

Identification and Characterization of a Fibroblast Marker: FSP1

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Abstract. We performed subtractive and differential hybridization for transcript comparison between murine fibroblasts and isogenic epithelium, and observed only a few novel intracellular genes which were relatively specific for fibroblasts. One such gene encodes a filament-associated, calcium-binding protein, fibroblast-specific protein 1 (*FSP1*). The promoter/enhancer region driving this gene is active in fibroblasts but not in epithelium, mesangial cells or embryonic endoderm. During development, *FSP1* is first detected by in situ hybridization after day 8.5 as a postgastrulation event, and is associated with cells of mesenchymal origin or of fibroblastic phenotype. Polyclonal antiserum raised to recombinant *FSP1* protein stained the cytoplasm of fibroblasts, but not epithelium. Only occasional cells stain with specific anti-*FSP1* antibodies in normal parenchymal tissue. However, in kidneys fibrosing from persistent inflammation, many fibroblasts could be identified in interstitial sites of collagen deposition and

also in tubular epithelium adjacent to the inflammatory process. This pattern of anti-*FSP1* staining during tissue fibrosis suggests, as a hypothesis, that fibroblasts in some cases arise, as needed, from the local conversion of epithelium. Consistent with this notion that *FSP1* may be involved in the transition from epithelium to fibroblasts are experiments in which the in vitro overexpression of *FSP1* cDNA in tubular epithelium is accompanied by conversion to a mesenchymal phenotype, as characterized by a more stellate and elongated fibroblast-like appearance, a reduction in cytokeratin, and new expression of vimentin. Similarly, tubular epithelium submerged in type I collagen gels exhibited the conversion to a fibroblast phenotype which includes de novo expression of *FSP1* and vimentin. Use of the *FSP1* marker, therefore, should further facilitate both the in vivo studies of fibrogenesis and the mapping of cell fate among fibroblasts.

FIBROBLASTS are traditionally viewed as pedestrian interstitial cells normally responsible for tissue infrastructure and organ remodeling. Fibroblasts synthesize an extracellular matrix comprising collagen types I and III, fibronectin and proteoglycans (10, 27, 40). Overproduction of these moieties during fibrogenesis, after chronic inflammation or injury, leads to excessive collagen deposition in parenchymal tissues that eventually accelerates organ insufficiency (24, 45).

Recent evidence also suggests that fibroblasts acquire a functional heterogeneity from the microenvironment in which they develop (5, 65). Cultured fibroblasts, for example, synthesize different types of collagen according to their site of origin (9) and respond differently to fibrogenic cytokines (5). Fibroblasts are easy to culture (9) and hence have been well characterized in vitro. However, the basis for their origin, heterogeneity, and abundance during in

vivo fibrogenesis has not been well studied because of a lack of good markers. Studies on the formation of interstitial fibrosis in organs such as lung (13), liver (57), heart (76), or kidney (54) have had to rely on the often imprecise technique of in situ hybridization using probes recognizing interstitial collagens.

Here we describe the isolation of several fibroblast-specific genes by comparative transcript analysis. We find that one gene, *FSP1*, is highly specific for fibroblasts and is associated with the conversion of epithelial cells to a fibroblast phenotype.

Materials and Methods

Cell Culture

Murine cell lines were all previously established and grown at recommended conditions: renal tubulointerstitial fibroblasts (TFB)¹ and dermal

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1. *Abbreviations used in this paper:* DFB, dermal fibroblasts; ES, embryonic stem cells; *FSP1*, fibroblast-specific protein 1; MCT, murine proximal tubular epithelial cells; MMC, mesangial cells; TFB, tubulointerstitial fibroblasts.

fibroblasts (DFB) (5), NIH/3T3 fibroblasts, murine proximal tubular epithelial cells (MCT) (34), murine distal tubular cells (NP-1) (gift of Dr. Fuad Ziyadeh, University of Pennsylvania, PA), mesangial cells (MMC) (78), M30 T cells (53), BAL-17 B cells (3), microvascular endothelial cells (60) (a generous gift of Dr. Auerbach, University of Wisconsin), hepatocytes (67), pancreatic islets (28), osteoblasts (OB) and thymic fibroblasts (ThyFB) (gift of Dr. Barbara Knowles, Jackson Laboratories, Bar Harbor, ME), F9 teratocarcinoma cells (73) and PYS-2 (47), and embryonic stem cells (ES) (11). In some experiments, NP-1 epithelium were grown as cultures submerged in collagen type I (Collaborative Research, Bedford, MA) using previously established methods (31).

Comparative Transcript Analysis

A cDNA library was prepared using transcripts from TFB fibroblasts (5), packaged in Lambda Zap II™ (Stratagene, La Jolla, CA), and plated on *E. coli* strain SURE for screening. A total of 180,000 clones were screened by double lift differential hybridization using [³²P]cDNA probes prepared from 5 µg of poly(A)⁺ RNA isolated from TFB fibroblasts or MCT epithelium and primed with oligo(dT)₁₂₋₁₈/random primers in the presence of reverse transcriptase (6). Typically, 2 × 10⁶ cpm/ml of probe were used per filter. Approximately 1.65 × 10⁶ clones from the TFB cDNA library were also screened with a subtracted probe (69) using mRNA from MCT epithelium according to protocol for the Subtractor I™ kit (Invitrogen, San Diego, CA). All clones obtained at final purity were independently isolated at least two times. Inserts were sequenced by chain termination (3) and subsequently used as probes on Northern blots using total cellular or organ RNA, as well as poly A⁺ mRNA (3). Up to 30 µg of RNA were loaded onto a 1.3% RNase-free agarose gel in 2.2 M formaldehyde and transferred to Genescreen™ membrane (New England Nuclear, Boston, MA) in 10 × SSC buffer. All cDNA probes (2 × 10⁶ cpm/ml), including the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled with random primers (3). Hybridizations were performed at high stringency in 0.1 × SSC at 65°C (34).

Assembly of Shuttle Vectors

A genomic clone (7cG), encoding *FSP1*, was isolated from an NIH/3T3 genomic library in λ FIX II (Stratagene). An EcoRI fragment (containing ~2,500 bp 5' to the transcription start site, the first two introns and the first two and parts of the third exon) was subcloned into pBSKSII (Stratagene) and subjected to further restriction analysis and partial sequencing. A luciferase reporter construct (pBK1800luc) was assembled by ligating the BglII (ca.-1,800 bp) and NheI (+65 bp) genomic fragment into the BamHI and KpnI sites of pXP-2 (59). This places the luciferase gene under the control of the 5' flanking region of the *FSP1* gene. Transient transfections were carried out using CaPO₄ (3). 10 µg of plasmid DNA was cotransfected with 2 µg of pCH110 (Pharmacia LKB Biotechnology, Piscataway, NJ), a vector expressing β-galactosidase, into 5–10 × 10⁵ cells plated on 60-mm dishes. pSV₂luc containing the SV40-promoter/enhancer served as a positive control (59). Medium was changed 24 h later and cells were harvested 48 h after transfection by lysis in KPO₄-DTT with 1% Triton X-100. Supernatants were assayed for luciferase activity in a Lumat LB 9501 luminometer. β-Galactosidase activity was measured (63), and luciferase activity was normalized accordingly. cDNA encoding *FSP1* containing the ATG start codon was also cloned into pcDNA-Neo (Invitrogen) for transfection into MCT cells. 24 h after transfection, cells were subjected to selective medium (DMEM + 700 mg/ml Geneticin™) for 14 d and then subcloned by limiting dilution. MCT cells stably transfected with pcDNA-Neo alone served as negative control. *FSP1* expression was verified in selected clones by Northern analysis (15, 27).

Expression and Purification of Recombinant FSP1

The coding region of *FSP1* (p48b1) was amplified with 5'-BamHI/in-frame and 3'-HindIII/termination primers and Taq polymerase in a standard cyler protocol (3). The 5'-primer contained an additional sequence coding for the tetrapeptide Ile-Glu-Gly-Arg, as a specific cleavage site for the blood coagulation factor X_a (56). The amplification product was subcloned into the TA cloning vector pCR™II (Invitrogen) for sequencing. Double cuts with BamHI and HindIII produced a forced in-frame ligation into pDS-MCS (gift of Dr. Tom Genetta, Howard Hughes Medical Institute at the University of Pennsylvania) for protein expression in MC 1061 *E. coli* containing helper plasmid pDMI-1 (58). The expressed *FSP1* fu-

sion protein contains 6 tandem histidine residues in the fusion sequence that allowed for affinity purification over a nickel resin column employing a step gradient elution with 25–200 mM imidazole into 8 M urea using protocols available from Quiagen (Chatsworth, CA). The purity of the expressed protein was determined on an 18% SDS-PAGE gel using Coomassie blue staining. Polyclonal antiserum against purified recombinant *FSP1* was generated in a New Zealand white rabbit.

