Aberrant expression of SPARC and its impact on proliferation and apoptosis in ADPKD cyst-lining epithelia

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Abstract

Background. Autosomal dominant polycystic kidney disease (ADPKD) results from a combination of environmental and genetic factors. Secreted protein acidic and rich in cysteine (SPARC) can be expressed by many different cell types and is associated with development, remodelling, cell turnover and tissue repair. The analysis of SPARC would help evaluate the effect of the unique matricellular glycoprotein on renal disease progression in ADPKD.

Methods. The concentration of SPARC was measured with an enzyme-linked immunosorbent assay (ELISA); distribution and expression levels were measured with in situ hybridization, immunohistochemistry, reverse transcription–polymerase chain reaction (RT–PCR) and western blot assays. Apoptosis was assessed by morphological observation and fluorescence-activated cell sorting (FACS) apoptosis index (AI) analysis. Cell cycle phase was examined by FACS analysis. Cell proliferation was studied using bromodeoxyuridine (BrdU) incorporation ELISA.

Results. The SPARC level in the renal cyst fluid of patients with ADPKD was greater than that in patients with simple renal cyst (SRC), and also greater than that found in the plasma and urine of patients with either ADPKD or SRC and normal subjects. SPARC mRNA and protein levels in polycystic renal tissue were greater than that in normal renal tissue. Additionally, SPARC could inhibit cyst-lining epithelial cell proliferation, bring about cell cycle arrest in the G0/G1 phase and induce apoptosis in vitro. SPARC treatment resulted in decreased mRNA levels of PCNA (proliferating cell nuclear antigen), MCM2 (minichromosome maintenance protein 2), ClnD1 and Bel-2, but an increased mRNA level of p21Waf1 in cyst-lining epithelial cells.

Conclusion. Our findings suggest that the increased SPARC expression in ADPKD renal tissue may provide negative feedback in ADPKD patients.

Keywords: ADPKD; proliferation; SPARC

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common life-threatening monogenic hereditary diseases with an incidence rate of ~1:1000 afflicting 5–6 million people in the world [1]. The occurrence and development of cysts are due to a ‘two hit’ of the PKD gene, resulting in an abnormality of cell cycle regulation and intercellular metabolism. This dysregulation of the cell cycle and cellular metabolism subsequently results in epithelial hyperplasia and accumulation of liquid in the renal tubule, and finally leads to the development and gradual enlargement of bilateral renal cysts [2,3]. The abnormalities of the extracellular matrix, such as fibrin and laminin, also accelerate the development of ADPKD.

About 50% of PKD patients develop end-stage renal failure (ESRF) by late middle age [4], accounting for 5–10% of patients requiring haemodialysis [1]. ADPKD can have effects on other organs, including polycystic liver, pancreatic and bile duct dilation, colonic diverticula, intracranial aneurysm and abnormality of the cardiac valve [5]. Despite the severity and incidence of ADPKD, there unfortunately remains a lack of effective therapeutic methods [6].

Secreted protein, acidic and rich in cysteine (SPARC), also termed osteonectin or BM-40, is a calcium-binding matricellular glycoprotein with antiproliferative and counteradhesive properties that can interact with specific cytokines and growth factors such
as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), bind to structural proteins and interact with cells directly. It plays important roles in embryogenesis, tissue remodelling, wound repair, morphogenesis, cellular differentiation, cell migration and angiogenesis [7]. SPARC, together with thrombospondin 1, tenascin C, TSP2, tenascin X, syndecans and osteopontin, comprise a non-homologous functional group of secreted matricellular proteins that interact with cell surface receptors, the extracellular matrix, growth factors and/or proteases but do not in themselves subserve exclusively structural roles [8,9].

Recent findings indicate that SPARC is involved in glomerular remodelling and repair [10,11]. Furthermore, SPARC has been implicated in various renal diseases such as passive Heymann nephritis, mesangio proliferative glomerulonephritis and diabetic nephropathy [10,11]. However, little is known about the role of SPARC in the progression of ADPKD.

We previously found by cDNA expression microarray that SPARC is upregulated in ADPKD kidney tissues (unpublished observation). Thus, in this study, we proceeded to investigate the role of SPARC in ADPKD. Body fluid concentrations of SPARC were measured and the distribution of SPARC in kidney tissues of patients with ADPKD was observed. In addition, ADPKD cyst-lining epithelial cells were treated directly with exogenous SPARC and their growth and apoptosis were studied. The effects of SPARC treatment on the mRNA levels of proliferation-related genes [proliferating cell nuclear antigen (PCNA) and minichromosome maintenance protein 2 (MCM2)], cell cycle regulating genes (ClnD1 and p21^waf1) and apoptosis-related genes (Bax and Bcl-2) were assessed.

