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Progressive renal fibrosis in murine polycystic kidney disease: An immunohistochemical observation

HIROKAZU OKADA, SHINICHI BAN, SHIZUKO NAGAO, HISAHIDE TAKAHASHI, HIROMICHI SUZUKI, and ERIC G. NEILSON

Department of Nephrology, and Second Department of Pathology, Saitama Medical College, Irumagun, and Laboratory Animal Center, Fujita Health University, Toyoake, Japan; and Division of Nephrology and Hypertension, Departments of Medicine and Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Progressive renal fibrosis in murine polycystic kidney disease: An immunohistochemical observation.

Background. The appearance of interstitial fibrosis in polycystic kidneys is emblematic of progressive disease. Matrix forming this scar tissue is derived from local renal cells in response to cystogenesis. We investigated the phenotype of collagen-producing cells in the cystic kidneys of DBA/2-pcy mice to better characterize the spectrum of interstitial cells associated with renal fibrogenesis.

Methods. The extent of interstitial fibrosis and the number of fibroblasts in cystic kidneys were first quantitated over time using computer-assisted image analysis. Subsequently, antisera to four cell protein markers were studied by coexpression immunohistochemistry during progression of fibrosis using confocal microscopy. The antisera included fibroblast-specific protein 1 (FSP1) for fibroblast phenotype, α-smooth muscle actin (α-SMA) for contractile phenotype, vimentin (VIM) for mesenchymal phenotype, and heat shock protein 47 (HSP47) for interstitial collagen-producing phenotype.

Results. Interstitial fibrosis in cystic kidneys gradually increased throughout the 30-week observation period of our study. With progression of cystogenesis, most of the tubules in pcy mice either dilated or disappeared with time. $FSP1^+$ fibroblasts were distributed sparsely throughout the renal interstitium of young pcy and wild-type mice. Their number increased in the widening fibrotic septa by 18 weeks of age and persisted through 30 weeks of the study interval. Some epithelia among remnant tubules trapped within fibrotic septa around adjacent cysts also acquired the phenotype of $FSP1^+$, $HSP47^+$ collagen-producing fibroblasts, suggesting a possible role for epithelial-mesenchymal transformation (EMT) in this process. Most $FSP1^+$ fibroblasts were $\alpha\text{-SMA}^-$, but $HSP47^+$, suggesting they were producing collagen proteins for the extracellular matrix. $\alpha\text{-SMA}^+$, $FSP1^-$, $HSP47^+$ or $HSP47^-$ cells were also observed, and the latter tended to distribute independently in a linear pattern, reminiscent of vasculature adjacent to forming cysts. VIM^+ expression was not observed in $\alpha\text{-SMA}^+$ cells.

Conclusions. Many nonoverlapping as well as fewer overlapping populations of $FSP1^+$ and $\alpha\text{-SMA}^+$ cells shared in the collagen expression associated with progressive fibrogenesis in pcy mice undergoing cystogenesis. Some $FSP1^+$ fibroblasts are likely derived from tubular epithelium undergoing EMT, while αSMA^+, VIM^- cells probably represent vascular smooth muscle cells or pericytes surviving vessel attenuation during the chaos of fibrogenesis. Importantly, not all interstitial cells producing collagens are $\alpha\text{-SMA}^+$.

Human polycystic kidney disease is a genetic disorder that typically expresses as autosomal dominant polycystic kidney disease (ADPKD) in adults [1–3] and as autosomal recessive polycystic kidney disease (ARPKD) in children [4, 5]. Progression to renal failure is variable in both disorders [1, 5]. Cysts in the kidney typically begin as small out-pouchings from any tubular segment and gradually increase in number and size over time [6, 7]. ADPKD is genetically heterogeneous with at least three genes involved: *PKD1*, which accounts for approximately 85 to 95% of all ADPKD and maps to chromosome 16p13.3 [2, 3]; *PKD2*, located on chromosome 4, which accounts for most of the remaining cases [8, 9]; and the hypothesized *PKD3*, which is not yet mapped [10]. The *PKHD1* gene responsible for ARPKD also remains elusive on chromosome 6 [11].

There are a number of animal models representing polycystic kidney disease [12, 13]. In addition to several models of hereditary cystogenesis, null alleles of murine *PKD1* and *PKD2* have also been used to create mouse homologues of human cyst formation [14–16]. Such null mice, especially when homozygous, develop severe cystic disease with many dying in utero or during early adulthood [14–16]. Among recessive models of spontaneous cystogenesis, the DBA/2-pcy mouse (pcy mouse) exhibits a slowly progressive form of disease with a phenotype

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reminiscent of human polycystic kidneys [12, 17]. Although the *pcy* gene localizes to chromosome 9 and is not murine *PKD1* and *PKD2*, the pcy phenotype has been useful in exploring the potential pathophysiology of human cystogenesis [12, 17, 18].

The initial morphologic changes seen in the kidneys of newborn pcy mice consist of segmental dilations along distal tubules and collecting ducts and, by four weeks of age, include the beginning of random cysts along proximal tubules and ascending limbs [18]. While the cystogenesis itself is thought to be a primary driver of organ injury [12, 17], several laboratories have also stressed the important relationship between the appearance of interstitial fibrosis and the progression of disease [19–22]. Progression to end-stage cystic disease is associated with the accumulation of interstitial collagens and an increase in cellularity, presumably because of proliferation of fibroblasts [23]. The expression of genes encoding extracellular matrix in pcy kidneys includes increased amounts over time of mRNA encoding fibronectin and α1(III) procollagen [24], although the phenotypes of the cells expressing these moieties are not yet known.