Immunoblot Analysis

One-dimensional immunoblots were performed using lysates from 3T3, TFB, and MCT cells obtained by lysis with a detergent based buffer (66). 100 µg of total cellular protein and 1 µg *FSP1*-fusion protein were run on an 18% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by electroblotting. 1 µg of murine RANTES-fusion protein containing an identical leader sequence was used as a negative control. To control for equally loaded amounts and adequate transfer, the membrane was stained with panceau red. After blocking with blotto/tween solution, the membrane was incubated with the primary polyclonal antibody in a 1:10,000 dilution followed by the secondary antibody (donkey-anti-rabbit, horseradish peroxidase linked; Amersham, Arlington Heights, IL). Positive reaction products were identified by chemiluminescence (ECL, Amersham) according to protocols available from the manufacturer.

Immunohistochemistry

Cells were grown on gelatin-coated coverslips or glass slides for 24 h, fixed

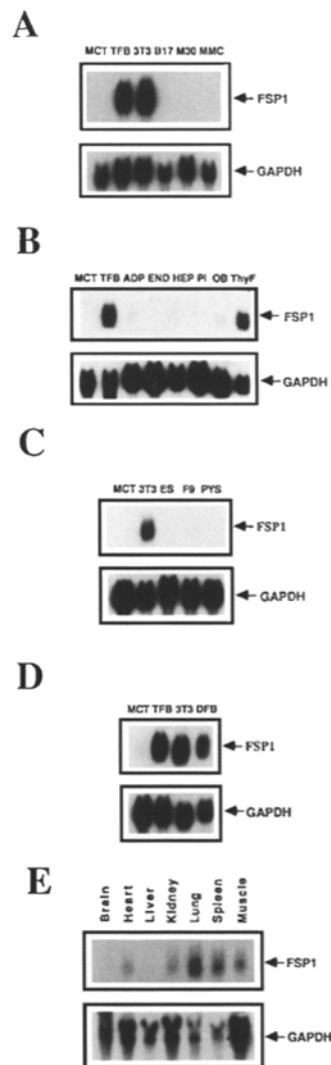


Figure 1. Northern analysis of *FSP1*. The transcript size for *FSP1* was 0.65 kb. (A) MCT epithelium, TFB fibroblasts, BAL B-lymphocytes, M30 T-lymphocytes, MMC mesangial cells. (B) MCT epithelium, TFB and 3T3 fibroblasts, ADI adipocytes, END endothelial cells, HEP hepatocytes, PI pancreatic islet cells, OB osteoblasts, and ThyFB thymic fibroblasts. (C) MCT epithelium, 3T3 fibroblasts, ES embryonic stem cells, and F9 and PYS-2 cells. (D) MCT-epithelium, TFB-, 3T3-, and dermal fibroblasts (DFB). (E) RNA from whole organs.

in acetone/methanol for 30 min at -20°C and stained with various primary antibodies in dilutions of 1:50–1,000; rabbit anti-FSP1; rabbit anti-vimentin, and rat anti-cytokeratin. After several washes in PBS, cells were incubated with optimal concentrations of secondary antibody FITC-goat anti-rabbit IgG (F(ab')₂-fragment) or FITC-rabbit anti-rat IgG (F(ab')₂-fragment) (35). Normal organs from 6–8 wk SJL/J mice were fixed in 1% paraformaldehyde and embedded in paraffin. Sections of 4–6 μm were cut and mounted on microscope slides. Before antibody incubation, the paraffin sections were deparaffinized in xylene and ethanol, rehydrated, and digested with proteinase K (6 $\mu\text{g}/\text{ml}$) for 20 min at room temperature (39). The polyclonal anti-FSP1 antibody was used in a concentration of 1:50–1:200. The preimmunization sera served as negative control. Positive reaction products were identified using the DAKO PAP KIT™ according to protocols available from the manufacturer (Dako, Carpinteria, CA). Sections were counterstained with Harris's hematoxylin. Kidneys demonstrating interstitial inflammation were also harvested and stained as above. Sections from all kidneys underwent trichrome staining to localize collagen.

In Situ Hybridizations

In situ hybridizations were performed on 8.5, 12.5, and 13.5-d-old mouse embryos that were fixed in modified Carnoy's fixative and embedded in paraffin (35). FSP1 sense/antisense riboprobes were synthesized by in vitro transcription of FSP1-cDNA in pBS SK– in the presence of [³⁵S]rUTP (800 mCi/mmol; Amersham). Each slide was hybridized with 5×10^6 cpm of probe in 100 μl of solution containing 50% formamide/10% dextran sulfate/0.3 M NaCl/10 mM Tris, pH 7.5/10 mM sodium phosphate/5 mM EDTA/10 mM dithiothreitol/1 \times Denhardt's/10 mM thio-dATP/1.5 $\mu\text{g}/\text{ml}$ yeast tRNA at 55°C overnight (19). The slides were then treated with RNase, washed, dipped in Kodak NTB2 emulsion, and developed after 21 d of exposure using Kodak Dektol developer. The slides were then stained with toluidine blue and mounted with Permount™ resin.

Results

Assessment of Unique Fibroblast Transcripts by Comparative Hybridization

To identify new markers in fibroblasts, we screened TFB fibroblast cDNA by differential and subtractive hybridization with isogenic transcripts from MCT epithelium. In the first series of experiments, 180,000 plaques were screened

by differential hybridization yielding four differentially expressed clones: the $\alpha 1$ - and the $\alpha 2$ - chains of collagen type I, osteopontin, and a fourth clone which demonstrated by Northern analysis the highest fibroblast specificity and was subsequently named fibroblast-specific protein (FSP1). All four clones were obtained in several isolations. Northern hybridization of these four clones registered signals of 4.7 and 5.7 kb for $\alpha 1(I)$ collagen, 5.3 kb for $\alpha 2(I)$ collagen, 1.5 kb for osteopontin, and 0.65 kb for FSP1. The cDNAs for the two collagen chains were partially sequenced and showed identity to the published genes (23, 62) and were not characterized further. The cDNA for osteopontin was sequenced in its entire length (1,372 bp) and proved to be identical to the published murine gene (55). Northern blot analysis revealed a high level expression of osteopontin in tubulointerstitial (TFB) and 3T3 fibroblasts, and adipocytes, and low level expression in tubular epithelium (MCT) and hepatocytes, osteoblasts, thymic (ThyFB) fibroblasts, embryonic (ES) stem cells, and F9 cells (data not shown). There was no expression detectable in endothelial cells, pancreatic islets, B and T cells, PYS-2 cells or dermal (DFB) fibroblasts. Thus, the expression of osteopontin confirms the phenotypic distinctions between fibroblasts of different origin, but is not highly specific.

The fourth clone, FSP1, was detected in high abundance on Northern blots in all fibroblast cells examined, including TFB and 3T3, ThyFB, and DFB fibroblasts (Fig. 1 D), with a transcript size of ~ 0.65 kb. There were little or no transcripts encoding FSP1 in all nonfibroblast cells examined, including proximal tubular cells (MCT), mesangial cells (MMC), B and T lymphocytes, adipocytes, endothelial cells, hepatocytes, pancreatic islets, and osteoblasts (Fig. 1, A and B). Furthermore, no transcripts were detectable in ES, F9, and PYS-2 cells of early embryonic origin (Fig. 1 C). In RNA isolated from whole organs, transcripts encoding FSP1 were readily detectable in lung, kidney, and spleen (Fig. 1 E), weakly present in muscle and heart,

