Early role of Fsp1 in epithelial-mesenchymal transformation

HIROKAZU OKADA, THEODORE M. DANOFF,
RAGHURAM KALLURI, AND ERIC G. NEILSON
Penn Center for Molecular Studies of Kidney Disease, Renal-Electrolyte and Hypertension Division, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6144

Okada, Hirokazu, Theodore M. Danoff, Raghuram Kalluri, and Eric G. Neilson. Early role of Fsp1 in epithelial-mesenchymal transformation. *Am. J. Physiol.* 273 (*Renal Physiol.* 42): F563–F574, 1997.—A seamless plasticity exists among cells shifting between epithelial and mesenchymal phenotypes during early development and again later, in adult tissues, following wound repair or organ remodeling in response to injury. *Fsp1*, a gene encoding a fibroblast-specific protein associated with mesenchymal cell morphology and motility, is expressed during epithelial-mesenchymal transformations (EMT) in vivo. In the current study, we identified several cytokines that induce Fsp1 in cultured epithelial cells. A combination of these factors, however, was most efficacious at completing the process of EMT. The optimal combination identified were two of the cytokines classically associated with fibrosis, i.e., transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF). To confirm that it was the induction of Fsp1 by these cytokines mediating EMT, we used antisense oligomers to block Fsp1 production and subsequently measured cell motility and markers of EMT phenotype. The antisense oligomers suppressed Fsp1 expression and epithelial transformation; therefore, we conclude that the appearance of Fsp1 is an important early event in the pathway toward EMT.

transforming growth factor-β1; epidermal growth factor; antisense; fibrosis; fibroblast; cell motility

VERTEBRATES ACHIEVE THEIR structural complexity during early development, in part, by undergoing primary epithelial-to-mesenchymal transformations (EMT). EMT at this stage permits primitive cells of the skeleton, connective tissue, and organ anlagen to redistribute in the body plan as a phenotype capable of movement (33, 34). Many of these repositioned mesenchymal cells are subsequently induced back into secondary epithelium, where they enter cell fate maps for pattern formation, spatial position, and stationary organ structure. Mature secondary epithelium assembles into functional units in these organ tissues using extracellular contact junctions between neighboring cells as well as attachments to underlying basement membrane (18). These specialized cell attachments help determine cell polarity and transport vectors built out from a rigging of highly organized cytoskeletal fibers and signaling networks. Mesenchyme not utilized to reform epithelium is attenuated by apoptosis (40, 52, 72). The molecular programs that guide these events, to the extent they are known, are typically restrictive, decisional, tissue specific, and in most cases reflect changes in transcription modulated by morphogenic cues (13, 43, 48, 75, 80).

Adult fibroblasts appear late in vertebrate development, probably the result of EMT from secondary epithelium (50, 76), and remain quiescent in the interstitial and perivascular spaces of organ and connective tissues. The process of local conversion and stimulation of new fibroblasts can be accelerated during wound healing or tissue inflammation (33, 34, 36, 57, 77, 88). However, these repair responses at maturity typically disturb the structure of complex epithelial units by inundating that microenvironment with excessive connective tissue. During these responses, it appears that new fibroblasts formed by EMT (77) retain a permanent mesenchymal state, as long as inciting stimuli persist (16, 70, 85). When this happens, fibrogenesis in adult tissues can be relentless.

EMT has also been observed in cell culture systems (3, 35, 36). Epithelium in culture can lose polarity, adherence to adjacent cells and basal lamina, convert into elongated fusiform shapes, and gain mesenchymal properties including motility (36). Growth factors (9, 24, 38, 61, 71, 86), oncogenes (6, 7, 74), and cell surface adhesion molecules (6, 46, 94) have been proposed as modulators of EMT; however, the sequential coordination of events has not been established.

Recently, we cloned Fsp1, a murine fibroblast-specific protein (77) that belongs to the calmodulin-S100-troponin C superfamily of intracellular calcium-binding proteins (77). The members of this family have been implicated in microtubule dynamics (17, 21, 53, 68), cytoskeletal-membrane interactions (4, 21, 25, 31, 59, 64), calcium signal transduction (21, 28), cell-cycle regulation (49), and cellular growth and differentiation (11, 15, 41, 58, 59). Although the precise functions of Fsp1 and its homologs are not entirely clear, their interaction with nonmuscle myosin II (23), nonmuscle tropomyosin (83), actin (27, 82, 87), or tubulin (53, 67), as well as the inducibility of a migratory or metastatic phenotype when transfected into nonmetastatic cells in vitro (14, 22, 30, 68), suggests that Fsp1 may be associated with mesenchymal cell shape and motility. Tubular epithelium transfected with cDNA encoding *Fsp1* exhibited several properties of EMT, including a reduction in cell adhesion, cytokeratin, and new expression of vimentin (77). In this report, we describe the cytokine inducers of Fsp1 that promote EMT.

MATERIALS AND METHODS

Cell culture. Renal proximal tubular epithelial cells (MCT cells), NIH-3T3 fibroblasts (3T3), and tubulointerstitial fibroblasts (TFB cells) have been maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml enicillin, and 100 μg/ml streptomycin (1, 32). In EMT experiments, the medium was replaced with serum-free K-1 medium (50:50 Ham's F-12/DMEM with 5 μg/ml transferrin, 5 μg/ml insulin, and 5 × 10^{-8} M hydrocortisone) containing various concentrations of cytokines. For the assay of secreted collagens, K-1 medium was additionally supplemented with ascorbic acid (50 μg/ml)

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and β1-aminopropionitrile (50 μg/ml). Microscopic examination was performed during each experiment to assess the morphological changes of MCT cells prior to sample analysis.

Reagents. Recombinant human transforming growth factor (TGF)-α, tumor necrosis factor-α (TNF-α), TGF-β1, epidermal growth factor (EGF), platelet-derived growth factor-β1 (PDGF-β1), basic fibroblast growth factor (basic FGF), hepatocyte growth factor (HGF), interleukin-2 (IL-2), Mullerian inhibitory factor (MIF), RANTES, murine IL-1β, IL-4, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from R & D Systems (Minneapolis, MN), and phorbol 12-myristate 13-acetate was purchased from Sigma (St. Louis, MO). The following antibodies were used: rabbit anti-Fsp1 (77); rabbit anti-vimentin (77); TROMA-1 and -3, rat monoclonal anti-cytokeratins (45); mouse monoclonal anti-α-smooth muscle actin (anti-α-SMA; Sigma); rat monoclonal anti-mouse syndecan-1 (Pharmingen, San Diego, CA); rat anti-ZO-1 (Chemicon, Temecula, CA); alkaline phosphatase (ALP)-conjugated goat anti-rabbit immunoglobulin G (IgG), anti-rat IgG (Sigma); fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-rabbit IgG, anti-mouse IgG, and anti-rat IgG (Zymed). Anti-type I collagen and anti-NC1 domain of type IV collagen were raised in rabbit to acid solubilized rat tail collagen and bovine NC1 domain of type IV collagen, respectively (44). FITC-conjugated phalloidin (Sigma) was used to detect F-actin. Cell culture plates coated with rat tail type I collagen, Engelbreth-Holm-Swarm type IV collagen, mouse laminin, human fibronectin, and Matrigel basement membrane matrix were obtained from Collaborative Research (Bedford, MA).