The cell(s) driving interstitial collagen formation in renal fibrosis has not been easy to characterize, although a tissue fibroblast seems a likely candidate [20]. Identification of interstitial cells producing collagens in vivo has been particularly hampered by low copy numbers of mRNA encoding interstitial collagens and low levels of secreted proteins. Fortunately, a number of new phenotypic markers have appeared in recent literature. The observation that the more abundant heat shock protein 47 (HSP47) is coexpressed as an intracellular chaperon during the synthesis of interstitial collagens has made the identification of collagen-producing cells more practical in tissues [25]. We have also cloned fibroblast-specific protein 1 (FSP1), a murine fibroblast-specific protein belonging to the S100 superfamily of intracellular calcium-binding proteins [26]. FSP1 is a cytoplasmic marker of resting and activated fibroblasts as well as of epithelium undergoing epithelial-mesenchymal transformation (EMT) [26, 27]. We wanted to compare the coexpression of these new markers with α-smooth muscle actin (α-SMA), which is normally synthesized by vascular smooth muscle cells, activated vascular pericytes, or contractile fibroblasts in granulation tissue known as myofibroblasts [20, 28, 29], as well as vimentin (VIM), which is expressed ubiquitously in mesenchymal cells [20, 30]. With this collection of reagents, we have characterized the phenotype of collagen-producing cells in polycystic kidneys of pcy mice during their fibrosis.

METHODS

Murine model of ADPKD

Male DBA/2-pcy mice were obtained from a breeding colony maintained in the Laboratory Animal Center, Fujita Health University (Toyoake, Japan). At weeks 4, 8, 18, and 30, four pcy mice were sacrificed at each interval, and kidneys were harvested from each mouse at sacrifice.

Histologic, immunohistochemical, and immunofluorescence examination

A part of each kidney was fixed in 4% paraformaldehyde and was embedded in paraffin. Another part of it was directly snap frozen for immunofluorescent examination. Sections were cut 4 μm in thickness from paraffin blocks and processed for hematoxylin-eosin (HE), Masson's trichrome (MT), and periodic acid-methenamine-silver (PAM), and indirect immunoperoxidase staining. For the latter, after deparaffinization and rehydration, the sections were treated with proteinase K and boiled in citrate buffer under microwave for unmasking antigenicities [31], and then they were immersed in 3% H_2O_2 in methanol for inhibition of endogenous peroxidase and flooded with 5% bovine albumin in PBS for inhibition of nonspecific reactions. Rabbit anti-FSP1 (1:500) [26] and mouse anti–α-SMA (1:500; Sigma, St. Louis, MO, USA) were applied as primary antibodies. Following primary immunoreaction, labelling was performed using relevant biotin-conjugated, secondary antibodies and a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA), and was visualized using diaminobenzidine (DAB) as a substrate.

Indirect immunofluorescence was performed on 4 μm in thickness, 4% paraformaldehyde-fixed cryostat sections with rat anti–E-cadherin (1:100; Sigma) and rat anti-cytokeratins (TROMA-1 and 3, 1:500 [32]) as primary antibodies with FITC-conjugated anti-rat IgG (1:500; American Qualex, San Clemente, CA, USA) as secondary antibodies [27]. These sections were observed with a laser microscope (Provis Ax; Olympus, Tokyo, Japan) equipped with appropriate filters. For dual staining, reaction with anti-FSP1 was followed by Rhodamine-conjugated antirabbit IgG (1:500; Chemicon International Inc., Temecula, CA, USA), and then reaction with FITC-conjugated anti–α-SMA (1:500; Sigma) was carried out.

HSP47 is a collagen-binding stress protein found in cells producing collagens [25]. To characterize further $FSP1^+$ or α-SMA^+ cells expressing HSP47, several different costainings were performed. Mouse anti-HSP47 (StressGen Biotechnologies, Victoria, Canada) conjugated with FITC using a FITC Protein Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany) and/or goat-anti-VIM (VIM; Sigma) were used. The representative sections were stained with anti-FSP1 in combination with Rhodamine-conjugated anti-rabbit IgG or Cy3-conjugated anti–α-SMA (1:100, Sigma) as primary antibodies, and each was then treated with FITC-conjugated anti-HSP47 (1:100) or anti-VIM (1:50) followed by FITC-conjugated anti-goat IgG (1:500; Chemicon). These dual-stained sections were analyzed by a confocal microscope

(MRC600; Bio-Rad, Hercules, CA, USA). All of the second antibodies had been isolated by immunoaffinity chromatography and absorbed for dual labeling. Controls included omitting the primary antibody and/or substituting the primary antibody with normal murine IgG.

Quantitation of collagenous fibrotic area and FSP1$^+$ cells in the tubulointerstitium

Interstitial areas of fibrosis and numbers of fibroblasts were quantitatively evaluated by computer-assisted image analyzer (Mac SCOPE, Version 2.5; Mitani Corp., Hukui, Japan). Thirty cortical fields were randomly analyzed at ×200 magnification from each kidney sample evaluated. Luminal areas of cysts were subtracted from each field, and then the collagenous fibrotic area in blue and FSP1$^+$ cells in brown was quantitatively determined; each was expressed as a mean percentage area or mean cell count per field.

RESULTS

Renal fibrosis in pcy mice became progressively more severe over the study interval of 30 weeks. The intensification of interstitial fibrosis is demonstrated in Figure 1 using a quantitative index based on area involvement. Table 1 further collates the longitudinal overview of phenotypic markers expressed during fibrosis in these cystic kidneys over time. At four weeks of age, the tubulointerstitium was largely normal with only sporadic dilation of distal tubular segments and collecting ducts. FSP1$^+$ fibroblasts were sparsely distributed in the renal interstitium, especially around dilated tubules (Fig. 2A). Dilated tubules and cysts increased in number by eight weeks of age. Many areas adjacent to these cysts remained relatively normal with minimal interstitial expansion or loss of vessels and tubules, while other areas had become slightly fibrotic (data not shown). An increasing number of FSP1$^+$ cells were also present in those areas of interstitium changed by fibrosis (Fig. 2B). In these areas, only vascular smooth muscle cells were α-SMA$^+$ (Fig. 2C). At this stage, epithelia in most normally sized tubules and most of the epithelia lining dilated tubules were positive for cytokeratin (Fig. 2D).

