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Significance of α-SMA in myofibroblasts emerging in renal tubulointerstitial fibrosis

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Summary. Myofibroblast transdifferentiation plays a crucial role in the development and progression of renal tubulointerstitial fibrosis. However, the significance of α-smooth muscle actin (α-SMA) expression, which is the major morphological characteristic of myofibroblasts, remains to be determined in detail. The effect of α-SMA expression on fibrosis tissue was examined by using a fibrosis model (collagen gel) *in vitro*. The transdifferentiation of fibroblasts into myofibroblasts was triggered in the culture medium with 0.5% fetal bovine serum (FBS)+transforming growth factor (TGF)-ß1, but not with 10% FBS+TGF-ß1. The TGF-ß1-induced gel contraction caused by myofibroblasts was greater than that by fibroblasts. Gel contraction by myofibroblasts involved the Ca^{2+}-dependent myosin light chain kinase pathway, as well as the activation of Rho kinase and p38 mitogen-activated protein kinase (MAPK). Taken together, these findings suggest that α-SMA expression in renal interstitial fibroblasts, i.e., myofibroblast transdifferentiation, accelerates the contraction of the tubulointerstitial fibrosis tissue via the Ca^{2+}-dependent pathway, in addition to the pathways involved in fibroblast contraction; this event may lead to renal atrophy and renal failure.

Key words: Renal fibrosis, Myofibroblast, α-smooth muscle actin, Transforming growth factor-ß1, Gel contraction

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Introduction

The number of cases in which hemodialysis therapy for renal fibrosis, e.g., diabetic nephropathy, is introduced has been increasing. Currently, the therapeutic purpose in renal fibrosis is not only to prevent development and progression of the disease, but also to promote its remission and regression.

Tubulointerstitial fibrosis of kidney diseases was closely correlated with a progressive decline in renal function (Bohle et al., 1991; Lane et al., 1993). The mechanism by which tubulointerstitial fibrosis occurs has been demonstrated as follows. TGF-ß1, which is a profibrogenic cytokine, is overexpressed in the interstitial cells and proximal tubular epithelial cells. Then, the interstitial cells, as well as tubular epithelial cells transdifferentiate into myofibroblasts in response to the cytokine (Fan et al., 1999). The transdifferentiation brings about the condition which leads to the development of fibrosis. Myofibroblasts, referred to as activated fibroblasts, overproduce the extracellular matrix (ECM) in response to TGF-ß1, and also increase the production of plasminogen activator inhibitor-1 and the tissue inhibitor of metalloproteinase, which attenuate the degradation of the ECM followed by its accumulation, especially of collagen fibers (Edwards et al., 1987; Laiho et al., 1987).

Although α-SMA expression, which is the most pronounced characteristic of myofibroblasts, has been considered to cause tissue contraction, the mechanism has not yet been determined in detail. Myofibroblasts were first described in wound-healing granulation tissue (Gabbiani et al., 1971). They were referred to as "modified fibroblasts" on the basis of ultrastructural identification in transmission electron microscopic studies. They were characterized as having constituents implicated in cell contraction followed by tissue

contraction, as well as containing a well-developed rough endoplasmic reticulum (RER) and a great number of mitochondria, just as in fibroblasts. They include a fibrillar system corresponding to stress fibers, nuclear indentation implying cell contraction, and cell-to-ECM and cell-to-cell junctions. Thus, modified fibroblasts (myofibroblasts) were described as an intermediate type of cell between fibroblasts and smooth muscle cells due to their ability to produce an ECM and contract. Later investigators have noted that α-SMA expression in the stress fibers was the major characteristic of myofibroblasts, since six actin isoforms have been isolated (Rayan and Tomasek, 1994; Tomasek and Layan, 1995). Now, cells with α-SMA-positive stress fibers in the interstitium, except for the vascular components, are recognized to be myofibroblasts. In vertebrate tissues, six actin isoforms can be differentiated based on differences in acidity and amino acid sequence. Four actin isoforms, including the α-skeletal, α-cardiac, α-vascular and γ-enteric isoforms, are tissue-restricted and involved in cell contraction. Two other actin isoforms are ubiquitous: the ß-cytoplasmic and γ-cytoplasmic isoforms. These have been considered to constitute noncontractile cytoskeletons. α-vascular actin is the SMA isoform, referred to as "α-SMA". In myofibroblast transformation, the actin isoform transits, at least in part, from the cytosolic actin to α-SMA. It has been proposed that myofibroblasts have the ability to contract due to α-SMA. On the other hand, although fibroblasts express cytosolic actins exclusively, they also show cellular contraction activity.

What is the difference in contraction between fibroblasts and myofibroblasts? Both vascular smooth muscle cells (VSMCs) and non-muscle cells contract through the phosphorylation of the myosin regulatory light chain (MLC) in response to various stimuli (Taylor and Stull, 1988; Parizi et al., 2000; Ina et al., 2007). In VSMCs, the increased level of intracellular Ca^{2+} plays a dominant role in contraction. An increase in the level of intracellular Ca^{2+} activates Ca^{2+}-dependent MLC kinase, followed by the phosphorylation of MLC (Taylor and Stull, 1988). In fibroblasts, cell contraction predominantly occurs in a Ca^{2+}-independent manner (Kimura et al., 1996). MLC is phosphorylated by Rho kinase in these cells, independently of MLC kinase, as we previously described (Ina et al., 2007). The mechanism of myofibroblast contraction remains to be determined.

The origins of myofibroblasts in renal fibrosis have been demonstrated to be the interstitial fibroblasts and the epithelial cells (Simonson, 2007). Recently, bone marrow-derived progenitors, circulating fibrocytes, were shown to transdifferentiate into myofibroblasts in post-ischemic renal interstitium (Martine et al., 2007). Moreover, adult stem cells in the kidney are also possible candidates. The contribution rate of these original cells to myofibroblast transdifferentiation remains unknown in detail. In the current study we used interstitial fibroblasts, because fibroblasts are possibly easier to transdifferentiate than epithelial cells due to intradermal transdifferentiation.

The present study demonstrates the mechanism of fibrosis tissue contraction accompanying α-SMA expression by using myofibroblasts originating from renal fibroblasts (NRK 49F cells); they were populated on a collagen lattice (Grinnell, 1994) as an *in vitro* model of fibrosis tissue (Miura et al., 2006).

Materials and methods

Materials

The following materials and chemicals were purchased: porcine type-I collagen solution (Nitta Gelatin, Osaka, Japan), recombinant human TGF-ß1 (R and D Systems, MN), the specific Rho kinase inhibitor Y27632 (Calbiochem, CA), hydroxyfasudil (Sigma-Aldrich, MO), a potent and selective Rho kinase inhibitor, p38 MAPK inhibitor SB203580 (A.G. Scientific, Inc, CA), p44/42 MAPK kinase inhibitor PD98059 (Calbiochem), phosphatidyl- inositol 3-kinase (PI3-K) inhibitor wortmannin (Upstate, CA), calmodulin antagonist N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide-HCl (W-7; Alexis Biochemicals, CA), and myosin light chain kinase inhibitor 1-(5-iodonaphthalene- 1-sulfonyl)-1H- hexahydro- 1,4-diazepine-HCl (ML-7; Sigma-Aldrich). Rhodamine-isothiocyanate (RITC)-conjugated phalloidin (Cytoskeleton, CO) was used to stain the actin filaments. Mouse monoclonal antibody against α-SMA or vinculin, which is one of the constituents of focal adhesion, was obtained from Sigma-Aldrich. Fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse IgG secondary antibody against these monoclonal antibodies was purchased from Caltag Laboratories (CA).