Materials and methods

Plasma, urine and cyst fluid samples

Samples of plasma, urine and cyst fluid were obtained from 37 Han subjects (16 males, 21 females; aged 18–78 years, mean age 47.41 ± 12.63 years) with ADPKD and 10 Han subjects with simple renal cyst (SRC) (four males, six females; aged 33–65 years, mean age 49.35 ± 13.26 years old). Control samples of plasma and urine were obtained from 20 Han volunteers (nine males, 11 females; aged 35–70 years, mean age 51.75 ± 14.07 years). All SRC subjects had unilateral renal cysts without hypertension, proteinuria or renal dysfunction. The subjects with ADPKD included out-patients and in-patients of Shanghai Changzheng Hospital. Chronic kidney disease (CKD) classification was evaluated by the Kidney Disease Outcomes Quality Initiative (K/DOQI) [12]. Glomerular filtration rate (GFR) was estimated using the MDRD prediction equation and given in ml/min/1.73 m² [170 × (creatinine)^–0.999 × (age)^–0.276 × (urea)^–0.170 × (albumin)^1.210] if female] based on age and levels of serum creatinine, urea and albumin [13]. None of the subjects had undergone dialysis or renal transplantation. ADPKD patients produced 1000–2000 ml of urine per day and proteinuria was negative or only trace. Each patient gave informed consent before the collection of body fluid samples.

Cyst fluid samples were generally obtained through percutaneous drainage of multiple renal cysts under ultrasonic guidance, and some through laparoscopic renal cyst decortication. The day before blood and urine samples were taken, the subjects ceased eating at 8 p.m. and drinking at 10 p.m. Peripheral blood and mid-stream urine samples were collected in the morning. Blood samples were treated with the anticoagulant agent sodium citrate and female urine samples were not harvested during menstruation. Blood was centrifuged at 3000 g for 10 min. Plasma was isolated and treated with Protease Inhibitor Cocktail Set (EMD Biosciences, Inc., San Diego, CA) and stored at −80°C. Fresh urine and cyst fluid were centrifuged at −80°C at 4000 g for 10 min, and the supernatant was treated with a protease inhibitor and stored at −80°C.

Human kidney specimens

Normal human renal tissue (n = 5) was obtained from kidneys surgically excised for a localized neoplasm. Tissue samples were obtained from macroscopically normal portions of kidney, located at some distance from the neoplastic process. ADPKD kidneys (n = 6) were excised for repeated haemorrhage. These patients were diagnosed and treated at Changzheng Hospital, Shanghai, China. The diagnosis of ADPKD was based on family medical history, ultrasonic and computed tomography (CT) scan. Patients provided informed consent before tissue specimens were collected.

Tissues were fixed in 4% paraformaldehyde (for in situ hybridization) and 0.1% diethylpyrocarbonate (DEPC) or in 10% neutral buffered formalin (for immunohistochemistry) for ≥12 h, processed, paraffin embedded and sectioned at 3 µm. Additionally, some tissue samples from the nephrectomy specimens were snap frozen and utilized for RNA and protein preparation.

ELISA assays of SPARC levels in body fluids

The concentration of SPARC in body fluids was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Hematologic Technologies, Essex Junction, VT). ELISAs were performed according to the manufacturer’s instruction manual. The absorbance at 492 nm was determined using a microplate reader (Labsystems Dragon Wellscan MK3, Finland).

In situ hybridization

Oligonucleotide probes (5’-GCCTGGATCCTTTTCTCTCTTTGCTTGCCGAC-3’, 5’-CTTCGACTCTTCTGCACCTTTG-3’ and 5’-TGAAGAAGATCCATGAGAAATGAGAAGGCCG-3’antisense to SPARC mRNA were synthesized and digoxigenin-UTP labelled at the 3’-terminus (Boehringer Mannheim, Germany). All the reagents and consumed materials used in RNA in situ hybridization processing were previously treated with 0.1% DEPC. The paraffin-embedded 3 µm thick sections were dewaxed in xylene and hydrated via a gradient of alcohol immersions. Endogenous peroxidase activity was blocked in 3% H₂O₂ at room temperature for 30 min and tissue was digested in proteinase K for 15 min to expose mRNA fragments.
Sections were treated with pre-hybridization buffer [42% deionized formamide, 10% dextran sulfate, 0.1% DEPC, 0.5 mM phosphate-buffered saline (PBS)] at 38°C for 2h. For subsequent hybridization, each section was then treated overnight with 20 μl of labelled probes (1.0 μg/ml) under a nuclease-free coverslip in a humid chamber at 38°C. Hybridized sections were washed and blocked and then incubated with biotinylated mouse anti-digoxigenin antibody, SABC-POD and biotinylated peroxidase. 3,3′-Diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich, MO) was used as the chromogen. The slides were counterstained with haematoxylin, dehydrated and coverslipped. Treatment with the same oligonucleotide probes without the digoxigenin label served as a negative control.