Cystogenesis in pcy mice progressed further by 18 weeks of age to where numerous cysts were distributed randomly throughout the kidney cortex. They varied in size, with large ones often insinuated between banded tissues of fibrotic scar (Fig. 3A). Such intersecting areas of interstitial fibrosis contained smaller cysts, atrophic tubules, vessels, residual glomeruli, and cells positive for FSP1 (Fig. 3B). Tubular epithelia in transition areas adjacent to these fibrotic septa also become FSP1$^+$, as did occasional epithelia among involved cysts (Fig. 3C; Fig. 3D is a negative control). In tubules trapped and

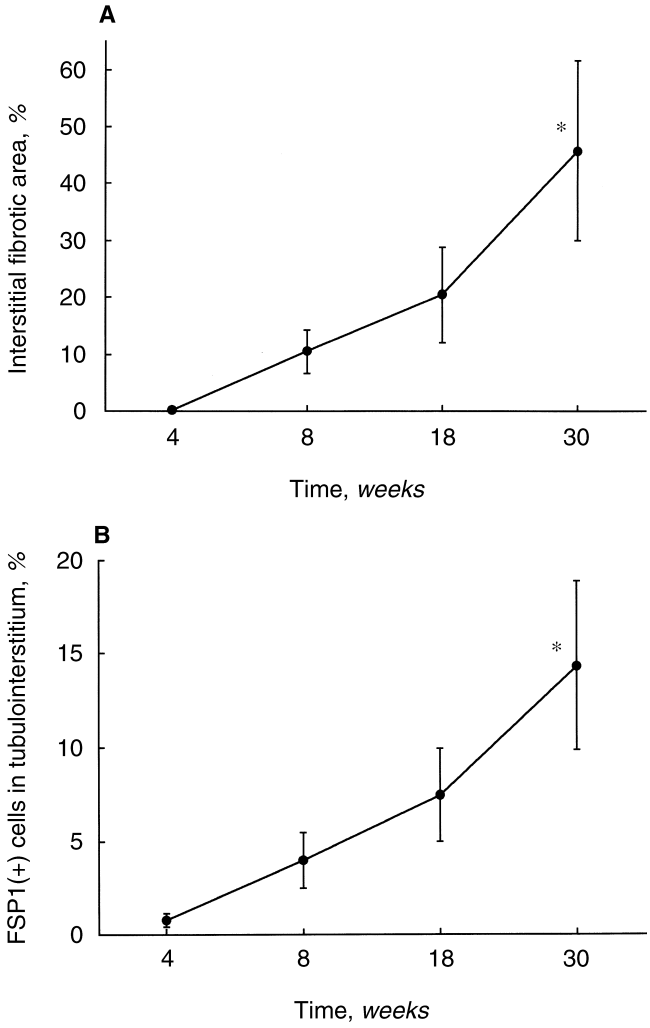

Fig. 1. Analysis of renal interstitial fibrosis and fibroblasts in pcy mice. The percentage area of collagenous involvement (*A*) and mean number of FSP1$^+$ cells (*B*) in the kidneys of pcy mice gradually increased in amount or number in time-dependent fashion over the 30-week study interval. Each point represents the mean ± SEM for groups of four pcy mice. *$P < 0.05$ vs. four-week-old pcy mice.

partially dismantled by septal fibrosis, there were disruptions in tubular basement membrane (Fig. 3E). Renal vessels were also trapped in these fibrotic septa (Fig. 3F), and their remaining cells stained positive for α-SMA (Fig. 3G). Cytokeratin staining at this stage identified most cystic epithelia but not all remnant tubular epithelia trapped within expanding segments of scar (Fig. 3H) [33].

Since the number of FSP1$^+$ interstitial cells was significantly greater in 18-week-old mice, and interstitial fibrosis itself progressed even further between 18 and 30 weeks of age (Fig. 1), we chose to phenotype interstitial cells in 18-week tissue that still possessed familiar anatomic landmarks. Double-stained sections of pcy kidney revealed that FSP1$^+$ fibroblasts and α-SMA$^+$ cells were

Table 1. Alterations in phenotypic markers in the kidneys of pcy mice

Marker	Site	4-week-old mice	8-week-old mice	18-week-old mice	30-week-old mice
FSP1	INT	+[a]	+[a]	++	++
	TE	−	+[b]	+[b]	±[c]
α-SMA	INT	+[d]	+[d]	++	++
	TE	−	−	−	±[c]
E-cadherin	TE	+	+	±[b], −[e]	−
Cytokeratin	TE	+[f]	+[f]	+[e], ±[f]	+[e]

Abbreviations are: INT, interstitium; TE, tubular epithelium.
[a]Remarkable around cysts
[b]Epithelium of some tubules adjacent to expanding cysts
[c]Segmental epithelium of expanded cysts
[d]Restricted to cells of the renal vasculature
[e]Epithelium lining some dilated tubules or expanding cysts
[f]Epithelium of remnant tubules

typically nonoverlapping (Fig. 4 A, B). Some FSP1$^+$ interstitial fibroblasts were also HSP47$^+$ (Fig. 4C; yellow/arrows), and also some of the α-SMA$^+$ interstitial cells were HPS47$^+$ (Fig. 4D; yellow/arrows). Thus, FSP1$^+$, α-SMA$^-$ fibroblasts and FSP1$^-$, α-SMA$^+$ interstitial cells both seemed capable of producing interstitial collagens. Of note, a few epithelial cells in some tubules were dual positive for FSP1 and HSP47 (Fig. 4C; arrowheads). Some FSP1$^+$ fibroblasts were also VIM$^+$ (Fig. 4E; yellow/arrows), but α-SMA$^+$ interstitial cells were VIM$^-$ (Fig. 4F).

By 30 weeks of age, large cysts were abundant, and normal tubules were in a minority (Fig. 5A). Resulting fibrous septa contained an abundance of FSP1$^+$ interstitial fibroblasts (Fig. 5B), while α-SMA$^+$ interstitial cells tended to distribute linearly around cysts (Fig. 5C). In addition, epithelial cells lining expanded cysts were occasionally negative for E-cadherin (Fig. 5D; arrowheads), but all of them were still strongly positive for cytokeratins (Fig. 5E), suggesting that cystic epithelium share a dynamic and shifting phenotype. Reactivity for each experimental control was mostly negative, and background activity was minimal (Fig. 3D).

DISCUSSION

Polycystic kidney diseases are a group of disorders recognized by the emergence of numerous cysts throughout the nephron mass. Four types of epithelial cell disturbances have been implicated in the process of cystogenesis: (*1*) Renal tubules undergo cystic dilation while changing their polarization [34–38]; (*2*) epithelia begin to proliferate abnormally [39–41]; (*3*) apoptosis reduces epithelial cell mass [42–44]; and (*4*) cystic segments synthesize aberrant basement membranes [23, 24, 35, 45–47]. While this process is likely facilitated by the absence of polycystin in null mice or humans with ADPKD [7], the genetics and mechanism of cyst formation in mouse strains with hereditary cystic disease are still uncertain. In pcy mice, the model we have studied in this report, cysts spontaneously arise as dilations from any segment of the nephron [1, 12, 17], and their number over time can rise to several hundred in a murine kidney of 5 to 6×10^5 nephrons.

