RhoA, Rac1 and Cdc42 differentially regulate αSMA and collagen I expression in mesenchymal stem cells

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Running title: Rho GTPase regulation of myofibroblast differentiation

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Abstract
Mesenchymal stem cells (MSC) are suggested to be important progenitors of myofibroblasts in fibrosis. To understand the role of Rho GTPase signaling in TGFβ-induced myofibroblast differentiation of MSC, we generated a novel MSC line and descendants of it lacking functional Rho GTPases and Rho GTPase signaling components. Unexpectedly, our data revealed that Rho GTPase signaling is required for TGFβ-induced expression of αSMA, but not of collagen I α1 (col1a1). While loss of RhoA and Cdc42 reduced αSMA expression, ablation of the Rac1 gene had the opposite effect. Although actin polymerization and MRTFa were crucial for TGFβ-induced αSMA expression, neither Arp2/3 dependent actin polymerization nor cofilin dependent severing and depolymerization of F-actin were required. Instead, F-actin levels were dependent on cell contraction and TGFβ-induced actin polymerisation correlated with increased cell contraction mediated by RhoA and Cdc42. Finally, we observed impaired collagen I secretion in MSC lacking RhoA or Cdc42. These data give novel molecular insights into the role of Rho GTPases in TGFβ signaling and have implications for our understanding of MSC function in fibrosis.

Introduction
Fibrosis, the excessive production of collagen I and other extracellular matrix (ECM) proteins, is an important clinical problem with few treatment options (1,2). In liver, fibrosis caused by viral hepatitis, alcohol, or obesity can lead to liver failure and increase the likelihood for liver cancer (3). However, fibrosis occurs also in many other tissues such as kidney, heart, and lungs and leads to organ failure due to functional impairment. The major cell type responsible for the pathological ECM production is the myofibroblast, which can be described as a contractile, collagen I producing, fibroblastoid cell, mostly characterized by high expression of α-smooth muscle actin (αSMA; gene name ACTA2) (4). The biology of myofibroblasts is complex, since apparently many different precursor populations are able to differentiate into myofibroblasts including tissue resident fibroblasts and epithelial cells, which might result in different subtypes of myofibroblasts with different biological
properties. The extent to which each progenitor population contributes to myofibroblasts in disease is debated and might be dependent on the tissue as well as on the stimulus. Recently, vascular mesenchymal stem cell-like cells (MSC) were described as critically important myofibroblast precursors in different murine fibrosis models (5). This originally small population, present in all vascularised tissues, is expanding strongly during disease and gives rise to a large share of the differentiated myofibroblasts. Moreover, elimination of this population strongly reduced development of kidney and heart fibrosis. It is important therefore to understand the mechanisms that trigger the differentiation of MSC into myofibroblasts. In vitro, TGFβ can induce myofibroblast differentiation of MSC derived from adipose or prostate tissue as characterized by αSMA and collagen I expression (6,7). TGFβ signaling is complex and can be divided into a canonical part dependent on the Smad2/3/4 transcription factors, and a less characterized non-canonical part, which includes activation of the small Rho GTPase RhoA (8). The latter was shown to regulate actin polymerization via ROCK (9), triggering nuclear translocation of the transcription co-factor MRTFa, which then binds to the transcription factor SRF triggering the expression of αSMA and collagen I in fibroblasts (10-12). How TGFβ signaling activates RhoA, how ROCK is controlling F-actin formation, and how SRF-MRTF synergizes with Smad2/3/4 canonical signaling is not well understood. It is also not clear to what extent other major regulators of actin polymerization such as the small GTPases Rac1 and Cdc42 might play a role in MRTFa activation during myofibroblast differentiation.

To increase the understanding of the molecular pathways underlying fibrotic disease, we set out to investigate how TGFβ-induced myofibroblast differentiation is regulated in the pathophysiologically relevant precursor population of MSC. Importantly, we tested how the Rho GTPases RhoA, Rac1 and Cdc42 are affecting MSC differentiation into myofibroblast, and which signal transduction pathways are involved downstream of Rho GTPases. Our results indicate important roles for RhoA, Rac1, and Cdc42 in controlling TGFβ-induced expression of the myofibroblast differentiation marker αSMA in MSC, but surprisingly little effect on the regulation of col1a1 mRNA. TGFβ-induced regulation of αSMA expression was dependent on contraction, but independent of cofilin, in contrast to previous suggestions. These data give important insights into myofibroblast differentiation of a disease-relevant progenitor population that might help to find novel treatments preventing fibrosis in different tissues.