Direct enzyme-linked immunosorbent assay. Cells (5×10^4 cells/well) were plated in 12-well plates and grown in DMEM with 10% FCS overnight. Then the medium was replaced with serum-free K-1 media containing various concentrations of cytokines. After 48 h culture, cells were harvested by trypsin/EDTA, spun down and suspended in phosphate-buffered saline (PBS). The number of cells was counted in a hemocytometer. A quantity of 5×10^4 cells were pelleted, then lysed in 500 μl of 6 M guanidine hydrochloride, buffered to pH 7.5 with 50 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride; 96-well multititer enzyme-linked immunosorbent assay (ELISA) plates were coated in triplicate with 100 μl of cell lysate (44). The plates were incubated overnight at room temperature. After coating, the plates were washed three times with 0.15 M NaCl and 0.05% Tween 20 washing solution. After washing, the plates were blocked with 2% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS incubating buffer for 30 min at 37°C. After blocking, the plates were again washed with the washing solution and then incubated with 1:1,000 dilution of anti-Fsp1 or in some experiments with 1:500 dilution of anti-vimentin or anti-cytokeratin in the incubation buffer. Preimmune serum or IgG was used as control. The plates were incubated for 1 h at room temperature. After primary antibody incubation, the plates were again washed and subsequently incubated with 1:1,000 dilution of ALP-conjugated secondary antibodies in the incubation buffer for 1 h at room temperature. Subsequently, the plates were again washed thoroughly, and substrate, disodium *p*-nitrophenyl phosphate (5 μg/ml), was added. After color development, the absorbance was measured with an ELISA autoreader at 450 nm.

For assay of the secreted collagens, 10^5 cells/well were plated in six-well plates and grown overnight in DMEM with 10% FCS. At that point the medium was replaced with serum-free K-1 medium supplemented with ascorbic acid and β1-aminopropionitrile containing various concentrations of cytokines. After 72 h, the supernatants and the cells were harvested separately, and the number of cells was counted on hemocytometer. A volume of 200 μl of the supernatants/well in triplicate was applied into the ELISA plates and dried under negative pressure for 48 h at room temperature. The direct ELISA assay was carried out with 1:1,000 dilution of anti-type I collagen or type IV collagen as described above. The results were normalized for the cell numbers.

Immunocytochemistry. Cells were grown on Lab-Tek slides (Nunc) and stimulated with cytokines for 48–72 h. The medium was removed, and the cell layer was rinsed with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in PBS. For the immunostaining of Fsp1, cytokeratins, vimentin, and α-SMA, the cells were fixed and permeabilized with acetone-methanol for 20 min at −20°C. For the staining of syndecan-1, ZO-1, and the detection of F-actin, the cells were fixed with freshly prepared 4% paraformaldehyde in 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 4 min. The cells were rehydrated with PBS, blocked with 5% BSA in PBS for 1 h, and incubated with a primary antibody for 1 h at room temperature. After washing with PBS, bound antibodies were detected using FITC-conjugated secondary antibodies described above and analyzed by fluorescence microscopy (77). Negative controls were performed using nonimmune serum or IgG instead of first antibodies. F-actin was detected directly using FITC-conjugated phalloidin.

Flow cytometric analysis. Cytokine-treated cells were lifted from the surface of the culture plate by gentle pipetting of monolayers incubated in 1 mM EDTA-Tris-buffered saline (25 mM Tris·HCl, pH 7.6, and 150 mM NaCl) on ice. The cells were spun down and washed with ice-cold washing buffer, 1% BSA in 0.5 mM EDTA-PBS. This washing step was repeated four times. A quantity of 10^6 cells were suspended in 50 μl of staining buffer, 1% BSA-PBS, and incubated with 1:100 dilution of anti-syndecan-1 for 20 min on ice. The cells were then washed two times with washing buffer. Subsequently, the cells were resuspended in 50 μl of staining buffer and incubated with 1:500 dilution of FITC-conjugated goat anti-rat IgG for 20 min on ice. The cells were then washed two times with washing buffer and fixed with 1% paraformaldehyde in PBS. The fixed cell samples were stored at 4°C under shade until assay, and the assay was carried out within 1 wk. FACScan analysis (Becton-Dickinson) was performed on 10^4 cells using CellQuest software (32). Controls were performed using isotype-matched rat IgG and FITC-conjugated antibodies.

Inhibition of in vitro EMT by Fsp1 antisense oligodeoxynucleotides. Phosphorothioate-capped oligodeoxynucleotides (oligomers) were synthesized on an automated synthesizer (Applied Biosystems, Foster City, CA). After deprotection, oligomers were dissolved in water, extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and redissolved in water. The Fsp1 sense oligomers sequence comprised 5′ CACGGTTACCATGGCAAGAC 3′, and antisense oligomers sequence comprised 5′ GTCTTGCCATGGTAACCGTG 3′; these sequences were chosen as likely to corrupt ribosomal docking by their location near the initial site of translation. Another oligomers used as a mismatch oligomers was a degenerate antisense sequence (5′ GTCNTGNCATGGNAANCGNG 3′). Oligomers were introduced into cells by permeabilization with streptolysin O (5). Briefly, 2×10^5 cells were suspended in 0.5 ml of permeabilization buffer [137 mM NaCl, 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 7.4, 5.6 mM glucose, 2.7 mM KCl, 2.7 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM sodium ATP, 0.1% BSA] containing 0.2 U/ml streptolysin O (Sigma) and 60 μM oligomers. After 5 min

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incubation at room temperature, 5 ml of DMEM with 10% FCS was added. The cells were immediately pelted, seeded into two wells of six-well plates, and grown overnight in DMEM with 10% FCS. At that point the medium was replaced with serum-free K-1 medium supplemented with EGF and TGF-β1. In case of collagen synthesis assay, ascorbic acid and β1-aminopropionitrile were also added. After 36 h, morphological alterations were checked, and the cell layer was partly scratched by a sterile razor blade. After subsequent 12 h, cell migration across the scratched area was evaluated. Supernatant and cells were collected, and biochemical assay for Fsp1, cytokeratins and collagens were carried out described above.

Statistics. Results are presented as means ± SE. The analysis of variance (Scheffé *t*-test) was performed where appropriate; significance of results was indicated when $P < 0.05$.