**Immunohistochemistry**

Tissue sections were dewaxed and endogenous peroxidase was inactivated. The sections were immersed in 0.01 M citrate buffer (pH 6.0), heated in a microwave oven to retrieve antigenicity and blocked for non-specific binding with 5% bovine serum albumin (BSA) in PBS for 20 min. Blocked sections were then incubated at 4°C overnight with mouse anti-SPARC monoclonal antibody (AON-5031, Hematologic Technologies). After washing in PBS, the sections were treated with horseradish peroxidase-conjugated goat anti-mouse IgG primary antibody served as the negative control. Incubation with an irrelevant non-immune IgG antibodies, SABC-POD and biotinylated peroxidase. After subsequent washing with TBST, signals were detected with 10% fetal bovine serum (Gibco) at 37°C with 5% CO2.

**Western blot analysis**

Western blot assays were performed with lysates from snap-frozen renal tissue samples. Human kidney tissue lysate was prepared by homogenization in modified RIPA buffer (150 mM sodium chloride, 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 5 μg/ml of aprotinin, 5 μg/ml of leupeptin). The protein concentration of each sample was determined by a Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). Lysates were fractionated on a 12% polyacrylamide gel that contained 0.1% SDS and then transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, USA) by electroblotting. The blots were blocked with TBST (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk for 2 h at room temperature, and then incubated with 2 μg/ml monoclonal mouse anti-SPARC antibody (AON-5031) at 4°C overnight. The membrane was subjected to three 3 min TBST washes, and incubated at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG. After subsequent washing with TBST, signals were detected with enhanced chemiluminescence (Amersham, UK).

**Cell proliferation analysis**

Cell proliferation was studied using bromodeoxyuridine (BrdU) incorporation ELISA (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The exogenous human platelet SPARC was from Hematologic Technologies. ADPKD cyst-lining epithelial cells (5 × 10⁵ cells/well) from the logarithmic growth phase were plated in three 96-well plates. When they had proliferated to 80% confluence, the cells synchronously entered a quiescent phase after the medium was changed to a serum-free medium for 24 h. After discarding the supernatant, the cells were treated with 10% fetal calf serum (FCS) medium containing SPARC at various concentrations (0, 1, 2.5, 5 and 10 μg/ml) for 48, 72 or 96 h. Each experimental group included six wells, and all experiments were repeated three times.

After incubation, the cells were labelled with BrdU (10 μmol/l) at 37°C for 6 h. DNA synthesis was assessed by measuring the amount of BrdU incorporation into the DNA, which was detected by immunoassay. Cellular proliferation was determined by measuring the magnitude of absorbance (A value) at 405 nm (reference wavelength 492 nm). The inhibition rate was expressed by the following equation:

\[
\text{Inhibition rate} = \frac{-100}{A} \text{value of control group} - A \text{value of SPARC-treated group}/ A \text{value of control group} \times 100\%
\]

**Cell cycle analysis**

Cell cycle phase was examined by fluorescence-activated cell sorting (FACS) analysis. Cyst-lining epithelial cells in a logarithmic growth phase were plated in 6-well plates and treated with SPARC at various concentrations (0, 1, 2.5, 5 and 10 μg/ml in media) for 72 h. The cells were trysinized, washed with PBS and fixed in 70% pre-cooled ethanol overnight at −20°C. After washing twice with PBS, the cells were stained with propidium iodide for FACS analysis in the dark. FACS analysis was performed under an excitation wavelength of 488 nm and a detection wavelength of 630 nm.
with Coulter Epics XL (Beckmen, USA). The FACS data were analysed by Multi-Cycle software (DNA Content and Cell Cycle Analysis Software, San Diego, CA). The proliferation index (PI%) was expressed by the following equation:

$$PI% = (S + G_2/M) \cdot (G_0/G_1 + S + G_2/M) \times 100\%$$

Analysis of apoptosis

Induction of apoptosis in cyst-lining epithelia after exogenous SPARC treatment was assessed by morphological observation under an inverted microscope (Olympus CK2) and use of an apoptosis index (AI). After 72 h of SPARC treatment, cells were harvested by trypsin digestion, fixed in 4% paraformaldehyde and 2% glutaraldehyde for ≥ 4 h and washed in PBS. Cell were clotted by addition of plasma, 1% 2.5 ml were carried out in 25 µl of reaction mixture [1.0 M primers, 1.0 M dNTP. The mixture was incubated at 25°C for 10 min, 42°C for 1 h and 52°C for 15 min. Reverse transcriptase was then inactivated by a 15 min incubation at 70°C. The primer sequences employed in RT–PCR are shown in Table 1. PCRs were carried out in 25 µl of reaction mixture [1.0 µl of cDNA, 2.5 µl of 10× buffer, 0.3 µl of 250 mM Mg²⁺, 0.3 µl of 25 mM dAGCU, 1.0 µl of 10× µM primers, 1.0 µl of 10⁻³ × calibration buffer, 0.8 µl of 25× SYBR Green (BioRad Laboratories, Inc., Hercules, CA), 1.25 U of Taq polymerase and 0.4 U of UNG]. The following cycle conditions were used: pre-denaturation at 95°C for 90 s, five cycles of denaturation at 95°C for 5 s, annealing at 55°C for 15 s, extension at 72°C for 20 s; 35 additional denaturing cycles at 95°C for 5 s, annealing at 60°C for 30 s to reach the fluorescent signal detection point; another 40 cycles of denaturation at 95°C for 1 min, and annealing at 55°C for 1 min (increasing 0.5°C cycle every 10 s). The amount of target gene expression was calculated from the respective standard curves, and quantitative expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with an iCycler Thermal Cycler (BioRad Laboratories, Inc., Hercules, CA).

Statistical analysis

The data are presented as means ± SD. Comparisons between results from different groups were performed using Student's t-test or one-way analysis of variance (ANOVA), as appropriate. Statistical significance was defined as $P < 0.05$ in all cases. The software package was SAS 9.1.3.

Results

The concentration of SPARC in plasma, urine and renal cyst fluid

The SPARC concentrations measured in the plasma, urine and renal cyst fluid samples of the ADPKD and SRC patients, and that in the plasma and urine samples of the normal subjects are summarized in Table 2. The influence of demographics such as age and sex was excluded. SPARC levels in renal cyst fluid of ADPKD patients (3628.75 ± 1445.90 ng/ml) were greater than that in renal cyst fluid of SRC patients (154.14 ± 41.28 ng/ml) ($P < 0.01$). SPARC levels in urine of ADPKD patients (1253.16 ± 544.81 ng/ml) were greater than that in urine of both SRC patients (134.08 ± 30.00 ng/ml) and normal control subjects (123.91 ± 28.37 ng/ml) ($P < 0.01$); however, the mean SPARC level in urine of the SRC group did not differ from that of the normal control group ($P > 0.05$).

<table>
<thead>
<tr>
<th>Table 1. PCR primers</th>
<th>Genes</th>
<th>Forward and reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPARC</td>
<td>F: 5'CAAGAAAGCCTGCGATGAGA-3'</td>
<td>R: 5'GGGGGTTTGCAGTCATGCAGC-3'</td>
</tr>
<tr>
<td>PCNA</td>
<td>F: 5'TTATCAAGTGAAGCCCGGAG-3'</td>
<td>R: 5'TGAGATCTCTGACCCAAGG-3'</td>
</tr>
<tr>
<td>MCM2</td>
<td>F: 5'GGAGGAGAGAGAGGATGATT-3'</td>
<td>R: 5'GGTGCTTGCCGAGATGATG-3'</td>
</tr>
<tr>
<td>ClnD</td>
<td>F: 5'GAGGAGGAGGAGGACTCAGT-3'</td>
<td>R: 5'GGGAGGAGGAGGACTCAGT-3'</td>
</tr>
<tr>
<td>p21Waf1</td>
<td>F: 5'TGTCGCTATGGATGGCAGGA-3'</td>
<td>R: 5'ATGAGGACCGCGGACCAAG-3'</td>
</tr>
<tr>
<td>Bax</td>
<td>F: 5'AGGCGCCTATCCCGTCTTG-3'</td>
<td>R: 5'GGGGGTGTTGTTCTCATCCAG-3'</td>
</tr>
<tr>
<td>Bel-2</td>
<td>F: 5'ATGCCGTGAGCCGATGCTG-3'</td>
<td>R: 5'GGGGGTGTTGTTCTCATCCAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'GGTATCGTGGGATGACTGAC-3'</td>
<td>R: 5'ATGCCGTGAGCCGATGCTG-3'</td>
</tr>
</tbody>
</table>

According to the 2002 updates for the K/DOQI clinical practice guidelines for chronic kidney disease (Table 3), of the ADPKD cases were
classified as stage CKD1, nine as stage CKD2–3 and 14 as stage CKD4–5. With ADPKD disease stage progression, the patients' renal function was aggravated gradually and the SPARC concentration in urine and cyst fluid tended to increase. However, SPARC concentrations in plasma, urine and cyst fluid did not differ among the patients of the different stages ($P > 0.05$).

The distribution of SPARC and its expression levels in renal tissues

*In situ* hybridization and immunohistochemistry were employed to detect SPARC distribution in normal and polycystic renal tissues. As shown in Figure 1, positive staining appeared as brown granules throughout the cytoplasm. Very weak expression of SPARC was observed in normal renal tubules. In renal tissues of adult patients with ADPKD, SPARC was localized mainly in cyst-lining epithelial cells, dilated tubules and collecting ducts, and presented strongly positive staining in cytoplasm.