Results

Establishment of a spontaneously immortalized MSC line

Standard preparations of primary mouse MSC are contaminated with other cell types at low passage numbers and prone to senescence upon longer passaging, only leaving a small window for experimentation. We therefore established a spontaneously immortalized murine MSC line from bone-derived MSC. After 58 passages, the MSC line showed high expression of the murine MSC surface markers CD105, CD44 and Sca1, while no contaminating hematopoietic cells expressing CD45 or macrophages expressing CD11b could be detected by FACS (Fig. 1A). Light microscopy revealed a fibroblast-like triangular cell morphology, similar to primary MSC (Fig. 1B). Moreover, the MSC showed efficient differentiation into adipocytes and osteoblasts in the respective differentiation medium (Fig. 1C). Moreover, no differentiation into these cell types was detectable in normal growth conditions.
These data show that the MSC line established displays major hallmarks of primary MSC at least up to passage 58. Further experiments were carried out with cells from passage 42 to 47.

**Canonical TGFβ signaling triggers myofibroblast differentiation of MSC**

TGFβ is promoting differentiation of fibroblasts into myofibroblasts characterized by expression of αSMA and collagen I. MSC are assumed to behave similarly, but have to date only been tested for αSMA expression in response to TGFβ1. Treatment of our MSC line with TGFβ1 indeed resulted in the upregulation of both αSMA and col1a1 mRNA after 24h (Fig. 2A, B). To investigate the dependency of this differentiation on canonical, Smad-related TGFβ signaling, we established MSC lines lacking Smad2, Smad3, or Smad4 using lentiviral, CRISPR/Cas-mediated genome editing, and confirmed the absence of respective protein by Western blotting (Fig. 2C). Deletion of Smad2 or Smad3 alone showed little effect, but ablation of the Smad4 gene effectively abrogated TGFβ-induced expression of αSMA or col1a1 (Fig. 2D-F). Basal expression of αSMA and col1a1 was not significantly altered by the loss of Smad4. These results demonstrate an essential role for canonical, Smad4-dependent TGFβ signaling for myofibroblast differentiation of MSC, while Smad2 or Smad3 are not required and probably have redundant functions, in line with earlier observations in other cell types.

**Rho GTPases control myofibroblast differentiation of MSC**

Treatment of MSC with TGFβ resulted in increased phosphorylation of Smad2 and the Rho GTPase downstream effectors MLC2 (in particular RhoA, but also Rac1 and Cdc42), Pak1 (Rac1, Cdc42) and Pak4 (Cdc42), suggesting that TGFβ is inducing Rho GTPase activation in parallel with canonical TGFβ signaling (Fig. S7). Kinetic analysis indicated a high and significant activation of Smad2, PAK1 and Pak4 after 24h and of MLC2 after 2h.

To analyze the functional role of basal and TGFβ-induced Rho GTPase activation in TGFβ-induced myofibroblast differentiation of MSC, we established MSC lines lacking RhoA, Rac1 or Cdc42 by CRISPR/Cas9-mediated genome editing. While RhoA and Cdc42 were efficiently deleted, loss of Rac1 protein was not complete, indicating the presence of a minor amount of non-recombined cells (Fig. 3A). This was most likely due to a severely reduced growth of Rac1-null MSC, giving non-recombined cells a competitive advantage in the polyclonal cell mixture (data not shown). MSC suppressed for RhoA, Rac1 or Cdc42 expression showed morphologies similar to fibroblastoid cells lacking these Rho GTPase genes (Fig. 3B). Time lapse migration assays revealed that Rac1-null MSC are hardly motile, while the long, thin extensions of Cdc42-null cells seen in still images (Fig. 3B) were observed to rupture during migration, leaving behind small patches of cytoplasm on cell tracks (Suppl. movies 1-4). RhoA-null MSC were also more elongated than wildtype cells, and at least a subpopulation of cells displayed extended rear edges. With respect to their differentiation potential, we found that loss of Rho GTPases did not prevent or induce differentiation of MSC to adipocytes or osteoblasts (Fig. S6). Furthermore, none of the Rho GTPase KO MSC lines showed altered phosphorylation of Smad2 after 24h treatment with TGFβ (Fig. 3C, D), suggesting normal activation of TGFβR kinase activity, crucial for both canonical and non-canonical signaling. TGFβ-induced expression of αSMA was significantly decreased in RhoA ko and Cdc42 ko MSC after 24h stimulation, while loss of Rac1 increased basal αSMA expression strongly (Fig. 4A). With respect to fold-change, only Cdc42 ko resulted in a significantly reduced induction (Supp. Table 1). After 3d TGFβ treatment, protein levels of αSMA were significantly lower
in RhoA ko and Cdc42 ko cells compared to wildtype MSC, while no clear difference was detectable in Rac1 ko MSC (Fig. 4B, C).

Surprisingly, deletion of RhoA in MSC showed no influence on TGFβ-induced col1a1 mRNA expression (Fig. 4D), although RhoA was reported to be required for collagen 1 expression in fibroblasts (12). Loss of Rac1 or Cdc42 resulted in slightly reduced col1a1 levels after 1d (Fig. 4D). However, after 3d stimulation, deletion of Cdc42 did not cause any change in col1a1 mRNA expression (Fig. 4E).