RESULTS

Cytokines and extracellular matrix molecules affect expression of Fsp1 protein in MCT epithelium. All experiments were carried out using syngeneic TFB and tubular epithelium (MCT) as cell lines (1, 32). Expression of Fsp1 mRNA and protein in TFB or other fibroblasts is abundant under basal culture conditions but absent in MCT epithelium by Western and North-

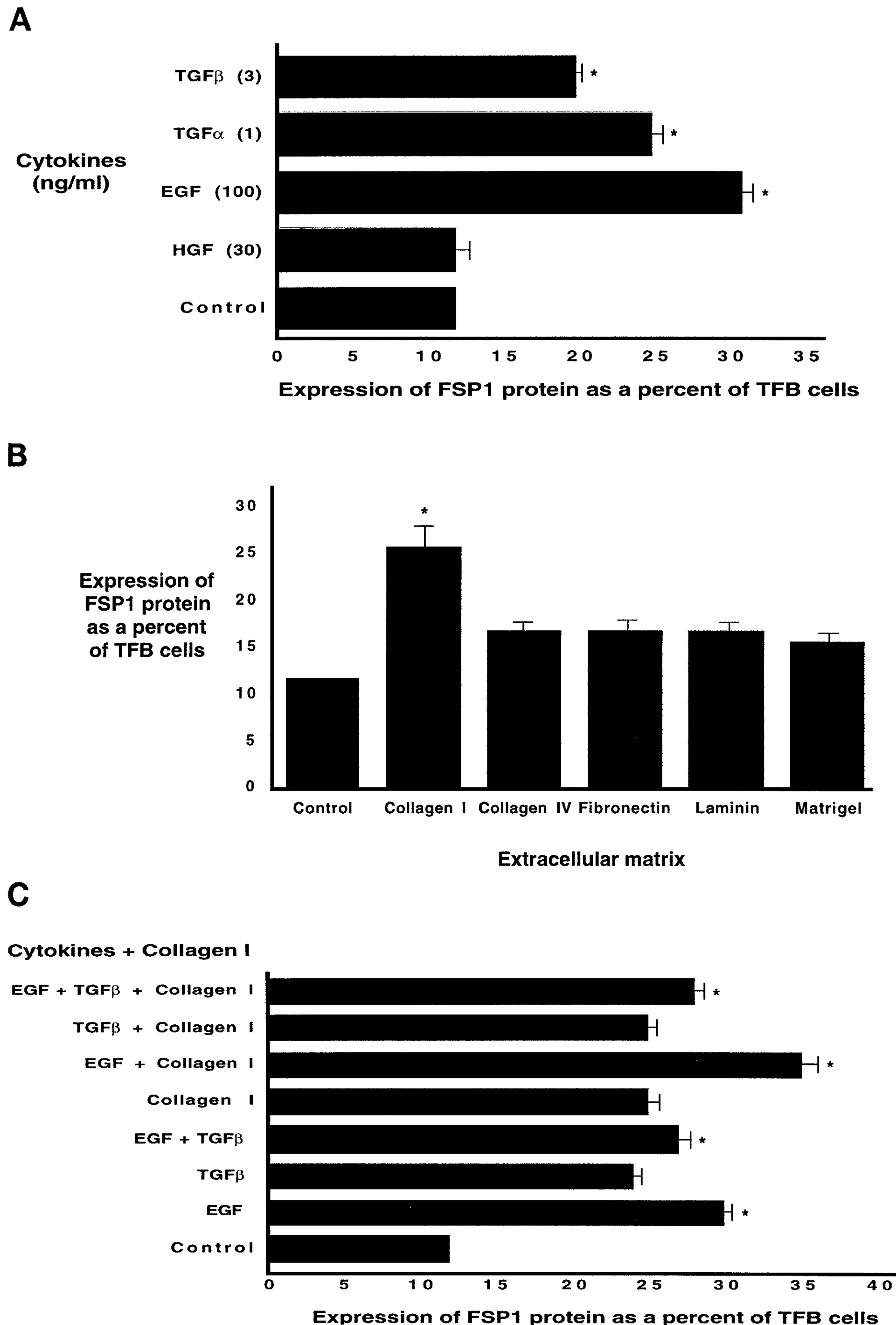

Fig. 1. De novo expression of Fsp1 by renal proximal tubular epithelial cells (MCT cells). *A*: Fsp1 protein level of MCT treated with humoral factors determined by direct enzyme-linked immunosorbent assay (ELISA). Values are relative to tubulointerstitial fibroblast (TFB) cells. Transforming growth factor-β1 (TGF-β1), TGF-α, and epidermal growth factor (EGF) induce de novo expression of Fsp1. *B*: Fsp1 protein level of MCT grown on different extracellular matrix molecules determined by direct ELISA. MCT grown on type I collagen express Fsp1. *C*: Combined effects of potent humoral factors and type I collagen determined by direct ELISA. HGF, hepatocyte growth factor. *Statistically significant, $P < 0.05$.

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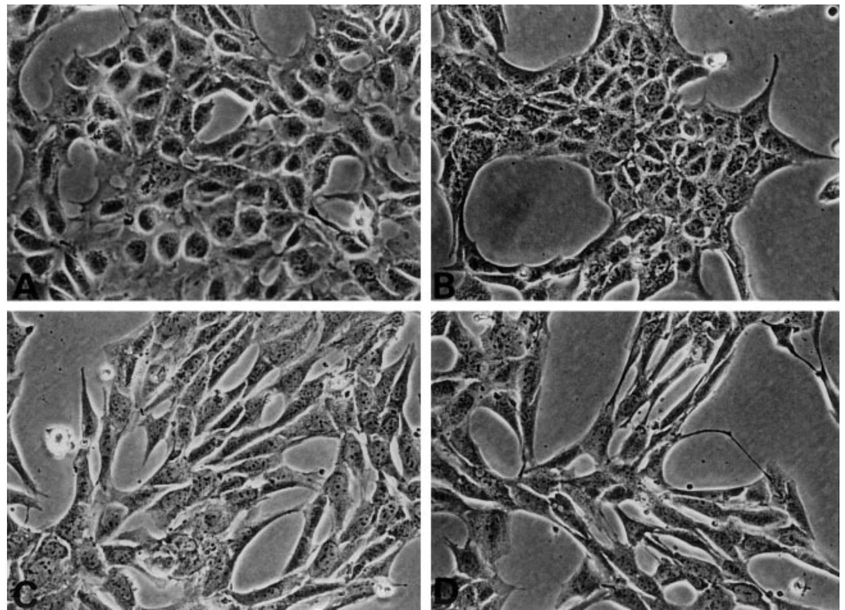

Fig. 2. Fsp1-inducible cytokines affect MCT morphology. *A*: control MCT grown on plastic surface without any treatment. They grow as monotonous, cuboidal cell sheet. *B*: MCT treated with EGF show slightly elongated, less cuboidal appearance. *C*: MCT treated with TGF-β1 become fusiform in shape, losing cell-cell adhesion. *D*: TGF-β1 in combination with EGF induces drastic morphological change in MCT, suggesting more complete epithelial-to-mesenchymal transformation (EMT). Magnification for *A–D*, ×70.