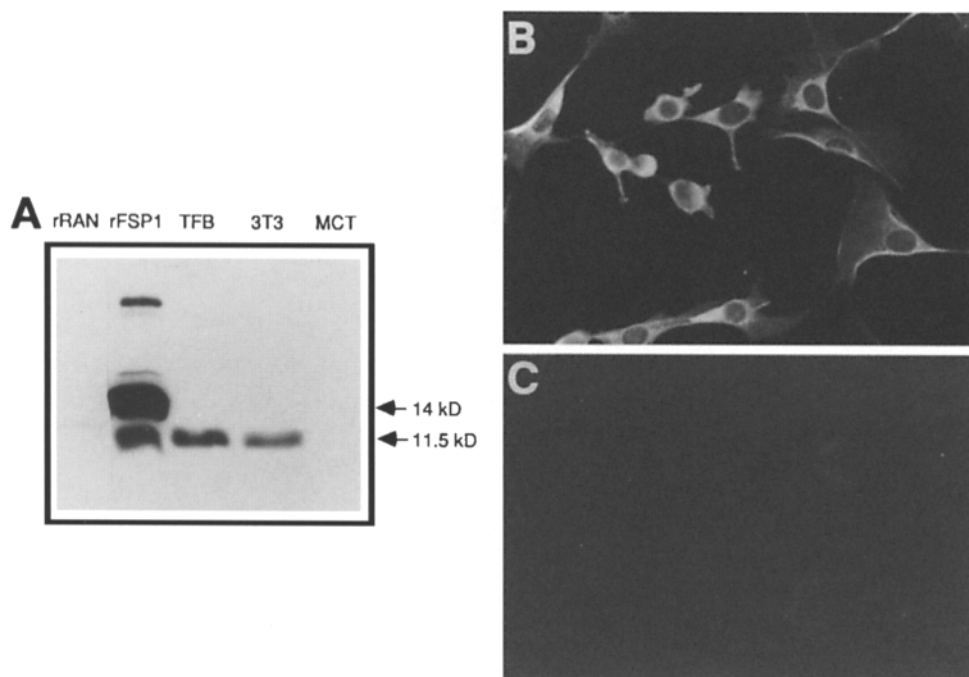
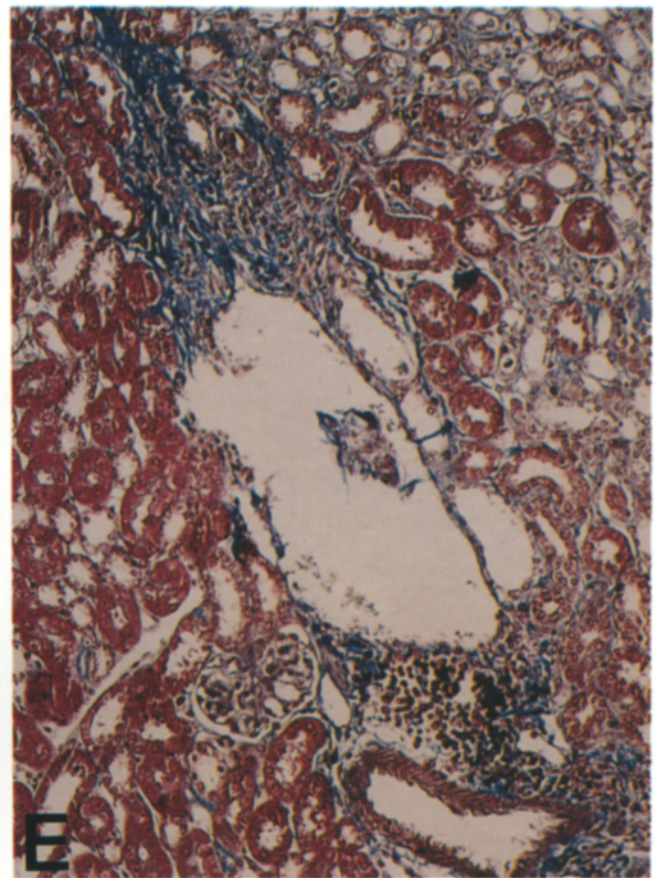
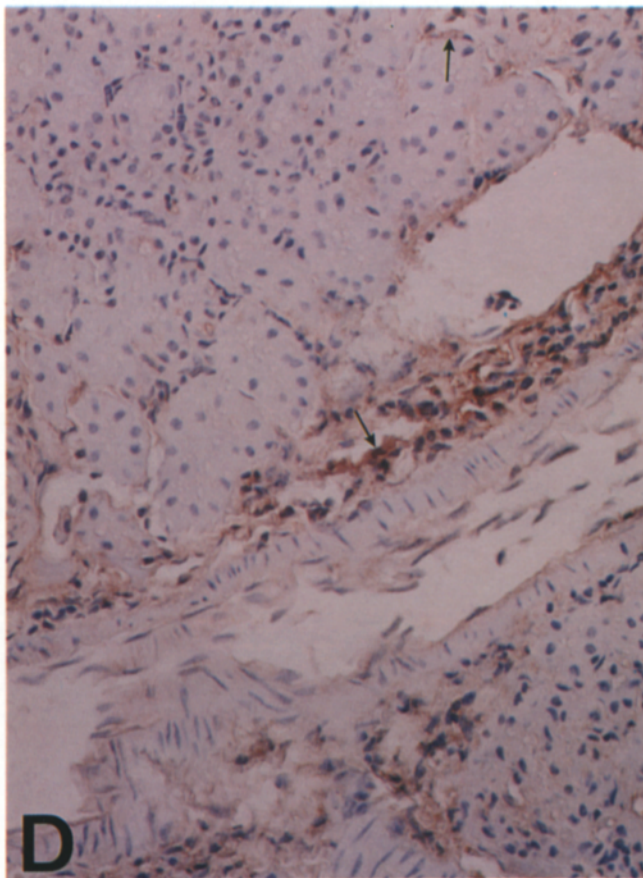
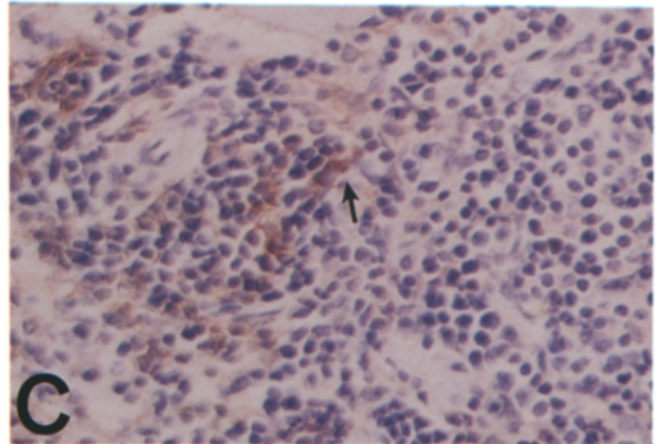
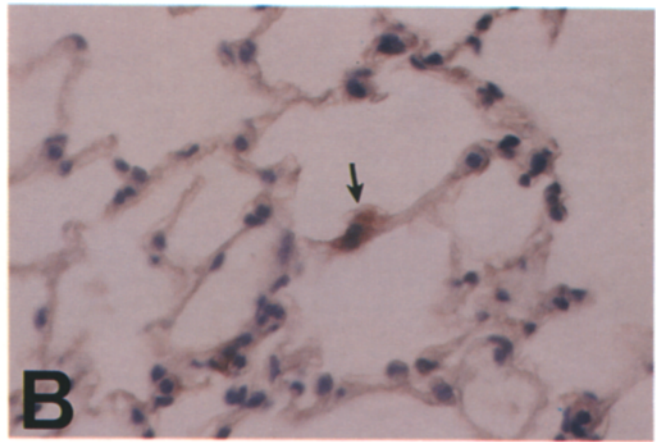
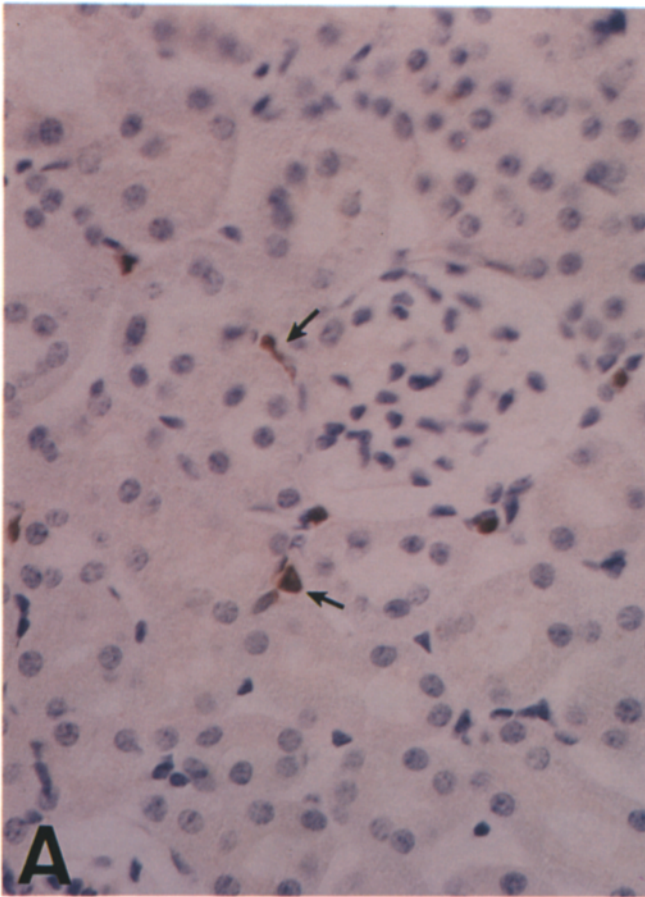


Figure 2. Expression of FSP1 as a fusion protein. (A) Immunoblot with the polyclonal anti-FSP1 antibody. Recombinant RANTES fusion protein (*rRAN*) served as negative, recombinant and FSP1 (*rFSP1*) as positive control. Protein extracts from TFB and 3T3 fibroblasts are positive for FSP1, while MCT epithelium is not. The main band in the *rFSP1* lane has a slightly larger size due to the fusion leader sequence. (B) TFB cells with the polyclonal anti-FSP1 antibody demonstrate strong staining in the cytoplasm of the fibroblast. (C) Immunostaining of MCT epithelium with anti-FSP1 antibody is negative.



