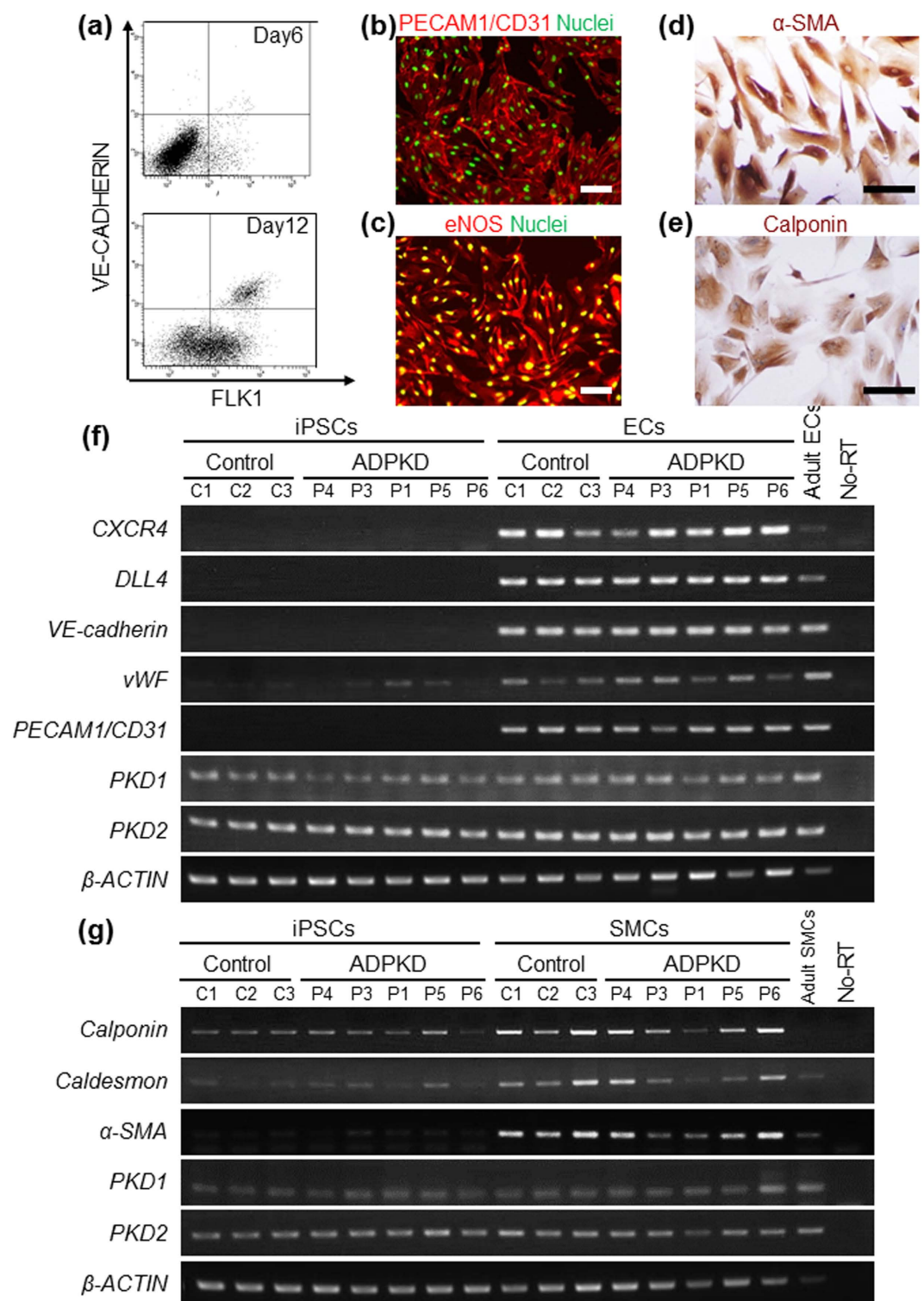


Figure 2. Directed Differentiation of ADPKD-iPSCs into Vascular Endothelia and Smooth Muscle Cells. (a) Flow cytometric analyses of ADPKD iPSC-derived cells on culture days 6 (upper) and 12 (lower). (b,c) Immunostaining of vascular endothelial cells (ECs) on day 12 differentiated from P4-iPSC-derived VE-CADHERIN (+) FLK1 (+) cells for the endothelial markers PECAM1/CD31 (b) and endothelial NO synthase (eNOS) (c). (d,e) Immunostaining of vascular smooth muscle cells (SMCs) on day 25 differentiated from P4-iPSC-derived VE-CADHERIN (-) FLK1 (+) cells for the smooth muscle cell markers α-smooth muscle actin (αSMA) (d) and Calponin (e). (f) The expression of marker genes (*CXCR4*, *DLL4*, *VE-cadherin*, *vWF* and *PECAM1/CD31*) and *PKD1* and *PKD2* in ECs derived from three control-iPSC (C1-3) and five ADPKD-iPSC lines (P4, P3, P1, P5 and P6). (g) The expression of marker genes (*Calponin*, *Caldesmon* and *α-SMA*) and *PKD1* and *PKD2* in SMCs derived from the control- and ADPKD-iPSC lines. Scale bars, 100 μm. See also Figures S7–S9.

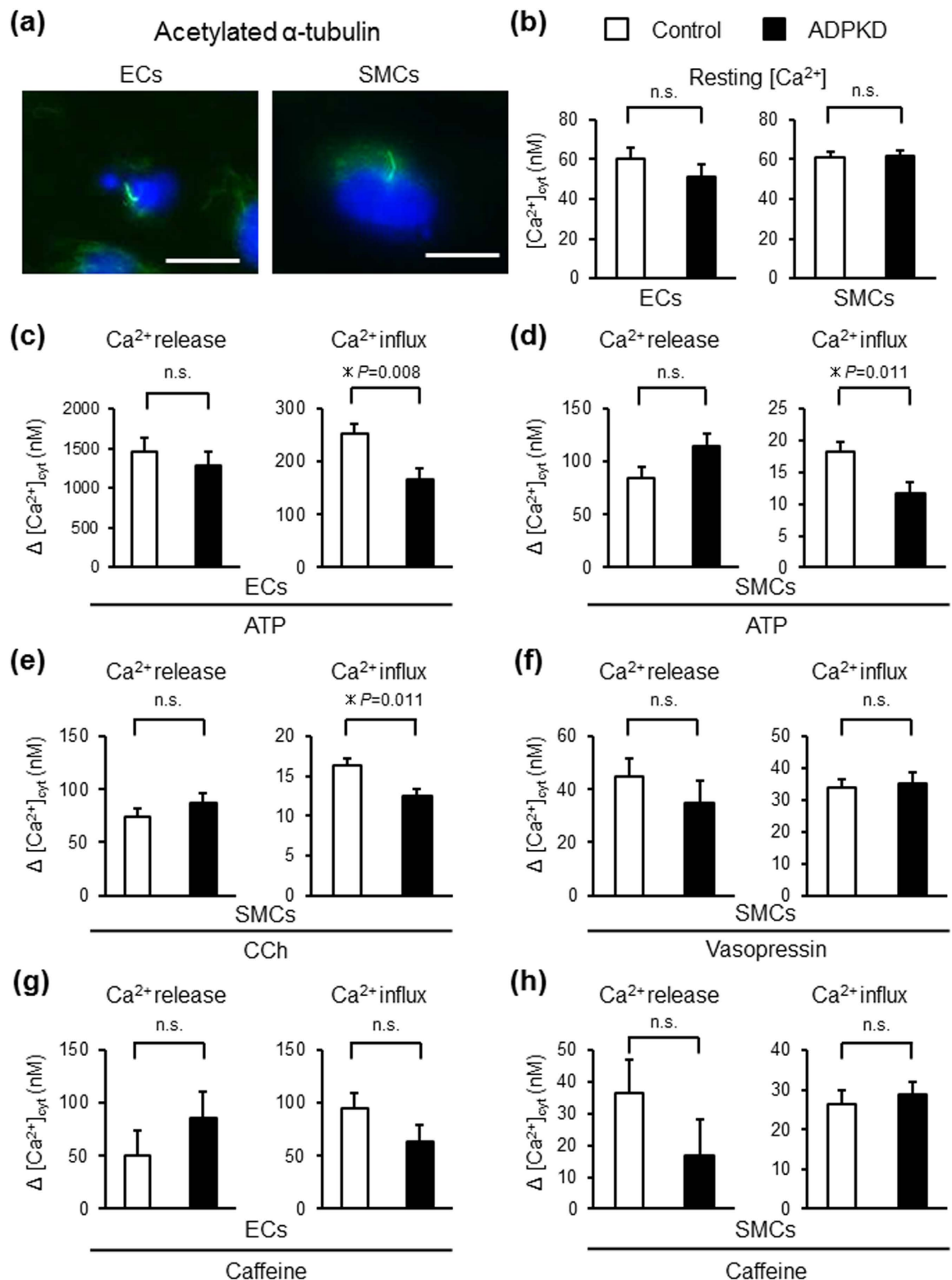


Figure 3. Intracellular Ca^{2+} Regulation in Vascular Endothelia and Smooth Muscle Cells Derived from Control- and ADPKD-iPSCs. (a) Anti-acetylated α -tubulin immunostaining of vascular endothelia (ECs) on culture day 12 and smooth muscle cells (SMCs) on day 25 differentiated from P4-iPSCs shows primary cilia (green). (b) The resting cytosolic Ca^{2+} concentration of ECs and SMCs derived from control-iPSCs (white bars) and ADPKD-iPSCs (black bars). (c–h) ATP (c,d), carbachol (CCh) (e), vasopressin (f) and caffeine (g,h) induced Ca^{2+} release and influx in ECs (c,g) and SMCs (d–f,h) derived from control- and ADPKD-iPSCs. Ten control- and seven ADPKD-iPSC lines and eight to ten control- and seven ADPKD-iPSC lines were used for the analyses of ECs (b,c,g) and SMCs (b,d–f,h), respectively. The results are expressed as the means $\pm$ SEM. *Statistically significant, n.s.; not significant. See also Tables S4 and S5.

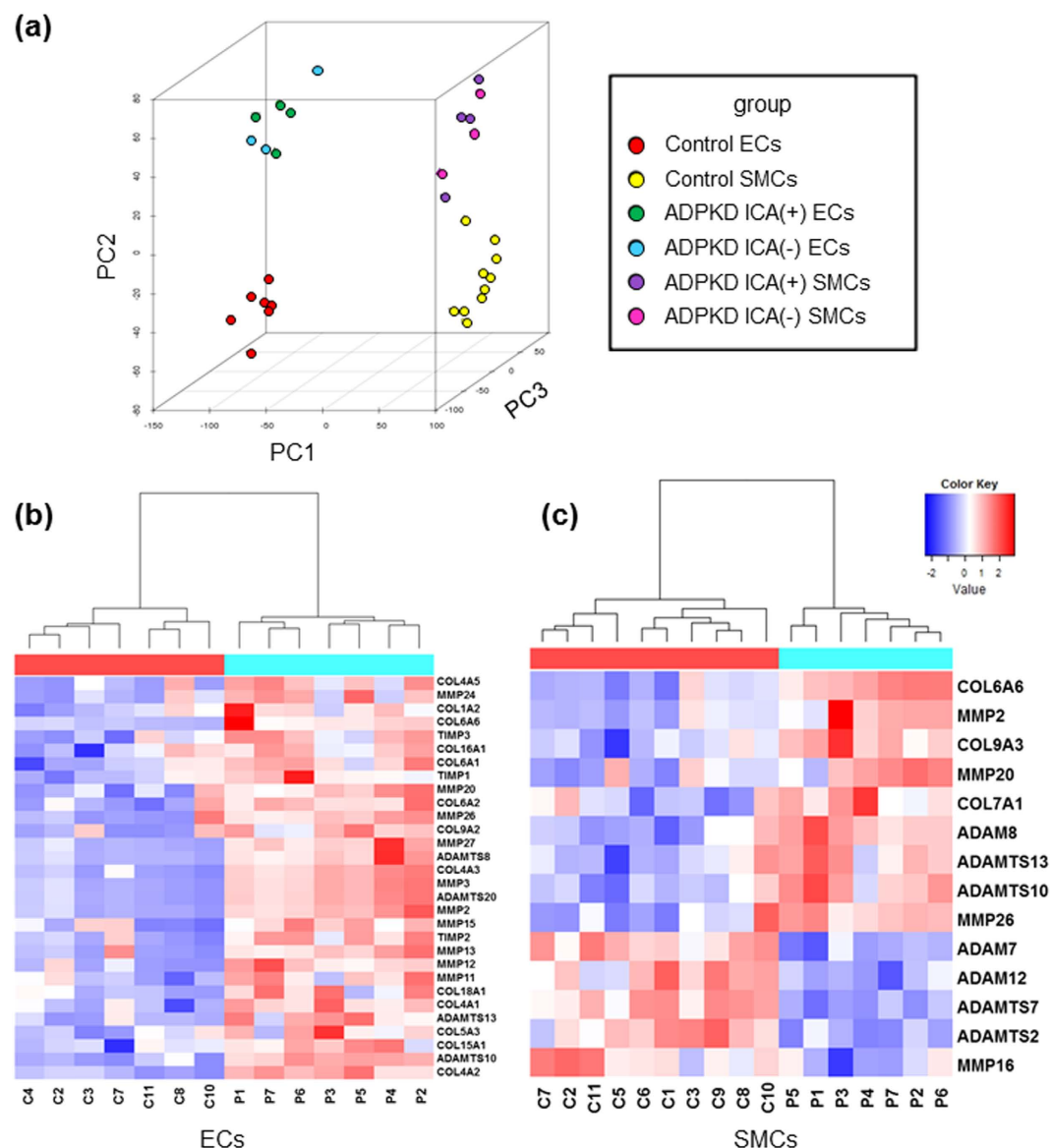


Figure 4. Altered Gene Expression Profiles of Vascular Endothelia and Smooth Muscle Cells Differentiated from ADPKD-iPSCs. (a) The gene expression profiles of vascular endothelia (ECs) and smooth muscle cells (SMCs) derived from ADPKD- and control-iPSCs were analyzed using a principal component analysis (PCA). The first, second and third principal components' contribution ratios were 0.404, 0.103 and 0.036, respectively. (b,c) The expression levels of multiple genes responsible for extracellular matrix metabolism were altered in ECs (b) and SMCs (c) derived from ADPKD-iPSCs compared with those derived from control-iPSCs. Genes with *P*-values less than 0.05 were listed and heat maps were generated. The z-scores were calculated from the average and standard deviation of all scores. High and low scores are indicated by red and blue, respectively. Microarray analysis was performed with one sample for each cell line in (a–c). PC, principal component; ICA, intracranial aneurysm.

compared each of the four ADPKD patients with ICAs with each of the three patients without ICAs for each gene and selected candidates that had an average fold change in expression of more than two. We thus found 17 and 5 candidates that had their expression upregulated in iPSC-derived endothelia and smooth muscle cells from the ADPKD patients with ICAs compared to those from patients without ICAs (Tables S6 and S7). In contrast, no genes were consistently downregulated.

Next, we sought to identify a serum marker specific for ADPKD patients with ICAs, and analyzed the protein sequence of the candidates. We selected seven (*MMP10*, *MMP1*, *PCSK1*, *BMP6*, *EDN1*, *CTGF* and *TFPI2*) and two (*MMP1* and *TFPI2*) out of the 17 and 5 candidates for endothelia and smooth muscle cells, respectively, that encoded secreted proteins (where secreted proteins were defined as having a signal peptide sequence and no or one transmembrane region). qRT-PCR analyses of the vascular cells from all seven ADPKD-iPSCs were performed to examine the reproducibility of the microarray data and showed that only the *MMP1* expression was significantly upregulated in the iPSC-derived endothelia from the four ADPKD patients with ICAs compared

with the three patients without ICAs ($P = 0.007$; Figs 5a and S10). In contrast, the expression level of *MMP1* in smooth muscle cells was very low, as evaluated by the qRT-PCR analyses (Fig. 5a). Volcano plots drawn with fold changes and uncorrected P -values of microarray data showed that *MMP1* was remarkably plotted (Figure S11). We also confirmed that both the expression and secretion of the *MMP1* protein, as analyzed by Western blotting analysis and enzyme-linked immunosorbent assay (ELISA), respectively, were significantly higher in endothelia from ADPKD-iPSCs derived from patients with ICAs compared with those from patients without ICAs ($P = 0.029$ for protein expression and $P = 0.043$ for secretion; Fig. 5b–d).

We then evaluated the diagnostic accuracy of *MMP1* assays to predict the presence of ICAs in ADPKD patients. Serum samples from 97 ADPKD patients with ICAs (cases) and 257 patients without ICAs (controls) were collected and analyzed for their *MMP1* levels using an ELISA (Table S8). All patients undergo MRA examinations every three years for those without ICAs and every year for those with ICAs. The results showed that ADPKD patients with ICAs had higher *MMP1* serum concentrations than those without (mean $\pm$ SD; 17.6 ± 14.9 vs 14.0 ± 12.1 ng/mL; $P = 0.043$; Fig. 5e). Using receiver operating characteristic (ROC) analyses to determine the highest sensitivity and specificity values that could be obtained, we set the serum *MMP1* level to 15 ng/mL as the cutoff for ADPKD patients to indicate the likelihood of having ICAs (odds ratio (95% CI); 2.031 (1.260–3.274); $P = 0.0036$; Tables 2 and S9). The sensitivity and specificity of *MMP1* were low at 0.48 and 0.68, respectively. However, in a multivariate analysis where the patients' age, sex, family history of ICAs or subarachnoid hemorrhage (SAH), renal function (serum creatinine level), dialysis therapy and *MMP1* levels were taken into account as potential confounding factors, the serum *MMP1* level was confirmed to be one of the risk factors associated with ICAs formation in ADPKD patients (adjusted odds ratio (95% CI); 1.891 (1.139–3.138); $P = 0.014$; Table 2). The addition of serum *MMP1* levels to the multivariate logistic regression analysis increased the values of the area under a ROC curve (AUC) from 0.653 to 0.672 and decreased the value of Akaike's information criterion (AIC) from 403.1 to 399.0 (Table 2). Family history of ICAs or SAH, which is reported to be risk factors for the complication of ICAs in ADPKD, sex (female), which is a known to be a risk factor for ICAs in the generalized population, and serum *MMP1* level were significantly associated with the complication of ICAs in ADPKD in our clinical study (Table 2)^{44,45}. These results suggest that the vascular cells derived from ADPKD-iPSCs may be used to identify novel disease-related molecules.