ern blotting (77). Numerous cytokines at varying concentrations were cocultured with MCT cells so that the expression of Fsp1 protein could be analyzed by ELISA; only peak doses are reported. In Fig. 1*A*, where the largest incremental response, if any, of each cytokine at peak concentration is illustrated, EGF (10 ng/ml), TGF-α (1 ng/ml), and TGF-β1 (3 ng/ml) robustly increased Fsp1 expression in MCT cells at 48 h. Northern analysis confirmed these results at the RNA level (data not shown). The effects observed with EGF or TGF-α were not likely the result of cell proliferation, because HGF was not effective, despite the strong promotion of cell proliferation (proliferation data not shown). GM-CSF (0.1 ng/ml), basic FGF (1 ng/ml), PDGF-β1 (1 ng/ml), IL-6 (1 ng/ml), IL-1β (1 ng/ml), and phorbol 12-myristate 13-acetate (30 ng/ml) also were coincubated with MCT cells, but no significant Fsp1 response was observed (data not shown).

MCT cells were also tested for an Fsp1 response after culturing on various extracellular matrix (ECM) molecules. Type I collagen was most effective in elevating Fsp1 expression by ELISA in MCT cells (Fig. 1*B*). The effects of EGF, TGF-β1, and type I collagen were also tested to observe the additive action of these Fsp1 inducers. The effects of cytokines EGF or TGF-β1 were not much different than the effects of type I collagen alone on the Fsp1 expression in MCT epithelium, and the inductive effect was not intensified when combined in coculture (Fig. 1*C*); the combination of type I collagen and EGF, however, seemed slightly more effective.

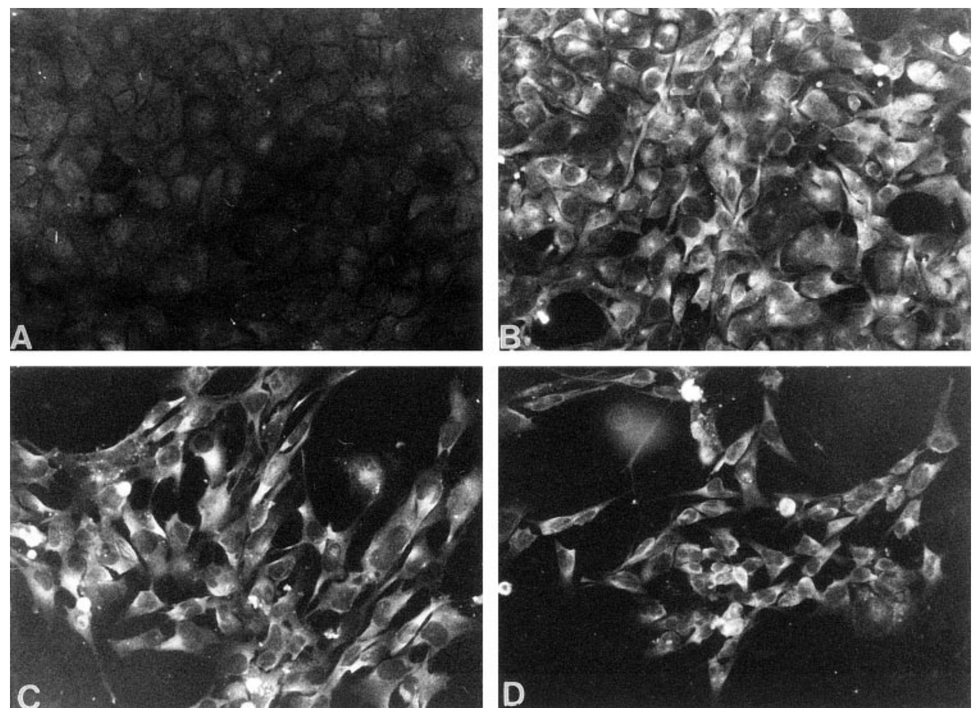

Fig. 3. De novo expression of Fsp1 of MCT detected by immunocytochemistry. *A*: control MCT are negative for Fsp1. *B*: MCT treated with EGF are strongly positive for Fsp1. *C* and *D*: MCT treated with TGF-β1 alone (*C*) and in combination with EGF (*D*) are also positive for Fsp1. Magnification for *A–D*, ×160.

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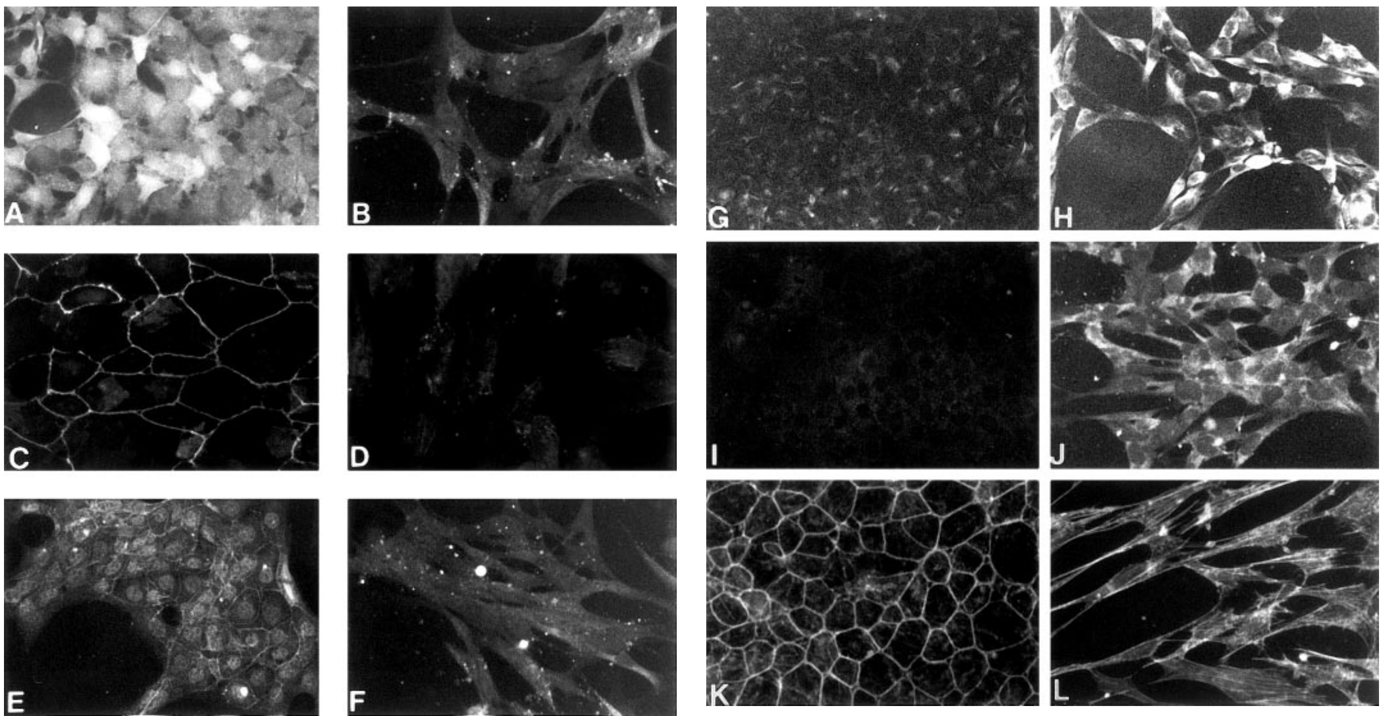

