Smooth Muscle α-Actin Deficiency in Myofibroblasts Leads to Enhanced Renal Tissue Fibrosis*

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Masanobu Takeji†§, Toshiki Moriyama‡¶, Susumu Oseto§, Noritaka Kawada§, Masatsugu Hori‡¶, Enyu Imai¶, and Takeshi Miwa†

From the †Genome Information Research Center, Research Institute for Microbial Diseases, and ‡Department of Nephrology, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871 and ¶Health Care Center, Osaka University, Toyonaka, Osaka 560-0043, Japan

Myofibroblasts are a major source of proinflammatory cytokines and extracellular matrix in progressive tissue fibrosis leading to chronic organ failure. Myofibroblasts are characterized by de novo expression of smooth muscle α-actin (SMαA), which correlates with the extent of disease progression, although their exact role is unknown. In vitro cultured myofibroblasts from kidney of SMαA knock-out mice demonstrate significantly more prominent cell motility, proliferation, and type-I procollagen expression than those of wild-type myofibroblasts. These pro-fibrotic properties are suppressed by adenovirus-mediated SMαA re-expression, accompanied by down-regulation of focal adhesion proteins. In interstitial fibrosis model, tissue fibrosis area, proliferating interstitial cell number, and type-I procollagen expression are enhanced under SMαA deficiency. In mesangio proliferative glomerulonephritis model, cell proliferation in the mesangial area is also enhanced in SMαA knock-out mice. Adenoviral SMαA introduction into renal interstitium obviously ameliorates tissue fibrosis in interstitial fibrosis model. These results indicate that SMαA suppresses the pro-fibrotic properties of myofibroblasts, highlighting the significance of smooth muscle-related proteins in moderating chronic organ fibrosis under pathological conditions.

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†To whom correspondence should be addressed: Genome Information Research Center, Research Inst. for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-8369; Fax: 81-6-6879-8372; E-mail: miwa@gen-info.osaka-u.ac.jp.

‡The abbreviations used are: SM, smooth muscle; SMαA, smooth muscle α-actin; FA, focal adhesion; FAK, focal adhesion kinase; MT, Masson’s trichrome; UUO, unilateral ureteral obstruction; VSMC, vascular smooth muscle cell; FCS, fetal calf serum; HVGN, Habu-venom glomerulonephritis; which is physiologically expressed in vascular smooth muscle cells (VSMCs) and has the function of regulating vascular tone in cooperation with myosin. Myofibroblasts are a specific cell population that has both fibroblastic and SM-like properties, thought to be a source of inflammatory cytokines and extracellular matrix in diseased conditions of various organs. These properties of myofibroblasts are common regardless of the affected organs; however, origins of myofibroblasts are quite diverse: stellate cells in liver and pancreas (2, 3), interstitial fibroblasts in lung (4), glomerular mesangial cells (5), and renal interstitial fibroblasts (6). Recently, bone marrow-derived cells have been implicated as a substantial source of myofibroblasts in some organs (7–9). Epithelial-to-mesenchymal transition from tubular epithelial cells is also supposed as a pathway for renal interstitial myofibroblast generation (10). SMαA expression is the most well known characteristic and widely used marker of myofibroblasts. In vitro SMαA molecules incorporated into actin filaments have functions in contracting collagen gel (11) and retarding motility by increasing cell adhesion onto extracellular matrix (12). These contractile functions serve wound repair processes of skin or connective tissues by generating a closing force; however, for other visceral organs, such as lung, liver, and kidney, the role of their myogenic properties is not understood in pathological states.

We have investigated the transcriptional mechanism of SMαA gene (Acta2) in renal myofibroblasts (13, 14). Rodent unilateral ureteral obstruction (UUO), characterized by a diffuse and extensive emergence of renal interstitial myofibroblasts in a short period, initiated by a simple procedure (15), is a good model for studying tissue fibrosis. Using the UUO model, this study aims to elucidate the exact function of SMαA in myofibroblasts in the course of tissue fibrosis, using SMαA knock-out mice and the adenoviral SMαA gene transfer method. Its unanticipated importance against progressive tissue fibrosis is reported.

