Expression of α-Smooth Muscle Actin Determines the Fate of Mesenchymal Stromal Cells

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SUMMARY

Pro-fibrotic microenvironments of scars and tumors characterized by increased stiffness stimulate mesenchymal stromal cells (MSCs) to express α-smooth muscle actin (α-SMA). We investigated whether incorporation of α-SMA into contractile stress fibers regulates human MSC fate. Sorted α-SMA-positive MSCs exhibited high contractile activity, low clonogenicity, and differentiation potential limited to osteogenesis. Knockdown of α-SMA was sufficient to restore clonogenicity and adipogenesis in MSCs. Conversely, α-SMA overexpression induced YAP translocation to the nucleus and reduced the high clonogenicity and adipogenic potential of α-SMA-negative MSCs. Inhibition of YAP rescued the decreased adipogenic differentiation potential induced by α-SMA, establishing a mechanistic link between matrix stiffness, α-SMA, YAP, and MSC differentiation. Consistent with in vitro findings, nuclear localization of YAP was positively correlated in α-SMA expressing stromal cells of adiposarcoma and osteosarcoma. We propose that α-SMA mediated contraction plays a critical role in mechanically regulating MSC fate by controlling YAP/TAZ activation.

INTRODUCTION

Human mesenchymal stromal cells (MSCs) are being used in cell therapy to support organ regeneration after injury, e.g., by injection into the heart after myocardial infarction (Behfar et al., 2014). However, the outcomes of MSC therapy have been variable and the reasons for success or failure are a matter of ongoing debate (Behfar et al., 2014; Bianco et al., 2013). First, the potential of MSC therapy to support organ regeneration depends on the intrinsic character of the transplanted cell population, which is often ill-defined (Bianco et al., 2013; Mishra et al., 2009; Prockop et al., 2014). Second, engraftment success, survival, phenotype, and activity of MSCs strongly depend on the microenvironment present at the site of delivery (Forbes and Rosenthal, 2014). This microenvironment often shares features of a healing wound, including inflammatory cells, neo-vasculature, and pro-fibrotic cytokines such as TGF-β1 (Forbes and Rosenthal, 2014). Tissue repair and tumor microenvironment can convert MSCs into contractile myofibroblasts (MFs) that de novo form α-smooth muscle actin (α-SMA)-containing stress fibers (Hinz, 2010a; Hinz et al., 2012). The most prominent examples are “cancer-associated fibroblasts” (CAFs) which originate at least in part from bone marrow-derived MSCs (Karnoub et al., 2007; Mishra et al., 2009; Öhlund et al., 2014; Quante et al., 2011).

Acute and transient MF activation is part of the body’s normal wound healing program, but persistent MFs contribute to fibrosis by excessively producing and contracting collagenous extracellular matrix (ECM) into stiff scar tissue (Hinz et al., 2012). In turn, the stiffness of mature scar promotes mechanical activation of MFs (Hinz, 2010b). In cell therapy, MSCs engrafted into early stages of organ fibrosis were shown to improve healing, but delivery into stiff mature scar further enhanced fibrogenesis in fibrotic lung, kidney, liver, and heart (Breitbach et al., 2007; di Bonzo et al., 2008; Nagaya et al., 2005; Ninnichuk et al., 2006; Yan et al., 2007). Substrate mechanics in conjunction with intracellular tension have also been shown to determine the preference of naive MSCs toward specific lineages (Engler et al., 2006; Winer et al., 2009; Yang et al., 2011, 2014), but the functional consequences of MF activation (fibrogenesis) on MSC clonogenicity and lineage differentiation potential have not been systematically investigated.

We hypothesize that acquisition of MF contractile features, most notably expression of α-SMA, will determine the fate of bone-marrow-derived human MSCs (hMSCs). Our results show that α-SMA-positive hMSCs exhibit low self-renewal and lineage differentiation potential, in contrast to α-SMA-negative hMSCs, which are clonal and multi-potent. Soft culture substrates that suppress the pro-fibrotic MF phenotype also enhance the lineage differentiation potential of α-SMA-positive hMSCs. We identify...
α-SMA incorporation into hMSC stress fibers and downstream translocation of YAP/TAZ transcription factors into the nucleus as a key event in regulating genes associated with self-renewal and differentiation. Inhibition of α-SMA may thus be a potential strategy to improve the therapeutic potential of MSCs and reduce the risk of MSC fibrogenesis.

RESULTS

MF Activation Results in Reduced Clonogenicity and Differentiation Potential of hMSCs
Independently of MSC origin, MF activation occurs spontaneously in standard cell culture on rigid tissue culture plastic in serum-containing media. Cultured hMSCs derived from adipose tissue, umbilical cord perivascular, and bone marrow all developed MF characteristics, including α-SMA-positive stress fibers, extradomain-A fibronectin (ED-A FN) in the ECM, and high contractile activity, which were enhanced by TGF-β1 (Figure S1).

To test whether MF activation affected stem cell features, we focused on bone-marrow derived hMSCs (Figure 1A). In standard culture, 17% ± 4.2% of hMSCs expressed α-SMA in F-actin-positive stress fibers, which increased to 32% ± 5.1% after TGF-β1 treatment, associating with 3.5-fold higher contraction (Figures 1A, 1B, and S1). TGF-β1 treatment reduced the number of colony forming units-fibroblasts (CFU-F) by 3-fold (Figure 1B) and the ability of hMSCs to differentiate into adipogenic (10-fold) and osteogenic (7-fold) cell lineages in conventional lineage-induction assays supplemented with TGF-β1 (Figure 1B).

TGF-β1 treatment resulted in ~1.5-fold increase in median fluorescence intensity of common MSC markers such as CD44, CD73, and CD90, but did not change levels of CD105 and CD166 (Figure S2). This supports the onset of a fibrogenic program since CD44, CD73, and CD90 have all been identified on fibroblasts and are upregulated in conditions of fibrosis (Fernández et al., 2013; Koumas et al., 2003; Li et al., 2011; Maring et al., 2012; McQualter et al., 2013).