Discussion

Although ICAs are one of the most serious cardiovascular complications in ADPKD, little is known about the pathogenesis of these vascular lesions, and novel cellular assay systems to elucidate the disease mechanisms are needed. In this study, we demonstrated that the vascular cells differentiated from patient-derived iPSCs could be used to identify molecules associated with the vasculopathy of ADPKD.

Vascular cells derived from ADPKD-iPSCs showed altered Ca^{2+} entry under stimulation with ATP or CCh, but not with vasopressin or caffeine. One possible explanation is that the receptors or downstream signaling molecules for these agonists were not sufficiently expressed in vascular cells derived from iPSCs. This is consistent with the finding of this study that a small percentage of cells responded to the vasopressin or caffeine stimulus. Although the difference of Ca^{2+} entry in iPSC-derived smooth muscle cells was small between controls and ADPKD patients, it has been reported that an altered local Ca^{2+} increase just below the channels can exert significant physiological effects on cell behavior^{46,47}, which suggests that the small differences observed between smooth muscle cells derived from control- and ADPKD-iPSCs may lead to different phenotypes. While vascular smooth muscle cells from *Pkd1*^{+/-} and *Pkd2*^{+/-} mouse aortas showed a statistically significant decrease in the resting $[\text{Ca}^{2+}]_i$ levels and agonist-induced Ca^{2+} release in previous studies^{24,25,27,39–41}, we found human vascular cells derived from ADPKD-specific iPSCs did not. One possible explanation for the inconsistency is the species difference in the correlation between the gene expression levels of *PKD1* or *PKD2* and the phenotypic presentation. Other possibilities include the functional differences between vascular cells differentiated from iPSCs and primary cultured vascular cells. Nevertheless, the present findings suggest that vascular cells derived from ADPKD-specific iPSCs can recapitulate one aspect of *in vivo* disease phenotypes.

The expression levels of multiple genes involved in extracellular matrix metabolism were significantly altered in vascular cells derived from ADPKD-iPSCs compared to those derived from control-iPSCs, as shown by the microarray analyses. These data are in line with the results of expression profiling studies of the kidney from rat and zebrafish PKD models^{42,43}. Other reports have also described the abnormal expression of matrix metalloproteinase genes, including *MMP9* in cell lines from the liver cysts of ADPKD patients⁴⁸, *MMP2* and *MMP14* in kidneys of PKD murine and rat models^{49–51}, and higher serum *MMP1* and *MMP9* levels in ADPKD patients⁵².

In this study, the mRNA and protein expression and protein secretion levels of *MMP1* for each ADPKD-iPSC line were not consistent and had large variation. One possible reason for the inconsistency and variation is that the samples were collected in different experiments because of the limited number of cells obtained in one experiment. Nevertheless, *MMP1* was consistently and significantly upregulated in the iPSC-derived endothelia from ADPKD patients with ICAs compared with the patients without ICAs in multiple assays. On the other hand, the expression level of *MMP1* in iPSC-derived smooth muscle cells was very low, suggesting that the upregulated *MMP1* expression by endothelia is the main associate of ICA formation.

Despite the altered expression of multiple genes involved in extracellular matrix metabolism, a differential expression of *MMP1* was not observed between the endothelia derived from ADPKD- and control-iPSCs, but was observed between iPSC-derived endothelia from ADPKD patients with ICAs and those from ADPKD patients without ICAs. An increased expression of matrix metalloproteinase genes, especially *MMP2* and *MMP9*, has been reported in aneurysmal tissues, which occurred in the absence of ADPKD, compared to normal arteries^{53–56}. Extending these findings to our results indicates that *MMP1* may be associated with the pathogenesis of ICA formation in ADPKD. It is possible that the elevation of *MMP1* may reflect inflammatory conditions at the site of the aneurysms. Another possibility is that the systemic elevation of *MMP1* may reflect generalized endothelial cell

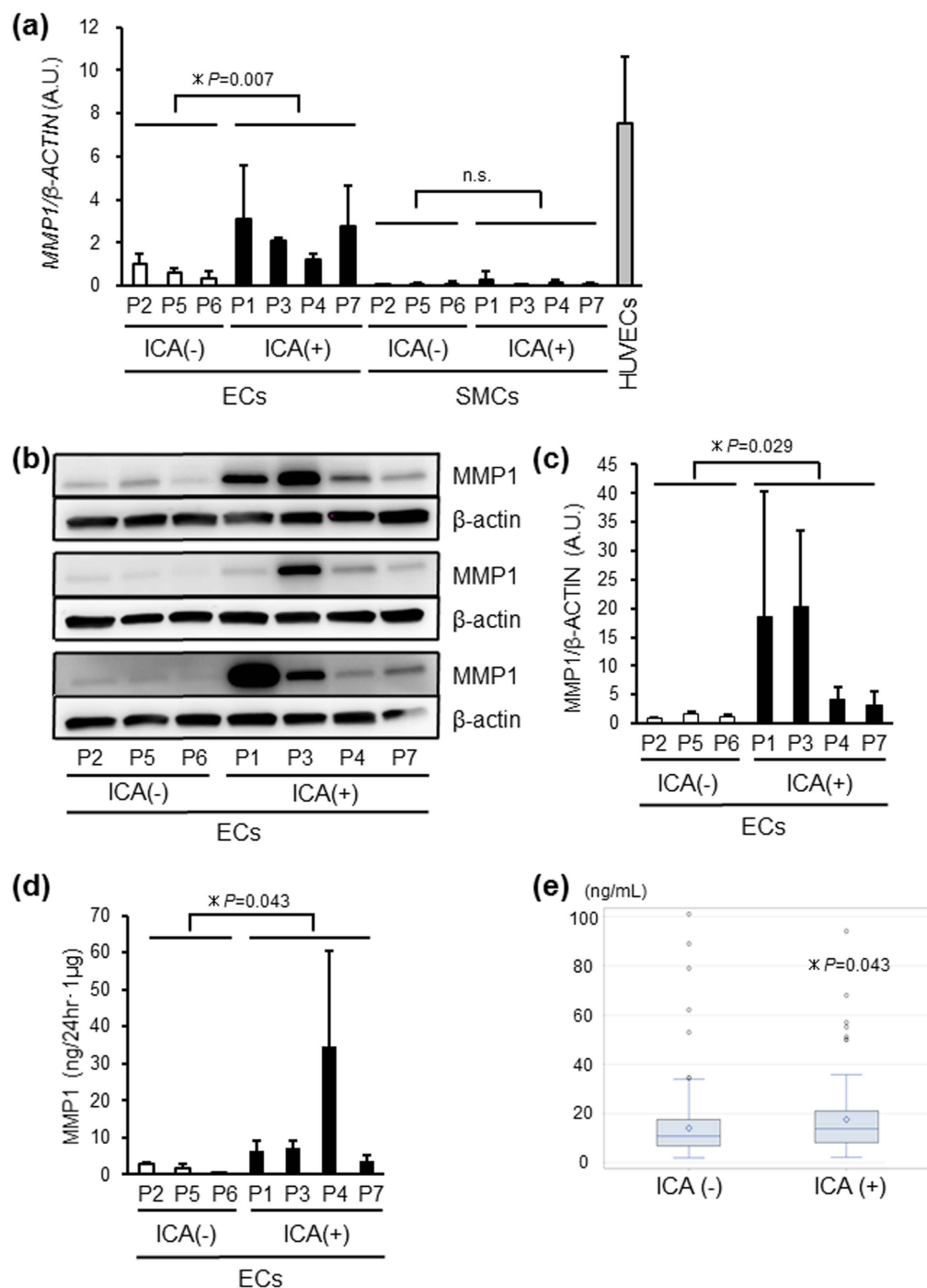


Figure 5. MMP1 Expression is Specifically Upregulated in iPSC-derived Endothelia from ADPKD Patients with ICA. (a) The expression levels of *MMP1* in iPSC-derived endothelial cells (ECs) from the four ADPKD patients with intracranial aneurysms (ICAs; P1, P3, P4 and P7) were significantly higher than those from the three patients without ICAs (P2, P5 and P6). Each value was normalized to the average of the iPSC-derived EC samples from P2. (b,c) There was significant upregulation of *MMP1* protein expression in iPSC-derived ECs from the four ADPKD patients with ICAs compared to those from the three patients without ICAs, as confirmed by a Western blotting analysis of total cell lysates. Each value was normalized to the average of the iPSC-derived EC samples from P2. (d) The secretion of the *MMP1* protein into the culture media from iPSC-derived ECs from the four ADPKD patients with ICAs was significantly higher than those from the three patients without ICAs as assessed by ELISAs. The secreted *MMP1* protein level was normalized to the total cellular protein weight of each cell lysate sample from iPSC-derived endothelial cell cultures. (e) A comparison of the serum *MMP1* levels from the 97 ADPKD patients with ICAs and the 257 patients without ICAs. The data from three (a,c) and five (d) independent experiments are presented as the means $\pm$ SD. SMCs; smooth muscle cells, HUVECs; human umbilical vein endothelial cells, *statistically significant, n.s.; not significant. See also Figures S10, S11, Tables 2 and S6–S9.

Risk factors	Multivariate analysis		
	Adjusted odd ratio	95% CI	P-value
(a) Model without serum MMP1 levels			
Age ≥ 65 Y	1.505	0.853–2.657	0.16
Sex (female)	1.809	1.086–3.013	0.023
Family history of ICAs or SAH	2.089	1.207–3.615	0.0084
Creatinine ≥ 2 mg/dL	1.133	0.533–2.410	0.75
Dialysis therapy	1.987	0.893–4.419	0.092
(b) Model including serum MMP1 levels			
MMP1 ≥ 15 ng/mL	1.891	1.139–3.138	0.014
Age ≥ 65 Y	1.537	0.864–2.735	0.14
Sex (female)	1.851	1.104–3.103	0.020
Family history of ICAs or SAH	2.165	1.243–3.768	0.0063
Creatinine ≥ 2 mg/dL	1.018	0.475–2.185	0.96
Dialysis therapy	1.918	0.857–4.290	0.11

Table 2. Multiple logistic regression analysis of MMP1, age, sex, family history, dialysis and renal function as risk factors for the incidence of intracranial aneurysms. (a) AUC: 0.653 (95%CI: 0.589–0.716), AIC: 403.11. (b) AUC: 0.672 (95%CI: 0.609–0.734), AIC: 399.04. ICA, intracranial aneurysm; SAH, subarachnoid hemorrhage; AUC, area under the curve; AIC, Akaike's information criterion.

abnormalities which increase the vulnerability of the vascular wall to mechanical stresses from the blood flow in the circle of Willis, thus eventually leading to the formation of ICAs. Our findings could also potentially be more broadly applied to aneurysm formation in the non-ADPKD general population.

Although intra-familial variability in ADPKD is much lower than inter-familial variability and some genotype-phenotype correlations have been reported in the development of ICAs in ADPKD patients, the finding that not all ADPKD individuals of high-risk families develop ICAs indicates that other genetic variants may be involved besides the *PKD1* or *PKD2* mutations^{14,15,45}. In our study, vascular cells from ADPKD-iPSCs showed impaired intracellular Ca^{2+} homeostasis, which may result from a *PKD1* or *PKD2* mutation, compared with those from control-iPSCs, but no significant differences were observed between vascular cells from ADPKD patients with and without ICAs, in spite of the differences in the expression of some genes, including *MMP1*. These results suggest that other genetic modifiers besides *PKD1* and *PKD2* are associated with the development of ICAs in ADPKD. *MMP1* may be a disease-related molecule involved in the pathogenesis of ICA formation in ADPKD, and its expression is likely regulated by such genetic modifiers.

In our clinical study, we examined the serum MMP1 levels of 354 ADPKD patients, and found that ADPKD patients with ICAs had higher MMP1 serum concentrations than those without ICAs although the difference was small. One possible explanation for the small difference is that the non-ICA group may include patients who will develop ICAs in the future because our study was cross-sectional. We examined multiple clinical parameters, such as age, sex, family history of ICAs or subarachnoid hemorrhage (SAH), serum creatinine level and the presence or absence of dialysis therapy, in addition to the serum MMP1 levels (Tables 2, S8 and S9). We found the serum MMP1 level is the second strongest parameter among the 6 factors examined by univariate analysis. It is stronger than family history of ICAs or SAH and age, which are reported to be risk factors for the complication of ICAs in ADPKD, and sex, which is a known risk factor for ICAs in the general population^{44,45}. Although univariate analysis found the serum creatinine level was stronger than the serum MMP1 level, multivariate analysis of all 6 factors found the serum creatinine level not significant (Table 2). Considering that we examined 5 common clinical parameters, our analysis shows that the serum MMP1 level is one risk factor for the development of ICAs in ADPKD patients. These results support organizing a prospective cohort study in the future to properly evaluate the diagnostic performance of serum MMP1 levels for predicting the development or current presence of ICAs in ADPKD patients. Additionally, a low-cost serum test would help determine which patients should undergo a MRA examination, which is currently used as a primary diagnostic method for complicated ICAs in ADPKD patients.

We could not identify the genetic mutation of P1 in the exons of *PKD1*/*PKD2*, but intronic mutations 8161 + 38G > A and 2986-15C > T were found in P1, which are described in ADPKD Mutation Database [PKDB] (<http://pkdb.mayo.edu/>). In the *PKD1*/*PKD2* mutation analysis, we could not specifically amplify the sequences of exons 8–12 of the *PKD1* gene from the genomic DNAs because of the presence of six highly homologous sequences. Thus, one possible explanation for not identifying the exonic mutations of P1 is that the mutations may be in the exons 8–12 of the *PKD1* gene. On the other hand, two recent papers showed that some ADPKD patients in Japan and China do not harbor patient-specific mutations in the exonic regions of either *PKD1* or *PKD2*^{57,58}. Although we cannot exclude the possibility of the mutations in the *PKD1* or *PKD2* genes of P1, there may be other causative mutations in ADPKD patients including P1. There is also a possibility that intronic mutations deposited in ADPKD Mutation Database might behave as causal mutations in ADPKD pathogenesis. Future investigation using next-generation whole genome sequencing analysis with long read techniques, which have the advantage of sequencing repetitive sequences, should provide reliable genotyping of the *PKD* genes and may disclose other candidate causative genes.

One of the advantages of disease modeling research using patient-derived iPSCs is that it makes it possible to address the symptomatic variability of human diseases, whereas mouse disease models generated by genetic manipulation show relatively uniform disease phenotypes, which do not reflect the variability of disease phenotypes among patients. The characteristic features of ADPKD include the intra- and inter-familial symptomatic variability, and a typical example is complicated ICAs, the prevalence of which was reported to be around 8%¹. We generated iPSCs from ADPKD patients with and without ICAs, and identified MMP1 as a possible molecule associated with this variability in the complication of ICAs among the ADPKD patients. iPSC models reduce the importance of age in comparative studies, as the telomere length of iPSCs is independent of the subject's age⁵⁹. In fact, we found in the present study larger differences in the gene expression patterns between vascular cells derived from ADPKD- and control-iPSCs than those among control-iPSC lines derived from subjects with a wide range of age. These results suggest that the impact of age is less than that of the genetic background. Future studies to create an *in vitro* model for aneurysm formation using vascular cells derived from ADPKD-iPSCs would lead to a better understanding of the mechanisms of aneurysm formation in ADPKD patients. These approaches using patient-derived iPSCs can also be applied to other disorders to elucidate the mechanisms underlying the symptomatic variability among patients.

In conclusion, the vascular cells derived from ADPKD-specific iPSCs can be used as a powerful tool for elucidating the mechanisms underlying the development of vascular complications in ADPKD. More broadly, our results suggest the usefulness of cellular disease models developed using patient-specific iPSCs for identifying novel disease-related molecules that may be used in clinical practice.

Materials and Methods

Ethics Statement. This study was approved by the Ethics Committees of Kyoto University and Toranomon Hospital and conducted according to the guidelines of the Declaration of Helsinki. All patients provided written informed consent. Animal experiments were approved by the CiRA Animal Experiment Committee, and conducted in accordance with the institutional guidelines.

In Vitro Differentiation into Vascular Cells. The differentiation of iPSCs into vascular cells was carried out as described previously^{36,37}. Briefly, undifferentiated human iPSCs were harvested and transferred to a collagen I-coated dish after adjusting the colonies to an appropriate size. On the second day of incubation, the culture medium was replaced with Primate ES medium (ReproCELL) without basic fibroblast growth factor (bFGF), supplemented with N2 supplement (Thermo Fisher Scientific)/B27 supplement (Thermo Fisher Scientific) and 6-bromindirubin-3'-oxime (BIO, 5 μ M, SIGMA). Thereafter, the cells were incubated for another 3 days, at which time the culture medium was replaced with StemPro-34 SFM (Thermo Fisher Scientific) supplemented with recombinant human vascular endothelial growth factor (VEGF, 50 ng/ml, PeproTech). After another 4–8 days of incubation, FLK1 (+) VE-cadherin (+) and FLK1 (+) VE-cadherin (–) cells were sorted individually using a FACSaria flow cytometer (BD) and were used for the subsequent experiments. Sorted FLK1 (+) VE-cadherin (+) cells were confirmed to remain VE-cadherin positive during the subsequent cell culture and analyses. On the other hand, the sorted FLK1 (+) VE-cadherin (–) cells were differentiated into vascular smooth muscle cells after an additional 18–22 days of differentiation on collagen I-coated dishes supplemented with recombinant human platelet-derived growth factor-BB (PDGF-BB, 20 ng/ml, PeproTech). Anti-FLK1 monoclonal antibody was kindly provided by Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan and used at 1:200. Anti-VE-cadherin monoclonal antibody (560410, BD Pharmingen) was used at 1:8.

Measurement of Changes in the $[Ca^{2+}]_i$. Fura-2 fluorescence was measured in HEPES-buffered saline (HBS) containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, and 20 mM HEPES, adjusted to pH 7.4 with NaOH. The fluorescent images were analyzed with a video image analysis system (AQUACOSMOS, Hamamatsu Photonics). Ratio images of 340:380 were obtained on a pixel-by-pixel basis. Fura-2 measurements were carried out at 25 ± 1 °C in HBS adjusted to pH 7.4. The 340:380 ratio images were converted to Ca^{2+} concentrations by *in vivo* calibration using 5 μ M ionomycin, as described previously⁶⁰. To measure Ca^{2+} release from the intracellular stores, the cells were incubated with Ca^{2+} -free solution containing 0.5 mM EGTA without $CaCl_2$ before Ca^{2+} release was evoked by adding 100 μ M ATP, 100 μ M CCh, 10 ng/ml vasopressin or 10 mM caffeine in Ca^{2+} -free solution. The Ca^{2+} entry was measured after the re-addition of Ca^{2+} into the solution.

