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Biochemical and biomechanical regulation of the myofibroblast phenotype

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α II-SPECTRIN AND β II-SPECTRIN DO NOT AFFECT FIBROBLAST MECHANOSENSING AND TGF β 1-INDUCED MYOFIBROBLAST DIFFERENTIATION

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Submitted for publication

ABSTRACT

Mechanosensing of fibroblasts plays an important role in the development of fibrosis. So far, no effective treatments are available to treat this devastating disorder. Spectrins regulate cell morphology and are potential mechanosensors in a variety of non-erythroid cells, but hardly anything is known about the role of spectrins in fibroblasts. We investigated whether α - and β -spectrins are required for the phenotypic properties of adult human dermal (myo)fibroblasts. Knockdown of α - or β -spectrin in fibroblasts did not affect cell adhesion, cell size and YAP nuclear/cytosolic localization. We further investigated whether α - and β -spectrins play a role in the phenotypical switch from fibroblasts to myofibroblasts under the influence of the pro-fibrotic cytokine TGF β 1. Knockdown of spectrins did not affect myofibroblast formation, nor did we observe changes in the organization of α SMA stress fibers. Focal adhesion assembly was unaffected by spectrin deficiency, as was collagen type I mRNA expression and protein deposition. Wound closure was unaffected as well, showing that important functional properties of myofibroblasts are unchanged without α - or β -spectrin. In fact, fibroblasts stimulated with TGF β 1 resulted in significantly lower endogenous mRNA levels of α - and β -spectrin. Taken together, despite the diverse roles of spectrins in a variety of other cells, they do not seem to be suitable candidates to interfere in fibrotic processes.

INTRODUCTION

Chronic organ injury often results in the development of fibrosis: an excessive production, post-translational modification, and stiffening of extracellular matrix (ECM) components¹. Pathological stiffening of the ECM creates a pro-fibrotic feedback loop², but how mechanical cues are transduced to change cell function and fate, remain incompletely understood. Driving the fibrotic response are activated fibroblasts or pericytes that acquire the myofibroblast phenotype, which is characterized by a well-developed endoplasmic reticulum and an extensive contractile actomyosin cytoskeleton³. Decades of research have been devoted to the contractile apparatus in the regulation of the myofibroblast phenotype. More recently, structural proteins belonging to the spectrin family were found to act as functional adaptors between the actomyosin cytoskeleton and the plasma membrane, and are thought to regulate transduction of mechanical signals^{4,5}.

Spectrins form a major component of the cytoskeleton at the membrane-cytoskeleton interface^{6,7}, and play an important role in maintaining cellular integrity⁸. Spectrins form tetrameric flexible heterodimers, which contain two alpha and two beta subunits^{9,10} and have been evolutionarily conserved in species as different as echinoderms¹¹, Sphophora^{6,12,13}, birds¹⁴, and humans¹⁵⁻¹⁷. They were first discovered in metazoan erythrocytes where they support the membrane cytoskeleton^{6,8}. In erythrocytes two different spectrin genes are found, *SPTA1* (α -spectrin) and *SPTB1* (β -spectrin). Both subtypes are uniquely expressed in erythrocytes and thus not found in other cell types¹⁴. More recently, other spectrin proteins were identified in non-erythrocyte cells. *SPTAN1* encodes several isoforms of the non-erythrocyte all-spectrin polypeptide that are generated through alternative splicing. In addition, non-erythrocyte β -spectrins are encoded by four similar genes: *SPTBN1* (β II-spectrin), *SPTBN2* (β III-spectrin), *SPTBN4* (β IV-spectrin) and *SPTBN5* (bV-spectrin (β Heavy)). Here, we focus on all-spectrin and β II-spectrin, since they have been reported to provide mechanical stability and maintaining cell integrity, plasma membrane stability and morphology—key features of cellular mechanosensing^{5,18-20}. Furthermore, all-spectrin and β II-spectrin regulate cell adhesion²⁰ and cell spreading^{5,18,21} and contain domains which function in protein sorting, vesicle trafficking and endocytosis^{18,22,23}.