To test whether low ECM stiffness, a powerful inhibitor of MF activation (Goffin et al., 2006), improves hMSC clonogenicity and lineage differentiation potential, we cultured hMSCs on silicone substrates with different Young’s elastic modulus simulating different scar maturation stages (Figure 1C). Expression of α-SMA and ED-A FN decreased after 5 days culture on “fibrosis-rigid” (65 kPa) over “wound-stiff” (26 kPa) to “normal tissue-soft” (3 kPa) substrates (Figures 1D and 1E). With percentages of α-SMA stress fiber-positive hMSCs decreasing from 21.9% ± 3.4% (65 kPa) to 17.3% ± 2.4% (26 kPa) and 5.7% ± 3.1% (3 kPa) (Figure 1F), the number of CFU-F increased (Figure 1G). Treatment with TGF-β1 enhanced α-SMA expression on 26 kPa and 65 kPa substrates compared to non-treated controls, but also reduced CFU-F capacity independently of α-SMA content (3 kPa). This finding is consistent with previous studies reporting inhibitory effects of TGF-β1 on mesenchymal cell proliferation (Kim et al., 2014) and MSC self-renewal capacity by inducing osteogenesis and chondrogenesis (Watabe and Miyazono, 2009). Consistently, our hMSCs exhibited ~8-fold increase in the early chondrogenic marker SOX9 and ~50-fold increase of the late marker ACAN (aggrecan) upon TGF-β1stimulation. Whereas ACTA2 was also increased by TGF-β1 treatment in conventional culture conditions (~8-fold), expression levels remained low in complete chondrogenesis medium (containing TGF-β) and pellet culture that induced SOX9 (~7-fold) and substantially ACAN (~180-fold) (Figure S1). Hence, using TGF-β1 as a stimulus is not suitable to test whether MF activation is directing hMSC stem cell potential.

α-SMA-Positive hMSCs Exhibit Low Clonogenicity and Bias toward Osteogenic Potential

To answer whether loss of hMSC stem cell features on stiff substrates is a consequence of MF activation and α-SMA expression, we sorted α-SMA-positive and α-SMA-negative hMSC from heterogeneous hMSC using flow cytometry (Figure 2A). Because no cell surface marker reliably identifies MFs, we sorted based on the observation that α-SMA-expressing hMSCs exhibited about six times larger spreading area than α-SMA-negative cells (Figure 2A). After live-sorting the lower and higher quartile of hMSCs by size, the large-size-sorted fraction contained no α-SMA-negative cells, whereas only 2.3% of the small-sorted population were α-SMA-positive (Figure 2B). For the remainder of this study, size-sorted hMSCs were thus termed SMA(−) (small) and SMA(+) (large). The pro-fibrotic character of SMA(+) hMSCs extended beyond expression of α-SMA as shown by higher mRNA levels of pro-fibrotic markers ACTA2, COL1A1, COL3A1, CTGF, and TGFβ2 as compared to SMA(−) hMSCs (Figure 2C).

SMA(−) hMSCs contained 8-fold more CFU-Fs compared with SMA(+) hMSCs (Figure 2D) and in single cell cloning assays, formed ~8-fold more clones than unsorted hMSCs; SMA(+) hMSCs did not produce any clones (Figure 2D). Consistently, mRNA levels of SOX2, OCT4, and DNMT1 genes that are indicative and instrumental for stem cell self-renewal and clonogenicity in MSCs (Arnold et al., 2011; Tsai et al., 2012; Yannarelli et al., 2013; Yu et al., 2012b), were ~5-fold lower in SMA(+) compared with SMA(−) hMSCs (Figure 2E). When cultured under respective lineage induction conditions, SMA(−) hMSCs...
differentiated into both lipid-producing and PPARγ transcript expressing adipocytes and osteoblasts that expressed RUNX2 and formed mineralized nodules (Figure 2F). In contrast, SMA(+) hMSCs exhibited substantially reduced adipogenic potential, but enhanced osteogenesis (~4-fold more mineralized nodules) (Figure 2F). Collectively, these results indicate that the α-SMA-positive fraction of hMSC populations is fibrogenic, non-clonal, and biased toward osteogenesis.

Mechanical Deactivation Restores the Differentiation Potential of SMA(+) hMSCs

We next addressed whether expression of α-SMA in hMSCs hallmarks reversible MF activation or terminal fibrogenic differentiation by culturing pure SMA(+) hMSCs for 5 days on soft culture substrates. α-SMA protein levels and sizes of cell-ECM focal adhesions decreased with decreasing substrate stiffness (Figure S3). Cell culture on 3 kPa soft substrates resulted in 2-fold reduced expression
Figure 2. Clonal and Differentiation Potentials Are Higher in Enriched α-SMA-Negative Than in α-SMA-Positive hMSC Populations

(A) hMSCs spontaneously acquire the MF phenotype, resulting in a heterogeneous population of α-SMA-positive (red) and -negative cells (nuclei only, blue). The scale bar represents 50 μm. The cell spreading area was measured as a function of α-SMA expression.

(B) Heterogeneous hMSC populations were sorted into small (lower 25%) and large hMSCs (upper 25%) using forward scatter in FACS and then immunostained for α-SMA in suspension or analyzed by western blotting.

(C) Small size-sorted hMSC, now defined as "SMA(−)/C0" and large-size sorted "SMA(+)" hMSCs, were cultured for 1 day on stiff substrates to assess α-SMA (red) and stress fibers (F-actin, green). Transcript levels were quantified for pro-fibrotic markers ACTA2 (α-SMA), TGFB2 (TGF-β2), COL1A1 (collagen type I), COL3A1 (collagen type III), and CTGF (CCN2) using quantitative (q)RT-PCR.

(D and E) Sorted SMA(−)/C0 and SMA(+) hMSCs were assessed for clonogenicity in functional CFU-F and single cell cloning assays and assessed for (E) levels of self-renewal markers OCT4, SOX2, and DNMT1.

(F) Sorted SMA(−) and SMA(+) hMSCs were lineage-induced in adipogenic and osteogenic induction medium and assessed for adipogenesis using oil red O staining and qRT-PCR analysis for PPARG and osteogenesis using Alizarin Red S staining and qRT-PCR analysis for RUNX2. The scale bars represent (A) 50 μm and (C and F) 50 μm. The graphs show averages ± SD from at least five independent experiments (*p ≤ 0.05, **p ≤ 0.005, and ***p ≤ 0.0005 using Student’s t test). See also Figure S2.
of α-SMA (Figures 3A and 3B), disappearance of α-SMA from stress fibers (Figure 3C), and generally reduced levels of fibrotic marker transcripts compared with hMSC(+) grown on stiff substrates (Figure 3D). The culture time required to deactivate MFs mechanically was 5 days (Figure S4). Reversibility of the fibrotic phenotype was not dependent on the degree of MF pre-activation. SMA(+) hMSCs lost the MF phenotype on soft substrate even when being sorted from TGF-β1-pre-treated heterogeneous populations and were not re-activated on soft substrate by adding TGF-β1 (Figure S4).