SPARC mRNA and protein levels in normal and polycystic renal tissues were further compared with real-time fluorescent quantitative RT–PCR and western blot assays. The real-time fluorescent quantitative RT–PCR and western blot (Figures 2 and 3) experiments indicated that the SPARC mRNA level in ADPKD kidney tissue was $(3.61 \pm 0.24) \times 10^2$ copies per million GAPDH, significantly higher than that in normal tissue $(1.72 \pm 0.09) \times 10^2$, and the SPARC protein level in ADPKD kidney tissue was also elevated relative to normal. The optical density ratio of SPARC protein and the corresponding GAPDH band in normal renal tissue and polycystic renal tissue were 44.68 and 81.25%, respectively. These results are consistent with our previous observation that SPARC is upregulated in ADPKD kidney tissue detected by cDNA expression microarray (unpublished findings).

**Increased SPARC secretion in cyst-lining epithelial cells**

Because SPARC is a secreted protein, it may affect growth of cyst-lining epithelia in an autocrine and/or paracrine manner. Therefore, SPARC secretion levels from HKC and cyst-lining epithelial cells were analysed. While western blot assay revealed no detectable SPARC from the concentrated protein in culture media of the control group, culture media from separate cultures of HKC cells or cyst-lining epithelial cells were found to have SPARC protein, and the level of the latter exceeded that of the former. The ratios of optical density values of SPARC protein secreted by HKC cells and cyst-lining epithelial cells to that of their corresponding GAPDH internal controls were 35.56 and 71.15%, respectively (Figure 4).

**Exogenous SPARC inhibits the proliferation of cyst-lining epithelia and affects the mRNA levels of PCNA and MCM2**

To investigate the autocrine/paracrine effects of SPARC on cyst-lining epithelial cell growth, epithelial cells were cultured for 48–96 h in medium containing different concentrations of exogenous human platelet SPARC. This result is important as it indicates whether platelet SPARC produces the same growth inhibitory effect on the cells used in this study as it does on other cells, e.g. endothelial, retinal pigment

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>SPARC concentration (ng/ml)</th>
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<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Urine</td>
<td>Cyst fluid</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>496.71 ± 169.98</td>
<td>123.91 ± 28.37</td>
<td></td>
</tr>
<tr>
<td>SRC</td>
<td>10</td>
<td>494.86 ± 173.30</td>
<td>134.08 ± 30.00</td>
<td></td>
</tr>
<tr>
<td>ADPKD</td>
<td>37</td>
<td>571.46 ± 250.53$^{b,c}$</td>
<td>1253.16 ± 544.81$^{a,c,d}$</td>
<td></td>
</tr>
</tbody>
</table>

Compared with the normal control group, $^aP > 0.05$, $^bP < 0.01$; compared with the SRC group, $^cP > 0.05$, $^dP < 0.01$, $^eP < 0.01$; compared with the SPARC concentration in urine of ADPKD group, $^fP < 0.01$.

**Table 3. The concentration of SPARC in plasma, urine and cyst fluid of the ADPKD group**

<table>
<thead>
<tr>
<th>CKD stage</th>
<th>n</th>
<th>SPARC concentration (ng/ml)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
<td>Urine</td>
<td>Cyst fluid</td>
</tr>
<tr>
<td>CKD1</td>
<td>14</td>
<td>548.02 ± 203.32</td>
<td>1063.21 ± 300.29</td>
<td></td>
</tr>
<tr>
<td>CKD2–3</td>
<td>9</td>
<td>527.44 ± 134.84</td>
<td>1220.53 ± 364.82</td>
<td></td>
</tr>
<tr>
<td>CKD4–5</td>
<td>14</td>
<td>623.20 ± 242.88</td>
<td>1464.10 ± 672.91</td>
<td></td>
</tr>
</tbody>
</table>

SPARC concentrations did not differ among groups of ADPKD patients by CKD stage ($P > 0.05$).
epithelial cells and ovarian cancer cells [15–17]. BrdU incorporation ELISA (described in Materials and methods) indicated that proliferation was progressively inhibited by treatment with increasing concentrations of SPARC. At 10 µg/ml, 48 h treatment with SPARC effectively inhibited proliferation of cyst-lining epithelial cells relative to controls ($P < 0.05$). This inhibitory effect was time dependent and reached its peak at 96 h of treatment. After 96 h of SPARC treatment, all tested concentrations of SPARC were sufficient to inhibit cyst-lining epithelial cell proliferation relative to the control group ($P < 0.01$) and relative to the same concentration after 48 and 72 h of treatment ($P < 0.05$). The inhibition rate of SPARC at the maximum tested concentration of 10 µg/ml reached 49.19%. The above proliferation inhibition data are summarized in Table 4, and Figures 5 and 6.