Microarray Analysis. Total RNA from skin fibroblasts, human iPSCs (hiPSCs), hESCs and vascular cells derived from hiPSCs was isolated using the RNeasy Mini Kit (Qiagen). One hundred nanograms of total RNA from each sample was processed using the WT Expression Kit (Ambion) and the WT Terminal Labeling Kit (Affymetrix). Samples were prepared for hybridization by labeling cDNA in a 1X hybridization cocktail according to the manufacturer's instructions. GeneChip arrays (Human Gene 1.0 ST) were hybridized in a GeneChip Hybridization Oven at 45 °C for 16 h at 60 RPM. Washing was done with a GeneChip Fluidics Station 450, according to the manufacturer's instructions, with the buffers provided in the Affymetrix GeneChip Hybridization, Wash, and Stain Kit. The arrays were scanned on a GeneChip Scanner 3000 7G plus (Affymetrix), and raw data processing was performed using AGCC software (version 2.0, Affymetrix). Raw data in the form of CEL files were imported into the GeneSpring GX (version 11.0) software program (Agilent Technologies). The normalization and probe summarization were performed by the Robust multi-array average (RMA) method. The prediction of the signal peptide sequence was performed using the Signal P 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>). Transmembrane regions were predicted by the SOSUI algorithm (<http://bp.nuap.nagoya-u.ac.jp/sosui/>).

The gene expression profiling of vascular cells derived from ADPKD- and control-iPSCs was performed using the SurePrint G3 Human Gene Expression $8 \times 60K$ v2 Microarray Kit (Agilent Technologies). The data were normalized using GeneSpring GX 12.6.1 software as follows: (i) threshold raw signals were set to 1.0, (ii) log base 2 transformation was performed, and (iii) 75th percentile normalization was chosen. Genes differently expressed between two conditions were listed using a multiple statistical test controlled by a false discovery rate (FDR) at 0.05 by Tukey's range test. The microarray data are available at Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>) with series accession number GSE74453.

Serum Specimen Collection from ADPKD Patients. A total of 354 ADPKD patients were analyzed for their serum MMP1 levels. All patients were Japanese. The presence of intracranial aneurysms (ICAs) was tested by magnetic resonance angiography (MRA) in all the patients. The profiles of the patients are shown in Table S8.

Testing for MMP1 Levels. MMP1 concentrations in the serum samples were measured by SRL, Inc., Japan using a commercially available enzyme-linked immunosorbent assay (Daiichi Fine Chemical CO., LTD, Japan). The serum samples obtained were stored at -80°C until the analysis.

Statistical Methods. For the baseline variables, summary statistics were constructed employing frequencies and proportions for categorical data, and the means, standard deviations and ranges for continuous variables. The comparability of the baseline characteristics between the two groups was assessed by means of a two-sample *t*-test for continuous variables or by using Fisher's exact test for categorical variables. For the repeated measurement data in the Ca^{2+} experiments, after a variance stabilizing transformation, a linear mixed model was applied to test for differences between cases and controls over time, and a compound symmetry covariance structure was used to model the data. A multiple logistic regression analysis was performed to examine the effects of MMP1, age, sex, family history, dialysis and renal function on the incidence of cerebral aneurysms. Adjusted odds ratios (OR) and their 95% confidence intervals (CI) were estimated by the model.

All comparisons were planned, and the tests were two-sided. A *P*-value <0.05 was considered to indicate a statistically significant difference. For the multiple testing problem, we applied the Holm method on the computed *P*-values to reduce the risk of type I errors. All statistical analyses were conducted using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

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Author Contributions

M.S., Y.S., Y.U., E.M., A.F., T.N., Y.M., A.K., K.N., S.Y. and K.O. designed the study. T. Ameku, D.T., T.N., F.S., T.T., S.M., T. Araoka, T. Yasuno, S.-I. M., H.K., N.K., S.A., T.I., N.M., S.I., A.W., K.T., I.A. and K.O. collected the data. D.T., M.S., T.N., F.S., T.T., F.K., N.A., M.N., A.W., T. Yamamoto, K.T., Y.S., Y.Y. and K.O. analyzed and interpreted the data. M.S., T.N., Y.M., A.K., K.N. S.Y. and K.O. supervised the study. T. Ameku and K.O. searched the literature and wrote the manuscript.

Additional Information

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SUPPLEMENTARY INFORMATION

Identification of MMP1 as a novel risk factor for intracranial aneurysms in ADPKD using iPSC models

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Table S1. Characterization of established iPSC clones, Related to Figure 1.

Patient	Clone number	Factors used	Marker expression		Pluripotency		Karyotype	STR	BS
			RT-PCR	IC	EB	Teratoma			
P1	CiRA00004	3F	✓	✓	✓	✓	46,XX[20]	✓	✓
P2	CiRA00005	3F	✓	✓	✓	✓	46,XX[20]	✓	✓
P3	CiRA00006	4F	✓	✓	✓	✓	48,XY,+X,+12[2] /46,XY[18]	✓	✓
P4	CiRA00007	4F	✓	✓	✓	✓	46,XY[20]	✓	✓
P5	CiRA00008	4F	✓	✓	✓	✓	46,XY[20]	✓	✓
P6	CiRA00009	4F	✓	✓	✓	ND	46,XX[20]	✓	✓
P7	CiRA00010	4F	✓	✓	✓	✓	46,XX[20]	✓	✓

IC, immunocytochemistry; EB, embryoid body; STR, short tandem repeat analysis; BS, bisulfite sequencing of OCT4 and NANOG promoters; 3F, iPSCs generated with three reprogramming factors, OCT4, SOX2 and KLF4; 4F, iPSCs generated with four factors, OCT4, SOX2, KLF4 and c-MYC; ND, not done.

Table S2. STR analyses of ADPKD-iPSCs and their parental fibroblasts, Related to Figure 1.

Locus/Cells	P1- fibroblast		P1- iPSC		P2- fibroblast		P2- iPSC		P3- fibroblast		P3- iPSC		P4- fibroblast		P4- iPSC		P5- fibroblast		P5- iPSC	
D3S1358	16	17	16	17	15	16	15	16	15	17	15	17	16	17	16	17	15	16	15	16
TH01	6		6		9		9		7	9	7	9	7	9	7	9	7		7	
D21S11	29	33.2	29	33.2	29	31	29	31	29	30	29	30	29		29		31	31.2	31	31.2
D18S51	13	22	13	22	14	16	14	16	18	19	18	19	12	13	12	13	17	21	17	21
Penta_E	19	21	19	21	12	16	12	16	19	20	19	20	9	19	9	19	15	21	15	21
D5S818	12	13	12	13	11	12	11	12	9	13	9	13	10		10		7	12	7	12
D13S317	8		8		8	11	8	11	9	11	9	11	10	11	10	11	11	12	11	12
D7S820	12		12		10	12	10	12	9	13	9	13	8		8		8	14	8	14
D16S539	12	13	12	13	9	12	9	12	12	14	12	14	9	12	9	12	9	10	9	10
CSF1PO	12		12		7	9	7	9	10		10		12		12		9	11	9	11
Penta_D	9	12	9	12	11		11		9	14	9	14	9	11	9	11	9		9	
AMEL	X		X		X		X		X	Y	X	Y	X	Y	X	Y	X	Y	X	Y
vWA	14		14		16	18	16	18	16	17	16	17	18		18		16	17	16	17
D8S1179	11	15	11	15	11	16	11	16	12	15	12	15	14	17	14	17	12	14	12	14
TPOX	9	10	9	10	8	11	8	11	8	12	8	12	8	10	8	10	8	11	8	11
FGA	21	22	21	22	20	22	20	22	23	24	23	24	23	24	23	24	22		22	

Locus/Cells	P6- fibroblast		P6- iPSC		P7- fibroblast		P7- iPSC	
D3S1358	15	17	15	17	15	16	15	16
TH01	7	9	7	9	7	9	7	9
D21S11	30	30.2	30	30.2	30	32.2	30	32.2
D18S51	15	17	15	17	14	15	14	15
Penta_E	5	15	5	15	11	15	11	15
D5S818	10	12	10	12	10	11	10	11
D13S317	11	12	11	12	11		11	
D7S820	8	11	8	11	12		12	
D16S539	10	11	10	11	12	13	12	13
CSF1PO	11		11		9	12	9	12
Penta_D	9	11	9	11	9	12	9	12
AMEL	X		X		X		X	
vWA	14	17	14	17	17	18	17	18
D8S1179	10	15	10	15	10		10	
TPOX	9	11	9	11	8	9	8	9
FGA	20	22	20	22	20	25	20	25

Table S3. Profiles of the eleven control iPSC lines.

Clones		Age	Sex	Original Cells	Derivation Method	Reference
C1	TIG107 4F1	81Y	Female	Fibroblasts	Retrovirus OSKM	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013
C2	TIG114 4F1	36Y	Male	Fibroblasts	Retrovirus OSKM	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013
C3	TIG118 4F1	12Y	Female	Fibroblasts	Retrovirus OSKM	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013
C4	TIG119 4F1	6Y	Male	Fibroblasts	Retrovirus OSKM	
C5	TIG120 4F1	6Y	Female	Fibroblasts	Retrovirus OSKM	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013
C6	TIG120 3F7	6Y	Female	Fibroblasts	Retrovirus OSK	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013
C7	TIG121 4F4	6M	Male	Fibroblasts	Retrovirus OSKM	
C8	TIG975E4	6Y	Female	Fibroblasts	Episomal OSK, L-MYC, LIN28, and shRNA for TP53	
C9	TIG975E2	6Y	Female	Fibroblasts	Episomal OSK, L-MYC, LIN28, and shRNA for TP53	
C10	585B1	30s	Male	T cells	Episomal OSK, L-MYC, LIN28, and shRNA for TP53	Okita et al.,2013
C11	TIG107 3F1	81Y	Female	Fibroblasts	Retrovirus OSK	Tomoda et al., 2012; Koyanagi-Aoi et al., 2013

O, OCT4; S, SOX2; K, KLF4; M, C-MYC.

Table S4. The percentage of cells responding to each agonist, Related to Figures 3 and S10.

	Endothelial cells			Smooth muscle cells		
Agonist	ADPKD	Control	<i>P</i> -value	ADPKD	Control	<i>P</i> -value
ATP	97.2±2.0	99.3±2.0	0.45	52.5±9.7	59.2±8.5	0.61
CCh	ND	ND	ND	53.1±8.5	51.3±7.9	0.88
Vasopressin	ND	ND	ND	6.3±1.2	7.8±NA	0.24
Caffeine	2.9±9.1	13.2±7.6	0.40	2.6±1.3	5.1±1.3	0.20

The results are expressed as the means ± SEM. CCh, carbachol; ND, not done; NA, not applicable.

Table S5. The result of Ca²⁺ experiments using iPSC-derived vascular cells, Related to Figures 3 and S10.**(a) iPSC-derived endothelia**

	Resting [Ca ²⁺] _i (nM)	ATP				Caffeine			
		Release Δ[Ca ²⁺] _i (nM)	Influx Δ[Ca ²⁺] _i (nM)	Resp (%)	Number of analyzed cells (Number of experiments)	Release Δ[Ca ²⁺] _i (nM)	Influx Δ[Ca ²⁺] _i (nM)	Resp (%)	Number of analyzed cells (Number of experiments)
P1	52.4	1253.8	204.3	100.0	419(5)	67.5	52.7	2.5	394(5)
P2	41.4	1139.4	159.9	100.0	157(2)	0.0	34.8	0.0	171(2)
P3	42.1	2426.6	185.3	100.0	188(2)	160.2	46.4	1.5	200(2)
P4	50.0	1199.8	177.8	100.0	225(3)	48.8	92.6	2.8	258(3)
P5	78.1	898.8	130.6	84.2	300(5)	136.7	94.9	7.5	200(3)
P6	27.0	1262.0	177.7	99.0	100(1)	0.0	78.8	0.0	100(1)
P7	57.8	761.6	103.2	100.0	100(1)	228.7	44.3	6.0	100(1)
C1	32.3	1485.6	468.7	100.0	100(2)	288.3	174.2	100.0	100(2)
C2	48.0	902.0	161.3	99.0	100(1)	0.0	152.1	0.0	100(1)
C3	66.6	1646.4	289.2	100.0	188(4)	0.0	87.8	0.0	182(2)
C4	85.8	1330.1	192.9	98.8	337(4)	26.1	65.2	2.7	337(4)
C5	83.2	1006.1	219.4	100.0	100(3)	105.5	50.7	21.0	100(1)
C6	54.7	1270.3	212.8	100.0	100(1)	0.0	41.9	0.0	100(1)
C7	64.9	2751.3	276.6	100.0	87(1)	33.6	239.8	2.0	100(1)
C8	58.1	2234.9	277.6	98.7	177(2)	28.7	39.5	0.7	171(2)
C9	39.8	1077.0	253.7	99.0	300(3)	80.0	109.8	5.7	300(3)
C10	56.5	990.7	203.8	100.0	100(1)	0.0	61.3	0.0	82(1)

(b) iPSC-derived smooth muscle cells

	Resting [Ca ²⁺] _{cyt} (nM)	ATP				CCh			
		Release Δ[Ca ²⁺] _i (nM)	Influx Δ[Ca ²⁺] _i (nM)	Resp (%)	Number of analyzed cells (Number of experiments)	Release Δ[Ca ²⁺] _i (nM)	Influx Δ[Ca ²⁺] _i (nM)	Resp (%)	Number of analyzed cells (Number of experiments)
P1	56.3	106.8	10.3	67.0	96(1)	91.4	10.2	45.0	95(1)
P2	63.6	157.6	4.4	53.1	100(2)	79.7	11.9	64.0	75(1)
P3	56.7	117.9	14.2	78.0	100(1)	67.3	14.9	47.0	87(1)
P4	80.2	123.6	18.1	59.5	82(1)	120.9	15.4	87.0	89(1)
P5	55.9	135.5	10.7	92.0	100(2)	101.5	10.3	91.0	100(1)
P6	62.9	113.7	10.8	5.0	100(1)	71.8	12.6	21.0	100(1)
P7	63.3	44.5	13.0	12.5	88(1)	76.1	12.6	17.0	98(1)
C1	64.5	105.2	16.8	78.5	100(2)	57.3	14.0	37.0	91(2)
C2	53.5	110.3	18.9	55.0	62(2)	119.3	14.2	60.0	96(2)
C3	50.8	63.6	18.1	73.0	100(1)	71.3	22.5	71.0	100(1)
C4	78.8	102.4	11.7	69.0	36(1)				
C5	54.5	64.3	16.3	49.4	100(1)	65.8	16.8	43.0	89(3)
C6	54.4								
C7	58.0	79.6	15.7	56.0	100(2)	88.1	16.6	62.0	100(4)
C8	61.3	58.8	23.1	47.0	100(1)	58.8	15.7	55.0	100(1)
C9	55.2	45.9	15.9	22.0	100(1)	33.1	14.3	28.0	200(2)
C10	83.7					99.8	16.6	55.0	94(1)
C11	69.3	127.9	27.9	83.0	100(1)				

	Vasopressin				Caffeine			
	Release $\Delta[\text{Ca}^{2+}]_i$ (nM)	Influx $\Delta[\text{Ca}^{2+}]_i$ (nM)	Resp (%)	Number of analyzed cells (Number of experiments)	Release $\Delta[\text{Ca}^{2+}]_i$ (nM)	Influx $\Delta[\text{Ca}^{2+}]_i$ (nM)	Resp (%)	Number of analyzed cells (Number of experiments)
P1	44.1	39.3	4.7	438(5)	6.9	29.8	2.4	478(5)
P2	21.9	27.3	7.4	353(4)	13.7	14.7	3.3	350(4)
P3	39.7	40.1	8.2	462(5)	23.6	27.1	2.8	500(6)
P4	0.0	26.5	0.0	36(1)	13.9	23.8	1.0	36(1)
P5	41.1	32.4	15.7	290(4)	35.8	35.7	6.5	200(3)
P6	33.3	42.3	3.7	486(5)	13.0	35.3	1.9	583(6)
P7	40.4	26.3	3.2	210(3)	10.4	35.0	0.4	204(3)
C1	102.2	43.7	16.7	180(3)	7.8	24.1	3.0	114(2)
C2	53.5	25.0	7.9	339(4)	63.0	18.3	2.8	78(2)
C3	50.8	23.7	7.8	664(8)	62.7	29.9	5.0	248(4)
C4	6.0	41.4	11.7	310(4)	0.0	40.5	0.0	56(2)
C5	43.2	31.2	4.7	384(8)	3.5	19.5	1.1	237(4)
C6	37.3	43.5	3.1	200(2)	27.6	23.8	11.0	200(2)
C7	72.1	45.3	11.1	252(6)	22.0	17.3	15.2	79(2)
C8	22.3	39.8	8.7	394(4)	15.5	47.4	1.1	178(2)
C9	32.4	26.9	3.9	385(4)	139.8	16.0	4.7	92(1)
C10	0.0	19.8	0.0	54(1)				
C11								

Resp (%); the percentage of cells responding to each agonist.

Table S6. The results of the microarray analyses comparing the gene expression profiles of iPSC-derived vascular endothelia from ADPKD patients with intracranial aneurysms and those from ADPKD patients without ICAs, Related to Figure 5.

The gene expression profiles of iPSC-derived vascular endothelia from four ADPKD patients with ICAs (P1, P3, P4 and P7) were individually compared with those from three patients without ICAs (P2, P5 and P6). From these 12 comparisons, genes with an average fold change in expression of more than two were selected.

