

INTRACELLULAR SIGNALING PATHWAYS REGULATING ALPHA-SMOOTH MUSCLE ACTIN EXPRESSION IN RENAL TUBULAR CELLS DURING EPITHELIAL-MESENCHYMAL TRANSITION

PhD dissertation

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TABLE OF CONTENTS

ABBREVIATIONS	4
I. REVIEW OF THE LITERATURE	8
I.1. Chronic kidney disease and the role of tubulointerstitial fibrosis.....	8
I.2. Characterization of myofibroblasts	11
I.3. Characterization of epithelial- mesenchymal transition.....	12
I.4. Alpha- smooth muscle actin (SMA) as the marker of myofibroblasts and EMT	14
I.5. Epithelial mesenchymal transition in LLC-PK1/AT1 cells	15
I.6. The “two hit” model.....	16
I.7. Role of intracellular junction proteins during EMT.....	19
I.8. The actin cytoskeleton and its components: actin, MLC, cofilin, LIMK, HSP27	21
I.9. Transforming Growth Factor beta1	23
I.10. TGF- β 1 and the Smad family of signaling proteins.....	24
I.11. Non-Smad TGF signals.....	25
I.12. The Rho family GTPases	26
I.13. The p38 MAP kinase.....	29
I.14. Serum response Factor (SRF)	30
I.15. Myocardin related transcription factors (MRTF).....	31
II. AIMS OF THE STUDY	34
III. MATERIALS AND METHODS	35
III.1. Materials and reagents	35
III.2. Cell culture and treatments	35
III.3. Antibodies.....	36
III.4. Plasmids.....	37
III.4.1. Promoter constructs	37
III.4.2. Expression vectors.....	37
III.5. Transient transfections and luciferase promoter activity assays	38
III.6. Recombinant adenoviruses	39
III.7. Infection of cells with recombinant adenoviruses	39
III.8. Rho activity assay	40
III.9. Rac1/Cdc42 activity assay.....	40
III.10. Western Blotting.....	41
III.11. Immunofluorescence microscopy.....	41

III.12. Wounding assay.....	42
III.13. Nuclear extraction	42
III.14. Statistical analysis	43
III.15. Quantification of nuclear/cytoplasmic distribution of proteins.....	43
IV. RESULTS	44
IV.1. Smad2 and Smad3 are involved in the regulation of TGF- β 1 induced SMA promoter activation and protein expression in renal tubular cells.....	44
IV.2. Rho and ROK are key mediators of contact disassembly- induced activation of the SMA promoter. Contact disassembly induces Rho/ROK dependent myosin phosphorylation	47
IV.4. Cell contact disassembly induces nuclear accumulation of Serum Response Factor in a Rho- and MLC dependent manner	54
IV.5. Rac, Cdc42 and PAK are stimulated by contact disassembly and contribute to the injury-dependent activation of the SMA promoter.....	57
IV.6. p38 MAPK is a potent and important modulator of SMA expression, and is regulated by both TGF- β 1 and disruption of cell contacts	62
IV.7. Localization of MRTF and its nuclear-cytoplasmic transfer is regulated by TGF- β 1, cell contact disassembly, Rho, MLC, Rac1, Cdc42, PAK and p38	73
IV.8. MRTF is an important regulator of the cell contact–regulated and TGF- β 1–modulated SMA promoter activation and SMA synthesis.....	81
IV.9. Distinct regulation of SMA promoter activity by small GTPases: the role of H-Ras.....	84
IV.10. Cell-cell contact status regulates SMA expression independently of receptor availability	85
V. DISCUSSION	87
VI. CONCLUSIONS	99
VII. SUMMARY	101
VIII. ÖSSZEFOGLALÁS	102
IX. REFERENCES	103
X. LIST OF PUBLICATIONS	137
XI. ACKNOWLEDGMENTS	139

ABBREVIATIONS

ADF	Actin depolymerising factor
AMH	Anti-Müllerian hormone
AP-1	Activator protein-1
aPBMC-CM	Activated human peripheral blood mononuclear cells-conditioned medium
Arp2/3	Actin-related proteins 2/3
AT1	Angiotensin 1
BMP	Bone morphogenic protein
bp	Base pair
BSAC	N-terminal basic, SAP {SAF-A/B, Acinus, PIAS}, and coiled-coil domains
CA	Constitutive active
CArg	CC A/T rich GG
Cdc42	Cell division cycle 42
CKD	Chronic kidney disease
CTGF	Connective tissue growth factor
Cy3	Cyanine dye
DAPI	4',6-diamidino-2-phenylindole
Dia	Diaphanous-related formin
DMEM	Dulbecco's modified Eagle Medium
DN	Dominant negative
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EMT	Epithelial-mesenchymal transition
ERK	Extracellular regulated kinase
ESRD	End-stage renal disease
FAST	Forkhead activin signal transducer
FBS	Foetal bovine serum
FGD	Faciogenital dysplasia protein
FGF-2	Fibroblast growth factor 2

FITC	Fluorescein isothiocyanate
FSP1	Fibroblast specific protein 1
GAP	GTPase activating protein
GDF	Growth differentiation factor
GDI	GDP dissociation inhibitors
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GFR	Glomerular filtration rate
GST	Gluthatione-S-transferase
GTP	Guanosine triphosphate
HA	Haemagglutinin
HSP27	Heat shock protein 27
IF	Immunofluorescence
IL	Interleukin
JNK	Jun N-terminal kinase
KLF4	Krüppel-like factor 4
LAP	Latency associated protein
LEF-1	Lymphocyte-enhancer factor-1
LIMK	LIM kinase
LZ	Leucine-like zipper
MAL	Megakaryocytic acute leukemia
MAPK	Mitogen activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MDCK	Madine Darby canine kidney
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MH domain	MAD homology domain
MK2	MAP kinase activate protein kinase 2
MKK	MAPK kinase
MKL	Megakaryoblastic leukemia
MLC	Myosin light chain
MMP	Matrix metalloproteinase

MOI	Multiplicity of infection
MRTF	Myocardin related transcription factor
NF κ -B	Nuclear factor κ -B
NTD	N-terminal domain
OptiMEM	Optimal minimal essential medium
p120ctn	p120 catenin
PAI-1	Plasminogen activator inhibitor-1
PAK	p21 activated kinase
Par6	Partitioning-defective protein 6
PBS	phosphate buffered saline
PDGF	Platelet-derived growth factor
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occuldens-1 protein (zo-1)
PI3K	Phosphoinositide 3-kinase
POR-1	Partner of Rac-1
Rac	Ras-related C3 botulinum toxin substrate
RAd	Recombinant adenovirus
Ras	Rat sarcoma oncogene
RBD	Rho binding domain
Rho	Ras homologous protein
ROK (ROCK)	Rho kinase
RPGN	Rapidly progressive glomerulonephritis
RRT	Renal replacement therapy
SAP domain	Scaffold attachment factor domain
SAP-1	SRF associated protein 1
SARA	Smad anchor for receptor activation
SBE	Smad binding element
SDS	Sodium-dodecyl-sulphate
SF-1	Splicing factor 1
SMA	Alpha smooth muscle actin
Smad	Sma-MAD protein
Smurf	Smad ubiquitination regulatory factor
SRE	Serum response element

SRF	Serum response factor
TAD	Transcription activation domain
TAK1	TGF β activated kinase 1
TATA	Thymidine adenine thymidine adenine
TCE	TGF- β 1 control element
TCF/LEF	T cell factor/Lymphocyte-enhancer factor
TCF	Ternary complex factor
TGF- β 1	Transforming growth factor β 1
Tiam	T-cell lymphoma invasion and metastasis
TIF	Tubulointerstitial fibrosis
TIMP	Tissue inhibitor of metalloproteinases
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP-like Verprolin-homologous protein
WB	Western blot
Wnt	Wingless homologue
WT	Wild type
ZO-1	Zonula occludens-1

I. REVIEW OF THE LITERATURE

I.1. Chronic kidney disease and the role of tubulointerstitial fibrosis

Progressive chronic kidney diseases (CKD) lead to end-stage renal disease (ESRD). ESRD patients require renal replacement therapy (RRT) with maintenance dialysis throughout the rest of their lives or kidney transplantation. The severity of their state is accentuated by several complications from chronic renal failure and co-morbid conditions. Quality of life of RRT patients is impaired and their life expectancy is shorter. The number of patients with ESRD is increasing each year, so does the number of dialyzed or transplanted patients. In Hungary 4,404 patients were enrolled in dialysis programs in 2000, and there were 132 new patients per million population yearly recruited to such interventions. 1,568 patients lived with a transplanted kidney in 2000. The number of new patients in a year is about 1,320, whereas 265 is the number of kidney transplanted patients per year (Mogyorosy et al. 2003). In Canada, the number of incident ESRD RRT patients was 159/million in 2003, the number of ESRD patients starting RRT was 5,178 during 2004 (source: CIHI, 2006).

Considering the soaring number of kidney disease-affected population and the increasing number of RRT patients, it is imperative to better understand the cellular and molecular mechanisms leading to progression of renal fibrosis in order to design effective and specifically targeted therapies to treat ESRD.

Diabetes mellitus, hypertension, chronic glomerulonephritis, vascular diseases and polycystic kidney disease are the leading causes of CKD. Irrespective of the pathological background and the initial cause, a progressive renal fibrosis is the key finding for CKDs. The degree of fibrosis is the most important predictor for organ prognosis and kidney excretory function. The histological characteristics and regulatory mechanisms of renal fibrosis correspond to those observed in other organs (Wynn 2007). All renal compartments are involved during the progressive fibrosis that leads to glomerulosclerosis, tubular atrophy, interstitial fibrosis and arteriolosclerosis.

The progression of renal fibrosis from an initial injury to renal scarring includes several steps (Remuzzi and Bertani 1998). Renal injury leads to the reduction of nephron mass which in turn increases angiotensin II levels, followed by TGF- β 1 upregulation, tubular cell hypertrophy, and increased synthesis of collagen type IV. The reduction of nephron mass causes glomerular-capillary hypertension that increases

glomerular permeability for macromolecules and filtration of plasma proteins, manifested by proteinuria. Excessive tubular reabsorption of protein leads to accumulation of proteins in endolysosomes and endoplasmic reticulum that activates NFkB dependent and independent inflammatory and vasoactive genes. The subsequent release of endothelin, chemokines, and cytokines triggers the transformation of tubular cells into fibroblasts. Fibroblast proliferation, interstitial inflammatory reaction, together with the newly formed collagen IV result the renal fibrogenesis, and cause renal scarring. As a result, excessive matrix deposition and thus the destruction of kidney structure leads to irreversible impairment of organ function.

Renal fibrogenesis can be described by three phases (Zeisberg et al. 2001). During the induction phase chemokines are released by tubular epithelial cells, pro-fibrogenic cytokines are released and resident fibroblasts are activated. Next inflammatory matrix is synthesized and deposited during the continued release of pro-fibrogenic cytokines by infiltrating cells. The post-inflammatory matrix synthesis phase is characterized by the cessation of the primary inflammatory stimulus, continued secretion of pro-fibrogenic cytokines by tubular epithelial cells, proliferation of fibroblasts and possible epithelial- mesenchymal transition (EMT) of tubular epithelial cells.

The leading role of tubulointerstitial fibrosis (TIF) during CKD was recognized when it was established that there is a strong correlation between tubulointerstitial fibrosis and the decrease of the glomerular filtration rate (GFR) (Risdon et al. 1968). Tubular epithelial cells play an important role in this process. Proteinuria, high glucose, growth factors, reactive oxygen species and direct interaction with mononuclear cells are well characterized stimuli that lead to pro-inflammatory reactions in tubular epithelial cells and thus to the induction of interstitial fibrosis. Interstitial fibroblasts are still believed to be the main effector cells in renal fibrogenesis. However, in regard of the identification and characterization of a fibroblast marker, FSP1, it was suggested that fibroblasts in some cases arise, as needed, from the local conversion of epithelium (Strutz et al. 1995). New evidence was published discussing the heterogeneity of interstitial fibroblasts in regard of the overlapping and non-overlapping populations of FSP1 and SMA positive cells (Okada et al. 2000). Moreover, in a transgenic mouse model of TIF nearly 40% of fibroblasts have been shown to originate from the tubular epithelium (Iwano et al. 2002). Myofibroblasts- fibroblast-like contractile cells exhibiting several ultrastructural features of smooth muscle cells, such as the presence

of microfilament bundles (Gabbiani 1992)- have been shown to participate in this process, being recognized as the principal effector cells that are responsible for the excess deposition of interstitial extracellular matrix (ECM) under pathologic conditions (Roberts et al. 1997, Powell et al. 1999).

Recently epithelial mesenchymal transition (EMT) has emerged as a central mechanism underlying TIF. EMT is a key process in tissue development, carcinogenesis and organ fibrosis (Lee JM et al. 2006). During this process tubular cells lose their polygonal shape and epithelial markers (e.g. E-cadherin), acquire fibroblast specific proteins (e.g. FSP1), increasingly synthesize ECM (e.g. fibronectin) and ultimately differentiate into α - smooth muscle actin (SMA) - positive myofibroblasts (Kalluri and Neilson 2003). Epithelial cells are reshaped for movement through the rearrangement of F-actin stress fibers, and the formation of lamellopodia and filopodia. Through the disassembly of basal membranes by matrix metalloproteinases (Yang and Liu 2001, Zeisberg et al. 2002) cells acquire migratory characteristics and can migrate through a damaged basal membrane (Ng et al. 1998). In obstructive nephropathy induced by unilateral ureteral obstruction Yang and Liu (Yang and Liu 2001) showed abundant cells co-expressing SMA and tubular markers, indicating a transition state between epithelia and mesenchyme. EMT was observed in human renal biopsies, in different renal diseases, independently of histological diagnosis. It was demonstrated that the number of tubular epithelial cells with EMT features was associated with serum creatinine and the degree of interstitial damage (Rastaldi et al. 2002). This process is regulated by several cytokines and growth factors (Hay and Zuk 1995), from which TGF- β 1 seems to be the most important regulator.

TGF- β 1 was shown to induce EMT in normal mammary epithelial cells by signaling through receptor serine/threonine kinase complexes (Miettinen et al. 1994). TGF- β 1 induced cell proliferation (Moses et al. 1987) and stimulated extracellular matrix production, regulating fibronectin and type I collagen mRNA levels (Ignatz et al. 1987). Moreover, TGF- β 1 and its receptors are expressed in the areas of tissue fibrosis (Border and Noble 1997). Renal expression of TGF- β 1 was shown to be elevated in human diabetic nephropathy (Yamamoto et al. 1993) and TGF- β 1 was found to correlate with impaired renal function (Hellmich et al. 2000). Importantly, targeted disruption and inhibition of TGF- β 1 signaling protected against renal tubulointerstitial fibrosis and epithelial mesenchymal transition (Sato et al. 2003, Zeisberg et al. 2003).

I.2. Characterization of myofibroblasts

Myofibroblasts were identified to play a crucial role during wound healing, pathological organ remodeling and organ fibrosis, atheromatous plaque formation (Hinz et al. 2007), or tumor progression (Nakayama et al. 2002). Contractile myofibroblasts express within a single cell phenotypes that are to be found separately in other cells (fibroblasts and smooth-muscle cells). Myofibroblasts have a surface characterized by prominent fibronectin fibrils and fibronexus junctions, and are positive for vimentin and SMA. The main features for defining the myofibroblasts are abundant rough endoplasmic reticulum, myofilaments with focal densities (stress fibers) (Eyden 2001). Myofibroblasts synthesize a series of inflammatory and anti-inflammatory cytokines, chemokines, growth factors, inflammatory mediators, as well as extracellular matrix proteins and proteases (Powell et al. 1999). Vimentin, desmin, and SMA are the three filaments most often used to classify myofibroblasts. At least three local events are needed to generate SMA-positive differentiated myofibroblasts: accumulation of biologically active TGF- β 1, the presence of specialized ECM proteins like the ED-A splice variant of fibronectin, and high extracellular stress, arising from the mechanical properties of the ECM and cell remodeling activity (Tomasek et al. 2002).

Renal fibroblasts form a heterogeneous population, and subsets of fibroblasts are the myofibroblasts which were identified and defined by their expression of SMA (Badid et al. 2001). Myofibroblasts are the sites for extracellular matrix production during fibrosis in the kidney (Tang et al. 1997) and other tissues, such as in the lung (Zhang et al. 1996). There is excellent correlation between the appearance of interstitial SMA-positive myofibroblasts and the development of interstitial fibrosis in human and experimental glomerulonephritis, and interstitial SMA immunostaining is the best prognostic indicator of disease progression (Alpers et al. 1992, Badid et al. 2002).

In the normal kidney the number of fibroblasts is minimal, and there is no trace of interstitial SMA expression, however, these features show a strong correlation with progressive fibrosis (Essawy et al. 1997). It has been suggested that myofibroblasts may be derived from the differentiation of fibroblasts, the migration of perivascular smooth muscle cells or local proliferation. However, there is growing evidence showing that myofibroblasts originate from tubular epithelial cells following their epithelial mesenchymal transition. During tubulointerstitial fibrosis epithelial cells lose their polarity and adhesions to neighboring cells and basal membrane. Cells becoming motile

infiltrate the peritubular space and differentiate to myofibroblasts (Liu 2004). Loss of E-cadherin expression showed correlation with SMA expression in a unilateral ureteral obstruction model (Yang and Liu 2001). In a study regarding tubular EMT in progressive tubulointerstitial fibrosis in human glomerulonephritis it was demonstrated that the transformed tubular epithelial cells showing co-expression of cytokeratin and SMA are co-localized with upregulation of TGF- β 1 and FGF-2 and collagen matrix production (Jinde et al. 2001). Impaired kidney function was found to strongly correlate with the number of myofibroblasts and SMA expression during tubulointerstitial fibrosis in diabetic nephropathy (Essawy et al. 1997), IgA nephropathy and rapidly progressive glomerulonephritis (RPGN) (Jinde et al. 2001), chronic renal allograft dysfunction (Badid et al. 2002) or membranous nephropathy (Roberts et al. 1997).

The presented data describes the pathomechanisms leading to progressive renal fibrosis. We can conclude that tubulointerstitial fibrosis, epithelial mesenchymal transition and myofibroblasts play a critical role in the progression of CKD. Our goal was to decipher new insides of EMT and to assess intracellular signaling pathways regulating this process.

I.3. Characterization of epithelial- mesenchymal transition

The term “epithelial mesenchymal transition” was introduced after the phenomenon was previously inappropriately described as “transformation”, “transdifferentiation”, “interaction”. “Transformation” is used to describe the oncogenic conversion of epithelia. “Transdifferentiation” refers to differentiated cells changing to other differentiated cells. “Interaction” refers to cross-talks between tissue epithelia and stromal fibroblasts. In this regard, the term transition names a variant of transdifferentiation, and describes the mechanism of dispersing cells in vertebrate embryos, forming fibroblasts in injured tissues or initiating metastases in epithelial cancer (Kalluri and Neilson 2003).

EMT was shown to play important roles during embryonic development, cancer progression and fibrotic disorders of mature organs.

EMT has been described in embryonic morphogenesis and organ formation. The role of EMT has been established in lung development and palate fusion (Karttinen et al. 1995). EMT occurs during the development of endocardial cushions in the atrioventricular canal of the chicken heart (Romano and Runyan 2000).

EMT plays an important role in tumor progression and metastasis formation. During EMT, malignant cells lose their epithelial markers and become motile, EMT being linked to metastasis in a model of breast cancer progression (Huber et al. 2004).

“Fibrogenic” EMT has been shown to contribute to progressive fibrosis of the kidney (Yoshikawa et al. 2007), thyroid gland (Grande et al. 2002), lens (Stump et al. 2006), liver (Sicklick et al. 2006), lung (Kim et al. 2006), and of some rheumatic diseases (Zvaifler 2006). EMT is a response of highly differentiated cells to cellular stress caused by hypoxia (Manotham et al. 2004), reactive oxidative species (Rhyu et al. 2005), inflammatory stimuli (Fan et al. 2001), metabolic factors (Oldfield et al. 2001) and injury (Tanaka et al. 2004).

EMT is a complex mechanism which requires sequential activation and repression of expression of many sets of genes in a coordinated way. Several key events could be necessary for the completion of EMT in vivo. Four steps have been identified that are crucial during tubular epithelial to mesenchymal transition: 1. loss of epithelial adhesion properties, 2. de novo expression of SMA and actin reorganization, 3. disruption of the basal membrane, 4. enhanced cell migration and invasion (Yang and Liu 2001). These steps are well orchestrated by TGF- β 1, which induces tubular epithelial cells to undergo all four steps.

Tubular epithelial cells under normal conditions are polygonal in shape and tightly attached to each other form an epithelial sheet through cell adhesion mechanisms. One of the first changes in the TGF- β 1 induced EMT is the suppression of E-cadherin expression. Similarly to E-cadherin, the tight junction component ZO-1 is also suppressed. Following these events cells dissociate from their neighbors and lose polarity. During the second stage, de novo expression of mesenchymal markers SMA and FSP1 occurs. Actin structure and cytoskeleton is reorganized. Epithelial cells are characterized by a cortical actin ring that is anchored to intercellular adherent junctions through specific structural proteins. After the first stage of EMT this cortical actin ring disappears and actin containing stress fibers are formed, which are also bundled with myosin filaments. SMA and the reorganization of actin structures are necessary for the acquisition of the motile phenotype and the capacity of contraction. Concomitantly focal adhesions are also formed that mediate communication with the ECM. The following event is the disruption of the basal membrane which enables the cell to leave the layer and migrate towards interstitium. This step involves the activation of matrix metalloproteases (MMPs), especially the role of MMP-2 has been discussed in this

regard. Finally, in the last step, a newly formed cell type, the myofibroblast, showing enhanced migratory and invasive potential is released to the interstitium. The new elongated shaped cell lost epithelial phenotype, and acquired fibroblast-like characteristics showing migratory potential.

I.4. Alpha- smooth muscle actin (SMA) as the marker of myofibroblasts and EMT

Myofibroblasts are identified by de novo SMA expression. SMA expression is an excellent marker of EMT and myofibroblasts. The role of SMA expression is to upregulate contractile activity of cells and increased expression of SMA is directly correlated with increased force generation by myofibroblasts (Hinz et al. 2001). SMA is required for the initial formation of cortical filament bundles in spreading rat lung myofibroblasts and SMA is enriched in stress fibers (Hinz et al. 2003). In some myofibroblast cell lines, SMA comprises up to 14–18% of total actin content (Arora and McCulloch 1994).

In mammalian cells six actin types have been identified: two striated muscle actins (alpha-skeletal, alpha-cardial), two smooth muscle actins (alpha-vascular= SMA, gamma-enteral) and two non-muscle, cytoplasmic types (beta and gamma actin) (Vandekerckhove and Weber 1981). These isoforms have different functions, and as such, transfected SMA is differentially sorted to stress fibers (Mounier et al. 1997). SMA expression has been shown in muscle cells, fibroblasts, lens epithelial cells, mesangial cells, and tubular epithelial cells. SMA expression is regulated by several extracellular stimuli involved in modulation of progressive tissue fibrosis, such as FGF-2, angiotensin II, TGF- β 1, while PDGF-BB and EGF inhibited its expression.

The SMA promoter contains a number of highly conserved, putative regulatory elements. Next to a TATA box CArG-A, CArG-B and CArG- domains were identified along with two E boxes (CAnnTG) in the close vicinity of the first exon, mutations of these domains leading to loss of promoter reporter construct activity in smooth muscle cells (Shimizu et al, 1995). CArG elements are the SRF responsive regions of the SMA promoter, while E-boxes are responsible for SMA expression in skeletal muscle cells. Interactions have been described between the two E-boxes and a cis-acting TGTTTATCCCCA element (Jung et al. 1999). In close proximity to the TATA box a TGF- β 1 control element (TCE) was identified (Hautmann et al. 1997). SMA promoters with mutations in the TCE region were not responsible to TGF- β 1 treatments in rat

aortic smooth muscle cells. The Krüppel-like factor 4 (KLF4) was identified as a component of the protein complex binding to the TCE domain, KLF4 expression and binding being increased by TGF- β 1 in vascular smooth muscle cells (King et al. 2003). At least two Smad binding element (SBE) regions were also described. Mutation of one of the SBEs decreased SMA promoter activity significantly, indicating a functional role for this SBE (Hu et al. 2003).

Previous work from our group (Masszi et al. 2003) identified the CARG-B box as the essential element for the Rho inducibility of the SMA promoter. In LLC-PK1/AT1 cells TGF- β 1 is a potent regulator of SMA expression. TGF- β 1 and β -catenin are both essential regulators of SMA expression.

I.5. Epithelial mesenchymal transition in LLC-PK1/AT1 cells

Our group established a tubular cell model to study EMT and the development of myofibroblasts (Masszi et al. 2003). In this model TGF- β 1 induced EMT and SMA expression, which can be reliably analyzed as an EMT marker. This EMT model was established on porcine proximal tubular epithelial cells (LLC-PK1, clone CL4), which stably express the rabbit AT1 receptor (LLC-PK1/AT1 cells). EMT features of these cells are presented below.

Resting LLC-PK1 cells show typical polygonal shape, cells are tightly attached to each other. Cells treated with 4 ng/ml of TGF- β 1 started to show morphological changes already 24 hours after the treatment. However, 3 days after the treatment, the effect was clearly visible in 80% of the cells. These cells became elongated, and many cells lost their contacts with neighboring cells. Changes first appeared at the edges of cellular islands, and by the end of the experiment, most of the cells showed fibroblast-like shape. Many cells exhibited lamellopodia. When resting cells were immunostained for adherent and tight junction proteins, these were located at cellular peripheries in narrow lines. However, after the TGF- β 1 treatment, the peripheral stainings of ZO-1, E-cadherin, β -catenin became discontinuous and reorganized (ZO-1), delocalized (β -catenin) from the membrane to the nucleus, or even disappeared (E-cadherin) (Figure 1A.).

The next effect of TGF- β 1 treatment was cytoskeletal reorganization. Control cells exhibited a strong peripheral F-actin ring, with fade stress fibers, which became very thick upon TGF- β 1 treatment with a concomitant decrease of the marginal F-actin.

Stainings for diphosphorylated MLC also showed strong cytosolic filaments in the treated cells, whereas this feature was absent in the control cells. Further aspect of a motile phenotype is characterized by leading edge formation. To examine this, cells were stained for cortactin. TGF- β 1 treated cells exhibited lamellopodia with cortactin staining.

To assess extracellular matrix production, cells were also analyzed for fibronectin expression. Control cells already exhibited a basal fibronectin expression, which became more intense upon TGF- β 1 treatment.

SMA expression is a marker of myofibroblasts, and EMT was assessed through this marker in our model. SMA protein expression appeared 3 days after TGF- β 1 treatment of LLC-PK1 cells. Similarly, in immunofluorescent assays an intense labeling was observed in cells treated 3 days with TGF- β 1, when SMA was organized in thick fibers. Furthermore, SMA gene transcription was assessed in transient transfection experiments using a construct encoding a 756 bp. sequence of the rat SMA promoter. When cells were transfected with the promoter, and then treated with TGF- β 1 for 24 hours, TGF- β 1 induced a 3-6 fold increase in SMA promoter activity.

These data show that our model is viable, where TGF- β 1 induced the EMT of LLC-PK1 cells.

I.6. The “two hit” model

During further assessment of our model it was observed that cell contact integrity is an important regulator of EMT (Masszi et al. 2004), when cell confluence levels seemed to play an important role during TGF- β 1 induced EMT in LLC-PK1 cells. To address this observation, three models were employed: confluent and subconfluent conditions, disruption of cell contacts in low extracellular Ca^{2+} containing medium, and wounding. The Ca^{2+} -free model consisted of the first step described during the Ca^{2+} switch model (Denker and Nigam 1998), where the normal medium was changed to a Ca^{2+} -free medium. In the absence of extracellular Ca^{2+} , the dimers of the Ca^{2+} -dependent cell-cell adhesion molecule E-cadherin uncouple. This leads to the disassembly of the other cell contact molecules, and the separation of neighboring cells.

Cells were grown in confluent and sparse cultures, corresponding to cells having mature or less developed intercellular contacts. Interestingly, when cells were treated 3 days with TGF- β 1, only the cells seeded at 30% confluence and then treated showed

staining for SMA, confluent layers showed no SMA expression upon the same treatment (Figure 1B.). When followed by Western blot, TGF- β 1 treatment failed to induce SMA expression even after 5 days. Moreover, in confluent cultures TGF- β 1 was unable to downregulate E-cadherin as it did in cells treated before reaching confluence. Cells were then subjected to wounding. Confluent monolayers showed no expression of SMA upon wounding; however the exposure of wounded monolayers to TGF- β 1 resulted in SMA expression in the cells located at the wound edge. The other model for disassembly of cell-cell contact was Ca^{2+} removal, which did not cause SMA expression alone, but when combined with TGF- β 1, it led to SMA expression and E-cadherin elimination. Similar results were obtained when assessing the activity of the SMA promoter. In confluent layers both TGF- β 1 and Ca^{2+} removal stimulated the SMA promoter activity, but the combination of the two treatments led to a marked activation of the promoter.

When searching for the molecular mechanisms that can mediate the effect of cell contact injury on the reprogramming of the cells during EMT, the main candidate was a protein located at the intracellular side of the adherent junction complex. β -catenin was shown to have a dual function in epithelial cells, as an adherent junction component and as a transcriptional co-activator, and it was found to redistribute to the nuclei of LLC-PK1 cells upon TGF- β 1 treatment. TGF- β 1 was shown to stimulate β -catenin dependent transcription. When cells were subjected to Ca^{2+} removal and then were treated with TGF- β 1, the combined treatment prevented the degradation of β -catenin that occurred in Ca^{2+} -deprived cells not exposed to TGF- β 1 (Figure 1C.). β -catenin was shown to be involved in TGF- β 1 induced SMA promoter activation and protein expression.

The “classical” sequence of EMT events was described starting with TGF- β 1 effects on the epithelial cells. TGF- β 1 was thought to first mediate the loss of epithelial adhesion by down regulating the cell contact proteins (Yang and Liu 2001). However, based on these data, our group introduced the “two hit” model for EMT. Apparently, in order for EMT to occur there is a need for an initial loss of epithelial integrity (first hit), which might be induced by immunocomplex deposition, hypoxia, ureteral obstruction, or physical injury. When these injured sites are exposed to TGF- β 1 (second hit), they serve as foci for EMT. These local groups of cells undergo EMT, leading to enhanced TGF- β 1 production and ECM deposition, which in turn disrupts neighboring areas leading to the potential propagation of EMT.

In the present work the author presents results of experiments that were aimed at dissecting signaling mechanisms that are involved in the cell contact dependent and the TGF- β 1 dependent regulation EMT.

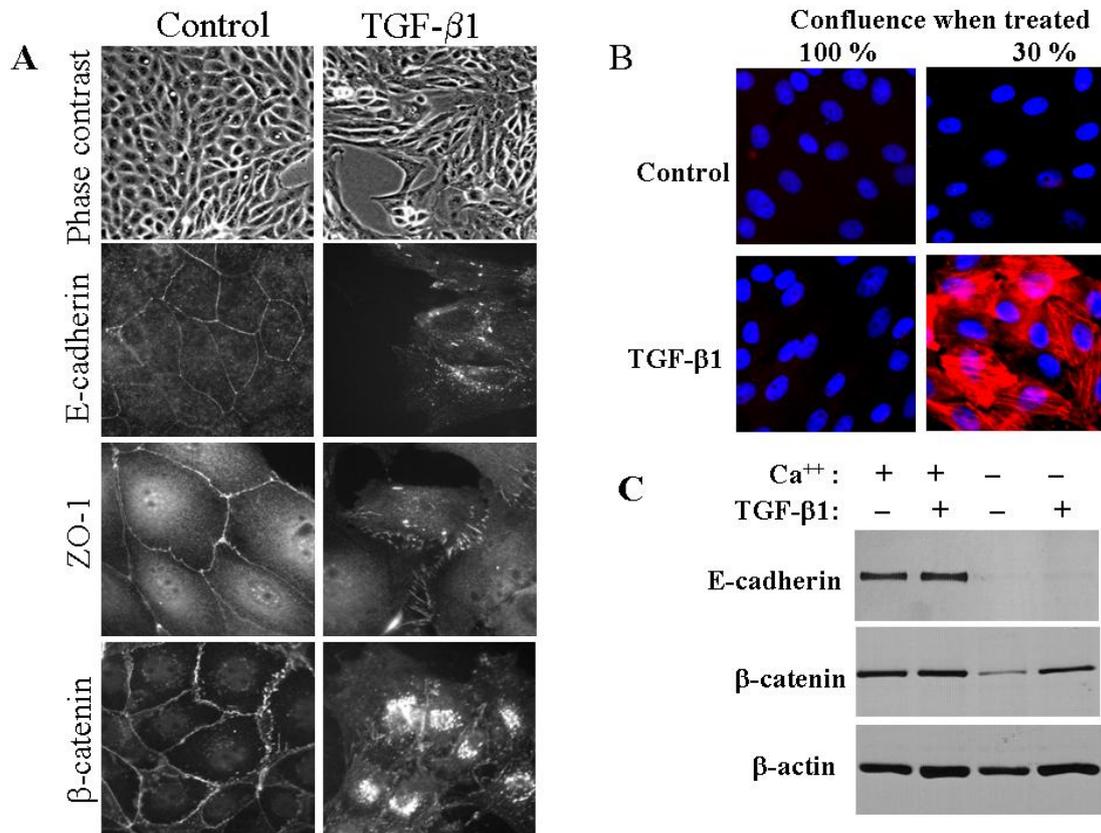


Figure 1. EMT in LLC-PK1 cells and the “two-hit model” was described by Masszi and coworkers (Masszi et al. 2003, Masszi et al. 2004). **(A).** EMT in LLC-PK1 cells is characterized by a change of cell forms, and reorganization of cell contact proteins. **(B).** Expression of SMA is dependent on cell density at treatment. **(C).** Cell contact disassembly induces degradation of junction proteins but TGF- β 1 selectively rescues β -catenin.