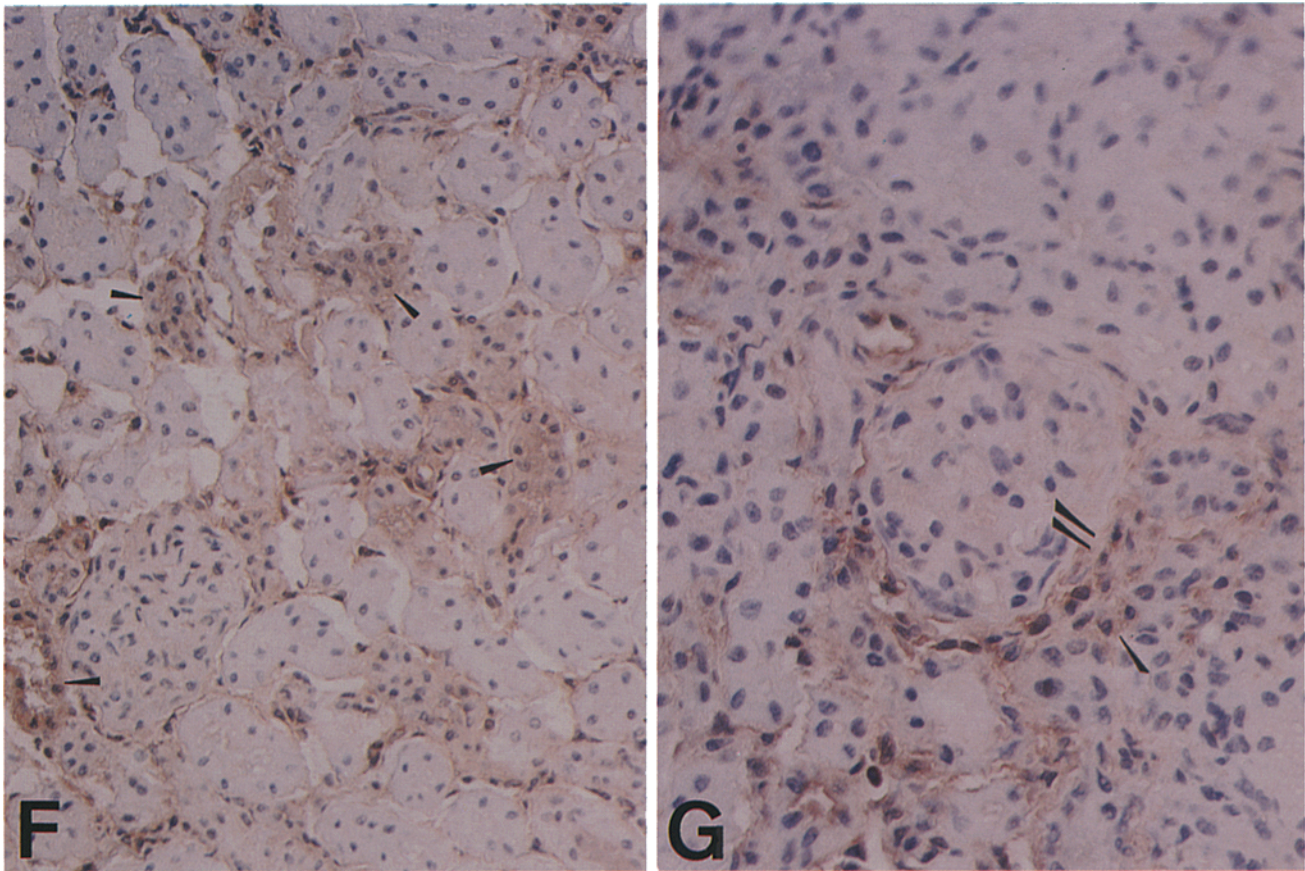


Figure 3. Immunohistology with the polyclonal anti-FSP1 antibody in normal tissues and fibrotic kidney. Staining of normal tissues demonstrate rare, occasional fibroblasts (marked with arrows) in kidney (A) and lung (B) interstitium, and in splenic pulp (C). Immunostaining with the polyclonal anti-FSP1 antibody in kidneys with fibrosis from anti-TBM disease at 8 wk (D) and 13 wk (F and G) after induction. In D there are occasional tubular conversions as well as new perivascular fibroblasts (see arrows). E illustrates collagen deposition by trichrome staining in the same kidney as D. F demonstrates progressive kidney involvement from anti-TBM disease at higher magnification; there are many tubules undergoing fibroblast conversion with new expression of FSP1 (see arrowheads). In G from the same kidney there are peri-glomerular tubules (single arrowhead) and new adjacent peritubular cells which have become FSP1⁺ (double arrowheads). The adjacent glomerulus, while surrounded by early fibrogenesis, has not as yet undergone any phenotypic conversion.

and not detectable in liver and brain; findings supported by immunochemistry in selected tissues (infra vide). The cDNA encoding FSP1 was 480 bp in length and was sequenced in its entirety. A search of the NCBI database showed that the sequence was identical to *pEL-98* (30), *18A2* (42), and *mts1* (20) of the S-100 superfamily.

We next performed subtractive hybridization to look for rare transcripts missed by differential screening. This search

yielded only one additional new clone, *S44a1*. However, of all clones found, it was not as stringently specific for fibroblasts as FSP1 (data not shown).

Antiserum Against FSP1 Detects Fibroblasts In Vitro and In Vivo

To further characterize fibroblasts in vivo, we generated a

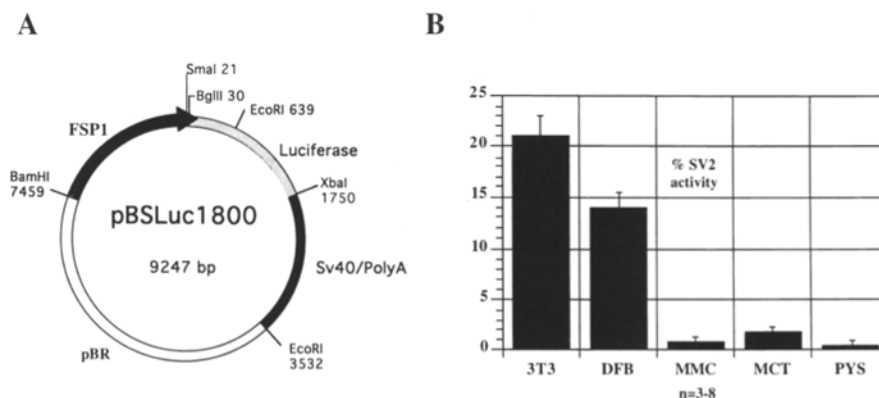


Figure 4. FSP1 promoter is active only in fibroblasts. (A) Luciferase reporter construct pBSK1800Luc for the analysis of the promoter activity of the 5' flanking region of the FSP1 gene. (B) Results of transient transfection in DFB and 3T3 fibroblasts, MCT epithelium PYS cells and MMC mesangial cells reveals the FSP1 promoter is only active in fibroblasts.