Fig. 4. Transdifferentiated MCT treated with EGF and TGF-β1 show reduced epithelial markers, increased mesenchymal markers, and reorganized F-actin bundles. *A*: control MCT are positive for cytokeratins in the cytoplasmic pattern. *B*: MCT treated with EGF and TGF-β1 lose cytokeratin expression. *C*: control MCT are positive for ZO-1 at their cell-cell boundaries. *D*: transdifferentiated MCT by EGF and TGF-β1 are negative for ZO-1. *E*: control MCT are positive for syndecan-1 at their cell-cell boundaries. *F*: treatment with EGF and TGF-β1 abolish syndecan-1 expression of MCT. *G*: control MCT are negative for vimentin. *H*: MCT treated with EGF and TGF-β1 are positive for vimentin. *I*: control MCT are negative for α-smooth muscle actin (α-SMA). *J*: treatment with EGF and TGF-β1 induce α-SMA in MCT. *K*: F-actin distributes at the cell-cell boundaries of control MCT. *L*: F-actin bundles reorganize into the stress fiber pattern in MCT treated with EGF and TGF-β1. Magnification for *A*–*L*, ×150.

EGF and TGF-β1 synergistically induce EMT in MCT epithelium. Although the addition of EGF (10 ng/ml) or TGF-β1 (3 ng/ml) alone to MCT cells in culture increased Fsp1, EMT-related morphological changes on light microscopy were not complete, as treated cells only showed a somewhat elongated appearance compared with controls (Fig. 2*A* vs. *B* and *C*). However, cotreatment of MCT cultures with EGF (10 ng/ml) and TGF-β1 (3 ng/ml) together produced a more obvious and consistent alteration in the shape of MCT cells, resulting in elongated, spindle-shapes characteristic of fibroblasts (Fig. 2*D*). All these alteration in morphology, even partial changes, accompanied the de novo expression of Fsp1 (Fig. 3, *A*–*D*). The EMT effect of peak doses of EGF (10 ng/ml) or TGF-β1 (3 ng/ml) did not produce more Fsp1, probably because the cytokine doses had been titrated to produce near maximal effects when used alone.

To further characterize the EMT of MCT cells following treatment with EGF and TGF-β1, we examined whether changes in other phenotypic markers besides cell shape correlated with morphological transformation; the two cytokines, EGF and TGF-β1, were used together, because alone they did not consistently pro-

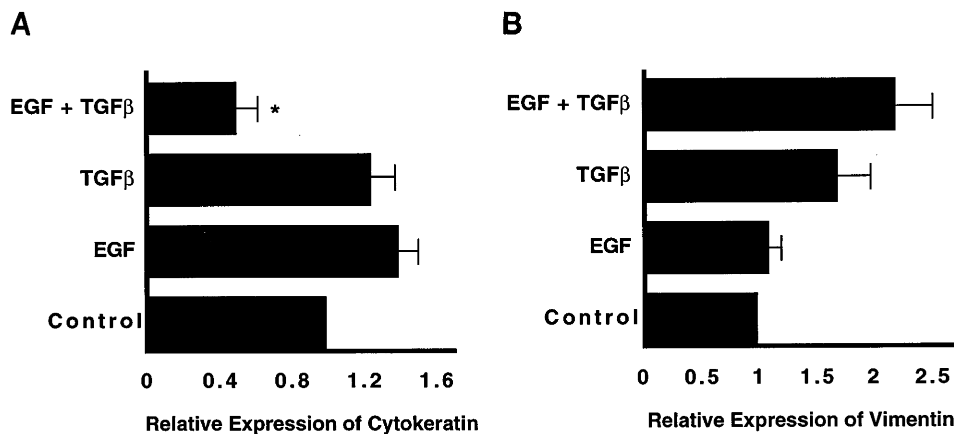

Fig. 5. Quantitative assay of phenotypic markers by direct ELISA. *A*: Effects of EMT related cytokines on cytokeratin expression of MCT. Treatment with EGF and TGF-β1 significantly reduce epithelial cytokeratin expression in MCT during EMT. *B*: TGF-β1 alone and in combination with EGF increases mesenchymal vimentin expression in MCT. * Statistically significant, $P < 0.05$.

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duce predicted changes. After exposure to cytokine, we stained MCT cells for the expression of cytokeratins, ZO-1, and syndecan-1 as epithelial markers using immunocytochemistry, and vimentin, α-SMA, and the intracellular distribution pattern of F-actin as mesenchymal markers.

The expression of cytokeratins were observed in control MCT cells and cells treated with EGF, but MCT cells treated with EGF and TGF-β1 were negative for cytokeratins (Fig. 4, *A* and *B*). MCT cultures treated with TGF-β1 alone contained cytokeratin-positive cells, and these findings on immunocytochemistry were confirmed by direct ELISA (Fig. 5*A*). Control MCT cells were positive for ZO-1 and syndecan-1 (Fig. 4, *C* and *E*), which appeared as circumferential staining at the boundaries between neighboring cells. However, following treatment with TGF-β1 and EGF, staining for ZO-1 and syndecan-1 largely decreased in parallel with the loss of formation of cuboidal sheets of MCT cells during EMT (Fig. 4, *D* and *F*). The phenotypic transformation of MCT cells following treatment with TGF-β1 alone, again, was uneven and incomplete; residual immunostaining of ZO-1 and syndecan-1, for example, partially remained at the end of the culture period (data not shown). Quantitative analysis of syndecan-1 expression was also performed, and fluorescence-activated cell sorting (FACS) analysis indicates that the total cell surface expression of syndecan-1 was not changed by these treatment (data not shown).

Meanwhile, the expression of vimentin and α-SMA in MCT cells increased following treatment with TGF-β1 in combination with EGF (Fig. 4, *G* to *J*). Direct ELISA assays were also performed to analyze the level of expression of vimentin in MCT cells. These studies demonstrated that the level of expression of vimentin increased during EMT (Fig. 5*B*). In addition, F-actin is generally connected with zonula adherens in epithelial cells and was detectable as a continuous line at the cell-cell boundaries in the untreated MCT cells (Fig. 4*K*). Following treatment with TGF-β1 and EGF, F-actin was reorganized during EMT into longitudinal stress fibers (Fig. 4*L*). All the data regarding changes in phenotypic markers are summarized in Table 1.

