c-Src inactivation reduces renal epithelial cell-matrix adhesion, proliferation, and cyst formation

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Elliott J, Zheleznova NN, Wilson PD. c-Src inactivation reduces renal epithelial cell-matrix adhesion, proliferation, and cyst formation. Am J Physiol Cell Physiol 301: C522–C529, 2011.—c-Src is a non-receptor tyrosine kinase whose activity is induced by phosphorylation at Y418 and translocation from the cytoplasm to the cell membrane. Increased activity of c-Src has been associated with cell proliferation, matrix adhesion, motility, and apoptosis in tumors. Immunohistochemistry suggested that activated (pY418)-Src activity is increased in cyst-lining autosomal dominant polycystic kidney disease (ADPKD) epithelial cells in human and mouse ADPKD. Western blot analysis showed that SKI-606 (Wyeth) is a specific inhibitor of pY418-Src without demonstrable effects on epidermal growth factor receptor or ErbB2 activity in renal epithelia. In vitro studies on mouse inner medullary collecting duct (mIMCD) cells and human ADPKD cyst-lining epithelial cells showed that SKI-606 inhibited epithelial cell proliferation over a 24-h time frame. In addition, SKI-606 treatment caused a striking statistically significant decrease in adhesion of mIMCD and human ADPKD to extracellular collagen matrix. Retained viability of unattached cells was consistent with a primary effect on epithelial cell anchorage dependence mediated by the loss of extracellular matrix (ECM)-attachment due to αβ3-integrin function. SKI-606-mediated attenuation of the human ADPKD hyperproliferative and hyper-ECM-adhesive epithelial cell phenotype in vitro was paralleled by retardation of the renal cystic phenotype of Pkd1 orthologous ADPKD heterozygous mice in vivo. This suggests that SKI-606 has dual effects on cystic epithelial cell proliferation and ECM adhesion and may have therapeutic potential for ADPKD patients.

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shown that inhibition of c-Src activity can block cell proliferation and decrease cell migration associated with metastasis, thereby suggesting c-Src as an attractive molecular target for anticancer therapy (20, 32). Since the progressive expansion of cysts in ADPKD is characterized by increased epithelial cell proliferation and increased cell-matrix adhesion coupled with abnormalities in cellular migration (8, 26, 29, 45, 49), this study was designed to examine the role of c-Src in these processes with a view to assessment of its value as a therapeutic target. We show that the Wyeth c-Src inhibitor SKI-606 inhibits mouse renal collecting duct and human ADPKD cyst lining epithelial cell proliferation and reduces cell-matrix adhesion in vitro and can retard cystic expansion in a Pkd1 heterozygous mouse model of ADPKD in vivo.

METHODS

Cell lines and tissues. The mouse inner medullary collecting duct (mIMCD) cell line was procured from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium-F-12 containing 4.5 g/l glucose and 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 g/ml streptomycin. Normal and ADPKD kidney tissues were procured at source by National Disease Research Interchange and prepared for microdissection; primary and conditionally immortalized [temperature sensitive (ts)] epithelial cell culture and characterization are as previously described (30, 46, 48). ADPKD cells were cultured in HEPES-buffered Click-RPMI media (Quality Biologicals, Gaithersburg, MD) containing transferrin (5 mg/ml, Sigma, St. Louis, MO), dexamethasone (5 –10–8 M, Sigma), insulin (5 mg/ml, BD Biosciences, Bedford, MA), tri-iodothyronine (5 –10–12 M, Sigma), and 1% FBS (Crystralgen, Plainview, NJ) (48). Conditionally immortalized ts-ADPKD cells were grown at 33°C and transferred to the nonpermissive 37°C for 3 days for differentiation before experimentation.