The functional domain in the all-spectrin subunit is the highly conserved Src Homology 3 (SH3) domain²⁴, which initiates Rac activation during cell adhesion and spreading¹⁸. In addition, all-spectrin contains a calmodulin binding site^{13,15}, which might be involved in cell contraction and migration. Furthermore, all-spectrin is reported to be involved in regulation of actin dynamics¹⁸ and β II-spectrin is involved in TGF β 1 signaling, where it functions as a Smad adaptor protein²⁵⁻²⁷. Additionally, spectrins associate with, as well as regulate, Yes-associated protein 1 (YAP)^{28,29}. YAP acts as a transcriptional regulator of genes involved in proliferation and suppression of apoptotic genes and is regulated by both Hippo and TGF β 1 signaling^{30,31}. YAP is a phosphoprotein involved in mechanotransduction³²⁻³⁴. Whether spectrins play a role in the myofibroblast

phenotypical switch remains unknown. Here we studied the role of all-spectrin and β II-spectrin in stiffness-induced cell spreading and adhesion, YAP translocation and wound closure with human dermal fibroblasts. Furthermore, we examined the role of all-spectrin and β II-spectrin in TGF β 1-induced myofibroblast differentiation.

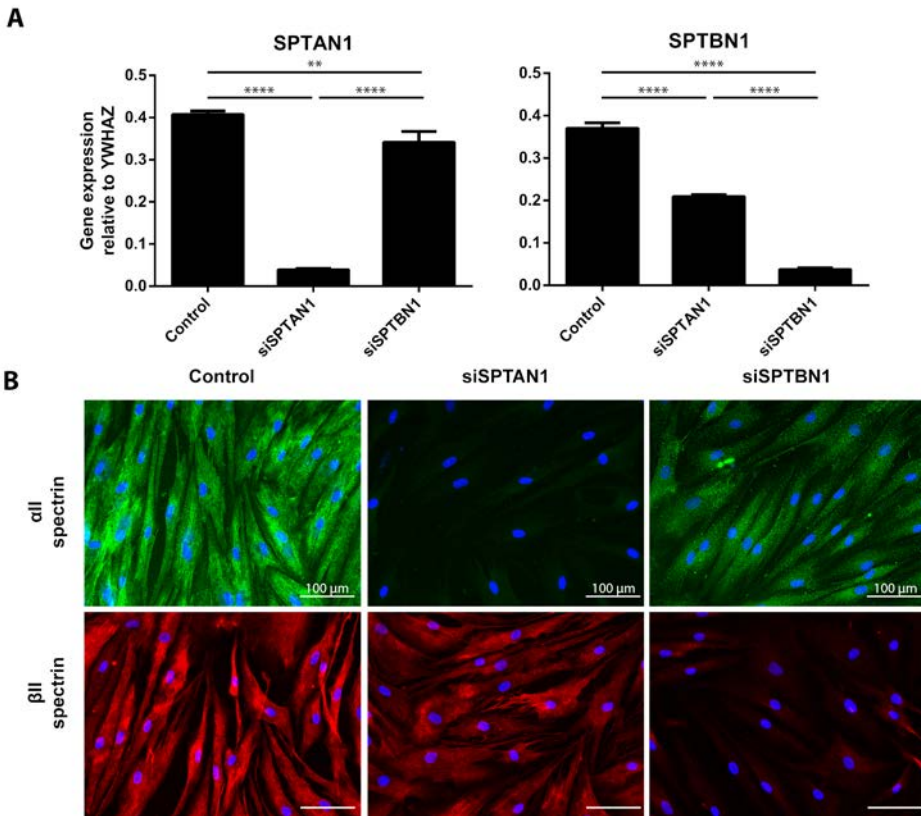


Figure 1. all-spectrin and β II-spectrin knockdown with esiRNA. (A) mRNA expression of all-spectrin (*SPTAN1*) and β II-spectrin (*SPTBN1*) 7 days after esiRNA transfection. One-way ANOVA; ** $p < 0.01$, **** $p < 0.0001$ (B) Representative immunofluorescent images of all-spectrin and β II-spectrin 7 days after esiRNA transfection. Original magnification 200 \times .