Alteration in collagen synthesis during EMT. Fibroblasts are a major source of interstitial ECM (50), particularly collagen types I and III and to a much lesser extent type IV collagen; the collagen secretory profiles of cultured fibroblasts derived from different tissues are heterogeneous (76). In the present study, we examined the changes in the intracellular content of types I and IV collagen in MCT cells following EMT and compared the results to TFB fibroblasts. In Fig. 6, the collagen ratio of type I to type IV increased in the presence of TGF-β1; in data not shown, both increased, but type I increased more, whereas with EGF, type I decreased slightly relative to unchanged type IV content. Quantitatively, untreated MCT cells secrete more type IV than type I collagen (1, 32).

Fsp1 antisense oligomers can inhibit cell motility and phenotype. Fsp1 antisense oligomers suppressed de novo expression of Fsp1 protein in MCT cells treated

Table 1. *Summary of EMT conversion of MCT cells in culture*

EMT Phenotype	Control	EGF	TGF-β1	EGF + TGF-β1
Cell morphology				
Shape	Cuboidal	Elongated	Fusiform	Spindle
F-actin distribution	Boundary	Boundary	Stress fiber	Stress fiber
Epithelial markers				
Cytokeratins	+	+	±	−
ZO-1	+	+	±	−
Syndecan-1	+	+	−	−
Mesenchymal markers				
Fsp1	−	+	+	+
Vimentin	−	−	+	+
α-SMA	−	−	+	+

EMT, epithelial-mesenchymal transformations (+, strong; ±, weak; −, negative); MCT, renal proximal tubular epithelial cells; EGF, epidermal growth factor; TGF, transforming growth factor; α-SMA, α-smooth muscle actin.

with EGF and TGF-β1, but sense and mismatch oligomers were without effect (Fig. 7). Trypan blue exclusion assay showed no measurable toxicity (data not shown). EGF and TGF-β1 also induced EMT morphology, decreased cytokeratin proteins, and increased type I collagen synthesis in MCT cells treated with sense or mismatch oligomers but not with antisense (Fig. 8). Finally, since mesenchymal cells are typically motile while epithelium is not, we examined the effect of these Fsp1 oligomers in a scratch motility study. In this experiment, control MCT epithelium did not move into the cell-free region of the slide during a 12-h interval, regardless of the presence of any oligomers (Fig. 9, *A* and *B*). MCT cells induced toward EMT with EGF and TGF-β1 migrated a visible distance over 12 h even in the presence of sense oligomers (Fig. 9, *C* and *D*) or mismatch oligomers (data not shown); however, migration was substantially attenu-

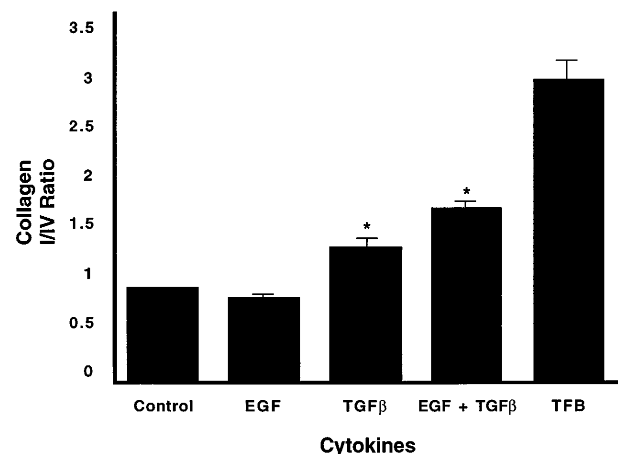

Fig. 6. Quantitative assay of collagen synthesis by direct ELISA. Relative secretion of types I and IV collagen in MCT cells was determined after stimulation of cultures by EGF and TGF-β1 alone or in combination. Combination of EGF and TGF-β1 best shifts the ratio of collagens toward the profile observed in TFB cells. *Statistically significant, $P < 0.05$.

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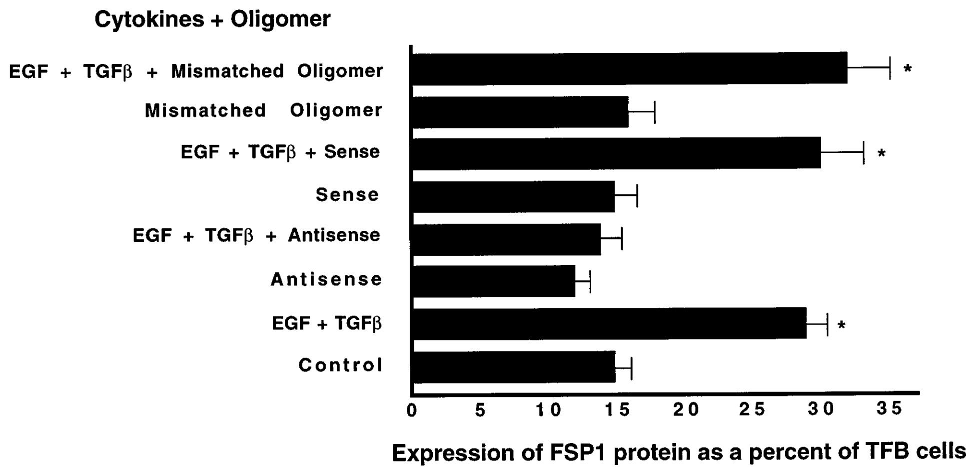


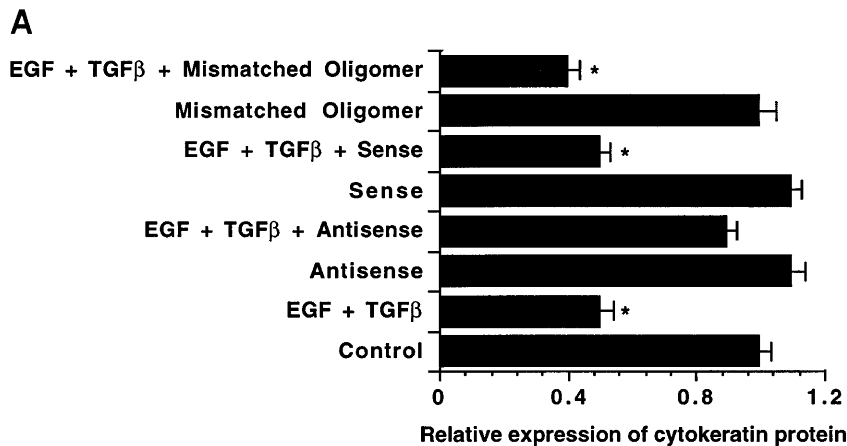
Fig. 7. Effects of Fsp1 antisense oligomers on Fsp1 protein expression. Fsp1 protein expression was determined with cell lysates by direct ELISA assay. Significant increases in Fsp1 expression by EGF and TGF-β1 treatment were observed in control MCT epithelium, MCT cells treated with Fsp1 sense oligomers, and MCT cells treated with mismatch oligomers. In contrast, de novo expression of Fsp1 was blocked by antisense oligomers. *Statistically significant, $P < 0.05$.

ated in the presence of Fsp1 antisense oligomers (Fig. 9, *E* and *F*).