The real-time fluorescent quantitative RT–PCR data describing SPARC treatment-induced changes in PCNA and MCM2 mRNA expression were consistent with those obtained from the BrdU incorporation ELISA. Treatment with 10 µg/ml SPARC for 72 h
decreased the epithelial mRNA levels of both PCNA and MCM2. The mRNA level of PCNA decreased from \((8.26 \pm 1.01) \times 10^3\) copies per million GAPDH to \((3.58 \pm 1.16) \times 10^3\) \((P < 0.01)\), and MCM2 decreased from \((5.23 \pm 1.01) \times 10^3\) to \((1.39 \pm 0.54) \times 10^3\) \((P < 0.01)\) (Table 5).

Exogenous SPARC inhibits cell cycle progression of cyst-lining epithelia and affects the mRNA levels of cell cycle regulatory genes

FACS was applied to evaluate the effects of SPARC on the cell cycle and PI% of cyst-lining epithelial cells. SPARC treatment for 72 h reduced the amount of cyst-lining epithelial cells in S\(+\)G2/M phases so that cells remained at G0/G1 longer, in a concentration-dependent manner. The PI% similarly gradually decreased with increasing SPARC treatment concentration (Table 6). Moreover, real-time fluorescent quantitative RT–PCR analysis revealed that 10\(\mu\)g/ml SPARC treatment for 72 h decreased the mRNA level of ClnD1 from \((7.50 \pm 0.99) \times 10^4\) copies per million GAPDH to \((4.25 \pm 1.38) \times 10^4\) \((P < 0.01)\), but increased that of \(p21^{Waf1}\) from \((4.25 \pm 1.38) \times 10^3\) to \((7.72 \pm 0.85) \times 10^3\) \((P < 0.05)\) (Table 7).
SPARC induces apoptosis in cyst-lining epithelia and affects the mRNA levels of cell apoptosis-related genes

Recent studies have confirmed that there are imbalances of proliferation and apoptosis in ADPKD cystic cells [18]. Thus, to determine whether exogenous SPARC can induce cyst-lining epithelial cell apoptosis, SPARC treatment effects on cyst-lining epithelial cell morphology were assessed and their AIs were measured. After 48 h treatment with SPARC at 2.5, 5 and especially 10 μg/ml, some previously irregularly shaped cells became elliptical or round and some became suspended with intact cellular membranes from adherent cells. Examination by electron microscopy revealed typical apoptotic changes with SPARC treatment, i.e. numerous cytoplasmic vacuoles and fissures emerged, nuclei became irregular with concentrated skirted chromatin and typical apoptotic bodies were observed in some cells (Figure 7).

Apoptotic peaks and AIs were detected by FACS after 48 h of 2.5, 5 and 10 μg/ml SPARC treatment. As shown in Figure 8, the typical hypodiploid peak (blue) was observed in the DNA histograms of all groups. The area of the apoptotic peak increased with increased SPARC treatment concentration. The corresponding AIs of all groups were 8.2, 12.7, 13.3 and 29.5%, respectively.

Real-time fluorescent quantitative RT–PCR measurements of changes in the apoptosis-related genes Bax and Bel-2 after SPARC treatment at 10 μg/ml for 72 h revealed a selective effect of

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Fig. 7. Ultrastructural changes of SPARC-treated cyst-lining epithelial cells. (A) Normal structure of control cells. (B) SPARC at 2.5 μg/ml. Note the numerous cytoplasmic vacuoles (arrow e). (C) SPARC at 5 μg/ml. Many cytoplasmic fissures (arrow f) are apparent. (D) SPARC at 10 μg/ml. Concentrated and skirted chromatin (arrow g) and typical apoptotic bodies (arrow h) in cytoplasm are visible.

Fig. 8. Apoptosis index of cyst-lining epithelial cells after SPARC treatment with 0 (A, control), 2.5 (B), 5 (C) and 10 (D) μg/ml.
the treatment on **Bcl-2**. While the mRNA level of **Bax** was not affected, **Bcl-2** was decreased from $(8.05 \pm 0.72) \times 10^3$ to $(6.50 \pm 0.80) \times 10^3$ copies per million **GAPDH** ($P < 0.05$). This selective decrease in **Bcl-2** resulted in a decreased **Bcl-2** to **Bax** ratio $(1.67 \pm 0.09$ vs $2.06 \pm 0.09$, $P < 0.05$) (Table 8).