Pkd1 null heterozygous (+/−) mice (23) were backcrossed onto the C57BL6 background to reduce variability and accelerate cystic development that had previously been noted after 12–16 mo of age (Dr. J. Zhou, personal communication). For characterization studies, at least six animals [Pkd1+/− and wild-type (WT) littermate] were euthanized for pathology, cystic, and marker analysis at 2, 3, 4, 5, 6, 8, 11, and 12 mo of age. Renal cysts (defined as a >3-fold increase in tubule lumen diameter) were seen in some mice by 3 mo of age and in all mice by 5 mo of age, and this development was accompanied by an increase in renal weight (WT 0.45 ± 0.03 g vs. Pkd1+/− 0.64 ± 0.06 g, n = 10). Some mice were monitored for longer, but since by 14 mo 20% had died, all animals were euthanized at 16 mo of age. All animal studies were approved by the Institutional Animal Care and Use Committee of Mount Sinai School of Medicine.

Cell and animal treatments. mIMCD and ADPKD cells were plated at confluent, 75% or 50% subconfluent density in standard cell-type media, in media containing 2.5 µM SKI-606 (Wyeth, generous gift of Dr. E. Avner, Medical College of Wisconsin), dissolved in minimal volume of 0.05% DMSO; or in media containing the vehicle (0.05% DMSO) alone. Wild-type (WT, +/+) and heterozygous Pkd1 (+/−) mice were identified by genomic PCR, and six groups of five animals were treated for 3 mo from 5 mo of age and euthanized at 8 mo, and from 8 mo of age and euthanized at 11 mo. Pkd1 (+/−) littermates were treated with SKI-606 (30 mg·kg−1·day−1) or vehicle alone (0.2% DMSO) in their daily drinking water and compared with untreated mice. After 3 mo, the mice were euthanized and the kidneys were fixed for 4 h at 4°C in 4% paraformaldehyde (electro microscope copy grade, EM Sciences, Pasadena, CA) in phosphate-buffered saline (PBS, pH 7.4), dehydrated, and embedded in low-temperature paraffin before sectioning at 5 µm.

Cell-matrix adhesion assays. mIMCD and ADPKD cells were subjected to matrix attachment assays for 2 h to 4.5,000–8,000 cells per well in 96- or 24-well plates were seeded onto collagen type I matrix (300 µg/ml, BD Biosciences) in 100 µl of the media in the presence or absence of SKI-606 or DMSO vehicle. After 4 h of attachment, unattached cells were gently removed by aspiration, and numbers of attached cells were determined either by cell counting of fixed, hematoxylin and eosin (H&E)-stained culture plates or by an automated colorimetric plate reader assay after 4 h of incubation in MTS (Cell Titer 96 AQ, Promega, Madison, WI). Color development was read at 492 nm (model 550, Bio-Rad, Bucks, UK) and values were subtracted from parallel control plates lacking cells. Each experiment was repeated three to six times with four to eight replicates of each condition. In addition, unattached cells released into the media were collected by aspiration and low-speed centrifugation and were counted using a hemocytometer. Attached cells from the same plates were collected by trypsinization and counted, total cell numbers were summed, and percentages of unattached floating cells were calculated.

Cell viability assays. Trypan blue exclusion assays were carried out to determine viability of attached and unattached cells. Trypan blue (0.1 ml, 0.4%) was added to 0.5 ml of cell suspension and left to stand at room temperature for 30 min before hemocytometer counting of viable (unstained) and nonviable (blue) cells.

Cyst proliferation assays. Multiple replicates of 2,000 cells were plated in 100 µl per well in 96-well plates and left to attach in standard media for 24 h and in serum-free media for a further 24 h. Cyst proliferation was then measured in the presence or absence of SKI-606 (2.5 µM) or vehicle (DMSO, 0.05%) at 4, 8, and 24 h by the addition of 20 µl MTS (Cell Titer 96 AQ), incubation at 37°C for 4 h, microplate reader determination of color development at 492 nm, and subtraction of no cell blanks. These experiments were repeated three times with eight replicates in each group.