I.7. Role of intracellular junction proteins during EMT

The adhesive elements linking the individual epithelial cells can be classified into three groups: gap junctions, tight junctions, and adherent junctions.

Gap junctions are intercellular structures that allow the passive diffusion of ions and small molecules between two neighboring cells (Kumar and Gilula 1996). Gap junctions are specialized regions of the cell membrane in which each gap junction pore is formed by connexins, the connexin family comprising over a dozen distinct connexin genes (Kausalya et al. 2001). Interestingly, connexin45 has been linked to EMT during heart development (Kumai et al. 2000).

Tight junctions form impermeable barriers to fluids holding cells together while maintaining the different composition of proteins and lipids between the apical and the basolateral plasma membrane domains. Tight junctions can regulate the growth and differentiation of cells. Various signaling proteins (protein kinases, small GTP-binding proteins) are either localized at the cytoplasmic plaque domain of tight junction, or they have a central role in the assembly or function of junctions (Tsukita et al. 2001). Similarly to connexin45, ZO-1 was also found to have an important role during EMT, when mutants of the TJ protein zonula occludens protein-1 (ZO-1), which encode the PDZ domains (ZO-1 PDZ) but no longer localize at the plasma membrane, induce a dramatic epithelial to mesenchymal transition of MDCK cells (Reichert et al. 2000).

Cadherins and catenins are the major proteins that form the adherent junction group of intercellular contact proteins. In epithelial cells the cadherin-based cell-cell contact is a specialized region of the plasma membrane, where cadherin molecules of the adjacent cells interact in a calcium-dependent manner. The extracellular part interacts with cadherins of the neighboring cells, and the intracellular part of E-cadherin is bound to proteins involved in the formation of the junctional structure. Catenins γ and β bind to the intracellular domain of E-cadherin, whereas α -catenin links actin cytoskeleton and β -catenin (Conacci-Sorrell et al. 2002). Through a site near its transmembrane domain, E-cadherin binds directly to a special catenin, the p120ctn. Actomyosin contractility may also play a role in cell-cell adhesion (Shewan et al. 2005). New evidence, however, showed that α -catenin cannot bind to β -catenin and actin simultaneously (Lien et al. 2006). Since the cadherin/catenin complex does not interact directly with actin, there are several candidate molecules (Weis and Nelson 2006) which could anchor actin to the adherent junction.

The function of cadherins is not only limited to formation of protein complexes inside the cells and linkage of the cells together, but they also regulate the signaling events during differentiation (Kan et al. 2007), proliferation (Zhang X et al. 2006) and migration (Strumane et al. 2006). It was also shown that E-cadherin is downregulated in a Slug and Snail dependent manner during EMT (Bolos et al. 2003) and in carcinomas (Castro Alves et al. 2007).

Being part of the armadillo proteins, β -catenin has a dual function. First, it is a key component of cell-cell adhesion linking cadherin receptors to the cytoskeleton. Moreover, in non-adherent cells E-cadherin and associated β -catenin, which binds strongly to cadherin, appears to be required for transport of cadherin to the cell surface (Chen et al. 1999).

β -catenin is also part of the Wnt/Wingless signaling pathway that controls numerous events during development, including differentiation, proliferation and morphogenesis (Wodarz and Nusse 1998). β -catenin can be released from the adherent junction upon downregulation of E-cadherin (Eger et al. 2000), and upon β -catenin phosphorylation, a phosphorylation which dissociates β -catenin and E-cadherin (Behrens et al. 1993). In the presence of Wnt signals non-phosphorylated β -catenin regulates gene expression through its association with transcription factors LEF-1 (lymphocyte-enhancer factor-1) and TCF (T cell factor), commonly named as TCF/LEF (Seidensticker and Behrens 2000). In the absence of Wnts β -catenin is phosphorylated and degraded in proteasomes. In tumors degradation of β -catenin is blocked due to a mutation of β -catenin or tumor suppressor gene APC (adenomatous polyposis coli). This leads to formation of TCF/ β -catenin complexes and activation of oncogenes (Seidensticker and Behrens 2000). β -catenin bypassing degradation is translocated to the nucleus and forms a complex with TCF/LEF, complex which regulates several genes involved in renal fibrosis, such as: connective tissue growth factor (Luo et al. 2004), fibronectin (Gradl et al. 1999). Moreover, TGF- β 1 and β -catenin were shown to have auxiliary effects. Eger and colleagues (Eger et al. 2004) showed that loss of E-cadherin can contribute to an increase in LEF/TCF- β -catenin signaling, which in turn cooperates with TGF- β 1 signaling to maintain an undifferentiated mesenchymal phenotype during EMT. Moreover, β -catenin was shown to modulate transcription and alternative splicing in colon cancer cells (Lee HK et al. 2006).

I.8. The actin cytoskeleton and its components: actin, MLC, cofilin, LIMK, HSP27

Actin is an integral component of the cytoskeleton and contributes to the control of cellular shape, movement, division and secretion. The ability of cells to move is largely based on the formation of actin filaments from actin monomers near the plasma membrane and on myosin motors that contract the filaments. Actin generation predominantly depends on the number of free barbed ends, which act as actin nuclei and receive new monomers. Therefore, regulation of actin polymerization depends on how new barbed ends are generated. Three major mechanisms have been described (Condeelis 2001): de novo nucleation, F-actin severing and uncapping of capped barbed ends.

Nucleation is regulated by the Arp2/3 complex. Upon activation, it binds to the sides of actin filaments and initiates actin nucleation by forming daughter filament branches in a 70° angle. This occurs at the leading edge of migrating cells and allows the push of the lamellopodia ahead. Activation of the Arp2/3 complex is mediated by the various members of the Wiscott-Aldrich Syndrome Protein (WASP) superfamily. These proteins are activated by key signal transducing elements, such as the Rho family GTPases Cdc42 (Rohatgi et al. 1999) and Rac (Miki et al. 1998).

Severing generates new barbed ends, generating a build-up of actin filaments, or in case of an extensive severing it can lead to actin depolymerization and loss of actin filaments. Cofilin (actin depolymerizing factor, ADF) is one of the most important severing proteins. Its activity is regulated by phosphorylation, phosphorylated cofilin being the inactive form, which does not bind G-actin or depolymerize F-actin (Agnew et al. 1995). Phosphorylation by LIM-kinase 1 inactivates cofilin, leading to accumulation of actin filaments (Arber et al. 1998). LIM kinase is regulated on its turn by two pathways: Rac and Cdc42 through their downstream effector PAK stimulate LIMK (Edwards et al, 1999), and the Rho-ROK pathway is also involved in LIMK regulation (Maekawa et al. 1999).

Uncapping, that is the release of actin capping proteins, is also a significant contributor to free barbed end generation (Barkalow et al. 1996). Major uncapping proteins are several phosphoinositides. Interestingly, the heat shock protein HSP27 has been also identified as a barbed-end filament capping protein that is inhibited by its

phosphorylation (Piotrowicz et al. 1997). This activity of HSP27 is controlled by p38 (Pichon et al. 2004).

TGF- β 1 is an important regulator of actin cytoskeleton. TGF- β 1 treatments induced both an early and a late reorganization of the actin filament system: the initial rearrangement of actin filaments resulted in membrane ruffling, and TGF- β 1 also induced the formation of stress fibers (Edlund et al. 2002). LIM-kinase 2 and cofilin phosphorylation were shown to mediate this TGF- β 1 effect on actin. TGF- β 1 induced LIMK2 phosphorylation, which phosphorylated the actin depolymerizing cofilin, leading to its inactivation and thus permitting actin polymerization (Vardouli et al. 2005).

The cytoskeletal actin-myosin complex is regulated by myosin light chain (MLC), which upon phosphorylation regulates myosin ATPase activity that leads to an increase in cell motility. Being the regulatory element of the complex, MLC is the mediator of several upstream signals.

Recently MLC was shown to be implicated in wound healing. Epithelial wound-induced MLC phosphorylation and acto-myosin ring formation is believed to be critical for wound closure (Darenfed and Mandato 2005). Phosphorylation of MLC turned out to be regulated by two pathways. MLC was shown to be phosphorylated upon hyperosmotic stress in a Rho/Rho kinase-dependent manner in LLC-PK1 cells (Di Ciano-Oliveira et al. 2003). Cdc42 dependent PAK is also able to monophosphorylate MLC (Chew et al. 1998), which leads to increased contractility and permeability in endothelial cells. PAK induces monophosphorylation of MLC at Ser-19, while MLCK induces MLC diphosphorylation at Ser-19/Thr-18 sites. Rac-induced activation of PAK2 resulted in its phosphorylation and translocation to intercellular junctions, where it locally facilitated MLC phosphorylation (Stockton et al. 2004). p38 was also shown to mediate MLC phosphorylation and endothelial permeability upon TGF- β 1 treatment (Goldberg et al. 2002).

The pathways involved in MLC regulation and the control of MLC on the cytoskeletal acto-myosin complex raise the possibility of a connection between cell contacts, TGF- β 1 and the regulation of SMA expression. Therefore we proposed to assess its role in this mechanism.

I.9. Transforming Growth Factor beta1

The TGF superfamily of growth factors consists of more than 35 members, such as the three highly similar TGF isoforms (TGF- β 1, TGF- β 2 and TGF- β 3), activins, inhibins, anti- müllerian hormone (AMH), bone morphogenic proteins (BMP), growth differentiation factors (GDF) and others (Piek et al. 1999a).

TGF- β 1 controls a variety of cellular processes. TGF- β 1 is involved in regulating cell proliferation, differentiation, apoptosis, migration, ECM production, and modulation of immune responses (Shi and Massague 2003). TGF- β 1 is involved in a multitude of kidney diseases by inducing such pathomechanisms, as tubular atrophy, podocyte depletion, loss of capillary endothelial cells, progressive nephron loss, and TGF- β 1 is a potent inducer of EMT (Böttinger and Bitzer 2002).

TGF- β 1 plays a key role in regulating ECM, upregulating the expression of various ECM components, such as collagens and fibronectin, and the expression of protease inhibitors, such as PAI-1 and TIMPs. Due to its effects on ECM deposition, TGF- β 1 has an important role in wound healing. Exogenous administration of TGF- β 1 improves wound healing (Schiller et al. 2004). TGF- β 1 is involved in regulating tissue fibroses, which is considered to occur due to a failure of normal wound healing to terminate (Leask and Abraham 2004). TGF- β 1 expression was shown to strongly correlate with kidney fibrosis. Intraglomerular TGF- β 1 mRNA levels were found to be elevated in renal biopsy specimens from diabetic nephropathy patients (Iwano et al. 1996) and within tubular epithelial cells in patients with nephrotic syndrome (Goumenos et al. 2002). In NMuMG TGF type I receptor was shown to mediate EMT (Piek et al. 1999b).

Platelets and bone are the major sources of human TGF- β 1. TGF- β 1 is synthesized as a biologically inactive precursor called latent TGF. TGF is activated when released from its binding to the latency-associated peptide (LAP) (Annes et al. 2004), activation occurring in vitro upon changes in pH, heat, irradiation, and under physiological conditions upon acidic cellular microenvironment, reactive oxygen species, plasmin, MMP-2 and MMP-9, thrombospondin, α v β 6-integrin. After activation, TGF- β 1 is able to bind to its specific serine/threonine receptor, which consists of two distinct transmembrane proteins, known as type I and type II receptors. Ligand binding occurs to type II receptor, and it induces association of type I and type II receptors,

when type II receptor phosphorylates type I receptor activating its kinase domain. The activated type I receptor then signals to the Smad family of intracellular mediators (Attisano and Wrana 2002). Smad2 and Smad3 are phosphorylated directly by the TGF type I receptor kinase and after partnering with the common mediator, Smad4, translocate to the nucleus, where they regulate transcription of target genes (Massague et al. 2000).

I.10. TGF- β 1 and the Smad family of signaling proteins

The Smad family of intracellular mediators was named following the combination of the name of two proteins: “MAD” (mothers against decapentaplegic) identified from the TGF- β 1 homologue dpp signaling in *Drosophila melanogaster* (Sekelsky et al, 1995), and “Sma” originating from the word “small”, denominating a *C. elegans* protein, mutation of which causing developmental abnormalities (Savage et al. 1996). These proteins were found very similar to the ones described in vertebrates; therefore the name Smad originates from the fusion between Sma and MAD (Derynck et al. 1996).

The Smad family consists of 8 members which can be divided into three groups according to their function: receptor-activated Smads (R-Smads, Smad1, -2, -3, -5, -8), common-mediator Smads (Co-Smads, Smad4), and inhibitory Smads (I-Smads, Smad6, Smad7) (Shi and Massague 2003). TGF- β 1 signals are mediated by Smad2, Smad3, Smad4 and Smad7. SARA (Smad Anchor for Receptor Activation), a FYVE domain membrane bound protein that directly interacts with Smad2 and Smad3, facilitates their recruitment to the activated receptor complexes by controlling the subcellular localization of the two R-Smads (Tsukazaki et al. 1998). The MH2 domain (MAD homology domain) of the R-Smads contains the SSXS receptor phosphorylation site, which I-Smads and Co-Smads lack. After activation, R-Smads associate with each other and with Smad4, and the active Smad complex (Chacko et al. 2004) containing Smad2, Smad3 and Smad4 translocates to the nucleus. In the nucleus R-Smads exert their transcriptional effects in different ways. Smad3 directly contacts DNA at CAGAC sequences with its MH1 domain (Zawel et al. 1998). These sequences are located within the target gene promoters and are called Smad binding elements (SBE) (Jonk et al. 1998). The presence of a 30 amino acid insertion within the MH1 domain of Smad2 as compared to that of Smad3 prevents its direct interaction with DNA. Smad2 dependent

gene transcription requires the recruitment of putative transcription factors like FAST1 and FAST2 which allows the binding of the Smad2/Smad4/FAST1 complexes to specific response elements (Chen et al, 1997, Liu et al, 1999).

Inhibitory Smads have the ability to form stable associations with TGF receptor type I, to interfere with the phosphorylation of R-Smads and their complex formation with Smad4 (Nakao et al. 1997). Smad7 was also shown to interact with the E3 ubiquitin ligases Smurf1 and Smurf2, recruiting them to the TGF receptor complexes and inducing the degradation of the activated type I receptor (Kavsak et al. 2000, Ebisawa et al. 2001).

Involvement of Smads in regulating EMT was proved in a number of papers. Smads induce EMT by mediating TGF- β 1 effects in NMuMG breast epithelial cells (Piek et al. 1999b). Several authors tried to distinguish between Smad2 and Smad3 in their EMT modulating effects. Smad3 was shown to have a differential effect in lens EMT (Saika et al. 2004a). In another paper Li and his colleagues (Li et al. 2002) showed that TGF- β 1 signals through Smad2 to mediate tubular EMT and collagen matrix production, which is blocked by overexpression of the inhibitory Smad7. TGF- β 1-induced increases in MMP-2 expression were Smad2-dependent, increases in CTGF and decreases in E-cadherin expression were Smad3-dependent, and increases in alpha-SMA expression were dependent on both Smad2 and Smad3 in human proximal tubule epithelial cells (Phanish et al. 2006), indicating that Smad signaling plays a key role in EMT. Smads were also shown to stimulate formation of β -catenin/LEF-1 complexes that induce EMT (Medici et al. 2006). However, overexpression of Smads was not enough to induce EMT in renal proximal tubular epithelial cells (Tian et al. 2003).

I.11. Non-Smad TGF signals

Although the TGF- β 1 signaling through the Smad system is well described, there is growing cellular and genetic evidence for Smad independent TGF- β 1 signaling pathways. Three distinct signaling mechanisms can be identified: 1. non-Smad signaling pathways that directly modify Smad function, 2. non-Smad proteins whose function is directly modulated by Smads and which transmit signals to other pathways, and 3. non-Smad proteins that directly interact with or become phosphorylated by TGF- β receptors and do not necessarily affect the function of Smads (Moustakas and Heldin 2005). Non-Smad dependent TGF- β 1 signaling pathways have been described during apoptosis, cell

proliferation and differentiation, matrix regulation, embryonic development and EMT. Mutant TGF- β type I receptors that lack the Smad-docking site can activate endogenous p38 or JNK signaling (Yu et al. 2002, Itoh et al. 2003). There is a direct link between TGF receptors and the Rho GTPase through the polarity protein Par6 (Ozdamar et al. 2005), providing a novel mechanism by which TGF- β 1 induces EMT. In prostate cancer cells, TGF- β 1 mobilizes RhoA and Cdc42 and their downstream effector p38 MAPK to induce membrane ruffling (Edlund et al. 2002). Similarly, TGF- β 1 was shown activate Rac1 in NIH 3T3 cells (Mucsi et al. 1996) and in a human breast epithelial model (Ueda et al. 2004).

TGF- β 1 signaling and Smads play an important role during EMT. However, it seems that Smads are necessary, but insufficient to solely induce EMT. It is plausible to speculate that TGF- β 1 and Smad dependent and independent signaling pathways might play important roles during EMT.

I.12. The Rho family GTPases

Approximately one percent of the human genome encodes proteins that either regulate or are regulated by direct interaction with members of the Rho family of small GTPases. These highly conserved molecules control some of the most fundamental processes of cell biology, common to all eukaryotes (Jaffe and Hall 2005).

GTPases are GTP/GDP dependent molecular switches: they are in active state when bound to GTP and inactive when bound to GDP. Under basal conditions these proteins are bound to the guanine nucleotide dissociation inhibitors (GDI), which inhibit their binding to cellular membranes. Dissociation of GDP and binding of GTP is enhanced by guanine nucleotide exchange factors (GEF), while GTP-ase activating proteins (GAP) induce GDP binding by hydrolyzing GTP. Interestingly, some GEFs can potentially act on multiple GTPases, such as Vav on Cdc42, Rac and Rho (Olson et al. 1996), while others are more specific: lbc and p115-RhoGEF act on Rho (Hart et al. 1996), Tiam-1 acts on Rac (Michiels et al. 1995) and FGD1 acts on Cdc42 (Olson et al. 1996).

The Ras superfamily of GTPases number over 60 members, which form five major groups: Ras, Rho, Rab, Arf, Ran. Rho GTPases have over 20 members, of which only Rho, Rac and Cdc42 have been studied in detail (Etienne-Manneville and Hall 2002). The classical view of the cellular function of these proteins is that Rho induces

assembly of contractile actin and myosin filaments (stress fibers) (Ridley and Hall 1992), Rac1 induces formation of actin- rich surface protrusions (lamellopodia) (Ridley et al. 1992), while Cdc42 was found to promote the formation of actin-rich finger-like membrane extensions (filopodia) (Kozma et al. 1995). Rho, Rac and Cdc42 mediate signaling pathways linking plasma membrane receptors to the assembly of distinct filamentous actin structures. They regulate cell polarity, gene transcription, cell cycle, microtubule dynamics, enzymatic activities, morphology, cell migration and contraction. Rho GTPases are important regulators of the actin cytoskeleton and cell-cell contacts, and, as such, influence the shape and movement of cells. In addition, Rac1, Cdc42 and RhoA also regulate transcription factors, such as SRF (Hill et al. 1995) or NF- κ B (Perona et al. 1997). Small GTPases regulate the activity of MAPkinases, such as ERK, JNK, p38. In Swiss 3T3 fibroblasts these GTPases activate each other in a hierarchical cascade in which Cdc42 activates Rac1, which in turn activates RhoA (Nobes and Hall 1995). RhoA, Cdc42 and Rac1 were shown to be important regulators of EMT in HK2 cells (Patel et al. 2005).

There are several extracellular stimuli that activate these GTPases, such as growth factors, hormones, physical and chemical stimuli. It is also known that TGF- β 1 activates Rho, Cdc42 (Edlund et al. 2002) and Rac1 (Mucsi et al. 1996). It has been long speculated that the small GTPases are activated by TGF- β 1 in Smad dependent or Smad independent manner. However, interesting data suggests that Rho was able to modulate Smad activation while regulating TGF- β 1-induced smooth muscle cell differentiation (Chen et al. 2006).

One of the main Rho functions is the regulation of the cytoskeleton. Active Rho induces stress fiber assembly, through two major downstream effectors, ROK (Rho kinase) and mDia. Two substrates of ROK involved in this effect are myosin light chain (MLC) (Amano et al. 1996) and the myosin binding subunit of MLC phosphatase (Kawano et al. 1999). MLC phosphatase is inhibited by phosphorylation, indirectly leading to an increase in MLC phosphorylation. Phosphorylation of MLC occurs at Ser-19 and promotes the assembly of actin-myosin filaments. Another ROK target is LIM kinase (LIMK), which upon phosphorylation phosphorylates and inhibits cofilin, leading to stabilization of filamentous actin structures (Maekawa et al. 1999). While ROK does not induce correctly organized stress fibers, when combined with an activated version of Dia, stress fibers are induced (Watanabe et al. 1999). This finding means that activation of both Dia and ROK by Rho are required to induce stress fibers.

Further, TGF- β 1 was shown to activate ROK (Bhowmick et al. 2001a), and subsequently TGF- β 1 phosphorylated cofilin through LIMK2 (Vardouli et al. 2005).

WASP is the main effector of Cdc42 implicated in actin reorganization. It was shown that WASP (Wiscott-Aldrich syndrome protein) binds to Cdc42 (Kolluri et al. 1996) and overexpression of these two molecules induced formation of very long microspikes (Miki et al. 1998). WASP binds to profilin (Suetsugu et al. 1998) and Arp2/3 complex (Machesky and Gould 1999), inducing actin polymerization. Rac is implicated in actin reorganization through POR-1 (Partner of Rac), involved in Rac-induced lamellopodia formation (Van Aelst et al. 1996), and WAVE (WASP-like Verprolin-homologous protein, also known as Scar), which has been shown to activate the Arp2/3 complex (Machesky et al. 1999). However, the main, common downstream of Rac1 and Cdc42 is the Ser/Thr kinase member p21-activated kinase (PAK). PAK requires autophosphorylation in order to become active. The inactive, auto-inhibited kinase is arranged in a head-to-tail fashion through PAK-interacting exchange factor (PIX) dimers. Upon Rac1/Cdc42 binding, the kinase undergoes conformational change that allows autophosphorylation. Autophosphorylation at Ser-144 contributes to kinase activation, while autophosphorylation at sites Ser-198/203 downregulates the PIX-PAK binding (Chong et al. 2001). PAK1 was shown to activate LIMK (Edwards et al. 1999). PAK was similarly shown to be involved in the regulation of EMT (Wiggin et al. 2006). TGF- β 1 activates PAK1 (Wang et al. 2006) and PAK2 (Wilkes et al. 2003).

Rho family GTPases are required for cadherin-mediated cell-cell adhesion. Rac1 and Rho are required for localization of E-cadherin to sites of cell-cell contact in keratinocytes. The effects of Rac1 and Rho on the localization of cadherin probably depend on the maturation status of the junction and the cell types (Braga et al. 1999). Cdc42, Rac1 and Rho are required for E-cadherin-mediated cell-cell adhesion in MDCK cells (Kuroda et al. 1997). Cdc42 and Rac1 negatively regulate the IQGAP1 function by inhibiting the interaction of IQGAP1 with β -catenin, leading to stabilization of the cadherin-catenin complex (Fukata et al, 1999). Moreover, adherent junctions are specifically protected by Rac1 signaling (Gopalakrishnan et al. 2002). However, Rac seems to have a more complex role, hyperactivation of Rac in keratinocytes leading to junction disassembly, and activation of Rac in MDCK cells plated on collagen promoted migration rather than cell-cell adhesion (Braga et al. 2000, Sander et al. 1998). Rac is involved in two seemingly opposing activities, namely cell-cell junction

assembly and cell migration; therefore it is likely that its effects will be greatly influenced by environmental factors and cell type.

Another member of the small GTPases, Rap1 was shown to be activated upon adherent junction disassembly that is triggered by E-cadherin internalization (Balzac et al. 2005). Interestingly, parallel to the activation of Rap1, Rac1 was shown to be inactivated by cell contact disassembly. Small GTPases are involved in the regulation of cell contact formation. On the other hand, cellular adhesion also regulates small GTPases.

Members of the Ras GTPase superfamily are regulated switches that control many intracellular pathways. The Ras family, which includes H-, K-, and N-Ras and other closely related isoforms, has been particularly associated with the control of proliferation in cells such as fibroblasts and epithelia. Ras was found to suppress SMA expression in vascular smooth muscle cells (Li et al. 1997). Moreover, H-Ras has been shown to inhibit Rho/ROK effects (de Godoy et al. 2007).

I.13. The p38 MAP kinase

Cellular behavior in response to extracellular stimuli (such as mitogenic stimuli, growth factors, cytokines, oxidative and osmotic stress) is mediated through the mitogen- activated protein kinase (MAPK) family, which contains four distinct subgroups: extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK/big MAP kinase 1 (BMK1), and p38 MAPK. MAPKs have both cytoplasmic and nuclear targets. MAPK cascades are composed of three sequentially activated kinases: MAPKs are activated upon phosphorylation by MAP kinase kinases (MAPKKs), which in turn are activated by MAP kinase kinase kinases (MAPKKKs). MAPK phosphatases reverse the phosphorylation and return the MAPKs to their inactive state.

p38 is involved in regulating cellular events during inflammation, apoptosis, cell cycle, development, cell differentiation and tumor suppression (Zarubin and Han 2005). p38 is activated by different cellular stresses, such as UV, heat shock and osmotic shock. TNF- α , IL, TGF- β 1, VEGF can also activate p38 (Ono and Han 2000). The p38 MAPK family consists of four isoforms: alpha, beta, gamma and delta. p38 isoforms have a determinant role in p38 signal specificity, as shown in the case of AP-1-dependent transcription (Pramanik et al. 2003). p38 MAPK was shown to mediate

Smad- independent TGF- β 1 signaling (Yu et al. 2002), however evidence of Smad-p38 cross-talks was also found (Leivonen et al. 2002). p38 is involved in regulation of SMA expression by TGF- β 1, as stated by Hu and colleagues, based on results obtained in human fetal lung fibroblasts (Hu et al. 2006). TGF- β 1 signaling requires p38 during TGF- β 1 induced fibroblastic transdifferentiation and cell migration, which is mediated by Rac1 (Bakin et al. 2002). Bagrodia and his colleagues showed earlier, that PAK (p21-activated kinase) and its upstream activator Cdc42 are potential regulators of p38 (Bagrodia et al. 1995).

p38 has an essential role in the PAK-p38 α MAPK-MAPKAP-K2-HSP27 signaling pathway in mediating the effects of chemotactic stimuli on cell migration (Rousseau et al. 2006) and control of cell cytoskeleton through the phosphorylation of HSP27 (Hedges et al. 1999). Keratinocyte migration has been also shown to be dependent on p38, a migration regulated by the Rho-ROCK-MEKK1-p38 pathway (Zhang et al. 2005).

Finally, SMA expression and myofibroblast differentiation are regulated by TGF- β 1 in an MK2 dependent manner, MAP kinase activated protein kinase 2 (MAPKAP2 or MK2) being a substrate of p38 that mediates p38 effects on actin cytoskeleton (Sousa et al. 2007).

I.14. Serum response Factor (SRF)

Serum response factor (SRF) is a nuclear transcription factor, which acts through binding to a consensus DNA sequence, the serum response element (SRE) (Treisman 1987). SREs contain the CArG domain (CC(A/T)₆GG), which is also found in the promoter region of more than 30 signaling molecules, transcription factors, cytoskeletal components and several muscle specific genes. Through binding to these sites in different promoters, SRF has been implicated in control of proliferation, migration, cytoskeletal dynamics and muscle differentiation. SRF exerts its regulatory effect through regulated nuclear translocation (Camoretti-Mercado et al. 2000), Rho being a regulator of its nuclear- cytoplasmic shuttling (Liu et al. 2003). SRF contains a unique and highly efficient nuclear localization signal (SRF-NLS) located at the N-terminal part of the protein (Gauthier-Rouviere et al. 1995).

SRF is activated by a variety of agents, such as serum, cytokines, TNF α . Several mechanisms have been shown to regulate its activity: association with cofactors

(Treisman 1994), phosphorylation-dependent changes in DNA binding (Manak and Prywes 1991), regulated nuclear translocation (Camoretti-Mercado et al. 2000), and alternative RNA splicing (Belaguli et al. 1999).

There are two principal pathways regulating SRF: the TCF, Ras dependent and the MAL dependent pathway (Posern and Treisman 2006). The ternary complex factor (TCF) family consists of Elk-1, SAP-1 and Net1. Activation of the MAP kinase pathway through Ras, Raf, MEK and ERK phosphorylates TCFs, which bind to their own Ets DNA recognition site and SRF.

SRF target genes are known to be governed by dynamic changes in the actin cytoskeleton (Miano et al. 2007). The small GTPase RhoA can activate SRF-mediated gene expression, the increase in SRF activity via RhoA occurred simultaneously with the depletion of globular (G) actin during filamentous (F) actin polymerization (Sotiropoulos et al. 1999). TGF- β 1 also regulates SRF activity, TGF- β 1-enhanced SRF-dependent transcription being inhibited by Smad7 (Camoretti-Mercado et al. 2006).

SMA was shown to contain contains two SREs (Kim et al. 1993), and is a target of SRF (Kim et al. 1994). Our group previously identified Rho as modulating SMA in an SRF dependent manner (Masszi et al. 2003).

Although SRF effects are well described, it remained intriguing how exactly SRF regulation occurs, since SRF is mainly localized in the nuclei of cells under basal conditions (Liu et al. 2003). The mechanisms by which Rho-actin signaling controls SRF remained unknown until the recent identification of MRTF.

I.15. Myocardin related transcription factors (MRTF)

The expression of smooth muscle specific genes in muscle cells and fibroblasts is controlled by serum response factor (SRF) and its recently discovered co-activators, myocardin and the myocardin-related transcription factors (MRTF), also called MAL (megakaryocytic acute leukemia), BSAC (composed of N-terminal basic, SAP {SAF-A/B, Acinus, PIAS}, and coiled-coil domains) or MKL (megakaryoblastic leukemia).

SRF regulates transcription of numerous muscle and growth factor-inducible genes. Since SRF is not muscle specific, it activates muscle genes by recruiting myogenic accessory factors. Myocardin was identified as a highly potent transcription factor, which is expressed in cardiac and smooth muscle cells. Myocardin is the founding member of a class of muscle transcription factors and provides a mechanism

whereby SRF can convey myogenic activity to cardiac muscle genes in *Xenopus* embryos (Wang et al. 2001). After myocardin, myocardin-related transcription factors A and B (MRTFs) were described to interact with SRF and stimulate its transcriptional activity (Wang et al. 2002). Cen et al. showed that megakaryoblastic leukemia 1 (MKL1), a potent transcriptional co-activator for serum response factor (SRF), is required for serum induction of SRF target genes in cellular models, as activation of SRF target genes may contribute to leukemogenesis (Cen et al. 2003). After mammalian cells, MRTF and SRF interaction was also shown in *Drosophila* proving that the interaction of MRTFs with SRF represents an ancient protein partnership (Han et al. 2004). MRTF-B was shown to be essential during smooth muscle differentiation (Oh et al. 2005), while MRTF-A is regulatory element for development of mammary myoepithelial cells in mice (Li S et al. 2006).

Myocardins were found to be implicated in several pathological conditions and diseases. Myocardin transcriptional activity is negatively regulated via phosphorylation of myocardin by glycogen synthase kinase-3 β , a known suppressor of hypertrophic signaling (Badorff et al. 2005). Moreover, myocardin transcript levels were found up-regulated in failing heart (Torrado et al. 2003). In leukemia MRTF-A is translocated and is involved in uncontrolled cell proliferation (Hsiao et al. 2005).

The members of the myocardin/MKL gene family contain a number of conserved domains: N-terminal domain (NTD), containing three RPEL motifs, basic domains B1 and B2, a glutamine-rich region (Q), a SAP domain (Scaffold attachment factor), a leucine zipper-like region (LZ) and a transcription activation domain (TAD).

The RPEL domains are critical for actin-MAL association, which are also required for the response to Rho signaling. Basic region B1 is essential for interaction with SRF, while both basic regions, B1 and B2, are required for effective nuclear accumulation of MAL. The Q box might mediate either export of MAL from the nucleus or its retention in the cytoplasm (Miralles et al. 2003), the Q-box being described as enhancing MAL-SRF interaction (Zaromytidou et al. 2006). SAP domains are found in a variety of nuclear proteins and have DNA binding properties (Kipp et al. 2000). Indeed, MAL is able to bind DNA and MAL-SRF complex formation is facilitated by direct MAL-DNA contact (Zaromytidou et al. 2006). The LZ domains are implicated in protein dimerization. In HeLa cells MKL2 could oligomerize with itself or MKL1 through the LZ domain (Selvaraj and Prywes 2003). TAD serves a general

function in transcriptional activation, but does not contribute to the specificity of these factors for SRF coactivation (Wang et al. 2001).

Rho mediated actin cytoskeleton reorganization has been long recognized as a key activator of SRF (Mack et al. 2001), however the underlying mechanisms remained undefined. New data suggests that SRF is activated by the Rho-dependent nuclear translocation of MRTF (Miralles et al. 2003). MAL was found to predominantly localize to the cytoplasm in serum-starved fibroblasts but is rapidly translocated to the nucleus upon serum stimulation. Moreover, a muscle-specific actin-binding protein striated muscle activator of Rho signaling (STARS) that activates SRF through a Rho-dependent mechanism, has been shown to activate SRF by inducing the nuclear translocation of MRTFs (Kuwahara et al. 2005). According to the current view, in quiescent cells MRTF is associated with monomeric (G) actin, a binding mediated by RPEL motifs in MAL (also required for Rho induced nuclear translocation), and its complex cannot enter the nucleus. Dissociation of MRTF upon actin polymerization leads to its nuclear translocation. Two Rho effector pathways have been involved in the mediation of this effect: increased actin polymerization via formin proteins (Copeland and Treisman 2002) and reduced F-actin severing by the LIM kinase-cofilin pathway (Geneste et al. 2002).