### Discussion

**Elevated SPARC in ADPKD**

Recent studies have indicated that **SPARC** has antitumour activity. Notably, tumour growth and metastasis in several tumour models were enhanced in **SPARC** gene knockout mice [19], and tumour-suppressing properties of **SPARC** have been reported in *in vitro* studies with ovarian cancer cells and neuroglioma cells [17,20]. The present study of the distribution and expression of **SPARC** in body fluids and renal tissues of ADPKD patients complements previous research on **SPARC**’s antitumour activity and is consistent with our previous observation (unpublished findings) that **SPARC** is upregulated in ADPKD kidney tissues. The present findings indicate that **SPARC** is expressed strongly and mainly in cyst-lining epithelial cells and dilated renal tubules of ADPKD patients. In addition, we found that **SPARC** levels were elevated in urine of ADPKD patients relative to SRC patients and normal subjects. **SPARC** levels in cyst fluid for ADPKD patients was markedly higher than that in urine.

**SPARC** is a secreted glycoprotein with a molecular weight of 32 kDa after cleavage of the signal sequence. Micromolecular proteins with a molecular weight of 32 kDa in urine would be expected to be derived from glomerular filtration. However, in this study, the ADPKD patients were negative or only mildly positive on urine protein tests. Moreover, **SPARC** mRNA and protein were found to be located in cyst-lining epithelial cells, dilated renal tubules and renal collecting ducts. Previous studies have shown that **SPARC** is expressed in the renal tubular epithelium, collecting duct, urethra and bladder epithelium [21]. Therefore, **SPARC** in ADPKD patients’ urine is probably from the urine outflow duct including the renal tubule, collecting duct, bladder and urethra. **SPARC** originating from the kidney is probably derived mainly from the renal tubule and collecting system, as well as the epithelial cells of little cysts along the renal tubule and collecting duct. Because the kidney is the main organ affected in ADPKD and neither the bladder nor the urethra were found to have pathological changes in our ADPKD patients, we did not include an analysis of the urethra and bladder in this study. Our findings suggest that the markedly elevated **SPARC** concentration in the urine of patients with ADPKD relative to normal subjects is due to increased **SPARC** secretion from dilated renal tubules and epithelial cells of renal tubule cysts.

Our findings with cultured cyst-lining epithelial cells and normal renal tubular cell supernatant are consistent with this view. As ADPKD develops, little cysts associated with the collecting ducts gradually lose contact as the ducts dilate. Therefore, **SPARC** in renal cyst fluid may be derived mainly from cyst-lining epithelial cells. Moreover, the finding that cyst-lining epithelial cells secreted more **SPARC** than tubular cells *in vitro* may explain, at least in part, the higher **SPARC** levels found in the cyst fluid of ADPKD patients compared with that in urine and the obvious difference in cyst fluid **SPARC** levels between the ADPKD group and SRC group. However, as the present study included relatively few SRC patients and histological data about **SPARC** in the renal tissue of SRC patients were not obtained, it is not known whether cyst fluid **SPARC** levels would differ between large samples of SRC and ADPKD patients.

Besides, we did find that urine and cyst fluid **SPARC** levels had a trend to increase along with the progression of renal function aggravation. However, this effect was not statistically significant in the present study. Therefore, further research focused on this question with larger group sizes will be required to determine whether there is a real relationship between **SPARC** levels in urine and cyst fluid and deterioration of renal function.

**SPARC inhibition of cyst-lining epithelial cell proliferation and the expression of PCNA and MCM2**

Recent studies have demonstrated that the cyst-lining epithelial cells of a polycystic kidney have the characteristic of high multiplication similar to that seen in benign tumours, which can promote the emergence and dilation of more cysts [22]. The antiproliferative activity of **SPARC** has been demonstrated in endothelial cells [23]. Domain IV of **SPARC**, which contains an EF-hand-like loop and has a high-affinity Ca$^{2+}$-binding site, is sufficient to induce **SPARC**-mediated growth inhibitory functions [24]. Furthermore, mesangial cells, fibroblasts and smooth muscle cells isolated from **SPARC-null** mice were found to grow faster than their respective wild-type counterparts [25].

The present study provides an *in vitro* demonstration that exogenous **SPARC** can reduce epithelial cell proliferation in a concentration- and time-dependent manner. Microgram concentrations of **SPARC** inhibited cyst-lining epithelial cell proliferation and

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**Table 8. The effects of 72 h SPARC treatment at 10 μg/ml on the expression of apoptosis-related genes in cyst-lining epithelial cells**

<table>
<thead>
<tr>
<th>Group</th>
<th>mRNA levels (copies/million <strong>GAPDH</strong>)</th>
<th>Bcl-2/Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$(4.05 \pm 0.18) \times 10^3$ to $(8.05 \pm 0.72) \times 10^3$ copies per million <strong>GAPDH</strong></td>
<td>$2.06 \pm 0.09$</td>
</tr>
<tr>
<td><strong>SPARC</strong>-treated</td>
<td>$(3.88 \pm 0.31) \times 10^3$ to $(6.50 \pm 0.80) \times 10^3$ copies per million <strong>GAPDH</strong></td>
<td>$1.67 \pm 0.09^a$</td>
</tr>
</tbody>
</table>

$^aP < 0.05$. 