**EXPERIMENTAL PROCEDURES**

SMαA−/− Mice and Cultured Mesangial Cells—Wild-type (WT) and SMαA knock-out (SMαA−/−) mice were produced by mating SMαA heterozygous (SMαA+/−), 129-background mice generously provided by Dr. R. J. Schwartz (Baylor College of Medicine, cytomegalovirus; RT-PCR, reverse transcription PCR; WT, wild type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
of Medicine) (16). Handling and surgical manipulation of all experimental animals were carried out according to the guidelines of the Committee on the Use of Live Animals in Teaching and Research of Osaka University. Primary culture of mesangial cells was established from kidneys of these mice as described previously (17), maintained in RPMI 1640 medium with 17% FCS unless otherwise mentioned, and utilized as cultured myofibroblasts.

**Cell Proliferation and Migration Assay**—Cell proliferation was evaluated by determining viable cell number colorimetrically, using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega Corp., Madison, WI), generally according to the product instructions. 2 × 10² cells were seeded in each well of 96-well plates and incubated in 17% FCS-RPMI 1640 medium for 24 h, followed by 0.5% FCS incubation to stop their proliferation for 24 h. Medium was then replaced by fresh 10% FCS or 0.5% FCS RPMI 1640, and 24 h later absorbance at 490 nm was measured. Cell migration was evaluated by scrape wounding assay. Subconfluent cells on a 35-mm-diameter dish were scraped to make a straight-edged wound using a plastic tip, incubated in RPMI 1640 medium 0.5% FCS to avoid the effect of proliferation, and monitored periodically by light microscope.

**Mouse Models for Renal Interstitial Fibrosis and Mesangio-proliferative Glomerulonephritis**—Ad as a model for progressive renal interstitial fibrosis, UUO was performed in 8-week-old male WT and SMαA−/− mice. The operation procedure was as described previously (13). Briefly, mice were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital and inhalation of diethyl ether. After making a small ventral incision, the left ureter was identified and ligated. Kidney samples were harvested 2, 3, 7, or 14 days after operation (n = 5–7 each). Mesangio-proliferative glomerulonephritis was made by Habu-snake (Trimeresurus flavoviridis) venom injection (Habu-venom glomerulonephritis; HVGN) as described previously (14). Briefly, 8-week-old male mice were hemi-nephrectomized and received an intravenous injection of lyophilized venom from Habu-snake (Wako, Japan) dissolved in saline at 1.5 mg/kg body weight. Kidney samples were harvested 7 days after injection.

**Western Blot Analysis**—Cultured cells were scraped, suspended in lysis buffer consisting of 150 mM sucrose, 50 mM Tris-HCl, 50 mM NaF, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 mM EDTA, and 2 mM EGTA and then homogenized. Protein concentration was measured by the Lowry method. Five μg of protein/well was electrophoresed in 8 or 10% SDS-polyacrylamide gel and then transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). Blots were probed with primary antibody; mouse monoclonal anti-SM α-actin antibody (clone 1A4) (DAKO), anti-β actin antibody (clone AC15) (Sigma), anti-panxillin antibody (clone 5H11) (Lab Vision, Fremont, CA), anti-focal adhesion kinase (FAK) antibody (clone 4.47) (Upstate Biotech., Lake Placid, NY), or rabbit anti-actin antibody (Sigma), goat anti-vinculin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Tyr397-phospho-FAK antiserum (Upstate Biotechnology). Antibodies were reacted with a secondary antibody, horseradish peroxidase-linked goat anti-mouse immunoglobulin antibody, donkey anti-rabbit immunoglobulin antibody, or rabbit anti-goat immunoglobulin antibody (Amersham Biosciences), detected by chemiluminescence of ECL Plus Western blotting detection reagents (Amersham Biosciences), and then exposed to Hyperfilm.