(a) List of 17 candidate genes

Gene Symbol	Description	RefSeq	Probe ID	Localization		Gene chip score							uncorrected <i>P</i> -value (t-test)
				Signal peptide	14	P1	P2	P3	P4	P5	P6	P7	
SPOCD1	SPOC domain containing 1	NM_144569.4	7914467	No	0	537.5	360.5	541.9	806.3	265.6	335.3	1546.5	9.78E-03
PLXNA2	Plexin A2	NM_025179.3	7923991	Yes	3	1800.6	1703.1	2112.7	2299.3	579.0	1122.0	2756.8	1.50E-02
MYEOV	Myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)	NM_138768.2	7942118	No	3	153.4	152.1	160.3	327.9	148.4	126.2	629.0	9.95E-02
MMP10	Matrix metalloproteinase 10 (stromelysin 2)	NM_002425.2	7951259	Yes	1	197.6	120.3	135.1	140.6	65.9	78.7	1042.8	6.36E-02
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	NM_002421.3	7951271	Yes	0	6827.3	1050.7	4954.3	4125.5	1487.5	506.8	12831.2	5.27E-04
E2F7	E2F transcription factor 7	NM_203394.2	7965094	No	0	519.6	463.1	791.4	1132.5	431.7	348.9	1716.8	1.49E-02
SLC30A3	Solute carrier family 30 (zinc transporter), member 3	NM_003459.4	8051050	No	5	362.9	220.6	490.6	630.4	215.3	239.0	524.0	4.25E-04
SNAI1	Snail homolog 1 (Drosophila)	NM_005985.3	8063382	No	0	939.7	223.1	463.3	792.3	279.9	314.0	352.6	1.32E-02
NR2F1	Nuclear receptor subfamily 2,	NM_005654.4	8106923	No	0	930.3	427.0	933.4	1066.8	553.2	614.7	1258.0	5.99E-04

	group F, member 1												
PCSK1	Proprotein convertase subtilisin/kexin type 1	NM_000439.4	8113234	Yes	0	1629.9	569.6	691.7	1169.1	670.8	370.1	2188.3	1.21E-02
BMP6	Bone morphogenetic protein 6	NM_001718.4	8116818	Yes	0	3127.2	1772.9	2943.1	3535.6	1475.8	1403.5	3026.6	6.07E-05
EDN1	Endothelin 1	NM_001955.4	8116921	Yes	0	7147.1	4570.0	9345.0	8857.1	3981.2	2327.3	9656.2	8.33E-04
CTGF	Connective tissue growth factor	NM_001901.2	8129562	Yes	0	10662.0	7513.3	8142.4	9285.0	2361.1	3284.2	11640.2	7.81E-03
TFPI2	Tissue factor pathway inhibitor 2	NM_006528.3	8141016	Yes	0	9092.9	2006.5	2415.7	3074.4	1457.4	1849.8	3198.4	3.44E-02
NRCAM	Neuronal cell adhesion molecule	NM_001037132.2	8142270	Yes	2	551.3	139.1	285.1	310.7	54.8	90.7	1129.2	2.10E-03
CD274	CD274 molecule	NM_014143.3	8154233	Yes	2	228.3	111.0	135.3	122.0	53.8	77.8	517.2	2.59E-02
FRMD3	FERM domain containing 3	NM_174938.5	8161964	No	1	128.2	127.2	223.6	367.1	93.3	117.7	258.0	1.66E-02

(b) Fold change in the 12 comparisons.

	SPOCD1			PLXNA2			MYEOV			MMP10			MMP1			E2F7		
	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6
P1	1.49	2.02	1.60	1.06	3.11	1.60	1.01	1.03	1.22	1.64	3.00	2.51	6.50	4.59	13.5	1.12	1.20	1.49
P3	1.50	2.04	1.62	1.24	3.65	1.88	1.05	1.08	1.27	1.12	2.05	1.72	4.72	3.33	9.78	1.71	1.83	2.27
P4	2.24	3.04	2.40	1.35	3.97	2.05	2.16	2.21	2.60	1.17	2.13	1.79	3.93	2.77	8.14	2.45	2.62	3.25
P7	4.29	5.82	4.61	1.62	4.76	2.46	4.14	4.24	4.98	8.66	15.8	13.2	12.2	8.63	25.3	3.71	3.98	4.92
	SLC30A3			SNAI1			NR2F1			PCSK1			BMP6			EDN1		
	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6
P1	1.65	1.69	1.52	4.21	3.36	2.99	2.18	1.68	1.51	2.86	2.43	4.40	1.76	2.12	2.23	1.56	1.79	3.07
P3	2.22	2.28	2.05	2.08	1.66	1.48	2.19	1.69	1.52	1.21	1.03	1.87	1.66	1.99	2.10	2.04	2.35	4.02
P4	2.86	2.93	2.64	3.55	2.83	2.52	2.50	1.93	1.74	2.05	1.74	3.16	1.99	2.40	2.52	1.94	2.22	3.81
P7	2.38	2.43	2.19	1.58	1.26	1.12	2.95	2.27	2.05	3.84	3.26	5.91	1.71	2.05	2.16	2.11	2.43	4.15
	CTGF			TFPI2			NRCAM			CD274			FRMD3					
	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6			
P1	1.42	4.52	3.25	4.53	6.24	4.92	3.96	10.1	6.08	2.06	4.25	2.94	1.01	1.37	1.09			
P3	1.08	3.45	2.48	1.20	1.66	1.31	2.05	5.20	3.14	1.22	2.52	1.74	1.76	2.40	1.90			
P4	1.24	3.93	2.83	1.53	2.11	1.66	2.23	5.67	3.43	1.10	2.27	1.57	2.89	3.93	3.12			
P7	1.55	4.93	3.54	1.59	2.19	1.73	8.12	20.6	12.5	4.66	9.62	6.65	2.03	2.76	2.19			

Table S7. The results of the microarray analyses comparing the gene expression profiles of iPSC-derived vascular smooth muscle cells from ADPKD patients with intracranial aneurysms and those from ADPKD patients without ICAs, Related to Figure 5.

The gene expression profiles of the iPSC-derived vascular smooth muscle cells from four ADPKD patients with ICAs (P1, P3, P4 and P7) were individually compared with those from three patients without ICAs (P2, P5 and P6). From these 12 comparisons, genes with an average fold change in expression of more than two were selected.

(a) List of 5 candidate genes

Gene Symbol	Description	RefSeq	Probe ID	Localization		Gene chip score							uncorrected <i>P</i> -value (t-test)
				Signal peptide	SOSUI	P1	P2	P3	P4	P5	P6	P7	
MYPN	Myopalladin	NM_032578	7927827	No	0	78.9	58.3	231.8	67.5	50.2	54.5	101.5	5.98E-02
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	NM_002421	7951271	Yes	0	481.6	243.1	1209.4	565.0	137.3	126.2	556.5	1.32E-03
HMGA2	High mobility group AT-hook 2	NM_000274	7956867	No	0	2892.3	916.3	2866.5	2468.6	1245.0	2167.0	2584.4	9.37E-03
RPPH1	Ribonuclease P RNA component H1	NR_002312.1	7977507	*	*	2034.5	1223.7	3236.0	3408.7	954.7	1125.7	1804.0	2.56E-03
TFPI2	Tissue factor pathway inhibitor 2	NM_006528	8141016	Yes	0	433.1	280.8	3923.8	1776.3	130.2	310.4	1706.2	8.28E-03

*: not available.

(b) Fold change in the 12 comparisons.

	MYPN			MMP1			HMGA2			RPPH1			TFPI2		
	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6	P2	P5	P6
P1	1.35	1.57	1.45	1.98	3.51	3.82	3.16	2.32	1.33	1.66	2.13	1.81	1.54	3.33	1.40
P3	3.97	4.61	4.25	4.97	8.81	9.59	3.13	2.30	1.32	2.64	3.39	2.87	14.0	30.1	12.6
P4	1.16	1.34	1.24	2.32	4.12	4.48	2.69	1.98	1.14	2.79	3.57	3.03	6.33	13.6	5.72
P7	1.74	2.02	1.86	2.29	4.05	4.41	2.82	2.08	1.19	1.47	1.89	1.60	6.08	13.1	5.50

Table S8. Profiles of ADPKD patients whose serum MMP1 levels were examined, Related to Figure 5.

	ICA (+)	ICA (-)
N	97	257
MMP1 (ng/mL), mean $\pm$ SD (range)	17.6 $\pm$ 14.9 (2.1-94)	14.0 $\pm$ 12.1 (1.8-101)
Age (Y), mean $\pm$ SD (range)	58.8 $\pm$ 10.6 (38-83)	54.8 $\pm$ 12.3 (22-81)
Male, n (%)	31 (32)	114 (44)
Family history of ICAs or SAH, n (%)	32 (33)	54 (21)
Creatinine (mg/dL), mean $\pm$ SD (range)	4.92 $\pm$ 3.5 (0.4-13.1)	4.02 $\pm$ 3.6 (0.5-15.9)
Dialysis therapy, n (%)	54 (56)	103 (40)

ICA, intracranial aneurysm; SAH, subarachnoid hemorrhage.

Table S9. Correlation between complicated intracranial aneurysms and serum MMP1 levels in ADPKD patients, Related to Figure 5.

	Univariate analysis				
Risk factors	Odd ratio	95% CI	<i>P</i> -value	AUC	AIC
MMP1 $\geq$ 15 ng/mL	2.031	1.260-3.274	0.0036	0.584	410.67
Age $\geq$ 65Y	1.799	1.072-3.020	0.026	0.557	414.27
Sex (female)	1.709	1.044-2.797	0.033	0.562	414.41
Family history of ICAs or SAH	1.842	1.096-3.095	0.021	0.559	413.91
Creatinine $\geq$ 2 mg/dL	2.096	1.281-3.429	0.0032	0.588	410.05
Dialysis therapy	1.865	1.163-2.991	0.0096	0.577	412.34

ICA, intracranial aneurysm; SAH, subarachnoid hemorrhage; AUC, area under the curve; AIC, Akaike's information criterion.

Table S10. Sequences of primers used in this study.

Gene	Forward primer; 5'-to-3'	Reverse primer; 5'-to-3'
hOCT4 Tg	GCTCTCCCATGCATTCAAAGTGA	CCC TTT TTC TGG AGA CTA AAT AAA
hSOX2 Tg	TTCACATGTCCCAGCACTACCAGA	GACATGGCCTGCCCCGGTTATTATT
hKLF4 Tg	CCACCTCGCCTTACACATGAAGA	GACATGGCCTGCCCCGGTTATTATT
hcMYC Tg	ATACATCCTGTCCGTCCA AGCAGA	GACATGGCCTGCCCCGGTTATTATT
hOCT4 Total	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
hSOX2 Total	TTCACATGTCCCAGCACTACCAGA	TCACATGTGTGAGAGGGGCAGTGTGC
hKLF4 Total	GATTACGCGGGCTGCGGCAAAACCTACACA	TTAAAAATGTCTCTTCATGTGTAAGGCGAG
hcMYC Total	ATACATCCTGTCCGTCCAAGCAGA	TCACGCACAAGAGTTCCGTAGCTGTTCAAG
hPKD1	AGGACTACGAGATGGTGGAGTT	CATCCCTTCAAAGCGGACTT
hPKD2	GATTATGGAGCGAGCCAAAC	AGCGTTCCAACTCTTCACGTA
hMMP1	CTGGGAGCAAACACATCTGA	AGTTCATGAGCTGCAACACG
hTFPI2	GTCGATTCTGCTGCTTTTCC	CACTGGTCGTCCACACTCAC
hMMP10	CCAGTCTGCTCTGCCTATCC	AACGTCAGGAAGTCCACACC
hCTGF	ACTGTCCCGGAGACAATGAC	TGCTCCTAAAGCCACACCTT
hNRCAM	CCAGTCCATTCTGGGTCCTA	TGGCATGCTGTTGTATGCTT
hCD274	GCCACCAGCTGTCATCACTA	CCAACACCACAAGGAGGAGT

hSLC30A3	TGCAGGTCGAGCAGTATCAG	GGTGGTAGGAGGGAGAGAGG
hNR2F1	AGAAGCTCAAGGCGCTACAC	CCTACCAAACGGACGAAGAA
hBMP6	TTCGTGCTGGAGTTTTGTTG	TGTGGCGTGGTATGCTGTAT
hEDN1	CCATGAGAAACAGCGTCAAA	ATGGAAGCCAGTGAAGATGG
FRMD3	GAGGGTGTTCCATTGCCTAA	CATAGCTCCACGACAAAGCA
hPLAXNA2	TGCAAAGTGGCTTCACAGAC	GCTGTGATCGTGTCTCAGGA
hPCSK1	CATGGCAACAGTTAGCTGGA	TGCGGGTAGTTTGT TTTTCC
hE2F7	CGGGTTGGTTGTTCTAGCAT	GGAAGGAGGGAAGGAAGAGA
hSNAI1	GGTTCTTCTGCGCTACTGCT	TAGGGCTGCTGGAAGGTAAA
hSPOCD	GCCGCCTGCTCTACTCATAC	AAGGAGAGGGGAGACAGGAA
hHMGA2	AGCAGAAGCCACTGGAGAAA	GTGCCATATGGGGTAGCAGT
hRPPH1	GTCACTCCACTCCCATGTCC	GGCGGAGGAGAGTAGTCTGA
hCXCR4	GCCTTATCCTGCCTGGTATTGTC	GCGAAGAAAGCCAGGATGAGGAT
hDLL4	GTGGACTGTGGCCTGGACAA	AGCATATCGCTGATATCCGACACTCTG
hVE-cadherin	ACACCTCACTTCCCCATCA	GACCTTGCCACATATTCTCC
hVWF	GGGGTCATCTCTGGATTCAA	AGGCAAACATCTCCCACAAC
hPECAM1/CD31	CAACGAGAAAATGTCAGA	GGAGCCTTCCGTTCTAGAGT
hCalponin	CTTCATGGACGGCCTCAAAGA	GTAGTTGTGTGCGTGGTGGTT
hCaldesmon	AGACAAGGAAAGAGCTGAGGCA	GCTGCTTGTTACGTTTCTGCTC

h α -SMA	CACTGCCTTGGTGTGTGACAAT	CGTAGCTGTCTTTTTGTCCCATTC
hNANOG	CCTGAAGACGTGTGAAGATGAG	GCTGATTAGGCTCCAACCATAC
hDPPA4	AGTGCCTGTTGCTTTGTGAGT	TGCACTGAACTGAGATTGCAC
hREX1	AAAGCATCTCCTCATTCATGGT	TGGGCTTTCAGGTTATTTGACT
hGDF3	CACCGTCACCAGCTATTCATTA	GTAGAGCATGGAAATGGGAGAC
hTERT	TGAAAGCCAAGAACGCAGGGATG	TGTCGAGTCAGCTTGAGCAGGAATG
β -ACTIN	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
PKD1(G3818R)	TAGTTCTCCAGGAGTGCCGC	CCGAGGTGAGCAGAGGCAG
PKD1(Q3895X)	AGGTGTGCCTGCTGCTGTT	AGTGAGGGCGTACAGCTGA
PKD1(G3818R), RFLP	TGCTGTTTCGCCGTGCACTTCGCCGT	TAGTGAAGCGGCGCGGGC
PKD1(Q3895X), RFLP	TAGTTCTCCAGGAGTGCCGC	CCGAGGTGAGCAGAGGCAG
F26	AGCGCAACTACTTGGAGGCCC	
R2LR	GCAGGGTGAGCAGGTGGGGCCATCCTAC	
PKDex15.14	ATCATTGAGGGTGGCTCATAC	(6-Fam)TTCTCTGGGCTCATGGGTGT
PKD1 exon1	CGCAGCCTTACCATCCACCT	TCATCGCCCCTTCCTAAGCA
PKD1 exon2-7	CCCCGAGTAGCTGGAACTACAGTTACACACT	CGTCCTGCTGTGCCAGAGGCG
PKD1 exon8-12	ACGTCTGCGAGCTGCAGCCC	CTGCAGGGACAGGCGTCAGTGA
PKD1 exon13-15	TGGAGGGAGGGACGCCAATC	GTCAACGTGGGCCTCCAAGT
PKD1 exon15-21	ATCCCTGGGGGTCCTACCATCTCTTA	ACACAGGACAGAACGGCTGAGGCTA

PKD1 exon22-26	ATGCTTAGTGAGGAGGCTGTGGGGGTCCA	CGCTTAAGGGGAATGGCTTAAACCCG
PKD1 exon27-34	CGGGTCACCGGTTGTGGCA	ATGAGGCTCTTTCCACAGACAACAGAGGTT
PKD1 exon35-41	CAAGAGGCTCAAGAAACTGCCCCG	GGGCTGTGGAAGCCGCCTA
PKD1 exon42-46	GAGTAGTTCTCCAGGAGTGCCG	ATTCTGCCTGGCCCTCGGCCTT

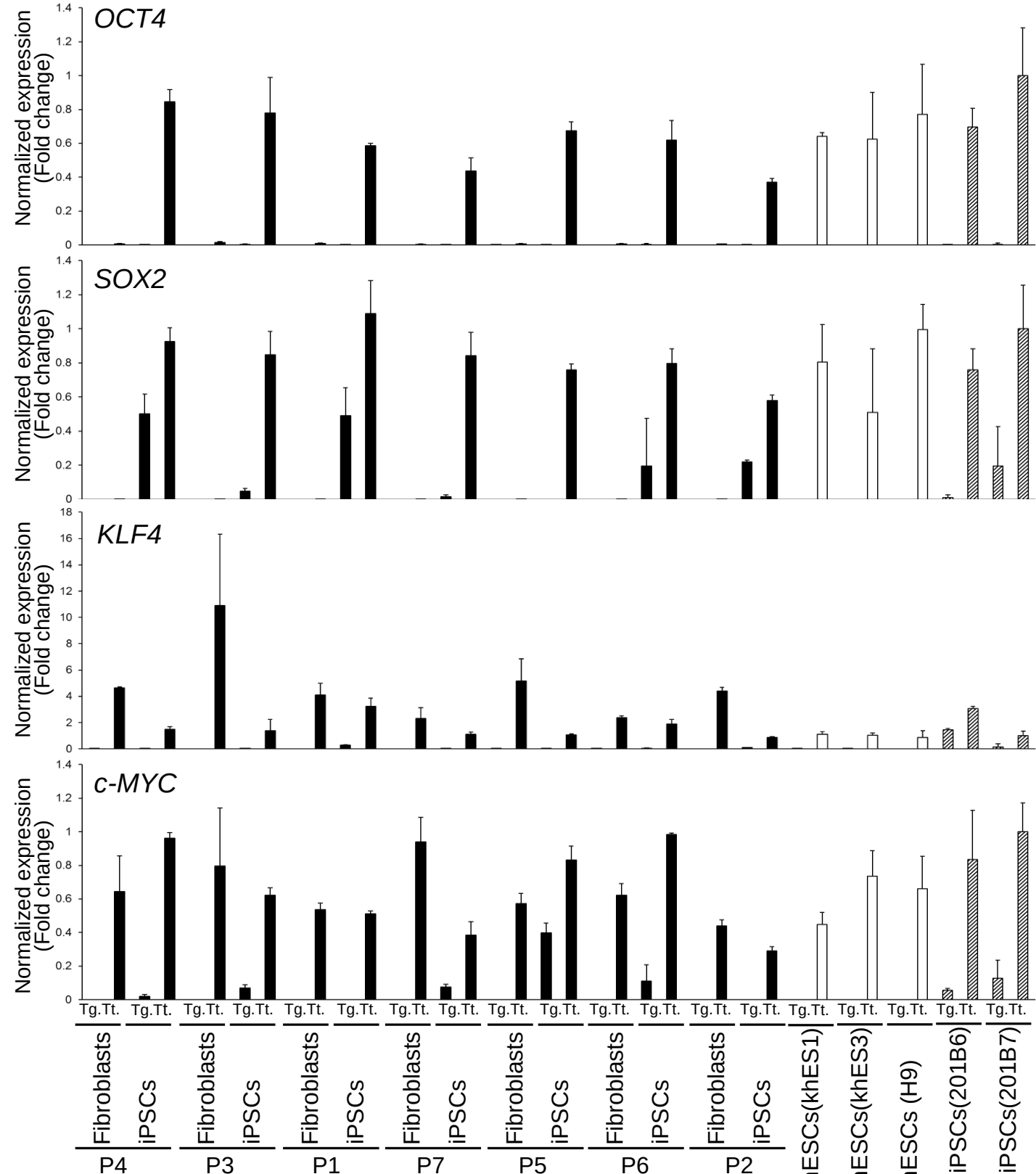


Figure S1. Retroviral transgene silencing in ADPKD-iPSC lines, Related to Figure 1. The results of the qRT-PCR analyses for the retroviral transgene (Tg) and total (Tt; the sum of the transgene and endogenous) expression of *OCT4*, *SOX2*, *KLF4* and *c-MYC* in ADPKD-iPSC lines and their parental fibroblasts from all seven patients (P1-7). hESC lines (khES1, khES3 and H9) and hiPSC lines (201B6 and 201B7) were used as controls. Each value was normalized to the samples from 201B7. Note that the expression level of Tg was so low in many samples that the histogram bars are at the baseline. All values are the means $\pm$ SD of triplicate samples from three independent experiments.