---
markedly reduced PCNA and MCM2 mRNA expression. PCNA is a nuclear protein that is expressed specifically during cell proliferation. Its expression starts at the end of G1 phase, reaches its peak during S phase and early G2 phase, and ceases during M and G0 phases [26]. MCM2 has a key role in DNA replication. The expression of MCM mRNAs and proteins selectively increases during proliferation and this increase is proportional to the degree of cell proliferation [27]. These findings suggest that SPARC may also inhibit cyst-lining epithelial cell proliferation in vivo.

**SPARC regulation of the cell cycle via influences on ClnD1 and p21<sub>Waf1</sub> expression**

SPARC treatment increased the number of cells at G<sub>0</sub>/G<sub>1</sub> phase and decreased the number of cells at S phase to a similar degree, indicating that SPARC inhibition of cell proliferation may be mediated through a regulatory effect on the G<sub>1</sub> phase. The cell cycle is the final pathway for cell proliferation and has several checkpoints. The G1–S checkpoint is key because this is when cells integrate a convergence of signals to determine whether to initiate cell division, to enter into a resting state (G0 phase) or to undergo apoptosis.

Cyclin D1 encoded by ClnD1 is an important cyclin associated with G<sub>1</sub> phase regulation that can control cyclin-dependent kinase (CDK) activity by phosphorylation and enhance the expression of some genes whose products promote the passage of cells through the G1–S checkpoint and hence the commencement of cell division [28]. The first CDK inhibitor identified in mammalian cells, p21<sub>Waf1</sub>, suppresses the activity of CDK or the cyclin D1–CDK complex through dephosphorylation and inhibits cell proliferation [29]. PKD1 can activate the JAK–STAT pathway, thereby upregulating p21<sub>Waf1</sub> and inducing cell cycle arrest in G<sub>0</sub>/G<sub>1</sub>. Increased expression of p21<sub>Waf1</sub> is primarily responsible for mediating the growth-suppressing effects of PKD1 in an experimental system [30]. In this study, we found that SPARC treatment decreased ClnD1 mRNA levels but increased p21<sub>Waf1</sub> mRNA levels in cyst-lining epithelial cells. These data suggest that SPARC may regulate the cell cycle in cyst-lining epithelial cells by concurrently inhibiting ClnD1 expression and augmenting p21<sub>Waf1</sub> expression. By this mechanism, SPARC may prevent cyst-lining epithelial cells from entering into S phase and keep them in a resting state.

**SPARC regulation of the apoptotic regulatory factors Bcl-2 and Bax**

Both epithelial cell apoptosis and proliferation are dysregulated in ADPKD [31,32]. Dysregulation of the balance between pro- and anti-apoptotic Bcl-2 family members correlates with increased apoptosis in ADPKD [33]. The ratio of Bcl-2–Bax heterodimers to Bax–Bax homodimers is a critical factor in determining apoptosis susceptibility [34]. We found that SPARC treatment could induce cyst-lining epithelial cell apoptosis and simultaneously reduce the ratio of Bcl-2 to Bax. Therefore, the regulation of Bcl-2 and Bax expression may be a key mechanism underlying SPARC induction of apoptosis in cyst-lining epithelial cells.

**Putative SPARC receptors**

The antiproliferative and apoptotic effects of exogenous SPARC on cyst-lining epithelial cells are consistent with the presence of cell surface receptors for SPARC. This hypothesis is supported by the results obtained from studying the mediators through which exogenous SPARC exerts its counteradhesive and antiproliferative effects on endothelial cells. Pretreating endothelial cells with protein tyrosine kinase inhibitors protected them against the inhibitory effect of SPARC on cell spreading. Moreover, inhibition of cell cycle progression by SPARC was blocked by treatment with heterotrimeric G protein inhibitors such as pertussis toxin and cholera toxin [35]. A recent study showed the first direct evidence that SPARC binds to putative SPARC receptors on the cell surfaces of HOSE and ovarian cancer cells using a fusion protein containing SPARC and human placental alkaline phosphatase [19]. To date, however, neither a putative SPARC receptor nor an intracellular signalling pathway triggered by SPARC has been identified.

**Summary**

In this study, we showed that exogenous SPARC could reduce proliferation and induce apoptosis in cyst-lining epithelial cells. The antiproliferative effect may be mediated by an inhibition of ClnD1 and stimulation of p21<sub>Waf1</sub> expression, thereby preventing the passage of cells through the G1–S checkpoint. SPARC may also induce apoptosis of epithelial cells by decreasing the ratio of Bcl-2 to Bax gene expression. The findings of the SPARC distribution in kidney tissues and in ADPKD patient body fluids indicate that SPARC may play a role in negative feedback. All these suggest that SPARC may have a protective effect in ADPKD.

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