**Adenovirus-mediated Gene Transfer**—Ad-mSMαA was developed generally according to the method of Graham and Prevec (18). Briefly, we first amplified murine SMαA cDNA (from bp +63 to bp +1268, fully including the translated region between bp +72 and bp +1205) using pCR-Blunt II-TOPO cloning vector (Invitrogen) and cloned downstream of the CMV promoter region of pACCMV.pLpA shuttle vector. Next, pACCMV.pLpA with SMαA cDNA inserted and pM17 vector (a replication-defective adenoviral genome vector with deleted E1 and mutated E3 promoter regions) were co-transfected into human embryonic kidney 293 cells to induce recombination to develop CMV promoter-driven SMαA cDNA-expressing adenoviral vector. The vector was then amplified and purified, and its infectivity was measured by plaque assay. When Ad-mSMαA was transfected into SMαA−/−-cultured myofibroblasts at a multiplicity of infection of 300 PFU/cell, more than 90% of cells expressed SMαA protein with no apparent cytotoxity. Another adenoviral vector, Ad-LacZ, was a generous gift from Dr. T. Mano (Osaka University) (19), used as a control for nonspecific effects of adenoviral infection. To transfer genes in vitro, subconfluent mesangial cells were rinsed twice with PBS(+), incubated with serum-free medium containing adenovector for 1 h at room temperature. After removing the adenoviral medium, cells were cultured in medium with 0.5% FCS for 7 days, and then RNA and cell lysates were collected. For in vivo gene transfer when operating for UUO, adenoviral vector solution (6 × 10² PFL in 50 μl of saline/individual kidney) was injected intraparenchymally with a 30-gauge needle separately at two points while clamping the renal artery and renal vein for 3 min to minimize adenoviral vector flowing immediately out of the kidney (n = 5–6 each).

**RNA Preparation and RT-PCR**—RNA from mouse kidneys and cultured cells was collected as described previously (14). Reverse transcription was performed using 1 μg of RNA, MuLV reverse transcriptase (Applied Biosystems, Foster City, CA), dNTP, and random hexamer. Sequences of primers for semi-quantitative PCR are indicated in Table 1A. Semi-quantitative
PCR was carried out with 1 μl of template cDNA, primers (10 pmol each), dNTP, and ExTaq DNA polymerase (Takara Bio Inc.) in a final volume of 20 μl. Thermal cycler conditions were as follows: denaturation at 95 °C for 45 s, annealing at 56–59 °C for 45 s, and extension at 72 °C for 1 min with appropriate cycle numbers. To perform real-time quantitative PCR, ABI PRISM 7700 Sequence Detection System (Applied Biosystems) was used with 50 μl of final volume containing 5 μl of template cDNA, solution, 10 pmol primers, labeled probe, 25 μl of Platinum Quantitative PCR Supermix-UDG (Invitrogen). PCR primer and probe sequences for real-time PCR are shown in Table 1, A and B. Thermal cycler conditions were 2 min at 50 °C, 10 min at 95 °C, and 50 cycles of 15 s at 95 °C, followed by 1 min at 60 °C.

RESULTS

Myogenic Gene Expression in SMαA<sup>−/−</sup> Myofibroblasts—SMαA<sup>−/−</sup> mice had no leaky expression of SMαA mRNA in all checked tissues, and primary kidney cells cultured by our method demonstrated positive staining for desmin, which is regarded as one of the markers for mesangial cells (data not shown). Cultured mesangial cells show general characteristics of myofibroblasts; therefore, they were used as cultured myofibroblasts for the following experiments. Cultured myofibroblasts from SMαA<sup>−/−</sup> mouse kidney had more sparse stress fibers in cytoplasm than WT, which seemed due to lack of SMαA (Fig. 1A). SMαA<sup>−/−</sup> myofibroblasts showed complete disappearance of SMαA expression but enhancement of other SM-related gene expression, including SM-γ-actin (SMγA), SM myosin heavy chain isoform 1 (SM1), and another actin isofrom, skeletal muscle α-actin (SkαA) (Fig. 1B). This result suggests the existence of some compensatory mechanism for maintaining SM phenotype and cellular actin content. Cysteine-rich LIM-only protein (CRP) 1 and myocardin, SM-specific transcriptional cofactors (20, 21), were not detected in either WT or SMαA<sup>−/−</sup> myofibroblasts by RT-PCR, whereas expression of CRP2 was observed in WT myofibroblasts but was down-regulated under SMαA deficiency (Fig. 1C).