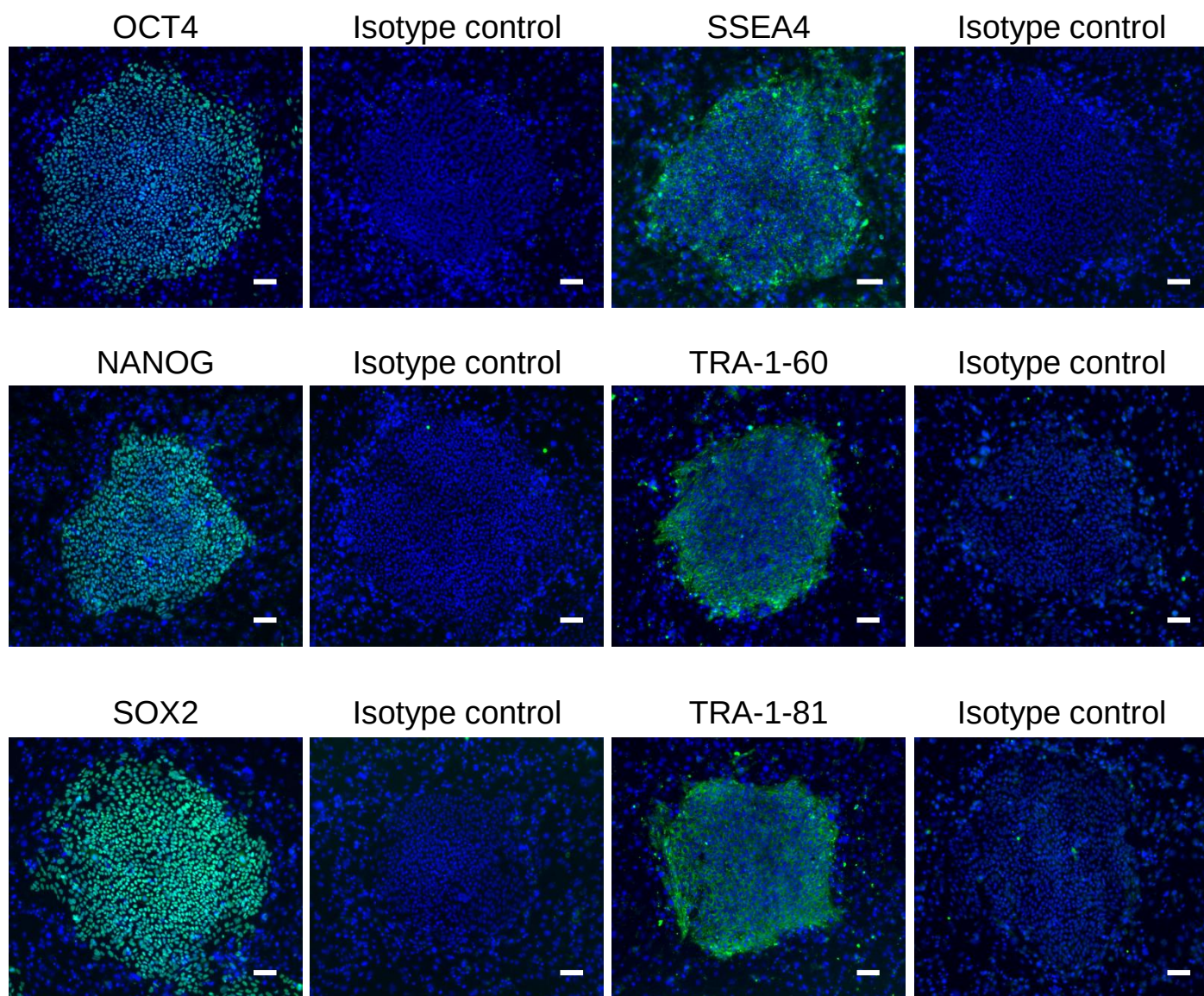


Figure S2. Expression of pluripotent marker genes, Related to Figure 1. The expression of pluripotency markers, such as OCT4, NANOG, SOX2, SSEA4, TRA-1-60 and TRA-1-81 in P4-iPSCs. Isotype control antibodies were used at the same concentrations as the tested antibodies. Scale bars, 100 μm.

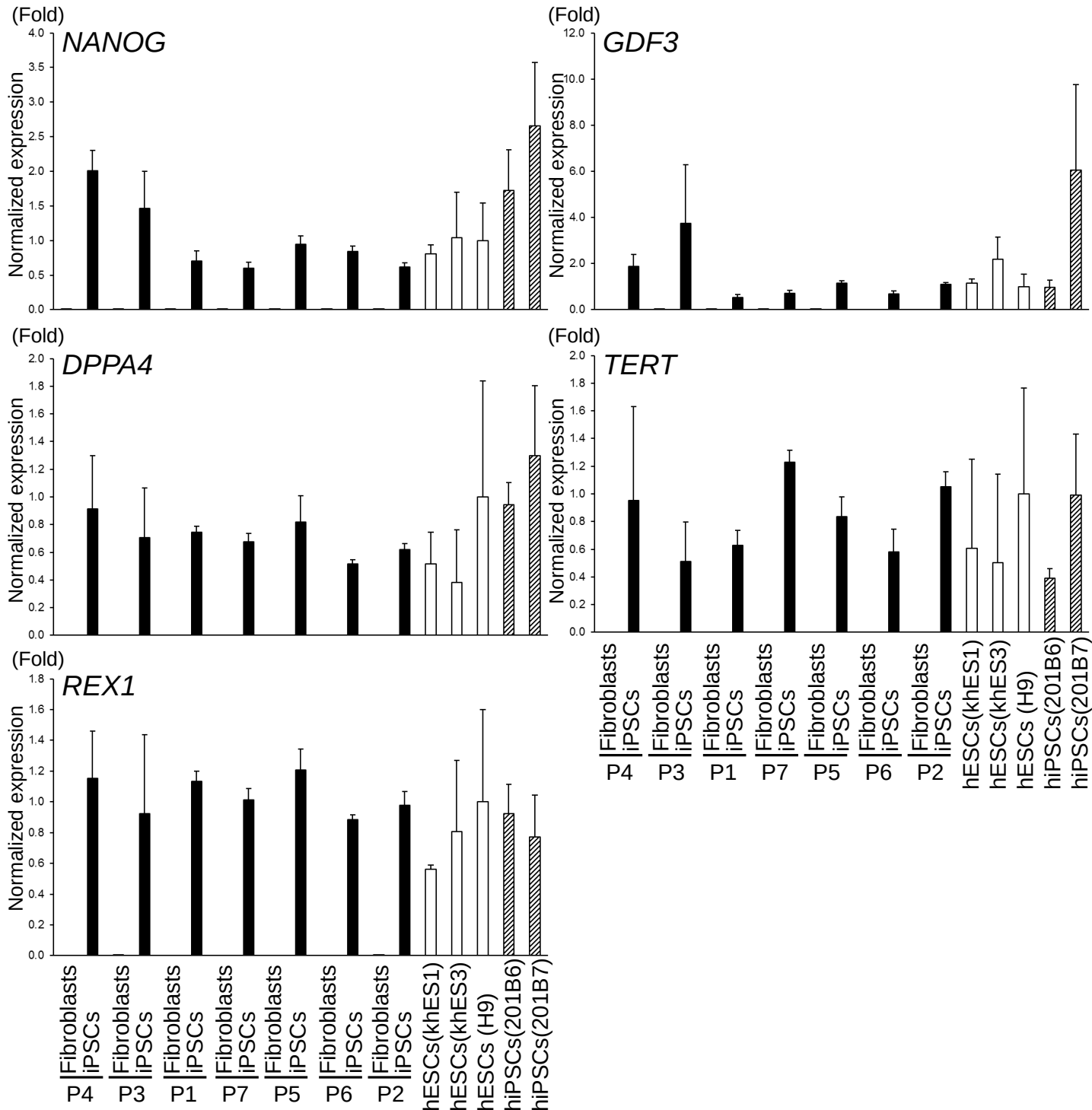


Figure S3. Gene expression analysis of ADPKD-iPSC lines, Related to Figure 1. qRT-PCR analyses were used to evaluate the expression of *NANOG*, *DPPA4*, *REX1*, *GDF3* and *TERT* in ADPKD-iPSC lines from all seven patients (P1-7) and their parental fibroblasts. Three hESC lines (H9, khES1 and khES3) and two hiPSC lines (201B6 and 201B7) were used as positive controls for the pluripotency gene expressions. Each value was normalized to the samples from H9. Note that the expression level of the five genes in fibroblast samples was so low that the histogram bars are at the baseline. All values are the means $\pm$ SD of triplicate samples from three independent experiments.

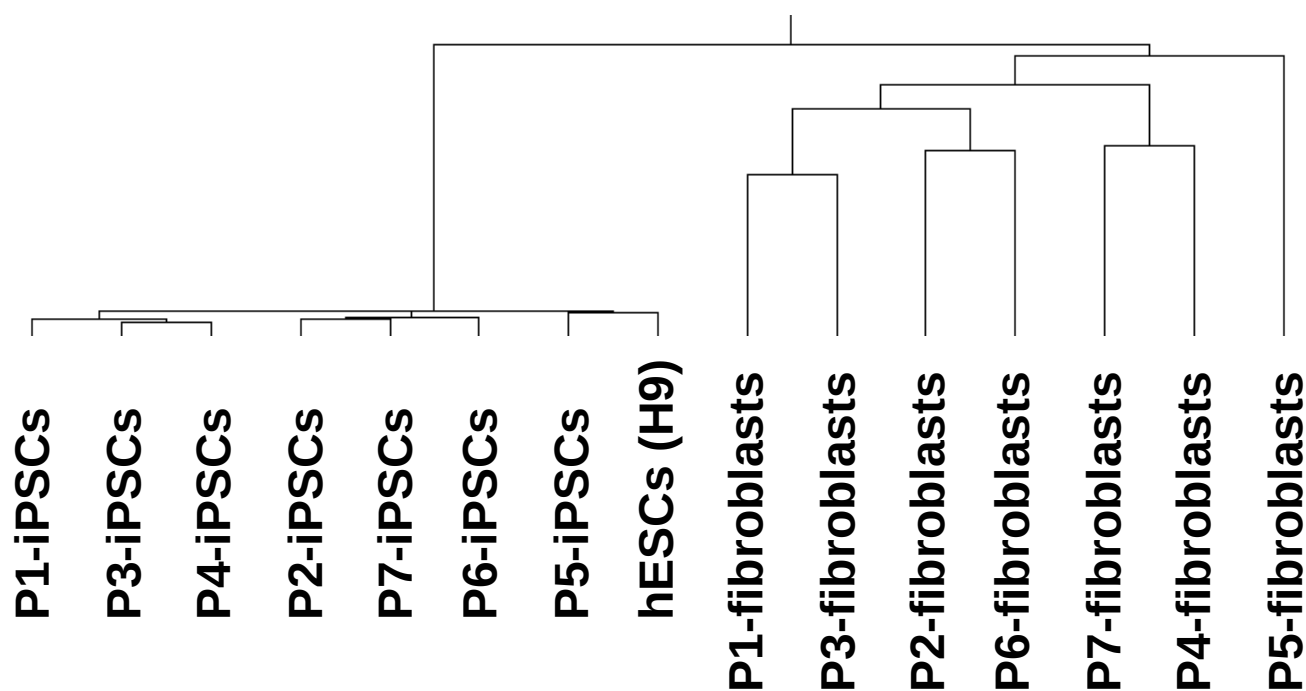
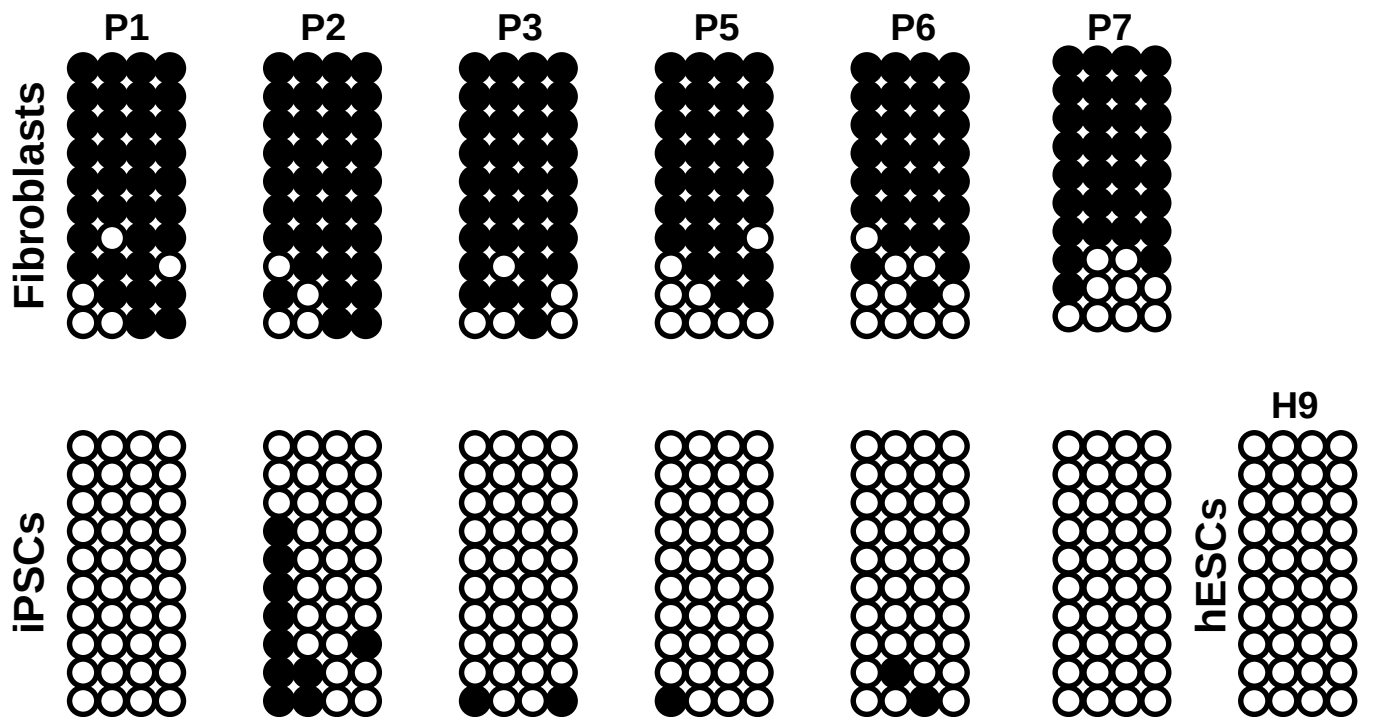


Figure S4. Global gene expression analyses of ADPKD-iPSC lines, Related to Figure 1. The microarray analysis and hierarchical clustering showed that the global gene expression profiles of ADPKD-iPSCs were more similar to those of hESCs (H9) than to those of the parental fibroblasts.

POU5F1 (OCT4)



NANOG

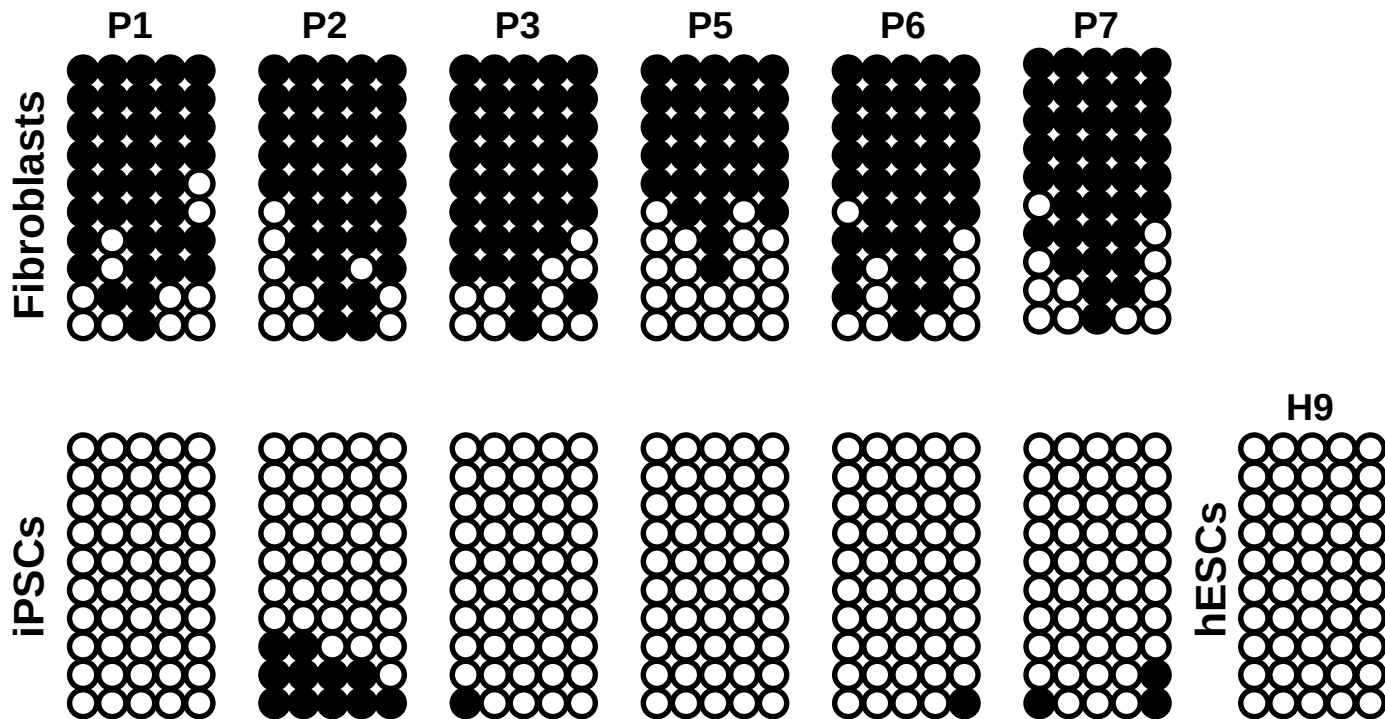


Figure S5. Methylation analyses of the *OCT4* and *NANOG* promoters in ADPKD-iPSC lines, Related to Figure 1. Bisulphite sequencing analyses of the *OCT4* and *NANOG* promoters in ADPKD-iPSC lines from six patients (P1-3 and P5-7) and their parental fibroblasts. The hESC line (H9) was used as a control.