Analyzing cellular and secreted collagen 1 protein, both RhoA ko and Cdc42 ko MSC displayed an intracellular accumulation of cellular collagen 1 (Fig. 4F, G). Moreover, secreted collagen 1 was significantly reduced in the supernatant of Cdc42-null MSC (Fig. 4F, H).

These data indicate that both RhoA and Cdc42 are required for TGFβ-induced αSMA expression, while Rac1 is playing an inhibitory role. They furthermore suggest a role for RhoA and Cdc42 in collagen I secretion, but no or only a minor role in the regulation of col1a1 mRNA.

Defective αSMA induction in Rho GTPase ko correlates with changes in F-actin and pMLC2

To identify changes in downstream components of Rho GTPase signaling that might correlate with the defective induction of αSMA, we first analyzed F-actin levels by quantifying cell stainings with fluorescently labelled phalloidin. TGFβ treatment of control MSC induced a clear increase in F-actin in control, but not in in RhoA ko and Cdc42 ko MSC (Fig. 5A; Fig. S1). Rac1 ko MSC, on the other hand, showed increased levels of F-actin in both presence and absence of TGFβ (Fig. S1). These changes in F-actin correlated with changes in pMLC2, which is a marker for cell contraction (Fig. 5B).

ADF/cofilin family members are important regulators of F-actin by severing it and promoting depolymerisation with cofilin-1 (cofilin) as the major form in non-muscle tissues. Cofilin is known to be inactivated by phosphorylation downstream of RhoA, Rac1, and Cdc42. However, we could not detect significant changes in cofilin phosphorylation in MSC lacking these Rho GTPases after 24 h treatment with TGFβ (Fig. 5C, Fig. S2). p38, Erk and JNK are described to be phosphorylated and activated by non-canonical TGFβ signaling. Testing pErk, pp38 after 24 h TGFβ treatment, no significant TGFβ-dependent stimulation and no Rho GTPase-dependent alteration was detectable (Fig. 5D, E, Fig. S2). Unexpectedly, the p54 isoform of pJNK was increased in untreated and TGFβ treated Cdc42 ko MSC, while RhoA ko cells showed no change (Fig. 5F, G, Fig. S2).

TGFβ-induced actin polymerization controls αSMA, but not col1a1 expression

In fibroblasts, actin polymerization promotes nuclear translocation of the transcription factor MRTFa which induces expression of both αSMA and collagen 1 (10,11). In MSC, TGFβ treatment increased the formation of F-actin stress fibers over 24h, visualized by fluorescent LifeAct (Fig. S8). We therefore investigated whether this pathway is playing a related role in MSC. Inhibition of actin polymerization in MSC by latrunculin abrogated basal as well as induced αSMA expression and caused a partial reduction of TGFβ-induced col1a1 expression (Fig. 6A, B). Moreover, jasplakinolide, which stabilizes F-actin, dramatically increased basal αSMA levels (Fig. 6A), but TGFβ treatment did not result in a further increase in αSMA. By contrast, jasplakinolide did not affect basal col1a1 expression and even inhibited the TGFβ1 induced increase of col1a1 (Fig. 6B).

Actin polymerization downstream of Rac1 and Cdc42 is prominently promoted by the Arp2/3 complex and downstream of RhoA by formins of the mDia family. To probe the role of Arp2/3 mediated F-actin formation during myofibroblast differentiation, we deleted Arpc2, which is an essential component of the Arp2/3 complex(13,14). Although Arpc2 was
efficiently deleted (Fig. 6C), we did not observe an alteration in TGFβ-induced expression of αSMA or col1a1 (Fig. 6D, E). CRISPR/Cas9-mediated coflin targeting strongly increased stress fiber formation and αSMA levels, but did not affect inducibility of αSMA by TGFβ1 (Fig. 6F, G), confirming the notion that coflin is not required for the TGFβ-induced increase of αSMA (Fig. 5C, 6G). CRISPR/Cas9 mediated deletion of the coflin gene however did not affect col1a1 expression (Fig. 6H). Downstream of RhoA, Rac1, and Cdc42, LIMK is inhibiting coflin by phosphorylation. Loss of LIMK should therefore decrease coflin phosphorylation, increase coflin activity, decrease F-actin, and decrease αSMA, at least if assuming that coflin expression is unaltered. LIMK ko were made and frameshifts in the coding region of the respective genes (LIMK1 and -2) were confirmed by sequencing of genomic PCR fragments (Fig. S3A). Paradoxically, loss of LIMK1 increased basal and TGFβ-induced expression of αSMA, while LIMK2 ko had no influence (Fig. 6I, J). Interestingly, only LIMK2 ko reduced pCofilin levels, indicating that it is the major regulator of coflin phosphorylation in MSC (Fig. 6L). LIMK1 ko, on the other hand, resulted in an increase of pCofilin, implying increased activation of LIMK2. Indeed, double ko of LIMK1 and LIMK2, restored αSMA levels to that of wildtype cells (Fig. 6K). Conceivably, deletion of LIMK1 is overcompensated by LIMK2, which is rescued by additional deletion of LIMK2. Correspondingly, LIMK1 ko increased, and LIMK2 or LIMK1/2 ko decreased F-actin in MSC (Fig. S3B, C).