In addition to forming cysts, all species with polycystic kidney disease develop other structural alterations to their interstitium that disturb important functional relationships between and among nephrons. Destruction of the tubulointerstitium by fibrogenesis is the most obvious, but the least well studied in detail. In this observational report, we have begun to characterize the cellular components contributing to this fibrogenesis.

Based on our findings, the origin of collagen-producing cells in the interstitium during cystogenesis potentially includes several phenotypes: pre-existing FSP1$^+$ fibroblasts that multiply in number, FSP1$^+$ fibroblasts that migrate into the interstitium from external sources, FSP1$^+$ fibroblasts that change their phenotype into α-SMA$^+$ myofibroblasts, α-SMA$^+$ vascular smooth muscle cells that cut loose in the interstitium, or new FSP1$^+$ fibroblasts that appear following EMT [26, 27].

While fibroblasts undoubtedly exist in the bone marrow, previous experiments in parabiotic rats suggest that tissue fibroblasts, when needed, do not accumulate from hematogenous sources [48], as once thought [49]. Many FSP1$^+$ fibroblasts in the kidneys of four week-old pcy mice are thought to be daughters of native fibroblasts, since FSP1$^+$ fibroblasts were especially apparent around dilating tubules absent other pathological alterations, like cell infiltration, tubular atrophy, or widening of the interstitium (Fig. 1A). This coincidence suggests that the proliferation of native fibroblasts in cystic kidneys by fibroblast growth factors like platelet-derived growth factor, angiotensin II, endothelin, or FGF2 may be stimulated by resident somatic cells from within the same microenvironment of dilating tubules or cysts [50].

From 8 to 18 weeks of age, the number of FSP1$^+$ fibroblasts in the kidneys of pcy mice continued to in-

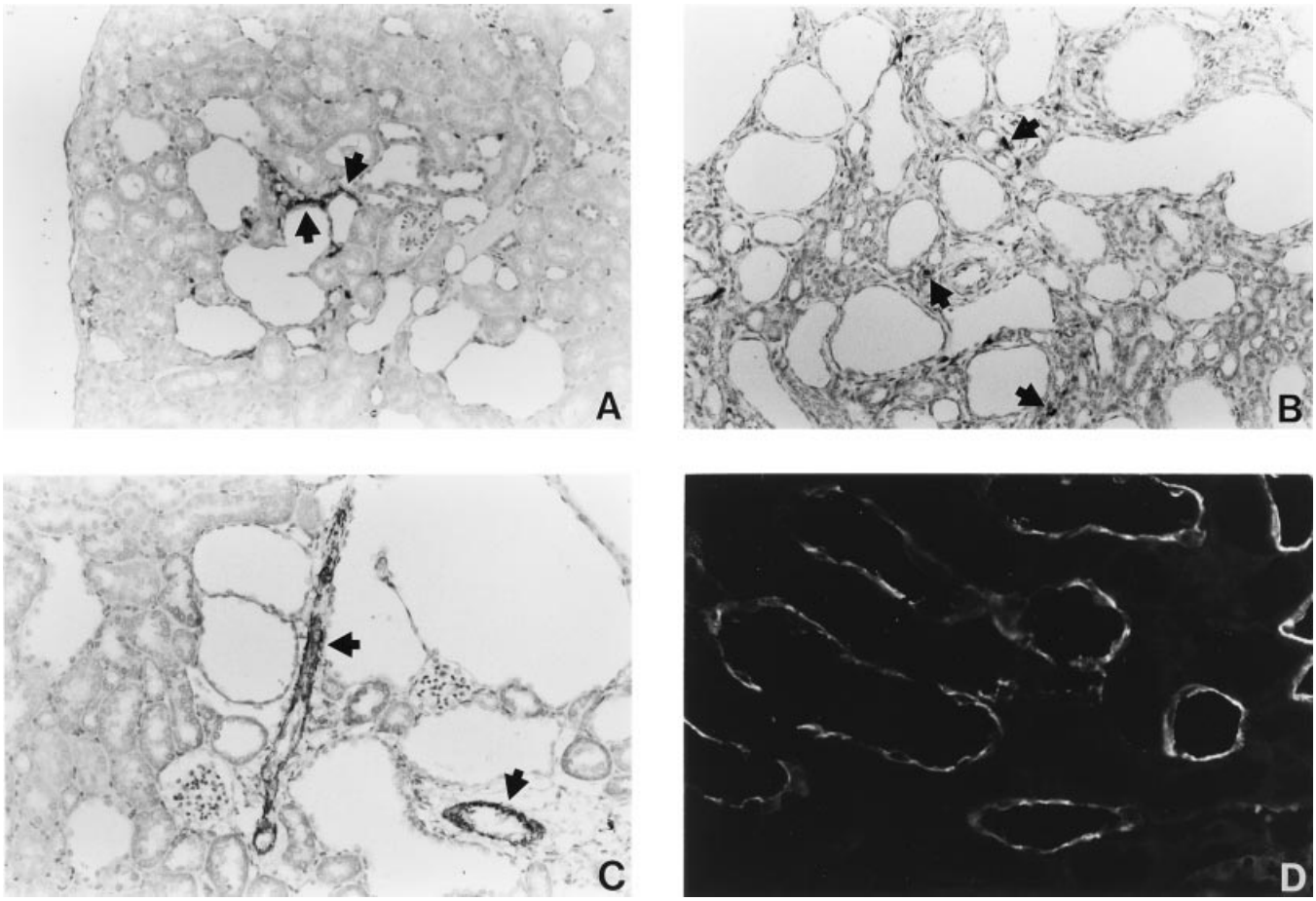

Fig. 2. Renal histopathology of 4- and 8-week-old pcy mice. (*A*) FSP1$^+$ fibroblasts in four-week-old pcy mice distributed sparsely in the interstitium but increased especially around dilating tubules (arrows; (DAB, ×200). (*B*) An increasing number of FSP1$^+$ cells in eight-week-old pcy mice reside in the widening interstitium (arrows; DAB, ×200). (*C*) All of the α-SMA positivity in eight-week-old pcy mice expressed exclusively within vascular smooth muscle cells (arrows; DAB, ×200). (*D*) Epithelia in most of normal-sized tubules in eight-week-old pcy mice and almost all the dilating tubules were positive for cytokeratins (FITC, ×200).