Transfer of SMA(+) hMSCs that had lost MF characteristics on soft substrates to conventional lineage induction media resulted in 1.3-fold higher levels of PPARγ mRNA and ~10-fold increase in lipid-production than SMA(+) hMSCs harvested from control stiff culture (Figure 3E). Conversely, the osteogenic capacity of soft-grown SMA(+) hMSCs was reduced by ~2-fold compared

Figure 3. Soft Substrate Culture Restores Lineage Differentiation of SMA(+) hMSCs
(A–D) Sorted SMA(+) hMSCs were cultured on stiff and soft substrates and assessed for MF activation after 5 days, clonogenicity after 10 days, and differentiation potential after 14 days. MF activation was assessed by (B) western blotting, (C) immunofluorescence for α-SMA (red) and stress fibers (F-actin, green) (the scale bar represents 20 μm), and (D) qRT-PCR for fibrotic markers.
(E) Self-renewal potential was quantified from CFU-F assays and qRT-PCR analysis of OCT4, SOX2, and DNMT1 transcripts.
(F) Sorted SMA(+) were grown for 5 days on stiff and soft culture substrates and then transferred to conventional culture dishes for induction into adipogenic lineage (oil red O, PPARγ) and osteogenesis (Alizarin Red S, RUNX2). The scale bar represents 50 μm. The graphs show averages ± SD from at least five independent experiments (*p ≤ 0.05, **p ≤ 0.005, and ***p ≤ 0.0005 using Student’s t test). See also Figure S3.
Expression of α-SMA Directly Controls hMSC Fate

Expression of α-SMA and incorporation into stress fibers upregulate contraction of fibroblastic cells (Hinz et al., 2001, 2002). It is conceivable that α-SMA directly regulates hMSC stem cell character because actin-myosin generated intracellular tension guides hMSC fate decision (Engler et al., 2006; MacQueen et al., 2013; McBeath et al., 2004; Swift et al., 2013). To elucidate the role of α-SMA protein in directing hMSC fate, we first transfected SMA(−) hMSCs with α-SMA-GFP (Figure 4A) that localized to stress fibers and GFP control that remained cytosolic (Figure 4B). Expression of SMA-GFP (67 kDa) resulted in upregulation of endogenous α-SMA (42 kDa) (Figure 4B), consistent with a positive feedback loop of enhanced cell contraction and mechanically induced α-SMA expression (Hinz et al., 2002). Overexpression of α-SMA-GFP reduced the high clonogenicity of α-SMA(−) hMSCs and transcript levels of SOX2, OCT4, and DNMT1 by maximally 4-fold compared to GFP control (Figure 4C). α-SMA-GFP-overexpressing SMA(−) hMSCs exhibited ~5-fold decreased adipogenesis (Figure 4D), but ~2-fold increased osteogenic potential compared to controls (Figure 4D).

Next, we knocked down α-SMA in SMA(+) hMSCs using short hairpin (sh)RNA directed against α-SMA, which resulted in ~3-fold higher numbers of CFU-Fs and dramatic upregulation of SOX2, OCT4, and DNMT1 transcripts (up to 40-fold, OCT4) than in controls (scrambled shRNA with GFP reporter) (Figure 4G). In cell lineage induction assays, SMA(+) hMSCs showed ~25-fold increased formation of lipid-producing adipocytes, ~5.6-fold higher PPARγ mRNA levels (Figure 4H), and ~2.2-fold decrease of mineralized nodule area, as well as ~100-fold decrease in RUNX2 mRNA levels after α-SMA knockdown compared with controls (Figure 4H). Our results showing that knockdown of α-SMA alone confers clonogenicity and adipogenic/osteogenic differentiation suggest that the MF is an activated phenotype and not a terminal differentiation state of SMA(+) hMSCs.

Expression of α-SMA in hMSCs Controls YAP/TAZ Nuclear Localization

We next addressed how α-SMA regulates hMSC fate and clonogenicity. Cell contractility and actin filament bundle assembly both stimulate activation and translocation of YAP/TAZ to the nucleus (Dupont et al., 2011; Gaspar and Tapon, 2014). YAP/TAZ transcriptionally regulate genes associated with stem cell fate decision and self-renewal (Varelas, 2014). To test whether α-SMA expression affects YAP/TAZ localization in hMSCs, we first co-analyzed α-SMA stress fiber intensity with the ratio of nuclear/cytoplasmic YAP on an individual cell basis and demonstrated a clear correlation (Figure S5). The low baseline percentage of SMA(−) hMSCs with predominantly nuclear YAP (25%, nuclear/cytoplasmic YAP > 1) was increased to 90% after transfection with α-SMA-GFP, corresponding to the percentage of SMA(+) hMSCs with constitutive nuclear YAP (Figure 5A). Knockdown of α-SMA with shRNA reduced the percentage of SMA(+) hMSCs with predominantly nuclear YAP to 7% (Figure 5B).

To decipher the mode of action of α-SMA, contraction, or action polymerization, we used the α-SMA-specific fusion peptide SMA-FP in combination with cytoskeletal drugs (Figure 5C). SMA-FP selectively targets α-SMA in stress fibers, inhibits α-SMA-mediated contraction, and selectively depolymerizes α-SMA from stress fibers (Clément et al., 2005; Hinz et al., 2002). SMA-FP removed α-SMA from persisting stress fibers, in contrast to control (DMSO and skeletal actin fusion peptide, SKA-FP) SMA(+) hMSCs (Figure S5). SMA-FP treatment resulted in low levels of nuclear YAP and reduced hMSC contraction on wrinkling elastomer substrates (Figure 5C). SMA(+) hMSCs treated with the myosin II inhibitor blebbistatin showed similar low levels of YAP in the nucleus and low contraction, but disassembled all actins from stress fibers, as evident from phalloidin staining (Figures 5C and S5).

Next, we stabilized stress fibers by pre-treating SMA(+) hMSCs with jasplakinolide, followed by addition of SMA-FP (Figure 5D). With jasplakinolide being present, the SMA-FP was unable to depolymerize α-SMA from stress fibers (Figure 5D) and hMSCs contraction was unaltered (Figures 5D and 5E), indicating that the SMA-FP reduces MF contraction primarily by depolymerizing α-SMA from stress fibers. Whereas YAP nuclear localization remained high under jasplakinolide/SMA-FP treatment (Figures 5D and 5F), addition of blebbistatin to jasplakinolide-treated SMA(+) hMSCs resulted in the loss of α-SMA from stress fibers, reduced cell contractility, and reduced YAP nuclear localization (Figures 5D–5F). Control SMA(+) hMSCs treated with jasplakinolide alone were similar to untreated cells (Figures 5D–5F). In combination, these experiments suggest that enhanced contractile activity mediated by α-SMA upregulates YAP/TAZ nuclear localization.
To show that YAP/TAZ activation is indeed responsible for reduced lineage differentiation and self-renewal capacity downstream of α-SMA-expression in hMSCs, we co-transfected SMA(−) hMSCs with small interfering (si) RNA directed against YAP1 together with α-SMA-GFP. Transfection of YAP1 siRNA substantially reduced YAP expression levels (Figure 6A) and resulted in reduced SOX2 and OCT4 levels in control cells (Figure 6A, GFP). Knockdown of YAP1 in α-SMA-overexpressing hMSCs restored expression of PPARγ mRNA and strongly reduced RUNX2 mRNA expression (Figure 6A). These results confirmed that the effect of high α-SMA expression on hMSC lineage differentiation potential is mediated by YAP. However, knockdown of YAP1 in α-SMA-overexpressing hMSCs did not restore high expression of SOX2 and...
Figure 5. Presence of α-SMA in hMSC Stress Fibers Results in YAP/TAZ Nuclear Accumulation

(A) Sorted SMA(−) hMSCs were transfected with control GFP and α-SMA-GFP constructs.