LIMK deletion did not influence TGFβ-induced col1a1 expression (Fig. 6M). Finally, deletion of the MRTFa gene, confirmed by genomic sequencing of the targeted genomic region (Fig. S3), blocked TGFβ induction of αSMA, but had no influence on col1a1 mRNA (Fig. 6N, O). Deletion of MRTFa did not interfere with the differentiation of MSC to adipocytes or osteoblasts (Fig. S6).

These data demonstrate that Arp2/3 complex-independent, but F-actin/MRTFa-dependent signaling is crucial for TGFβ-induced αSMA expression in MSC. However, F-actin/MRTFa signaling is again dispensable for the stimulation of col1a1 expression by TGFβ. Moreover, Rho-GTPase dependent regulation of ADF/cofilin activity is not essential for TGFβ-induced expression of αSMA.

**TGFβ-induced cell contraction is required for increased actin polymerization**

To understand the relationship between TGFβ-induced cell contraction, F-actin distribution and αSMA expression, we inhibited cell contraction directly by blebbistatin and indirectly by inhibition or ko of contraction promoting Rho GTPase effectors. Blebbistatin strongly reduced F-actin levels and prevented TGFβ-induced αSMA expression, while not affecting col1a1 expression (Fig. 7A, B, E). Similarly, inhibition of the RhoA effectors ROCK1 and ROCK2 by Y27632 inhibited stress fiber formation and effectively reduced TGFβ-induced αSMA expression, but not col1a1 expression (Fig. 7C, D, E). Individual ko of ROCK1 and ROCK2 showed a partial reduction of pMLC2, F-actin, and αSMA, while combined deletion of ROCK1 and ROCK2 had a stronger effect, indicating redundancy between ROCK1 and ROCK2 (Fig. 7F, G, I, J, K; Fig. S4A). Combined deletion of ROCK1 and ROCK2 did not interfere with the differentiation of MSC to adipocytes or osteoblasts (Fig. S6).

Aside from Rock, the Cdc42 effectors MRCKα and MRCKβ were also reported to regulate cell contraction in a fashion similar to ROCK. However, deletion of these genes showed little influence on pMLC2, F-actin or αSMA (Fig. 7F, H, L, M, N; Fig. S5A), indicating that Cdc42 is affecting cell contraction in MSC in a non-classical manner. Importantly, none of the manipulations described above led to a
significant change in basal or TGFβ-induced col1a1 mRNA, corroborating the regulation of αSMA and col1a1 expression in MSC to be distinct (Fig. S4B, 5B).

Discussion

This study on the TGFβ-induced differentiation of MSC to myofibroblasts revealed several surprising findings (Fig. S10): First, it showed that TGFβ-induced expression of αSMA and collagen 1 are differentially regulated in MSC, in contrast to fibroblasts; Secondly, it indicated that Rac1 and Cdc42 control TGFβ-induced αSMA expression in an opposite manner and independent of Arp2/3 complex-mediated actin polymerization; Thirdly, it demonstrated that regulation of cofilin activity is not important for TGFβ-induced αSMA expression, contrary to earlier expectations; Fourthly, the data suggest that RhoA, Rac1, and Cdc42 control αSMA expression by a contraction-dependent pathway; and finally, this study reveals that Cdc42 and RhoA affect cytoplasmic retention and secretion of collagen I in MSC.

While MRTFa is required for collagen I expression in lung fibroblasts and in myofibroblasts derived from scleroderma (10,11), collagen I expression in our MSC line is clearly independent of MRTFa, although TGFβ-induced αSMA expression is highly dependent on MRTFa. On the other hand, canonical Smad4 dependent TGFβ signaling is essential for both collagen I as well as αSMA expression. This fits to an earlier report on mesangial cells, showing that Smad4 is required for TGFβ-induced collagen I expression (15). It is well known that the regulation of TGFβ-dependent genes is strongly context-dependent and cell-type specific (8). Synergistic interaction of MRTFa with Smad4 signaling at the collagen I promoter is required in fibroblasts and myofibroblasts (10,11), but, as our data reveal, not in MSC. Wildtype MSC will therefore be resistant to fibrosis-inhibiting drugs targeting the RhoA/ROCK/MRTFa pathway. It will be interesting to investigate, why MSC do not require MRTFa activation for col1a1 expression. It is possible that MRTFa is removing an inhibitory transcription factor or epigenetic mark from the col1a1 gene locus in fibroblasts, but not in MSC. Such a mechanism was found in genes for master regulators of ES cell differentiation, where the Smad3 binding factor TRIM33 is disabling repressive histone marks (16).