Enhanced Interstitial Fibrosis and Mesangial Proliferation in Renal Disease Models of SMαA<sup>−/−</sup> Mice—There were no overt macroscopic or microscopic differences between WT and SMαA<sup>−/−</sup> kidney. Therefore, UUO, which causes artificial hydronephrosis and is the most common experimental model for renal interstitial fibrosis (15), was performed on both WT and SMαA<sup>−/−</sup> mice. For WT kidney under physiological conditions, SMαA immunostaining was only seen in VSMCs of blood vessels but was newly observed in renal interstitium on UUO day 7, indicating the emergence of myofibroblasts (Fig. 2A). In contrast, no SMαA immunostaining was seen in either
physiological or UUO kidneys of SMαA<sup>−/−</sup> mice. The proportion of fibrosis area calculated from the blue-stained area of MT staining was more extensive in SMαA<sup>−/−</sup> mice than WT mice (Fig. 2, A and B). Interstitial proliferating cells estimated from proliferating cell nuclear antigen (PCNA)-positive cell count in UUO kidney on day 7 was significantly higher in SMαA<sup>−/−</sup> mice than WT mice (Fig. 2C). In fibrotic tissue, type-I collagen is the main extracellular matrix protein, composed of procollagen α1(I) and α2(I) chains processed from the products of genes Col1a1 and Col1a2. In the UUO model, expression of type-I procollagen (α2) mRNA increased with time. Type-I procollagen (α2) mRNA in SMαA<sup>−/−</sup> mouse UUO kidney on days 7 and 14 was raised to about twice the level found in WT UUO kidney (Fig. 2D). These results demonstrate that SMαA deficiency promotes the increase in myofibroblasts and progression of renal interstitial fibrosis.

Next, we performed HVGN to see the difference in the progression of glomerulonephritis between SMαA<sup>−/−</sup> and WT mice. In HVGN, normal mesangial cells transdifferentiate into SMαA-positive myofibroblasts and exhibit prominent cell proliferation (14). Glomerular mesangial cell number was significantly more increased in SMαA<sup>−/−</sup> mice than WT mice (39.3 ± 5.0 versus 31.7 ± 5.3) (Fig. 3). Therefore, SMαA deficiency also promotes progression of mesangioproliferative glomerulonephritis.

**Myofibroblast Activity Is Diminished by Forced SMαA Re-expression**—Both WT and SMαA<sup>−/−</sup> cultured myofibroblasts show no evidence of proliferation in 0.5% FCS RPMI
medium. SMαA−/− myofibroblasts showed more vigorous proliferating activity than WT cells (153 ± 28% versus 117 ± 23%) after 10% FCS stimulation for 24 h compared with incubation without stimulation, as measured by formazan colorimetric assay. Their enhanced proliferation was blunted to a degree comparable with WT cells with forced SMαA re-expression by transfection of adenoviral vector (Ad-mSMαA), which effectively expresses murine SMαA downstream of the CMV promoter (Fig. 4A). Type-I procollagen (α2) mRNA expression was enhanced in SMαA−/− cultured myofibroblasts 2.2-fold more than in WT cells (Fig. 4B), consistent with in vivo UUO data. When SMαA was re-expressed in SMαA−/− myofibroblasts, type-I procollagen mRNA expression was reduced to a similar extent as in WT myofibroblasts.

Migration is an important property of active myofibroblast. In wound scrape assay, SMαA−/− cultured myofibroblasts also demonstrated more enhanced migration than quite slow migration of WT cells in minimum (0.5%) FCS medium, used to avoid effects from cell division. Forced re-expression of SMαA in SMαA−/− myofibroblasts slowed their enhanced migration (Fig. 4C). Enhanced myofibroblast migration is related to accelerated tissue fibrosis in several organs (22, 23) although the relation is still not shown in kidney UUO model. It seems that enhanced migration accelerates the accumulation of myofibroblasts to the inflammation site in kidney. Ad-LacZ, an adenoviral vector expressing β-galactosidase from the same CMV promoter, was used as a control for adenoviral infection. Transfection of Ad-LacZ into myofibroblasts at the same multiplicity of infection as Ad-mSMαA had no significant influence on their proliferation, type-I procollagen mRNA expression, or migration. These results indicate that expression of SMαA affects suppression of proliferation, procollagen synthesis, and migration of myofibroblasts.