(B) Sorted SMA(+) hMSCs were transfected with control scrambled (scr) and α-SMA-shRNA constructs. Localization of YAP/TAZ was visualized using immunofluorescence staining for YAP (red) and GFP (green).

(C–F) The ratio of nuclear over cytosolic YAP/TAZ was calculated by image analysis and the percentage of cells with predominantly nuclear YAP/TAZ localization was determined. Sorted SMA(+) cells were either pre-treated with (C) DMSO (control) or (D) jasplakinolide for 30 min, followed by the addition of the α-SMA N-terminal peptide AcEEED (SMA-FP), blebbistatin, or DMSO control for another 40 min. Cells were then stained for YAP and α-SMA or YAP and stress fibers as indicated and assessed for contraction using wrinkling silicone substrates. Quantified from these images were (E) cell contraction as percentage of cells associated with wrinkles, and (F) percentage of cells with predominant nuclear YAP/TAZ localization.

All scale bars represent 20 μm. The graphs show averages ± SD from at least three independent experiments (*p ≤ 0.05 and **p ≤ 0.005 using ANOVA followed by a post hoc Tukey’s multiple comparison test). See also Figure S5.
OCT4, as expected if α-SMA would suppress hMSC self-renewal by acting through YAP (Figure 6A). To test whether this result was due to the overall loss of YAP, we used verteporfin to selectively abrogate the nuclear activity of YAP by inhibiting binding to TEAD elements (Liu-Chitlenden et al., 2012) (Figure 6B). Drug action was controlled by low levels of the YAP1 downstream target CCN2 (CTGF). All cells were analyzed by qRT-PCR for mRNA expression of RUNX2, PPARG, SOX2, and OCT4. The graphs show averages ± SD from three different donors per condition (*p ≤ 0.05, **p ≤ 0.005, and ***p ≤ 0.0005 using ANOVA followed by a post hoc Tukey’s multiple comparison test).

Finally, to investigate whether α-SMA expression is relevant for YAP/TAZ activation and hMSC lineage determination in vivo, we correlated α-SMA expression and YAP nuclear localization in soft mesenchymal tumors (adiposarcoma) and stiff mesenchymal tumors (osteosarcoma) that involve MSC tumorigenesis and fibrogenesis (Mohseny et al., 2009; Rodriguez et al., 2012; Xiao et al., 2013). The levels of α-SMA expression were negligible in healthy fat control tissue and low in normal bone, but significantly higher in the activated stroma of the respective tumor (Figures 7A–7C). Increased α-SMA expression in adiposarcoma correlated with 3.5-fold higher percentages of cells with predominantly nuclear YAP staining (22%) compared to normal fat tissue (6%, Figures 7A, 7C, and 7D). The levels of nuclear localized YAP staining did not significantly increase in osteosarcoma compared to already high levels observed in healthy bone (Figures 7B–7D), supporting that high levels of YAP/TAZ in mesenchymal cells direct osteogenic differentiation.
**DISCUSSION**

MSCs are prone to MF activation by stiff ECM and TGF-β1, but the consequences on their stem cell potential and reversibility have not been systematically assessed. We establish a direct link between α-SMA expression/function, YAP/TAZ activity, and hMSC fate. Different actin isoforms promote specific types of actin organization levels and a shift in the ratios of actin isoforms can reprogram cell differentiation (Lechuga et al., 2014; Tondeleir et al., 2012). α-SMA incorporation into existing stress fibers has been shown to increase actin organization and intracellular tension (Goffin et al., 2006; Hinz et al., 2001). Actin organization as cortical filaments or incorporation into stress fiber bundles has been shown to differentially control YAP/TAZ activation, nuclear localization, and regulation of the Hippo pathway (Gaspar and Tapon, 2014; Halder et al., 2012; Yu et al., 2012a). In epithelia, cell-morphology-dependent actin organization provides positional information to individual cells in a multicellular layer (Aragona et al., 2013; Wada et al., 2011). YAP/TAZ are also central in regulating cell fate decision by interacting with RUNX2 and PPARγ (Hong et al., 2005; Hong and Yaffe, 2006; Varelas et al., 2008). Consistently, neo-expression of α-SMA alone was sufficient in our experiments to reduce hMSC differentiation potential, which was rescued by inhibition and downregulation of YAP1.

Whether the α-SMA-induced nuclear shift of YAP is responsible for reduced clonogenicity is unclear because YAP1 knockdown did not protect against the reduction of SOX2 and OCT4 transcript levels upon α-SMA-overexpression. Consistently, YAP binds to and is expected to activate gene transcription of SOX2 and OCT4 in embryonic stem (ES) cells (Lian et al., 2010). However, YAP is inactivated in ES cells during differentiation (Lian et al., 2010), whereas it drives specific lineage specification in MSCs (Dupont et al., 2011). Overexpression of OCT4 has been shown to result in increased MSC proliferation, whereas OCT4 knockdown had the opposite effect (Tsai et al., 2012). Similarly, knockdown of SOX2 reduces proliferation in MSC-derived osteoprogenitors that is restored by YAP1 overexpression, which acts downstream of SOX2 in this context.
model (Seo et al., 2013). Hence, the interplay between α-SMA, YAP/TAZ, and self-renewal genes is complex and not entirely understood at present.

YAP/TAZ transcription factors provide a direct link between cell mechanosensing and gene regulation (Halder et al., 2012). Inhibition of cell contraction has been shown to decrease nuclear localization of YAP/TAZ and transcription of downstream genes, whereas high intracellular tension drives YAP/TAZ into the nucleus (Calvo et al., 2013; Dupont et al., 2011). Expression of α-SMA is not essential for the localization of YAP/TAZ in the nucleus and ~10% of the hMSC retained predominantly nuclear YAP even in the presence of the SMA-FP. However, incorporation of α-SMA into existing stress fibers substantially increases contractile force and cell stress (Hinz et al., 2001) and thus accentuates fibrogenic transcription programs. YAP/TAZ have been shown to be involved in regulating α-SMA expression and fibrogenesis using an experimental model of epithelial-to-mesenchymal transition (Speight et al., 2013) and during lung fibroblast-to-MF activation by stiff ECM (Liu et al., 2015). These reports and our works suggest a positive feedback loop between α-SMA and YAP/TAZ signaling that amplifies fibrosis.