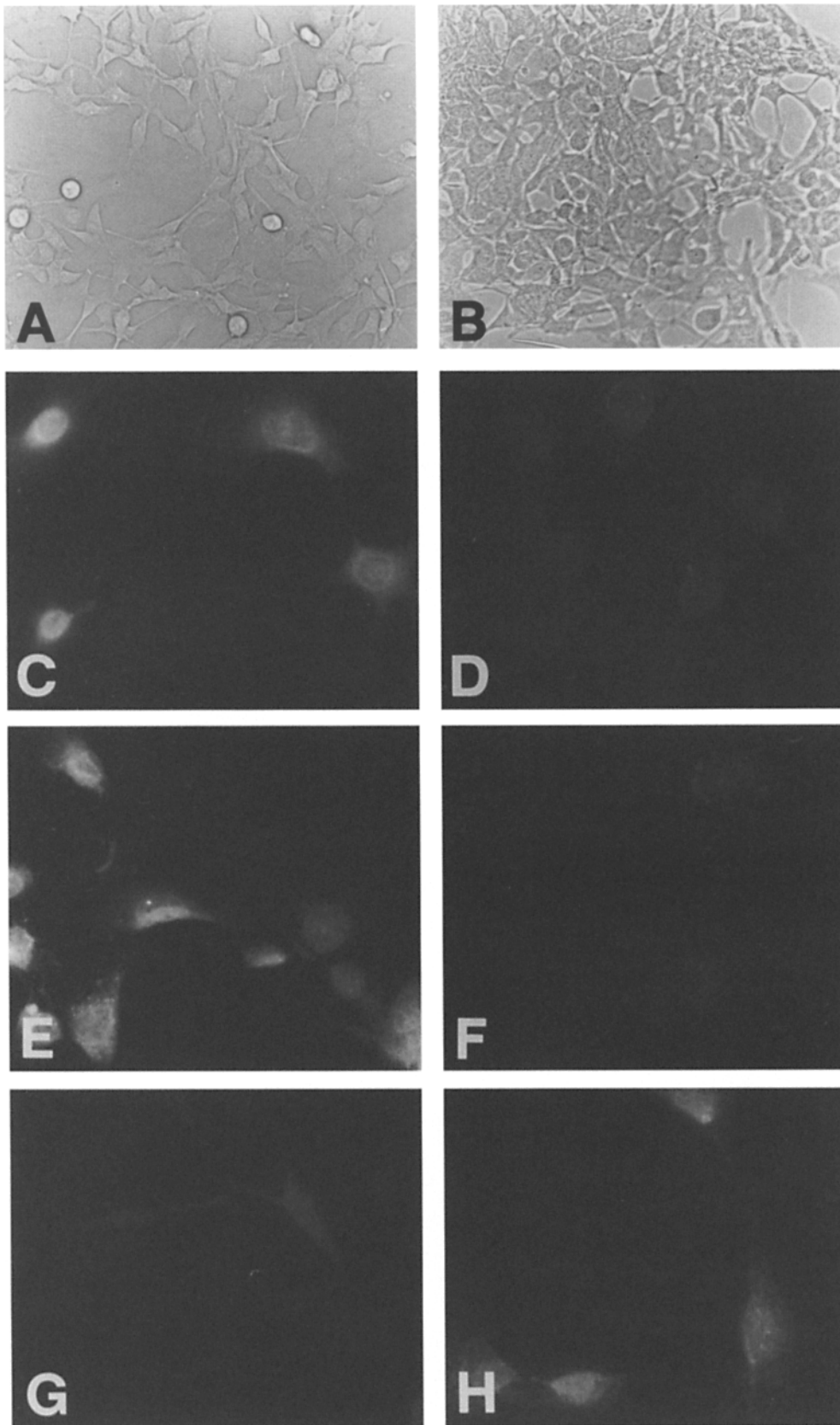


Figure 5. Overexpression of FSP1 cDNA in MCT.pFSP1.8 tubular epithelium converts these cells towards a fibroblast appearance. All plating densities were identical at the beginning of culture passage. (A) MCT.pFSP1.8 epithelium stably transfected with FSP1 do not form cuboidal sheets 72 h after passage in subconfluent cultures, but rather maintain a more stellate or fibroblast appearance by light microscopy. (B) MCT.pNeo epithelium stably transfected with the neomycin-resistance gene maintains a capacity to form cuboidal sheets in focal sites after subconfluent culture as early as 48 h. (C) Using immunofluorescence, MCT.pFSP1.8 epithelium express cytoplasmic FSP1. (D) MCT.pNeo epithelium stably transfected with the neomycin-resistance gene do not express FSP1. (E) MCT.pFSP1.8 epithelium now express vimentin. (F) MCT.pNeo epithelium do not express vimentin. (G) MCT.pNeo epithelium now express less cytokeratin. (H) MCT.pNeo epithelium continue to express cytokeratin.

polyclonal and several monoclonal antibodies to the FSP1 fusion protein. The studies presented here were performed with the polyclonal antibody. This antibody reacted specifically with the FSP1 fusion protein on immu-

noblots (Fig. 2 A) and was very specific for fibroblasts in cell culture (Fig. 2 B). On tissue sections of the kidney, it stained occasional interstitial cells (Fig. 3 A). Similar results were obtained in the lung and spleen tissues (Fig. 3, B

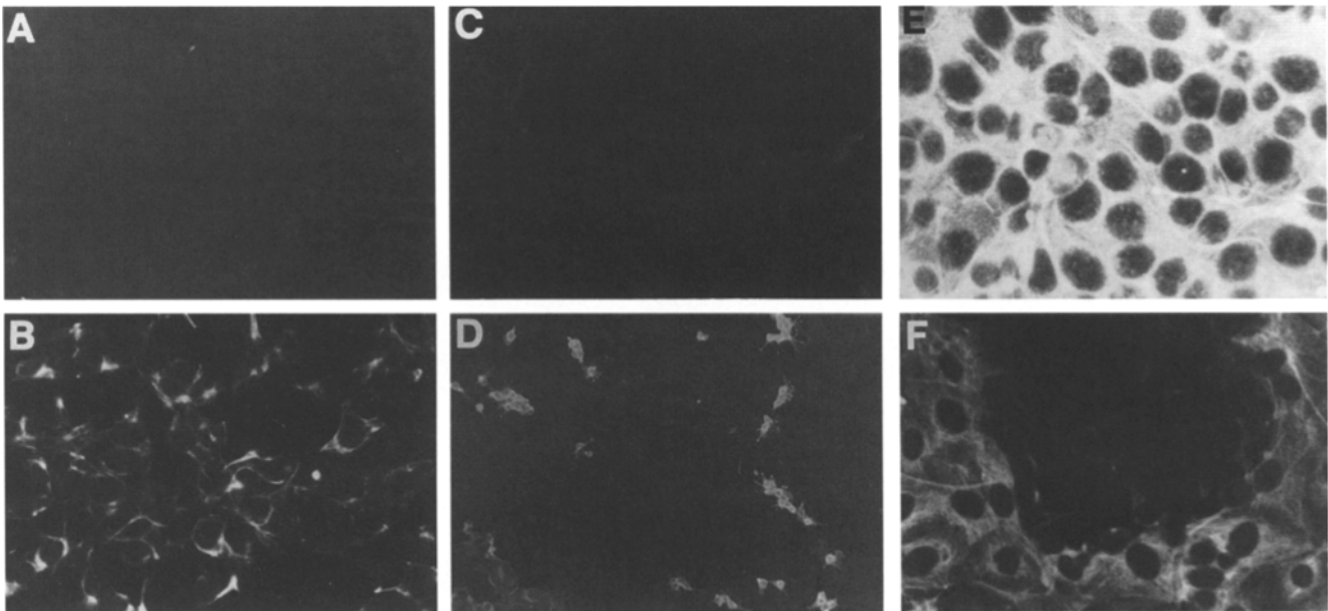


Figure 6. Cultures of NP-1 tubular epithelium submerged in collagen type I gels converts these cells to a fibroblast appearance. NP-1 cells grown in submersion gels or controls grown on the surface of collagen-coated plates were analyzed at 72 h by immunofluorescence. (A) NP-1 control cells grown on the surface did not express FSP1. (B) NP-1 epithelium grown in submersion gels began to express FSP1. (C) NP-1 control cells grown on the surface did not express vimentin. (D) NP-1 epithelium grown in submersion gels began to express vimentin. (E) NP-1 control cells grown on the surface continue to express cytokeratin. (F) NP-1 epithelium grown in submersion gels in focal areas began expressing less cytokeratin as they undergo phenotypic conversion; converted cells in the dark center are particularly devoid of cytokeratin.

and C, respectively). However, no staining was detectable on tissue sections of heart and liver (data not shown), a result in concordance with the Northern in Fig. 1 E, indicating that most cells in these organs do not normally express FSP1.