Through its muscle gene specificity and SRF activating effects, MRTFs came to our attention as potential regulators of the SMA expression, and thus, of EMT.

II. AIMS OF THE STUDY

The work described in this thesis focused on deciphering the complex regulation of SMA expression during EMT in tubular epithelial cells. Earlier our group described the “two-hit” model of EMT induction where the integrity of cell-cell contacts and TGF- β 1 together regulate mechanisms involved in this phenomenon. The main goal of the study was to study the intracellular pathways involved in the cell contact dependent and TGF- β 1 dependent modulation of SMA expression in renal tubular cells. The involvement of the Smad family of signaling proteins and p38 was analyzed during the TGF- β 1-dependent hit. On the other hand we hypothesized that cell contact disassembly would lead to an increase in contractility, an effect mediated by the Rho-ROK pathway. Therefore we analyzed the role of MLC in the regulation of SMA expression. Since small GTPases are major modulators of the cytoskeleton and cell contacts, we also analyzed the potential role of the Rho GTPase family (RhoA, Cdc42, Rac1) and the involvement of the cytoskeletal elements in SMA regulation. SRF and its regulation as a transcription factor by the synergistic effects of cell contact disassembly and TGF- β 1 was also examined in the context of the recently described transcriptional cofactor, MRTF.

The specific aims of the study were, as follows: in LLC-PK1 renal tubular cells we wished-

1. to study the role of Smads in regulating SMA expression upon TGF- β 1 treatment.
2. to study the role of Rho and ROK in mediating cell contact disruption induced SMA promoter activation.
3. to study the role of MLC phosphorylation in the Rho-ROK-SRF pathway.
4. to identify the role of the small GTPases Rac1, Cdc42 and their downstream effector, PAK, in mediating SMA expression.
5. to study the complex role of p38 in the cell contact and TGF- β 1 dependent regulation of SMA expression during EMT.
6. to investigate the role of MRTF in the regulation of SMA expression and SMA promoter activation.
7. to identify the potential signaling pathways through which small GTPases and the two hits regulate SMA promoter activity and expression.

III. MATERIALS AND METHODS

III.1. Materials and reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. Media, foetal bovine serum, trypsin-EDTA solutions, balanced salt solutions, penicillin/streptomycin solution were purchased from Gibco and Sigma. Human recombinant Transforming Growth Factor- β 1 (TGF- β 1) was purchased from Sigma-Aldrich. The ROK inhibitor Y-27632, the myosin ATPase inhibitor blebbistatin, the PAK inhibitor PAK18, the p38 inhibitor SB203580 and the actin-polymerizing agent jasplakinolide were purchased from Calbiochem (San Diego, CA). DAPI was obtained from Invitrogen. FuGENE6 was from Roche Molecular Biochemicals. ECL was from Amersham.

III.2. Cell culture and treatments

During our studies we used porcine proximal tubular epithelial cells (LLC-PK₁) stably expressing the rabbit angiotensin II receptor AT₁. The selected Cl4 clone of LLC-PK₁/AT₁ cells was a kind gift from Dr. R. Harris (Burns and Harris 1995). LLC-PK₁ cells were characterized by Hull and coworkers (Hull et al. 1976). EMT in this proximal epithelial tubular cell line was well characterized by our group in previous studies (Masszi et al. 2003). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4500 mg/l glucose), supplemented with 10% foetal bovine serum (FBS), 100 IU/ml of penicillin and 100 μ g/ml streptomycin. Cells were cultured at 37°C under 5% CO₂ in a humidified incubator.

Cells were grown on 6-well or 12-well plates, on glass coverslips for immunofluorescence microscopy, or 10 cm dishes, to either 100% confluence or subconfluence as indicated in the legend of the corresponding figures. Cells were then subjected to various treatments. For acute Ca²⁺ removal, cells were preincubated in an isotonic NaCl-based medium (140 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM glucose, 20 mM HEPES, pH 7.4) for 10 minutes and then the medium was replaced with the same basic solution lacking CaCl₂ and supplemented with 1 mM EGTA. For chronic Ca²⁺ deprivation, the cells were washed 4 times with Phosphate-buffered saline (PBS), and once with serum- and Ca²⁺-free DMEM followed by incubation in the latter

solution. Control samples were incubated with serum-free DMEM containing Ca^{2+} . Where applied, TGF- β 1 (5 or 10 ng/ml, vehicle for controls) was added to cells for times specified at the individual experiments. For inhibitor studies, cells were preincubated for 45 minutes or 1 hour with 10 μM Y-27163, 50-100 μM blebbistatin, 1, 5 or 10 μM SB203580, 20 μM PAK18. Jaspilakinolide was used in 0.5 μM concentration for 12 hours.

III.3. Antibodies

Antibodies were purchased from:

- Cell Signaling Technology (Danvers, MA): monophospho-MLC, phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-PAK1/2, phospho-cofilin, cofilin
- Chemicon (Temecula, CA): histones
- Cytoskeleton Inc. (Denver, CO): rhodamine-labeled phalloidin
- Jackson ImmunoResearch Laboratories (West Grove, PA): FITC- and Cy3-labeled, horseradish-peroxidase-conjugated anti-mouse, anti-rabbit, anti-goat secondary antibodies
- Roche Molecular Biochemicals: rat monoclonal anti-HA 3F10
- Santa Cruz Biotechnology (Santa Cruz, CA): SRF, Myc (9E-10), fluorescein isothiocyanate (FITC)- conjugated Myc, Cdc42
- Sigma: α -SMA (1A4), β -actin, FLAG, tubulin
- Upstate Biotechnology (Lake Placid, NY): Rac1
- Zymed Laboratories Inc. (San Francisco, CA): Smad2, Smad3

Antisera against phospho-Smad2 (PS2) and phospho-Smad1 (PS1) which shows cross-reactivity with phosphorylated Smad3, were kind gifts from Dr. A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden) (Piek et al. 1999c, Dooley et al. 2001).

The polyclonal anti- α -BSAC antibody raised against the mouse MKL1 protein was described previously (Sasazuki et al. 2002).

III.4. Plasmids

III.4.1. Promoter constructs

The p765-SMA-Luc vector was a kind gift from Dr. R. A. Nemenoff (Dep. of Medicine, University of Colorado). It contains a 765 bp. (-713/+52) long fragment from the rat α -smooth muscle actin promoter, subcloned into the PA₃-Luc firefly luciferase plasmid (Garat et al. 2000). The fragment contains several cis-elements including the serum response element binding motifs (CArG A and CArG B boxes), a TGF- β 1 control element (TCE), a TATA box, and two E-boxes. In certain experiments we used the pGL₃-SMA-Luc plasmid which harbors the same promoter region as the previous plasmid, along with its shorter, 152 bp. long version containing only a CArG A, CArG B, TCE and TATA box (provided by Dr. S. H. Phan, University of Michigan Medical School, Ann Arbor) (Hu et al. 2003).

The SBE4-Luc reporter plasmid which containing four tandem repeats of the SMAD-binding element was a kind gift of Dr. A. B. Roberts (National Institutes of Health, Bethesda) (Felici et al. 2003).

The thymidine kinase- driven Renilla luciferase vector (pRL-TK, Promega) was used as an internal control for transfection efficiency.

III.4.2. Expression vectors

The pSmad7 expression construct was a kind gift from Dr. E. P. Böttinger (Albert Einstein College of Medicine, Bronx, New York) (von Gersdorff et al. 2000). The vector encoding dominant negative (DN) Smad3 was described previously (Mucsi and Goldberg 1997).

Plasmids (pcDNA3.1) encoding the C-terminally His- and Myc-tagged wild type myosin regulatory light chain-2 (WT-MLC) and its dominant negative version in which T18 and S19 were replaced with alanine (DNMLC), were kind gifts from Dr. H. Hosoya (Dept. Biological Sciences, Hiroshima University) (Iwasaki et al. 2001, Di Ciano-Oliveira et al. 2005).

FLAG-tagged MRTF-A, MRTF-B and the dominant negative truncation mutant (Δ C585) of myocardin were kindly provided by Dr. E. N. Olson (Dept. Molecular Biology, University of Texas), and were described previously (Wang et al. 2001).

Vectors encoding for Myc-tagged constitutive active RhoA (Q63L, CA-Rho) and dominant negative RhoA (T19N, DN-Rho) were described and used in previous studies from our group (Masszi et al. 2003). GFP-tagged H-Ras and DN-H-Ras vectors were described previously (Choy et al. 1999). CA Rac1, DN-Rac1, CA Cdc42 and DN-Cdc42 plasmids were a kind gift from dr. G. Downey. The constitutively active (Q61L) and dominant-negative (T17N) mutants of both Rac1 and Cdc42 are NH₂ terminally Myc tagged and were previously described (Zhang et al. 1995). CA-PAK1 (H83,86L/T422E) and DN-PAK (H83,86L/K299R) plasmids were a kind gift from dr. A.S. Mak and were previously described (Sells et al. 1999, Webb et al. 2005). A DN form of p38, the p38AF (T180A) plasmid was a kind gift from dr. A. Klip and was previously described (Huang et al. 1997, Li Z et al. 2006).

III.5. Transient transfections and luciferase promoter activity assays

Cells were grown on 6-well plates and transfected at subconfluence or 100% confluence using 2.5 µl FuGENE 6 (Roche) reagent/ 1 µg plasmid DNA. Transfections were carried out using 0.5 µg of the pSMA-Luc (or pGL₃-SMA-Luc) luciferase reporter plasmid, 0.05 µg pRL-TK and 2 µg of either empty vector (pcDNA3.1) or constitutive active/ dominant negative/ wild type expression vector. The required amount of FuGene 6 was added to serum- and antibiotics- free OptiMEM medium and incubated for 5 minutes. This mixture was added to the mixed plasmid DNA and was further incubated for 15 minutes. 100 µl of the DNA-FuGene 6- OptiMEM cocktail was added to cells in each well. Cells were washed 16 hours later three times with PBS, and after 4 hours of serum depletion, cells were treated for 16 hours with TGF-β1 or its vehicle. When stimulating with Ca²⁺-free conditions, cells were washed 24 hours after transfection, and incubated for further 24 hours in serum-free medium either containing or lacking Ca²⁺. Cells were then washed on ice with cold PBS, and scraped in 500 µl Passive Lysis Buffer (Promega). Samples were then subjected to a cycle of freezing (-80°C)/ thawing (+37°C), and then clarified by centrifugation (12,000 RPM, 5 minutes at 4°C). Firefly and Renilla luciferase activities were measured from the supernatant by the Dual-Luciferase Reporter Assay Kit (Promega) according to the instructions of the manufacturer. The measurements were executed using a Berthold Lumat LB 9507 luminometer by adding 100 µl of each buffer to 20 µl of the sample. In order to minimize variability caused by difference in cell numbers or by transfection efficiency,

results were normalized by dividing the Firefly luciferase activity with the Renilla luciferase activity of the sample. For each condition duplicate or triplicate measurements were performed, and experiments were repeated at least three times.

In case of transfections for immunofluorescence microscopy, 1-2 μg of plasmid/coverslip was transfected under the same conditions.

III.6. Recombinant adenoviruses

Recombinant replication-deficient adenovirus RAdLacZ, which contains the *Escherichia coli* β -galactosidase gene under the control of the cytomegalovirus immediate early promoter, was kindly provided by Dr. Gavin W.G. Wilkinson (University of Cardiff) (Wilkinson and Akrigg 1992). Recombinant adenoviruses coding for dominant negative Smad3 (RAdSmad3DN) (Pardali et al. 2000) and the wild-type human SMAD7 (Fujii et al. 1999) adenovirus were kindly provided by Dr. A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). The adenovirus for constitutively active MEK1 (RAdMEK1CA) (Foschi et al. 1997) was provided by Dr. M. Foschi (University of Florence). Adenoviruses for constitutively active MKK3b (RAdMKK3bE), constitutively active MKK6b (RAdMKK6bE), dominant negative p38 α (RAdp38 α AF) (Wang et al. 1998) and dominant negative p38 β (RAdp38 β AF), dominant negative MKK3b (RAdMKK3bA) and MKK6b (RAdMKK6bA) were all kindly provided by Dr. Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA). Replication-deficient (E1- and E3-) adenoviruses RAdSmad2, RAdSmad3 harboring human Smad2 and Smad3 cDNAs, respectively, with an N-terminal hemagglutinin (HA) tag, were described by Leivonen et al. (Leivonen et al. 2002).

III.7. Infection of cells with recombinant adenoviruses

Cells were infected in suspension with the adenoviruses at 1 MOI (multiplicity of infection, e.g. in this case one viral particle infecting one cell) in DMEM with 1% FCS, then plated and incubated for 18 h. Subsequently the medium was replaced with fresh 1% FCS DMEM. 6 hrs later 5 ng/ml TGF- β 1 was added for the time indicated. The cells then were harvested in SDS sample buffer and analyzed by Western blotting.

III.8. Rho activity assay

Rho activation was assessed by an affinity pull-down assay. The preparation of glutathione-S-transferase-Rho-binding domain beads was described previously (di Ciano-Oliveira et al. 2003). Cells were grown on 10 cm dishes. After the indicated treatments, cells were lysed in 800 μ l of cold Rho lysis buffer (100 mM NaCl, 50 mM Tris-Base (pH 7.6), 20 mM NaF, 10 mM MgCl₂ and 1% Triton X-100) supplemented with 0.5% deoxycholic acid, 0.1% SDS, 20 μ l/ml protease inhibitor cocktail, 1 mM Na₃VO₄, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation (12,000 RPM, 1 minute at 4°C), glutathione-sepharose beads (10-15 μ g/sample) covered with GST-Rho-binding domain (RBD) fusion protein were added to the supernatants and incubated at 4°C for 45 min. The GST-RBD beads were pelleted by quickspin and washed three times with lysis buffer, then were boiled in 25 μ l of 2x Laemmli sample buffer. Samples were subjected to electrophoresis on 15% SDS-polyacrylamide gels followed by Western blotting using an anti-Rho antibody. Total Rho was assessed from samples obtained from the supernatant after lysis and centrifugation.

III.9. Rac1/Cdc42 activity assay

Rac1 and Cdc42 activity assay was performed using the PAK-GST Protein Beads from Cytoskeleton Inc. (Denver, CO), following the instructions of the manufacturer. Cells were grown on 10 cm dishes. After the indicated treatments, cells were scraped in 600 μ l of cell lysis buffer supplemented with 20 μ l/ml of protease inhibitor cocktail. Samples were then clarified by centrifugation (12,000 RPM, 5 minutes at 4°C). Supernatant was added 60 μ l loading buffer and 20 μ l of the PAK-GST beads. Samples were then rotated 1 hour at 4°C. After this step, samples were centrifuged at 8,000 RPM, 1 minute at 4°C, washed two times in 500 μ l of wash buffer, finally being boiled 5 minutes in 25 μ l of 2x Laemmli sample buffer. Samples stimulated with GTP γ S and GDP were added 6 μ l of the substances after the addition of the loading buffer, and incubated 15 minutes at RT. The preparation followed with the rotation after the addition of 60 μ l of stop buffer and 20 μ l of the beads. Total Rac was assessed from 15 μ l of supernatant with the addition of 15 μ l of 2x Laemmli sample buffer. Samples were subjected to electrophoresis on 12% SDS-polyacrylamide gels

followed by Western blotting using an anti-Rac1 antibody. The Cdc42 activity was determined by re-probing the membrane previously assessed for active Rac1.

III.10. Western Blotting

Cells were grown on 3 cm dishes to 100% confluence. Cells were stimulated either by TGF- β 1 or by Ca²⁺- removal. Cells were washed with cold PBS, and scraped into Triton Lysis Buffer (30 mM HEPES, (pH 7.4), 100 mM NaCl, 1 mM EGTA, 20 mM NaF, 1% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulphonyl fluoride, 20 μ l/ml protease inhibitory cocktail). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Samples were added 2x Laemmli sample buffer in 1:1 ratio and boiled for 5 min. For pMLC blots, the cells were lysed in ice-cold acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol, followed by centrifugation for 10 min at 12,500 rpm at 4°C. The resulting pellet was washed with pure acetone, allowed to air dry, and dissolved in 60 μ l of Laemmli sample buffer. Equal amounts of protein were separated on 10 or 12 % SDS-polyacrylamide gels using the Mini Protean II and III apparatuses (Bio-Rad). Samples were run at 70 and then at 100 V. Proteins were then transferred to nitrocellulose membranes at 350 mA for 90 minutes. Blots were blocked with Tris-buffered saline (TBS) containing 0.1% TWEEN 20 and 5% albumin for an hour. Membranes were incubated overnight at 4°C with the primary antibody (generally at 1:1000 dilution), washed 3 times 10 minutes and then incubated for 90 minutes with the corresponding peroxidase-conjugated secondary antibody (generally at 1:2000 dilution). After final washes immunoreactive bands were visualized by the enhanced chemiluminescence reaction.

III.11. Immunofluorescence microscopy

Cells grown on 25 mm sterile coverslips were fixed with 4% paraformaldehyde for 30 min, washed with PBS and incubated with 100 mmol/L glycine in PBS for 10 min. Cells were then permeabilized in PBS containing 0.1% Triton X-100, blocked for an hour with 3% albumin, and incubated with the primary antibody or antibodies (in case of co-staining) for 1 h. After extensive washes, fluorescently labeled secondary antibodies were added for another hour. Nuclei were visualized by DAPI staining. The coverslips were washed and then mounted on slides using Fluorescence Mounting

Medium (DAKO). When directly labeled, FITC-conjugated mouse anti-Myc antibody was used together with another mouse primary antibody, the cells were initially processed for staining with the unlabeled primary and corresponding secondary antibodies, blocked again with mouse serum (1:100), and then incubated with the directly labeled primary antibody for an hour. Samples were analyzed by an Olympus IX81 microscope (60x or 100x objectives) coupled to an Evolution QEi Monochrome camera, controlled by the QED InVivo Imaging software. Images were processed by the ImagePro Plus 3DS 5.1 software. Bars on the microscopic images correspond to 20 μm .

In case of Figure 15B, cells grown to subconfluence were pretreated with the indicated inhibitors and treated with TGF- β 1 for 3 days. After 4 days of incubation cells were washed with cold PBS and fixed with methanol at -20°C for 6 minutes. Cells were then stained with the appropriate primary and secondary antibody, and with Hoechst 33342 for nuclear visualization. Samples were analyzed by a Leica microscope.

III.12. Wounding assay

Cells were grown to total confluence on coverslips. After serum deprivation the surface of the coverslip was scraped with a rubber policeman under sterile conditions, leaving 1-3 mm wide gaps in the confluent monolayer. Cells were fixed 6 hours after wounding and then stained for immunofluorescent microscopy.

III.13. Nuclear extraction

Nuclear extracts were prepared from confluent layers of LLC-PK1 cells grown on 10-cm dishes, using the NE-PER® Nuclear Extraction Kit from Pierce Biotechnology (Rockford, IL) according to the manufacturer's recommendation. After sequential steps of extracting by vortexing and centrifuging with CER I and II (Cytoplasmic Extraction Reagent) and NER (Nuclear Extraction Reagent), nuclear extracts were collected, their protein concentration determined, and samples of equal protein content were analyzed by western blotting. Anti-histones antibody was used to check for equal loading of nuclear proteins.

III.14. Statistical analysis

All experiments were repeated at least three times. Data are presented as the means \pm SD for the number of experiments (n) indicated. In case of western blot and immunofluorescence experiments representative images are shown. Statistical significance was determined by Student's t-test or one-way ANOVA using the GraphPAD InStat software.

III.15. Quantification of nuclear/cytoplasmic distribution of proteins

Staining was quantified using the ImagePro Plus software: fluorescence intensities were determined at three random nuclear and cytoplasmic points along a line, or in 3 equal rectangular areas within the nucleus or the cytoplasm. An average of 3 determinations/ cell was used, and the nuclear/cytoplasmic ratio calculated. Ratios measured along lines or within rectangular areas were identical. Nuclei were independently visualized by DAPI staining. MRTF distribution was categorized as cytosolic or nuclear when the nucleus was clearly demarcated either by exclusion or accumulation of the label. Otherwise the distribution was regarded as even (or pancellular). To make these categories exact, distribution data were verified using the nuclear/cytoplasmic ratios as < 0.75 (cytosolic), $0.75-1.25$ (even) and > 1.25 (nuclear). In the vast majority of cells within the nuclear category the ratio was >2 .

IV. RESULTS

IV.1. Smad2 and Smad3 are involved in the regulation of TGF- β 1 induced SMA promoter activation and protein expression in renal tubular cells

Several TGF- β 1 induced effects are transmitted from the cell membrane to the nucleus through the Smad family of signaling proteins. The SMA promoter was shown to contain Smad dependent domains; therefore the role of receptor regulated Smad proteins in mediating the effects of TGF- β 1 on SMA expression and EMT was assessed.

First, we wished to address whether TGF- β 1 activates the receptor regulated Smad proteins in LLC-PK1/AT1 cells. For this confluent cells were serum-starved for 24 hrs, subsequently 5 ng/ml TGF β 1 being added for the indicated times. Smad2 and Smad3 were phosphorylated upon TGF- β 1 treatment, however showing different responses in time. Followed by Western blot on a short time course, both Smad2 and Smad3 were phosphorylated 15 minutes after the treatment, and the effect was at its maximum after 60 minutes, followed by a quicker (in case of pSmad3) or slower (in case of pSmad2) decrease to the baseline level. When followed on a longer time course, activation of Smad3 was transient, dropping and disappearing 4 hours after the treatment. In contrast, Smad2 activation followed a biphasic pattern. After the initial 1 hour peak of activation, the signal returned towards the baseline level, followed by a second activation wave 12 hours after the treatment, on up to 96 hours (Figure 2).

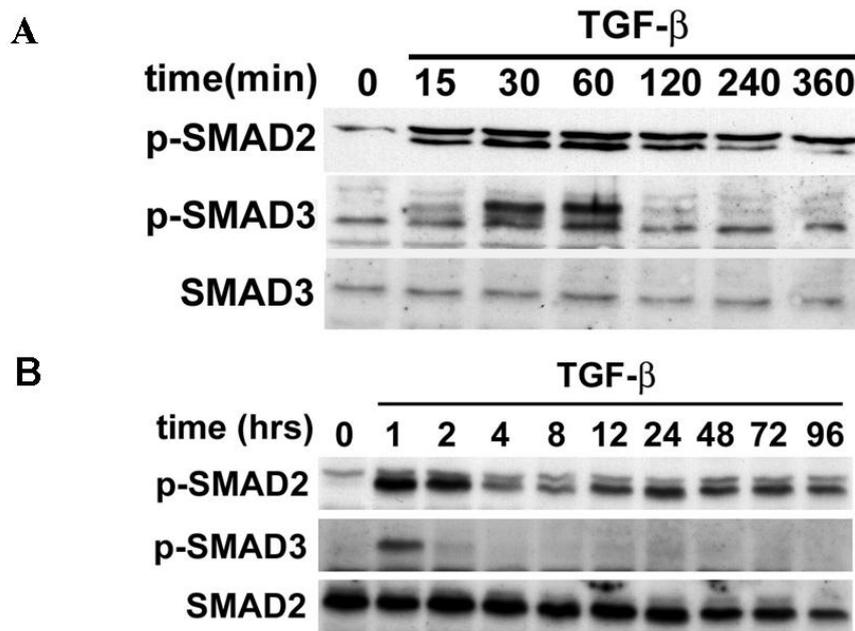


Figure 2. *Smad2 and Smad3 are phosphorylated upon TGF- β 1 treatment.* Cells grown on 6 cm dishes were treated with 5 ng/ml TGF- β 1 for the indicated times. Whole cell lysates were prepared and Western blots were performed using phospho- Smad2 and Smad1 (cross-reacting with phosphorylated Smad3) antibodies. Loading controls obtained by re-probing with the non-phosphorylated Smad3 (A) or Smad2 (B) confirmed equal loading. Activation of Smad2 and Smad3 is presented in a short (A) and a long (B) time course.

In order to establish the role of Smads in regulating α -SMA expression upon TGF- β 1 treatment, we used two different experimental approaches. Smad transcriptional activity can specifically be blocked by using dominant negative mutants of the R-Smad proteins, such as the DN-Smad3 construct. Expression, overexpression of wild type Smad7 also inhibits Smad signaling by preventing the phosphorylation of R-Smads. First, their role was assessed in transient transfection experiments, where the SMA promoter luciferase construct was cotransfected with expression vectors encoding for Smad7 and DN-Smad3. TGF- β 1 treatment induced a 5.7 fold increase in SMA promoter activity in subconfluent cells. This effect was inhibited by both Smad7 and DN-Smad3 (Figure 3).

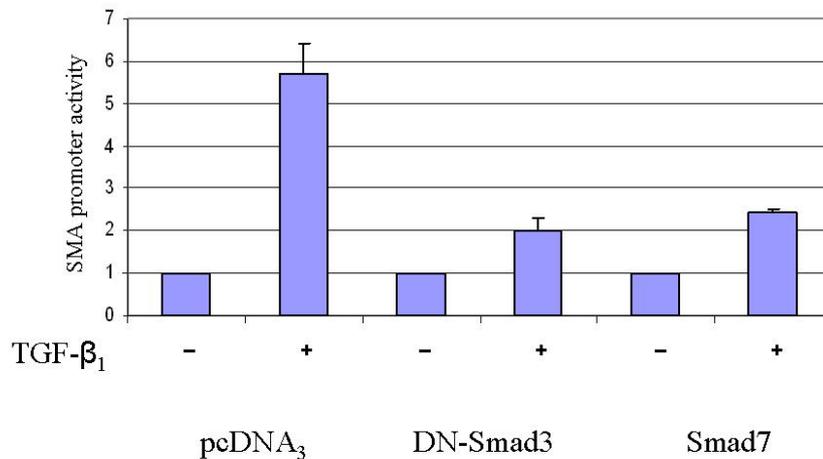


Figure 3. *Smads contribute to the transcriptional activation of the SMA promoter induced by TGF-β1.* Cells grown on 6-well plates were transiently co-transfected with the p765-SMA-Luc promoter, the Renilla luciferase vector (pRL-TK), and either the empty pcDNA3 vector, the DN-Smad3 expressing construct or the Smad7 expressing vector, using FuGene6 transfection reagent. Cells were treated with 5 ng/ml TGF-β1 for 20 hours. Results were normalized to values obtained by the Renilla measurements for each sample. Results are shown as fold stimulation by TGF-β1 in each group. The effect of TGF-β1 on the pcDNA3 transfected group was 5.68 ± 0.71 , while the presence of DN-Smad3 (1.99 ± 0.31) and Smad7 (2.45 ± 0.07) inhibited this effect ($n=6$, $p < 0.05$).

Subsequently we wished to confirm the effect of inhibitory Smads on SMA protein synthesis level. For this cells were infected with FLAG-tagged adenoviral expression vectors encoding Smad7 or DN-Smad3. Unlike the rather low efficiency of transient transfections, adenoviral vector experiments have the major advantage to theoretically infect all cells in a culture, and as such, to introduce the desired modifications to all cells.

Control cells were infected with the empty vector RAdLacZ. Three days after TGF-β1 treatment control cells exhibited SMA protein expression, assessed by Western blot. Over-expression of both FLAG-tagged Smad7 and FLAG-tagged DN-Smad3 completely abolished the TGF-β1 induced SMA protein synthesis (Figure 4). These results confirmed the proposed role of Smads in this mechanism.

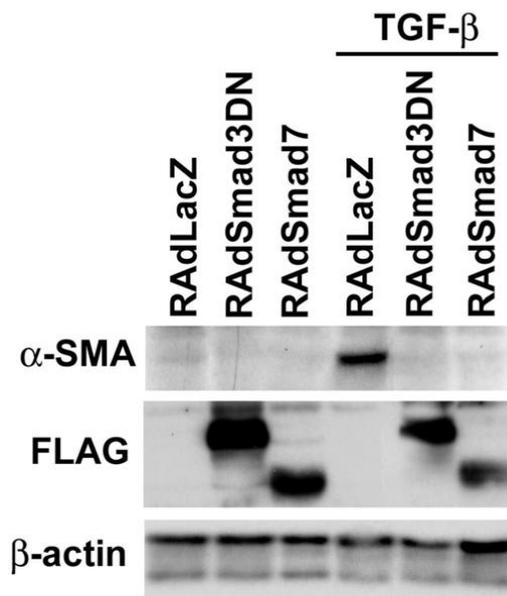


Figure 4. *Smads contribute to TGF- β 1-induced SMA protein expression.* Cells were infected in suspension with recombinant replication deficient adenoviruses RAdSmad3DN and RAdSmad7 (1 MOI) and were treated with 5 ng/ml TGF- β 1 or vehicle for 3 days. Cells were then harvested in SDS sample buffer and analyzed by Western blotting. Membranes were probed for α -SMA and re-probed for FLAG to visualize the presence of the adenoviruses and β -actin to confirm equal loading.

IV.2. Rho and ROK are key mediators of contact disassembly- induced activation of the SMA promoter. Contact disassembly induces Rho/ROK dependent myosin phosphorylation

Rho was shown to stimulate SMA expression in smooth muscle cells and fibroblasts (Mack et al, 2001) and its role in TGF- β 1 induced SMA expression during EMT was also described (Masszi et al, 2003). In order to dissect the role of Rho during EMT and the “two-hit” mechanism, we first examined whether constitutively active Rho can activate the SMA promoter and whether cell contact disruption can modulate Rho activity. Then we examined whether the interference with a dominant negative Rho construct can eradicate the induction of SMA promoter activation by cell contact disruption.

Confluent cells were transfected with the SMA promoter construct and the empty pcDNA3 vector for the control or with the CA-Rho construct for 24 hours and then subjected to serum removal for 24 hours. Indeed, cotransfection of the CA Rho

construct induced a 35.9 fold increase in SMA promoter activity, indicating that Rho is a potent modulator of SMA expression (Figure 5A).

Next we wished to assess whether the disassembly of cell-cell contacts by Ca^{2+} removal affects Rho signaling in LLC-PK1 cells. During this experiment, active (GTP-bound) Rho was detected with an affinity pull-down assay. Cells were subjected to an acute Ca^{2+} removal by adding a solution containing 1 mM EGTA for 5 minutes, which rapidly disrupted the intercellular contacts. Ca^{2+} -free environment caused a rapid and robust, 3 fold increase in Rho activation (Figure 5B).

Next confluent cells were transfected with the SMA reporter construct and the pcDNA3 empty vector, followed by serum removal and medium exchange to DMEM either containing or lacking Ca^{2+} . Cell-cell contact disruption by Ca^{2+} removal in confluent monolayers induced a 6-10 fold increase on the activity of the transfected SMA-Luc promoter. When cotransfecting a Myc epitope- tagged dominant negative (T19N) Rho construct (DN-Rho) with the promoter, it eradicated the effects of the Ca^{2+} removal on the promoter, while it had no significant effect on the basal promoter activity (Figure 5C). These data suggest that Rho is indeed involved in the regulation of the expression of SMA, not only in the TGF- β 1 induced effects, but also in the cell contact dependent effects.

The downstream effector of Rho, ROK was also examined in this context. When cells were pretreated with the specific ROK inhibitor, Y-27632, the effects of cell contact disruption on the SMA promoter by Ca^{2+} removal was also abolished (Figure 5D).

Myosin light chain came to our attention as a downstream effector of Rho and ROK. The disassembly of cell-cell contacts by Ca^{2+} not only activated Rho, but affected cells exhibited a large increase in their staining for the monophosphorylated myosin light chain (pMLC) following the same acute conditions (Figure 6A/b). Monophospho-MLC staining was observed predominantly at the periphery of Ca^{2+} removed cells. Cells were stained for pMLC after a chronic Ca^{2+} removal where the normal serum free medium was replaced with serum and Ca^{2+} -free DMEM for 24 hours. MLC showed intense phosphorylation under chronic Ca^{2+} removal conditions, too (Figure 6A/c). Characterization of the MLC phosphorylation revealed that this effect was a sustained response, since under Ca^{2+} -free conditions peripheral pMLC levels remained elevated in about 60% of the cells for days throughout the duration of the transfection and promoter studies (Figure 6B).

We hypothesized that Rho and ROK were required for monophosphorylation of MLC. First cells were transfected with the CA Rho construct. 24 hours after transfection cells transfected with CA Rho showed marked MLC phosphorylation, pMLC being organized in fiber-like structures along the cells (Figure 6A/d,d'). Then cells were transfected with the DN Rho construct, and 24h later cells were subjected to a 24h Ca^{2+} removal. In immunofluorescent studies we found that DN Rho prevented the injury-induced increase in pMLC: more than 60% of control Ca^{2+} removed cells showed peripheral myosin phosphorylation, whereas this response was negligible in DN-Rho expressing cells (Figure 6A/e,e'). Similarly, the Rho kinase inhibitor Y-27632 abolished the MLC phosphorylation (Figure 6A/f), indicating that the Rho-mediated ROK activation was indispensable in this mechanism.