Introduction of this col1a1 gene inhibitory signal to MSC will make their collagen I expression dependent on activation of RhoA/ROCK/MRTFa. This could increase the efficiency of ROCK inhibitors as antifibrotic drugs. ROCK inhibitors such as fasudil showed some efficacy in mouse and rat models of fibrosis (17), but no reports on clinical efficacy in humans have been published up to now.

Polymerization and depolymerisation of F-actin and thus MRTFa activation is not only regulated by RhoA, but also by Rac1 and Cdc42 (18). It was therefore expected that deletion of Rac1 or Cdc42 might decrease MRTFa dependent αSMA expression by reducing Arp2/3-dependent actin polymerization or by increasing actin depolymerisation via reduced phosphorylation of cofilin. Unexpectedly, our data revealed that Rac1 and Cdc42 modulate F-actin amounts and αSMA expression, but in an Arp2/3 and cofilin-independent manner. Moreover, Cdc42 and Rac1 had an opposite effect on actin polymerization, suggesting that they regulate F-actin in MSC in a rather unconventional manner, probably dependent on cell contraction. MRCKα and MRCKβ are ROCK-like effectors of Cdc42 that mediate contraction (19). In MSC, however, MRCKα and MRCKβ are apparently not involved in the regulation of myofibroblast differentiation. While the molecular details of regulation of contraction by Cdc42 and Rac1 in
MSC remain to be elucidated, our data clearly identify Cdc42 as a potential novel drug target for fibrosis therapy.

We observed increased JNK phosphorylation in Cdc42 ko MSC after 24h TGFβ stimulation, while control cells or cells lacking RhoA or Rac1 showed no effect, although a transient, earlier increase cannot be excluded. JNK activation leads among other pathways to phosphorylation and activation of c-jun, which was reported to inhibit canonical TGFβ signaling (20,21). Indeed, Cdc42-null MSC showed a certain reduction of collagen I expression, which might be related to the increased JNK activation. On the other hand, the JNK inhibitor CC-401 significantly inhibited renal fibrosis in a kidney fibrosis model (22), suggesting a profibrotic function of JNK. Moreover, JNK activation is a well-known part of non-canonical TGFβ signaling and was described to be crucial for TGFβ-induced myofibroblast development in vitro (23). The molecular mechanism of Cdc42 loss of function-dependent JNK activation is not clear, since the latter is so far considered as downstream effector of Cdc42 (24). JNK inhibitors employed in Cdc42-null MSC might reveal the relevance of JNK for myofibroblast differentiation.

Cofilin inhibition by phosphorylation commonly promotes F-actin formation (25), expected to result in increased MRTFa activation and increased MRTFa-dependent gene expression. Indeed, deletion of cofilin (cofilin1) in MSC increased F-actin and αSMA expression, suggesting that MRTFa is able to stimulate αSMA expression also in the absence of exogenous TGFβ. However, addition of TGFβ resulted in a similar fold change in αSMA expression as in wildtype cells, indicating that the TGFβ-induced induction of αSMA expression is largely independent of cofilin. Normal αSMA expression after deletion of both LIMKs, negative regulators of cofilin downstream of RhoA, Rac1 and Cdc42, supported this notion. Interestingly, deletion of RhoA, Rac1, or Cdc42 did not lead to changes in basal levels of TGFβ-induced phospho-cofilin, neither in the presence nor the absence of TGFβ (2h), although all three Rho GTPases affected F-actin levels. These data suggest that the importance of Rho GTPase-dependent cofilin regulation is highly cell type dependent.

A possible model explaining our data is that RhoA, Rac1 and Cdc42 regulate αSMA expression in MSC via a RhoA/ROCK/pMLC2/F-actin/MRTFa pathway. Apparently, increased contraction of F-actin is preventing its depolymerisation, as already suggested previously (26). Since Arp2/3 complex-dependent actin polymerization is not required for TGFβ-induced αSMA expression, formin-mediated stress fiber formation could be important for regulating MRTFa in MSC, which would fit the correlation between stress fiber formation and αSMA expression observed here, and the well-known correlation between contraction and stress fiber formation (27). Since Cdc42- and Rac1-dependent actin assembly operating in TGFβ-induced differentiation of MSC is not controlled through conventional regulation by Arp2/3, cofilin, or MRCK-dependent regulation of contraction, it is possible that they act upstream of RhoA. Cross-talk between Rho GTPases is well established in various conditions and cell types, and might involve competition for GDIs, GDFs, and GAPs (28,29).