Importantly, expression of α-SMA is not simply a marker for MF activation, but also the driver of cell function and fate. Increased expression of α-SMA directly reduces the clonal potential of hMSCs and guides their differentiation toward osteoblasts. Hence, α-SMA not only identifies osteoprogenitors in hMSC populations as shown by others before (Grgic et al., 2012; Kalajzic et al., 2008), but may be part of the mechanism driving differentiation. Analysis of bone marrow-derived hMSCs treated with TGF-β1 and/or exposed to fibrosis-stiff culture substrates has previously revealed a fibrogenic MF activation program (Park et al., 2011; Wang et al., 2004). Our results additionally demonstrate that neo-expression of α-SMA associates with reduced clonogenicity and lineage differentiation potential of hMSCs and that this program is reversible. SMA(+) hMSCs can be deactivated to lose fibrotic MF features by reducing substrate stiffness. Originally considered to be a terminal differentiation state of various precursor cells, deactivation of the MF has been shown in recent experimental models of kidney and liver fibrosis (Hecker et al., 2011; Kisseleva and Brenner, 2013). In vitro, the depletion of MF features in fibroblasts and MSCs is achieved by culture on soft silicone substrates (Achterberg et al., 2014; Balestrini et al., 2012; Goffin et al., 2006; Park et al., 2011) or by treatment with anti-fibrotic growth factors (Desai et al., 2014). Our results add to these findings that SMA(+) hMSC populations, deactivated to lose MF features, regain adipogenic lineage differentiation potential rather than turning into α-SMA-negative fibroblasts. Hence, α-SMA-positive cells are likely derived from previously α-SMA-negative hMSCs and expression of α-SMA reversibly reduced their clonogenicity and lineage potential.

Our findings have important implications for hMSC therapy in fibroproliferative diseases, including tumor formation and development of fibrosis. Our in vivo data show that the percentage of α-SMA-expressing MFs in sarcomas correlates with the degree of YAP/TAZ activation. A wide variety of different tumors have been shown to accumulate stromal cells that are positive for α-SMA and perform MF functions, including stiffening of the stroma and promoting tumor progression (Hinz et al., 2012; Öhlund et al., 2014). Consistently, YAP expressed in cancer-associated fibroblasts was recently shown to play an important role in controlling of cytoskeleton-regulating genes, as well as tumor cell invasion and ECM stiffening (Calvo et al., 2013). We propose a feedforward loop of MSC-to-MF activation in the tumor microenvironment, leading to higher contractile cells and stiffer ECM, which both lead to increased YAP/TAZ activity and conversion of regenerative MSCs into fibrotic MFs.

Interrupting this feedforward loop will have important consequences for hMSC potential in clinical applications. First, specific inhibitors of α-SMA such as the SMA-FP or shRNA strategies may be co-delivered with hMSCs to exert fibrosis-inhibitory effects on the resident fibroblastic cell population in the lesion. Second, suppressing MSC-to-MF activation during cell culture expansion will enhance the fraction of valuable stem cells and reduce the risk of fibrosis upon implantation. We have shown that explantation and continued culture on soft culture substrates renders populations of lung fibroblasts resistant to subsequent mechanical activation on stiff substrates over several consecutive passages (Balestrini et al., 2012). This concept of cells acquiring a “mechanical memory” has recently been confirmed for MSC lineage programs on a shorter timescale with YAP/TAZ being involved (Yang et al., 2014). It is yet unclear why hMSCs and other mesenchymal cells that have been cultured on conventional stiff culture dishes are not similarly primed and can at least acutely (up to 8 days in our experiments) lose fibrogenic character upon short-term exposure (5 days) to soft substrates. In our own studies, we found it to be essential to use substrates with a pathophysiological stiffness range (1–100 kPa) and to never expose cells to tissue culture plastic for mechanical priming to occur (Balestrini et al., 2012).

**EXPERIMENTAL PROCEDURES**

For detailed experimental procedures, see the Supplemental Experimental Procedures.

**Tissue, Cell Culture, and Drugs**

MSCs were obtained from the bone marrow of healthy donors (Davies et al., 2001), from umbilical perivascular tissue (Ennis et al., 2008)
and from adipose tissue (Vermette et al., 2007). Tissue sections of mesenchymal tumors and respective healthy control tissues were purchased from Biomax. Cell drugs used in these study are: blebbistatin (50 μM) (Tocris Bioscience, Cedarlane), jasplakinolide (50 nM) (Life Technologies), verteporfin (2 μM) (Sigma-Aldrich), SMA-FP and control SKA-FP (5 μM). Immunofluorescence, microscopy, and western blot were performed as described earlier with antibodies listed in the Supplemental Experimental Procedures (Klingberg et al., 2014). Adipogenesis, osteogenesis, and chondrogenesis were induced and assessed as described earlier (Majd et al., 2011). Elastic silicone culture substrates were purchased from Excellence Biotech SA and activated for cell adhesion by plasma oxygenation (PE-100, Plasma Etch), followed by coating with 2 μg/cm² FN (Millipore). Cell contractility was assessed using FN-coated wrinkling silicone substrates (Balestrini et al., 2012).

**Flow Cytometry and Cell Sorting**

For flow cytometry, fixed cells were immunostained using antibodies CD44-APC-H7, CD73-PE-CY7, CD90-FITC, CD105-PerCP-Cy5.5, CD146-V450, and respective isotype controls (BD Bioscience) and analyzed using a flow cytometer (LSR II, BD). To enrich for α-SMA-positive and α-SMA-negative hMSC populations, cells were trypsinized and sorted for cell size (upper and lower 25% in forward scatter) using fluorescence activated cell sorting (FACS) (FACS Aria III, BD).

**Plasmid Constructs**

α-SMA was overexpressed using α-SMA-GFP (Clément et al., 2005) and knockeddown using 29-mer shRNA targeting α-SMA transcripts in pGFP-V-RS vectors (OriGene Technologies). Human YAP1 27-mer siRNA were ordered from (OriGene Technologies). Cells were transfected using an electroporation device (Neon, Life Technologies).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at [http://dx.doi.org/10.1016/j.stemcr.2015.05.004](http://dx.doi.org/10.1016/j.stemcr.2015.05.004).

**AUTHOR CONTRIBUTIONS**

N.P.T. designed, performed, analyzed, and wrote the experimental data in manuscript format. B.H. supervised, designed, analyzed, and wrote the manuscript. J.E., J.E.D., and A.K. contributed to data analysis and writing of the manuscript.