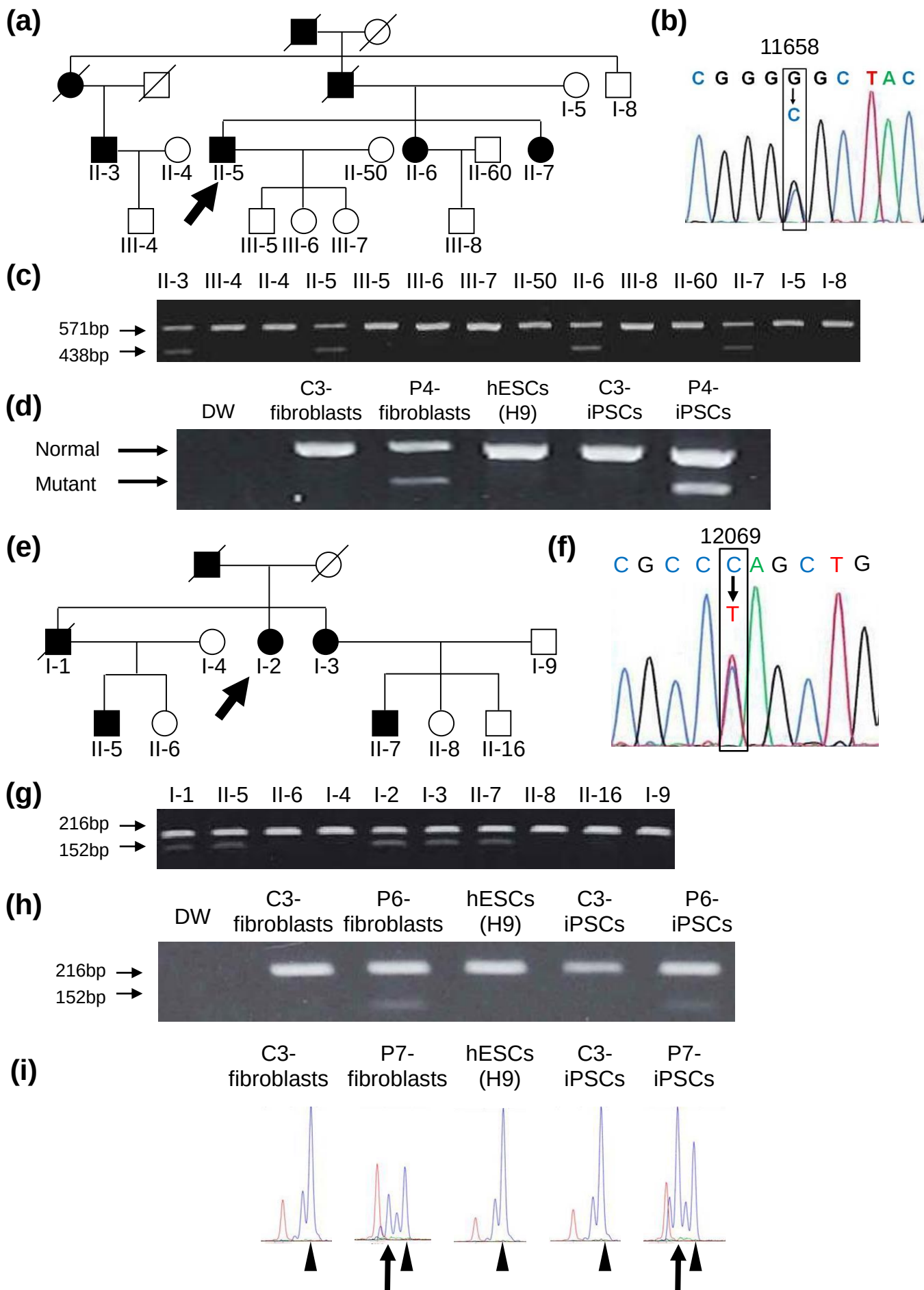


Figure S6. Mutation analyses, Related to Figure 1. (a) ADPKD patient P4 and pedigree. Solid symbols indicate affected members. The arrow indicates the patient (II-5). (b) Sequencing results showed the presence of 11658G>C mutation in *PKD1* in P4. (c) PCR RFLP verification of the mutation in genomic DNA from fresh peripheral blood from P4 and other members of the pedigree. A RFLP analysis was done using *BfaI*. (d) PCR RFLP verification of the mutation in P4-iPSCs, P4-fibroblasts, control (C3)-iPSCs, hESCs (H9) and C3-fibroblasts. (e) Patient P6 and pedigree. Solid symbols indicate affected members. The arrow indicates the patient (I-2). (f) The sequencing results showed the presence of 12069C>T mutation in *PKD1* in P6. (g) PCR RFLP verification of the mutation in genomic DNA from fresh peripheral blood from P6 and other members of the pedigree. A RFLP analysis was done using *HaeII*. (h) PCR RFLP verification of the mutation in P6-iPSCs, P6-fibroblasts, C3-iPSCs, H9 and C3-fibroblasts. (i) Detection of a 2-bp deletion (7024del AC) in exon 15 of *PKD1* in patient P7.¹ The expected size of the patient allele was 202 bp (arrows), while the normal allele was 204 bp (arrowheads). Both P7-iPSCs and P7-fibroblasts showed the same genotype of 202/204, while C3-iPSCs, C3-fibroblasts and H9 showed the genotype of 204/204. Red waves indicate 200 bp.

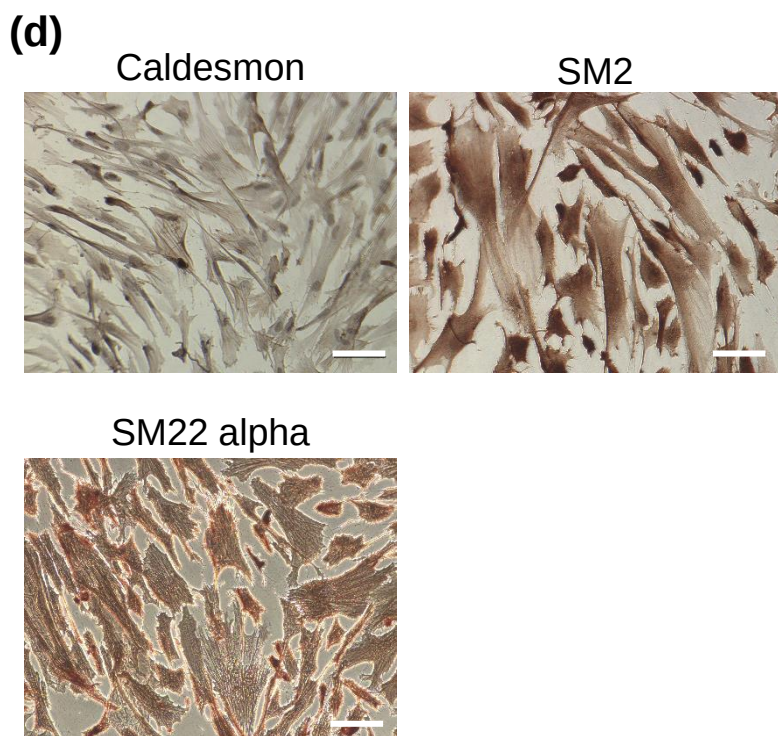
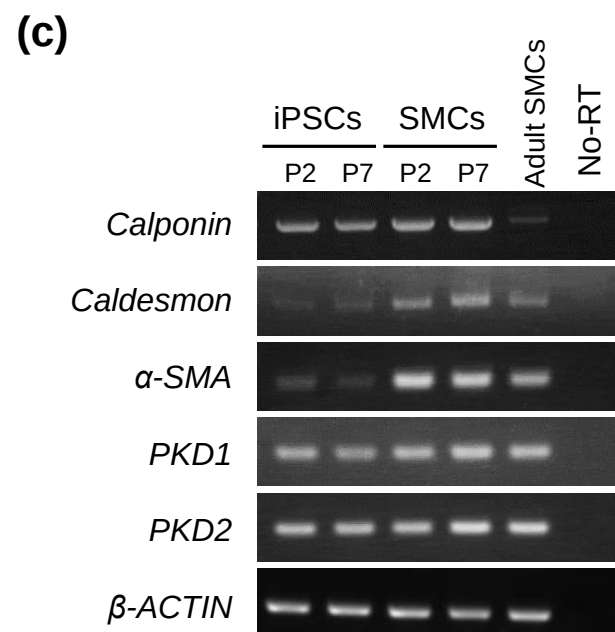
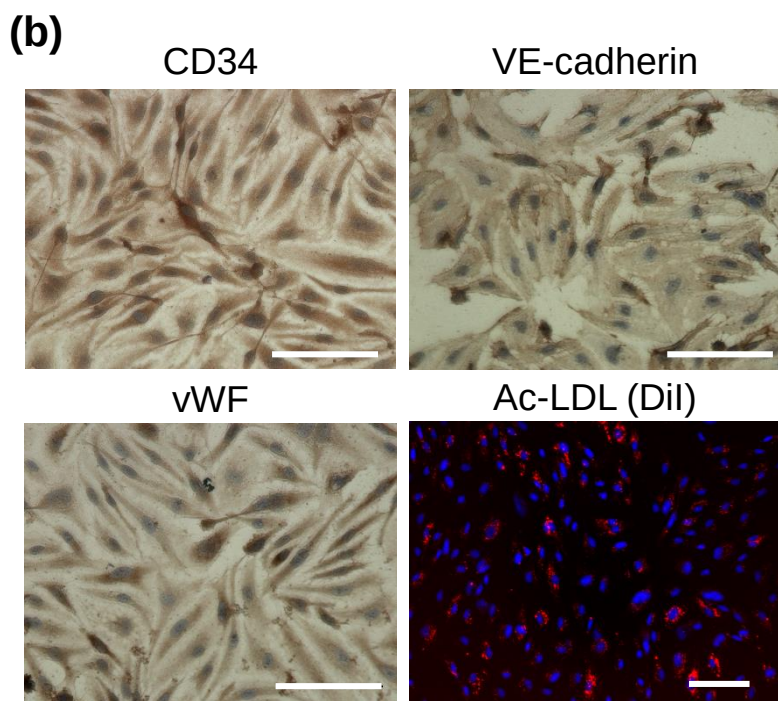
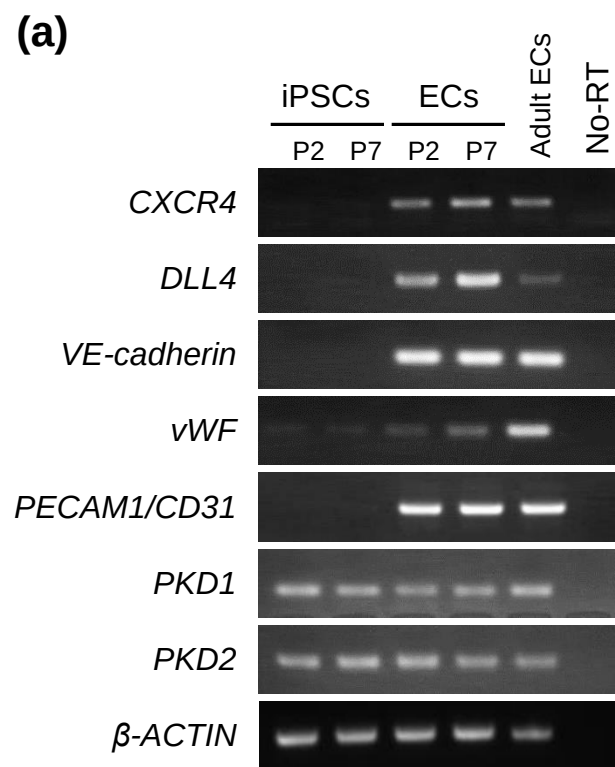
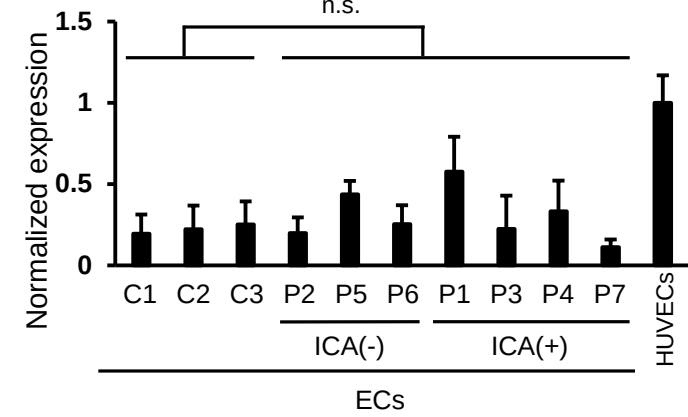


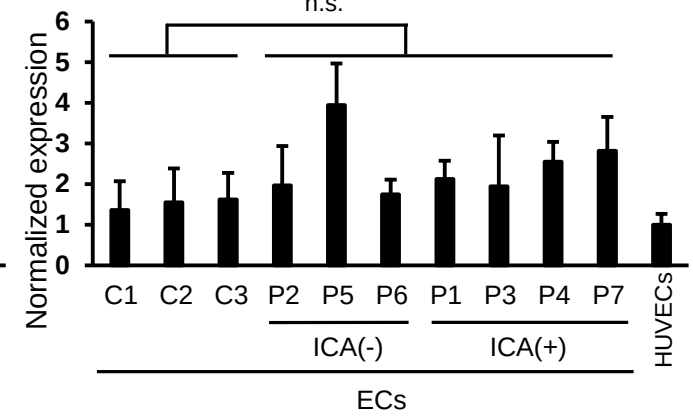
Figure S7. Directed differentiation of ADPKD-iPSCs into vascular endothelia and smooth muscle cells, Related to Figure 2. (a) The expression of endothelial marker genes (*CXCR4*, *DLL4*, *VE-cadherin*, *vWF* and *PECAM1/CD31*) and *PKD1* and *PKD2* in vascular endothelia derived from two ADPKD-iPSC lines (P2- and P7-iPSCs). (b) Immunostaining of vascular endothelia on culture day 12 differentiated from P4-iPSCs for endothelial markers, CD34, VE-cadherin and von Willebrand factor (vWF) , and the uptake of DiI labeled acetylated low density lipoprotein (DiI-Ac-LDL). (c) The expression of smooth muscle cell marker genes (*Calponin*, *Caldesmon* and *α -SMA*) and *PKD1* and *PKD2* in vascular smooth muscle cells derived from two ADPKD-iPSC lines (P2- and P7-iPSCs). (d) Immunostaining of vascular smooth muscle cells on culture day 25 differentiated from P4-iPSCs for smooth muscle cell markers, caldesmon, smooth muscle myosin heavy chain (SM2) and SM22 alpha. Scale bars, 100 μ m. ECs, endothelial cells; SMCs, smooth muscle cells.

(a)