Cell culture

Normal rat kidney fibroblasts (NRK 49F cells) were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in Dulbecco's minimal essential medium (D-MEM) (Nissui Pharmaceutical, Tokyo, Japan) containing 100 IU ml^{-1}penicillin, 100 μg ml^{-1} streptomycin, and 10% FBS (JRH Biosciences, KS) at 37°C in a humidified, 5% CO_2 atmosphere. FBS had been heat-inactivated at 56°C for 30 min prior to use. Cells from passage 3 to passage 8 were used in the experiments described below. Rat aortic smooth muscle cells were isolated as previously described (Diglio et al., 1989). Briefly, the full length of the thoracic aorta was removed and placed in Hanks' balanced salt solution (HBSS) without $Ca^{2+}$and Mg^{2+}. Then, the adherent periadventitial fat and connective tissue were removed using fine forceps. The aorta was irrigated with HBSS to remove the blood elements. The remaining aorta was then cut into ring segments (1- to 1.5-mm thick) using a sharp scalpel blade. The ring explants were placed in D-

MEM+10% FBS. In order to establish pure endothelial cell cultures, the ring explants were initially removed after 3 to 4 days of culture. The subsequent transfer of the same rings into new culture wells at 4-day intervals resulted in the progressive development of mixed cell populations consisting of endothelial and smooth muscle cells. Further transfer of the rings and cloning resulted in a highly pure population of smooth muscle cells. These VSMCs were used between passage 3 and passage 8 in all experiments.

Gel contraction assay

Seven ml of a collagen solution (3 mg/ml stock) were gently mixed with 2 ml of five-fold-concentrated D-MEM on ice. The mixture was adjusted to pH 7.4 with 1 ml of 200 mM HEPES plus 2.2% $NaHCO_3$ in distilled water. This collagen mixture (0.3 ml) was added to each well of a 24-well plate (Becton-Dickinson Labware, NJ). Then, the collagen gel was induced by the incubation of the mixture for 30 min at 37°C. After gelatination, NRK 49F cell suspension, to which D-MEM+10% FBS (1 ml) was added, was poured on the gel of each well. The initial number of cells in each well was determined according to the FBS concentration in the medium for the final incubation, as follows: in 0.5% FBS, $1.5x10^4$ cells ml^{-1}, and in 10% FBS, $0.5x10^4$ cells ml^{-1}. The two cell numbers converged after the final incubation. The distribution of cells on the gels was rendered ubiquitous. The initial number of VSMCs was $0.5x10^4$ cells ml^{-1}. Preincubation of the fibroblast- or VSMC-populated collagen lattice (FPCL or SPCL, respectively) was performed in D-MEM+10% FBS for 24 h. Next, the gels were rinsed with serum-free D-MEM three times for 30 min. Then, they were exposed to 10% FBS or 0.5% FBS+D-MEM with or without 5 ng/ml TGF-ß1 for 48 h at 37°C. In the medium containing TGF-ß1, the inhibitors Y27632 (10 μM), hydroxyfasudil (20 μM), SB203580 (10 μM), PD98059 (10 μM) and wortmannin (5 nM) were added or not. The gels were then detached from the lateral wall and bottom of each well with a microspatula and used for the experiments. The diameter of each gel was measured with a ruler at the indicated time points, to assess the gel contraction after treatment with the reagents. Ten μM W-7 or 2.5 μM ML-7 was added to the medium 30 min prior to the detachment of the gels.

Fluorescence microscopy

After the contraction experiments, the gels were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 10 min. After rinsing, the cells in the collagen lattices were permeabilized with 0.5% Triton X-100 in PBS for 20 min, washed with PBS, and incubated with the primary antibody against α-SMA or vinculin for 2 h at room temperature (RT). After washing, they were incubated with FITC-conjugated goat anti-mouse IgG secondary antibody for 1 h at RT. To obtain the characteristics resulting from merging with phalloidin, after rinsing with PBS, they were stained with RITC-conjugated phalloidin for 30 min. After staining, the lattices were rinsed, observed, and photographed using an Olympus BX 60 microscope equipped with epifluorescence optics. In order to identify the cell type, a monolayer culture of the cells was grown on a two-well Labtek chamber slide (AS ONE, Osaka, Japan). The monolayer culture procedure for immunostaining was similar to that of gel culture. As a negative control, the primary antibody was replaced with mouse non-immune serum.

Assessment of the percentage of cells with α-SMA-positive stress fibers

When there were one or more stress fibers with α-SMA-positive staining in a cell, the cell was considered to be α-SMA-positive and was referred to as a "myofibroblast". The percentage of myofibroblasts was calculated by counting the α-SMA-positive cells from among approximately 500 cells in ten or more fields.

Transmission electron microscopy (TEM)

The cells on the Labtek chamber slides were fixed in two-fold-diluted Karnovsky's fixative (Karnovsky, 1965) for 10 min at 4°C, and then washed with 0.1 M cacodylate buffer (pH 7.4) and postfixed in 2% osmium tetroxide+0.05% potassium ferrocyanide for 30 min at 4°C. The specimens were dehydrated in an ascending ethanol series and embedded in epoxy resin. Ultrathin sections were cut on an ultramicrotome (LKB 2088 Ultrotom V; LKB, Bromma, Sweden), mounted on copper grids, and stained with methanolic uranyl acetate and lead citrate. The sections were observed and photographed under a transmission electron microscope (TEM-1200 EX II; JEOL, Tokyo, Japan) at 80 kV.

Statistical analysis

The experimental data are presented as means±SD. Statistical analyses were performed using Student's *t*-test. Differences were considered to be statistically significant at $P<0.05$.