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Expression of $\alpha$-Smooth Muscle Actin Determines the Fate of Mesenchymal Stromal Cells

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**Figure S1: TGF-β1 activates myofibroblasts (MFs) from different hMSC sources**

hMSCs were obtained from human adipose tissue (‘a’), bone marrow (‘b’), and umbilical cord tissue (‘u’) and cultured in the presence or absence of TGF-β1 (2 ng/ml) for four days. (A) Cells were immunostained for MF markers α-SMA (red) and ED-A fibronectin (green) (scale bar=20 μm) and processed for (B) Western blotting. The (C) percentage of α-SMA stress fiber-positive cells was quantified from immunofluorescence images and (D) percentage of contractile cells on wrinkling silicone substrates was calculated by image analysis. (E) Bone marrow-derived hMSCs were cultured for 7 days in control culture medium (con), medium containing 10 ng/ml TGF-β1 (TGF) and chondrogenic medium (C.M.). Cells were extracted and lysates used for qRT-PCR to detect the chondrogenic markers SOX9 and aggrecan (ACAN) and α-SMA (ACTA2) Graphs show mean ± standard deviation from at least five independent experiments (*p≤0.05, **p≤0.005 and ***p≤0.0005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).
Figure S2: MF activation of hMSCs affects ‘MSC marker’ expression.

hMSCs were cultured in control and TGF-β1 (2 ng/ml) treated condition for four days to assess CD markers by flow cytometry. Unstained (red), control (blue), and TGF-β1-treated (green) plots are summarized for each CD marker. (B) Median fluorescence intensity was assessed for each population via FlowJo, and graphed. Graphs show mean ± standard deviation from at least five independent experiments (*p ≤ 0.05, **p ≤ 0.005 and ***p ≤ 0.0005 using Student t-test).
Figure S3: SMA(+) hMSCs lose the MF phenotype upon soft substrate culture.

A) Sorted α-SMA(+) hMSCs were cultured on fibrosis-rigid (65 kPa), wound-stiff (26 kPa) and normal-tissue-soft (3 kPa) silicone substrates for four and assessed for MF phenotype by B) immunofluorescence analysis of α-SMA (red) and supermature focal adhesions (vinculin, red) and C) Western blotting. D) The graph represents area of focal adhesions measured across different stiffness. Scale bar= 20 μm. Graphs shows focal adhesion quantification from atleast 100 cells on each stiffness (*p ≤ 0.05, **p ≤ 0.005 and ***p ≤ 0.0005 using ANOVA followed by a post-hoc Tukey’s multiple comparison test).
**Figure S4: Effect of culture time and activation intensity on MF deactivation.**

(A) Sorted α-SMA(+) hMSCs, pre-activated on conventional plastic culture substrates, were transferred to stiff and soft culture substrates. Cells were assessed by Western blotting after 1, 5, and 8 d for α-SMA expression, normalized to loading control vimentin. (B) MF activation of hMSCs was either mechanically stimulated by growth on conventional plastic culture substrates or mechanically/chemically by additional stimulation with 2 ng/ml TGF-β1 for 5 d. SMA(+) hMSC were then sorted as described for Fig. 2 and plated for another 5 d on stiff substrates, soft substrates, and soft substrates+TGF-β1 before assessing expression of α-SMA and vimentin (loading control) by Western blotting.
Figure S5: Correlating nuclear YAP with α-SMA expression and targeting components of actin-myosin stress fibers affects α-SMA localization.

(A) Heterogeneous populations of hMSCs were cultured on conventional plastic culture substrates for 5 days and immunostained for α-SMA (red), YAP (green), and nuclei (blue). Average intensities were measured for cytoplasmic YAP, nuclear YAP, and α-SMA per cell using image analysis and correlated in a scatter plot. Data are shown for 3 independent experiments and 10 cells were analyzed per experiment. Lines indicate threshold levels for ‘α-SMA-positive cell’ and a nuclear/cytoplasmic ratio of 1. (B) α-SMA(+) hMSCs were treated with DMSO control, blebbistatin, α-SMA-FP and α-SKA-FP non-targeting control peptide for 40 min and assessed for α-SMA expression (red) in immunofluorescence. Scale bars= 50 μm.
Supplementary Experimental Procedures

**Ethics statement**

The use of human bone marrow, umbilical cord perivascular, and adipose tissue MSCs were approved by the institutional review boards of the University of Toronto, McMaster University and Laval University, respectively. In each case, informed written consent was obtained from patients for use of the cells/tissue for scientific research.

**Tissue, cell culture, and drugs**

Bone marrow cells were harvested from healthy donors according to Davies et al. (Davies et al., 2001), from umbilical perivascular tissue according to Ennis et al. (Ennis et al., 2008) and from adipose tissue as described previously (Vermette et al., 2007). All hMSCs were expanded in α-MEM with supplemented with ribonucleosides and deoxyribonucleosides (Life Technologies, Burlington, Canada), 10% fetal bovine serum (Sigma-Aldrich, Oakville, Canada), and 1% penicillin/streptomycin (Life Technologies). Only cells between P1 to P3 were used for experiments. Tissue sections of human soft mesenchymal tumors (adiposarcoma/liposarcomas, T243), stiff mesenchymal tumors (osteosarcoma, T261) and respective healthy control tissues were purchased (Biomax, Rockville, MD) and immunostained as indicated. Actin-myosin contraction was blocked using 50 μM blebbistatin (Tocris Bioscience, Cedarlane Corp., Burlington, Canada). F-actin was stabilized with 50 nM jasplakinolide (Life Technologies). Fusion peptides containing a membrane-penetrating vector and the membrane-penetrating-terminal sequence AcEEED (SMA-FP) or the α-skeletal actin specific control sequence AcDEDE (SKA-FP) were kind gifts from Drs. Giulio Gabbiani and Christine Chaponnier (University of Geneva, Geneva, Switzerland) (Hinz et al., 2002). YAP activity in the nucleus was inhibited by adding verteporfin (2 μM) (Sigma-Aldrich, Oakville, Canada) for 5 days to the culture medium (Liu-Chittenden et al., 2012).

**Elastic cell culture substrates**

Deformable polydimethylsiloxane (PDMS) silicone (Dow Corning, Midland, MI) substrates were prepared with varying stiffness to mimic the elastic range of normal connective tissue (3 kPa), the stiffer immature scar of healing wounds fibrotic (26 kPa) dermis and rigid fibrotic tissue (65 kPa) as previously described (Achterberg et al., 2014; Balestrini et al., 2012; Goffin et al., 2006). Soft silicone substrates became commercially available (Excellness Biotech SA, Lausanne, Switzerland) during the course of this study and were used with identical results. To activate the elastomer surface for cell adhesion, surfaces were treated with vacuum plasma oxygenation (PE-100, Plasma Etch, Inc., Carson City, NV) for 25 sec followed by overnight coating with 2 μg/cm² human fibronectin (Millipore, Billerica, MA).