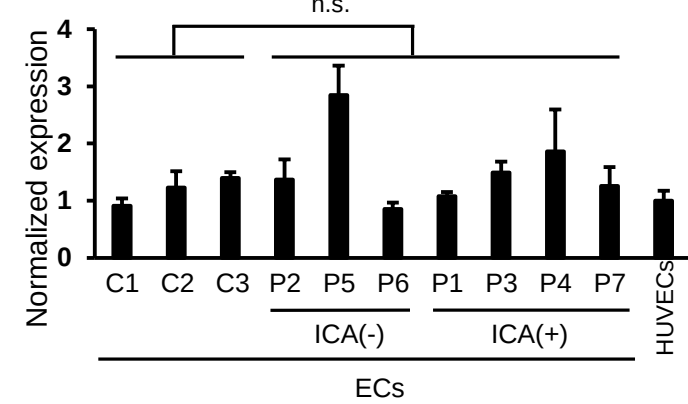
(Fold) *CXCR4*



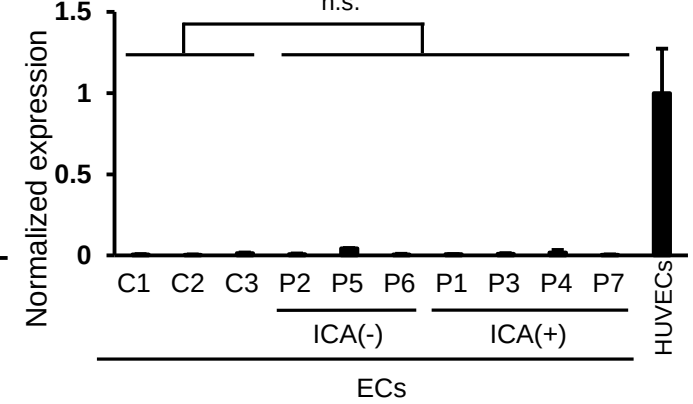
(Fold) *DLL4*



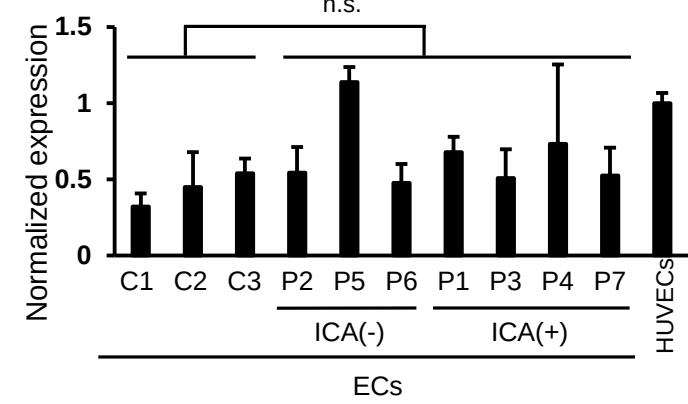
(Fold) *VE-cadherin*



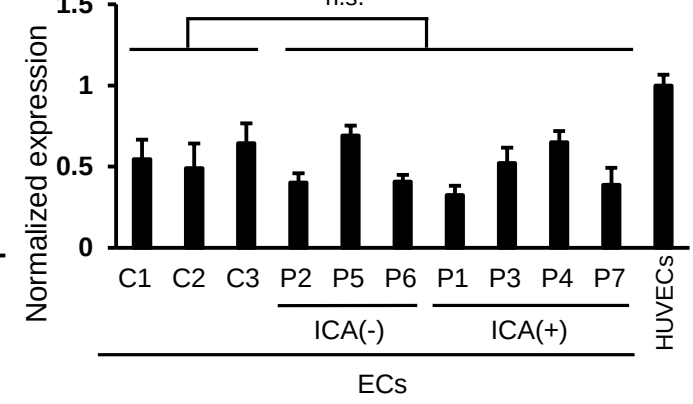
(Fold) *vWF*



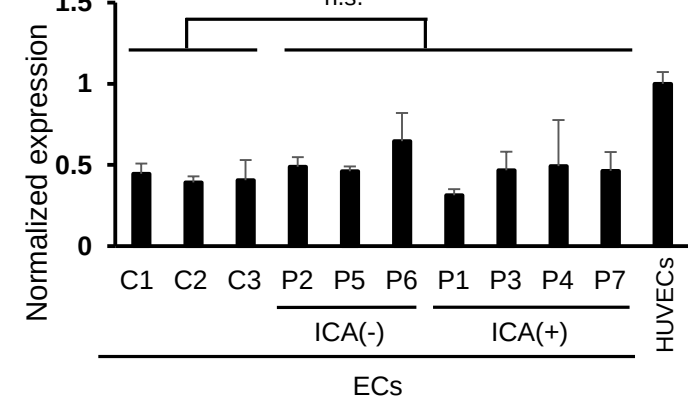
(Fold) *PECAM1/CD31*



(Fold) *PKD1*



(Fold) *PKD2*



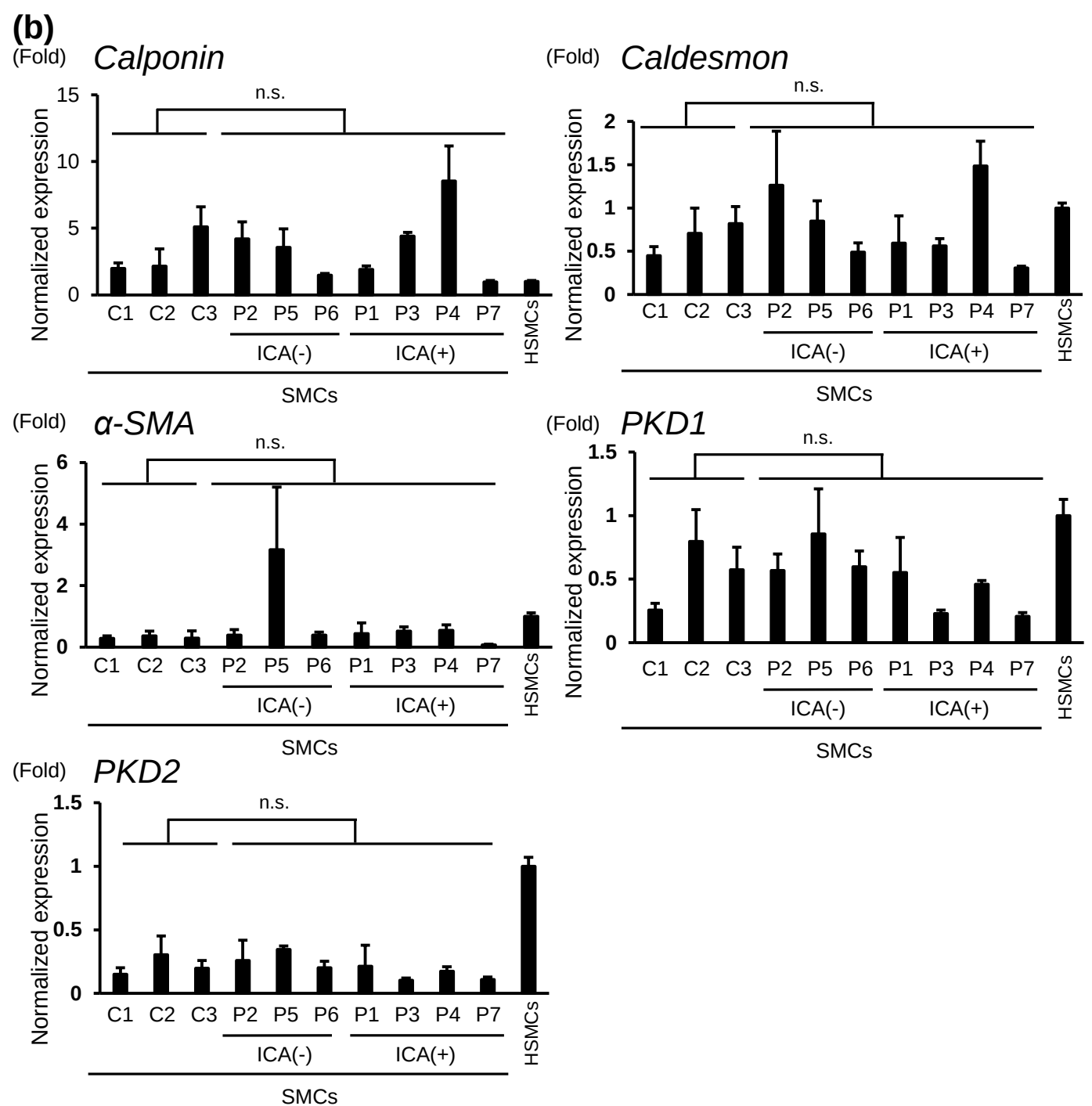


Figure S8. Comparison of marker gene expression between vascular cells derived from control- and ADPKD-iPSCs, Related to Figure 2. (a, b) qRT-PCR analyses were used to compare the expression of marker genes for vascular endothelia (a) and smooth muscle cells (b) and of *PKD1* and *PKD2* between endothelia (a) and smooth muscle cells (b) differentiated from control- and ADPKD-iPSCs. Each value was normalized to the average of those of HUVEC samples in (a) and HSMC samples in (b). All values are presented as the means $\pm$ SD of triplicate samples from three independent experiments. ECs, endothelial cells; SMCs, smooth muscle cells; ICA, intracranial aneurysms.

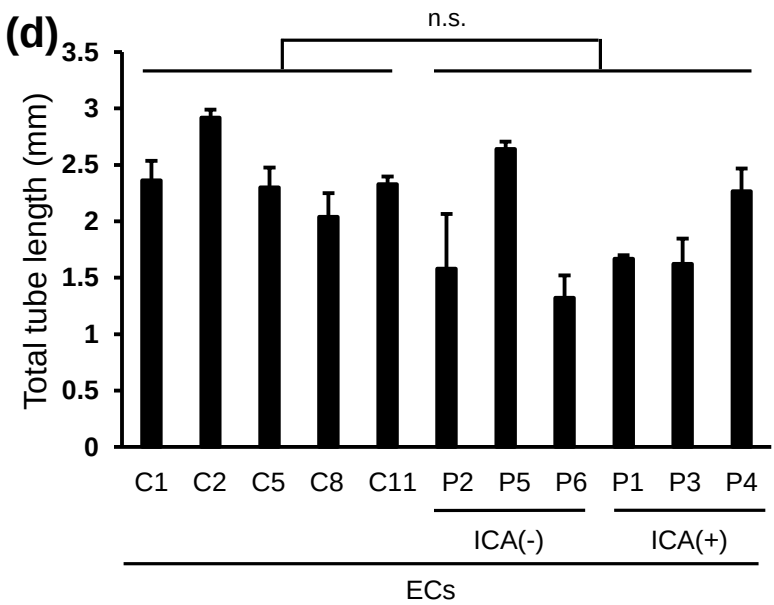
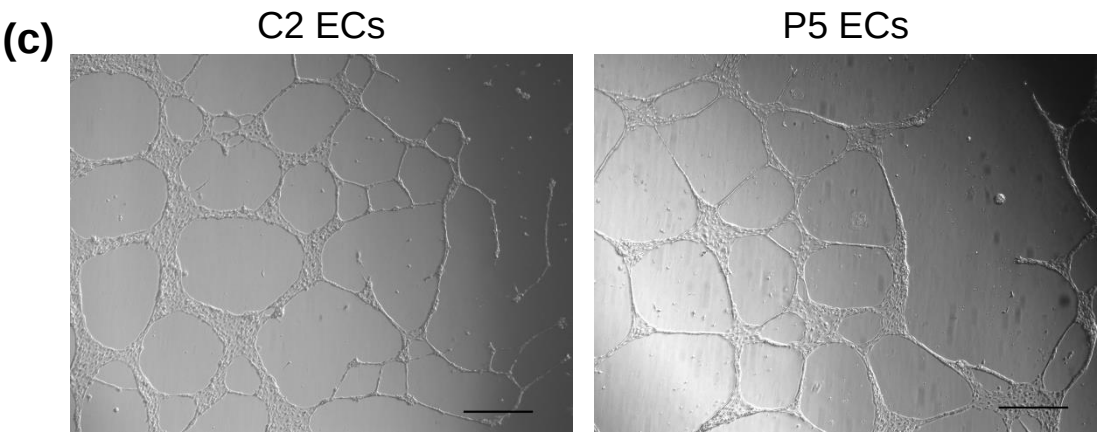
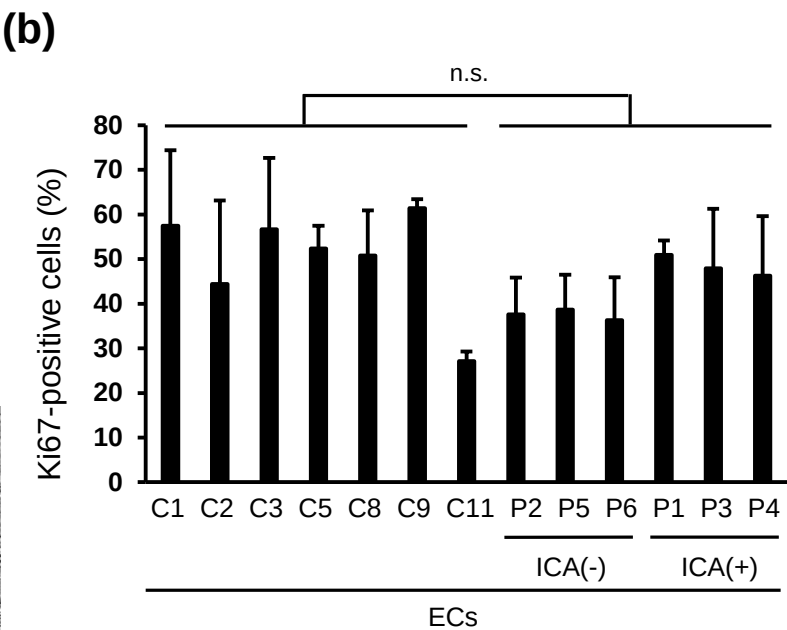
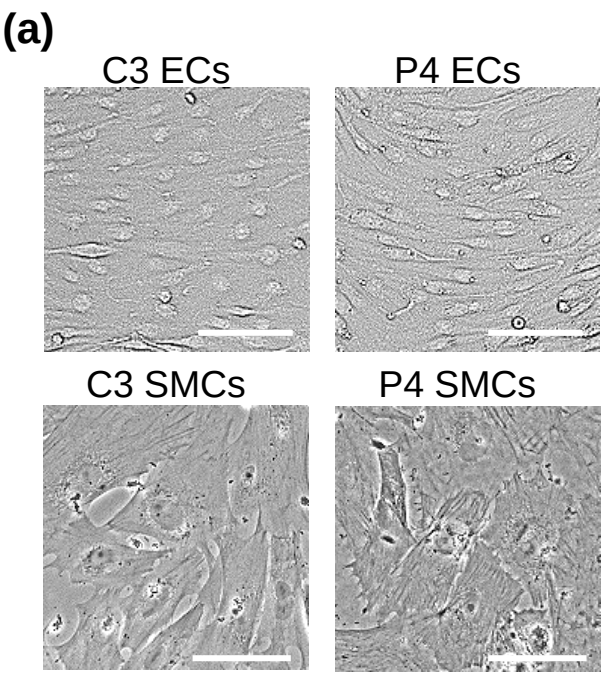
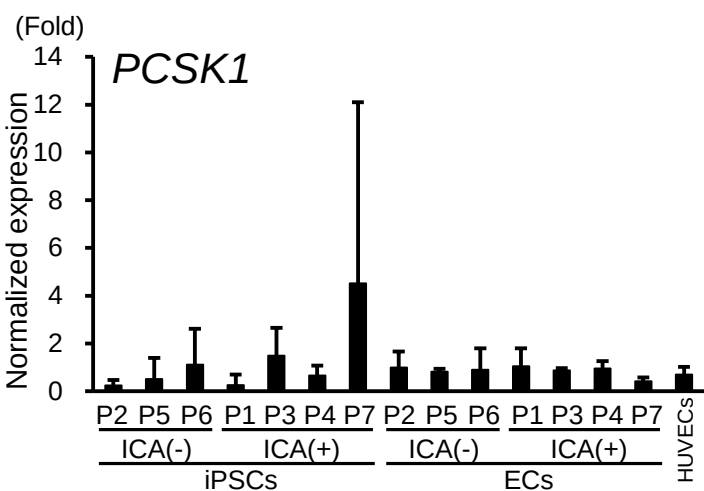


Figure S9. Comparison of proliferation and angiogenic potentials between vascular endothelia derived from control- and ADPKD-iPSCs, Related to Figure 2. (a) No obvious morphological differences were found between vascular cells differentiated from control- and ADPKD-iPSCs. Typical images of endothelial cells (ECs) and smooth muscle cells (SMCs) differentiated from control (C3)- and ADPKD (P4)-iPSCs. (b) Proliferation potential of hiPSC-derived endothelia was assessed by quantitative analyses of Ki-67 immunostaining images. (c, d) Tube formation assay was performed to evaluate the angiogenic activity of hiPSC-derived endothelia. Typical images of endothelia differentiated from control (C2)- and ADPKD (P5)-iPSCs. (d) Total tube length was calculated as the average of three fields. All values are the means $\pm$ SD of triplicate samples from three independent experiments. Scale bars, 100 μ m in (a) and 500 μ m in (c). ICA, intracranial aneurysms; n.s., not significant.



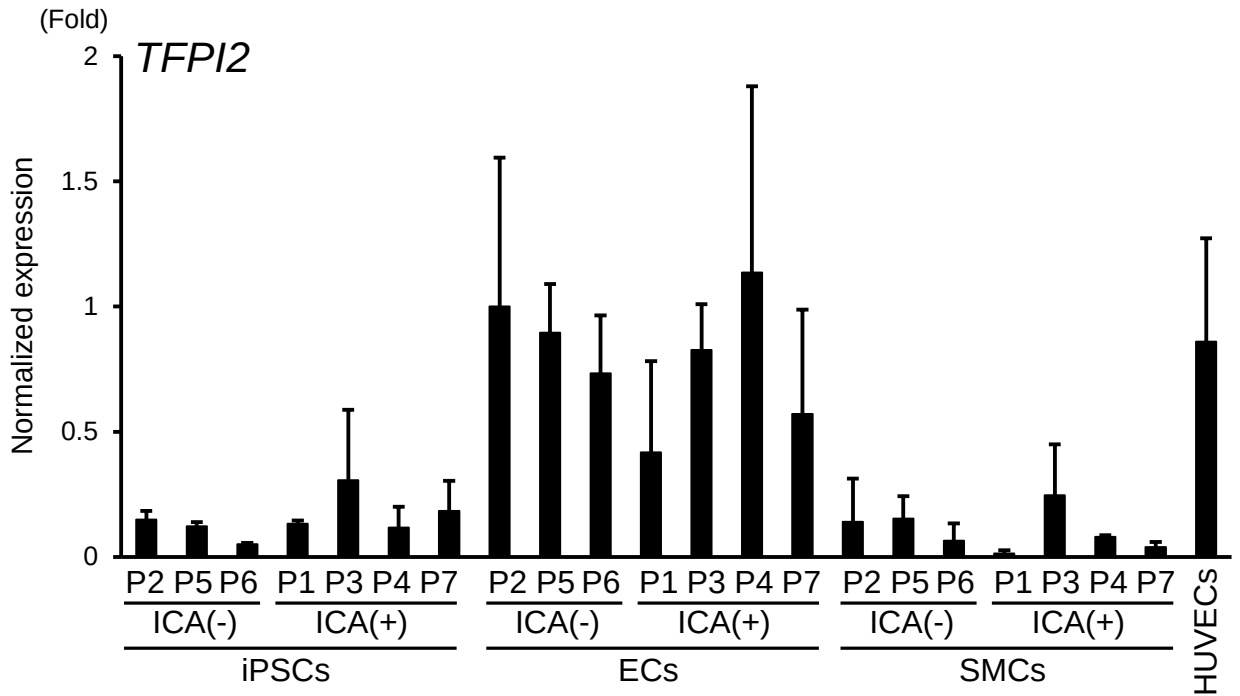


Figure S10. Gene expression analyses of iPSC-derived endothelia and smooth muscle cells from ADPKD patients with intracranial aneurysms (ICAs) and those without ICAs, Related to Figure 5. qRT-PCR validation of the microarray data for the expression of *MMP10*, *PCSK1*, *BMP6*, *EDN1* and *CTGF* in ADPKD-iPSCs and iPSC-derived endothelial cells (ECs), and that of *TFPI2* in ADPKD-iPSCs and iPSC-derived ECs and smooth muscle cells (SMCs) from the ADPKD patients with ICAs (P1, P3, P4 and P7) compared with the patients without ICAs (P2, P5 and P6). Each value was normalized to the average of iPSC-derived EC samples from P2. All values are the means $\pm$ SD of triplicate samples from three independent experiments. Note that the iPSC-derived ECs induced by our differentiation protocol have features of arterial ECs and therefore the expression profiles of some genes were different between iPSC-derived arterial ECs and human umbilical vein ECs (HUVECs).^{2,3} ICA, intracranial aneurysms.