Western immunoblot analysis. Total cell protein extracts were prepared by lysis in buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.2, 1 mM EGTA, 1 mM EDTA, and a comprehensive protease and phosphatase inhibitor cocktail (Sigma). Protein concentrations were determined using the Bradford assay (Pierce). Cell proteins were separated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Lysate (20–50 µg) was loaded per lane, and separated proteins were transferred to nitrocellulose or polyvinylidene difluoride membranes. After blocking in 5% nonfat milk in TBST (10 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 8) or 5% BSA, membranes were incubated in primary antibody for 1.5 h at room temperature or overnight at 4°C and washed in TBST. The primary antibodies used were as follows: anti-Src (1:1,500, mouse monoclonal, Upstate Biotechnologies, Lake Placid, NY), anti-pY181,Src (1:1,000, rabbit polyclonal, Biosource, Camarillo, CA), anti-EGFR (1:1,000, rabbit polyclonal, Cell Signaling, Danvers, MA), anti-pY1068 EGFR (1:500, mouse monoclonal, Cell Signaling); anti-ErbB2 (1:1,000, rabbit, AbCam, Cambridge, UK), anti-pY877 and 1021/2 ErbB2 (1:500 rabbit, Cell Signaling), and anti-β-actin (1:5,000, rabbit, Abcam). Conjugated horseradish peroxidase-coupled goat anti-mouse (1:2,000, Pierce) or goat anti-rabbit IgG (1:40,000, Sigma) were used as secondary antibodies and immunoreactivity detected by enhanced chemiluminescence (ECL, Thermoscientific, Rockford, IL).

Immunohistochemistry. Paraffin sections (5 µm) of age-matched human and mouse normal and ADPKD kidneys, fixed for 4 h at 4°C in 4% paraformaldehyde (EM Sciences) in PBS, pH 7.4, were subjected to optimized immunohistochemistry, as described previously (29) using an optimized indirect, avidin biotin-enhanced technique and aminoethylcarbazole detection yielding a red reaction product (Vectastain, Vector Laboratories). Anti-Src (1:150, GD1 monoclonal, Upstate) and anti-pY418-Src (1:100); AQP-1 (1:500, Alpha Diagnostic, Cagus); calbindin (mouse monoclonal D28K, 1:300, Sigma), and AQP-2 (1:75, Alpha Diagnostic) were used and compared with matched controls lacking primary antibody or incubated with preabsorbed and irrelevant antibodies. Some sections were also stained with the peroxidase-coupled lectins DBA, PNA, and LTA...
(Sigma) or for alkaline phosphatase using the nitro blue tetrazolium (NBT-BCIP) technique (Vector).

**Microscopy.** Sections were viewed under a Nikon Eclipse 800s microscope by bright field, or differential interference contrast (DIC) optics. At least 10 random fields were examined per section and 10 sections per block of normal, WT, or cystic kidneys for photography and counting of cysts (> 3-fold expansion of normal tubule diameter).

**Statistics.** Data were collected and plotted as means ± SE to compare pairwise the difference in the untreated and treated groups of cells. Statistical analysis was performed using Student’s t-test or analysis of variance, and significance was determined as a P value. P < 0.05 was considered significant.

**RESULTS**

Activated c-Src is increased in human and mouse ADPKD cyst-lining epithelia. Since an increase in expression and activation of c-Src by phosphorylation on its autophosphorylation site at Y418 is associated with increased cell proliferation, spreading, and migration in numerous tumors and cancer cell lines, we first analyzed its distribution and activation in human and mouse ADPKD. Immunohistochemistry was carried out using well-characterized, anti-pY418-Src antibodies (Fig. 1) and demonstrated strongly increased levels of active c-Src in cyst-lining ADPKD epithelial cells when compared with age-matched normal human kidneys (Fig. 1, B vs. A) as well as in C57/Pkd1 heterozygous (+/−) compared with WT (+/+) littermate mouse kidneys from 5 mo of age (Fig. 1, D vs. C).