We next examined the staining pattern of fibroblasts in a mouse model of renal interstitial fibrosis (34). 8 wk after the induction of interstitial inflammation, a robust increase in staining of the FSP1-reaction product was detectable (Fig. 3 D). This was accompanied by increased interstitial collagen deposition as demonstrated by trichrome staining (Fig. 3 E). 13 wk after induction the interstitial staining was even more intense (Fig. 3, F and G [higher magnification]). In the areas of fibrogenesis it is significant, and a surprise for us to note, that tubular epithelium engulfed in the local inflammatory process have now begun expressing FSP1 (see discussion for further comments).

The 5' Flanking Region of FSP1 Has Fibroblast-Specific Promoter Activity

To examine the promoter region of the *FSP1* gene, we isolated a genomic clone from a NIH/3T3 genomic library using a cDNA fragment from the 5' coding region (15). 1,800 bp of the 5' flanking sequence was placed in front of a luciferase reporter gene (Fig. 4 A depicts the reporter construct) to study its activity in fibroblasts and nonfibroblasts relative to the activity of the SV40-promoter/enhancer. Fig. 4 B demonstrates that elements in the 5' flanking region of *FSP1* are strongly active in DFB and 3T3 fibroblasts, but not in nonfibroblast cells, such as proximal tu-

bular cells (MCT), embryonic PYS-2 cells, nor mesangial cells (MMC). This pattern of activity is consistent with the Northern analysis showing the absence of FSP1 transcripts in these cells.

Expression of FSP1 in Epithelium Favors Conversion to a Fibroblast Morphology

To investigate the possible phenotypic effects of FSP1 expression, we performed two different experiments. In the first series of studies, we stably transfected MCT tubular epithelium with a shuttle vector containing FSP1 cDNA driven by a CMV promoter (MCT.pFSP1.8) maintained in G418 (confirming Northern analysis not shown); control MCT cells were stably transfected with pcDNA.Neo (MCT.pNeo). New expression of the FSP1 in MCT.pFSP1.8 cells led to a change in morphology to a more stellate and elongated fibroblast phenotype (Fig. 5 A) compared to MCT.pNeo epithelial cells which were still capable of forming early epithelial sheets in subconfluent culture (Fig. 5 B). Cells were also passaged onto gelatin-coated glass to stain for various cytoplasmic markers. MCT.pFSP1.8 cells stained positive for FSP1 by indirect immunofluorescence (Fig. 5 C) compared to MCT.pNeo which did not (Fig. 5 D). MCT.pFSP1.8 cells also began expressing new vimentin (Fig. 5 E), and had reduced staining for cytokeratin (Fig. 5 G) when compared to controls (Fig. 5, F and H, respectively).

In a second series of experiments, we cultured NP-1 tubular epithelium either submerged in a type I collagen gel or, as a control, on the surface of type I collagen-coated

E8.5

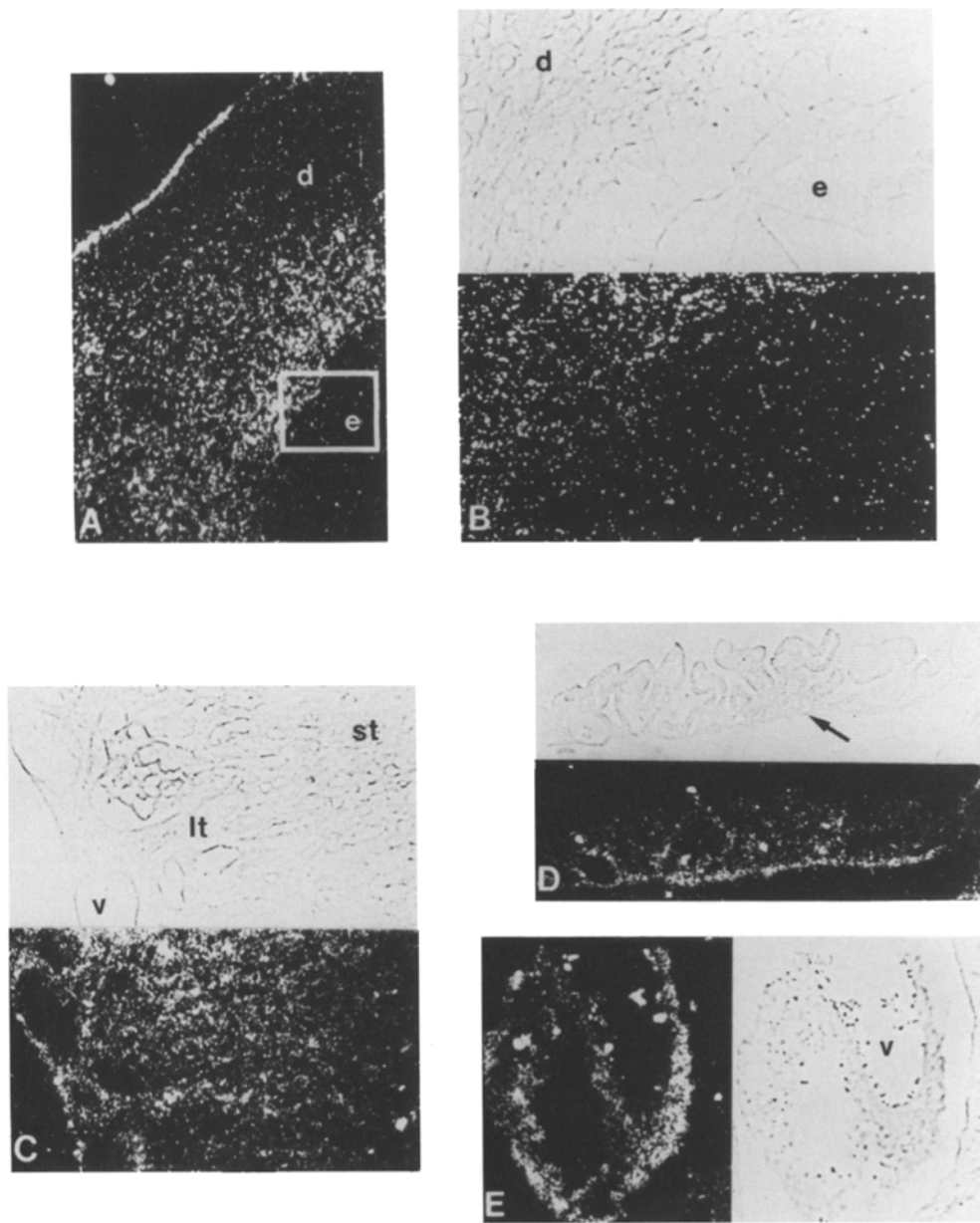


Figure 7.

dishes for 7 d (31). NP-1 tubular cells submerged in type 1 collagen began to convert into mesenchymal fibroblasts and this was associated with the expression of new FSP1 (Fig. 6 B) and vimentin (Fig. 6 D) when compared to controls (Fig. 6, A and C, respectively). Submerged NP-1 cells also express much less cytokeratin (Fig. 6 F), particularly as mesenchymalized cells start to detach and move away (*dark center*) compared to control NP-1 cells growing on the surface of the collagen gel (Fig. 6 E).

Detection of Fibroblasts in Developing Tissues

In situ hybridization of mouse embryos were probed with

FSP1 cDNA at days E8.5, E12.5, and E13.5 to investigate the expression of FSP1 in developing tissues. The results are shown in Fig. 7 (plates E8.5, E12.5, and E13.5). *FSP1* is not expressed in the embryo at day E8.5. Its message could be detected, however, in the decidua tissues (plate E8.5; panels A and B). Decidual cells are derived from uterine fibroblasts and retain many fibroblast features including the ability to synthesize collagen (1, 22). Spongrotrophoblasts, certain areas of the yolk sac and perivascular cells of the umbilical vessels (plate E8.5; panels C–E) also stained positive. At day E12.5, *FSP1* message can be detected in the embryonic dental mesenchyme, the leptomeningeal membrane, around the sclerotomes, the ura-