Finally, we observed accumulation of intracellular collagen I in Cdc42 ko and RhoA ko cells. In Cdc42-null MSC, this correlates with a decreased amount of collagen I in the cell supernatant, while this was not obvious in RhoA null cells. It was reported previously that in smooth muscle cells, Cdc42 regulates collagen I secretion via PKCθ (30). It appears that a similar mechanism might take place in MSC. Cdc42 is...
known to regulate transport between ER and Golgi (31), while RhoA has not been reported to be involved in constitutive secretion. In any case, the secretion defect in Cdc42-null MSC further increases the interest in Cdc42 as a potential drug target for fibrosis therapy.

In conclusion, not only RhoA, but also Rac1 and Cdc42 are important for non-canonical TGFβ signaling, at least in MSC differentiation into myofibroblasts. Non-canonical TGFβ signaling differentially regulates expression of αSMA and col1a1 in MSC, in contrast to fibroblasts and myofibroblasts, which might be of relevance for fibrosis therapies. Finally, Cdc42 emerges as an interesting drug target for fibrotic diseases, since it might interfere with both myofibroblast differentiation as well as collagen I secretion.

**Experimental Procedures**

**MSC isolation and culture**

Primary mouse MSC were isolated from bone as described (32). Primary and immortalized MSC were cultured in MSC growth medium (αMEM [Gibco, 32561-029], 10% FBS (Thermo Fisher, 12662011), 100 U/ml penicillin-streptomycin (P/S) [Gibco, 15140]) at 37°C and 5% CO2. Primary MSCs were split 1:3 (primary) or 1:4 (MSC line) after reaching 90% confluence. For detachment, MSCs were washed once in a 10 cm tissue culture dish (Greiner Cellstar, P7612) with 2 ml warm PBS, and then incubated with 1 ml 5% Trypsin-EDTA (Gibco, 25200-056) for exactly 2 min at 37 °C. Trypsin digestion was stopped by adding 1 ml of MSC growth medium. Since contaminating macrophages mostly will not detach during this time, MSCs will be enriched by passaging.

**MSC differentiation**

Differentiation potential of MSC to adipocytes or osteoblasts was measured using corresponding kits from STEMCELL (STEMCELL, #05503 & #05504). Myofibroblast differentiation of MSC was stimulated by 5 ng/ml human TGFβ1 (Chinese Hamster Ovary cell line-derived, Ala279-Ser390; R&D, 240-B-002) for 24 h or 72 h.

**CRISPR/Cas9-mediated genome editing in MSC**

Gene deletion in MSC was mediated by lentiviruses transducing CRISPR/Cas9 gene editing constructs (33). Guide RNAs (gRNA) that target the coding region close to the translation start site of respective gene of interest were designed using the CRISPRSCAN program (34): Non-target control:

- CACCGGTATTACTGATATTGGTGGG; RhoA;
- CACCCGATAGAGAAGGCGGCGAGG; Rac1;
- CACCAGCGTGGGGATGTACTCTCC; Cdc42;
- CACCGAAAGCTGGCGCGGGATCTGA; Rock1;
- CACCCGCCATTTGGGATCCCGACG; Rock2;
- CACCCGGGTTTCTGAATAACACAAGG; MRCKa;
- CACCAGGCCAGGAAGTGGCATTGG; MRCKb;
- CACCCGGGCGCCAGTTCTCGAGTG; Smad2;
- CACCCGATGAAAAATACGGCCG; Smad3;
- CACCCGGTCGCCGACTGGCACACAGG; Smad4;
- CACCAGATGTGCTACAGACAGGTG; MRTFa;
- CACCCGGAGAGCTATCATTGGTG; PAK1;
- CACCCGGGCGTGGGAGACTAAGACTGG; Limk1;
- CACCAGGGTGGGAGCCGGGATGTAGGC; Limk2;
- CACCCGGGTAGCATGAGGTGTG; cofilin;
- CACCCGGTTCTCCAGGCTCTTGCTG; Apc2;
- CACCCGGTTAGCGAAGTTGGGT; Ap2.

Annealed DNA oligonucleotides containing the GRNA sequence were then cloned into the lenticRISPR v2 vector (addgene #62988) as described earlier (35). When needed, the puromycin resistance was exchanged against a blasticidin resistance. Lentivirus was produced by transfection of subconfluent HEK293 cells with 0.8 µg lentiCRISPRv2 vector (addgene #62988) as described earlier (35). When needed, the puromycin resistance was exchanged against a blasticidin resistance. Lentivirus was produced by transfection of subconfluent HEK293 cells with 0.8 µg lentiCRISPRv2 described above or 0.8 µg pLenti.PGK.LifeAct-GFP (addgene #51010), 0.6 PAX2 (addgene #12260), and 0.6 µg VSVG plasmid (addgene, # 8454) in the presence of 8 µg polyethylenimine (PEI; Polynplus) in 200 µl opti-MEM (Thermo Fisher, 11058021). Virus-containing supernatant was collected after 24 h.
and 48 h, filtered through a 0.45 µm cellulose acetate membrane filter (VWR, 28145-481) to remove cells, and used directly for transduction of MSC. Supernatant of 40-60% confluent MSC seeded the previous day was aspirated and 1-2 ml filtrated virus supernatant and 4 µl 4µg/ml hexadimethrine bromide (polybrene; Sigma, 107689) in PBS were added to each well of a 6-well plate. After 1 d, supernatant was replaced with MSC growth medium. 48 hours after transduction, antibiotic selection was started with 4 µg/ml puromycin for 2 d or 8 µg/ml blasticidin for 5 d. MSC expressing fluorescent LifeAct were not selected by antibiotics, but identified by fluorescence.