MATERIALS & METHODS

Reagents and antibodies

Reagents were as follows: human plasma fibronectin (20 $\mu\text{g}/\text{mL}$, F1056; Sigma-Aldrich, Munich, Germany), human recombinant TGF β 1 (10 ng/mL, 100-21C; Peprotech, London, UK), all-spectrin siRNA (25 ng/cm 2 , EHU093741, Sigma-Aldrich), β II-spectrin siRNA (25 ng/cm 2 , EHU081451, Sigma-Aldrich), Renilla luciferase siRNA (25 ng/cm 2 , EHURLUC; Sigma-Aldrich), Alexa647 labeled-streptavidin (8 $\mu\text{g}/\text{mL}$, S32357; Thermo Fisher Scientific, Landsmeer, the Netherlands), TRITC labeled-Phalloidin (100 nM, P1951; Sigma-Aldrich). Antibodies used: mouse anti-all-spectrin (2 $\mu\text{g}/\text{mL}$, sc-376849; Santa Cruz Biotechnology, Dallas, USA), mouse anti- β II-spectrin (2 $\mu\text{g}/\text{mL}$, sc-376487; Santa Cruz), mouse anti- α SMA (0,28 $\mu\text{g}/\text{mL}$, M0851; DAKO, Glostrup, Denmark), mouse anti-collagen type I (1 $\mu\text{g}/\text{mL}$, ab90395;

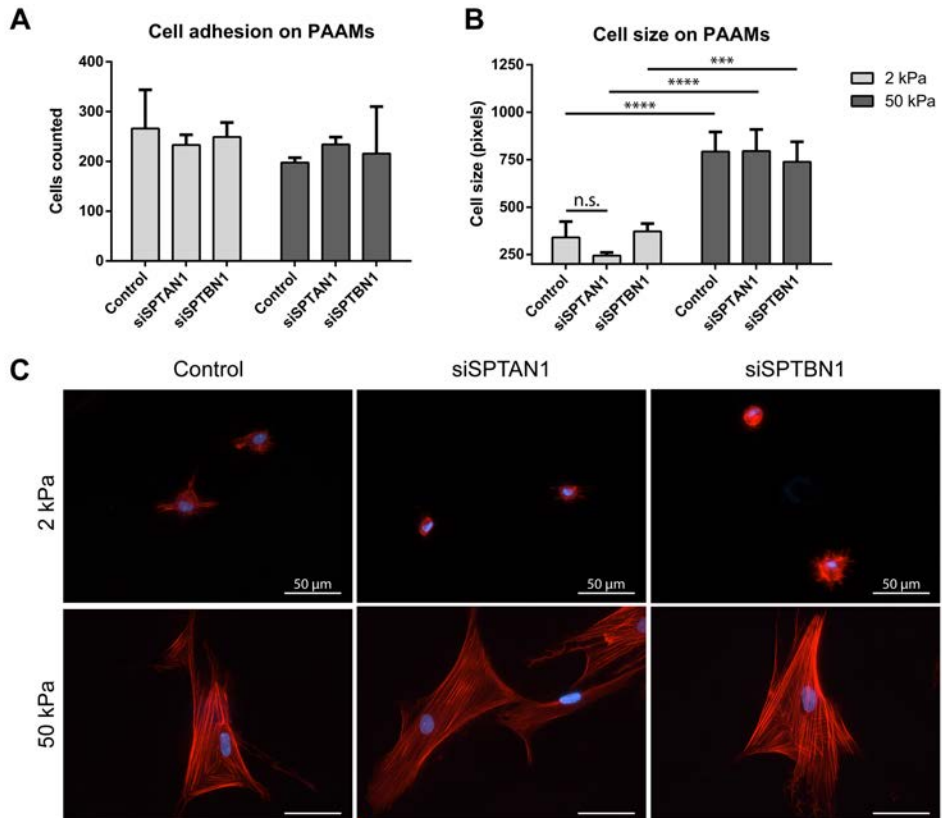


Figure 2. all- and β II-spectrin do not mediate fibroblast spreading and adhesion. (A) Cell adhesion on 2 kPa and 50 kPa polyacrylamide hydrogels. (B) Effect of hydrogel stiffness on cell morphology and cell spreading. Two-way ANOVA; *** $p < 0.001$, **** $p < 0.0001$ (C) F-actin (phalloidin) and nucleus (DAPI) staining to visualize cell size and cell adhesion. Original magnification 400 \times . DAPI, 4',6-diamidino-2-phenylindole; kPa, Kilo Pascal; PAAM, polyacrylamide.