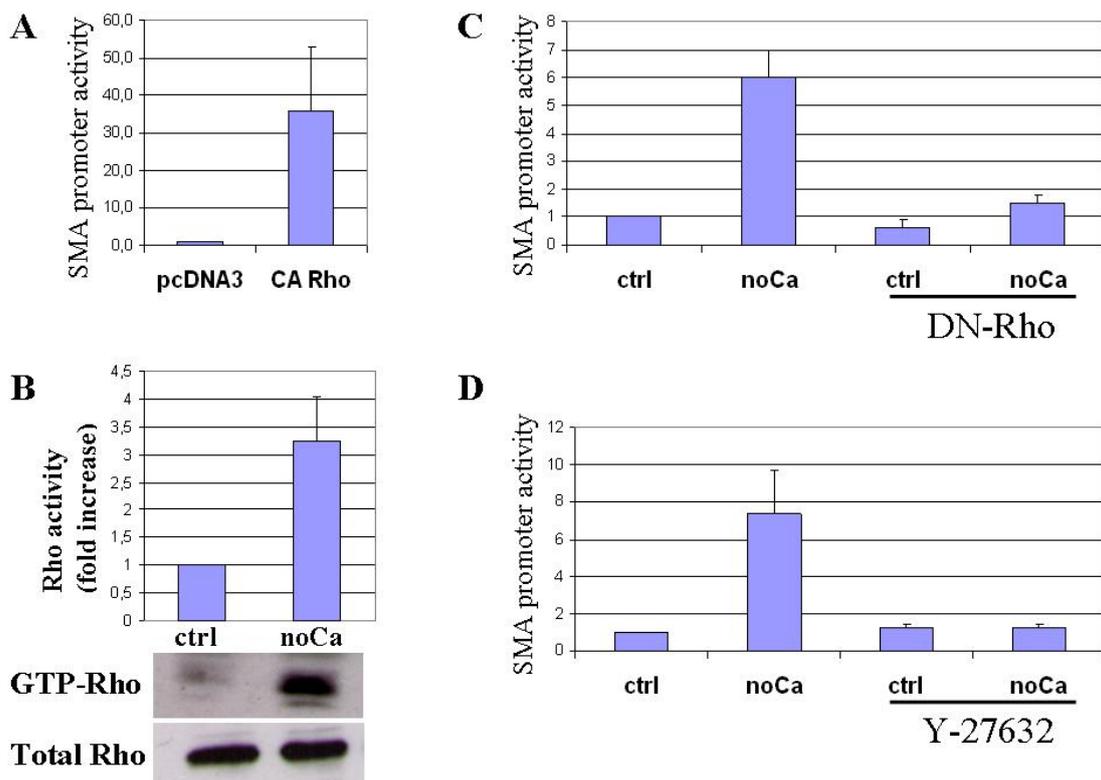


Figure 5. *Rho and ROK mediate contact disassembly induced SMA promoter activation.* (A). Confluent cells were transfected with pSMA-Luc and pRL-TK along with either empty vector (pcDNA3.1) or with CA Rho. After 24 hours cells were serum depleted and incubated for an additional 24 hours. CA Rho induced a massive activation of the SMA promoter. (B). Confluent LLC-PK1 cell cultures were serum-starved for 3 h and then pre-incubated with a Ca^{2+} containing NaCl-based medium for 10 min. Subsequently the medium was aspirated and either replaced with the same solution (control) or with a Ca^{2+} -free solution containing 1 mM EGTA (noCa) to rapidly disrupt the intercellular contacts. Five minutes later cells were lysed, and samples of equal

protein content were subjected to the Rho activity assay as described in Materials and Methods. Total Rho was determined from the same lysates. One representative blot of three separate experiments is shown. Densitometry (bars) was performed for each experiment, and Rho activation was expressed as fold increase compared to the control. **(C)**. Confluent cells were transfected with pSMA-Luc and pRL-TK along with either empty vector (pcDNA3.1) or with DN-Rho (see Materials and Methods). After 24 h the cells were incubated in serum-free (control-ctrl) or serum- and Ca^{2+} -free DMEM (noCa) for an additional 24 h, followed by determination of luciferase activity. Ca^{2+} removal induced a 6 ± 0.94 fold increase in SMA promoter activity, which was inhibited by DN-Rho, 1.5 ± 0.26 ($p < 0.05$). **(D)**. The same conditions as in **C**, except cells were pretreated for 30 min before Ca^{2+} depletion with vehicle or 10 μM Y-27632. The initial 7.36 ± 2.29 fold stimulation by Ca^{2+} removal was inhibited by the specific ROK inhibitor, 1.23 ± 0.15 ($p < 0.05$)

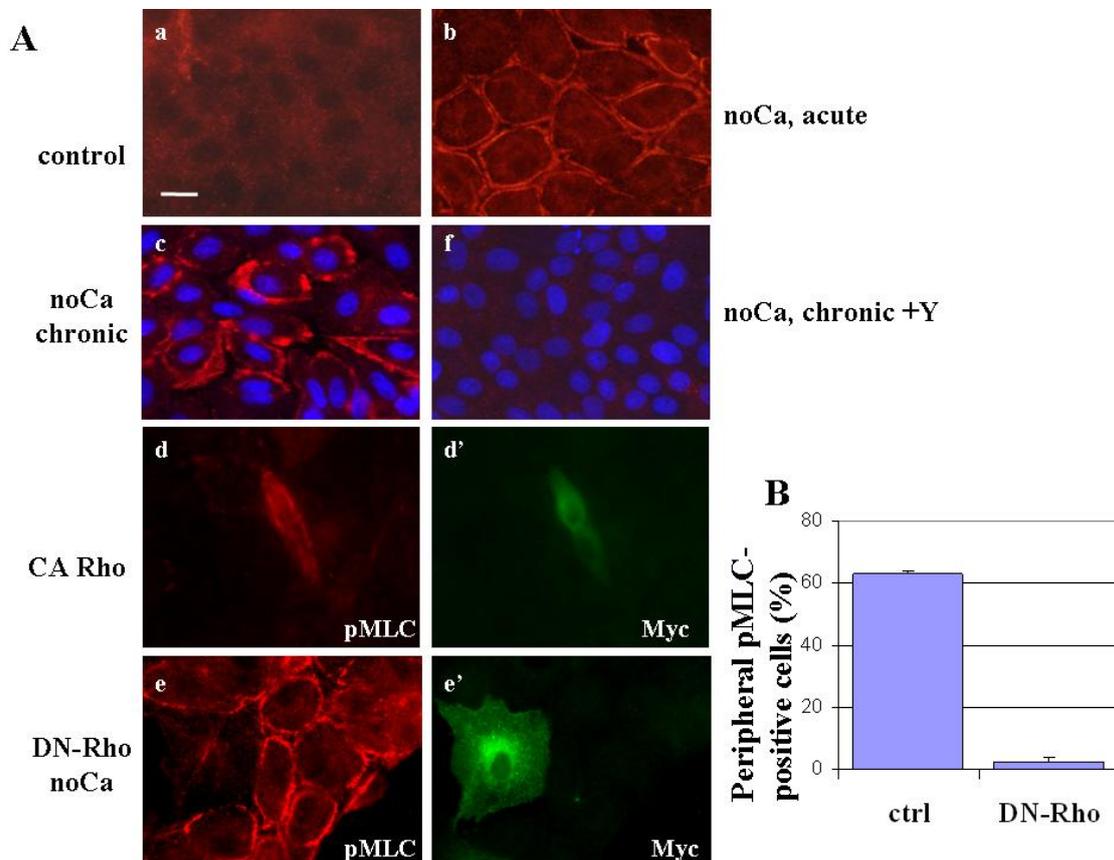


Figure 6. Contact disassembly induces Rho/Rho kinase-dependent myosin light chain phosphorylation. **(A)** LLC-PK1 cells were grown on coverslips to confluence, and after various treatments were stained with anti monophospho-MLC antibody: (a) No treatment; (b) cells were exposed to acute Ca^{2+} removal for 5 min using EGTA; (c and f) for chronic Ca^{2+} removal, the normal, serum-free DMEM was replaced with Ca^{2+} -free DMEM for 24 h. Thirty minutes before Ca^{2+} removal cells were pre-incubated with vehicle (c) or 10 μM Y-27632 (f), which remained present throughout the whole experiment. To visualize cells, nuclei were stained with DAPI; (d and d') cells grown to confluence were transfected with Myc-tagged CA Rho for 24 h, then cells were serum deprived for additional 24 hrs, and double stained for monophospho-MLC (red) and

Myc (green) to visualize the Rho transfected cells; (e and e') cells grown to confluence were transfected with Myc-tagged DN-Rho for 24 h, exposed to Ca^{2+} -free conditions for an additional 24 h, and then double stained for monophospho-MLC (red) and for the Myc epitope (green). **(B)** The frequency of peripheral phospho-MLC staining was quantified in control and DN-Rho expressing cells. More than 60% of controls cells showed peripheral myosin phosphorylation, whereas this response was negligible in DN-Rho expressing cells. (n=3, in each experiment >60 cells were counted in each cell population).

IV.3. Myosin phosphorylation plays an important role in the Ca^{2+} removal-induced activation of the SMA promoter and in the regulation of SMA protein expression

Rho is known to be involved in the regulation of SRF-dependent gene expression; however the downstream pathways mediating this effect have not been entirely elucidated. Particularly, the potential role of MLC activity or phosphorylation has not been addressed. By modulating cellular contractility, MLC was recently shown to be involved in wound healing. After showing the robust MLC activation upon contact disassembly and the role of Rho and ROK in this effect, we proposed to determine whether MLC phosphorylation contributes to the activation of the SMA promoter. First, pretreatment with blebbistatin, a specific inhibitor of myosin ATPase (Straight et al. 2003), prevented the activation by Ca^{2+} removal of the SMA promoter. Pretreatment with blebbistatin reduced the modest increase in SMA promoter activity upon TGF- β 1 treatment of confluent layers. The combined Ca^{2+} removal and TGF- β 1 treatment led to a larger increase in promoter activity, which in synergism is about the multiplication of the two effects. Blebbistatin fully eliminated the major activation of the promoter by the combined treatment (Figure 7A).

Next cells were transfected with a construct encoding for a Myc epitope- tagged, non-phosphorylatable myosin mutant, DN MLC, in which the critical target residues T18 and S19 were exchanged with phenylalanine (AA-MLC). This approach offers the advantage over blebbistatin in that it prevents myosin phosphorylation and activation without interfering with basal myosin ATPase activity. Transfection of cells with this construct prevented the Ca^{2+} removal induced peripheral MLC phosphorylation, proving that this DN MLC construct indeed functioned as dominant negative MLC (Figure 7B). Cotransfection of cells with DN MLC and SMA promoter led to the abolition of the Ca^{2+} deprivation induced increase in promoter activity. Moreover DN MLC reduced the

synergistic effect of the combination of Ca^{2+} removal and TGF- β 1 treatment (Figure 7C). To verify that the type of the reporter plasmid vector was not critical, and that the observed effect was indeed exerted on the promoter, we repeated these experiments using an alternative (pGL3) plasmid harboring the same 765-base pair promoter sequence. DN-MLC effectively inhibited the Ca^{2+} depletion-induced luciferase response in this system as well. To show that the mutation of MLC is indeed the determining factor for the inhibitory effect, cells were transfected with the Myc-tagged wild-type MLC. Overexpression of WT MLC had no effect on the basal promoter activity and did not alter its activation by Ca^{2+} removal (Figure 7D).

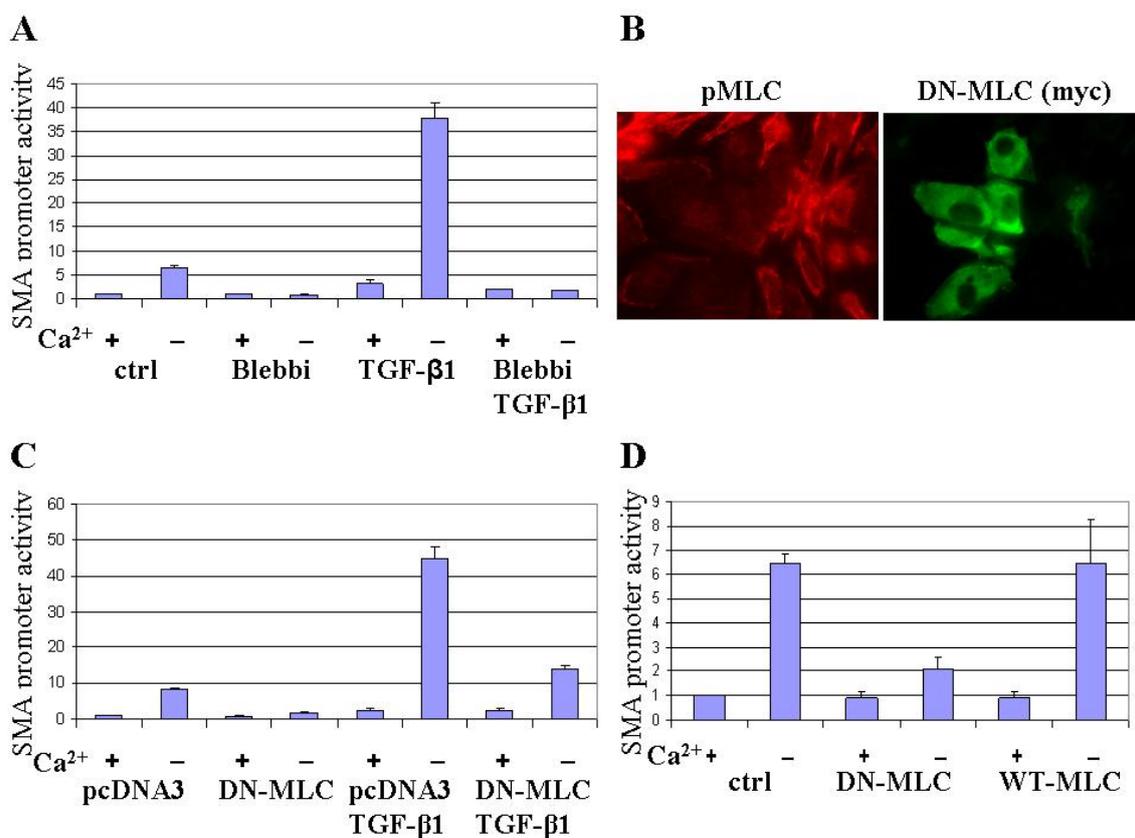


Figure 7. Inhibition of myosin ATPase activity or myosin phosphorylation strongly suppresses the contact disruption-induced activation of the SMA promoter and its enhancement by TGF- β 1. **(A)** Confluent monolayers were transfected with p-SMA-Luc and pRL-TK, and after 24 h were treated with vehicle or 100 μM blebbistatin for 2.5 h. Subsequently the cells were incubated in serum-free, Ca^{2+} containing or Ca^{2+} -free DMEM, in the presence or absence of blebbistatin. After 4 h, 10 ng/ml TGF- β 1 was added to the samples where indicated. Sixteen hours later the cells were lysed, and their luciferase activity was determined. Blebbistatin inhibited the effects of Ca^{2+} removal (6.5 ± 0.5 v. 0.8 ± 0.2 , $p < 0.05$, $n = 3$) and completely abolished the synergistic effect of contact disruption and TGF- β 1 treatment (37.8 ± 3.2 v. 1.8 ± 0.1 , $p < 0.05$, $n = 3$). **(B)** DN-

MLC inhibits the Ca^{2+} removal-induced MLC phosphorylation. Cells grown on coverslips in 6-well plates were transfected with Myc-tagged DN-MLC for 24 h, incubated in serum and Ca^{2+} -free DMEM for another 24 h, and then fixed and doubly stained for the Myc epitope (green) and phospho-MLC (red). **(C)** DN-MLC inhibits the contact disassembly induced activation of the SMA promoter. Confluent cells were cotransfected with SMA promoter and empty vector (pcDNA3) or DN-MLC, and after 24 h were subjected to Ca^{2+} removal where indicated. Four hours later, 10 ng/ml TGF- β 1 was added for 20 h to the indicated samples, followed by lysis and determination of luciferase activity. DN-MLC inhibited both the effects of Ca^{2+} depletion (8.3 ± 0.37 v. 1.82 ± 0.41 , $p<0.05$, $n=3$) and of the combined treatment (44.6 ± 3.71 v. 13.9 ± 1.04 , $p<0.05$, $n=3$). **(D)** Cells were transfected with pGL3-SMA-Luc, an alternative vector harboring the same 765bp. SMA promoter region as PA3-SMA-Luc. Other conditions were identical as in C. DN-MLC inhibited the effects of contact disruption on the promoter (6.45 ± 0.35 v. 2.1 ± 0.51 , $p<0.05$, $n=3$), while WT MLC did not alter this effect (6.45 ± 0.35 v. 6.45 ± 1.76 , $p<0.05$, $n=3$).

Since SMA protein expression upon TGF- β 1 is dependent on cell confluence levels, the question rose to assess the behavior of MLC phosphorylation upon TGF- β 1 under confluent and subconfluent conditions. When confluent, cells showed no staining for phospho-MLC in either control or TGF- β 1 treated conditions (Figure 8A/a,a'). However in subconfluent conditions the cells forming islands showed dim staining for pMLC at the periphery of the islands at the free edges of cells. The staining became more accentuated upon TGF- β 1 treatment in these areas, that corresponded to the same loci where cells are susceptible to TGF- β 1-induced SMA expression (Figure 8A/b,b'). Moreover, wounding of confluent layers also resulted in MLC phosphorylation at the edge of the wound suggesting that MLC is implicated in wound healing (Figure 8A/c,c').

After examining the potential role of MLC in regulating the SMA promoter by transfections, we next assessed the potential involvement of MLC regulation on protein level. We addressed this by interfering with myosin phosphorylation by expressing the DN MLC construct in non confluent cells before their TGF- β 1 treatment. The presence of DN MLC reduced the number of SMA expressing cells. In the control group TGF- β 1 treatment induced SMA formation in 22% of cells, however, in cells expressing DN MLC this number dropped more than four times (4%) (Figure 8B).

These data suggest that myosin activity and myosin phosphorylation are important contributors to the contact- dependent regulation of SMA.

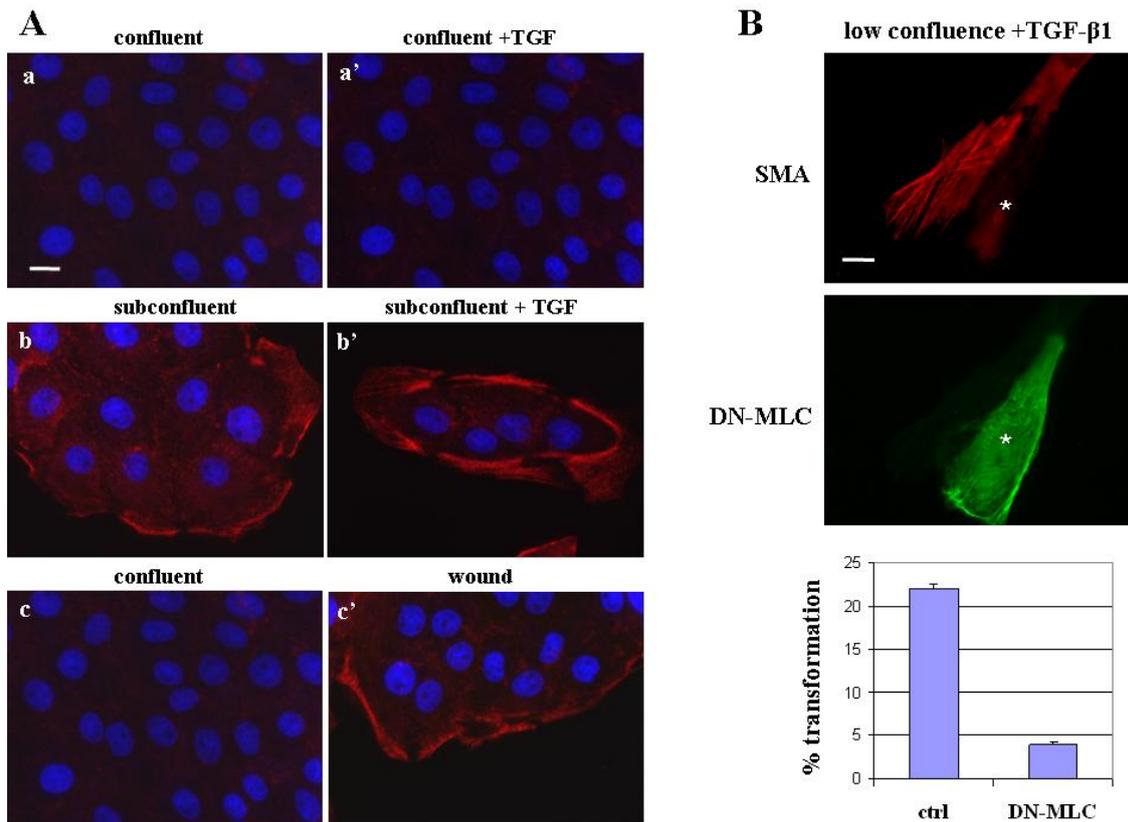


Figure 8. The effect of TGF- β 1 in confluent and subconfluent layers on MLC phosphorylation. SMA expression upon TGF- β 1 is dependent on MLC. **(A)** Confluent (a and a') or subconfluent (b and b') layers were left untreated or exposed to TGF- β 1 for 16 h and then fixed and stained for pMLC. A wound was generated in a confluent monolayer with a rubber policeman, and 6 h later the cells were fixed and stained for pMLC (c and c'). Nuclei were visualized by DAPI. **(B)** Cells grown in subconfluent conditions were transfected with Myc- tagged DN-MLC and were treated with 10 ng/ml TGF- β 1 for 3 days. Cells were then fixed and double stained for SMA and Myc. DN-MLC prevented the expression of SMA protein in the transfected cells. To quantify the effect, three separate experiments were performed, in which 910 randomly selected control (non-transfected) cells and 311 DN-MLC-expressing cells were assessed for SMA expression.

IV.4. Cell contact disassembly induces nuclear accumulation of Serum Response Factor in a Rho- and MLC dependent manner

Serum response factor (SRF) is the key *cis*-element driving SMA expression. Its activity could be regulated by nuclear-cytoplasmic shuttling (Camoretti-Mercado et al. 2000), although both this possibility and the involvement of the Rho pathway in this process remain controversial (Cen et al. 2004). Therefore we asked whether contact disruption affects SRF localization. Even in non-stimulated, resting cells SRF exhibited nuclear localization. Its nuclear distribution was more pronounced in subconfluent

cultures, and along with the progress of confluence nuclear labeling became less pronounced, while there was an increase in cytosolic staining. However, throughout the whole process nuclear labeling remained higher than the extra-nuclear signal. In cells subjected to Ca^{2+} removal, SRF showed a significant and time- dependent increase in nuclear accumulation, as assessed in immunofluorescent experiments (Figure 9A). We wished to verify that this was not an optical artifact due to cell contraction-associated cytosolic shrinkage. Therefore Western blots were performed on nuclear extracts from control and Ca^{2+} deprived cells. An increase in nuclear SRF upon Ca^{2+} removal was detected also by this technique (Figure 9B).

Next we assessed whether a Myc- tagged CA Rho construct can affect SRF localization. In cells transfected with the CA Rho construct an enhanced nuclear accumulation of SRF was observed (Figure 9C). Curiously however, this effect was clearly visible only in cells that showed a modest Rho expression (as visualized by Myc staining), whereas it was not apparent in cells with high level (and possibly longer lasting) expression of active Rho. This finding suggests that the increase in nuclear SRF accumulation may be transient, or various Rho-dependent pathways might be involved both in nuclear import and export processes. Moreover, expression of DN Rho significantly reduced or completely prevented the increase in nuclear SRF staining (Figure 9D). Cells were then transfected with DN MLC to test whether the inhibition of myosin had a similar effect on SRF. DN MLC also decreased the nuclear accumulation of SRF upon Ca^{2+} removal (Figure 9E). Further results were obtained by the quantification of fluorescent intensity of SRF staining in the nucleus and cytosol of individual (control or DN MLC expressing) cells. The nuclear-cytoplasmic ratio was calculated for the various conditions. In control cells there was a 1.4 fold nuclear accumulation of SRF over the cytosol, which increased to 2.1 fold upon Ca^{2+} removal. The expression of DN MLC did not affect the resting SRF distribution; however it reduced the effect of Ca^{2+} removal by 60% (Figure 9F).

Given the fact that there is a substantial amount of SRF in the nucleus even under resting conditions, and that the effect of DN-MLC was only partial, we continued to examine the contribution of other processes.

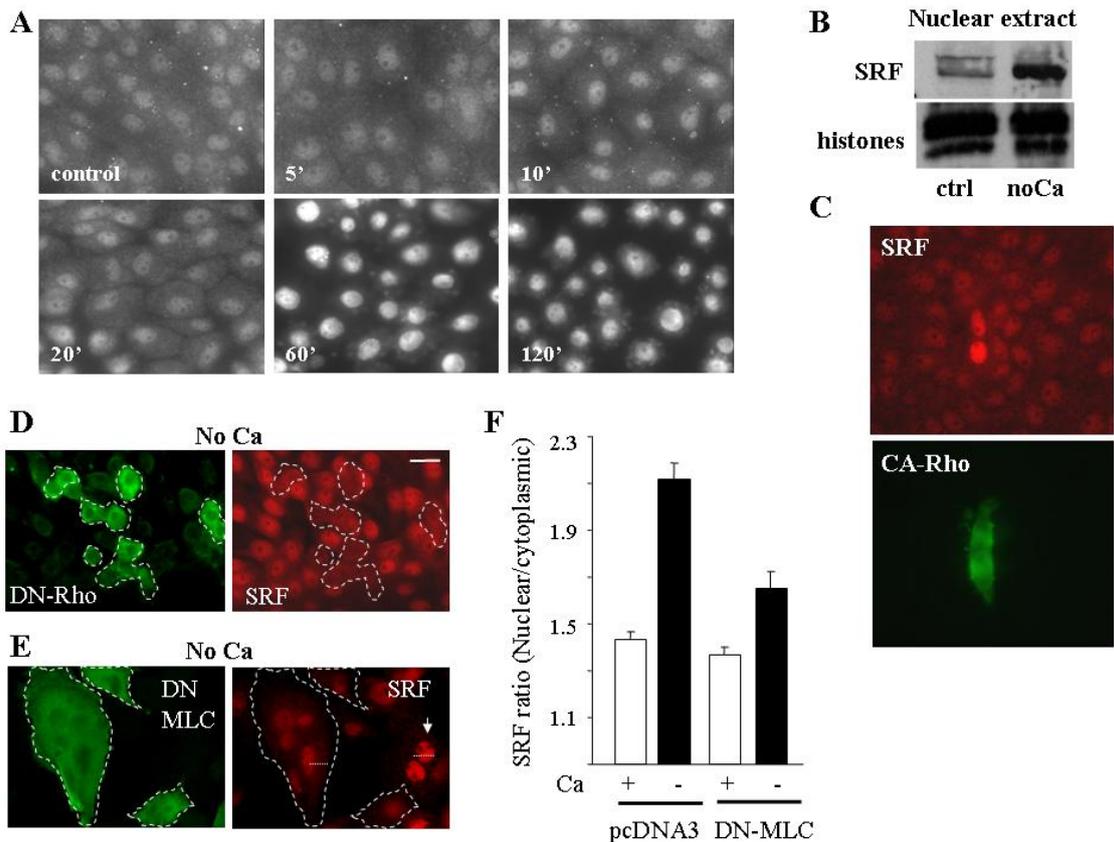


Figure 9. Contact disassembly facilitates the nuclear accumulation of serum response factor (SRF) in a Rho and MLC phosphorylation–dependent manner. **(A)** Confluent monolayers were serum-starved for 3 h and then medium was changed to Ca²⁺-free DMEM for the indicated times. Cells were then fixed and stained for SRF. **(B)** Nuclear extracts were prepared from Control or Ca²⁺ deprived (3 h) cells followed by Western blotting for SRF and H3 histones as a nuclear marker. **(C)** Confluent cells were transfected with constitutive active Myc-tagged Rho (CA-Rho) for 24 h, and then double stained for SRF (red) and Myc (green). **(D)** Cells were transfected with Myc-tagged dominant negative Rho (DN-Rho) for 24 h followed by incubation in Ca²⁺-free DMEM for another 24 h. Cells were then fixed and stained for SRF (red) and Myc (green). To facilitate the identification of the same cells on the two corresponding fluorescent images, successfully transfected cells or clusters of cells are circled with dashed lines. There is a substantial reduction in the nuclear SRF staining of DN-Rho–expressing cells. **(E)** Conditions were as in D, except the cells were transfected with Myc-tagged DN-MLC. **(F)** The intracellular distribution of SRF was quantified by measuring the nucleo-cytoplasmic ratio of the fluorescence intensity. For each cell determinations were made along lines drawn across the nucleus (see dashed line in E). The ratios were calculated for control (pcDNA3) and DN-MLC–transfected cells, which were incubated either in Ca²⁺ containing or Ca²⁺-free medium for a day. In each category at least 60 cells were analyzed. Ca²⁺ removal significantly enhanced the nuclear accumulation of SRF ($p < 10^{-10}$), and this effect was significantly suppressed ($p < 10^{-6}$) by DN-MLC.

IV.5. Rac, Cdc42 and PAK are stimulated by contact disassembly and contribute to the injury-dependent activation of the SMA promoter

Recently Patel et al. (Patel et al. 2005) showed through microarray analyzes that not only the Rho/ROK pathway, but also Rac1 and Cdc42 through their downstream effector, PAK (p21-activated protein kinase) seem to be essential in activated PBMC conditioned medium (aPBMC-CM) induced EMT in HK2 cells. Therefore we proposed to investigate the potential role of Rac1, Cdc42 and PAK in the cell contact and TGF- β 1 dependent EMT.

First, we investigated the effect of Rac1, Cdc42 and PAK overexpression on the SMA promoter. We transiently cotransfected the constitutively active constructs of these Rho family GTPases with the SMA promoter luciferase construct for 24 hours. Cotransfection of CA Rac1 with the SMA promoter induced a 9.6 fold increase in SMA promoter activity (Figure 10A). CA Cdc42 had an even more marked effect; it stimulated the activity of the promoter 22.8 fold (Figure 10B). Cotransfection of the CA PAK construct with the promoter showed a lower increase, yet the 4.3 fold increase in SMA promoter activity meant a strong activating potential of PAK (Figure 10C).

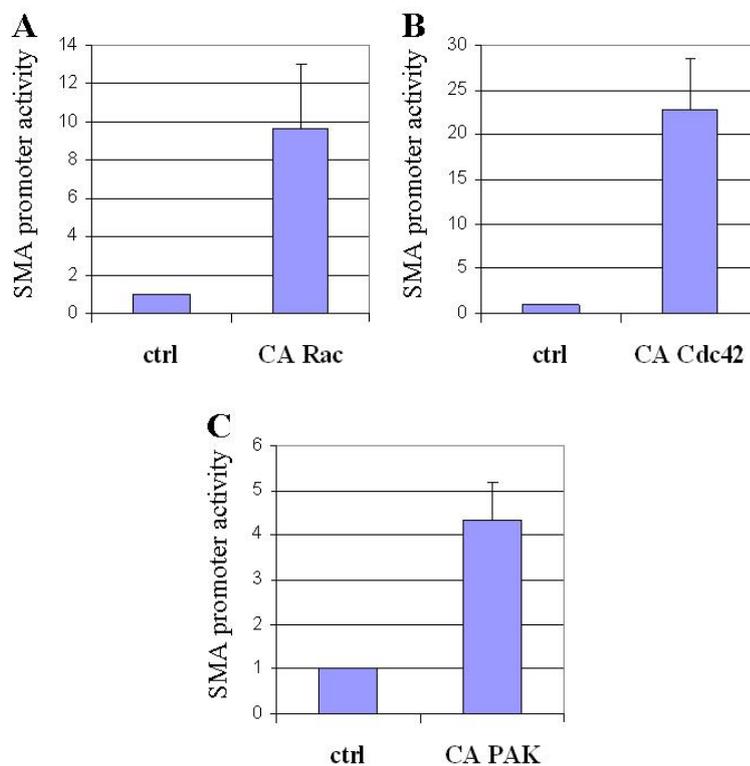


Figure 10. Overexpression of constitutive active Rac, Cdc42 and PAK induced activation of the SMA promoter. Confluent cells were cotransfected with the SMA promoter, pRL-TK, pcDNA3 for the control groups, and the constitutive active

plasmids for 24 hours. Cells were then harvested and luciferase assay was performed. **(A)** Overexpression of Rac1 induced a 9.6 ± 3.39 fold increase in the promoter activity ($n=4$, $p<0.05$). **(B)** Overexpression of Cdc42 induced a 22.8 ± 5.6 fold increase in the promoter activity ($n=3$, $p<0.05$). **(C)** Overexpression of PAK induced a 4.3 ± 0.9 fold increase in the promoter activity ($n=3$, $p<0.05$).

Besides looking at the potential activation of SMA promoter by the constitutive active constructs, we also tested whether their DN negative forms interfere and inhibit SMA promoter activation of contact disassembly by Ca^{2+} removal. Cotransfection of the SMA promoter with all three dominant negative constructs, DN Rac1, DN Cdc42, DN PAK, inhibited the initial ~ 6 fold increase in its activity induced by contact disassembly (Figure 11). This data suggests the strong involvement of Rac1, Cdc42 and PAK in regulating the expression of SMA.