crease with the envelopment of cystogenesis by septal fibrogenesis. In addition to growth factors, compression by expanding cysts and the attendant ischemia of adjacent renal parenchyma may accelerate fibroblast presence as well. The renin-angiotensin system is activated in the ADPKD [22, 51], and angiotensin II as a fibroblast growth factor and morphogenic cytokine can induce renal fibrosis in rats [52]. Furthermore, angiotensin-converting enzyme inhibitors are efficacious in reducing cyst size in rat model of ADPKD [53, 54]. Thus, fibroblasts by several potential mechanisms can be driven to expand fibrogenesis in cystic kidneys.

The notion that some interstitial fibroblasts may be derived from tubular epithelium is of particular interest to us. Renal tubular epithelium in distal tubules and collecting ducts typically stain positive for cytokeratin [33], so it was reasonable to expect that such tubules would be cytokeratin positive in young pcy mice (Fig. 2E). Most of the epithelia comprising seemingly uninvolved tubular segments, or enlarging cysts, continued to remain positive for cytokeratins. In contrast, residual tubular epithelium interwoven or trapped by forming fibrotic septa were increasingly negative for cytokeratin, suggesting as a hypothesis that such epithelium stop expressing cytokeratins as their units are dismantled by EMT and engulfed by scar. Epithelia in tubules adjacent to expanding cysts and septa were also double positive for FSP1 (Fig. 3C) and HSP47, suggesting they were producing interstitial collagens (Fig. 4C) at the edge of a fibrotic front. Tubular epithelial cells transformed by local paracrine factors benefit from the disruptions of basement membrane as they go through EMT (Fig. 3E) [24, 55]. These disruptions are probably accelerated by metalloproteases released by the epithelium as they begin this transformation (Strutz et al, manuscript in preparation). FSP1$^+$ fibroblasts can also be VIM$^+$ or VIM$^-$ (Fig. 4E) or HSP47$^+$ or HSP47$^-$ (Fig. 4C). This variability may reflect the degree of engagement by morphogenic