E12.5

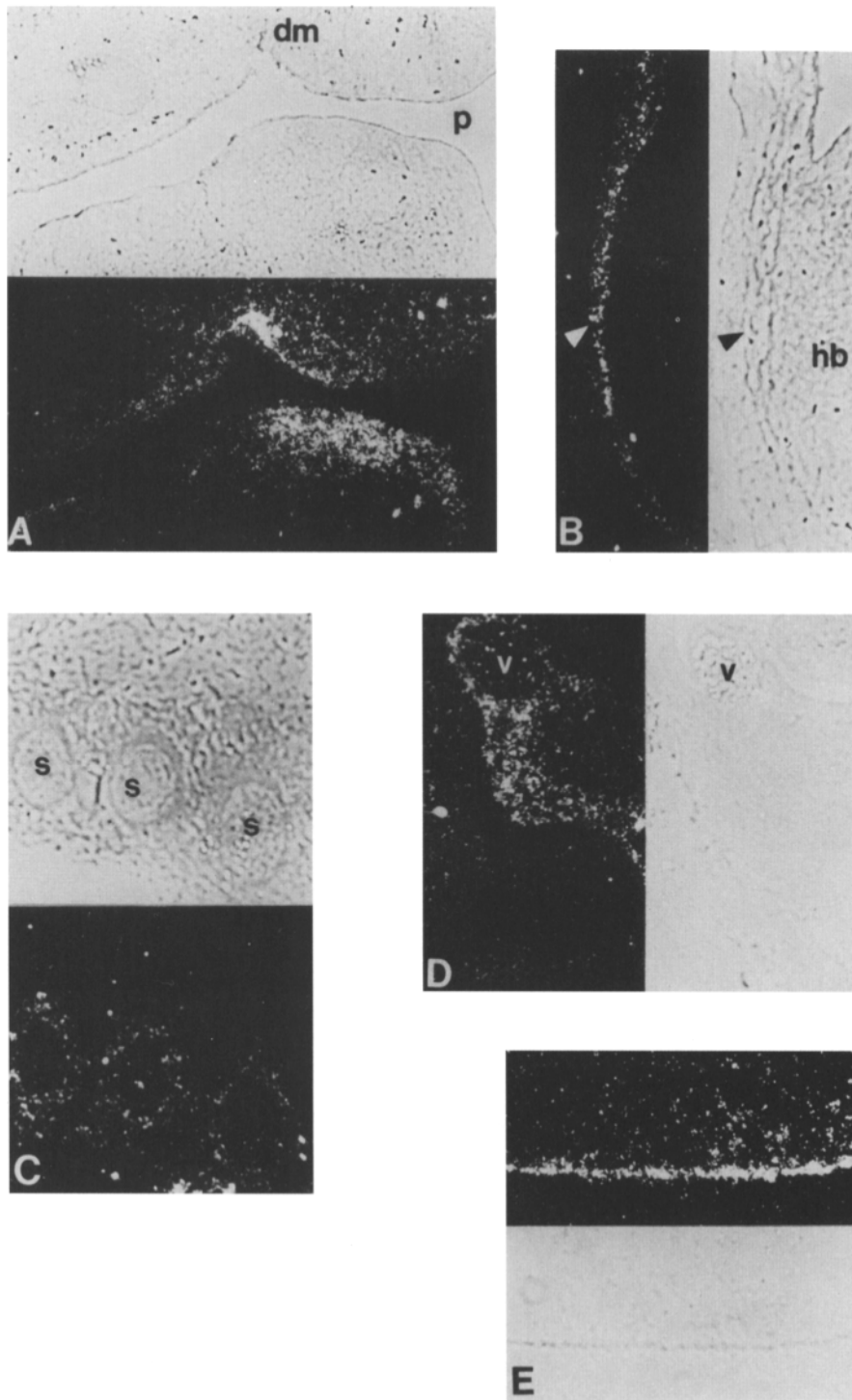


Figure 7.

chus and in the periderm covering the caudal aspect of the embryo (Fig. 7; plate E12.5; panels A–E). At day E13.5, the following regions stain positively for *FSP1*: again the periodontal mesenchyme (Fig. 7; plate E13.5; panel B) and the myotomes (Fig. 7; plate E13.5; panel C). The vast majority of kidney cells at that stage do not express *FSP1*,

however strong expression can be detected in the supporting capsules of the kidney and adrenal gland (Fig. 7; plate E13.5; panel E) as well as the thymus (Fig. 7; plate E13.5; panel D), which at that time is particularly rich in fibroblasts (64). Interestingly, intravascular cells in two blood vessels near the thymus and in the brain also stain positive,

E13.5

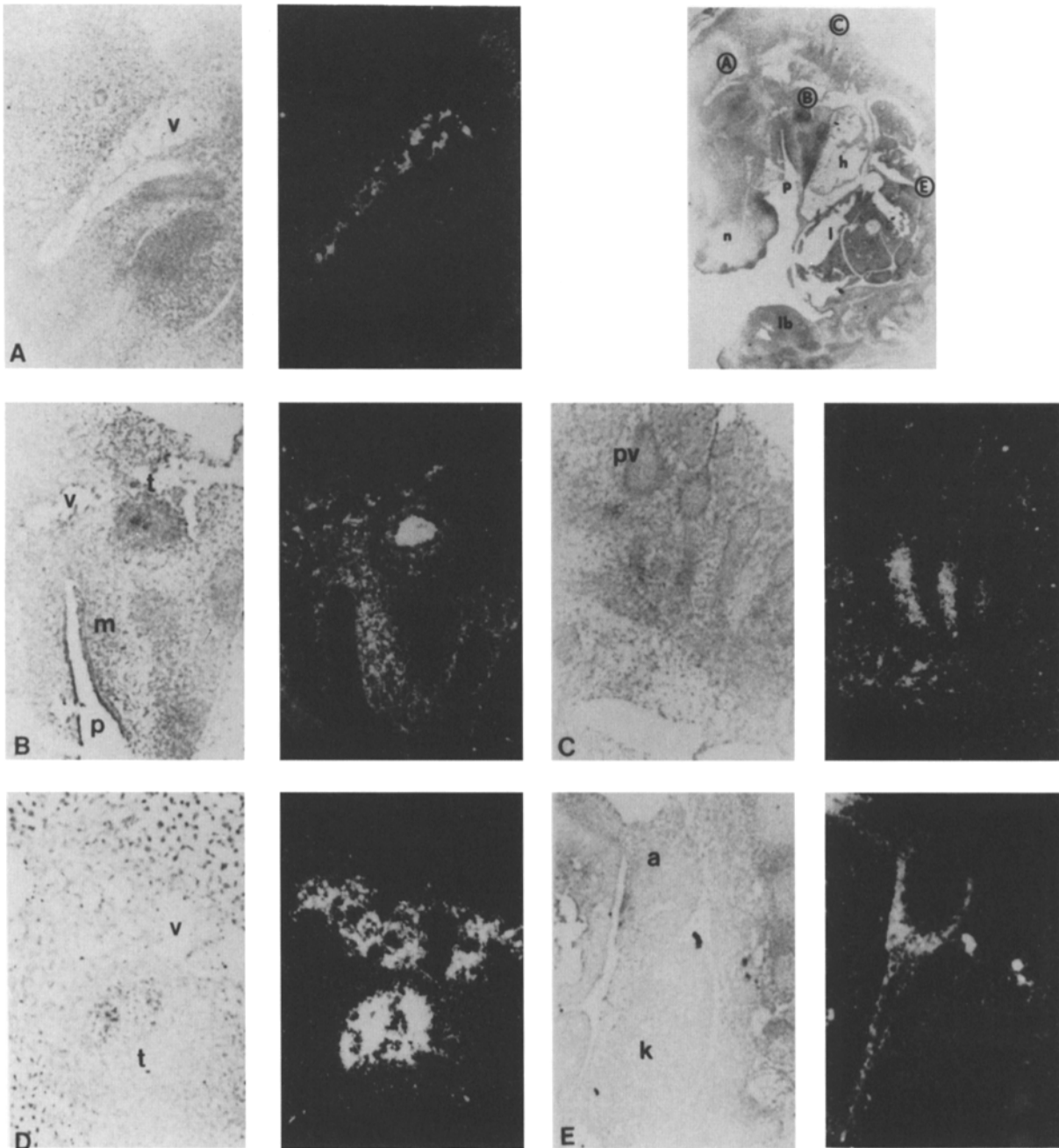


Figure 7. FSP1 expression in mouse embryos by in situ hybridization of sagittal sections. (*E8.5*) *A* (*d* marks the decidua) and *B* (shows the area *e* in *A* at a higher magnification; *d* again marks the decidua, *e*, embryo), the placenta *C* (*v*, placental vein, *lt*, labyrinth trophoblast, *st*, spongiotrophoblast), the yolk sac *D*, and the umbilical cord *E* (*v*, umbilical vein). Bright-field and corresponding dark-field micrographs. The embryo itself showed no detectable staining at day 8.5. (*E12.5*) FSP1 expression in organs and tissues of 12.5 d embryos. Sagittal sections of the periodontal mesenchyme *A* (*dm*, dental mesenchyme, *p*, pharynx), the leptomeningeal membrane *B* (*hb*, hind-brain), the sclerotomes *C*, the urogenital sinus *D* (*v*, umbilical vein) and the caudal part of the skin *E*. Bright-field and corresponding dark-field micrographs. (*E13.5*) (*A*) Sagittal sections of a cerebral vein (*v*); (*B*) the pharyngeal (*p*) area with periodontal mesenchyme (*m*); (*C*) the myotomes and prevertebrae (*pv*); (*D*) area around the thymus (*t*) with adjacent vein (*v*) at higher magnification; and (*E*) the kidney and adrenal gland (*a*).

while all other intravascular cells are completely negative (Fig. 7; plate E13.5; panels A and D).