Results

Pattern of α-SMA expression in the monolayer culture of NRK 49F cells and VSMCs on the glass slide

There were two kinds of staining patterns for α-SMA: a dot pattern and a fiber pattern (Fig. 1A). When NRK 49F cells were cultured with 0.5% FBS+5 ng/ml TGF-ß1 on Labtek chamber slides (glass slides) for 48 h, 97.4±3.0% of NRK 49F cells expressed α-SMA in stress fiber formation (Fig. 1Ad,B). Also, almost all stress fibers were composed of α-SMA (data not shown). As these cells were smaller in size and lower in α-SMA

density than the VSMCs derived from aorta ring cultures (Fig. 1Ad,Ae), they were referred to as "myofibroblasts". A few cells (19.2±3.7%) revealed stress fibers consisting of α-SMA in the medium containing 10% FBS+TGF-ß1 (Fig. 1Ab,B). The α-SMA was expressed in a dot pattern in the medium with 0.5% FBS in the absence of TGF-ß1 (Fig. 1Ac). Few cells (2.9±1.2%) were stained for α-SMA in the medium with 10% FBS (Fig. 1Aa,B). The α-SMA expression was stimulated by lowering the FBS concentration or adding TGF-ß1 in the monolayer culture on the glass plate. The pattern of α-SMA expression was dotted or indicative of stress fiber

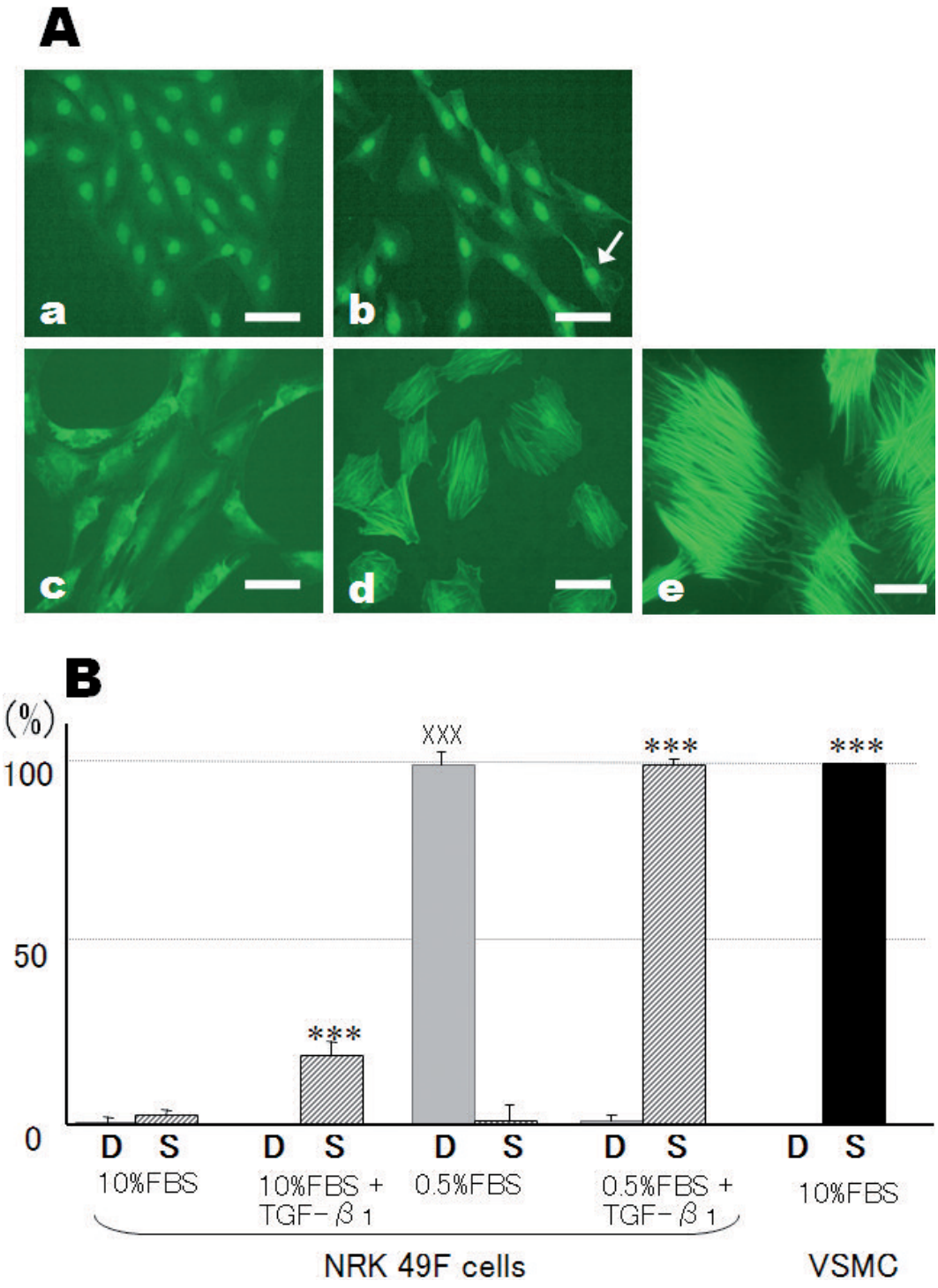

Fig. 1. α-SMA organization observed by fluorescence microscopy in a 2-day monolayer culture on the glass plate. **A.** Cells originating from NRK 49F cells and VSMCs were fixed and stained with antibody to detect α-SMA. NRK 49F cells in medium with 10% FBS **(Aa)**, with 10% FBS+TGF-ß1 **(Ab)**, with 0.5% FBS **(Ac)**, with 0.5% FBS+TGF-ß1 **(Ad)**, VSMCs in medium with 10% FBS **(Ae)**. **B.** Percentage of α-SMA- positive cells. Almost all cells reveal stress fibers with α-SMA in the medium with 0.5% FBS+TGF-ß1. To a lesser extent, a few cells (arrow in Fig. 1Ab) exhibit stress fibers with α-SMA in medium with 10% FBS+ TGF-ß1. α-SMA -positive stress fibers are absent in the cells in medium with 0.5% FBS. Most cells are positive for α-SMA in a dot pattern in the medium. All VSMCs reveal stress fibers with α-SMA in medium with 10%FBS. The density of their stress fibers is much higher than that of myofibroblasts originating from NRK 49F cells in medium with 0.5% FBS+TGF-ß1. Data are presented as mean±SD for the percentages of α-SMA-positive cells in each of ten or more fields. ***P<0.001 (stress fiber formation), xxxP<0.001 (dot pattern) vs. cells in medium with 10% FBS. D, dot pattern of α-SMA expression; S, stress fiber formation with α-SMA. Bar: 30 μm.

formation, respectively. TGF-ß1 was found to enhance the polymerization of α-SMA.

Features of the fibroblast, the myofibroblast, and VSMC in transmission electron microscopy

In transmission electron microscopy of cells from a 2-day monolayer culture on the glass plate, myofibroblasts (NRK 49F cells in medium with 0.5%FBS+TGF-ß1) possessed more mitochondria and a well-developed RER compared with fibroblasts (NRK 49F cells in medium with 10%FBS), and had bundles of actin microfilaments with dense bodies similar to those of fibroblasts (Fig. 2a,b). The density of actin bundles with dense bodies in these cells was far lower than in the VSMCs (Fig. 2b,c).