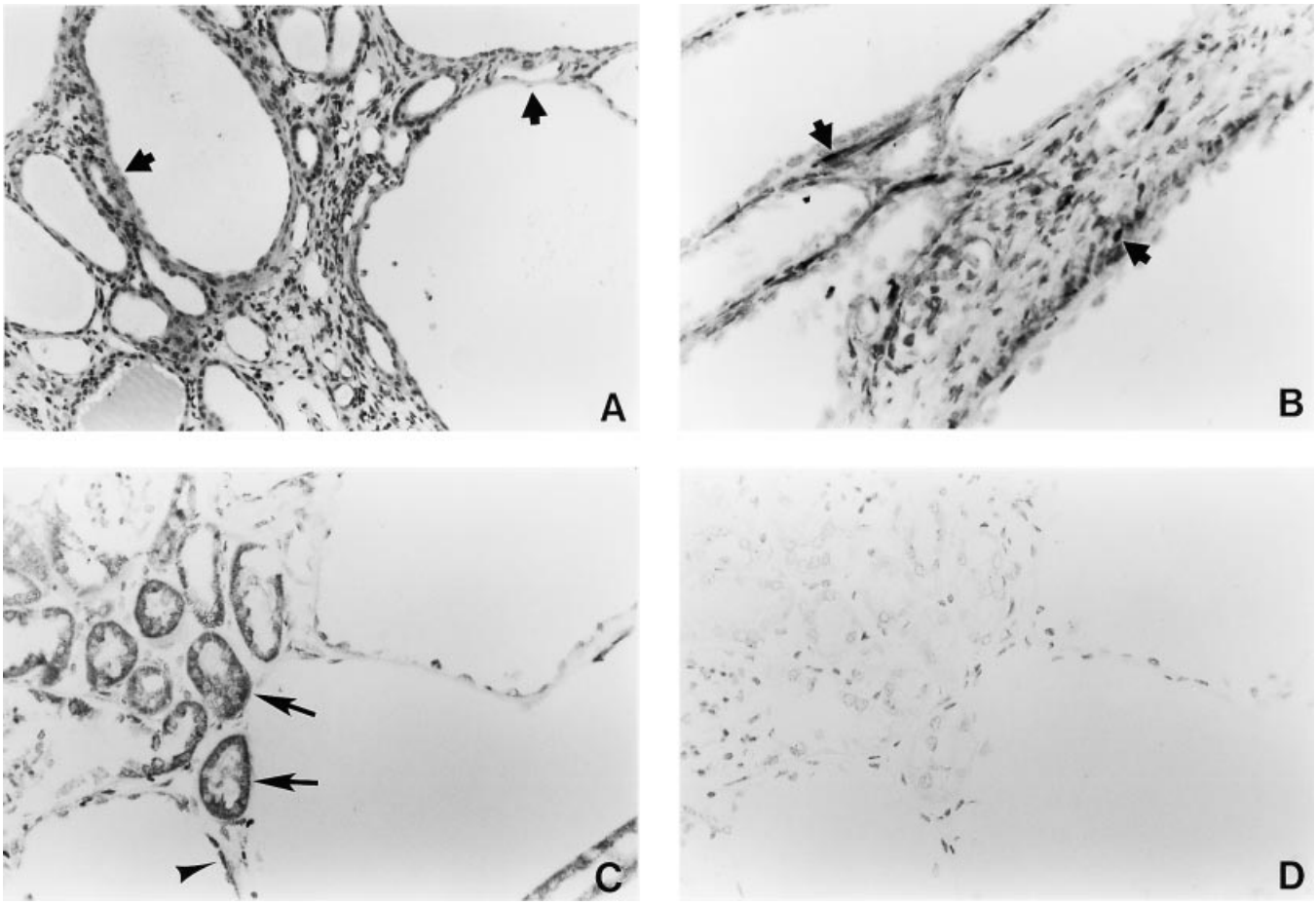

Fig. 3. Renal histopathology of 18-week-old pcy mice. (*A*) In HE-stained sections, the cysts varied in size, and widened fibrotic septa between those cysts contained intact to atrophic tubules, vessel, and infiltrates (arrows; ×200). (*B*) FSP1$^+$ fibroblasts existed in thin to thick walls of cysts (arrows; DAB ×400). (*C*) FSP1$^+$ tubular epithelial cells were found within reactive tubules adjacent to expanding cysts (arrows). A few of the epithelial cells lining cysts showed FSP1$^+$ as well (arrowhead; DAB ×400). (*D*) Negative control for (C) using a serial section (DAB ×400). (*E*) In PAM-stained sections, the interstitial areas surrounded by expanding cysts, the basement membrane of residual tubules and cysts were disrupted (arrows; ×200). (*F*) In HE-stained sections, the thin epithelial layer lining a cyst was immediately opposed to a small intrarenal artery (arrows; ×200). (*G*) α-SMA$^+$ cells in the interstitium were derived from vascular walls (arrowhead at interlobular artery) and tended to form lines encircling cysts (arrows; DAB ×200). (*H*) All epithelial cells lining cysts were positive for cytokeratins, while others among remnant tubules within fibrotic septa had become negative (arrows; FITC ×200).

cytokines. Such cells in cystic kidney for the most part were surprisingly α-SMA$^-$ (Fig. 4 A, B).

Only obvious vascular structures were α-SMA$^+$ in early cystic kidneys of pcy mice. These vascular smooth muscle cells remained α-SMA$^+$ as the kidney became more cystogenic, and the residual vasculature formed remnant ghosts suggestive of former vessels (Fig. 3F). Alternatively, vascular pericytes can become SMA$^+$ in certain situations [56] and may represent a possible origin of new α-SMA$^+$ interstitial cells. Their absence of VIM positivity noted previously in this article may reflect a nonmesenchymal origin.

α-Smooth muscle actin$^+$ cells in fibrotic tissue have also been referred to as activated fibroblasts or myofibroblasts [28, 30]. Some of the α-SMA$^+$ interstitial cells in cystic kidneys of pcy mice were HSP47$^+$ (Fig. 4D), indicating they were also synthesizing interstitial collagens [25]. Nevertheless, only occasional α-SMA$^+$ cells were FSP1$^+$ (Fig. 4 A, B). This latter finding suggests that detection of α-SMA$^+$ cells alone may significantly underestimate the actual fibroblast population. In preliminary experiments, we have also observed that α-SMA$^+$, FSP1$^-$ or α-SMA$^-$, FSP1$^+$ cells are more common than double-positive cells in the interstitial fibrosis associated with murine Goodpasture syndrome or transgenic HIV nephropathy (Okada; unpublished data).

Tubular epithelium in culture undergo EMT when grown in type I collagen and pressured by cytokines, particularly transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) [27]. The fibroblasts that

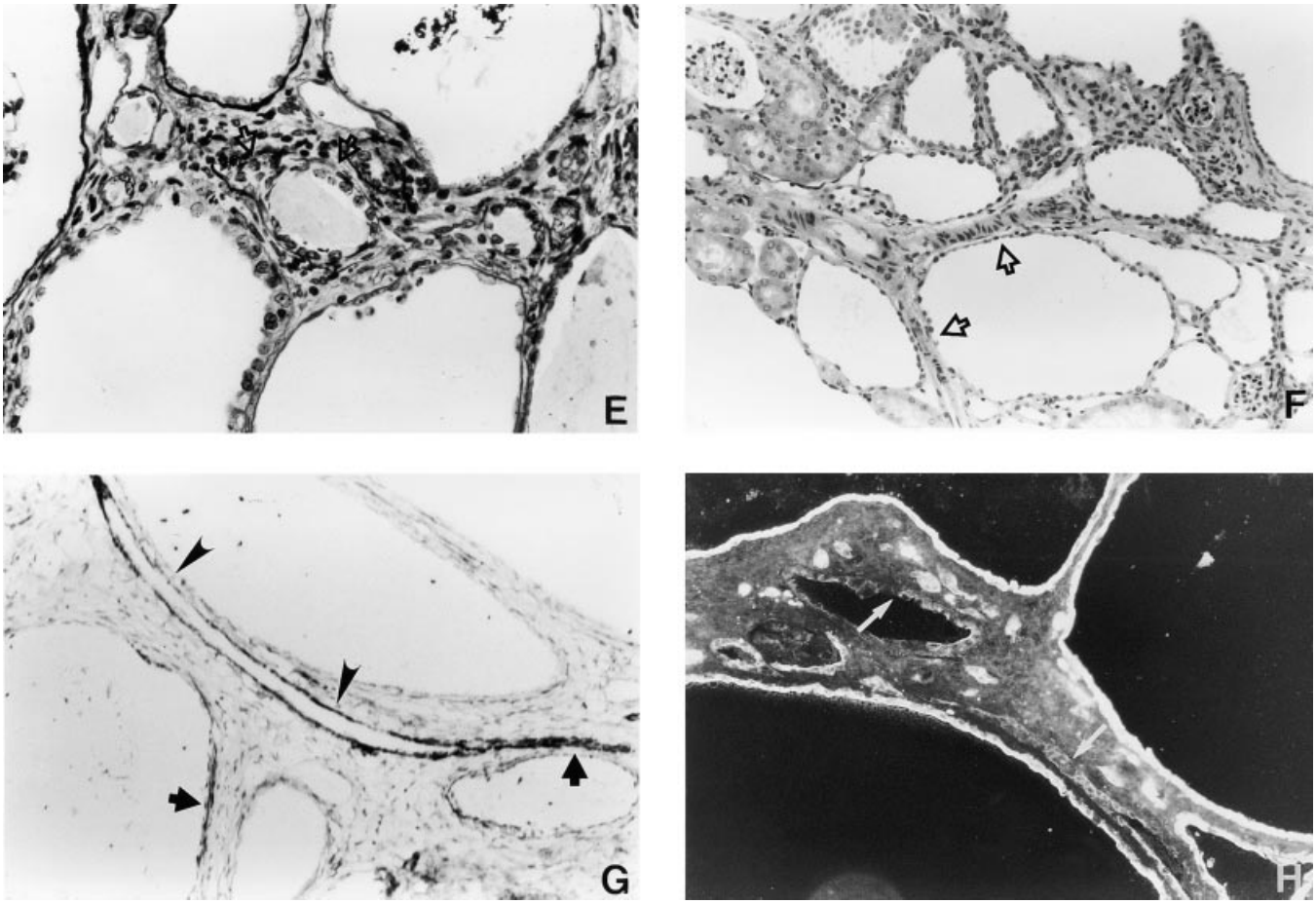

Fig. 3. (Continued)