**SKI-606 is a specific inhibitor of activated (p)-Src.** The recent development of cell-permeable, orally available, small molecule inhibitors of tyrosine kinases has led to advances in the analysis, understanding, and inhibition of proliferative conditions such as cancer. To determine the profile of specificity of the c-Src inhibitor SKI-606, with regard to ADPKD-related proliferative pathways, we carried out comparative Western blot analysis mIMCD cells (Fig. 2A). SKI-606 (2.5 μM in 0.05% DMSO-containing media) (lane 3 vs. lane 1), but not of DMSO (0.05%) vehicle alone (lane 2), resulted in specific and complete inhibition of active pY418-Src but had no effect on the levels of expression of the other important ADPKD-related receptor tyrosine kinases, EGFR or ErbB2. Levels of total c-Src protein, which would include active (phosphorylated) and inactive c-Src, were unchanged. SKI-606 also inhibited pY418-Src in cultured human ADPKD cells (Fig. 2B, lane 2 vs. lane 1).
Inhibition of active c-Src reduces normal renal tubule epithelial cell-matrix attachment and proliferation. Renal epithelial cell-matrix adhesion is essential for and mediates subsequent proliferative responses during normal tubule development and differentiation in vitro and in vivo. Matrix adhesion assays were carried out to determine the role of activated c-Src in normal renal epithelial cells by quantitative comparison of adherent cell numbers after incubation for 4 h in the presence or absence of the c-Src inhibitor SKI-606 (Fig. 3A). Although this compound is cell permeable and orally available, it must be solubilized in low concentrations (0.05%) of DMSO vehicle. Control studies showed that 0.05% DMSO alone caused no statistically significant alteration in adhesion properties (Fig. 3A, bar 2 compared with bar 1) while the presence of SKI-606 resulted in a statistically significant decrease in cell-matrix attachment (Fig. 3A, bar 3 compared with bars 1 and 2; P < 0.05). This result was confirmed by the demonstration of statistically significant increases of nonadherent floating cells collected from the media of SKI-606-treated cells (26 ± 6%) compared with DMSO (3.8 ± 0.4%) vehicle-treated or untreated cells (4.2 ± 0.7%; P < 0.05) (Fig. 3B). In addition, Trypan blue exclusion viability studies demonstrated that these effects were not due to a toxic or apoptotic effect of SKI-606 since only low levels (15.2 ± 3.5%) of nonviable mIMCD cells were seen in the unattached populations after 2 h of treatment with SKI-606, a value that was not significantly different from those measured for unattached mIMCD cells before treatment (12.5 ± 2.8%). These results are consistent with a specific effect of pY418-Src inhibition on cell-matrix attachment (anchorage dependence) of normal renal epithelial cells.

Longer-term studies were carried out to determine the effects of SKI-606 on proliferation in serum-starved mIMCD cells over a 24-h period (Fig. 4). These experiments demonstrated a statistically significant decrease in mIMCD cell proliferation after 8 h of treatment that was exacerbated after 24 h. DMSO vehicle alone had no effect of mIMCD cell proliferation under identical conditions.

Inhibition of active c-Src reduces ADPKD cyst epithelial cell-matrix attachment and proliferation. Since active pY418-Src is apparently highly expressed in ADPKD epithelia (Fig. 1) and human ADPKD epithelia had previously been shown to exhibit increased cell-matrix adhesion (29, 49) and increased epithelial cell proliferation (8), we next sought to determine whether SKI-606 could normalize the human ADPKD cell phenotype in vitro. Five thousand cells were plated per well, allowed to settle, and serum starved overnight before addition of the compound. Interestingly, significant decreases in ADPKD epithelial cell-matrix adhesion were seen after 4 h of attachment in the presence of 2.5 μM SKI-606 (bar 3 compared with bars 1 or 2, Fig. 5A). Corresponding significant increases in unattached ADPKD cells were measured in SKI-606-treated cells (29 ± 2.5%) compared with DMSO (4.2 ± 0.7%; P < 0.05).