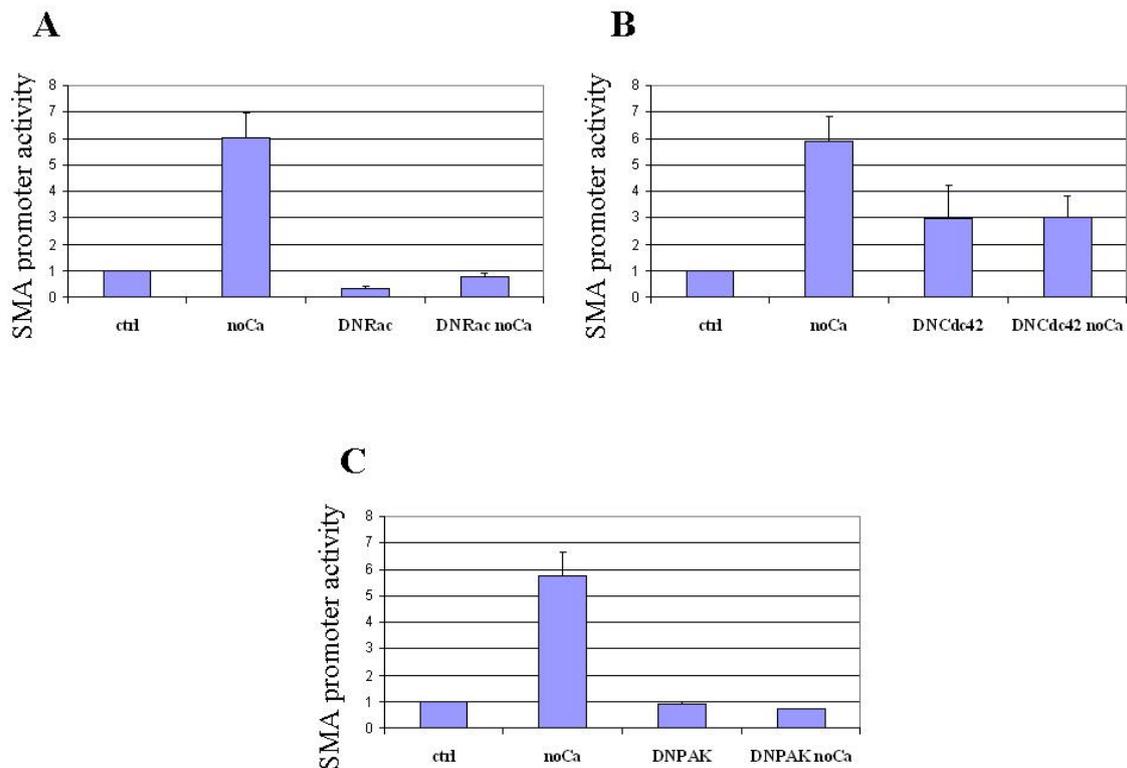


Figure 11. Dominant negative Rac, Cdc42 and PAK decrease the contact disassembly induced activation of the SMA promoter. Cells were cotransfected with SMA promoter, pRL-TK and DN-Rac, DN-Cdc42, DN-PAK for 16 hours, then controls were serum deprived and the other groups were Ca^{2+} deprived for 24 hrs. **(A)** DN-Rac inhibited the effect of contact disassembly on the SMA promoter (6.0 ± 0.94 v. 0.8 ± 0.15 , $n=3$, $p<0.05$). **(B)** DN-Cdc42 exerted a 50% decrease in Ca^{2+} removal induced SMA promoter activation (5.9 ± 0.95 v. 3 ± 0.8 , $n=3$, $p<0.05$). **(C)** DN-PAK inhibited the activation of the SMA promoter upon cell contact disruption (5.8 ± 0.9 v. 0.7 ± 0 , $n=3$, $p<0.05$).

The Rho-dependent region of the SMA promoter in LLC-PK1 cells was previously identified (Masszi et al, 2003). Next we tried to identify if there is a similar Rac1, Cdc42 or PAK responsive region in the SMA promoter. In this set of experiments besides the 752 bp. long SMA promoter we used a truncation of 152 bp., both sequences being inserted into the pGL3 vector. This second construct contains two CArG elements (B and A) that were identified as binding sites for SRF, a TGF- β 1 control element (TCE) and a TATA box. Ca^{2+} removal induced a 2.5 fold increase in both short and long SMA promoter's activity. As a control of these two constructs, we first tested the results of CA Rho cotransfection: cotransfection of CA Rho with the short or the long promoter induced a 6 fold increase in promoter activity. Cotransfection of CA Rac1 with the long promoter resulted in a 6.6 fold increase in promoter activity, while its cotransfection with the short promoter yielded a 22.7 fold increase. Similar to CA Rac1, cotransfection of CA Cdc42 with the long promoter showed a 13.8 fold increase in activity, while the short promoter yielded a 20 fold increase upon cotransfection with CA Cdc42. CA PAK induced a 6.4 fold increase in the long promoter activity, and a 8.3 fold increase in the activity of the short promoter. These results suggest that the CArG boxes are indispensable for the induction of the SMA promoter by the Rho GTPase family, and this effect is a SRF dependent signaling mechanism (Figure 12 A, B, C, D).

Moreover, when cells were transfected with CA Rac1 and CA Cdc42, and then stained for SRF, transfected cells exhibited an increased nuclear distribution of SRF (Figure 12 E, F).

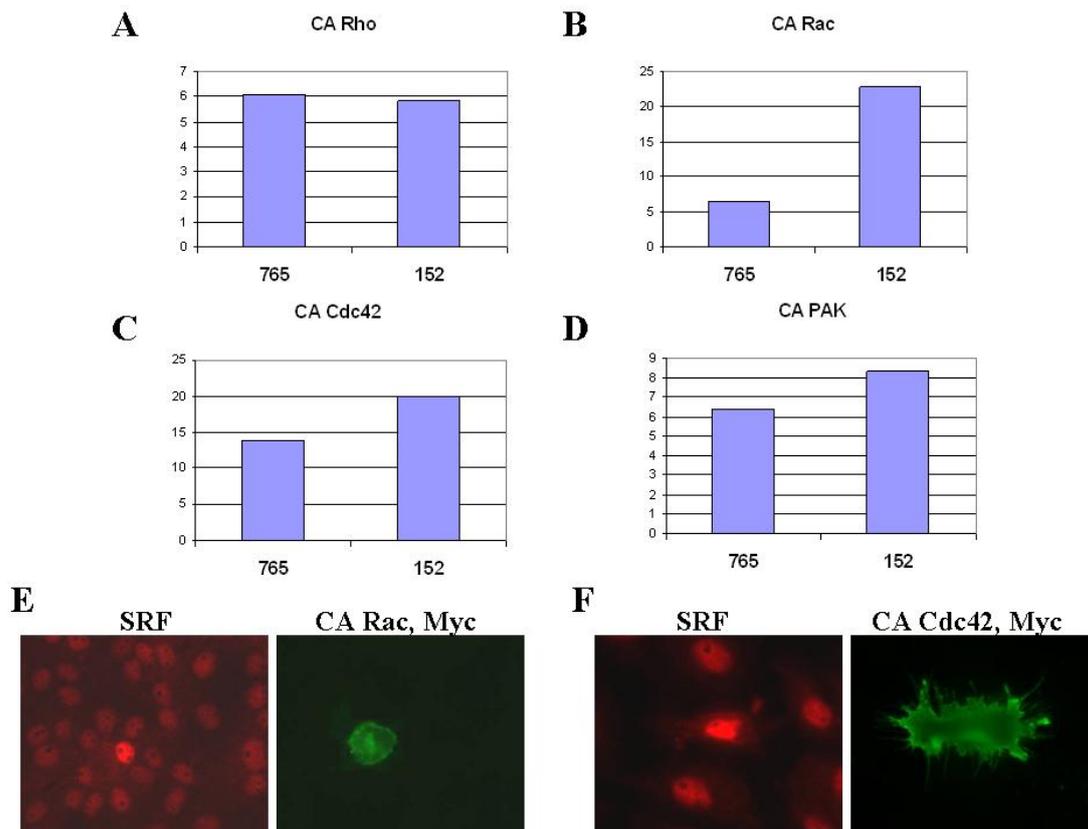


Figure 12. Regulation of the SMA promoter by Rac, Cdc42 and PAK are SRF dependent. Cells were transfected with pGL3-SMA-765 vector or pGL3-SMA-152 vector, pRL-TK and CA Rho, CA Rac, CA Cdc42, CA PAK as indicated. 24hrs after transfection cells were serum deprived for 24 more hrs, then harvested and luciferase assay was performed. **(A)** Cotransfection of Rho induced a 6 v. 5.6 fold increase on the 752bp. and the 152bp. promoters (n=2). **(B)** Cotransfection of Rac induced a 6.6 v. 22.7 fold increase on the 752bp. and the 152bp. promoters (n=2). **(C)** Cotransfection of Cdc42 induced a 13.8 v. 20 fold increase on the 752bp. and the 152bp. promoters (n=2). **(D)** Cotransfection of PAK induced a 6.4 v. 8.3 fold increase on the 752bp. and the 152bp. promoters (n=2). **(E)** Confluent cells grown on coverslips in 6-well plates were transfected with CA Rac for 24 hrs and then were fixed and stained for SRF and Myc to visualize the plasmid. **(F)** Similar as in E, for transfection of CA Cdc42.

Since SMA promoter activation is dependent on the integrity of cell-cell contacts, the next step was to address whether cell contact disassembly by Ca^{2+} removal could activate Rac1, Cdc42 and PAK. First Rac1/Cdc42 activity assays were performed as described in the Materials and methods section. Cells were subjected to Ca^{2+} removal and both Rac1 and Cdc42 were activated in a time dependent manner (Figure 13A). Already 1 minute after Ca^{2+} removal Cdc42 became activated, Rac1 showing activation 15 minutes after the treatment. Their activation decreased 60 minutes after the treatment. By binding to PAK, Rac1 and Cdc42 induce its autophosphorylation, and, as such, activation. Western blot experiments were executed to address the potential

activation of PAK by Ca^{2+} removal, using a phospho-specific antibody. Phosphorylation of PAK occurs 5 minutes after Ca^{2+} removal, levels of pPAK increasing with the time of exposure. The activation of PAK by contact disassembly was assessed on a 24 hrs time course (Figure 13B).

PAK1 was also an interesting target in regard of TGF- β 1 signaling. The next question addressed was whether TGF- β 1 can phosphorylate PAK. This was examined by Western blot experiments. TGF- β 1 induced phosphorylation of PAK in a time dependent manner, pPAK1 reaching its peak one hour after TGF- β 1 treatment and remaining activated 24 hours after the treatment (Figure 13C).

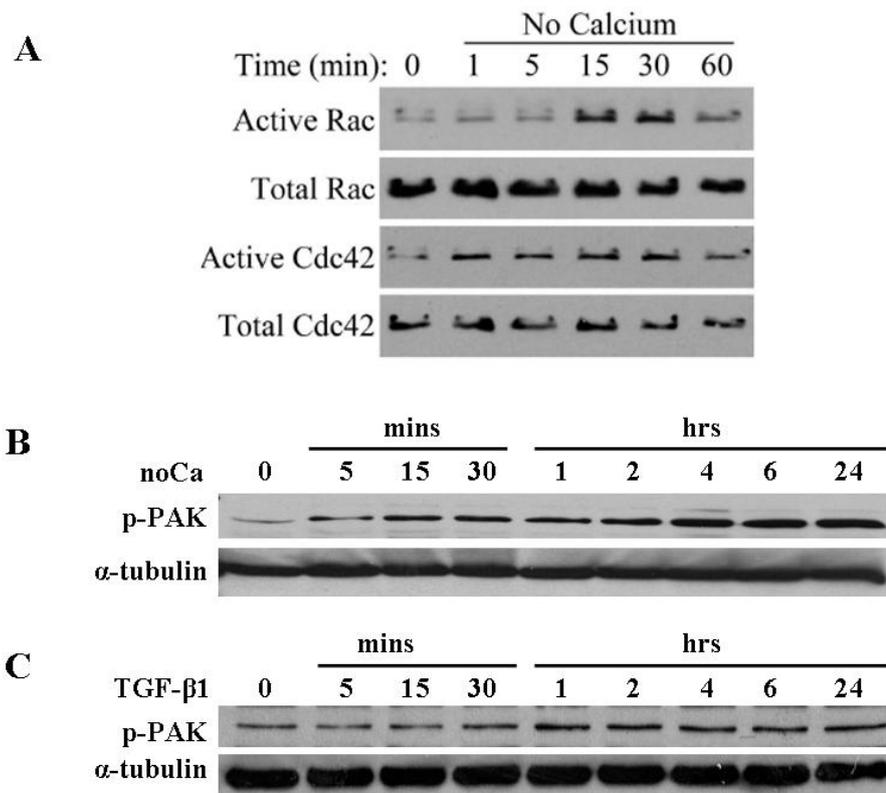


Figure 13. Cell contact disruption activates Rac1, Cdc42 and PAK. TGF- β 1 phosphorylates PAK. **(A)** Cells were grown to confluence on 10 cm dishes, 6 hrs serum deprived, and then Ca^{2+} containing medium was changed to Ca^{2+} -free DMEM, for the indicated times to disrupt intercellular contacts. Rac/Cdc42 assay was performed as indicated in the Materials and methods section. Cell lysates were examined by Western blot. Total cell lysates were examined for total Rac and total Cdc42 to prove that equal amount of proteins were loaded. **(B)** Cells grown on 3 cm dishes were subjected to 3 hrs serum removal and then Ca^{2+} was removed for the indicated times using Ca^{2+} -free DMEM. Cell lysates were prepared and examined by Western blotting. **(C)** Cells grown on 3 cm dishes were subjected to 3 hrs serum removal and then were treated with 10 ng/ml TGF- β 1 for the indicated times. Cell lysates were prepared and examined by Western blotting. Membranes were re-probed for α -tubulin to serve as loading control (B, C).

We concluded that cell-cell contact disassembly by Ca^{2+} removal activates Rac1, Cdc42 and phosphorylates their downstream effector, PAK. Not only Ca^{2+} removal, but TGF- β 1 also phosphorylates PAK1 in a time dependent manner. A pathway pointing to SRF could be also involved in the complex regulation of SMA expression; therefore we investigated possible downstream scenarios.

IV.6. p38 MAPK is a potent and important modulator of SMA expression, and is regulated by both TGF- β 1 and disruption of cell contacts

p38 MAPK was described as a potential target of Rac1 and Cdc42. Moreover, p38 was linked to Smad-independent TGF- β 1 signaling. We wished to examine its potential involvement in the regulation of SMA expression. Therefore we first analyzed p38 in regard of the two hits involved in EMT and SMA regulation, namely TGF- β 1 and cell contact disassembly.

Phosphorylation of p38 MAPK upon TGF- β 1 treatment was assessed in LLC-PK1/AT1 cells during Western blot experiments. p38 is phosphorylated by TGF- β 1 in a biphasic manner. There is a first peak of activation 60 minutes after the treatment, followed by a return to the baseline of the signal (Figure 14A). A second peak of activation followed 48 hours after the TGF- β 1 treatment, this second wave of activation being also present 72 and 96 hours after the treatment (Figure 14B).

To assess whether p38 mediates the effects of Ca^{2+} removal, and as such, play a role during cell-cell contact dependent EMT, confluent cells were subjected to Ca^{2+} removal. Under such conditions, p38 was also activated, phosphorylation occurring 30 minutes after the treatment, p38 remaining activated up to 4 hours, followed by a decrease of its phosphorylation levels (Figure 14C).

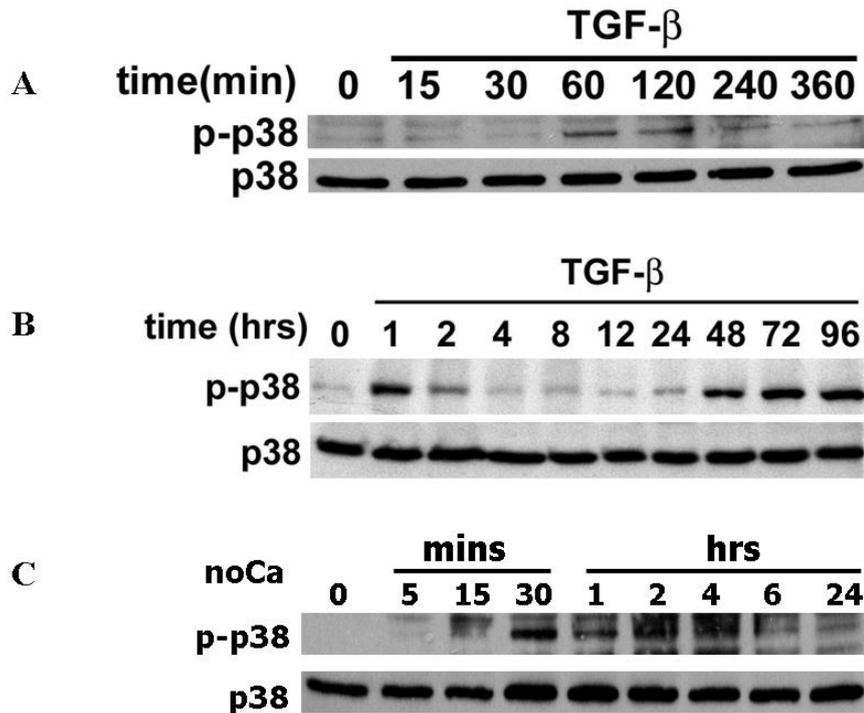


Figure 14. *p38 is phosphorylated by TGF-β1 and Ca²⁺ removal.* Cells were grown to confluence on 3 cm dishes and subjected to TGF-β1 (5 ng/ml) treatments (**A**, **B**) or Ca²⁺ removal (**C**) for the indicated times. Cell lysates were prepared and examined by Western blotting using a phospho-p38 antibody. Membranes were re-probed for p38 to show equal loading.

To assess the role of p38 in regulation of SMA expression, the specific p38 inhibitor, SB203580 was used. SMA synthesis was examined by Western blot and immunofluorescence experiments. Three days after TGF-β1 treatment, SMA was expressed by LLC-PK1 cells, as seen both on Western blot and immunofluorescence. Pretreatment of subconfluent cells with SB203580 before the TGF-β1 treatment completely abolished its effect; there was no SMA expression in the pretreated cells. SB203580 in 1 μM concentration reduced significantly the expression of SMA, and 5 μM of inhibitor completely abolished its expression as seen on Western blot (Figure 15A). 5 μM of the specific p38 inhibitor also prevented SMA expression as seen during immunofluorescence experiments (Figure 15B).

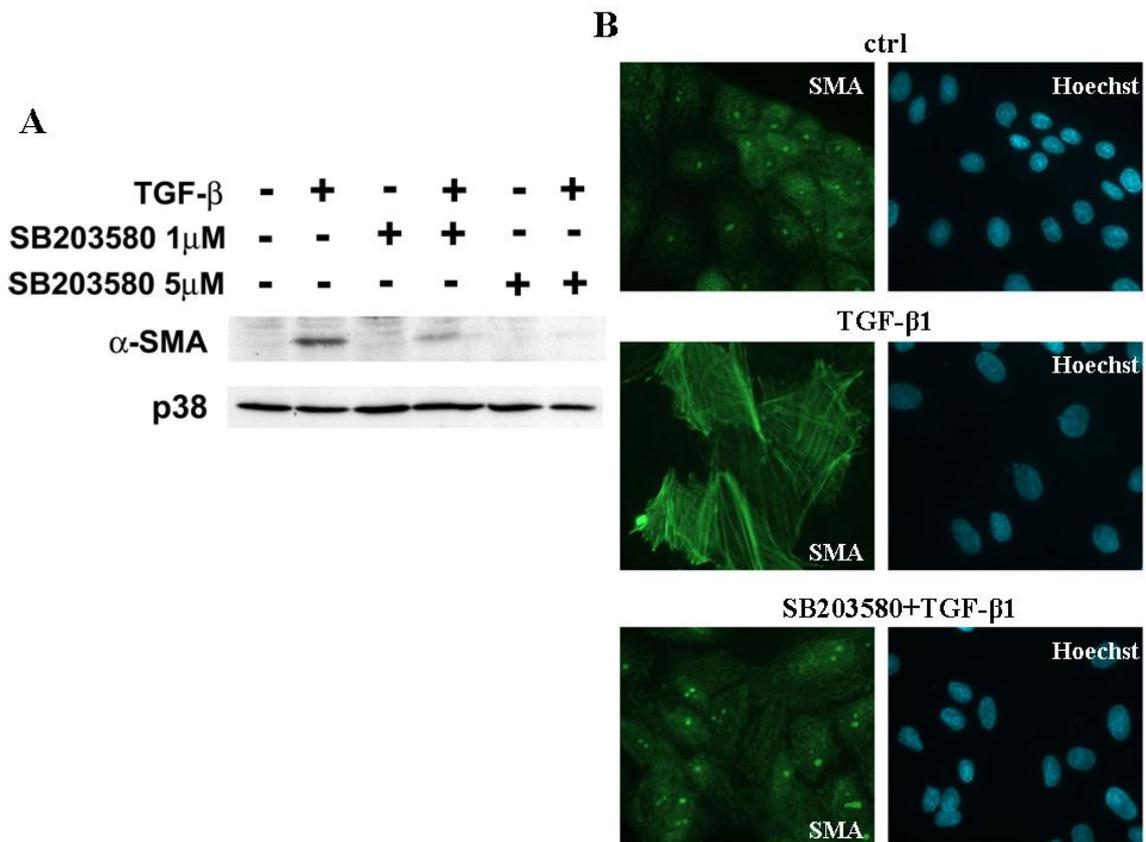


Figure 15. Inhibition of p38 MAPK prevents TGF- β 1 induced SMA synthesis in renal tubular cells. **(A)** LLC-PK1/AT1 cells were sparsely grown on 3 cm dishes and were treated with vehicle or 5 ng/ml TGF- β 1 for 96 hours. 45 minutes of 1 μ M and 5 μ M SB203580 pretreatment was used as indicated. Cell lysates were analyzed by Western blotting for SMA. Membranes were re-probed for p38 to demonstrate equal loading. **(B)** Cells grown on coverslips were treated with vehicle or 5 ng/ml TGF- β 1 for 4 days. Cells were pretreated with 5 μ M SB203580 for 45 minutes as indicated, and SB203580 was present through the whole duration of incubation. Cells were immunostained for SMA. Nuclei were visualized by Hoechst staining.

Next we wished to verify these results in transient transfection experiments. Confluent cells were transfected with the SMA promoter and with p38AF, a plasmid expressing a dominant negative form of p38 containing a T180A mutation. p38AF inhibited the Ca^{2+} removal induced SMA promoter activation (Figure 16A). Pretreatment with SB203580 also prevented the induction of the SMA promoter by Ca^{2+} removal, yielding a 75% inhibition (Figure 16B). Next p38AF was cotransfected with the SMA promoter in non-confluent cells. In this experimental setup the dominant negative plasmid induced a 50% inhibition of the TGF- β 1 effect on the promoter (Figure 16C). Much to our surprise, when trying to inhibit TGF- β 1 effects on SMA promoter by pre-treating the cells with SB203580, the inhibitor did not inhibit the SMA promoter activation, not even in higher concentrations, than the one used in the previous

WB and IF experiments (Figure 16D). These results might indicate the involvement of p38 in regulating mRNA stability through its downstream effector, MK2. This observation is dealt with in the “Discussion” chapter in regard to other publications concerning mRNA stability.

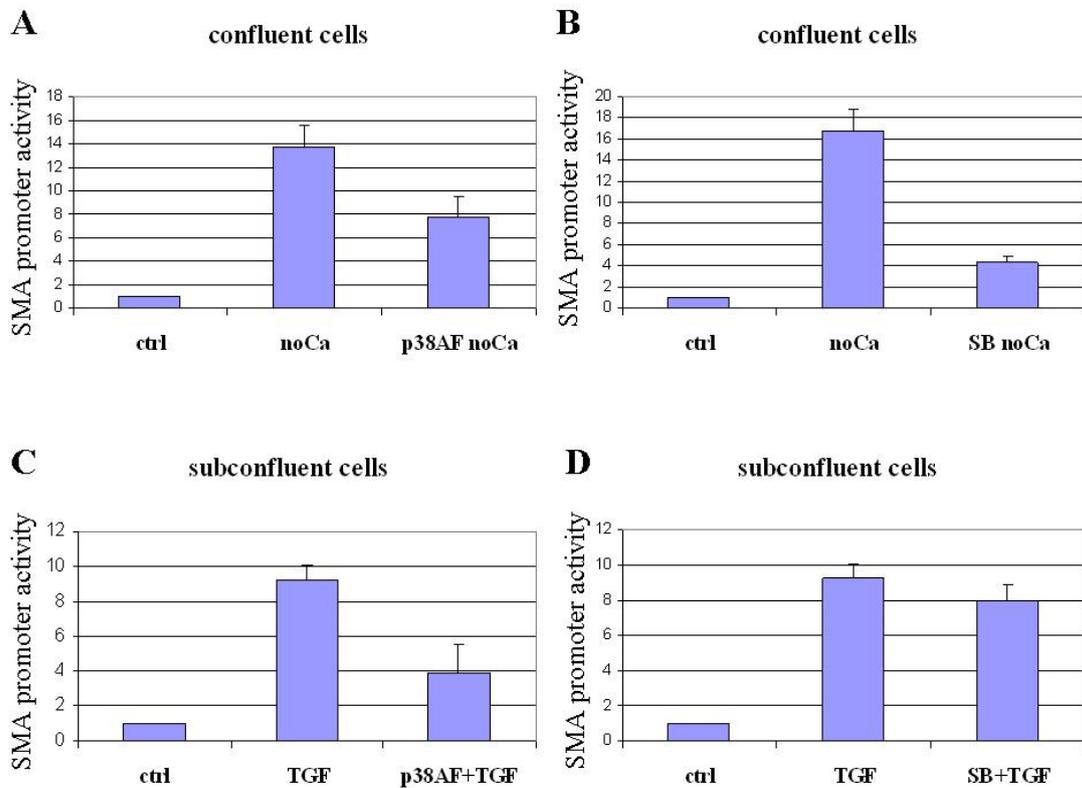


Figure 16. *p38* is an essential modulator of the SMA promoter. Cells were grown on 6-well plates to confluence or subconfluence, either cotransfected with SMA promoter, pRL-TK, pcDNA3 or p38AF and treated as indicated, or cotransfected with SMA promoter, pRL-TK and pcDNA3, being pretreated with 5 μ M SB203580 for 1 hour, the inhibitor being present through the rest of the experiment. Cell lysates were analyzed by luciferase assay. **(A)** Under confluent conditions p38AF induced a 43% inhibition of the Ca^{2+} removal induced SMA promoter activation (13.7 ± 1.93 v. 7.8 ± 1.67 , $n=3$, $p<0.05$). **(B)** SB202580 inhibited the Ca^{2+} depletion induced effect on the SMA promoter in confluent cells (16.8 ± 2.06 v. 4.4 ± 0.54 , $n=3$, $p<0.05$). **(C)** Cotransfection of subconfluent cells with p38AF decreased the TGF- β 1 induced SMA promoter activation (9.2 ± 0.88 v. 3.9 ± 1.66 , $n=3$, $p<0.05$). **(D)** In sharp contrast to the protein assay results obtained by Western blot and immunofluorescence, SB203580 did not inhibit the TGF- β 1 induced SMA promoter activation in subconfluent monolayers (9.2 ± 0.88 v. 8 ± 0.92 , $n=3$, $p<0.05$).

SB203580 was reported to inhibit p38 α and p38 β but not p38 γ or p38 δ (Davies et al. 2000), therefore the next step was to define if both the α and β isoforms played a role in the TGF- β 1 induced SMA expression. LLC-PK1/AT1 cells were infected with replication deficient adenoviral vectors expressing the dominant negative (DN) mutant

of p38 α and p38 β . Four day incubation in the presence of TGF- β 1 induced marked α -SMA protein expression in tubular cells infected with the control adenovirus, RAdLacZ. Dominant inhibitory p38 α (p38 α AF) caused a detectable decrease in the effect of TGF- β 1, whereas adenoviral expression of dominant negative p38 β (p38 β AF) inhibited SMA expression almost completely. No further increase in the inhibitory effect of DN p38 β was seen when the cells were infected with both DN p38 α and DN p38 β together. In order to gather further evidence for the predominant role of p38 β and to investigate the upstream mechanisms regulating p38 during the TGF- β 1 induced SMA regulating hit, experiments were designed with the upstream activators of p38, MKK3 and MKK6. MKK3 activates p38 α , p38 δ and perhaps p38 γ , while MKK6 activates all four isoforms. To explore the role of these kinases in the increased α SMA expression we also exploited adenoviral gene delivery of mutated signaling molecules. Infection of the cells with a vector harboring a dominant negative form of MKK6b (MKK6bA) caused a substantial inhibition of the TGF- β 1 effect. On the other hand, dominant negative MKK3b (MKK3bA), which is expected to inhibit all p38 MAPK isoforms except p38 β , had no significant effect on the SMA expression induced by TGF- β 1. Inhibition by MKK6bA indicates that the SMA expression inducing effects of TGF- β 1 are MKK6 dependent, not MKK3 dependent (Figure 17). These results together point towards the more important contribution of p38 β to the TGF- β 1 induced SMA expression.

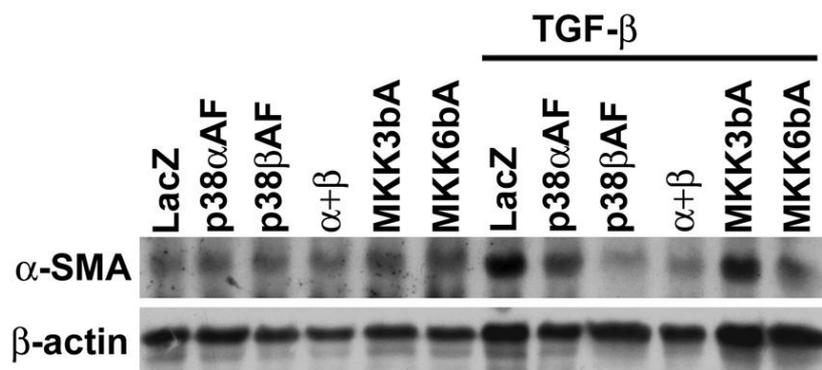


Figure 17. TGF- β 1 activates α -SMA through MKK6 and p38 β . Cells were infected in suspension with adenoviruses interfering with the p38 MAPK pathway at 1 MOI. 24

hours later 5 ng/ml TGF- β 1 was added for 4 days. Cells were then harvested in SDS sample buffer and analyzed by Western blotting for α -SMA. Membranes were re-probed for β -actin to demonstrate equal loading.

Next we wished to assess whether p38 regulates SMA in a SRF dependent pathway. For this we transfected confluent cells with the 765bp. and the 152 bp. pGL3-SMA promoters, and Ca²⁺ depleted the cells. The pretreatment with SB203580 induced a similar inhibition in the case of both promoters (Figure 18 A, B), which indicates that p38 acts through SRF when modulating SMA expression.

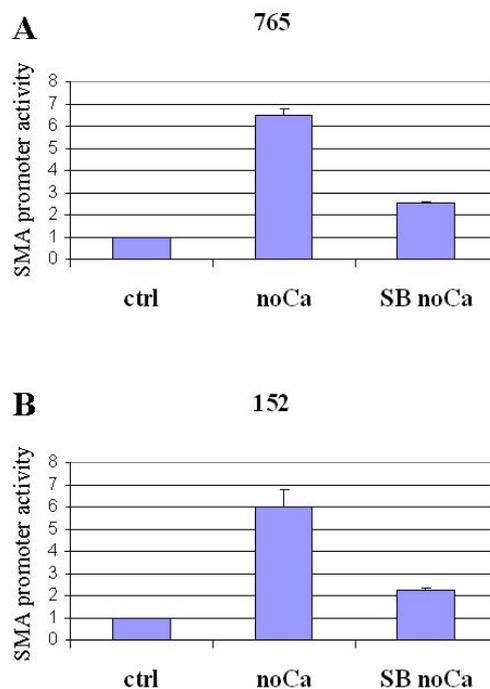


Figure 18. *p38 mediates the effects of contact disassembly on the SMA promoter through SRF dependent signaling.* LLC-PK1/AT1 cells were grown in 6-well plates to confluence. The pGL3-765 and pGL3-152 promoters were cotransfected with pRL-TK and pcDNA3. 24 hours later cells were subjected to Ca²⁺ removal for additional 24 hours. Some cells were pretreated for 1 hour with 5 μ M SB203580, which was present through the rest of the experiment. Cell lysates were analyzed by luciferase assay. SB203580 yielded a similar inhibition of cell contact removal induced activation in case of both promoters. **(A)** SB203580 inhibited the effect of Ca²⁺ removal on the long pGL3-SMA promoter (6.48 \pm 0.29 v. 2.51 \pm 0.06). **(B)** SB203580 inhibited the effect of Ca²⁺ removal on the short pGL3-SMA promoter (5.98 \pm 0.78 v. 2.23 \pm 0.12).

In order to further substantiate findings regarding the involvement of p38, we investigated its role in the cell contact dependent hit. After establishing the role of the Rho-ROK-pMLC-SRF pathway in regulating SMA expression, the potential role of pMLC in the Rac1/Cdc42-PAK pathway was addressed. All molecules involved in

these two pathways are important cytoskeletal regulators; therefore we next investigated the possible involvement of MLC and cofilin in these mechanisms.

PAK was shown to phosphorylate MLC (Kiosses et al. 1999) and p38 (Zhang et al., 1995), moreover, p38 was also found to phosphorylate MLC (Goldberg et al. 2002). We hypothesized that p38 is involved in regulating SMA expression and that Rac1/Cdc42 and PAK might activate MLC through the phosphorylation of p38, and regulating as such SMA expression. Since MLC phosphorylation is dependent on ROK, we also wished to assess whether there was a link between ROK and p38.