**Fast cycling Rho GTPase mutants**
cDNAs encoding HA-tagged fast cycling Rho GTPase mutants (RhoA (F30L), Rac1 (F28L), Cdc42 (F28L); 36) were cloned together with IRES-EGFP into the lentiviral expression vector pRRl5in kindly obtained by Dr. Didier Trono (EPFL, Lausanne, Switzerland). Lentiviral transduction of MSC was carried out as described above. Efficient transduction was confirmed by assessing EGFP expression using fluorescence microscopy.

**F-actin staining**
Sterile coverslips were placed into wells of a 24-well plate with 500 µl 50 µg /ml poly-L-lysine solution. After 1 h incubation at 37 °C, the coating solution was removed and the surface allowed to dry. 5000 MSCs were plated on each of the glass slides in 1 ml MSC growth medium. The next day, MSCs were stimulated with 5 ng/ml TGFβ1 (R&D, 240-B-002) for 24 hours, and then MSCs fixed with 4% PFA in PBS for 10 minutes at RT, and stored at 4 °C. To stain for F-actin, MSCs on coverslips were incubated with 1:200 diluted phalloidin atto 568 (Thermo Fisher, A12380) in PBS with 2% BSA and 0.5 % Triton for 1 hour at RT. Coverslips were washed with 1ml PBS for 10 seconds, and then mounted on glass slide using mounting buffer (Sigma, F6057). For each sample, 5 randomly chosen, non-overlapping images were taken. For a given experiment, all images were taken with the same exposure time using a fluorescent channel appropriate for detection of Alexa Fluor 568 by conventional fluorescence microscopy (Axio Imager 2, Zeiss). Total F-actin signal was measured on each slide using Image J, and each cell was selected manually and integrated density in each cell on the red channel measured and quantified.

**Flow cytometry**
MSC were detached by trypsinization and then resuspended in PBS containing 1% BSA at 1 million cells/ml. To block binding of antibodies to cellular Fc receptors, MSCs were pre-incubated with 1 µg of anti-mouse CD16/CD32 (eBioscience 14-0161-85) per 100µl MSC for 15 minutes at 4°C in 1.5 mL Eppendorf tubes. Then, fluorescently-labelled antibodies at indicated dilutions were added. The following rat anti mouse antibodies (all eBioscience) were used: CD105 (12-1051-81), CD11b (17-0112-81), CD44 (17-0441-81), CD45 (12-0454-82), Sca1 (17-5981-83), IgG2a isotype control APC, IgG2a isotype control PE (12-4321-81). After mixing, the solution was protected from light and incubated for at least 30 minutes at 4 °C. Cells were washed by adding three volumes of PBS/1%BSA, pelleted by centrifugation at 340g for 5 minutes, resuspended and fixed with 1% PFA in PBS for 10 minutes at RT, pelleted by centrifugation and resuspended in 500 µl PBS. Cellular fluorescence was measured using CellQuest software on a BD FACS Calibur. Data analysis was carried out with the FlowJo program (FlowJo 10.2, FlowJo, LLC).

**Western blot**
Cell lysate samples, around 10 -20 µg per lane, were separated by 12% SDS-PAGE and transferred at 4 °C to polyvinylidene fluoride membranes. The membranes were blocked using 5 % milk in Tris-buffered saline, 0.05%
TWEEN (TBS-T) for 60 min and incubated overnight at 4 °C with the indicated primary antibodies: RhoA (sc-418), GAPDH (sc-25778), Rock1 (sc-5560), Rock2 (sc-5561), MRCKa (sc-374568), MRCKb (sc-374597), pp38 (sc-17852-R, all Santa Cruz), Rac1 (610650), Cdc42 (610929, all BD Biosciences), p-MLC2 (#3674), p-Cofilin (#3311), pSmad2 (# 3108S), pJNK (#9251S), pERK (#9101S), PAK1 (#2602, all Cell Signaling), aSMA (GTX100034, GeneTex), Collagen I (ab34710), Smad3 (ab28379), Smad4 (ab40759), Arpc2 (ab133315; all Abcam), pPAK1 (PA5-12844), pPAK4 (PA5-36865; all Thermo Fisher). Following three 15-min washing steps with TBS-T, the membranes were incubated with corresponding secondary antibodies (Jackson Immunoresearch), LuminaTM Western HRP chemiluminescence substrate detection reagent (Millipore) for 1 minute at RT. Luminescence was detected with Medical X-Ray film (AGFA).