Fig. 3. Effects of SKI-606 on mIMCD cell adhesion to matrix. A: attached cells. B: unattached cells from culture media. White bars, untreated; gray bars, treated with 0.05% DMSO vehicle; black bars, treated with 2.5 μM SKI-606. 4-h Attachment to type I collagen. SRCI, c-Src inhibitor. *P < 0.05.

Fig. 4. Effects of SKI-606 on mIMCD cell proliferation. All cells were serum starved for 24 h before plating of 2,000 cells per well. Colorimetric MTS assay; 8 replicates per condition; 3 experiments. *P < 0.05.

Fig. 5. Effects of SKI-606 on human ADPKD epithelial cell adhesion to matrix. A: attached cells. B: unattached cells from culture media. Bar 1, untreated; bar 2, treated with 0.05% DMSO vehicle; bar 3, treated with 2.5 μM SKI-606. 4-h Attachment to type I collagen. *P < 0.05.
606-treated ADPKD cells compared with vehicle or untreated control cultures (bar 3 compared with bars 1 or 2, Fig. 5B). Trypan exclusion assessment of viability detected low levels of cell death (12.5 ± 3.1%) in these floating SKI-606-treated cells that were not significantly different from those values calculated for untreated controls (17.1 ± 2.5%). These results are again consistent with an effect of SKI-606 on ADPKD cell anchorage-dependence (loss of ECM attachment). In addition, 24 h studies on serum-starved monolayers showed an effect of SKI-606 on human ADPKD epithelial cell proliferation which was significantly reduced after 24 h of treatment (Fig. 6).

Inhibition of active c-Src reduces cystic development in ADPKD mice. Although complete knockout of the PKD1 gene and PC-1 protein causes embryonic lethality in mice and humans, its haplo-insufficiency and loss of function results in progressive renal cystic disease. Modification of an orthologous Pkd1-null mouse (23) by backcrossing onto a homogeneous C57/BL6 background and characterization of the heterozygous offspring has led to the development of a Pkd1 mouse model that closely recapitulates the progressive time scale and phenotypic properties of renal cystic (and interstitial fibrotic) development seen in human ADPKD (Fig. 7, A–C and L). By 6 mo of age, 100% of C57/Pkd1+/− mice had cystic kidneys with an average of 2-fold increases in kidney volume and 1.5-fold increases in kidney weight compared with their unaffected littermates. Immunohistochemical marker analysis demonstrated the presence of aquaporin 1 (AQP-1), alkaline phosphatase, calbindin, DBA, and AQP-2-positive cysts (Fig. 7, D–K, and data not shown), reflecting the patterns seen in human ADPKD in which cysts are derived from every segment of the nephron (45, 47).

Treatment of C57/Pkd1+/− mice with the pY418-Src inhibitor SKI-606 for 3 mo from 5 to 8 mo of age resulted in significant reduction of cyst numbers (defined as a ≥3-fold increase in lumen diameter) by comparison to untreated or vehicle-treated controls (untreated 89.45 ± 6.5; DMSO-treated 92.64 ± 7.9; SKI-606-treated 22.35 ± 4.2; P < 0.05 treatment vs. DMSO or treatment vs. untreated). Assessment was carried...
out by two blinded individuals and 10 fields were examined per section, with 5 sections per kidney from 5 mice per condition. Cystic reduction in the SKI-606-treated animals was associated with the normalization of kidney morphology (Fig. 8), volume, and weight (WT at 8 mo: untreated, 0.50 ± 0.05 g; vehicle-treated, 0.47 ± 0.03 g; and SKI-606-treated, 0.51 ± 0.08 g, vs. Pkd1 at 8 mo: untreated, 0.74 ± 0.06 g; vehicle-treated, 0.72 ± 0.07 g; and SKI-606-treated, 0.51 ± 0.08 g).