**Flow cytometry and cell sorting**

Cells were trypsinized using 0.05% trypsin and washed 3-times using phosphate buffered saline (PBS) without Ca²⁺/Mg²⁺, followed by fixation with 3% paraformaldehyde (PFA) on ice. The cells were then washed in PBS and incubated for 1h with fluorophore-conjugated antibodies directed against hMSC surface proteins, followed by 3-times washing.
in Hank’s Balanced Salt Solution (HBSS), 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), and 2% FBS. Antibodies were directed against CD44-APC-H7 (mouse IgG2b, 560532; BD), CD73-PE-CY7 (mouse IgG1, 561258; BD), CD90-FITC (mouse IgG1, 555595; BD), CD105-PerCP-Cy5.5 (mouse IgG1, 560819; BD) and CD166-PE (mouse IgG1, 559263; BD) (BD Bioscience, Burlington, Canada). BD™ Compensation beads, anti-Mouse Igκ and negative controls were utilized in compensation of above-mentioned antibodies. For α-SMA staining, suspended cells were fixed as above, permeabilized using 0.2% TX-100 for 10 min and incubated with antibodies against α-SMA (mouse IgG2a, clone αSM-1, a kind gift of Giulio Gabbiani, University of Geneva, Switzerland), followed by incubation with goat anti-mouse IgG Alexa Fluor 568 secondary antibodies (Life Technologies). Flow analysis was done using a flow cytometer (LSR II, BD).

To enrich for α-SMA-positive and α-SMA-negative hMSC populations, cells were trypsinized and suspended in 1x HBSS/2% FBS/20 mM HEPES. The cells were then analyzed and gated for larger cells size (upper 25% of forward scatter) using fluorescence activated cell sorting (FACS) (FACSAria™ III, BD), with 20 psi and 100 µM nozzle at 37°C and intermittent shaking conditions. the lower 25% in the forward scatter channel were collected as small cell fraction. Sorted small and large cells were tested for α-SMA levels by flow cytometry, Western blotting and qRT-PCR analysis for sorting stringency.

**Immunofluorescence, microscopy, and Western blotting**

For immunofluorescence, cell samples were fixed with 3% PFA for 10 min, washed with PBS and then permeabilized with 0.2% Triton X-100 for 30 min. We applied primary antibodies directed against α-SMA (mouse IgG2a, clone αSM-1), vinculin (rabbit IgG, ab11194, Abcam, Cambridge, MA), EGFP (rabbit IgG, ab290; Abcam), ED-A fibronectin (mouse IgG1, sc-59826; Santa Cruz Biotechnology, Dallas, TX), vimentin (mouse IgG1, M0725, Dako Canada Inc., Burlington, Canada), and YAP (rabbit IgG sc-101199, Santa Cruz Biotechnology, Inc.) for 1h in 0.02% Triton X-100 buffer, followed by 3-times washing. Secondary antibodies were goat anti-mouse IgG Alexa Fluor 568 (A-11004, Life Technologies), goat anti-mouse IgG1 FITC (1070-02, Southern Biotechnology), goat anti-mouse IgG2a TRITC (1080-03, Southern Biotechnology) and goat anti-rabbit-TRITC and -FITC (F9887, Sigma-Aldrich). To stain filamentous actin (F-actin) and nuclear DNA, phalloidin Alexa Fluor 488 and 568 (A-12379 and A-22287, Life Technologies) and 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (D9542, Sigma-Aldrich) were used, respectively. Secondary antibodies were incubated for 1h in 0.02% Triton X-100 PBS buffer, followed by 3-times washing and mounting in by polyvinyl alcohol (341584, Sigma-Aldrich). For immunohistochemistry staining of normal and sarcoma tissue samples, paraffin-embedded sections were deparaffinized in xylene, rehydrated in ethanol, and rinsed in distilled water, as described previously (Sarrazy et al., 2014). Antigens were revealed by boiling the slides in sodium citrate buffer (Dako, Burlington, ON) at 95-100 C for 40 min. After cooling to room temperature, sections were rinsed in PBS, blocked with 2% goat serum/1% BSA for 40 min and stained with primary and secondary antibodies applied sequentially for 1 h at room temperature.

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For Western blotting, proteins were isolated by scraping cultured cells from cell culture substrates in standard sample buffer. Proteins were equally loaded based on Protein assay kit, (PI-23221, Invitrogen) and run on 10% SDS-PAGE gel (200 mAmperes, 150 V, 90 minutes), under reducing conditions, followed by wet transfer (500 mAmperes, 100 V, 60 minutes) onto nitrocellulose membranes as described previously (Sarrazy et al., 2014). Membranes were blocked with 5% non-fat skim milk for 1 h, followed by incubation with primary antibodies. After washing with Tris-buffered saline (TBS)/0.1% Triton-X100, primary antibodies were detected using anti-mouse-680 nm fluorescently labeled secondary antibodies (Odyssey, LiCor Biosciences, Lincoln, NE), and detected using a digital imaging system (Odyssey, LiCor).

Multilineage differentiation and analysis of hMSCs

The multilineage potential of hMSCs was analyzed by differentiating cells into MFs, adipocytes and osteoblasts in accordance with established protocols (Pittenger et al., 1999). Briefly, adipogenesis was induced by plating cells at 25,000 cells/cm² in growth medium containing 100 μM indomethacin, 1 μM dexamethasone and 0.5 mM isobutyl-methylxanthine for 14 days. Osteogenesis was induced by plating cells at 25,000 cells/cm² in growth medium containing 100 μM ascorbate-2-phosphate, 10 nM dexamethasone, and 10 mM β-glycerophosphate (all Sigma-Aldrich) for 21 days. Chondrogenesis was induced by pelleting 50,000 hMSCs at 1500 RPM for 5 min and stimulating the cell pellet in growth medium for 7 days containing 10 μg/ml TGF-β3, 100 μM ascorbate-2-phosphate, 1% ITS+ supplement (Insulin-Transferrin-Selenium supplements (625 mg/ml insulin, 625 mg/ml transferrin, 625 ng/ml selenious acid) and 10nM dexamethasone (all Sigma-Aldrich).

MF activation was induced by culturing cells at 2,500 cells/cm² in the presence of TGF-β1 (2 ng/ml; R&D Systems, Cedarlane Corp.) for 4 days. Adipogenic differentiation was assessed by incubating 3% PFA fixed hMSCs using with Oil red O that stains lipid droplets (Sigma-Aldrich). Osteogenesis was assessed by incubating fixed hMSCs with freshly prepared 0.6% NH₄⁺-buffered Alizarin Red S solution that stains mineralized nodes (Sigma-Aldrich). Chondrogenesis was tested by assessing mRNA expression of ACAN and SOX9. The clonal character of hMSCs was assessed by plating 25 cells/cm² for 10 days and counting the number of crystal violet stained colonies (>16 cells).