DISCUSSION

We have begun to identify the conversion drivers of EMT in epithelium obtained from adult organ tissue. One of the markers of fibroblast formation is the expression of Fsp1, a 10-kDa cytoskeletal protein belonging to the calmodulin-S100-troponin C superfamily of intracellular calcium binding proteins associated with cytoskeletal fibers, cell motility, and a mesenchymal phenotype (93). We initiated the current study to better describe the early role of Fsp1 in EMT.

The process of EMT begins with destabilization of the differentiated state of candidate epithelium. The loss of epithelial adhesion properties is an early permissive event (46, 55) that is followed by the engagement of promoter factors that signal the transformation to



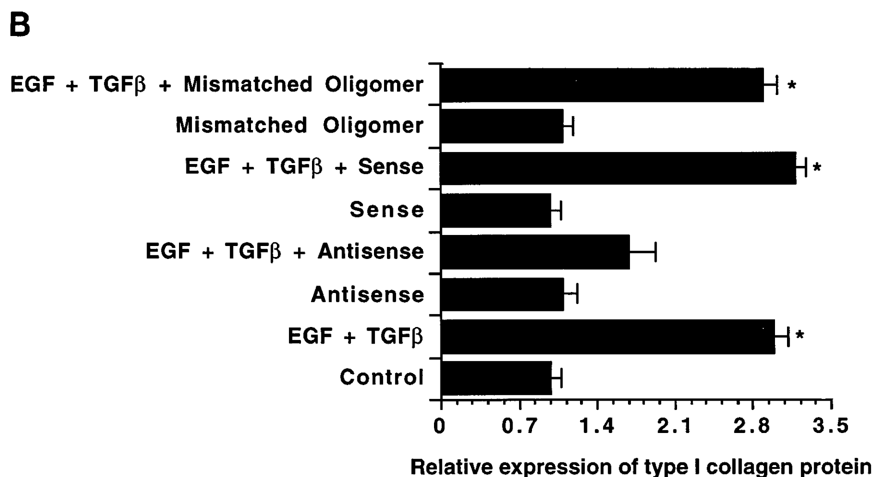


Fig. 8. Effects of Fsp1 antisense oligomers on cytokeratin and collagen type I expression in MCT cells induced by coculture with EGF and TGF-β1. Relative expression of cytokeratin and collagen type I was determined by direct ELISA assay in a representative experiment. *A*: expected levels of native cytokeratin expression were observed in control MCT epithelium, MCT cells treated with Fsp1 sense, antisense, or mismatch oligomers. In contrast, native expression of cytokeratin was inhibited by treatment with EGF and TGF-β1 and EGF and TGF-β1 plus sense or mismatched oligomers but not by EGF and TGF-β1 plus antisense oligomers. *B*: expected increases in collagen type I expression following treatment with EGF and TGF-β1 were observed in MCT epithelium and in MCT cells cotreated either with Fsp1 sense oligomers or mismatch oligomers. In contrast, more expression of collagen type I by treatment with EGF and TGF-β1 could be blocked by coincubation with antisense but not sense or mismatched oligomers. *Statistically significant, $P < 0.05$.

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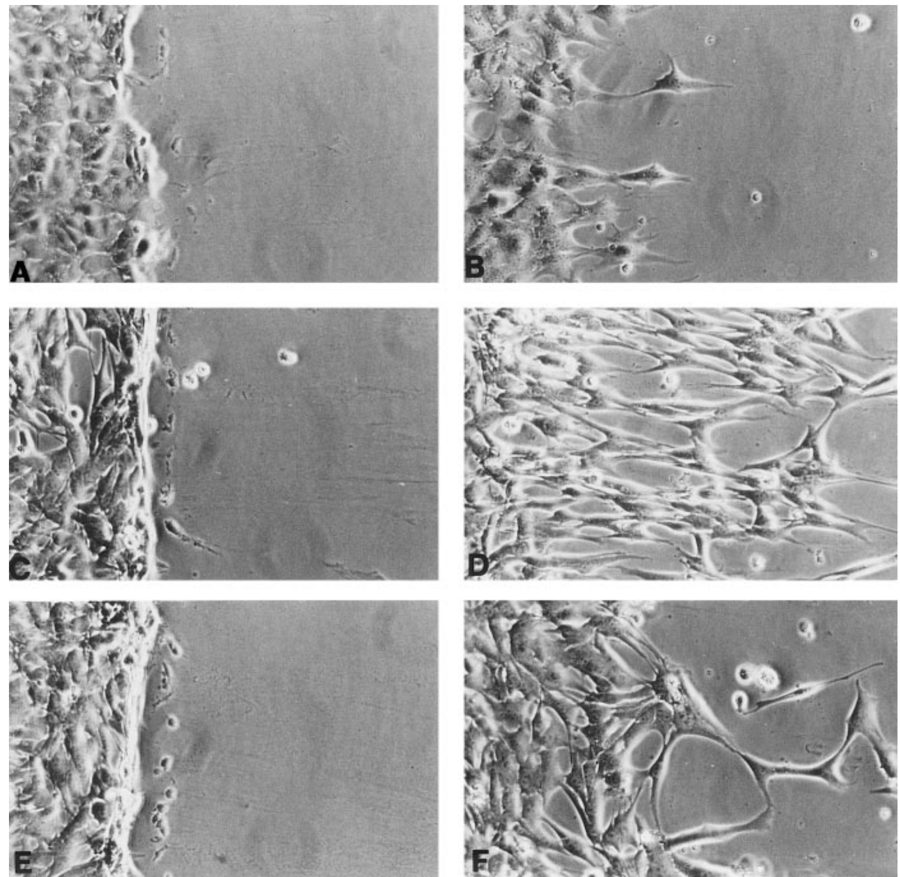

Fig. 9. Effects of Fsp1 antisense oligomers on cell motility induced by EGF and TGF-β1 treatment. *A* and *B*: MCT epithelium treated with Fsp1 sense oligomers barely moved in 12 h (*A*, 0 h; *B*-12 h). *C* and *D*: MCT cells transformed with EGF and TGF-β1 and treated with Fsp1 sense oligomers moved from their starting place (*C*, 0 h; *D*, 12 h). *E* and *F*: movement of MCT cells transformed with TGF-β1 and EGF was inhibited when the cells were treated with Fsp1 antisense oligomers (*E*, 0 h; *F*, 12 h). For *A*–*F*, all magnifications are ×100.

completion (36). Inducers of EMT have been examined in other systems. EGF (60) and TGF-β1 (61), for example, have shown some special promise. Other growth factors like TGF-α (24), MIF (86), acidic FGF (9), and a commercial serum substitute (10) have also been shown to induce EMT. These initiators of normal transformation trigger second-order signals that involve selected oncogenes, like v-*src*, (7) v-*ras*, v-*mos* (6), and c-*fos* (74); uncontrolled expression of these proteins facilitates oncogenesis (73) and may relate to the mesenchymal characteristics of some tumors. Furthermore, type I collagen gel can induce cultured epithelium to lose cell polarity and become fusiform in shape (95), and we have previously shown that renal epithelial cells submerged in type I collagen 3D gels lost cytokeratin expression while acquiring Fsp1 (77).