First, we proposed to investigate the link between PAK, ROK and p38. Phospho-p38 levels were assessed by Western blotting, cells being subjected to 1 hour of Ca^{2+} removal. p38 was phosphorylated upon Ca^{2+} removal, and this effect was inhibited by the presence of the PAK inhibitor, PAK18. Cells pretreated with PAK18 showed a much lower level of p38, PAK18 reducing phosphorylation of p38. This result indicates that Rac1/Cdc42-PAK pathway indeed signals through activating p38. Moreover, blocking of ROK by 10 μ M of its specific inhibitor, Y-27632, also partially inhibited phosphorylation levels of p38 upon Ca^{2+} removal. This data suggested a cross talk between the Rac/Cdc42-PAK pathway and the Rho-ROK pathway at p38 level. As expected, the specific p38 inhibitor SB203580 did not alter p38 phosphorylation, indicating that it only inhibits p38 effects (Figure 19).

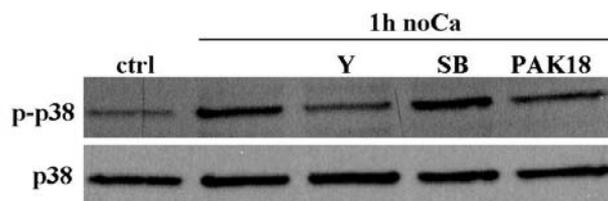


Figure 19. *Phosphorylation of p38 is dependent on both PAK and ROK.* Cells grown to confluence in 3 cm dishes were pretreated 1 hour with 10 μ M Y-27163, 10 μ M SB203580, 20 μ M PAK18 and then subjected to Ca^{2+} removal for an additional hour. Cell lysates were prepared and analyzed by Western blotting. Membranes were re-probed for p38 to demonstrate equal loading.

Next we tested the potential involvement of MLC in the effects of Rac1, Cdc42, PAK, p38. First, transfection of CA Rac1 to cells led to phosphorylation of MLC. This staining showed different characteristics than in the Ca^{2+} removed cells. pMLC showed both focal and peripheral staining, yet did not show a circular, ring-like shape. Transfection of CA Cdc42 induced a marked phosphorylation of MLC, similar to the pattern showed by Rho. pMLC was organized in fiber-like structures probably along the actin structures, throughout the transfected cells. Transfection of CA PAK also resulted in phosphorylation of MLC. pMLC was localized both at the cell periphery in ring-like structures, and also showed fiber-like accumulations (Figure 20).

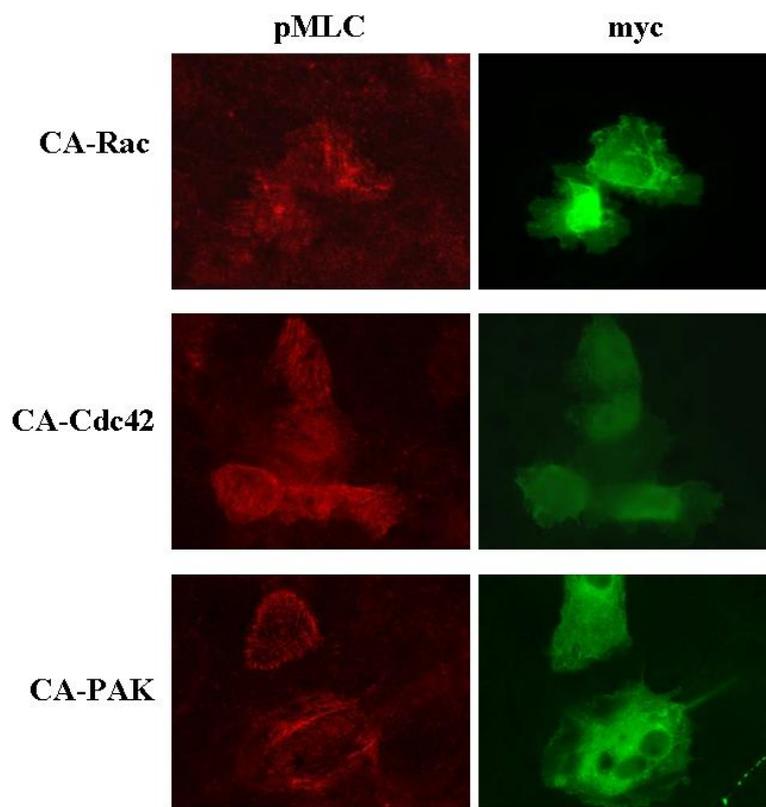


Figure 20. *Rac, Cdc42 and PAK phosphorylate MLC.* Cells grown to confluence on coverslips were transfected with CA Rac, CA Cdc42 and CA PAK for 24 hours, then were fixed and stained for pMLC and Myc to visualize the expression of the constitutive active plasmids. Rac, Cdc42 and PAK induced phosphorylation of MLC.

Next we assessed whether the blockage of Rac1, PAK or p38 can prevent phosphorylation of MLC upon cell contact disassembly. Therefore cells were transfected with DN-Rac or DN-PAK, and were then subjected to by Ca^{2+} removal. However, the dominant negative constructs did not inhibit the MLC phosphorylation upon Ca^{2+} removal (Figure 21 A,B). These results indicated, that indeed Rac1 and PAK

are able to phosphorylate MLC, however this mechanism is not involved in this particular signaling pathway. Next cells subjected to Ca^{2+} removal were pretreated with SB203580, a specific p38 inhibitor, and were stained for pMLC. Inhibition of p38 did not prevent MLC phosphorylation by Ca^{2+} removal (Figure 21C). These results indicate that despite the potential to phosphorylate MLC, the signaling pathway formed by Rac1/Cdc42-PAK-p38 does not include MLC in this particular mechanism.

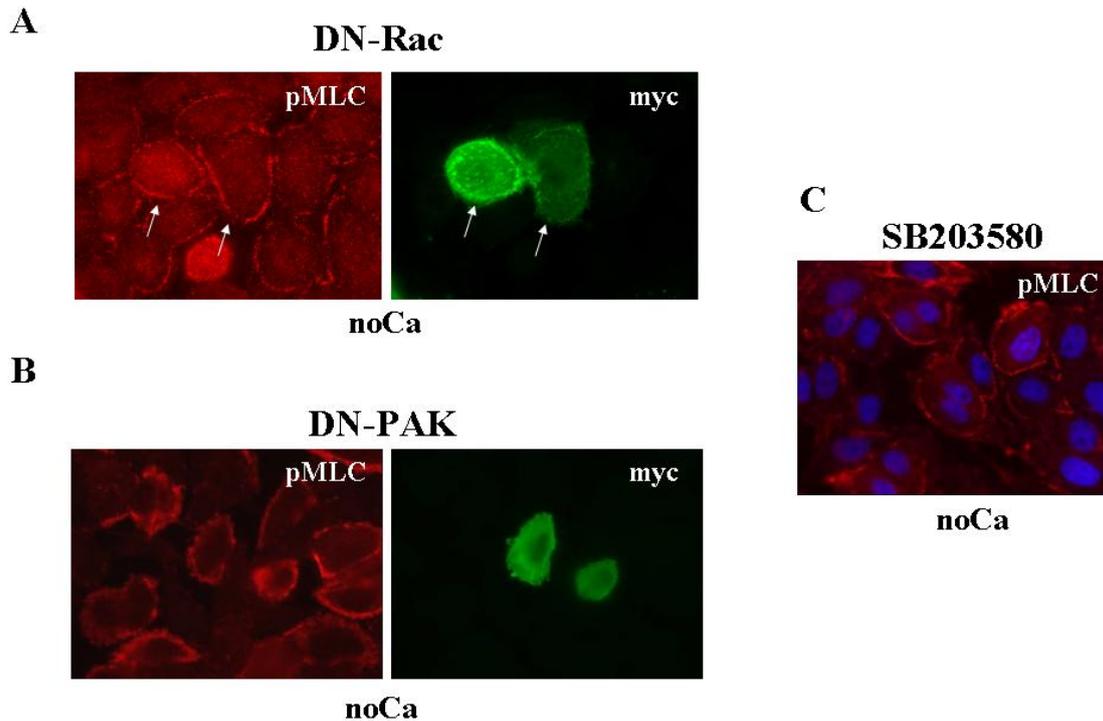


Figure 21. *DN-Rac, DN-PAK or pretreatment with SB203580 does not inhibit MLC phosphorylation induced by Ca^{2+} removal. (A,B)* Confluent cells grown on coverslips were transfected with DN-Rac or DN-PAK for 24 hours, and then were subjected to Ca^{2+} removal for 24 hrs. DN-Rac and DN-PAK did not inhibit MLC phosphorylation induced by cell contact disassembly. *(C)* Confluent cells were pretreated for 1 hour with 5 μM SB203580 and then were subjected to Ca^{2+} removal for 24 hours. The presence of the specific p38 inhibitor did not prevent the cell contact disassembly induced phosphorylation of MLC. DAPI was used for nuclear visualization.

To further dissect the downstream mechanisms of SMA regulation, we were looking for the involvement of other cytoskeletal regulators, which might be involved in the Rac1-PAK dependent responses. Interestingly, cofilin can be downstream not only from the Rho/ROK but also the Rac/PAK pathway (Bokoch 2003, Jaffe and Hall 2005). Cofilin is a regulator of cytoskeleton and, as such, actin polymerization: when

phosphorylated it becomes inactive and thus permitting actin polymerization. Cofilin is regulated by LIM kinase, which has been shown to be dependent on both ROK and PAK. Since small GTPases are strongly involved in cytoskeleton modulation, cofilin was analyzed under the two stimuli of the “two hit” model.

Ca²⁺ removal (Figure 22A) and TGF-β1 (Figure 22B) induced phosphorylation of cofilin, as shown by Western blot. The effect of TGF-β1 was discernable 5 minutes after the treatment and persisted up to 24 hours. Similarly, Ca²⁺ removal also resulted in cofilin phosphorylation 5 minutes after the change of the medium, the phosphorylation level reaching its peak after 30 minutes, the phosphorylation levels remaining elevated up to 24 hours. The role of ROK, PAK and p38 in regulating cofilin was assessed by Western blotting (Figure 22C). Cells were pretreated with the specific inhibitors Y-27632, SB203580 and PAK18, and then 1 hour of Ca²⁺ removal was used to stimulate cofilin phosphorylation. Interestingly, it was only the ROK inhibitor which reduced cofilin phosphorylation, indicating that the mechanism by which cofilin regulates cytoskeleton is Rho-ROK dependent.

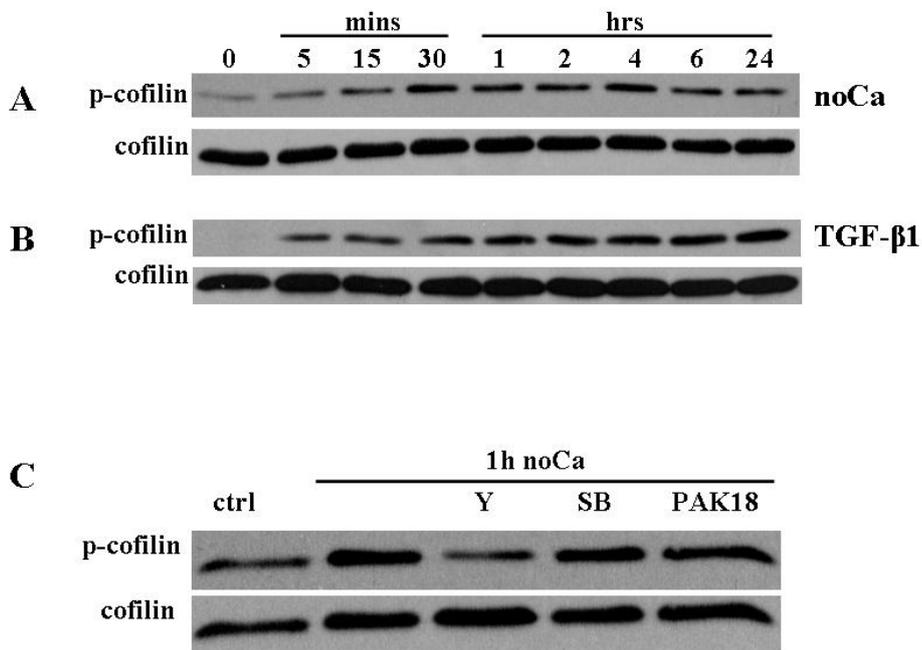


Figure 22. Cell contact disassembly induced phosphorylation of cofilin is ROK dependent. TGF-β1 phosphorylates cofilin. (A) and (B) Cells grown to confluence in 3 cm dishes were subjected to Ca²⁺ removal or TGF-β1 treatment for the indicated times. Western blotting was performed on cell lysates with a phospho-cofilin antibody. Cofilin was used as a loading control. (C) Cells grown to confluence in 3 cm dishes

were pretreated 1 hour with 10 μ M Y-27163, 10 μ M SB203580, 20 μ M PAK18 and then subjected to Ca^{2+} removal for an additional hour. Cell lysates were prepared and analyzed by Western blotting for phospho-cofilin. Membranes were re-probed for cofilin to demonstrate equal loading.

Next we wished assess the relationship of Rac1 and PAK to cofilin phosphorylation by the means of immunofluorescence. For this, cells were transfected with CA-Rac1 and CA-PAK, and then stained for phospho-cofilin. The active constructs did not induce phosphorylation of cofilin. Moreover, the presence of the dominant negative mutants Rac1 and PAK did not prevent phosphorylation of cofilin upon Ca^{2+} removal (Figure 23).

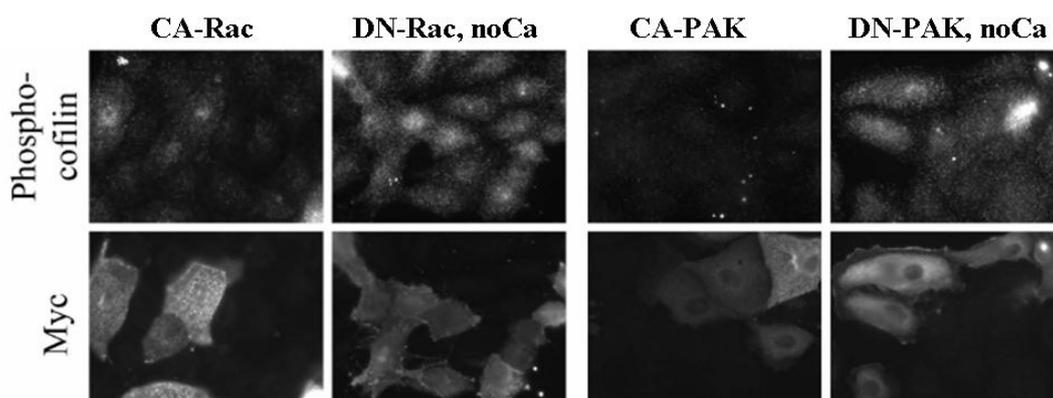


Figure 23. *Rac and PAK do not regulate cofilin phosphorylation.* Cells were transfected with CA-Rac, DN-Rac, CA-PAK or DN-PAK for 24 hours. Cells were then subjected to Ca^{2+} removal for 1 hour, as indicated, and double stained for p-cofilin and Myc. CA-Rac and CA-PAK did not induce cofilin phosphorylation, and their dominant negative forms did not prevent cofilin phosphorylation upon cell contact disassembly.

Here we showed that p38 MAPK is an important modulator of both TGF- β 1 and contact dependent hits. We also showed that besides the Rho-ROK-MLC-SRF pathway other signaling molecules might form another important mechanism: Rac1/Cdc42-PAK-p38-SRF. In our search for a common modulator that could merge the effects of these two pathways, we turned our attention towards another cytoskeletal actor, MRTF.

IV.7. Localization of MRTF and its nuclear-cytoplasmic transfer is regulated by TGF- β 1, cell contact disassembly, Rho, MLC, Rac1, Cdc42, PAK and p38

Since there is a substantial amount of SRF in the nucleus even under resting conditions, we continued to examine the mechanisms regulating SMA expression by evaluating the role of a SRF cofactor, MRTF.

In order to characterize MRTF in LLC-PK1 cells and to overcome the restricted availability of MRTF antibody, we established a model to examine MRTF isoforms by using constructs encoding FLAG epitope-tagged MRTF-A and MRTF-B, and followed their localization through staining with an anti-FLAG antibody. Staining intensities were recorded and nuclear/cytoplasmic ratios were calculated. Average ratios were established as follows: localization was considered cytoplasmic for ratios <0.75 , pan cellular, equal distribution was considered a ratio value between $0.75-1.25$, whereas ratios >1.25 indicated nuclear localization. In LLC-PK1 cells MRTF-A was mostly nuclear ($>70\%$), whereas MRTF-B was mainly cytosolic ($>70\%$). When we wished to verify that the changes in actin organization are indeed able to redistribute MRTF-B in kidney epithelial cells, jasplakinolide, a potent actin-polymerizing agent, was used. This drug provoked robust nuclear accumulation of MRTF-B (Figure 24).

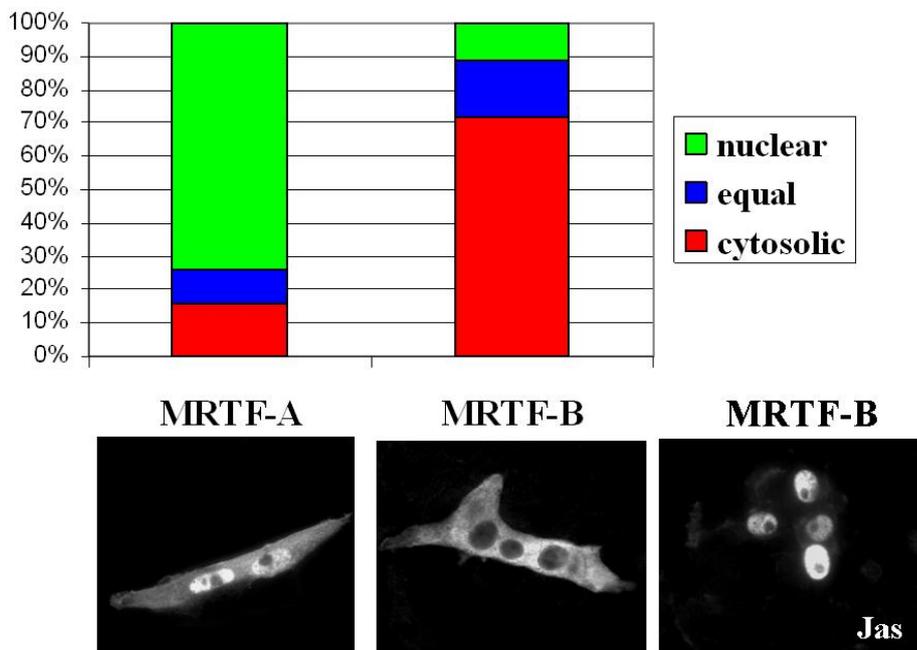


Figure 24. Distinct localization of MRTF isoforms in LLC-PK1 cells. LLC-PK1 cells were transfected with either FLAG-tagged MRTF-A or MRTF-B and two days later stained with an anti-FLAG antibody. FLAG-expressing LLC-PK1 cells (3940) were

counted for nuclear, even or cytosolic distribution. MRTF-A was mainly localized to the nuclei (74%), whereas equal (10%) or cytoplasmic (16%) localization was less frequent. MRTF-B was mainly localized in the cytoplasm (72%), 17% of cells exhibiting equal and 11% nuclear staining. Jasplakinolide (Jas) treatment (0.5 μ M, 12 hrs) induced strong nuclear accumulation of the transfected MRTF-B.

The localization of endogenous MRTF was assessed using a polyclonal antibody raised against BSAC, the mouse homologue of MKL1/MRTF-A. In resting LLC-PK1 cells endogenous MRTF showed entirely cytosolic distribution with strong nuclear exclusion. One hour after Ca^{2+} removal, MRTF became nuclear in small clusters of cells, however, 24 hours after cells were Ca^{2+} deprived, MRTF showed a marked nuclear accumulation: 16% of cells showed nuclear accumulation, 74% of cells exhibited equal distribution of MRTF in the nucleus and cytoplasm, and in 10% of cells the staining was entirely cytoplasmic (Figure 25A).

These findings raised the possibility that the contact-dependent regulation of MRTF distribution might play an important role in the differential responsiveness of confluent and non-confluent cultures to the EMT-inducing effect of TGF- β 1. In order to test this hypothesis, we compared MRTF distribution in confluent and non-confluent cultures exposed to TGF- β 1 for various times. Endogenous MRTF was entirely cytosolic in confluent cultures. Treatment of intact confluent layers with TGF- β 1 (0–24 h) did not induce nuclear translocation of MRTF, and most cells showed no change in MRTF localization at all, whereas some exhibited a punctate, perinuclear labeling. A radically different picture was observed in subconfluent cultures. Under resting condition, \sim 75% of the cells located at the free edges of cellular islands showed cytosolic MRTF staining, whereas \sim 17% showed clear nuclear accumulation and 8% had even cytosolic and nuclear distribution. The extent of the nuclear accumulation of MRTF in subconfluent layers was in good agreement with the values obtained in cells in which the contacts were disassembled by Ca^{2+} depletion. In cells located in the intact inner regions of these multicellular islands, MRTF was fully cytosolic. In subconfluent layers (as opposed to the confluent ones), TGF- β 1 exposure induced a dramatic change in MRTF distribution: in cells at the free edges, perinuclear MRTF condensation was apparent after 1 h treatment (not shown), whereas after 6 h, 95% of peripheral cells showed strong nuclear accumulation of MRTF. Cells in rows adjacent to the peripheral row also showed increased nuclear localization, whereas in the inner areas MRTF remained cytosolic. To our surprise nuclear accumulation of MRTF in the peripheral

cells was transient: after 24 h of TGF- β 1 treatment, the response significantly decreased: only 25% of the cells showed clear nuclear MRTF localization, whereas even distribution or punctate, perinuclear labeling was visible in 12% of the cells (Figure 25B, C). Next we assessed the effect of the combination of the two treatments on MRTF localization. When Ca²⁺ removal and addition of TGF- β 1 was combined for 6 hours, MRTF showed a massive nuclear translocation in almost all cells, a similar pattern to the result of 6 hours TGF- β 1 treatment of non-confluent layers (Figure 25D).

In addition to Ca²⁺ removal and subconfluence, a third, and from a pathological standpoint possibly the most relevant, model of contact disruption was mechanical wounding of a confluent monolayer. Cells located at the wound edge exhibited nuclear accumulation of endogenous MRTF (Figure 25E), whereas the next 2-3 rows of cells adjacent to the wound showed less and less nuclear staining, MRTF being localized solely to the cytoplasm in the rest of the cells.

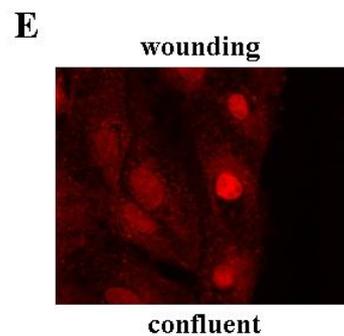
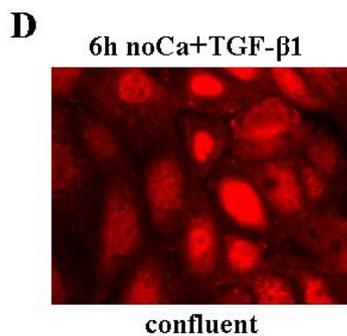
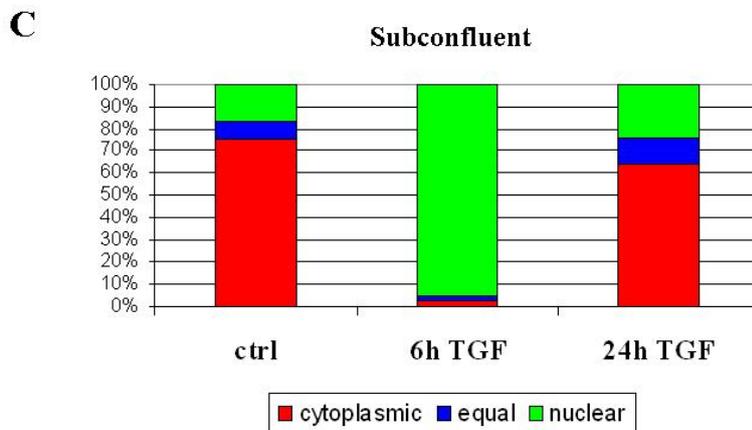
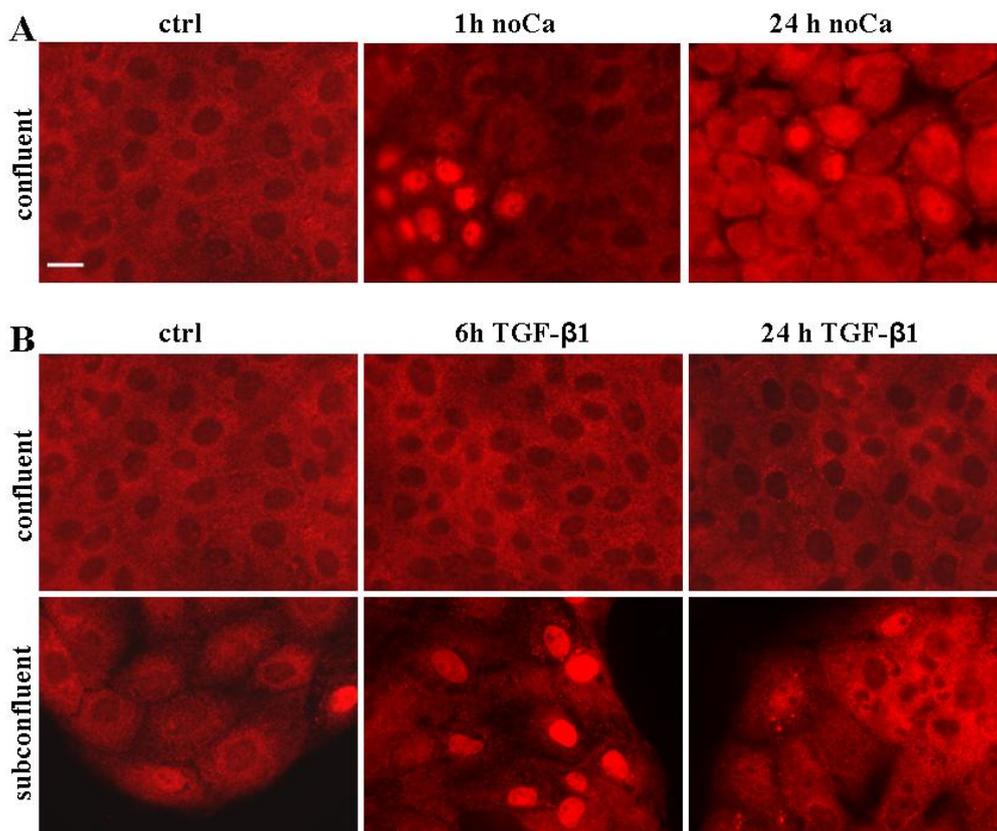


Figure 25. (A) Contact disassembly induces nuclear translocation of endogenous MRTF in LLC-PK1 cells. Cells were serum-depleted for 3 h and then placed into either

Ca²⁺ containing or Ca²⁺-free DMEM for 1h or 24 h. Cells were then fixed and stained for endogenous MRTF using a polyclonal antibody raised against BSAC, the mouse MKL1 or MRTF-A protein. **(B)** *TGF-β1 induces nuclear translocation of endogenous MRTF in subconfluent cells, without having such an effect on confluent layers.* Cells were grown to 100% confluence or approx. 30% confluence (subconfluent) and left untreated and fixed or treated with 10 ng/ml TGF-β1 for the indicated times and then fixed and stained for MRTF. **(C)** The bar diagram indicates the intracellular distribution of endogenous MRTF in cells at the periphery of cellular islands, under control conditions or after treatment for the indicated times with TGF-β1. **(D)** *The combined 6 h treatment with Ca²⁺ removal and TGF-β1 induces a massive nuclear translocation of MRTF in confluent cells.* Cells grown to confluence were serum starved for 3 hour, followed by Ca²⁺ removal and treatment with 10 ng/ml TGF-β1 for 6 hours. Cells were fixed and stained for MRTF. **(E)** *Mechanical wounding translocates MRTF to the nuclei of cells situated at the edge of the wound.* A wound was generated in a confluent monolayer with a rubber policeman, and 6 h later the cells were fixed and stained for MRTF.

Next we examined which upstream mechanisms regulate MRTF nuclear-cytoplasmic shuttling. First, LLC-PK1 cells were transfected with the CA Rho construct, cells being double stained for Myc and BSAC/MRTF. Expression of Rho redistributed the endogenous MRTF into the nucleus (Figure 26A, C), about 85% of Rho transfected cells showing intense nuclear labeling for MRTF, the rest of the cells exhibiting an even distribution in the cytoplasm and the nucleus. Next DN-Rho transfected cells were then subjected to Ca²⁺ removal. The expression of DN-Rho strongly inhibited the nuclear translocation of MRTF upon cell contact disruption. Moreover, cells transfected with DN-MLC also inhibited the nuclear translocation of MRTF after Ca²⁺ removal (Figure 26B, C).

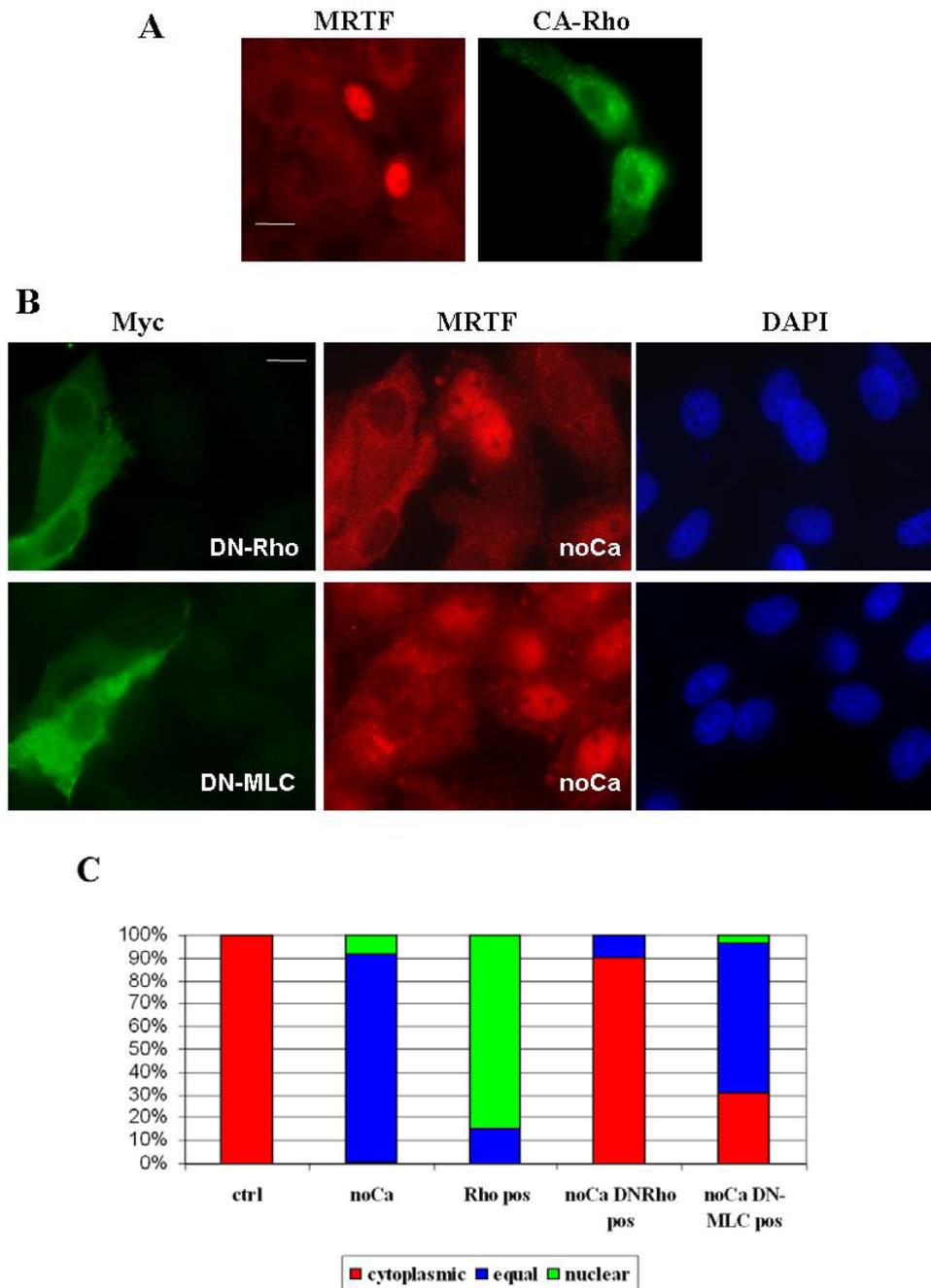


Figure 26. Contact disassembly induces Rho- and MLC phosphorylation-dependent nuclear translocation of endogenous MRTF in LLC-PK1 cells. **(A)** Cells were transfected with Myc-tagged CA Rho and 24 h later fixed and stained for endogenous MRTF and Myc. Transfected cells exhibited nuclear accumulation of MRTF. **(B)** Cells were transfected with Myc-tagged DN-Rho (upper panel) or Myc-tagged DN-MLC (lower panel) and 24 hours later subjected to Ca^{2+} removal for 24 h, fixed and stained for Myc, MRTF and DAPI. DN-Rho and DN-MLC transfected cells exhibit a reduced nuclear accumulation of MRTF upon contact disassembly as compared to their non-transfected neighbors. **(C)** Distribution of endogenous MRTF was quantified in each transfected group. The number of evaluated cells was: control 283, noCa 438, Ca Rho

52, DN-Rho 52, DN-MLC 224. (Rho pos= cells transfected with CA Rho, noCa DNRho pos= DN Rho transfected cells subjected to cell contact disassembly, noCa DNMLC pos= DN MLC transfected cells subjected to cell contact disassembly)

Similarly to Rho, overexpression of CA forms of Rac1, Cdc42 and PAK (Figure 27A) also induced the nuclear translocation of MRTF in almost all corresponding transfected cells. Accordingly, in cells transfected with the DN forms of Rac1, Cdc42 and PAK, nuclear translocation of MRTF upon Ca²⁺ removal was inhibited (Figure 27B, C).