Microscopy and image analysis

Immunofluorescence images were taken using an upright microscope (Axiolmager M2, Carl Zeiss, Oberkochem, Germany) equipped with a charge-coupled device digital camera (AxioCam HRm, Carl Zeiss) and structured illumination (Apotome2, Carl Zeiss), and image acquisition software (Zen SP1, Carl Zeiss). All figures were assembled using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, http://www.adobe.com). To quantify histological stains (Oil Red O and Alizarin Red S), 10 random images from three independent experiments were acquired and quantified on the basis of positive staining. To measure adipogenesis, the percentage of Oil Red O positive cells was calculated based on positive Oil red O staining and total number of cells quantified from images. To quantify osteogenesis,
images were thresholded to select and quantify Alizarin Red S positive area using ImageJ. The ratio between positively stained area and total image area was the converted into percentage. For quantification of YAP/TAZ images, the number of cells demonstrating (YAP_{nucleus}>YAP_{cytosol}) was related to total number of cells and expressed as percentage. To define α-SMA-positive MFs in immunofluorescence images, the intensity of α-SMA expressed in stress fibers was first measured in cells grown on culture plastic + TGF-β1 treatment, which represents maximal MF induction. The threshold for ‘α-SMA-positive’ was then set to at least 50% of the intensity measured in the positive controls run together with every experiment (Supplementary Figure S5a).

**Cell contraction assay**

Cell contractility was assessed using deformable (wrinkling) silicone substrates that were coated with plasma fibronectin (10 μg/ml) as previously described (Balestrini et al., 2012). Cells were plated for 4 h at 2,000 cells/cm² on wrinkling substrates and 10 random images were taken using a 20x objective mounted on Zeiss PrimoVert inverted microscope (Carl Zeiss Microscopy). Wrinkling was quantified using ImageJ software as previously described (Balestrini et al., 2012). Briefly, 8-bit monochrome images were thresholded for phase-bright wrinkles and converted into binary images applying the threshold. After excluding circular shapes, the area covered by the remaining highlighted structures was quantified using the ‘analyze particle’ function. Values were then normalized to cell numbers present in that image field. Contraction values obtained from this procedure depend on the percentage of cells producing wrinkles and intensity of wrinkles. For select experiments, the percentage of cells forming substrate wrinkles was manually quantified without considering wrinkle intensity.

**Statistical analysis**

Every experiment was performed at least three times. Data are presented as means ± standard deviation (SD). We assessed differences between groups with an analysis of variance (ANOVA) followed by a post-hoc Tukey’s multiple comparison tests and paired, two tailed, Student t-test as indicated. Statistics were performed and graphs produced using Graphpad Prism software.

**Plasmid constructs and qRT-PCR**

To overexpress α-SMA, we transfected hMSCs with α-SMA-GFP (Clement et al., 2005). To knock-down α-SMA expression, 29mer shRNA targeting α-SMA transcripts were obtained in pGFP-V-RS vectors (OriGene Technologies, Inc., Rockville, MD). Human YAP1 27mer siRNA were ordered from (OriGene Technologies). All constructs were transfected using an electroporation device (Neon®, Life Technologies) with settings: 1150 V, 20 ms and 2 pulses. GFP-positive cells (α-SMA-GFP, GFP, shRNA plasmid with GFP reporter) were sorted via FACS (FACS™ Aria™ III, BD) 2 days after transfection to 100% expression of transfected construct. For qRT-PCR experiments, cells were first washed with PBS for 3-times. Total RNA was isolated using RNeasy mini kit (Qiagen, Toronto, Canada), following manufacturer’s instructions, and quantified using Nanodrop 1000 (Thermo Fischer Scientific, Burlington, Talele et al., Supplementary Material, p. 10
Canada). cDNA synthesis was performed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Burlington, Canada) as per manufacturer’s instruction. qRT-PCR was performed as a two-step reaction using RT² SYBR® Green qPCR Mastermix (Qiagen) in SteponePlus Real-Time PCR system (Life Technologies). RN18S was used as housekeeping gene in all experiments. All the primers are tabulated. The qRT-PCR reaction was controlled as well as analyzed using system-provided StepOne software, and the results were exported to Graphpad Prism (Graphpad, La Jolla, CA) for analysis.

| Refseq   | Gene Symbol | Orientation | Sequence               |
|----------|-------------|-------------|------------------------|
| NM_001135 | ACAN        | FORWARD     | CCTTGGAGGTCGTGGTGAAAGG |
| NM_001135 | ACAN        | REVERSE     | AGGTGAACTTTCTCTGGCGACGT |
| NM_001141945 | ACTA2  | FORWARD     | CTTTCACAATGAGCTTTCGTT |
| NM_001141945 | ACTA2  | REVERSE     | ATTTGAGTATTTTCTCAGGC |
| NM_000088  | COL1A1      | FORWARD     | CAGGCTGGTGTGAGGGTACG |
| NM_000088  | COL1A1      | REVERSE     | CTCCATCTTTGCCAGCAGG   |
| NM_000090  | COL3A1      | FORWARD     | GAAAGGTAAGGGGAGGCCCT  |
| NM_000090  | COL3A1      | REVERSE     | GCCACGTTACCTTTGACAC   |
| NM_001901  | CTGF        | FORWARD     | CTTCTGTGACTCTGGATCT   |
| NM_001901  | CTGF        | REVERSE     | CTGGTACTTGCAGCTGCTCT  |
| NM_001130823 | DNMT1  | FORWARD     | CGTAAAGAAGGATTATCCGG  |
| NM_001130823 | DNMT1  | REVERSE     | GTTTTCTAGACGTCCATTCA  |
| NM_138712  | PPARG       | FORWARD     | GACTTCTCCAGCATTTTCTA  |
| NM_138712  | PPARG       | REVERSE     | TCCACTTGGATATTGCACTTT |
| NM_001173531 | OCT4   | FORWARD     | GACAGGGGAGGGAGAGGAG   |
| NM_001173531 | OCT4   | REVERSE     | CTTCCTCCACACAGTGGG    |
| NM_001015051 | RUNX2  | FORWARD     | AAGCTTGATGACTCTAAACC  |
| NM_001015051 | RUNX2  | REVERSE     | TCTGTAATCTGACTCTGTTCC |
| NM_003106  | SOX2        | FORWARD     | GGGAAATGGGAGGGGTCAAG  |
| NM_003106  | SOX2        | REVERSE     | TTGGGTGAGTGTGGATGGGA  |
| NM_000346  | SOX9        | FORWARD     | TACATGAAGATGCACGGACA  |
| NM_000346  | SOX9        | REVERSE     | CACACCATGAAGGCGTTTCC  |
| NM_001135599 | TGFBI   | FORWARD     | GCAGCTAATGACGACAGCA   |
| NM_001135599 | TGFBI   | REVERSE     | TGCAGCAAGAGCACTGAAG   |
| NR_003286  | RNA18S      | FORWARD     | ATCCGGGAGTGGCAATTATTC |
| NR_003286  | RNA18S      | REVERSE     | TCTCAGTAAACCATCAGAAT  |
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