From among many combinations of cytokines tested in our experiments, no one cytokine alone induced all characteristics of EMT in MCT epithelium. EGF alone did promote proliferation and an elongated shape in MCT cells, probably due to de novo expression of Fsp1. In other systems, immortalized mammary epithelial cell lines can transiently lose epithelial markers, desmoplakins, and become motile by EGF treatment (60). In our studies, however, the transformation was incomplete, as EGF-treated MCT cells were still rich in epithelial markers (20). The actions of EGF on MCT cells also seemed different from those of HGF, another potent epithelial cell growth factor that stimulates epithelial tubulogenesis and branching morphogenesis in vitro (78). HGF, in particular, failed to increase expression of Fsp1 in MCT cells. All this is consistent with the in vitro observation that EGF can facilitate the phenotypic drift toward stromal cells (89), and, if anything, HGF encourages the opposite effect (45).

TGF-β1 also appears to be one of the principal initiating factors for EMT (38, 61, 71). MCT epithelium treated with TGF-β1 became fusiform in shape and rich in mesenchymal markers including Fsp1. Even though several mesenchymal markers appear in MCT epithelium treated with TGF-β1, the response toward EMT also did not go to completion because the cells still expressed abundant epithelial markers, like cytokeratins and ZO-1, and continued synthesize type IV collagen. It clearly induces EMT in mammary epithelium (61), but in the case of renal epithelium, EMT initiated by TGF-β1 alone is not entirely sufficient (12, 38, 39, 61).

Because single cytokine effects were incomplete inducers of EMT, we also examined cytokine combinations. Combined treatment with EGF and TGF-β1 could fully effect EMT and together were strong inducers of Fsp1 protein and a mesenchymal phenotype, suggesting in renal epithelium that EGF and TGF-β1 compensate for each other and synergistically promote the transformation of epithelium.

Some interactions between EGF and TGF-β1 on epithelial biology have been published (38, 39, 42, 47), but they mostly focus on their conflicting effects on epithelial cell growth. The mechanism of the antiprolif-

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erative effect of TGF-β1 on EGF relates to inhibition of c-*myc* transcription (69), replacement of EGF receptor with lower affinity receptors, p53-mediated G_1 cell cycle arrest (42), or induction of ECM molecules that can affect target cell responses (90). The effect of TGF-β1 on Fsp1 in our experiments, for example, can be connected through new synthesis of type I collagen, which in turn stimulates the production of Fsp1. Both EGF and TGF-β1 can also induce the appearance of new forms of the adenosine 3′,5′-cyclic monophosphate response element binding proteins (CREB) in renal epithelial cells (2), which may explain the additive effect of those factors, if CREB is involved in transcriptional regulation of EMT-related molecules. TGF-β1 also increases EGF receptor mRNA in rat kidney fibroblasts and synergizes with EGF to stimulate growth in soft agar, a characteristic of the transformed phenotype (37). Finally, the antiproliferative effects of TGF-β on epithelium in culture is associated with apoptotic cell death, and this tendency is obviated by the presence of EGF (79). These observations suggest that collective interactions between these two cytokines may confer a stabilizing benefit or survival advantage to cells changing phenotype in a cytokine-rich environment. The threshold quantification of this effect is difficult, however, without absolute biological standards of measure.

MCT cells induced to undergo EMT with EGF and TGF-β1 also demonstrate an increase in vimentin and α-SMA, a reorganization of F-actin into stress fibers, and an altered collagen synthesis profile showing more type I collagen synthesis than type IV collagen. In addition to the morphological change, immunochemistry and FACS analysis for syndecan-1, a substrate adhesion molecule, indicated a reversion of expression from a cell-cell boundary to global cell-surface boundary, possibly as a result of loss in cell polarity.

In our study, we observed that de novo expression of Fsp1 preceded the EMT phenotype and capacity for movement in MCT epithelium, as it could be blocked with Fsp1 antisense oligomers. This finding supports our previous observation that overexpression of cDNA encoding Fsp1 in epithelium could effect a mesenchymal phenotype (77). Acquiring the capacity for motility is one cardinal feature of a metastatic phenotype (50, 54), and the expression of Fsp1 appears emblematic of that process as well (29, 30, 68, 81). Fsp1 protein participates in motile events probably by the interaction with nonmuscle myosin II (23), nonmuscle tropomyosin (83), or actin (27, 82, 87) by which protrusion of lamellipods, detachment of the cell rear, and translocation of the cell body forward occur (54).

Finally, several lines of evidence also point to TGF-β1 as a key modulator of organ fibrosis following tissue injury (8, 19, 56, 66, 76) with supportive contributions from TGF-α (51), TNF-α (62), PDGF (84), and GM-CSF (91). The focus of this notion has emphasized the role of TGF-β1 in promoting the infiltration and activation of inflammatory cells and stimulating fibroblasts to proliferate or produce fibrogenic proteins. Little has been said, however, of the origin of these fibroblasts (76). Early de novo expression of vimentin (63) or Fsp1 (66, 77) has been reported in resident epithelial cells of nephritic kidneys. EMT initiators identified in this study, like EGF and TGF-β1, are also quite common in the nephritic kidneys (26, 92). Single cells or loosely organized small cell clusters still positive for epithelial markers can be found in the widened interstitium of the advanced or end-stage kidney (65).

These observations collectively suggest, as a hypothesis, that renal epithelium sitting on basement membrane damaged during the inflammatory phase of tubulointerstitial nephritis and exposed to TGF-β1 and EGF in that microenvironment begin expressing Fsp1 and "mesenchymalize" into fibroblasts. These fibroblasts further flood the interstitium with fibrotic collagen types I and III, and this in turn, sustains the transformation of more epithelium leading to tubular atrophy and progressive renal failure.

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Address for reprint requests: E. G. Neilson, C. Mahlon Kline Professor of Medicine, 700 Clinical Research Bldg., Univ. of Pennsylvania, 415 Curie Boulevard, Philadelphia, PA 19104-6144.

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