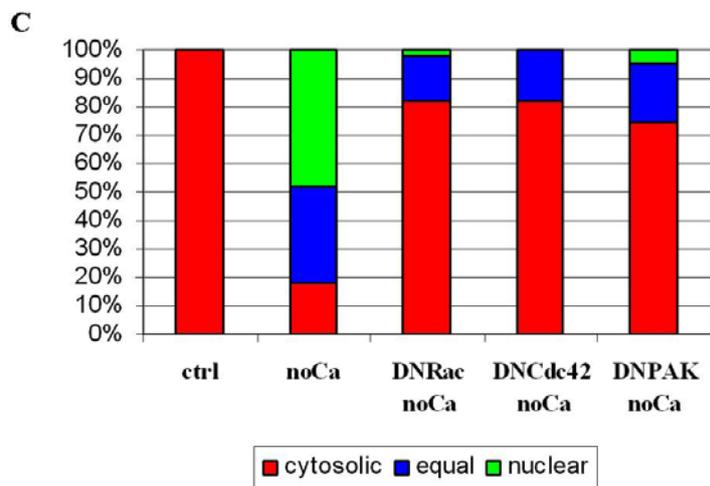
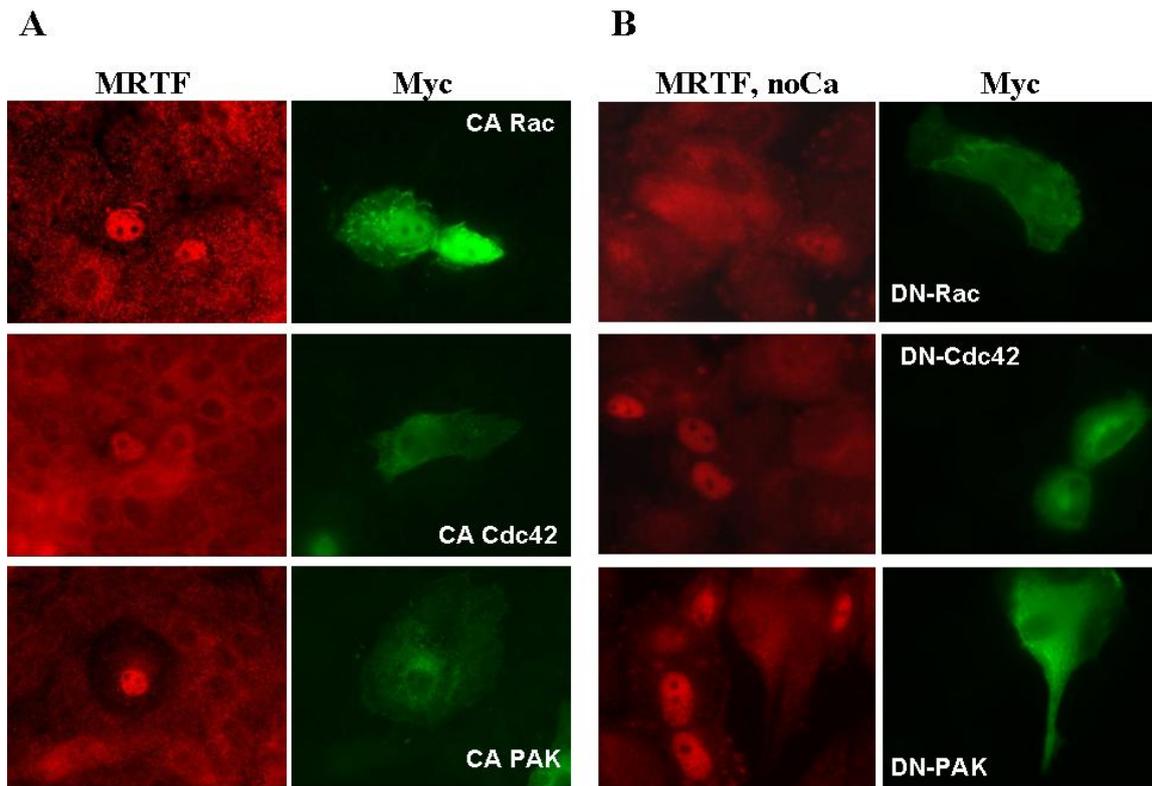


Figure 27. Contact disassembly induces *Rac*-, *Cdc42*- and *PAK*- dependent nuclear translocation of endogenous MRTF in LLC-PK1 cells. (A) Cells were transfected with Myc-tagged CA *Rac*, CA *Cdc42* and CA *PAK* and 24 h later fixed and stained for endogenous MRTF and Myc. Transfected cells exhibited nuclear accumulation of MRTF. (B) Cells were transfected with Myc-tagged DN-*Rac*, DN-*Cdc42* or DN-*PAK* and 24 hours later subjected to Ca^{2+} removal for 24 h, fixed and stained for Myc and MRTF. DN-*Rac*, DN-*Cdc42* and DN-*PAK* transfected cells exhibit a reduced nuclear accumulation of MRTF upon contact disassembly as compared to their non-transfected neighbors. (C) Distribution of endogenous MRTF was quantified in each transfected group. The number of evaluated cells was: control 127, noCa 307, DN-*Rac* 62, DN-*Cdc42* 77, DN-*PAK* 68.

Next we investigated whether p38 influences cellular distribution of MRTF. The pretreatment and presence of SB203580 in cells subjected to cell contact disruption dramatically reduced the Ca^{2+} removal induced nuclear translocation of MRTF, as revealed by immunoblots performed on nuclear extractions (Figure 28A) or by immunofluorescent microscopy (Figure 28B, C).

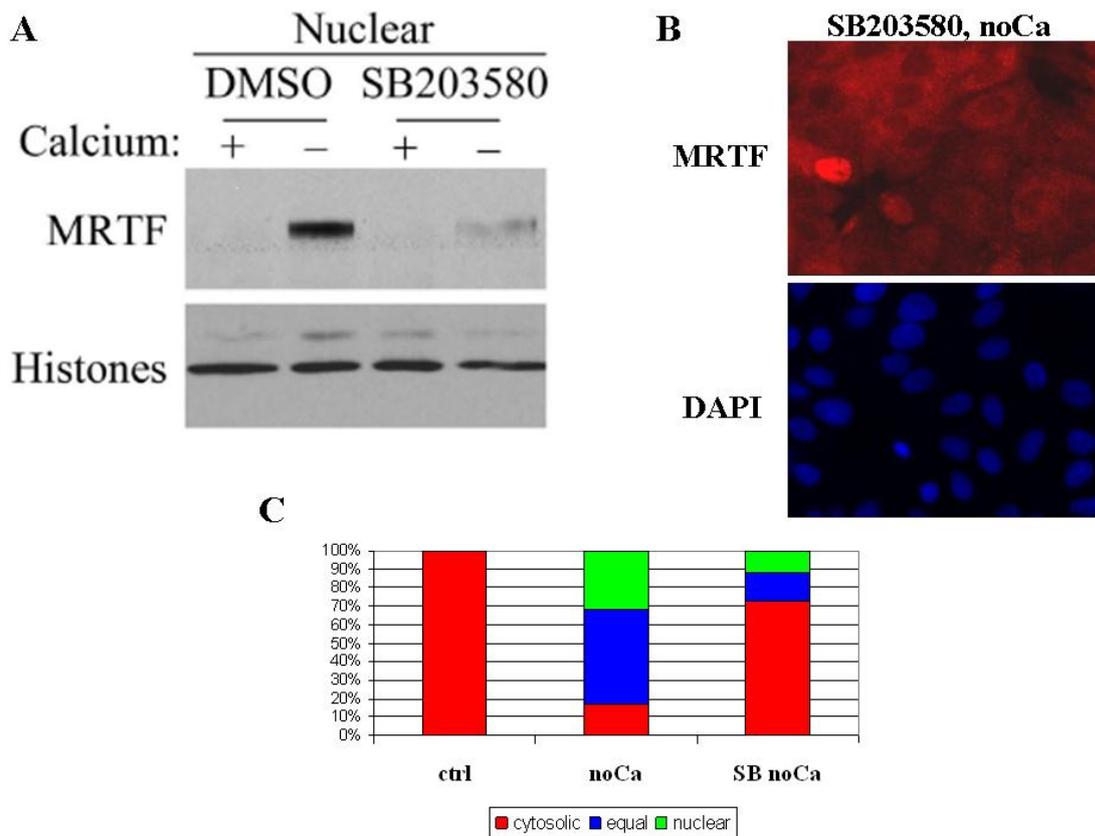


Figure 28. Contact disassembly induces p38- dependent nuclear translocation of endogenous MRTF in LLC-PK1 cells. (A) Confluent cells were pretreated with DMSO or 10 μ M SB203580 for 30 minutes prior to incubation with or without extracellular calcium for one hour. Nuclear extractions were prepared, and their MRTF content was analyzed by Western blotting. Membranes were re-probed with anti-histones to assess

equal loading. **(B)** Confluent cells grown on coverslips were pretreated with 10 μM SB203580 for an hour and then were subjected to 24 hours of Ca^{2+} removal, then fixed and stained for MRTF. SB203580 prevented the accumulation of endogenous MRTF in the nuclei. **(C)** Distribution of endogenous MRTF was quantified in the control, Ca^{2+} deprived with or without the SB203580 pretreatment. The number of evaluated cells was: control 142, noCa 490, SB noCa 625.

Based on these results, nuclear translocation of MRTF is regulated by cell contact disassembly and TGF- β 1. Small GTPases Rho, Rac, Cdc42, similarly to their downstream effectors PAK, MLC and p38 regulate cellular distribution/nuclear translocation of MRTF.

IV.8. MRTF is an important regulator of the cell contact–regulated and TGF- β 1–modulated SMA promoter activation and SMA synthesis

We wished to examine the possible direct link between MRTF and SMA. Therefore cells were transfected with the MRTF-A and MRTF-B. Consistent with an important role of MRTF in the regulation of SMA expression, transfection of MRTF isoforms led to a robust increase in the SMA promoter activity, which, in agreement with the localization data, was stronger in case of MRTF-A than MRTF-B (Figure 29A). Besides the marked effect on the SMA promoter, transfection of MRTF-A and MRTF-B resulted in actual SMA protein synthesis, as assessed by Western blotting (Figure 29B) and immunofluorescent experiments (Figure 29C). Cells transfected with the MRTF plasmids were harvested 48 hours after transfection, and the presence of α -SMA protein was detected by Western blot. Similarly, cells transfected for 48 hours with MRTF-A and MRTF-B showed de novo α -SMA protein expression. The expression of SMA was robust considering that transient transfection of a few percent of the cells with MRTF-A or B resulted in SMA synthesis that was readily detectable in total cell lysates by Western blotting. Control or mock-transfected epithelial cells did not express SMA.

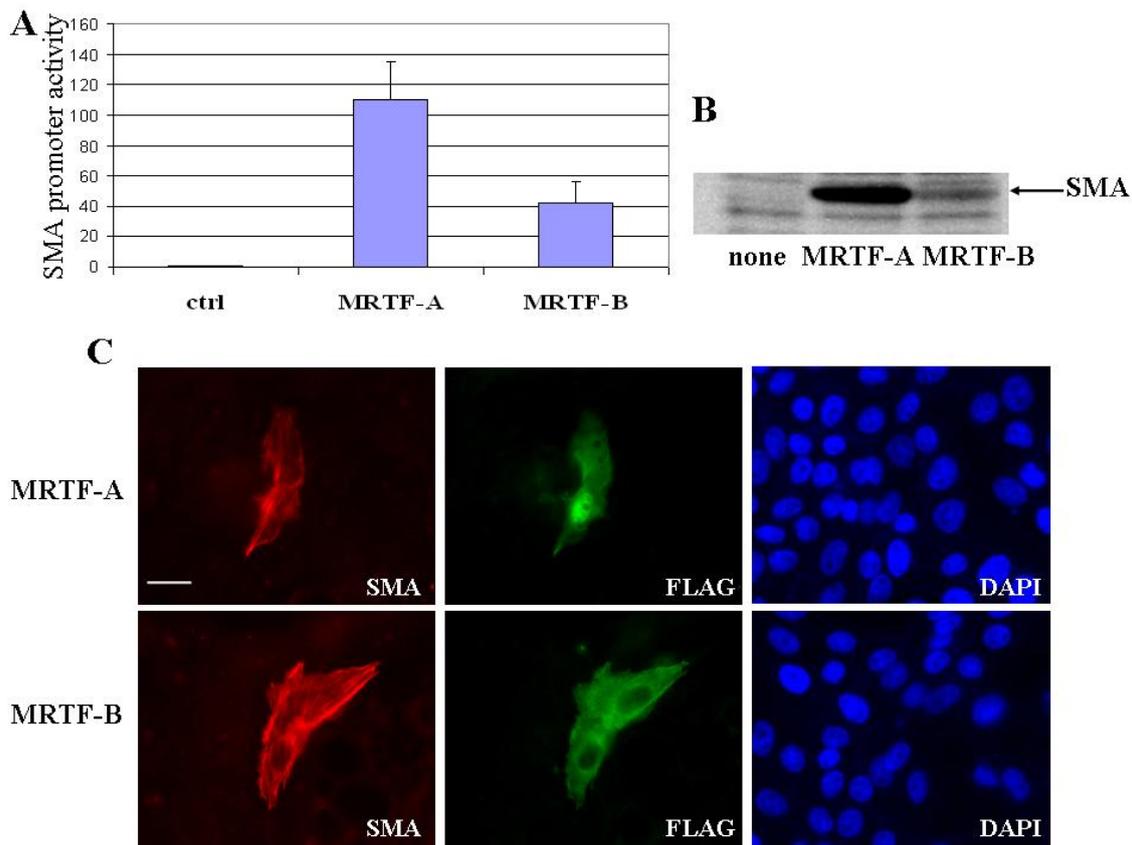


Figure 29. *MRTF* is a potent inducer of *SMA* promoter and *SMA* protein synthesis. **(A)** Cells grown in 6 well plates were cotransfected with pSMA-Luc, pRL-TK and either MRTF-A or MRTF-B. Twenty-four hours later SMA promoter activity was determined. **(B)** Non-transfected controls (none) or cells transiently transfected with MRTF-A or MRTF-B for 48 h were lysed and analyzed by Western blotting using an anti-SMA antibody. Control cells do not express SMA, whereas both MRTF-A and MRTF-B were able to induce SMA expression. The response was stronger in the case of MRTF-A in agreement with the strong nuclear localization and greater SMA promoter-activating capacity of this construct. **(C)** Cells were transfected with FLAG-tagged MRTF-A or FLAG-tagged MRTF-B, and after 48 h, fixed and stained for SMA and FLAG. DAPI was used for nuclear stainings.

Finally, to address whether MRTF has an ultimate causal role in the contact injury-dependent and TGF- β 1-dependent SMA promoter response, transient transfection experiments were carried out with a Δ C585 mutant FLAG-tagged myocardin, DN-MyoC, which lacks the transactivation domain, and has been shown to act as dominant negative against each member of the MRTF family (Wang et al., 2001). This mutant showed spontaneous accumulation in the nucleus and was present in the cytosol too, as revealed by immunostaining with an anti-FLAG antibody (Figure 30A). Expression of DN-MyoC abolished the Ca²⁺ deprivation-triggered increase in promoter activity and strongly suppressed the synergism between contact disassembly and TGF-

$\beta 1$ (Figure 30B). Moreover, DN MRTF also abolished the SMA activating effect of TGF- $\beta 1$ in non confluent layers (Figure 30C). These observations suggest that endogenous MRTF activity is a central target of the cell contact- and TGF- $\beta 1$ -dependent regulation of the SMA promoter, and it plays an indispensable role in myofibroblasts differentiation of kidney tubular cells.

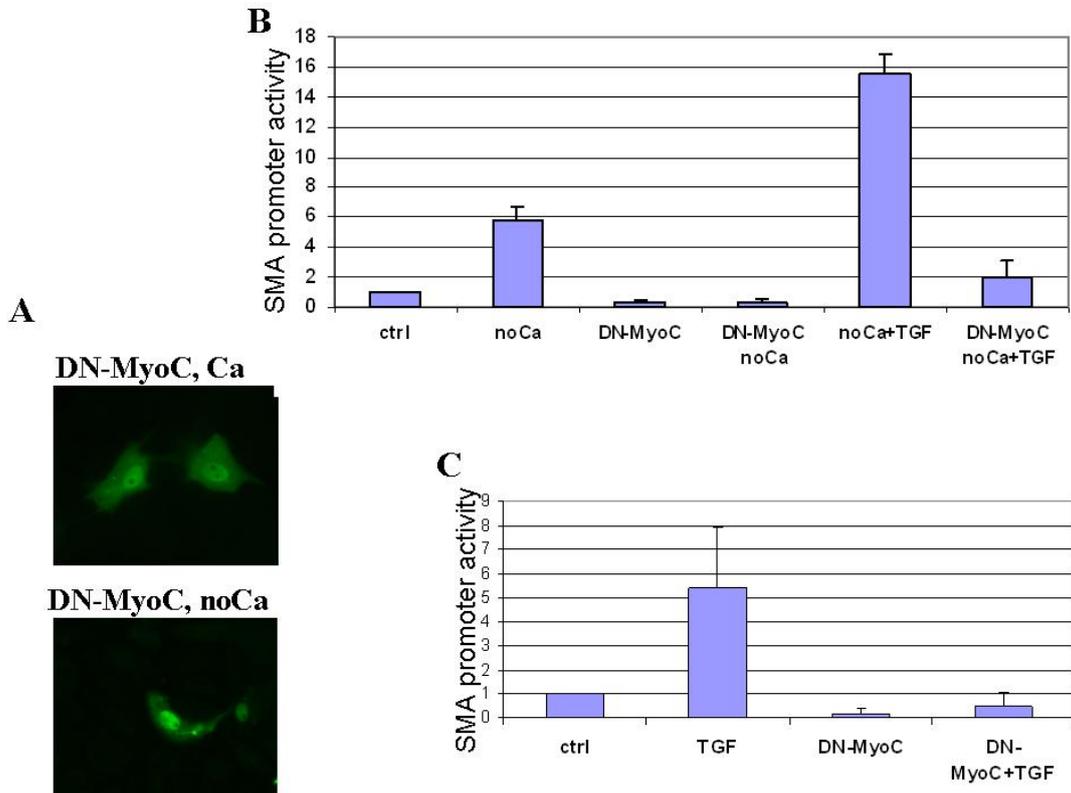


Figure 30. Dominant negative MRTF inhibits the contact disassembly- and the TGF- $\beta 1$ -induced activation of the SMA promoter and suppresses the synergism between contact disruption and TGF- $\beta 1$. **(A)** Cells transfected with FLAG-tagged DN-MyoC for 24 h were serum-starved, incubated in Ca^{2+} containing or Ca^{2+} -free medium for 24 h, fixed, and stained using an anti-FLAG antibody. DN-MyoC exhibited a predominantly nuclear localization irrespective of the state of the intercellular contacts. **(B)** Confluent cells were cotransfected with pSMA-Luc, pRL-TK along with either empty vector (pcDNA3) or DN-MyoC for 24 h, and then exposed to Ca^{2+} removal, 10 ng/ml TGF- $\beta 1$, or the combination of these treatments. Luciferase assay was performed from the cell lysates. **(C)** Subconfluent cells were cotransfected with pSMA-Luc, pRL-TK along with either empty vector (pcDNA3) or DN-MyoC for 24 h, and following 4 hours of serum starvation, 10 ng/ml TGF- $\beta 1$ was added as indicated. Luciferase assay was performed to analyze SMA promoter activity.

IV.9. Distinct regulation of SMA promoter activity by small GTPases: the role of H-Ras

The small GTPases Rac1, Cdc42 and Rho proved to be important regulators of SMA expression. We aimed elucidate potential roles of other GTPases in the regulation of SMA expression. The active Ras isoform, H-Ras, another member of this family, has been linked to oncogenic pathways. Recently, Kaplan-Albuquerque et al. (Kaplan-Albuquerque et al, 2003) investigated its potential role in regulating the SM22alpha expression in vascular smooth muscle cells. Expression of constitutively active Ras in these cells produced suppression on SM22alpha gene activity. We assessed its function in our system. Cotransfection of CA H-Ras with the SMA promoter had no effect on the promoter activity. On the other hand CA H-Ras strongly inhibited the Ca^{2+} removal induced promoter activation, from a 6.5 fold the promoter activity dropped to 1.7 fold. Parallel to this, DN H-Ras a positive effect on the promoter, its presence during Ca^{2+} removal induced a 12.5 fold increase in SMA promoter activity (Figure 31A).

Next, we addressed whether H-Ras effects have the same endpoint as Rho, Rac1, Cdc42, PAK effects have. Cotransfection of CA H-Ras with the long and the short promoter constructs showed parallel inhibition of the cell contact disassembly induced SMA promoter activation, from ~2.3 fold to 0.9. Based on these data we concluded that the inhibitory effect of H-Ras is also pointed to the SRF binding domain of the SMA promoter, which means that H-Ras has an inhibitory effect on SRF (Figure 31B).

Rho family GTPases RhoA, Rac1, Cdc42 and their downstream effectors, ROK and PAK respectively, are positively regulating SMA promoter activity through SRF dependent pathways. In contrast to the Rho family GTPases, H-Ras has an inhibitory effect on SMA promoter over the same SRF dependent pathway. This data was confirmed by the experiments with the dominant negative H-Ras construct, which arrested the inhibitory effects of H-Ras on the promoter.

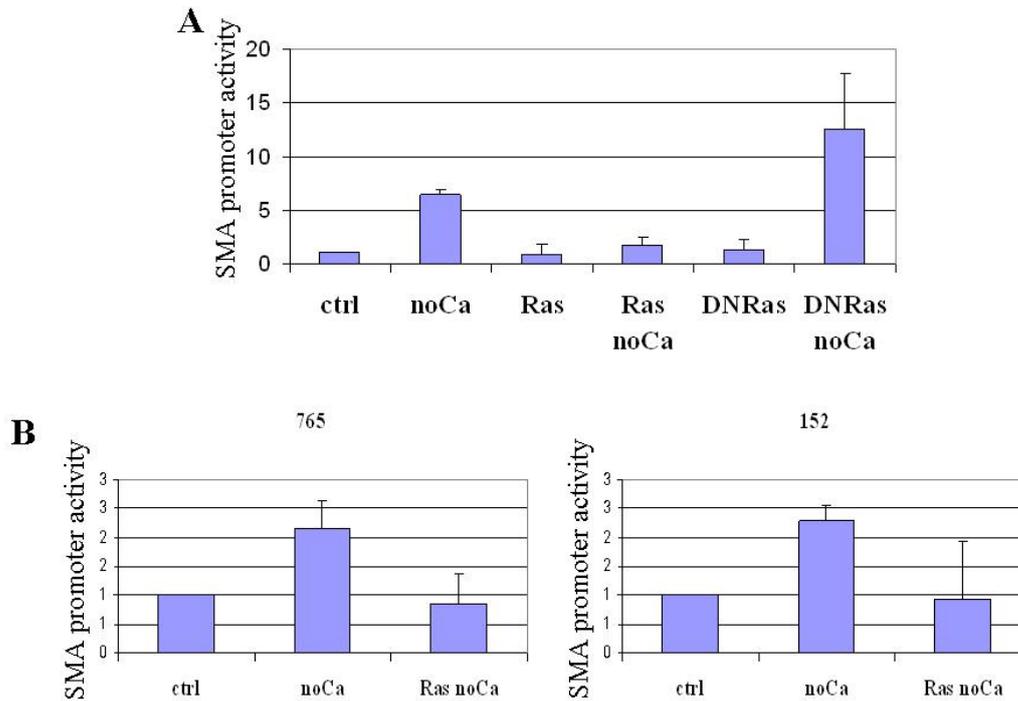


Figure 31. *H-Ras* negatively regulates the SMA promoter. **(A)** Confluent cells grown on 6-well plates were transfected with the SMA promoter, pRL-TK, and pcDNA3, H-Ras and DN-H-Ras as indicated. Control, H-Ras and DN-H-Ras transfected groups 24 hours after transfection were serum deprived for 24 hours. Groups stimulated with contact disassembly were subjected to 24 hrs of Ca^{2+} removal. The presence of H-Ras inhibited the effect of contact disassembly on the SMA promoter (6.5 ± 0.4 v. 1.7 ± 0.85 , $n=3$, $p<0.05$), while DN-H-Ras induced a more pronounced effect (6.5 ± 0.4 v. 12.5 ± 5.15 , $n=3$, $p<0.05$). **(B)** Cells were transfected with pGL3-SMA-765 vector or pGL3-SMA-152 vector, pRL-TK and H-Ras as indicated. 24hrs after transfection cells were Ca^{2+} deprived for 24 more hrs, then harvested and luciferase assay was performed. H-Ras inhibited the induction by Ca^{2+} removal of both promoters (2.2 ± 0.49 v. 0.9 ± 0.5 for the 765bp. promoter and 2.3 ± 0.27 v. 0.9 ± 0.71 for the 152bp. promoter, $n=3$, $p<0.05$).

IV.10. Cell-cell contact status regulates SMA expression independently of receptor availability

The experimental data presented here demonstrated that EMT and SMA are both dependent on cell contact integrity and TGF- β 1. The joint regulation of EMT and SMA expression by cell contact disassembly and TGF- β 1, the “two-hit” model, needs the interplay of several pathways that were dissected here. The last question addressed regarded the hypothesis that limited receptor accessibility might be a potential factor in the augmented effects of TGF- β 1 upon contact disruption. For this we tested the effect

of Ca^{2+} removal and TGF- β 1 on another TGF- β 1 induced effect, the activation of the Smad- binding element by using a TGF- β 1-responsive SBE4 reporter construct (p-SBE-Luc, Felici et al., 2003), a construct containing 4 tandem Smad-binding elements, which are induced by active Smad 3/4. First, the SBE4 promoter was transfected to subconfluent cells, where TGF- β 1 induced its 2.9 ± 0.13 fold activation ($n=3$, $p < 0.05$) (Figure 32A). Next SBE4 was transfected to confluent layers. Here Ca^{2+} depletion did not influence its activity. TGF- β 1 induced a similar stimulation of SBE4 both in the presence (3.0 ± 0.31) or absence of Ca^{2+} (2.7 ± 0.52) (Figure 32B), indicating that altered receptor accessibility does not play a key role in the observed effects, and that the Ca^{2+} removal-induced enhancement is specific for the SMA promoter. Furthermore, Ca^{2+} removal failed to change the effect of TGF- β 1 also at low TGF- β 1 concentrations, indicating the lack of the difference was not due to an already saturated response.

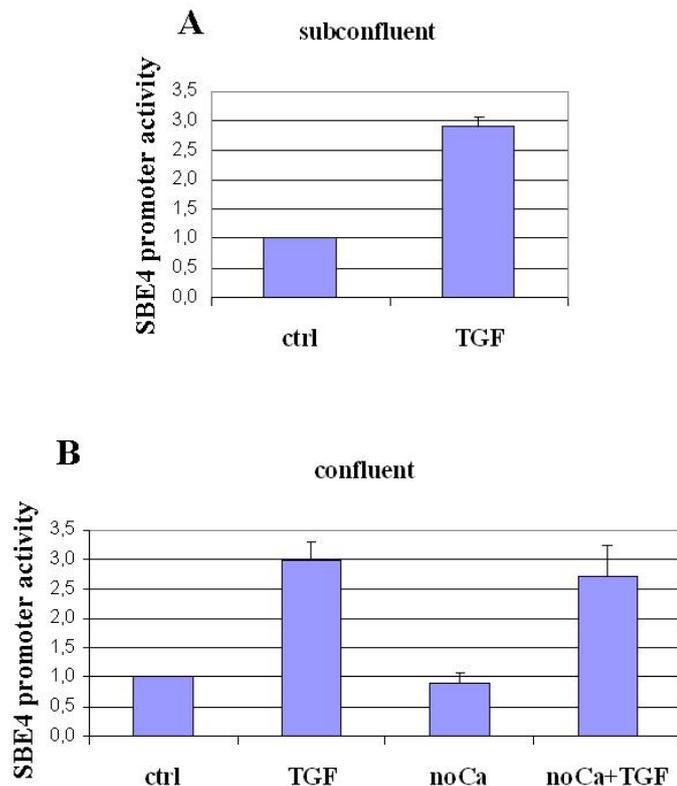


Figure 32. Ca^{2+} removal does not act through increasing receptor availability for TGF- β 1. **(A)** Subconfluent cells were transfected with the TGF- β 1-responsive SBE reporter (p-SBE4-Luc) and were either treated with 10 μM TGF- β 1 or its vehicle. SBE4 promoter activity was assessed by luciferase assay. **(B)** Confluent cells were transfected with the TGF- β 1-responsive SBE reporter (p-SBE4-Luc) and left untreated or challenged with Ca^{2+} removal, 10 μM TGF- β 1, or the combination of these stimuli. Luciferase assay was performed from the cell lysates obtained.

V. DISCUSSION

Epithelial-to-mesenchymal transition has been shown to be a highly relevant event during tubulo-interstitial fibrosis in the kidney. During this process tubular cells lose their epithelial markers, change their shape and become motile. As a result of this change a new, different cell type emerges, the so called myofibroblast. One of the most important markers of this process is the alpha-smooth muscle actin, a protein which is not present in the original epithelial cells, but it appears in cells upon the acquisition of the mesenchymal phenotype. Injury or absence of intercellular contacts exerts a permissive and enhancing effect on the transdifferentiation of epithelial cells to myofibroblasts (Masszi et al. 2004). This phenomenon may have a key importance from a patho-biologic standpoint: while intact epithelia may be partially resistant to the fibrogenic effect of TGF- β 1, an initial injury may render the wounded region susceptible for this cytokine, thereby generating focally transformed areas. From these foci the process can spread to neighboring regions.

The aim of this work was to describe mechanisms regulating SMA expression during renal EMT, in regard of the “two hit” model established by our group. SMA synthesis is dependent on both TGF- β 1 and the status of intercellular junctions. We proposed decipher new insights into regulation of TGF- β 1- induced, cell contact and small GTPase dependent SMA expression by approaching several pathways, that are discussed here. Beyond the experimental data, there is scientific evidence of several intriguing possibilities for cross-talk between these pathways, showing the complexity of EMT regulation. These findings are also reviewed here in regard of the presented experimental data to try to elucidate more of this complexity.

In the first chapter of the Results section we discussed the role of the Smad family of signaling proteins during the TGF- β 1 dependent EMT hit. We established that both Smad2 and Smad3 were phosphorylated by TGF- β 1, although presenting different activation patterns: while Smad2 exhibited a biphasic activation curve with an acute and a chronic peak, Smad3 showed activation only 1 hour after the treatment. Both Smads were shown to play a major role in mediating TGF- β 1 effects during SMA protein synthesis. In experiments using adenoviruses and plasmids expressing inhibitory Smad

constructs, Western blot and transient transfection approaches showed that both Smad2 and Smad3 are necessary during TGF- β 1 induced expression of SMA.

The differential role of Smad2 and Smad3 in mediating fibrotic effects has long been discussed in the literature. It has been suggested, that only Smad3 would be responsible for TGF- β 1 induced transdifferentiation (Saika et al. 2004a). It was also reported that Smad7 inhibits fibrotic effect of TGF- β 1 on renal tubular epithelial cells by blocking Smad2 activation (Li et al. 2002). This idea of Smad2 and Smad3 having differential role in regulation of EMT was supported with data by Phanish and coworkers (Phanish et al. 2005) where they suggested the differential role of Smad2 and Smad3 only in regulation of E-cadherin, MMP-2 and CTGF in proximal tubular epithelial cells, but not in the case of SMA expression. Their data suggests the involvement of both Smad2 and Smad3 in the regulation of SMA. Similarly, Valcourt and coworkers (Valcourt et al. 2005) presented data showing that both Smad2 and Smad3 are required in TGF- β 1 induced EMT in human and mouse epithelial cells.

The SMA promoter harbors several transcriptional regulatory elements, including the SRF/ MRTF- binding CArG-boxes, the Kruppel factor-binding TGF- β 1 control element (TCE) and the TGF- β 1-responsive Smad binding element (SBE). Accordingly the promoter can be regulated by both contact- and TGF- β 1- dependent pathways.

Smad2 and Smad3 may have a different subset of target genes and regulate distinct cellular processes. Smads must cooperate with other transcription factors to activate or repress target genes. Smad2 was shown to activate p38 and subsequently Rho during TGF- β 1 induced endothelial barrier dysfunction (Lu et al. 2006). On the other hand, RhoA was shown to modulate Smad2 and Smad3 phosphorylation during smooth muscle differentiation (Chen et al. 2006). Smad3 may interact with SRF-associated complexes to regulate SM22 expression during TGF- β 1 induce myofibroblast transdifferentiation (Qiu et al. 2003). Smad7 plays an important role in TGF- β 1 effects, competing with the R-Smads, acting as a general inhibitor of TGF- β 1. Although regarded as an I-Smads, Edlund and coworkers (Edlund et al. 2003) suggested that Smad7 is a positive regulator of the TGF- β 1-TAK1-MKK3-p38 pathway leading to apoptosis in PC-3U cells. The same group proved that Smad7 is required for TGF- β 1-induced activation of the small GTPase Cdc42, an upstream of p38 (Edlund et al. 2004).