**DISCUSSION**

Src was first identified as an oncogene and subsequently the cellular Src family of kinases has been implicated in cell growth regulation, particularly through the activation of the extracellular regulated kinase (MEK/ERK)/mitogen-activated kinase (MAPK) signaling pathway (31) and shown to be activated via cell-matrix adhesion or growth factor receptor activation. Both integrin-dependent adhesion- and growth factor induced-signaling leads to Src-mediated phosphorylation of FAK at Y925 that then creates a binding site for the SH2 domain of the adaptor protein Grb2 thus coupling Src to the activation of the Ras/MEK/ERK pathway (34). ADPKD is characterized by progressive expansion of renal tubular cysts due in part to increased cell proliferation and fluid secretion associated with abnormalities in cytoskeletal organization, cell-matrix adhesion, and growth factor-dependent migration (45). Increased EGFR, ErbB2, and ERK expression or overactivation of c-Src is often associated with abnormalities in cytoskeletal organization, morphogenesis and faulty renal tubule differentiation leading to cystic dilation (3, 29, 36). It is well established that overexpression or overactivation of c-Src is often associated with abnormal morphology and plays multiple roles in the regulation of proliferation, cell-ECM adhesion, invasion, and motility (52). Furthermore, the c-Src tyrosine kinase has been shown to interact with the catalytic domain of ErbB2, another major mediator of increased proliferation in ADPKD and tumors (19, 53). This led to the hypothesis that specific inhibition of activated pY418-Src could be therapeutic for ADPKD and carcinogenesis and plays multiple roles in the regulation of proliferation, cell-ECM adhesion, invasion, and motility (52). Therefore, c-Src is the only member of the Src kinase family that is significantly increased in ADPKD (data not shown), it is unlikely that other potential

![Fig. 8. Effects of SKI-606 on ADPKD cyst expansion in heterozygous C57/Pkd1 mouse model of ADPKD. A: unaffected littermate kidney. H&E. B: Pkd1+/- untreated mouse kidney untreated. H&E. C: Pkd1+/- SKI-606-treated mouse kidney. H&E. All mice were 8 mo of age and treatments were 3 mo in duration. Bars, 10 μm.](http://ajpcel.physiology.org/)

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targets for this drug such as Fyn, Yes, or Lyn play a significant role in its ability to restore normal cell function to ADPKD epithelia in vitro and retard cystic expansion in vivo (11).

This compound has previously been found to retard cystic expansion in rodent models of ARPKD including the phenotypic, nonorthologous Pck rat model of ARPKD (37) where its mechanism of action was concluded to be antiproliferative via the ERK/MAPK pathway. ARPKD in human patients is caused by mutational loss of the PKHD1 gene and shares some phenotypic features with ADPKD, particularly with regard to hyperproliferation of collecting duct proliferation and morphogenesis (25). Recent studies have also shown that ARPKD epithelial cell migration are characterized by hyperactive \( pY^{418} \)-Src, increased ECM adhesion, and reduced growth-factor-mediated directional migration (18) and that the PKHD1-encoded protein fibrocytin-1 participates in the PC-2/PC-1/integrin multiprotein complex (43). Furthermore, the PC-1-protein has been shown to be phosphorylated on Y4237 in its intracellular tail in normal renal collecting tubule cells, where phospho-activation of c-Src has also been detected, particularly in fetal stages of development (10, 22). Taken together, these results suggest a role for overactivation of c-Src in rodent models of both ARPKD and ADPKD.

Our studies suggest that SKI-606 may be a dual function inhibitor of renal epithelial cell proliferation and matrix-mediated cell anchorage and that it may provide a promising therapeutic agent for the future retardation therapy of cystic expansion in ADPKD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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