Statistics
Data were presented as mean±SEM. Unpaired, two-tailed Student’s t-tests were used for comparisons between two groups. For multiple comparisons, two-way ANOVA with Tukey’s multiple comparisons was applied. Statistical analysis was done using GraphPad Prism. The following indications of significance were used throughout the manuscript: *p<0.05, **p<0.01, ***p<0.001.

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Conflict of interest
There are no conflicts of interest.

Author contributions
JG designed and performed the experiments and analyzed the data. CB designed the research and wrote the manuscript. LB, MA, MQK, MS and KR provided technical and intellectual support. All authors discussed the results and commented on the manuscript.
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Figures and Legends

Fig. 1: Establishment of a spontaneously immortalized MSC line. A: FACS analysis of p58 MSC for surface markers of MSC (CD105, CD44, Sca1) and hematopoietic cells (CD45, CD11b). Autofluorescence shown in red (n=3). B: Bright field microscopy of MSC. C: Differentiation of p58 MSC to adipocytes and osteoblasts stained with Oil-red-oil (adipocytes; fat vesicles in red) and Alizarin red S (osteoblasts; orange-red) (n=4).
Fig. 2: Canonical TGFβ signaling is required for myofibroblast differentiation of MSC. A, B, D-F: qRT PCR analysis for indicated genes of WT, Smad2-KO, Smad3-KO, and Smad4-KO cells (black bars: 24h TGFβ; grey bars: untreated; n=3/3). C: Representative Western blots for the indicated proteins of MSC with ko of Smad2, Smad3, and Smad4.
Fig. 3: Rho GTPase-deficient MSC show clear morphological changes, but normal Smad2 phosphorylation in response to TGFβ. A: Representative Western blots for the indicated proteins of MSC with KO of RhoA, Rac1, and Cdc42. B: Bright field microscopy of MSC with indicated gene deletions. C, D: Representative Western blot and quantification of lysates of indicated cells for pSmad2. (black bars: 24h TGFβ; grey bars: untreated; n=3/3).
Fig. 4: Rho GTPases regulate TGFβ-induced expression of αSMA, but not of col1a1. A, D, E: qRT PCR analysis for indicated genes of RhoA-KO, Rac1-KO, Cdc42-ko and WT cells after treatment for 24h (A, D) or 3d (E) with TGFβ. (black bars: 24h TGFβ; grey bars: untreated; n=6/6). B, C, F-H: Representative Western blots and quantification of lysates of indicated cells for αSMA (B, C) and collagen I (F-H). (black bars: 24h TGFβ; grey bars: untreated; n=4/4).
Fig. 5: Cdc42-KO MSC display increased pJNK. A: F-actin of TGFβ1-treated and untreated MSC, detected by fluorescently-labelled phalloidin. B-G: Quantifications of Western blots of lysates of indicated MSC for pMLC2 (B), pCofilin (C), pErk (D), pp38 (E) JNK pp46(F), and JNK pp54 (G). (black bars: 24h TGFβ; grey bars: untreated; n=3/3).
Fig. 6: Cofilin is not required for TGFβ-induced expression of αSMA in MSC. A, B, D, E, G-K, M-O: qRT PCR analysis for indicated genes of WT cells treated with latrunculin A (latA) or jasplakinolide (jasplak) (A, B), Arpc2-KO (D, E), cofilin-KO (G, H), Limk1-KO (I, M), Limk2-KO (J, M), Limk1/2 KO (K, M), and MRTFa KO (N, O) (n= 4/4). C: Representative Western blots for the indicated proteins of MSC with ko of Arpc2 or cofilin. L: Western blot for pCofilin of MSC with indicated KO. (A-O: black bars: 24h TGFβ; grey bars: untreated).
Fig. 7: TGFβ-induced αSMA induction of MSC correlates with changes in contraction and F-actin. A, B, C, D: qRT PCR analysis for indicated genes of WT cells treated with blebbistatin (Blebb; A, B) or Y-27632 (C, D) (n= 3/3). E: F-actin of MSC treated with blebbistatin or Y-27632, detected by fluorescently-labelled phalloidin. F: Representative Western blots for the indicated proteins of MSC with KO of Rock1, Rock2, MRCKα, and MRCKβ. G-I, L: Representative Western blots and quantification of lysates of indicated cells for pMLC2 (n=6/6). J, M: Quantification of phalloidin stainings of indicated MSC (n= 5/5). K, N: qRT PCR analysis for αSMA of MSC with indicated KO (n= 5/5). (A-D, I-O): Black bars: 24h TGFβ; grey bars: untreated).
RhoA, Rac1 and Cdc42 differentially regulate αSMA and collagen I expression in mesenchymal stem cells
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