Signaling pathways involved in the expression of α-SMA

The expression of α-SMA in NRK 49F cells was

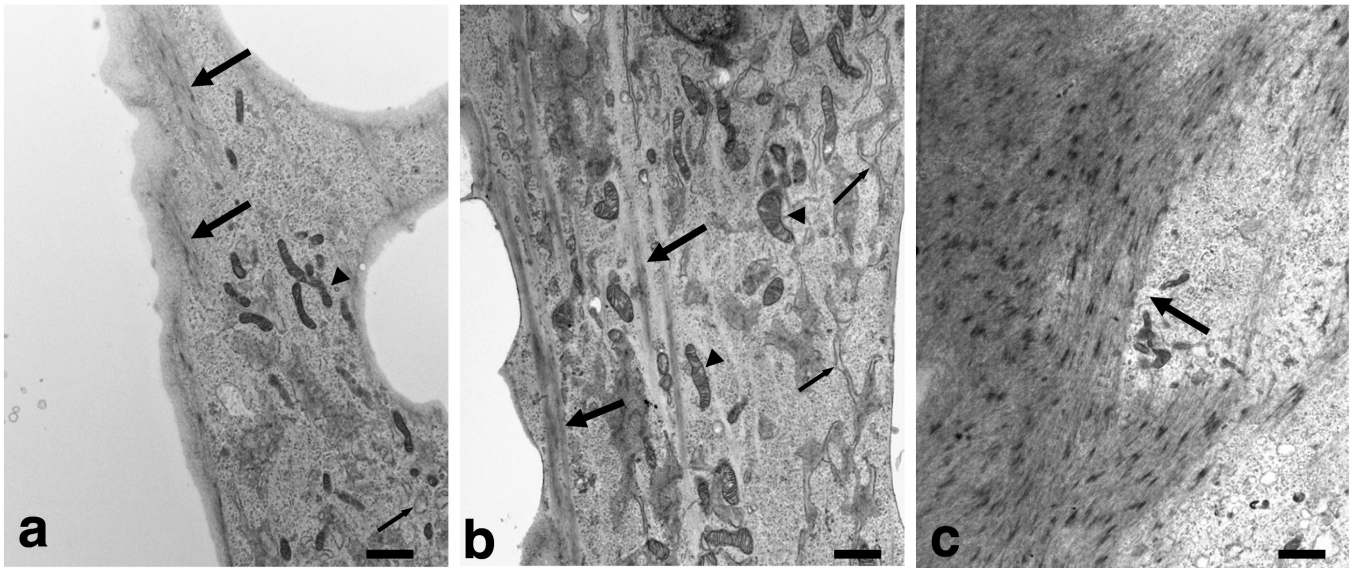

Fig. 2. TEM of three types of cells from a 2-day monolayer culture on the glass plate. **a.** A fibroblast (NRK 49F cell) in medium with 10% FBS. **b.** A myofibroblast originating from an NRK 49F cell in medium with 0.5% FBS+TGFß1. **c.** A VSMC in medium with 10% FBS. All three types of cells show bundles of actin microfilaments with dense bodies (large arrows). In the myofibroblast, a great number of mitochondria (arrowheads) and a well-developed RER (small arrows) are seen. In the VSMC, bundles of actin microfilaments with dense bodies are abundant. Bar: 1 μm.

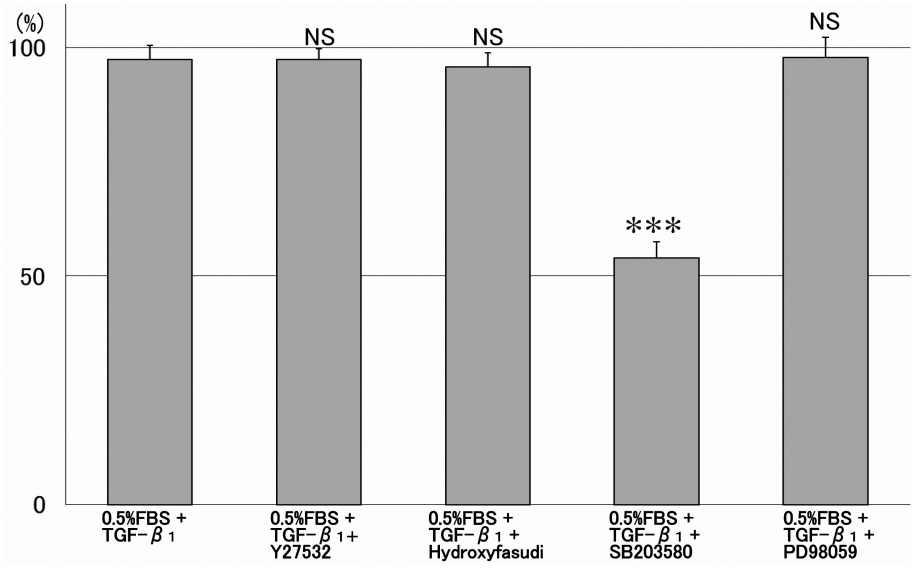

Fig. 3. Signaling pathways for α-SMA expression. α-SMA expression is repressed by SB203580 exclusively. Data are presented as mean±SD for the percentages of α-SMA-positive cells in each of ten or more fields. ***P<0.001, NS vs. cells in medium with 0.5% FBS+TGF-ß1.

repressed by the addition of SB203580 to the medium containing 0.5% FBS+TGF-ß1 in the glass slide culture (with or without SB203580; 53.9±3.6% or 98.7±1.4%, respectively), but not by addition of Y27632, hydroxyfasudil or PD98059 (Fig. 3). This demonstrates that the p38 MAPK pathway is involved in α-SMA expression.

Relationship between α-SMA expression on the gel and gel contraction

NRK 49F cells with α-SMA expression accounted for 22.9±2.0% on the anchored gel (MPCL) culture with 0.5% FBS+TGF-ß1 (Fig. 4B). This proportion was lower than that in the glass slide culture (Fig. 1B, 4B). Lowering the rigidity of the ECM repressed TGF-ß1-induced α-SMA expression. No α-SMA-positive cells were present on the FPCL culture under other culture conditions (Fig. 4B). On the other hand, almost all VSMCs exhibited α-SMA-positive stress fibers in the anchored SPCL culture in the conventional medium (10% FBS without TGF-ß1), similarly to the cells in the glass slide culture (Figs. 1B, 4B). Figure 4C shows gel contraction at 1 h after the detachment of the gels populated by either NRK 49F cells or VSMCs. The gels also contracted without α-SMA expression in the medium with 10% FBS only or 10% FBS+TGF-ß1 (Fig. 4B,C). Elevation of the FBS concentration induced gel contraction, while TGF-ß1 accelerated it. VSMCs strongly induced gel contraction in the conventional culture medium (Fig. 4C).