emerge from this transformation after several days in culture often have a phenotype not unlike what has been called a myofibroblast: They are FSP1$^+$, α-SMA$^+$, VIM$^+$. Why this double positivity can be recognized clearly in culture but not in tissue is unclear. We hypothesize that it may depend on availability or strength of the cytokine environment. EGF and TGF-β, for example, can be detected in the kidneys of pcy mice [50, 57], and we can detect type I collagen by immunohistochemistry in their interstitium (data not shown). The transcription of EGF and TGF-β genes, however, are down- and up-regulated, respectively, in these kidneys [50]. Therefore, one could speculate that in vivo, unlike in vitro [27], the cytokine pressure is less intense or complete, and fewer cells that undergo EMT become double positive for FSP1$^+$ and α-SMA$^+$. Although purely descriptive, our findings add new information regarding the fibrogenic response of polycystic kidneys in experimental mice. We now describe the chronological appearance of FSP1$^+$ fibroblasts in the renal interstitium, evaluate their coexpression of phenotypic markers like HSP47, α-SMA, and VIM, and conclude that fibrogenesis in this environment is quite complex: The heterogeneity of cell fate among participating cells is far greater than anticipated. Most important, overlapping positivity between FSP1 and α-SMA markers in the interstitium among collagen-producing cells is far less that originally suspected. α-SMA positivity cannot be construed as a gold standard for all collagen-expressing cells during fibrogenesis, and suggests that the further study of the mechanism of this phenotypic diversity will be an important facet to understanding progressive renal disease.

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Reprint requests to Eric G. Neilson, M.D., Department of Medicine, D-3100 MCN, Vanderbilt University Medical Center, Nashville, Tennessee 37232-2358, USA.
E-mail: Eric.Neilson@mcmail.vanderbilt.edu