**FIGURE 4. Cell proliferation and collagen synthesis are enhanced under deficiency of SMαA in myofibroblasts.** A, proliferation rate measured by formazan colorimetric assay. Ratios of A490 of cells incubated for 24 h in 17% FCS to those incubated in 0.5% FCS were calculated and indicated as percentage. Data are indicated as mean ± S.D. *, p < 0.05 versus WT; **, p < 0.01 versus SMαA−/− control. B, type-I procollagen mRNA of cultured myofibroblasts quantified by real-time PCR normalized by ratio to GAPDH mRNA. Data are indicated as mean ± S.D. *, p < 0.05 versus WT; **, p < 0.01 versus SMαA−/− control. C, wound scrape assay of cultured myofibroblasts showing enhanced cell motility in SMαA deficiency. Cells were scraped using a plastic tip and incubated for 7 days in 0.5% FCS medium. Upper photographs are at the time of scraping, and the lower photographs are 7 days after scraping. Scale bar, 200 μm.
clearly diminished. In these conditions, there was not a large difference in total actin proteins. These findings suggest that SMαA expression in myofibroblasts down-regulates cellular FA protein contents.

In Vivo Adenoviral SMαA Gene Transfer Ameliorates Renal Fibrosis—As the above in vitro results suggested the possibility of ameliorating tissue fibrosis by forced SMαA expression, Ad-mSMαA was introduced into mouse kidney and its effect on interstitial fibrosis was examined. Intraparenchymal Ad-mSMαA injection induced SMαA expression in interstitial fibroblasts of SMαA/−/− UUO kidneys as early as day 2, although WT non-injected UUO kidneys had no SMαA staining in the interstitium on the same day (Fig. 6A). SMαA expression induced by Ad-mSMαA was observed to persist until day 7 in SMαA−/− UUO kidneys (Fig. 6B).

Analysis of the extent of renal disease progression for SMαA−/− UUO kidneys with forced SMαA re-expression demonstrated a significantly diminished interstitial fibrosis area and type-I procollagen mRNA expression in renal tissue to almost half the level of non-injected UUO kidneys. Also, SMαA overexpression in WT UUO kidneys ameliorated interstitial fibrosis, and type-I procollagen mRNA expression in renal tissue was reduced to almost one-third of non-injected UUO kidneys (Fig. 6, D and E). Collectively, this amelioration of tissue fibrosis appears attributable to suppression of myofibroblast expansion and collagen synthesis by forced SMαA expression.

Interestingly, Ad-mSMαA-transfected WT kidneys 7 days after UUO showed paradoxically less total SMαA-positive area and SMαA mRNA expression compared with both non-injected and Ad-LacZ-injected WT UUO kidneys (Fig. 6, B and C). The CMV promoter in Ad-mSMαA vector drives SMαA gene expression strongly and constitutively, whereas the proper promoter in SMαA gene shows spatiotemporally regulated gene activation. The earlier expression of SMαA forced by Ad-mSMαA was observed compared with non-transfected WT UUO kidneys (Fig. 6A) that may have suppressed expansion and pro-fibrotic activity in interstitial fibroblasts.
DISCUSSION

Myofibroblasts, which express SMαA and other SM-related proteins, appear in various pathological states, including connective tissue granulation, fibrotic diseases, and stroma reaction to neoplasia (1); however, little is known about the role of SMαA in these conditions. In fibrotic diseases of several organs, SMαA expression of myofibroblasts is recognized as a hallmark of their emergence and an indicator of disease severity (1, 26). However, the role of SM-related proteins in the pathogenesis of fibrosis has been poorly elucidated. The present study demonstrates that SMαA deficiency enhances the progression of kidney diseases in two models, interstitial fibrosis (UUO) and mesangioproliferative glomerulonephritis (HVGN) (Figs. 2 and 3), suggesting the role of SMαA in myofibroblast common in both mesangial and interstitial lesions. Tissue fibrosis is ameliorated by forced expression of SMαA in renal interstitial myofibroblasts.

Recently, reports have shown that contractile, plus proliferative and/or collagen-producing, properties do not coincide or are differentially exhibited in myofibroblasts under certain conditions (27, 28). VSMCs, which physiologically express abundant SM contracton-related proteins including SMαA, modulate their phenotype from “contractile type” to “synthetic type” under pathological conditions such as atherosclerotic lesions (29). To date, several transcription factors related to VSMC differentiation have been demonstrated to inhibit VSMC proliferation, migration, and progression of arteriosclerosis (19, 30), suggesting that the contractile properties are distinct from proliferative, migratory, and synthetic properties. In addition, SMαA promoter has a target sequence of tumor suppressor protein p53 (31), and SMαA gene introduction into transformed fibroblasts reduces their proliferative properties (32), although the underlying mechanism has not been elucidated. Taking these findings together, we propose that the SM-like phenotype and productive phenotype are mutually opposed within myofibroblasts and that SMαA per se has the function of maintaining a cell in a static state.