Relationship between FBS concentration and TGF-ß1 stimulated gel contraction

TGF-ß1 accelerated the gel contraction process from 30 min to 2 h after the detachment of the gels, independently of the concentration of FBS in the culture medium (Fig. 5A). Contraction did not occur in the medium with 0.5% FBS, but it did occur with 10% FBS (Fig. 4C, 5A). There was no significant difference in gel contraction between 0.5% FBS+TGF-ß1 and 10% FBS+

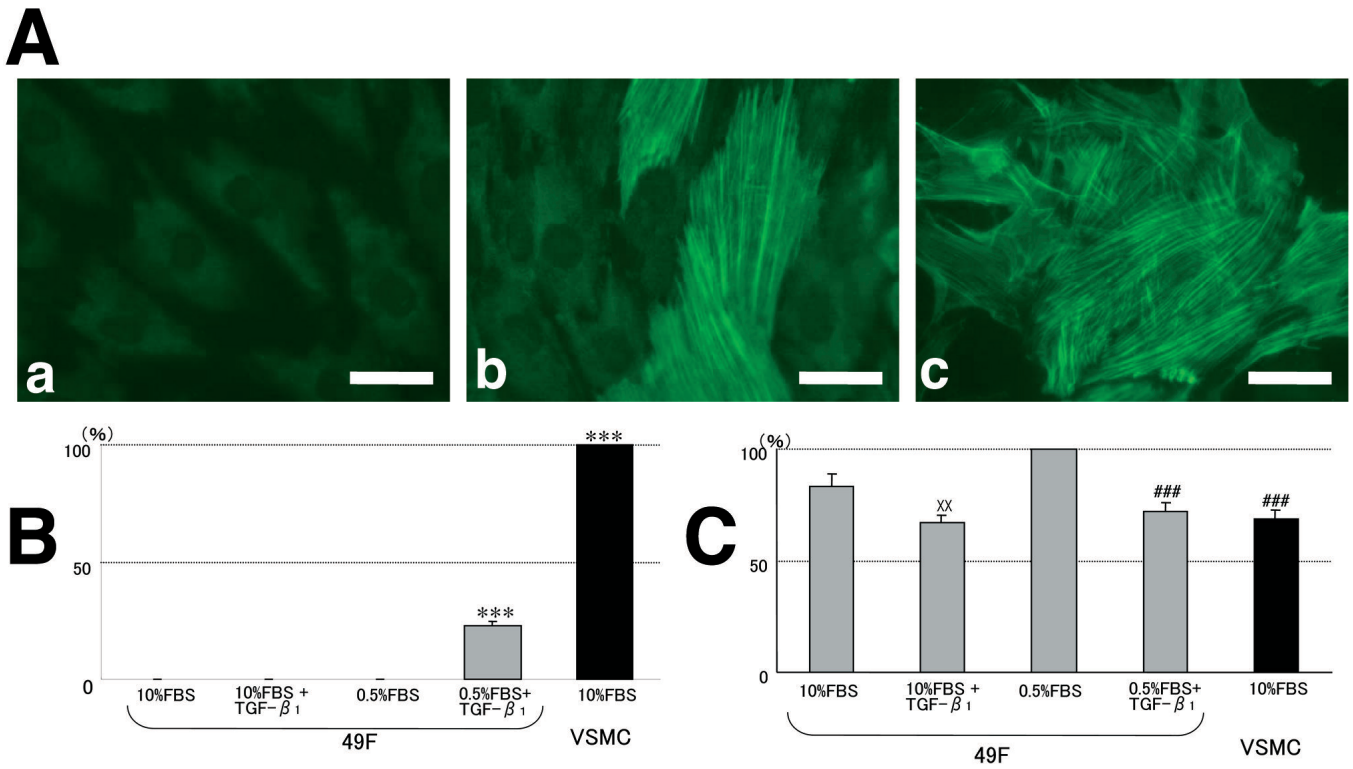

Fig. 4. Percentage of α-SMA-positive cells on the anchored gel and gel contraction. **A.** Immunofluorescence of α-SMA in a 2-day culture on the anchored gel. Cells derived from NRK 49F cells in medium with 0.5% FBS **(Aa)**, with 0.5% FBS+TGFß1 **(Ab)**, from VSMCs with 10% FBS **(Ac)**. **B.** The percentage of α-SMA-positive cells. **C.** Gel contraction at 1 h after gel release. α-SMA-positive NRK 49F cells are not found in the medium with 10% FBS, 10% FBS+TGF-ß1 or 0.5% FBS. Approximately a quarter of the cells originating from NRK 49F cells were positive for α-SMA in stress fibers in medium with 0.5% FBS+TGF-ß1. All VSMCs were α-SMA-positive. Data are presented as mean±SD for the percentages of α-SMA-positive cells in each of ten or more fields. ***P<0.001 vs. NRK 49F cells in medium with 10% FBS **(B)**. Degrees of gel contraction are shown by measuring the gel diameter at 1 h after gel release as percentages of the initial diameters. Gels both with and without α-SMA-positive cells underwent contraction. Values represent mean±SD (n=3). xxP<0.01 vs. FPCLs with 10% FBS alone. ###P<0.001 vs. FPCLs with 0.5% FBS alone **(C)**. Bar: 30 μm.

TGF-ß1 (Fig. 5A), but the contraction rate upon the addition of TGF-ß1 was greater in the medium with 0.5% FBS than in that with 10% FBS (Fig. 5B).

Signaling pathways involved in gel contraction

The contraction of MPCL was attenuated by Y27632, specific Rho kinase inhibitor or SB203580, p38 MAPK inhibitor, similarly to FPCL (Fig. 6A,B). Also, SPCL in the medium with 10% FBS gave similar results (Fig. 6C). The Rho kinase and p38 MAPK pathways were involved in the contraction of FPCL, MPCL and SPCL. The contraction of FPCL was not affected by W-7, calmodulin antagonist, or ML-7 or myosin light chain kinase inhibitor (Fig. 7A). On the other hand, the contraction of MPCL and SPCL was suppressed by W-7 or ML-7 (Fig. 7B,C). The gel with α-SMA-positive cells contracted through the activation of Ca^{2+}-calmodulin and MLCK, but not the gel without α-SMA-positive cells.

Relationship between the cells and the collagen in gel contraction

TGF-ß1 stimulated the formation of stress fibers and focal adhesions independently of the concentration of FBS in the culture medium (Fig. 8). Few vinculin molecules were observed in the fibroblasts on the anchored gel in the medium without TGF-ß1 (Fig. 8b, h). These findings are compatible with the result that TGF-ß1 accelerated the gel contraction.