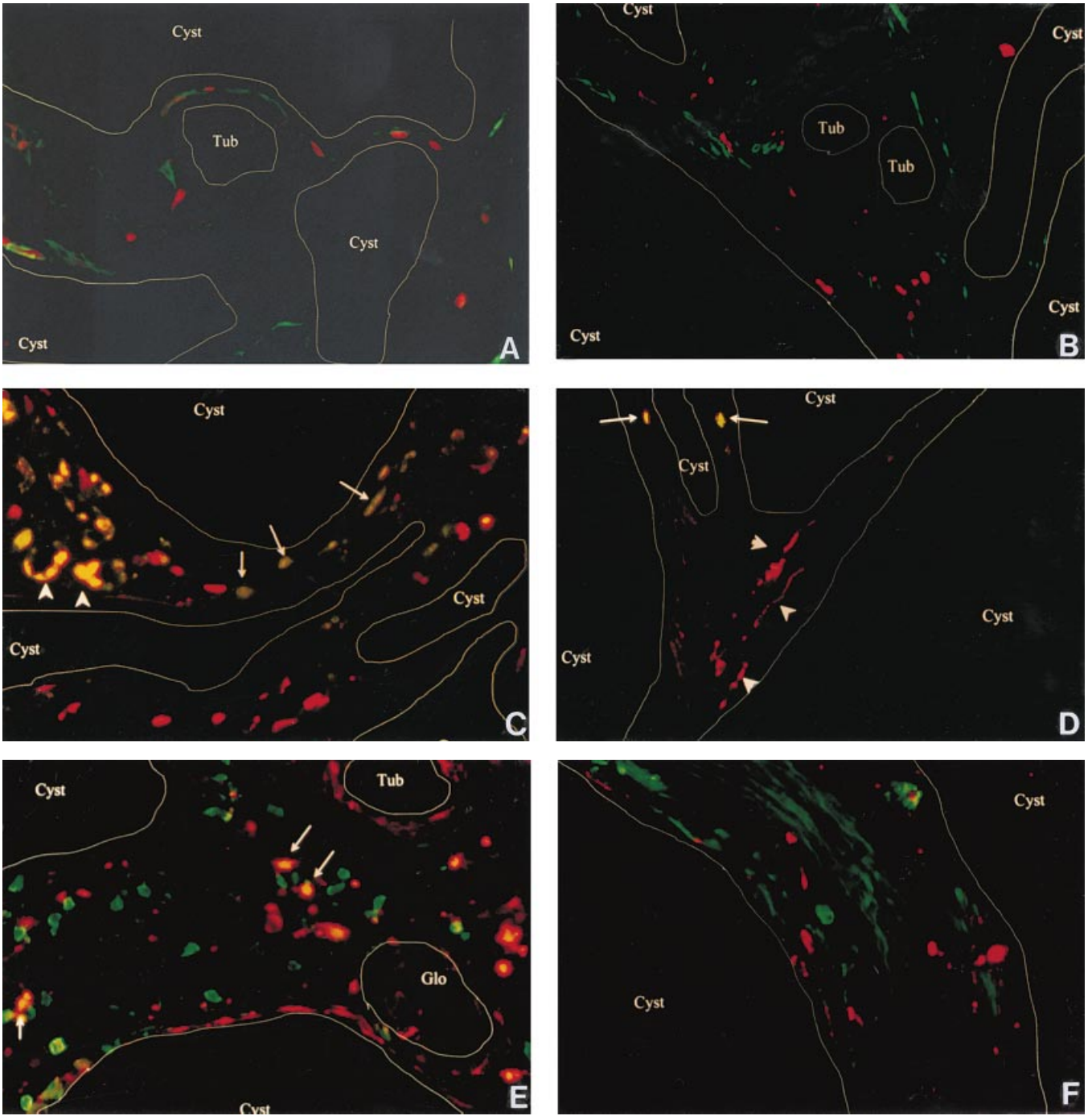

Fig. 4. Dual immunofluorescence on the fibrous interstitium of the kidneys of 18-week-old pcy mice. (*A* and *B*) FSP1$^+$ cells (in red) and α-SMA$^+$ cells (in green) were clearly different from each other, since there were no cells registered in yellow, indicating an absence of dual positive cells (FITC and Rhodamine ×400). (*C*) Some of the FSP1$^+$ cells registered yellow [FSP1$^+$ cells (in red) and HSP47$^+$ cells (in green)] because of dual positivity for HSP47 (arrows). The epithelial cells in atrophic tubules (arrowheads) were found to be dual positive for FSP1 and HSP47 (FITC and Rhodamine ×400). (*D*) Some of the α-SMA$^+$ cells registered yellow [α-SMA$^+$ cells (in red) and HSP47$^+$ cells (in green)] because of dual positivity for HSP47 (arrows). However, αSMA$^+$ cells distributing linearly around cysts (arrowheads) tended to stain red, indicating they were HSP47$^-$ (FITC and Cy3 ×400). (*E*) Some of FSP1$^+$ cells also registered yellow [FSP1$^+$ cells (green) and VIM$^+$ cells (red)], demonstrating dual positivity for VIM (arrows; FITC and Rhodamine ×400). (*F*) There were no dual-positive cells when stained for α-SMA$^+$ cells (green) and VIM$^+$ cells (red; (FITC and Rhodamine ×400).

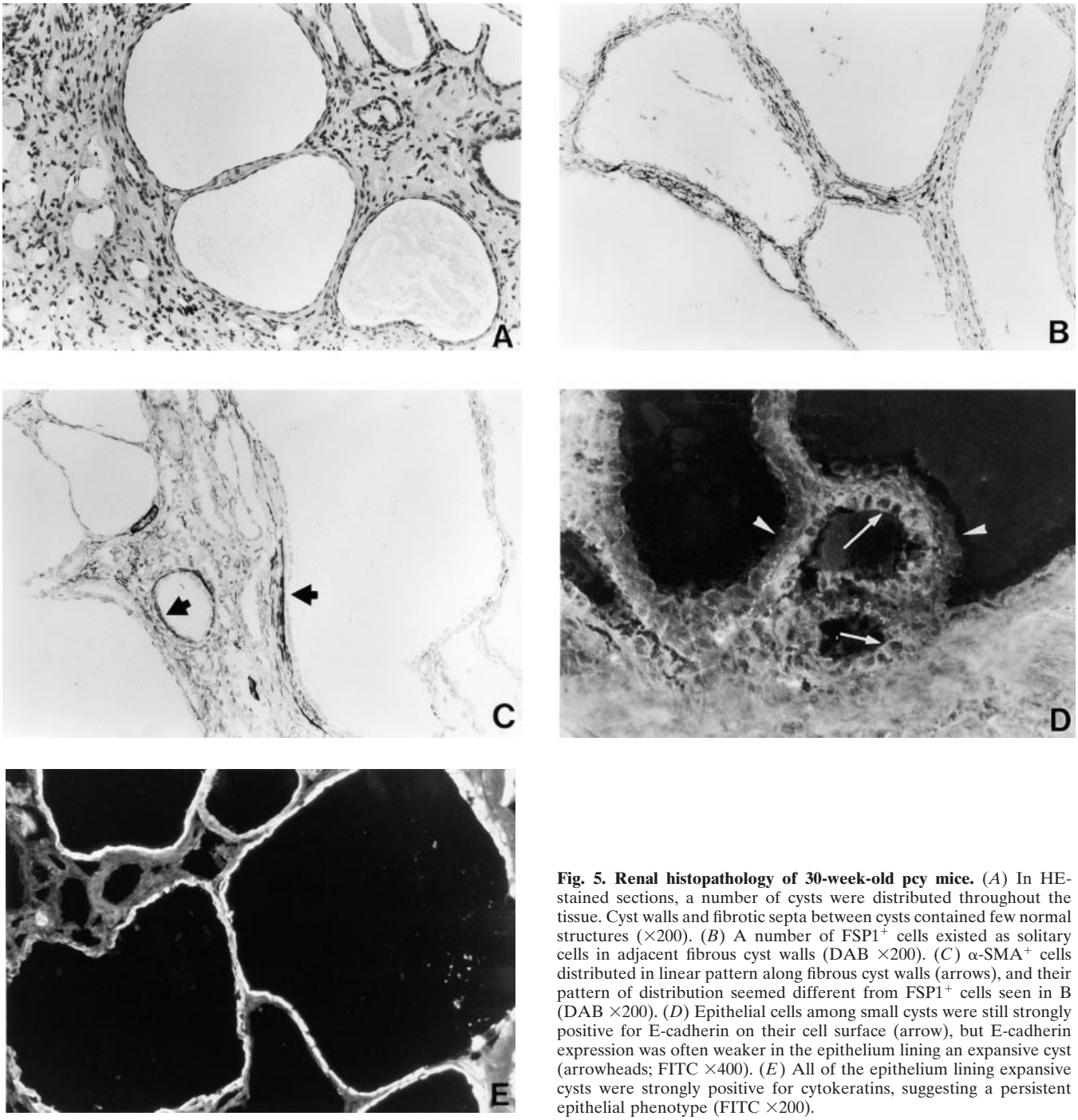

Fig. 5. Renal histopathology of 30-week-old pcy mice. (*A*) In HE-stained sections, a number of cysts were distributed throughout the tissue. Cyst walls and fibrotic septa between cysts contained few normal structures (×200). (*B*) A number of $FSP1^+$ cells existed as solitary cells in adjacent fibrous cyst walls (DAB ×200). (*C*) α-SMA^+ cells distributed in linear pattern along fibrous cyst walls (arrows), and their pattern of distribution seemed different from $FSP1^+$ cells seen in B (DAB ×200). (*D*) Epithelial cells among small cysts were still strongly positive for E-cadherin on their cell surface (arrow), but E-cadherin expression was often weaker in the epithelium lining an expansive cyst (arrowheads; FITC ×400). (*E*) All of the epithelium lining expansive cysts were strongly positive for cytokeratins, suggesting a persistent epithelial phenotype (FITC ×200).

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