Discussion

Microscopy studies have long created the impression that fibroblasts are a monotonous phenotype of limited complexity (27). Fibroblasts first appear phylogenetically in lower chordates that form a mesoderm during gastrulation (38). This mesodermal plane between the ectoderm and endoderm contains mesenchymal cells, and at some point, fibroblasts that create necessary fibrillar infrastructure forming loose tissue spaces for cell migration during organogenesis (36). It is quite likely, however, that fibroblasts also function as part of the integrated biology of mature organisms. Several studies, in fact, now suggest that morphogenic cues from tissue microenvironments confer a functional heterogeneity to fibroblasts harvested for culture (5, 44). Although several fibroblast markers have been described in the literature (4, 16, 18, 48), none of them have proven to be highly specific.

We initially approached the issue of determining fibroblast phenotype using a conventional antibody strategy that was not successful. This then led to the notion of making transcript comparisons (30, 41, 68, 69) between fibroblasts (TFB cells) harvested from the same microenvironment as differentiated isogenic epithelium (MCT cells). After differential and subtractive cloning, we were surprised to observe that fibroblasts produce very few unique transcripts compared to epithelium. Two transcripts encoded intracellular proteins (*FSP1* and *S44a1*), and three encoded secretory proteins ($\alpha 1$ and $\alpha 2$ collagen type I and osteopontin); none of them had extracellular domains as membrane-bound species.

The detection of the two collagen chains was to be expected, since abundant production of interstitial collagen type I is typical of fibroblasts (27), although not specific (34). The interstitial collagens consist mainly of types I and III (61). Other collagen chains were not detected possibly due to their low rate of transcription in fibroblasts in vitro (49) or their shared expression with epithelium (15). Osteopontin is a cell-matrix adhesion molecule (70) found in osteoblasts, macrophages, decidual cells, vascular smooth muscle cells, and distal tubular cells in the kidney, in addition to fibroblasts (14, 51, 68). By Northern analysis, *S44a1* was also expressed in several different cell types, and showed low specificity for fibroblasts. The usefulness of osteopontin and *S44a1* as fibroblast-specific markers seems unlikely.

The cDNA sequence encoding *FSP1* shares identity to clones of unknown function already in the database. Previous isolated transcripts 18A2 (placental calcium-binding protein) (42), *pEL98* (30), and *mts1* (20) were found in mice using differential hybridization. 18A2 was obtained by differential hybridization of serum stimulated vs unstimulated Balb/c 3T3 fibroblasts (42, 50). Although 18A2 was thought to represent a cell cycle-dependent transcript, the same group was unable to confirm their findings in a different line of embryonic fibroblasts or in primary cultures of embryonic fibroblasts (42). The term placental calcium-binding protein for 18A2 is probably due to its expression in decidual cells and spongiotrophoblasts, both

specialized fibroblasts (1, 22), as we observed on day E8.5 in confirmation using in situ hybridization. *pEL98* was also obtained by the differential hybridization of Balb/c3T3 fibroblasts against embryonic fibroblasts (30). While there was a notable difference in expression, embryonic fibroblasts clearly express the transcript encoding *FSP1*. Thus, these studies confirm our notion of fibroblast specificity for *FSP1*.

The fact that both of our cloning strategies together yielded only five differentially expressed genes was a surprise. There are several possible explanations: First, the cloning methods we used were not sensitive enough, and perhaps a form of differential display would have identified rarer transcripts than subtractive hybridization. Second, undifferentiated metanephric progenitor cells and renal fibroblasts harvested from mature tissues share similar properties (21)(68, 69), and consequently, the molecular difference between these mesenchymal cells and renal epithelium may lie in the added diversity of additional transcripts in the more specialized cell type.

Our culture experiments, using epithelium transfected with *FSP1* cDNA or epithelium submerged in collagen type I, suggest that *FSP1* is associated with, or may influence the morphogenic conversion of fibroblasts. Immunofluorescent stainings indicate that new expression of *FSP1* is associated with a loss of cytokeratin and a gain of vimentin. Two groups have recently reported the interaction of *FSP1*-like proteins with tubulin (46) and actin filaments (75), and a third group has described a correlation with cell motility (74). Although more work is needed, this suggests that *FSP1* in the cytoplasm may alter internal morphogenic cues that regulate the synthesis or assembly of other cytoskeletal proteins. In this regard, it has also been observed that other members of the S-100 protein family interact with components of the cytoskeleton (17). For example, S-100b protein itself interacts with the microtubule-associated τ protein inhibiting microtubule polymerization (8). Calpactin, another member of the family, acts as a modulator of membrane-cytoskeletal interaction by inhibiting tyrosine kinase-dependent phosphorylation (33). Such Ca^{++} -binding protein interactions with cytoskeletal filaments, as a hypothesis, may alter the morphology and migratory capacity of some differentiated cells.

Furthermore, the transition of epithelium into oncogenic cells is sometimes associated with the acquisition of mesenchymal properties (26, 37). For example, breast cancer cells can express either epithelial or fibroblastic phenotypes, as reflected by their morphologies and expression of intermediate filament proteins (71, 72). These fibroblastic tumor cells, which express vimentin, have a higher potential to metastasize. Overexpression of vimentin in an epithelial breast cancer cell line, however, failed to induce the fibroblastic phenotype (71). It, therefore, is not surprising that *FSP1*, like other proteins of the S-100 family, are up-regulated in some tumor cells, especially in those with high metastatic potential (17, 20). The use of the *FSP1* marker to study fibroblasts at the site of tumor metastases will have to be interpreted cautiously.

Finally, our original impetus to find a generally reliable fibroblast marker was to facilitate the study of fibrogenesis following tissue injury. While we are just starting to explore this issue in detail, we were struck by two observa-

tions made during the present investigation which bear further comment. First, fibroblasts have been traditionally thought to inhabit the interstitial spaces of normal organ tissue awaiting a reparative assignment. Our evidence, using FSP1 as a marker, suggests however, that fibroblasts are not an abundant phenotype in normal organ tissue. FSP1-positive fibroblasts are dispersed sparsely throughout organs. This is consistent with the observation that collagens found in the infrastructure of parenchymal tissues delicately lace the interstitial spaces in limited quantity (29, 77). Second, studies on the time course of interstitial fibrosis in injured organs demonstrate the initial deposition of collagen types I and III typically in close proximity to blood vessels (43, 52, 77), and as we observed, adjacent to occasional FSP1-positive fibroblasts in some perivascular spaces. Fibrogenesis, when it becomes fully active, however, occurs diffusely in many regions remote from large blood vessels. This spreading effect has been explained using *in vitro* observations supporting the notion that fibroblasts might migrate into areas of inflammatory injury by chemotaxis or haptotaxis and divide (2).

Alternatively, we suggest as a new hypothesis, that fibroblasts may at least be partly created at the site of injury by the conversion of parenchymal epithelium. In the current study we observed the new expression of FSP1 in typically negative tubular epithelium at the site of interstitial inflammation, the new expression of FSP1 in tubular epithelium undergoing a transition to fibroblasts in collagen gels, and the development of a fibroblast phenotype in tubular epithelium after the overexpression of FSP1. Epithelial-mesenchymal transitions are both economical and probable as operational processes in the early development of complex organisms (32, 37). In hepatic fibrogenesis, for example, the transition of lipocytes into myofibroblasts is thought to be a key feature in the perpetuation of the disease (7, 25). While these transitions could be considered as a form of cell activation, and FSP1 as an activation antigen, we believe phenotypic conversion is a more appropriate concept. Invoking an epithelial-mesenchymal transition for the origin of fibroblasts also provides an explanation for epithelial atrophy in areas of fibrogenesis, and the observed functional heterogeneity among cultured fibroblasts harvested from different microenvironments (5, 12). That is, fibroblasts that have transitioned from specialized epithelium might still harbor selected receptors and interactional programs that modulate according to this imprinted background.

FSP1 in mice appears to be a reliable marker for mapping the cell fate of fibroblasts among the mesenchymal anlagen. Transitions between normal cells are unexpected after organ development, and their presence in mature tissues reminds us of the conceptual links between the biology of development, repair, and oncogenesis.

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