The association of Smad3/4 and β -catenin was shown to play a major role in adherent junction disassembly (Tian and Phillips 2002) and EMT (Masszi et al. 2004). Moreover, the interaction between Smad7 and β -catenin is a key moment in TGF- β 1 induced apoptosis (Edlund et al. 2005). It has been shown that the liberation of β -catenin is a potent activator of EMT, and is regarded as a key step during EMT. Moreover, MRTF was also found to interact with Smads. In addition to forming ternary complex with SRF and CArG boxes, it was found to bind to the Smad proteins too, and thus it might facilitate transcription through the SBE (Qiu P et al. 2005).

The role of RhoA in regulating SMA expression and EMT was previously showed by Masszi and coworkers (Masszi et al. 2003). TGF- β 1 was shown to activate RhoA in a biphasic manner in LLC-PK1 cells, similarly to the activation of RhoA shown in PC-3U human prostate carcinoma cells during TGF- β 1 induced rearrangements of the actin filament system (Edlund et al. 2002). Here we showed that RhoA is also activated by Ca^{2+} removal induced cell contact disassembly followed by ROK-mediated MLC phosphorylation. Our finding that the Ca^{2+} removal-induced disruption of cell junctions activates Rho is in good accord with the reported converse phenomenon i.e. that during the Ca^{2+} triggered formation of intercellular junctions Rho activity is gradually downregulated (Noren et al. 2003). In tubular cells, contact disassembly led to rapid and long-lasting MLC phosphorylation, which was most prominent at the cell periphery. This response was mediated by the Rho/ROK pathway since it was inhibited by genetic or pharmacological interference with this signaling route. The same maneuvers abolished the Ca^{2+} removal-induced activation of the SMA promoter as well, indicating that the Rho/ROK pathway has a key role in cell contact-dependent regulation of gene expression. In addition to the spatially restricted activation of Rho, junctional ROK and/or myosin localization or accumulation may also contribute to the focal MLC phosphorylation. Indeed, a subpool of ROK was found to be associated with the adherent junctions (Walsh et al. 2001), and a peripheral myosin ring is present in epithelial cells (Ivanov et al. 2004, Ivanov et al. 2005). Thus, each component of the Rho/ROK/MLC pathways can be junction-associated, facilitating the preferential activation of this particular downstream Rho pathway at the contacts.

Rho has been shown to increase the transcriptional activity of SRF on those target genes, including SMA, whose promoter harbors CArG boxes (Hill et al. 1995,

Mack et al. 2001, Masszi et al. 2003). Elegant studies have revealed that the effect of Rho is mediated by cytoskeletal reorganization, a key component of which is enhanced F-actin polymerization (Miralles et al. 2003). So far two downstream Rho effector pathways have been implicated in SRF-dependent transcription: the activation of the formin protein mDia, which induces net F-actin polymerization (Copeland and Treisman 2002) and the activation of the Rho/ROK/LIM kinase/cofilin phosphorylation pathway, which stabilizes F-actin due to decreased severing (Geneste et al. 2002). The former mechanism was predominant in fibroblasts, whereas both were critical in neuron-like PC12 cells. Here a third Rho effector pathway, the ROK-dependent MLC phosphorylation, is shown to be an important modulator of SRF-dependent transcription. This mechanism, at least in our epithelial cells, seems to be an important contributor, since the myosin inhibitor blebbistatin or a phosphorylation incompetent DN myosin mutant abolished the contact disruption-provoked SMA promoter expression, eliminated the synergism between contact injury and TGF- β 1 on the promoter, and suppressed SMA protein expression. Peripheral myosin activity (junctional contractility) has been proposed to participate in the regulation of various functions including junction remodeling (Ivanov et al. 2004, Ivanov et al. 2005), cell scattering (de Rooij et al. 2005), morphogenesis (Bertet et al. 2004), and closure of epithelial wounds (Darenfed and Mandato 2005). Our data assign yet another critical role for this process: the regulation of SRF-dependent gene expression. This mechanism efficiently couples the mechanical and genetic responses to wounding: formation of actin-myosin complexes triggers contractile wound closure and at the same time initiates genetic reprogramming leading to enhanced generation of extracellular matrix proteins and contractile elements.

MLC regulates SMA synthesis through MRTF. There are at least two scenarios to explain how MLC can act through MRTF, and how myosin activity impacts on MRTF localization or activity. MRTF localization is regulated by the G/F actin ratio. Binding of monomeric actin (presumably through a yet unidentified protein) to MRTF prevents its translocation to the nucleus whereas actin polymerization removes G-actin from MRTF, thereby exposing its nuclear localization sequence (Miralles et al. 2003, Posern et al. 2004). It is conceivable that myosin activity, which promotes actin filament bundling, can engage monomeric actin from MRTF, or the formation of actin-myosin complexes may specifically reduce the MRTF-binding competent pool of actin. Another potential mechanism is that myosin, as a force-generating protein, might be

required for the efficient nuclear import or retention of MRTF. There is accumulating evidence that both the microtubule and the microfilament cytoskeleton are involved in the nuclear import of certain proteins (Campbell and Hope 2003). Myosin may affect other processes in addition to MRTF translocation. Ivanov and colleagues showed that myosin activity is essential for the contact disassembly induced internalization of E-cadherin, and blebbistatin maintains E-cadherin at the cell surface (Ivanov et al. 2004). Similarly, the Src-mediated delocalization of E-cadherin from the AJ also requires MLC phosphorylation (Avizienyte et al. 2004). Taken together, junction stabilization by myosin inhibition may contribute to the inhibition of the SMA promoter.

Small GTPases are involved in the regulation of the cytoskeleton. Rho was previously shown to be a key regulator of EMT and SMA expression. There is recent evidence supporting the role of other members of this family in regulating EMT. Parallel activation of Rho, Rac1 and Cdc42 induced by activated PBMC conditioned medium (aPBMC-CM) was shown to regulate EMT in HK2 cells, showing that Rho effects are mediated by Rho kinase and Rac1/Cdc42 signaling through their downstream effector PAK (Patel et al. 2005). It is intriguing though that in other cellular models a differential role of these proteins were shown when mediating EMT inducing effect. EMT of contact-inhibited corneal endothelial cells (CECs) is mediated by fibroblast growth factor (FGF)-2 through by active Rac and Cdc42 and inactive Rho (Lee and Kay 2006). Fibroblast-collagen matrix contraction was recently shown, on the other hand, to be regulated by both active Rac1 and Rho (Abe et al. 2007). When considering the role of cell-cell contacts in mediating EMT through small GTPases, this differential effect was also described. Factors that perturb cell-cell junctions, such that the cytoplasmic pool of p120-catenin is increased, are predicted to decrease RhoA activity but to elevate active Rac1 and Cdc42 (Noren et al. 2000). p120-catenin might also activate RhoA too, since ectopic expression of full-length p120 in epithelial cells promoted cytoskeletal changes, stimulates cell motility, and activated RhoA (Cozzolino et al. 2003). When LLC-PK1 cells were subjected to cell contact disassembly, RhoA, Rac1 and Cdc42 were activated, probably due to the activation by either release of certain junction proteins (such as p120-catenin) or by E-cadherin endocytosis. Similarly, TGF- β 1 was also shown to activate these GTPases (Edlund et al. 2002, Wilkes et al. 2003), indicating that both EMT controlling signals, cell contact disassembly and TGF- β 1, are mediated through these GTPases. Moreover, the downstream effector of Rac1 and

Cdc42, PAK was shown to regulate TGF- β 1 induced fibroblast responses in a Smad independent manner (Wilkes et al. 2005). Both Ca²⁺ removal and TGF- β 1 treatment induced PAK phosphorylation in LLC-PK1/AT1 cells.

Our finding that Rac is activated upon contact disassembly may seem somewhat unexpected, since earlier studies reported a decrease in Rac activity upon the addition of the Ca²⁺-chelator EGTA to epithelial cells (Balzac et al. 2005). However, a number of novel findings make a contact disassembly-induced Rac-activating mechanism likely. First, Rap, an upstream of Rac, is stimulated by contact disruption (Balzac et al. 2005). Further, the GDP/GTP exchange factor GEF-H1 that has recently been identified as the activator of Rho upon contact disassembly (Samarin et al. 2007), can also act as a Rac-GEF (Ren et al. 1998). Moreover, PAK activation was proposed to facilitate the Rac-activating potency of GEF-H1 (Callow et al. 2005). Thus, PAK activation, either downstream or independent of Rac may represent a positive feedback mechanism. In any case, our previous and current results show that acute contact injury leads to both Rho and Rac activation, and each of these is indispensable for the ensuing activation of the SMA promoter.

In contrast to Rho, Rac1 and Cdc42, H-Ras was found to have an opposite effect on SMA. This observation is in agreement with similar data described in vascular smooth muscle cells, where Ras modulates the suppression of platelet-derived growth factor (PDGF) induced SMA expression (Li et al. 1997). H-Ras was found to act in the same SRF-dependent manner as the other Rho GTPases.

Our results indicate that RhoA, Rac1, Cdc42 and PAK mediate EMT in a SRF-dependent manner, supported by a previous finding which demonstrated that RhoA, Cdc42 and Rac1 regulate transcriptional activation by SRF (Hill et al. 1995). Accordingly, SMA was found to be regulated through SRF by these molecules. When the downstream effector of Rac1, Cdc42 and PAK was examined, the first choice was MLC. We showed that the Rho-ROK induced SMA expression is indeed mediated by MLC. Rac1 was previously shown to mediate MLC phosphorylation through PAK (Brzeska et al. 2004, Kiosses et al. 1999). When constitutively active Rac1, Cdc42 and PAK were transfected to LLC-PK1 cells, these constructs induced the phosphorylation of MLC. However, the dominant negative forms of these proteins did not inhibit MLC phosphorylation upon Ca²⁺ removal, indicating that this mechanism is a viable one, without playing a role in our model.

There is wide evidence proving that another downstream of Rac1, Cdc42 and PAK is the p38 MAPK. Rac1-dependent cell spreading was found to be mediated by p38 kinases that act downstream of Rac1 to control the actin capping activity of heat shock protein 27 (Schindeler et al. 2005), cell migration being shown to be mediated by PAK through p38 (Rousseau et al, 2006). UV is known to induce activation of p38, activation shown to be Cdc42 dependent (Seo et al. 2004). Moreover, the Rho/ROK pathway was also shown to be involved in the regulation of p38, when RhoA pathway inhibitors attenuated leptin-induced p38 activation in cultured neonatal rat ventricular myocytes (Zeidan et al. 2006). p38 regulates migration and proliferation of healing corneal epithelium in its TGF- β 1 induced EMT (Saika et al. 2004b), and is required for fibroblastic transdifferentiation (Bakin et al. 2002).

We found that p38 was phosphorylated by both Ca^{2+} removal and TGF- β 1 in LLC-PK1 cells. This means that p38 MAPK is dependent on both, TGF- β 1- and cell contact injury- dependent, hits regulating SMA expression during EMT. Phosphorylation of p38 by cell contact disassembly was diminished by pretreating cells with ROK and PAK inhibitors, showing that not only do Rac1, Cdc42 and PAK regulate p38, but p38 might also act as an effector of the Rho/ROK pathway. ROK inhibition also prevented p38 activation in human tenon fibroblasts (Meyer-ter-vehn et al. 2006). Treatment of cells with the specific p38 pharmacological inhibitor, SB203580, abolished SMA synthesis induced by TGF- β 1 treatment. Moreover, using different adenoviral constructs we were able to show that TGF- β 1 activates SMA expression predominantly through MKK6 and p38 β . It was shown that the different p38 isoforms influence p38 signal specificity (Pramanik et al. 2003). Indeed, EMT in our model is more to be linked with the MKK6-p38 β pathway, MKK-s also playing a differential role in activating p38 isoforms. Indeed, p38 seems to play an important role in EMT. Recently it was shown, that p38 plays an important role in cell migration via the PAK-p38-MAPK-MAPKAP-K2-HSP27 signaling pathway (Rousseau et al. 2006). SRF, the transcription factor regulating SMA, is also activated by p38, result shown both in vitro and in vivo (Heidenreich et al. 1999), similar to the mechanism we showed here. Several authors have shown its role in regulating different smooth muscle marker genes (Deaton et al. 2005), so p38 is not only responsible for regulating expression of SMA, but it also modulates the expression of SM-MHC, SM22 α . p38 MAPK has been shown to contribute to the regulation EMT in different cells (Valcourt et al. 2005,

Bhowmick et al. 2001b). Recently p38 MAPK has been implicated in TGF- β 1 induced EMT in renal tubular cells (Rhyu et al. 2005). Although several authors (Yu et al. 2002) demonstrated that p38 signals in a Smad independent manner, there is also data showing a crosstalk between p38 and Smads in TGF- β 1 signaling (Leivonen et al. 2002). Smad2 was shown to activate p38 and subsequently Rho during TGF- β 1 induced endothelial barrier dysfunction (Lu et al. 2006).

Although we showed that inhibition of p38 by SB203580 abolished the synthesis of SMA upon TGF- β 1 treatment, the same treatment failed to inhibit SMA promoter activation by TGF- β 1. Interestingly, the activation of the promoter by Ca²⁺ removal was diminished following pretreatments with SB203580. A similar effect was described during transforming growth factor-beta1 autoinduction on proximal tubular epithelial cells (PTC). Inhibition of p38 inhibited de novo TGF- β 1 protein synthesis, but did not influence TGF- β 1 mRNA expression (Zhang M et al. 2006). When investigating the role of the p38- activator protein-1 (AP-1) signaling pathway in TGF- β 1 induced SMA expression in human fetal lung fibroblasts (HLF-02), the induction of SMA expression by TGF- β 1 was shown to be suppressed by SB203580 and the AP-1 inhibitor curcumin. However, SB203580 did not inhibit the AP-1 DNA binding activity induced by TGF- β 1 (Hu et al. 2006). These effects are due to the involvement of MK2, which are important regulators of gene expression at transcriptional and post-transcriptional levels. Recently experiments in mouse embryonic fibroblasts demonstrated that disruption of MK2 expression reduces SMA levels in response to TGF- β 1. TGF- β 1 causes even down-regulation of SMA in MK2 negative MEFs, instead of upregulation observed in wild type MEF. Down-regulation of SMA in MK2 negative cells is not due to the lack of activation of serum responsive promoter elements, but probably due to the reduced SMA message stability (Sousa et al. 2007). In this context we believe that p38 signaling is essential during SMA regulation through MK2, which further regulates HSP27 and SMA mRNA stability. The SMA mRNA stability might be also influenced by β -catenin, which not only mediates gene transactivation, but also regulates pre-mRNA splicing through splicing factor-1 (SF-1) (Shitashige et al. 2007).

The signaling steps previously described all converge towards SRF, and more importantly, to MRTF. The fact that SRF is expressed ubiquitously suggested the SRF cofactors might be involved in the regulation of SRF dependent genes. The current

work identifies and promotes MRTF as the ultimate regulator of SMA expression, during the “two-hit” model characterized EMT. This conclusion is supported by the findings that overexpression of MRTF is sufficient to induce SMA promoter activation and protein expression in tubular cells, induction of robust actin polymerization induces nuclear accumulation of MRTF concomitant with SMA expression, and DN-myocardin prevents the Ca^{2+} depletion- and TGF- β 1- induced promoter activation and the synergism between contact injury and TGF- β 1.

In non-stimulated LLC-PK1 cells endogenous MRTF (as visualized by the anti-BSAC antibody) was cytosolic. Interestingly, MRTF-A was predominantly nuclear, whereas MRTF-B localized mainly to the cytosol. MRTF cellular localization is regulated by Rho, Rac1, Cdc42, PAK, p38, and by TGF- β 1 and Ca^{2+} removal, which induce its nuclear accumulation. We observed that TGF- β 1 was unable to induce MRTF translocation in fully confluent layers, only in non confluent layers, and it enhanced nuclear accumulation after contact disassembly. This finding implies that MRTF localization is one of the key target mechanisms that underlie the synergy between TGF- β 1 and contact injury. Presumably, the strong, contact-dependent Rho activation is indispensable for the efficient nuclear accumulation of MRTF. On the other hand, moderate translocation of endogenous MRTF may not be sufficient to induce SMA expression, because cells adjacent to the wound are not transformed in the absence of TGF- β 1. The SMA promoter harbors several transcriptional regulatory elements, including the SRF/MRTF-binding CARG-boxes, the Krüppel factor- binding TGF- β 1 control element (TCE), and the TGF- β 1- responsive SBE. Accordingly, the promoter can be collectively regulated by contact-dependent (Rho-mediated) and TGF- β 1- dependent (partially Rho-independent) pathways. Interestingly MRTF may have multiple roles: in addition to forming a ternary complex with SRF and CARG boxes, it was found to bind to the SMAD proteins too, and thus it might facilitate transcription through the SBE (Qiu et al. 2005). These multiple inputs then can culminate in robust promoter activation.

Our results indicate that p38 is an important regulator of MRTF cellular localization. Since p38 is a general mediator of stress, it may link a variety of stresses (TNF- α , oxidative and osmotic stress) to MRTF regulation.

Besides ROK, another Rho downstream regulating actin polymerization are the diaphanous formins 1 and 2 (mDia 1 and 2). mDia 1 and 2 were recently shown to

stimulate endogenous SMA expression in 10T1/2 cells. The effects of mDia1 and mDia2 required the presence of SRF and the activity of the myocardin transcription factors and were dependent on changes in actin polymerization, mDia activation promoting nuclear localization of MRTF-A and MRTF-B (Staus et al. 2007). Other intriguing evidence linking mDia and MRTF was also published recently. mDia was shown to act as a nodal modulator of two pathways, resulting in reciprocal regulation of SRF and TCF/LEF, via reciprocal effects on the localization of their cytoplasmic co-activators, MAL and β -catenin, respectively (Gopinath et al. 2007).

Finally, we observed that even under the maximally effective two-hit conditions, MRTF accumulation in the nucleus is transient. Future studies should investigate the regulation of the nuclear export of MRTF, MRTF recently being reported to rapidly shuttle between the cytosol and the nucleus, and a reduction in its rate-limiting efflux was proposed to be the primary mechanism of regulation (Vartiainen et al. 2007).

In summary, we propose a “two-hit” model of SMA regulation during EMT that is dependent on TGF- β 1 and the integrity of cell contacts. These two hits converge in the same MRTF and SRF dependent modulation of the SMA gene.

TGF- β 1, as one of the hits, regulates SMA expression through the Smad family of signaling proteins, and through the p38 MAPK. TGF- β 1 is an important modulator of MRTF cellular localization.

The other hit, cell contact disassembly, is exerting its effects on the SMA gene through at least two well defined pathways. Rho dependent regulation includes ROK and MLC downstreams, which act as regulators of MRTF and SRF. The Rac1/Cdc42 dependent pathway includes PAK and p38 MAPK, and these molecules all regulate MRTF. p38 MAPK is the site of the cross-talk between the Rac1- and Rho- dependent pathways, and p38 also modulates both TGF- β 1- and cell contact- dependent effects through MRTF (Figure 33).

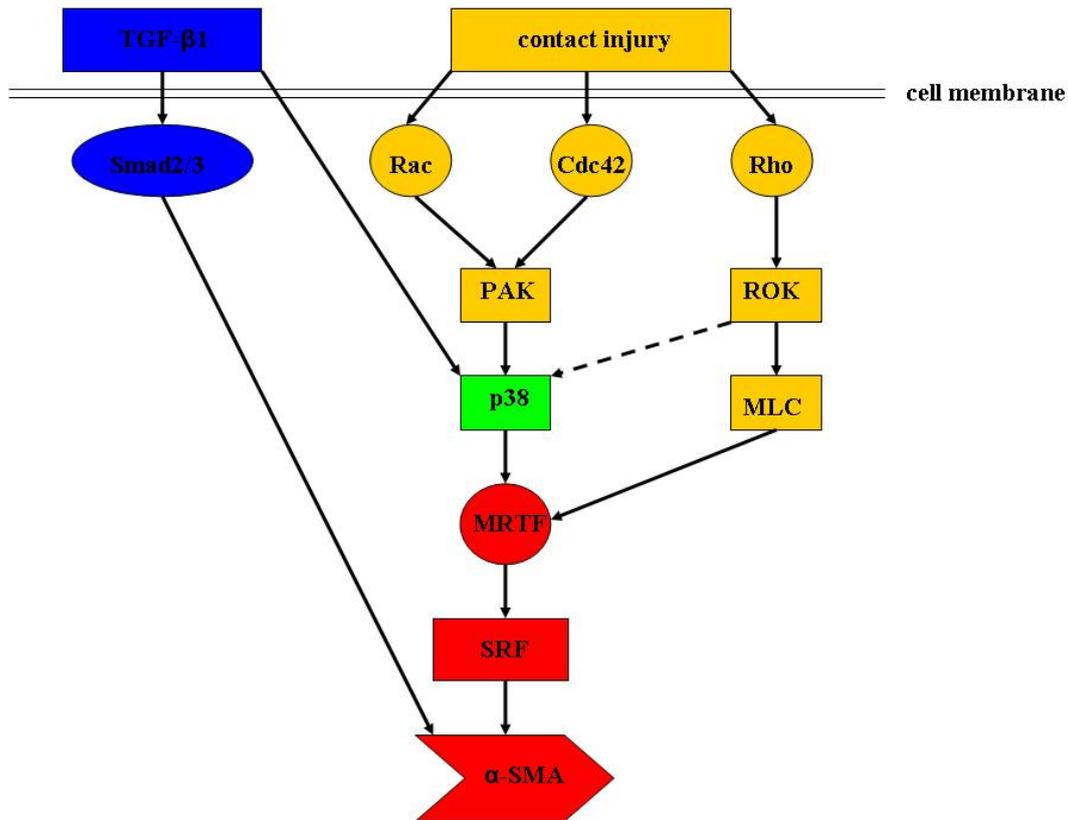


Figure 33. Intracellular signaling pathways involved in the TGF- β 1- and cell contact-dependent regulation of SMA expression during EMT. TGF- β 1, the first hit, regulates SMA expression through the Smad family of signaling proteins. It also regulates the p38 MAPK, and is an important inducer of MRTF nuclear translocation. The other hit, contact injury, regulates SMA expression through two pathways: Rho-ROK-MLC-MRTF/SRF, and Rac1/Cdc42-PAK-p38-MRTF/SRF. Further, a possible cross-talk between ROK and p38 might also be involved in this regulation.

In addition to the mechanisms studied here, several other steps might be involved in the synergistic effect of cell contact injury and TGF- β 1 in the complex regulation of the SMA promoter.

First, TGF- β 1 activates a multitude of signaling pathways, which via various transcription factors act on the TCE and SBE cis elements. Further, TGF- β 1 rescues dislocated β -catenin, which might form a complex with the Smad3/4 and as such regulate SMA expression.

TGF- β 1 and contact disassembly activate Rho which, in turn, stimulates mDia and ROK. mDia regulates SMA expression through the control of localization of β -catenin and MRTF. Downstream of ROK is MLC, which also regulates SMA expression through SRF and MRTF.

The contact dependent Rac1/Cdc42 pathway is signaling through PAK and p38 MAPK. p38 is the main link between the TGF- β 1- and contact- dependent pathway. Moreover, the possible cross-talk between p38 and ROK is also an interesting possibility in this regard. p38 MAPK regulates MRTF nuclear translocation, and as a general mediator of stress, it may link a variety of stresses (TNF- α , oxidative and osmotic stress) to MRTF regulation. Besides Rho, ROK, MLC, Rac1, Cdc42 and PAK, p38 MAPK is also involved in the regulation of the actin cytoskeleton through its downstream effector, HSP27. Finally, an intriguing regulation is probable on the level of SMA mRNA, where p38 might be involved in regulating mRNA stability, another potential regulator being β -catenin, which was shown to be involved in splicing events.

We assume that the questions raised by the complex regulation of SMA expression during EMT are far from being answered; other molecules might be also involved in this regulation. The investigation of the further signaling events during the “two-hit” model of SMA regulation will be addressed in future work.

VI. CONCLUSIONS

This study focused on the complex regulation of SMA during EMT in renal tubular cells within the framework of the “two hit” model described earlier by our group. These two hits, namely TGF- β 1 and cell contact disruption regulate SMA expression through distinct, synergizing pathways in LLC-PK1/AT1 cells. The major conclusions of this work are:

1. **Smad2 and Smad3 are important regulators of TGF- β 1 induced SMA expression.** We showed that Smad2 and Smad3 are important regulators of SMA expression, both Smad2 and Smad3 being activated by TGF- β 1 in LLC-PK1 cells. Inhibition of Smads in transient transfection and adenoviral infection experiments prevented TGF- β 1 induced SMA promoter activation and SMA protein expression in our model.
2. **Rho and ROK are key mediators of contact disassembly- induced activation of the SMA promoter.** Rho is activated by cell contact disassembly. Overexpression of Rho induced SMA promoter activation, while inhibition of Rho and ROK blocked cell contact disassembly induced SMA promoter activation.
3. **MLC is involved in the Rho-ROK-SRF pathway and regulates SMA expression.** In our tubular cells contact disassembly induced Rho/ROK dependent MLC phosphorylation. We demonstrated that myosin phosphorylation, in turn, is involved in the regulation of contact disassembly induced SMA promoter activation and SMA expression. Moreover, nuclear accumulation of SRF upon Ca²⁺-removal is Rho and pMLC dependent. TGF- β 1 also induced phosphorylation of MLC and TGF- β 1 induced SMA synthesis was MLC dependent.
4. **Rac1, Cdc42, PAK and H-Ras differentially regulate SMA promoter activity through SRF-dependent pathways.** In our experiments Rac1, Cdc42 and PAK activated, while H-Ras inhibited SMA promoter activity in SRF dependent manner. Rac1, Cdc42 and PAK were activated by cell contact disruption. Furthermore, PAK phosphorylation was also induced by TGF- β 1. Rac1 and Cdc42 increased the nuclear accumulation of SRF in LLC-PK1 cells.
5. **p38 is an important step in mediating SMA expression induced both by cell contact disruption and by TGF- β 1.** p38 MAPK phosphorylation was induced both by Ca²⁺-removal and by TGF- β 1. Its inhibition prevented SMA protein expression. p38 regulates SMA expression through SRF and MRTF.

6. **MRTF is an important regulator of SMA expression.** Its cellular localization is regulated by both cell contact disruption and TGF- β 1. It is translocated into the nucleus upon TGF- β 1 treatment in subconfluent cells, but not in confluent cultures. Cell contact disruption by Ca²⁺-removal or wounding induced its nuclear translocation in confluent cells. MRTF nuclear translocation is induced by Rho, Rac1, Cdc42, PAK, while its translocation upon Ca²⁺-removal is prevented by inhibition of Rho, Rac1, Cdc42, PAK, MLC and p38. MRTF overexpression leads to massive activation of the SMA promoter and to SMA protein expression.
7. **Rho-ROK-MLC-SRF/MRTF and Rac1/Cdc42-PAK-p38-SRF/MRTF are two major pathways regulating SMA expression, in a TGF- β 1 and cell contact dependent manner.** Rho dependent regulation includes ROK and MLC downstreams, which act as regulators of MRTF and SRF. The Rac1/Cdc42 dependent pathway includes PAK and p38 MAPK, and also regulated SMA expression through MRTF and SRF. p38 MAPK is a potential site of the cross-talk between the Rac1- and Rho- dependent pathways, and p38 also modulates both TGF- β 1- and cell contact- dependent effects through MRTF.

VII. SUMMARY

Epithelial-to-mesenchymal transition (EMT) of tubular cells into α -smooth muscle actin (SMA) expressing myofibroblasts is a central mechanism in the pathogenesis of tubulo-interstitial fibrosis. Tubular epithelial cells that undergo EMT express SMA in response to an injury or the absence of intercellular junctions and transforming growth factor- β (TGF β). The complex regulation of EMT requires the interplay of several intracellular signaling pathways.

We demonstrated that TGF β regulates SMA expression through several signaling molecules, such as the Smad family of signaling proteins and p38 mitogen activated protein kinase family (p38 MAPK).

In our experiments cell contact disruption activated Rho and induced Rho kinase (ROK) - mediated myosin light chain (MLC) phosphorylation. Rho, ROK and MLC were found to regulate SMA expression. Contact disassembly enhanced nuclear accumulation of the serum response factor (SRF), as well. Contact injury-dependent Rho activation also resulted in the nuclear translocation of myocardin- related transcription factor (MRTF), a cofactor of SRF. Our results suggest that MRTF and SRF act together to induce SMA promoter activation in a cell contact- and TGF β -dependent manner in renal tubular cells.

We showed that two additional Rho-family GTPases, Rac and Cdc42 also participate in the contact- and contractility-dependent regulation of the SMA promoter. Constitutive active Rac1, Cdc42 and their downstream effector p21-activated kinase (PAK) activated the SMA promoter in an SRF- and MRTF-dependent manner. Moreover, p38MAPK was also found to mediate cell contact disassembly- induced SMA promoter activation through MRTF.

Based on our results MRTF emerged as a key regulator of SMA expression in renal tubular cells. Its nuclear- cytoplasmic shuttling is regulated by both cell contact disassembly and TGF β , through several downstream effectors including Rho, Rac1, Cdc42, PAK, MLC, p38.

We showed here that the Rho-ROK-MLC-MRTF-SRF and Rac1/Cdc42-PAK-p38-MRTF-SRF pathways are important regulators of SMA expression and EMT in renal tubular cells.

VIII. ÖSSZEFOGLALÁS

A tubulo-interstitialis fibrosis pathogenesisének központi mechanizmusa a tubuláris sejtek átalakulása α -simaizom aktint (SMA) expresszáló myofibroblastokká (epithelialis-mesenchymalis transzformáció- EMT). E folyamat kiváltásában az intercelluláris kontaktusok sérülése vagy megszűnése mellett fontos szerepet játszik a transforming growth factor- β (TGF β). Az EMT szabályozásában számos intracelluláris jelátviteli folyamat szerepe igazolható, melyek egymással szoros kölcsönhatásban hangolják össze a komplex folyamat egyes lépéseit.

Kísérleteinkben kimutattuk, hogy a TGF β több jelátviteli rendszer, így a Smad fehérjék, valamint a Mitogén Aktiválta Protein Kinázok (MAPK) közül a p38 MAPK közvetítésével szabályozza az SMA expressziót.

A sejtkontaktusok dezintegrálása során a Rho kis G fehérje és Rho-kináz (ROK)-aktiválódása révén foszforilálódik a miozin könnyű lánc (MLC). A Rho, a ROK és az MLC szintén fokozzák az SMA expresszióját. A sejtkontaktusok sérülése fokozta a serum response factor (SRF) nuclearis akkumulációját is. A sejt kontaktusok szétesése továbbá Rho dependens módon kiváltotta a myocardin-related transcription factor (MRTF) nuclearis transzlokációját, amely az SRF kofaktora. Eredményeink arra utalnak, hogy az MRTF és az SRF együttműködve fokozzák a sejt kontaktus- és TGF β -dependens SMA promoter aktivációt vese tubulus sejtekben.

Eredményeink szerint két további kis G fehérje, a Rac1 és Cdc42 is szerepet játszik az SMA promoter kontaktus- és kontraktilitás- dependens szabályozásában. A Rac1, a Cdc42 és ezek effektora, a p21 activated kinase (PAK) overexpressziója SRF és MRTF dependens módon aktiválta az SMA promotert. Végül azt is kimutattuk, hogy a p38 MAPK az MRTF-en keresztül részt vesz a SMA promoter kontaktus dependens szabályozásában is.

Eredményeink azt mutatták, hogy az MRTF az SMA kulcsfontosságú szabályozója. A sejtmag és a citoplazma közötti mozgását a sejt kontaktusok szétesése és a TGF β szabályozza. E hatás közvetítésében számos jelátviteli molekula, köztük a Rho, Rac1, Cdc42, PAK, MLC, és a p38 MAPK vesz részt.

Vizsgálatainkban kimutattuk, hogy a Rho-ROK-MLC-MRTF-SRF és a Rac1/Cdc42-PAK-p38-MRTF-SRF jelátviteli utak kiemelkedő fontosságúak az SMA expresszió és az EMT szabályozásában vese tubulus sejtekben.

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X. LIST OF PUBLICATIONS

Publications referred to in this work:

Sebe A*, Fan L*, Peterfi Z, Masszi A, Thirone AC, Rotstein OD, Nakano H, McCulloch CA, Szaszi K, Mucsi I, Kapus A. Cell contact-dependent regulation of epithelial-myofibroblast transition via the rho-rho kinase-phospho-myosin pathway. *Mol Biol Cell*. 2007 Mar;18(3):1083-97. *- equal contribution

Sebe A, Leivonen SK, Fintha A, Masszi A, Rosivall L, Kähäri VM, Mucsi I: Transforming Growth Factor- β Induced Alpha-Smooth Muscle Cell Actin Expression in Renal Proximal Tubular Cells is Regulated by p38 β Mitogen Activated Protein Kinase, Extracellular Signal Regulated Protein Kinase1,2 and the Smad Signaling During Epithelial-Myofibroblast Transdifferentiation.

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Sebe A, Masszi A, Rotstein OD, Speight P, Nakano H, Mucsi I, Szaszi K, Kapus A: Rac, PAK and p38 regulate cell contact-dependent nuclear translocation of myocardin-related transcription factor.

manuscript under review

Other publications:

Terebessy T, Masszi A, Fintha A, **Sebe A**, Huszar T, Rosivall L, Mucsi I. Angiotensin II activates the human renin promoter in an in vitro model: the role of c-Jun-N-terminal kinase.

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manuscript under review

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Budapest, 2007

A handwritten signature in black ink, appearing to read 'Attila Sebe'. The signature is written in a cursive style with some loops and flourishes.

Attila Sebe