FA complex consists of paxillin, vinculin, talin, plasma membrane integrins, and FAK and is linked to cytoplasmic actin fibers (24). Several stimuli affecting integrin or changes in actin fiber status induce phosphorylation at several tyrosine residues of FAK, inducing active cell motility, cell cycle promotion, and protein (25, 33). Recent reports indicate the importance of FAK in the regulation of myofibroblast activation and collagen synthesis (34, 35). Our results, decreasing of FA complex in myofibroblasts by SMαA expression, suggest the relationship between SMαA in actin stress fibers and FA. We think additional investigations about protein-protein interaction between SMαA and FA complex are necessary.

Our results show no evident difference in other important receptor kinases for myofibroblast activity such as transforming growth factor β receptor-type-I and platelet-derived growth factor β-receptor (data not shown). In addition, Rho-GTPases are important downstream effectors of FAK on cell motility (36). Our previous report demonstrated that inhibition of Rho-associated kinase attenuated renal interstitial fibrosis in the UUO model (37), suggesting the importance of this pathway in the pathogenesis of fibrosis.

Recent evidence has emerged indicating that actin molecules are abundant in the nucleus and perform functions in chromatin remodeling and gene activation (38, 39). SMαA is reported to be a binding target of antioxidant-responsive element present in some genes (40), although it is still unclear whether SMαA exists and has a specific function in the nucleus. This mechanism might also take part in the type of gene expression and proliferation reported here.

SMαA−/− myofibroblasts interestingly demonstrated up-regulation of miRNA of other SM-related proteins and another myogenic isoform of actin, such as SMγA, SM1, and SkαA, presumably to compensate for the lack of SMαA. Similar compensatory expression systems have been reported in cardiomyocytes of cardiac α-actin knock-out mice (41) and in skeletal muscle of SkαA knock-out mice (42), although the underlying transcriptional mechanisms have not been clarified. In addition, from the present findings expression of CRP1, CRP2, and myocardin, transcriptional co-factors for SM-specific gene expression in VSMCs (20, 21), was not detected or up-regulated in SMαA−/− myofibroblasts (Fig. 1C); therefore, they do not seem responsible for the compensatory SM gene expression in these cells. These results suggest that different signaling systems from VSMCs predominate for other SM-related protein expression in myofibroblasts.

The limitation in this experiment is the usage of somewhat conventional knock-out mice and an adenoviral gene transfer system. SMαA−/− mice physiologically exhibit lower blood pressure and poor responsiveness of arteries to vasoconstrictor agents (16). As fibrosis in the UUO kidney is triggered by vasoconstriction and a decrease in renal blood flow (15), plus is ameliorated by several blood pressure-lowering agents (43, 44), these characteristics appear unlikely to contribute to renal fibrosis progression. It is also possible that the effect of SMαA deficiency is modulated by compensatory up-regulation of other SM-related proteins. However, this up-regulation seems not to contribute to the modulation of disease course, as SMαA gene transfer into UUO kidneys significantly ameliorated interstitial fibrosis in both WT and SMαA−/− mice. These results suggest a pivotal role for SMαA but not for other SM-related proteins. Inflammation evoked by adenovirus infection is reported as an important problem (45); however, there have been no reports on adenoviral vector-mediated fibrosis in mouse kidney and no apparent differences in mRNA expression levels of pro-inflammatory mediators such as transforming growth factor β1 and monocyte chemoattractant protein-1 between non-injected and Ad-LacZ-injected UUO kidneys (data not shown). Of note, the present study showed that forced SMαA expression ameliorates tissue fibrosis in a kidney UUO model. However, in some pathological conditions such as contraction of skin scars, contraction force generated by SMαA plays a certain role in disease deterioration. Those may be beneficially influenced by SMαA deficiency. Appropriateness of SMαA introduction needs to be considered on an individual disease basis.

In conclusion, SMαA in myofibroblasts appears to have a suppressing role in tissue fibrosis progression, demonstrated by
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both loss-of-function and gain-of-function analyses. These findings suggest several novel therapeutic approaches to myofibroblast-related fibrotic diseases: for example, enhancement of SMαA expression by gene transfer methods or by up-regulation of SM-related transcription factors.

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