Discussion

To our knowledge, this is the first study to establish

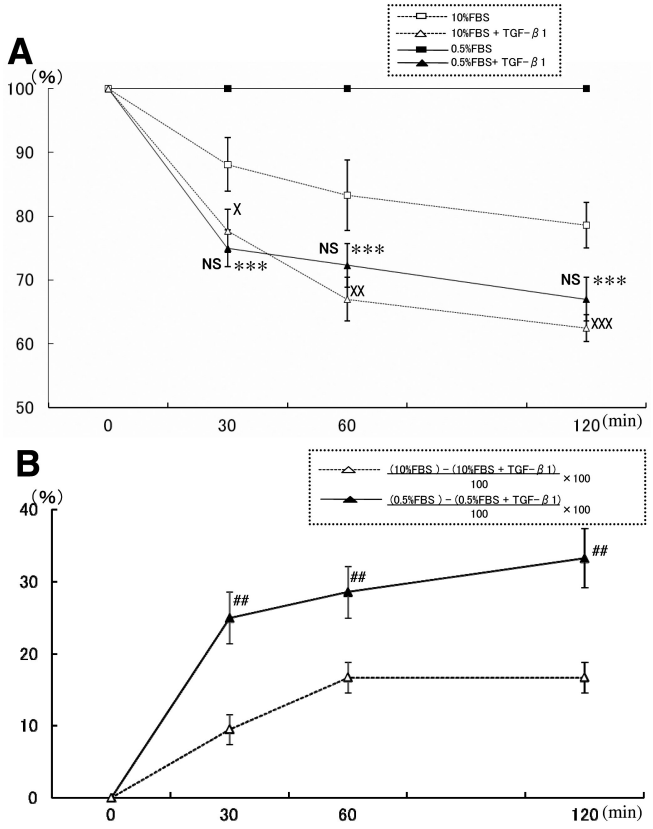

Figure 5. Gel contraction in response to FBS and TGF-ß1. The final diameters as percentages of the initial diameters were measured at each time point following gel release, after culturing in medium with 0.5% or 10% FBS in the presence or absence of TGFß1 for 2 days. **A.** The final diameters of the gels as percentages of the initial diameters under each condition. **B.** The contraction rate of the gels upon the addition of TGF-ß1 in medium with 0.5% or 10% FBS. Ten % FBS and TGF-ß1 stimulated gel contraction **(A).** The contraction rate of the gels upon the addition of TGF-ß1 was greater in the medium with 0.5% FBS (MPCL) than in that with 10% FBS (FPCL) **(B)**. Values represent mean±SD (n=3). xP<0.05, xxP<0.01, xxxP<0.001 vs. gels with 10% FBS alone at each time point after gel release. ***P<0.001 vs. gels with 0.5% FBS alone at each time point. NS (not significant) vs. gels with 10% FBS+TGF-ß1 at each time point. ##P<0.01 vs. gel contraction rates accelerated by TGF-ß1 in medium with 10% FBS at each time point.

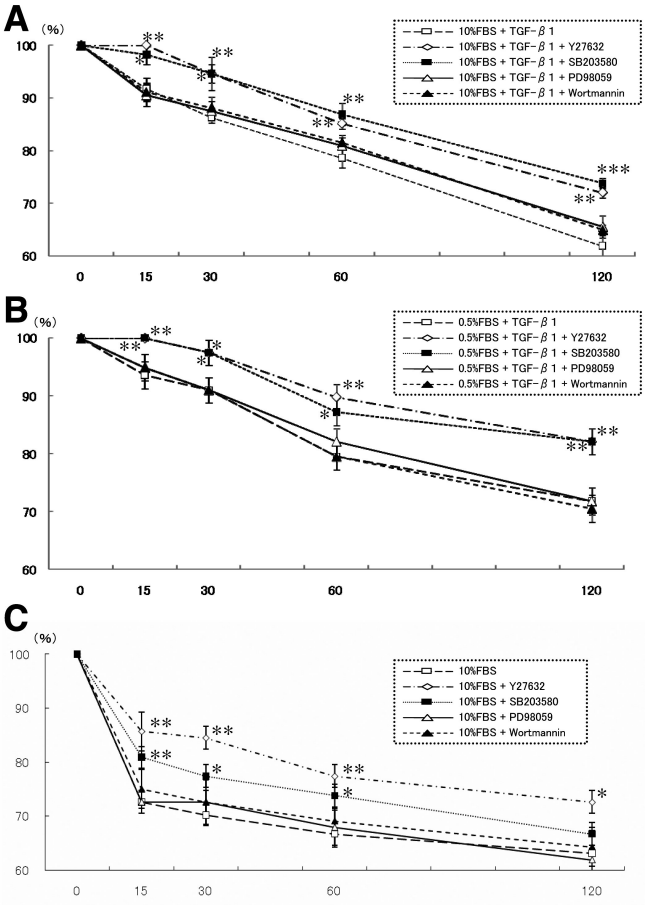

Fig. 6. Signaling pathways involved in gel contraction. **A.** FPCL. **B.** MPCL. **C.** SPCL. Gel contraction in the three groups of gels was similarly suppressed by Y27632 or SB203580 at each time point after gel release. Values represent mean±SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. time-matched controls.

myofibroblasts from the rat renal fibroblast cell line, NRK49F, with a very high induction rate. Myofibroblast transdifferentiation was carried out by lowering the FBS concentration and adding TGF-ß1 to the medium of the monolayer culture on the glass slide. The cells had α-SMA-positive stress fibers whose density was lower than those in VSMCs. Also, the cells were smaller than VSMCs. Furthermore, they contained a great number of mitochondria and a well-developed RER in their cytoplasm. Based on these findings, they were identified as myofibroblasts.

The α-SMA expression was stimulated by lowering the FBS concentration independently of TGF-ß1 in the monolayer culture on the glass plate. Therefore, FBS was suggested to contain substances suppressing the expression of α-SMA. The effect of lowering the FBS concentration on the α-SMA expression was limited to the dot pattern that likely corresponded to granular actin expression, not leading to formation of the stress fibers of α-SMA. TGF-ß1 could stimulate the polymerization of α-SMA and form its stress fibers. Also, α-SMA expression was strongly repressed in NRK 49F cells on the anchored gel, compared with that in the cells on the glass plate. The α-SMA expression is considered to be affected by the rigidity of the ECM, which transfers signals to the cell via integrins, as shown in in vitro studies (Arora et al., 1999; Kondo et al., 2004; Lygoe et al., 2004).

On the other hand, gel contraction was demonstrated to be induced by fibroblasts without α-SMA (FPCL), as well as by a cell mixture of fibroblasts and myofibroblasts with α-SMA (MPCL), as previously reported (Kolodney and Elson, 1993; Garcia et al., 1995; Chrzanowska-Wodnicka and Burridge, 1996). Gel contraction by fibroblasts was induced through the elevation of the FBS concentration in the medium or the addition of TGF-ß1 to the medium. It appeared that FBS contained the substances causing gel contraction, but these substances were not isolated. FBS was considered to contain the substances to repress α-SMA expression and the factors to stimulate cell contraction and control gel contraction. TGF-ß1 has been demonstrated to trigger the contraction of collagen lattices populated by a variety of cells: fibroblasts (Montesano and Orci, 1988; Fukamizu and Grinnell, 1990; Grinnell and Chin-Han, 2002), mesangial cells (Kitamura et al., 1992), etc. We have also shown the contraction of FPCL upon the addition of TGF-ß1 in a previous study (Ina et al., 2007). MPCL contraction was produced to the same extent as FPCL contraction. TGF-ß1-induced gel contraction was greater in MPCL than in FPCL. MPCL contraction by TGF-ß1 was suggested to be induced through a direct effect, as well as through the α-SMA expression effect of TGF-ß1.