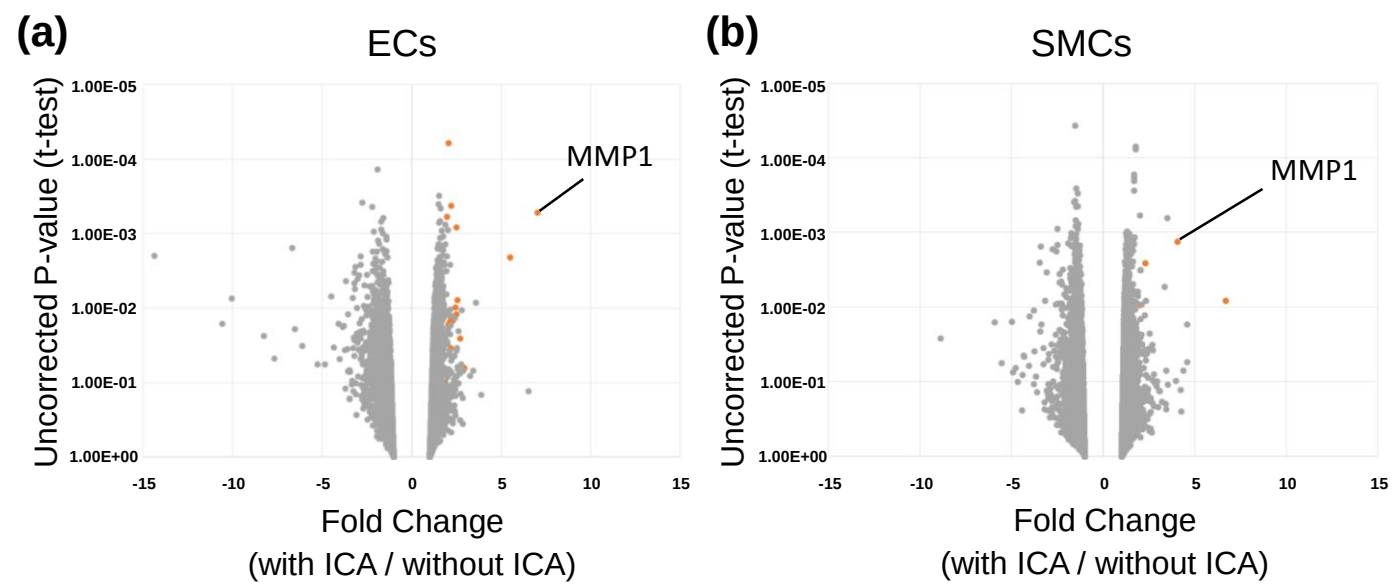


Figure S11. Comparison of gene expression profiles of iPSC-derived vascular cells from ADPKD patients with intracranial aneurysms (ICAs) and those from ADPKD patients without ICAs, Related to Figure 5. (a, b) Volcano plots were drawn with fold changes (with ICAs/without ICAs) and uncorrected *P*-values (t-test) of the microarray data for vascular endothelia (a) and smooth muscle cells (b). Orange plots were the candidate genes that are listed in **Tables S6** and **S7**. ECs, endothelial cells; SMCs, smooth muscle cells.

Supplementary Materials and Methods

Plasmid Construction and Lentivirus/Retrovirus Production.

The pMXs-based retroviral vectors for human *OCT4*, *SOX2*, *KLF4* and *c-MYC* have been described previously.⁴ A 20 bp random sequence, which was designated the N₂₀ barcode, was introduced into the *NotI/SalI* site of the *OCT4* expression vector. This unique barcode sequence was used in each experiment to avoid inter-experimental contamination. Supernatant containing the pMX retroviruses was generated by transfecting PLAT-E packaging cells with the pMXs vectors using the Fugene 6 transfection reagent (Roche).

The mouse *Slc7a1* ORF was transferred to pLenti6/UbC/V5-DEST (Thermo Fisher Scientific) by the Gateway cloning system (Thermo Fisher Scientific). Supernatant containing the mouse *Slc7a1* lentiviruses was produced by transfecting 293FT cells (Thermo Fisher Scientific) with pLenti6/UbC-*Slc7a1* and Virapower packaging mix using Lipofectamine 2000 (Thermo Fisher Scientific).

Generation of Patient-specific iPSCs

Fibroblasts derived from ADPKD patients were maintained and expanded in Dulbecco's

Modified Eagle Medium (DMEM, Nacalai Tesque) containing 10% fetal bovine serum (FBS, Japan Bioserum). The induction of iPSCs was performed as described previously.⁴ In brief, the patients' fibroblasts were seeded in six-well plates at 1.0×10^5 cells/well. The next day, the cells were infected with Slc7a1 lentiviruses with 4 μ g/ml polybrene (Nacalai Tesque). Then, the patients' fibroblasts expressing the mouse *Slc7a1* gene were seeded in six-well plates at 1.0×10^5 cells/well one day before transduction. Equal amounts of three or four retrovirus-containing supernatants were mixed and supplemented with 4 μ g/ml polybrene. Six days after transduction, the fibroblasts were replated onto mitomycin C-treated SNL feeder cells.⁵ Thirty days after transduction, iPSC colonies were selected for expansion.

Cell Culture

iPSCs were maintained in Primate ES medium (ReproCELL) supplemented with 500 U/ml penicillin/streptomycin (Thermo Fisher Scientific) and 4 ng/ml recombinant human basic fibroblast growth factor (bFGF, WAKO). For routine passaging, human iPSCs were washed once with phosphate buffered saline (PBS, Nacalai Tesque) and then incubated with CTK dissociation solution containing 0.1% collagenase IV, 0.25% trypsin, 20% knockout serum replacement (KSR, Thermo Fisher Scientific) and 1 mM

CaCl₂ in PBS. The split ratio was routinely between 1:3 and 1:6.

RT-PCR and Real-time Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's recommendations, followed by cDNA synthesis using standard protocols. Briefly, 1 µg of total RNA was treated with DNase I (Qiagen) for 15 min, and the cDNA was synthesized using ReverTra Ace (TOYOBO). The cDNA samples were subjected to PCR amplification using a thermal cycler (Veriti 96-well Thermal Cycler, Thermo Fisher Scientific). PCR was performed using the Ex-Taq PCR kit (Takara) according to the manufacturer's instructions. The PCR cycles were as follows: for *β-ACTIN*, initial denaturation was performed at 94°C for 2.5 min, followed by 25 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 30 s and a final extension at 72°C for 10 min. For the other genes, the cycles consisted of initial denaturation at 94°C for 2.5 min, followed by 30-40 cycles of 94°C for 30 s, 58-62°C for 30 s, 72°C for 30 s and a final extension at 72°C for 7 min. qPCR was performed using the Step One Plus Real-Time PCR System (Thermo Fisher Scientific) and SYBR Green PCR Master Mix (Takara). Denaturation was performed at 95°C for 10 min, followed by 45 cycles at 95°C for 5 s and at 60°C for 30 s. As recommended by the manufacturer, the threshold cycle method was used to

analyze the data for the gene expression levels and was normalized to those of the housekeeping gene, *β-ACTIN*. The PCR reactions were performed in triplicate for each sample. The primer sequences are listed in **Table S10**.

Short Tandem Repeat (STR) Analysis and Karyotyping

The STR analyses were performed at BEX CO. LTD., Japan, and the chromosomal G-band analyses were performed at the Nihon Gene Research Laboratories, Japan.

Bisulfite Sequencing

Sodium bisulfite conversion of genomic DNA (1 µg) was performed using the EZ DNA methylation kit (ZYMO Research), according to the manufacturer's instructions. The promoter regions of the human *OCT4* and *NANOG* genes were amplified by PCR as described previously.⁴ The PCR products were subcloned into the pCR4-TOPO TA vector (Thermo Fisher Scientific) and were sequenced.

Embryoid Body (EB) Formation

For EB formation, a 10 cm plate containing human iPSCs was rinsed with PBS and treated with 1 mg/ml type IV collagenase (Thermo Fisher Scientific) in DMEM for 10

min at 37°C. The collagenase was rinsed away with PBS and replaced with undifferentiation medium. The cells were then scraped off with a cell scraper (IWAKI), dissociated by pipetting, and distributed into a low attachment 6-well plate (Corning) containing knockout-DMEM (Thermo Fisher Scientific) supplemented with 20% KSR, 0.1 mM non-essential amino acids (Thermo Fisher Scientific), 2 mM glutamine (Thermo Fisher Scientific), 500 U/ml penicillin/streptomycin and 0.55 mM 2-mercaptoethanol (Thermo Fisher Scientific). After 8 days as a floating culture, the EBs were transferred to gelatin-coated plates and cultured in the same medium for another 8 days.

Teratoma Formation

The iPSCs were harvested using CTK solution, collected into tubes and centrifuged, and the pellets were resuspended in DMEM/F12 (Thermo Fisher Scientific). One quarter of the cells from a confluent 100 mm dish was injected into the testes of a NOD-SCID mouse (CREA). Nine to twelve weeks after injection, the tumors were dissected and fixed with PBS containing 4% paraformaldehyde (PFA). Paraffin-embedded tissues were sliced and stained with hematoxylin and eosin.

Mutational Analysis

Mutational analyses of the three families with ADPKD (P4, P6 and P7) were performed as described previously, with some modifications.¹ In brief, the pedigree structures were constructed based on interviews, and additional medical histories were obtained from patient records. Informed consent, in accordance with Kyoto University Hospital guidelines, was obtained from each patient and/or their family. Multipoint linkage analyses and haplotype constructions were performed to confirm the prevalent type of ADPKD as *PKD1* in all the three pedigrees using four microsatellite markers, D16S404, D16S3075 (*PKD1*), D16S103 and D16S3046, spanning 27 cM on chromosome 16p. To exclude linkage with the *PKD2* locus, we used six microsatellite markers, D4S392, D4S2964, D4S1534 (*PKD2*), D4S414, D4S1562 and D4S406, spanning 38.7 cM on chromosome 4q. Linkage studies were performed using GENEHUNTER software.⁶ The *PKD1* genomic DNA was isolated from either fresh or frozen peripheral blood. The DNA was extracted using two kinds of commercially available extraction kits, the Wizard Genomic DNA Purification Kit (Promega) and the QIAamp DNA Blood Mini Kit (Qiagen). The primer pairs used to amplify the *PKD1* (GenBank accession number L39891) genomic fragments are shown in **Table S10**. PCR amplification was performed in a PTC-100 programmable thermal controller (MJ Research) or in a Gene

Amp PCR System 9700 (Thermo Fisher Scientific). For the mutational analysis of the duplicated part of *PKD1*, long-range PCR (LR-PCR) followed by nested primer PCR was performed. LR-PCR was carried out using a TaKaRa LA PCRTM Kit Ver. 2.1 (Takara) with a hot start protocol, *PKD1*-specific primers (**Table S10**), and protocols that were reported previously.⁷⁻¹⁰ For nested PCR after LR-PCR, diluted (1/105) product was used to avoid any genomic influence. The GC-rich region was amplified using the GC buffer provided in the TaKaRa LA PCRTM Kit Ver. 2.1. The quality of the PCR-amplified products was assessed by agarose-gel electrophoresis. The bands were excised and purified using a Prep-A Gene DNA Purification Kit (Bio-Rad) or a QIAquick Gel Extraction kit (Qiagen). An ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit (Thermo Fisher Scientific) was used to sequence the purified PCR products, and automated sequencing was performed using an ABI PRISM 310 genetic analyzer (Thermo Fisher Scientific).

To elucidate the 2 bp deletion mutation of the family of patient P7, genotyping was performed using the GeneScanTM device (Thermo Fisher Scientific), which can detect an allele two bases shorter by semiautomatic electrophoresis of the PCR-amplified products using fluorescently-labeled primers. For patient P7, the 2 bp deletion in exon 15 of *PKD1* was amplified by LR-PCR, followed by nested-PCR. The

primer sets used were reported previously.⁵ Briefly, in the LR-PCR, a 3378 bp product was amplified with the F26 and R2LR primers (**Table S10**) and then the product was diluted to 10^{-5} . The diluted mixture was amplified with the nested primer set of PKDeX15.14F and PKDeX15.14R (**Table S10**). The expected size of the patient allele (7024del AC) was 202, while that of normal size was 204. The difference in size between the mutated allele and the wild allele was identified by the GeneScan™ system using the fluorescently-labeled reverse primer (PKDex15.14R).

The mutational analyses of the remaining four ADPKD patients (P1, P2, P3 and P5) were performed by whole-exome sequencing and amplicon-sequencing.

Whole-exome Sequencing

Exon capture and Illumina's library preparation were performed using the SeqCap EZ v3 (Roche) and a TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA) as reported previously.¹¹ Paired-end sequencing for 2 X 101 cycles was performed using a TruSeq SBS Kit v3 and a HiSeq2500 instrument (Illumina). Processing of the raw data, mapping and SNV/indel calling were performed as previously reported.¹¹

Amplicon Sequencing

PCR was performed using KAPA HiFi Hot Start Ready (KAPA Biosystems) for exon 1 of PKD1 or Prime STAR GXL DNA polymerase (TaKaRa, Japan) for the other regions. The PCR conditions when using KAPA HiFi Hot Start was 1 cycle of 95°C for 5 min, followed by 25 cycles of 98°C for 20 sec, 65°C for 15 sec and 72°C for 3 min. The PCR using Prime STAR GXL was 30 cycles of 98°C for 10 sec and 68°C for 30 min. The primer pairs used to amplify the *PKD1* genomic fragments are shown in **Table S10**. PCR products were then purified using a QIAquick PCR Purification Kit (QIAGEN) and Illumina's libraries were prepared using a TruSeq PCR-free Kit (Illumina). Paired-end sequencing for 2 X 151 cycles was performed using a MiSeq device (Illumina). Base calling was done by MiSeq Reporter, and quality filtering was performed by the Qcleaner software program (Amelieff Corporation) with option `-qp 20,80 -n 5 --trim 20 -length 32`. The sequenced reads were mapped to the region encoding the *PKD1* gene by BWA-MEM 0.7.10. The Genome Analysis Toolkit (GATK) v1.6 software program performed local re-alignment around indels, recalibration and SNVs/indel calling according to the Broad Institute's best-practice v3 guidelines [<https://www.broadinstitute.org/gatk/guide/best-practices>]. The Autosomal Dominant Polycystic Kidney Disease Mutation Database: PKDB (<http://pkdb.mayo.edu/>) was used to annotate SNVs/indels.

Immunocytochemistry and Alkaline Phosphatase Analysis

Alkaline phosphatase (AP) staining was performed using the Alkaline Phosphatase Detection Kit (Millipore). For immunocytochemistry, cells were fixed with 4% PFA/PBS at 4°C for 20 min after a brief wash with PBS. Fixed cells were washed with PBS and then blocked with PBST (PBS/0.1% Triton X-100)/5% normal donkey serum (Jackson ImmunoResearch Laboratories, JIRL) for 1 h at room temperature. The primary antibodies were diluted in blocking solution and incubated overnight at 4°C. The secondary antibodies were incubated for 1 h at room temperature. The following primary antibodies and dilutions were used: β III-tubulin (1:1000, MRB-435P, Covance Research Products), α -smooth muscle actin (1:500, A2547, Sigma), Vimentin (1:100, SC-6260, Santa Cruz Biotechnology), SOX17 (1:500, AF1924, R&D systems), OCT3/4 (1:200, AF1759, R&D systems), NANOG (1:200, 4903, Cell Signaling Technology), SOX2 (1:200, 3579, Cell Signaling Technology), SSEA1 (1:200, MAB2155, R&D systems), SSEA4 (1:200, MAB1435, R&D systems), TRA-1-60 (1:200, MAB4360, Millipore), TRA-1-81 (1:200, MAB4381, Millipore), CD31/PECAM1 (1:200, 14-0319-80, eBioscience), eNOS (1:200, 610296, BD Biosciences), Calponin (1:200, M3556, DAKO), CD34 (1:500, GTX28158, GeneTex), VE-cadherin (1:200, 560411,

BD Biosciences), Von Willebrand Factor (1:25, M061601, Dako), Caldesmon (1:100, sc-7574, Santa Cruz Biotechnology), Smooth Muscle Myosin Heavy Chain (1:180, 7601, YAMASA), SM22 alpha (1:200, ab14106, abcam), acetyl- α -tubulin (1:500, 5335, Cell Signaling Technology) and Ki67 (1:1000, NCL-Ki67p, Leica Biosystems). Purified mouse IgG, rabbit IgG and goat IgG whole molecules (Jackson ImmunoResearch) were used as negative controls. For immunocytochemical detection of α SMA, calponin, CD34, VE-cadherin, vWF, caldesmon, SM2 and SM22 alpha, cells were treated using a Dako Cytomation Envision+System–HRP(DAB) kit after incubation with primary antibodies, following the manufacturer's protocol (DakoCytomation). Nuclei were visualized by hematoxylin staining (Muto Pure Chemicals). For immunocytochemical detection of other antibodies, the secondary antibodies included Alexa488-conjugated donkey anti-mouse IgG, Alexa594-conjugated donkey anti-mouse IgG, Alexa488-conjugated donkey anti-rabbit IgG, Alexa488-conjugated donkey anti-goat IgG, Alexa594-conjugated donkey anti-goat IgG and Alexa594-conjugated donkey anti-rat IgG (1:1000, Thermo Fisher Scientific). Nuclei were stained with Hoechst 33342 (1:1000, Thermo Fisher Scientific). The rate of Ki67 positive cells were examined using In Cell Analyzer 6000 (GE Healthcare).

Dil Labeled Acetylated Low Density Lipoprotein (Dil-Ac-LDL) Uptake Assay

After pre-incubation with serum-free medium containing 0.1% BSA for 24 hours, cells were incubated with 10 µg/ml acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3',3'-tetramethylindo-carbocyanine perchlorate (Dil-Ac-LDL) (Alfa Aesar) for 5 hours at 37°C and subsequently fixed with 4% PFA/PBS at 4°C for 20 min after washing three times with PBS.

Tube Formation Assay

Tube formation assay was performed as described previously.² In brief, vascular endothelia derived from iPSCs (4.0×10^4 cells/well) were seeded onto matrigel-coated (Becton Dickinson) 24-well plates. Cells were incubated for 24 h, and digital images of the tubes that formed were captured. Total tube length was calculated using BZ-II analyzer software (KEYENCE) after three randomly selected fields were examined in each well of a 24-well plate. The total tube length was calculated as the average for the three fields.

Western Blot Analysis

Immunoblotting was performed for 10 µg of cell lysate extracted with radioimmune

precipitation assay (RIPA) buffer. The membranes were incubated with two primary antibodies, MMP1 (2 µg/ml, MAB901, R&D Systems) and β-Actin (1:1000, A1978, Sigma-Aldrich), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000, NA931, GE Healthcare). The ECL-PLUS Detection System (GE Healthcare) was used as the substrate for chemiluminescent detection. Blots were visualized and scanned using the ImageQuant LAS 4000 Mini (Fujifilm), and the density of bands was measured with Multi Gauge Ver. 3.0 (Fujifilm).

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