The intracellular signaling pathway of cell contraction resulting in gel contraction was shown to include the activation of Rho kinase and p38 MAPK in the contraction of FPCL, MPCL and SPCL, but not the activation of p44/42 MAPK kinase and PI3-K. On the other hand, the activation of Ca^{2+}-calmodulin and MLCK was involved in TGF-ß1-induced MPCL contraction and FBS-induced SPCL contraction, but not in TGF-ß1- and FBS-induced FPCL contraction. Cells with α-SMA-positive stress fibers, i.e., myofibroblasts and VSMCs, were suggested to contract as a result of an increase in Ca^{2+}-calmodulin and the activation of MLCK, as well as activation of Rho kinase and p38 MAPK. Tomasek and colleagues have previously obtained data similar to those in our present study by using human myofibroblasts from patients with Dupuytren's disease (Parizi et al., 2000). They documented that lysophosphatidic acid-promoted myofibroblast contraction was dependent on the inhibition of MLC phosphatase and MLCK. In the present study, the signaling pathway of TGF-ß1-induced myofibroblast contraction was considered to be as follows (Fig. 9). First, TGF-ß1 may activate Rho and subsequently Rho kinase, and cause actomyosin contraction through the phosphorylation of MLC, as

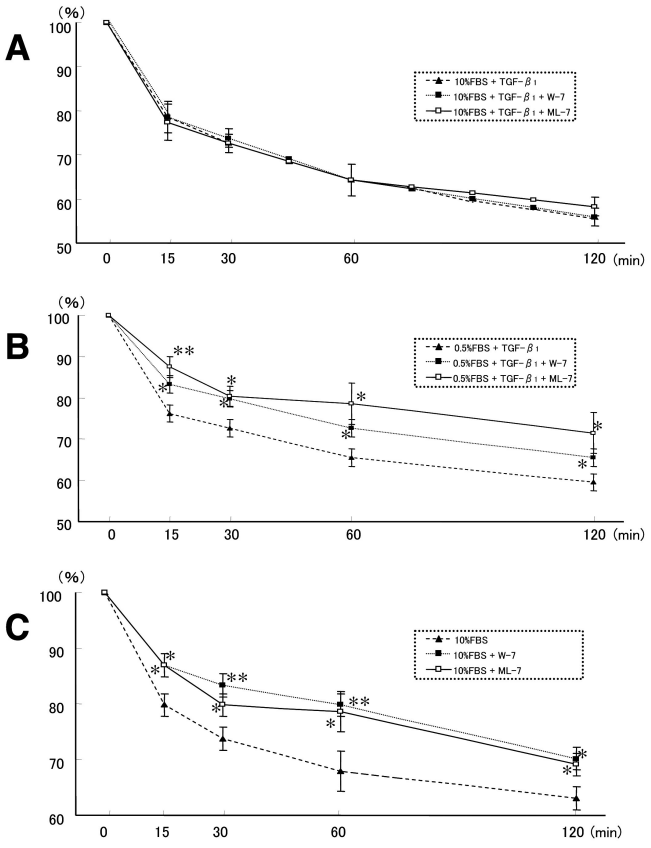

Fig. 7. Ca^{2+}-dependent signaling pathways involved in gel contraction. **A.** FPCL. **B.** MPCL. **C.** SPCL. Gel contraction was attenuated by W-7 or ML-7 at each time point after gel release in MPCL and SPCL, but not in FPCL. Values represent mean±SD (n=3). *P<0.05, **P<0.01 vs. time-matched controls.

described in our previous report (Ina et al., 2007). Second, the pathway of p38 MAPK activation is also involved in TGF-ß1-induced contraction. However, the molecular detail, which traces the pathway leading p38 MAPK to MLC phosphorylation, is still being studied. Third, distinctly from these pathways, TGF-ß1 increases the Ca^{2+} concentration in the cytoplasm of myofibroblasts, and Ca^{2+} binds with calmodulin, followed by MLC kinase activation. Then, MLC kinase phosphorylates MLC, leading to actin-myosin

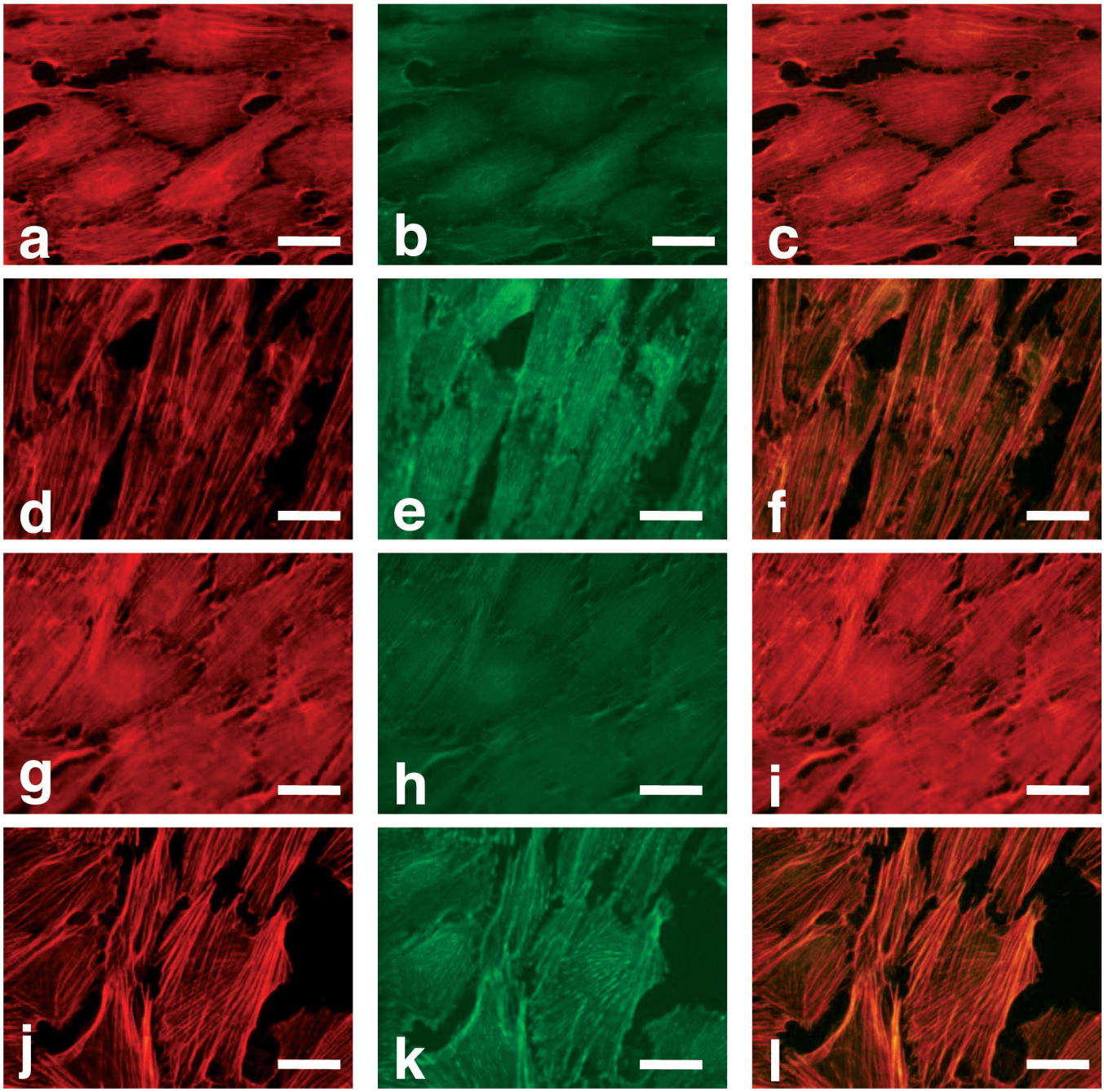

Fig. 8. Actin organization and vinculin expression observed by fluorescence microscopy in a 2-day culture on the anchored gels. Cells derived from NRK 49F cells were fixed and stained by RITC- phalloidin (**a, d, g, j)** or the antibody for vinculin (**b, e, h, k)**. Merged images of phalloidin and vinculin are shown **(c, f, i, l)**. Cells in medium with 10% FBS **(a-c)**, with 10% FBS+TGF-ß1 **(d-f)**, with 0.5% FBS (**g-i)**, with 0.5% FBS+ TGF-ß1 **(j-l)**. The stress fibers of F-actin are thin (**a, g)**, and few vinculin molecules are seen (**b, h)** in the medium without TGF-ß1. The formation of stress fibers of F-actin **(d, j)** and focal adhesions containing vinculin (**e, k**) are accelerated in response to TGF-ß1. Bar: 30 μm.

interaction. The pathway through which an increase in intracellular Ca^{2+} is triggered by TGF-ß1 was not determined in the present study. It may be the pathway involving p38 MAPK activation, as previously demonstrated in cardiac muscle cells (Magne et al., 2001). In the present study, the pathways involved in gel contraction were plainly shown. Cross-talk between signaling pathways that include the Smad pathway remains to be determined.

The expression of α-SMA induced by TGF-ß1 in the medium upon lowering the concentration of FBS involved the activation of p38 MAPK. Rho, however, did not contribute to α-SMA expression. This means that there was a difference in the intracellular signaling pathway between α-SMA expression and cell contraction. These findings are compatible with the study of Miura et al. (2006), in which retinal pigment epithelial cells were used. In contrast, Masszi et al. (2003) have found that the activation of Rho kinase was involved in α-SMA expression in renal tubular epithelial cells. The contrast between these findings might be due to the difference in the cells used.

Did cell contraction result in gel contraction? Meshel et al. (2005) have documented an elegant study in which the contraction of a single fibroblast caused the contraction of a single collagen fiber. To our knowledge, that is the only report describing the direct effect of cell contraction on ECM contraction. However, that study did not reveal that cell contraction led to the contraction of the whole tissue. In the present study, TGF-ß1-induced gel contraction in both FPCL and MPCL was accompanied by the accelerated formation of stress fibers and focal adhesion containing vinculin. It is well-known that actin filaments are linked to integrin via the actin-binding protein, vinculin. Also, integrin binds to collagen fibers. Thus, through the close relationship between actin filaments and collagen fibers, the contraction force involved in actin filaments may be transferred to collagen fibers, resulting in the traction of collagen fibers. In fact, when the cells were localized in a small area on the gel in the MPCL culture, the collagen gel showed indentation toward the cell mass; this did not occur in the FPCL culture without contraction (data not shown). This suggests the traction of collagen by the cells. These findings support the premise that cell contraction leads to tissue contraction. However, in 10% FBS-induced FPCL contraction, the formation of stress fibers and focal adhesion was not promoted. Taken together, these findings suggest that because TGF-ß1 accelerated the formation of stress fibers and focal adhesion, it could generate a stronger traction force than FBS only.

These results indicate that during the clinical course of renal fibrosis, when fibroblasts express α-SMA in stress fibers in response to TGF-ß1 and become myofibroblasts, they acquire the ability to contract in an accelerated manner, resulting in the accelerated contraction of the fibrosis tissue. In renal fibrosis, the contraction of the fibrosis tissue may lead to tissue ischemia (Ziyadeh and Goldfarb, 1991; Melin et al., 1997) and a decreased glomerular filtration rate resulting from the stenosis of the vascular system and the nephron, respectively. Furthermore, it is conceivable that renal atrophy, in addition to the renal damage described above, would also be induced, followed by renal failure. Thus, because myofibroblasts would not only develop fibrosis, but also intensify the contraction of the fibrosis tissue leading to renal failure, they would make a good

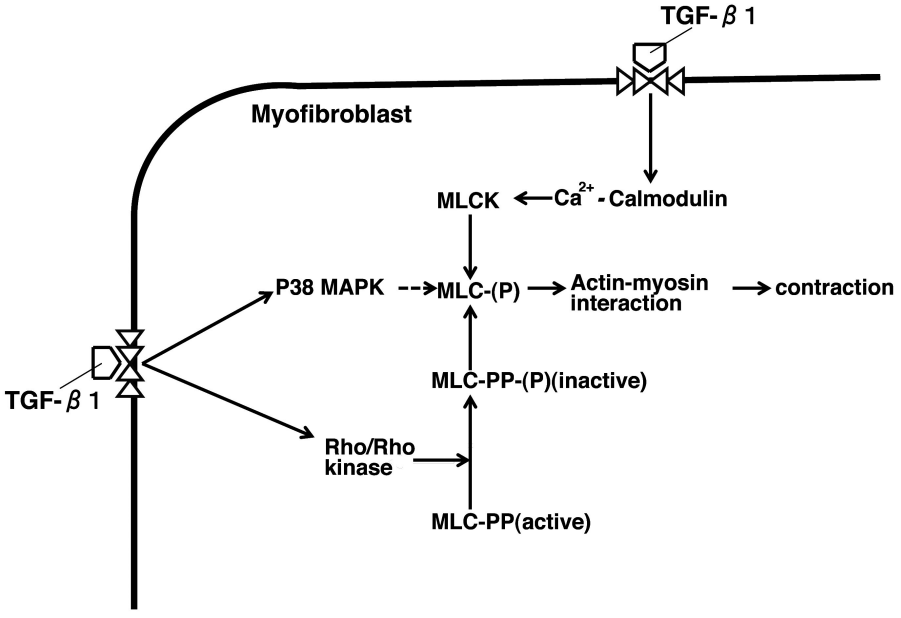

Fig. 9. Hypothetical regulation mechanism of TGF-ß1-promoted myofibroblast contraction. TGF-ß1 is proposed to promote myofibroblast contraction by three distinct signaling pathways. First, Rho/Rho kinase activation is associated with TGF-ß1-induced contraction. Activated Rho kinase phosphorylates MLC phosphatase and inactivates MLC phosphatase, resulting in increased phosphorylated MLC. This induces contraction. Second, TGF-ß1 induces cell contraction via p38 MAPK activation. p38 MAPK activation might result in MLC phosphorylation, leading to contraction. Third, TGF-ß1 increases intracellular Ca^{2+} and subsequently elevates the Ca^{2+}-calmodulin level, followed by MLCK activation. Activated MLCK phosphorylates MLC, leading to contraction. The last pathway is considered to involve α-SMA-associated contraction. MLC-(P), phosphorylated MLC; MLC-PP, MLC phosphatase; MLC-PP-(P), phosphorylated MLC phosphatase.

therapeutic target in renal fibrosis.

In conclusion, the significance of α-SMA expression in fibroblasts in renal fibrosis tissue was considered to be as follows: myofibroblasts accelerate the contraction of the fibrosis tissue and lead to renal failure via the Ca^{2+}-dependent signaling pathway, in addition to the pathways involved in fibroblast contraction.

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