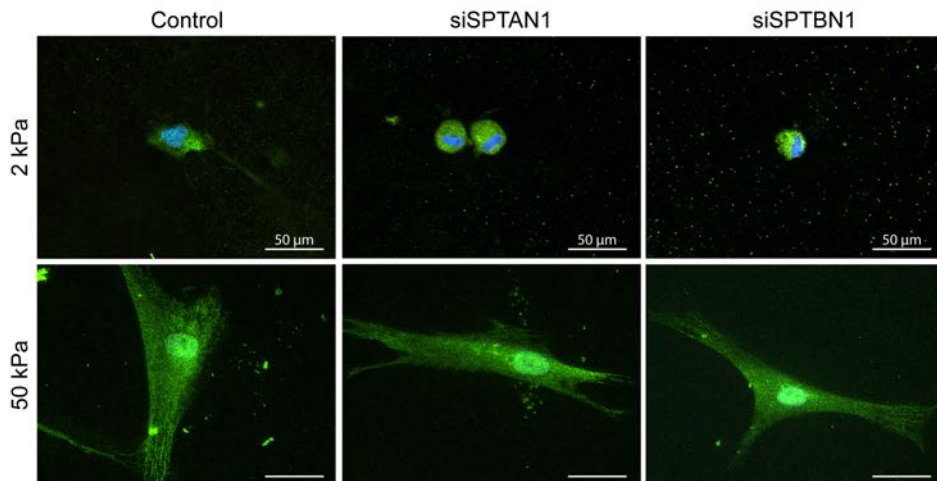


Figure 3. YAP nuclear accumulation is independent from α - and β -II-spectrin. Yes-associated protein 1 (YAP; green) translocation in spectrin KD fibroblasts cultured on either 2 kPa or 50 kPa polyacrylamide hydrogels. Nuclei are stained with DAPI. Original magnification 400 \times . DAPI, 4',6-diamidino-2-phenylindole; kPa, Kilo Pascal.

Abcam, Cambridge, UK), mouse anti-vinculin (9,3 μ g/mL, V9131; Sigma-Aldrich).

Cell manipulations

Before the onset of experiments, normal adult human dermal fibroblasts (CC-2511, nHDF-Ad-Der; Lonza, Basel, Switzerland) were propagated in Dulbecco's modified Eagle medium (DMEM, 12-604F; Lonza) supplemented with 2 mM L-glutamine, 50 U/L penicillin/streptomycin, and 10% FCS. For protein knockdown experiments, cells were seeded at 15.000 cells/cm² and transfected with siRNA using Lipofectamine RNAiMax reagent (Thermo Fischer Scientific) and incubated for 72 h in DMEM supplemented with 1.5 mM L-glutamine, 38 U/L penicillin/streptomycin, and 7.5% FCS. siRNA targeting Renilla luciferase was used as negative control. After the transfection period, cells were cultured for an additional 96 h in DMEM containing 0.5% FCS supplemented with 2 mM L-glutamine and 50 U/L penicillin/streptomycin to ensure elimination of the spectrin proteins, as they are relatively long-lived proteins. Efficiency of knockdown was subsequently determined by means of qPCR and immunofluorescence. For cell adhesion, cell spreading and YAP translocation studies, cells were reseeded on fibronectin-functionalized polyacrylamide gels for 24 h. Cell spreading was determined by measuring cell surface area with Nuance FX software (Perkin Elmer, Groningen, the Netherlands). Cell adherence was determined by quantifying the amount of cells in 25 FOVs. YAP translocation was measured by means of immunofluorescence.

For myofibroblast differentiation experiments and the wound healing assay, the trypsinized cells were reseeded on polystyrene culture wells (for mRNA measurements or wound healing) or slides (for immunostaining), and cultured

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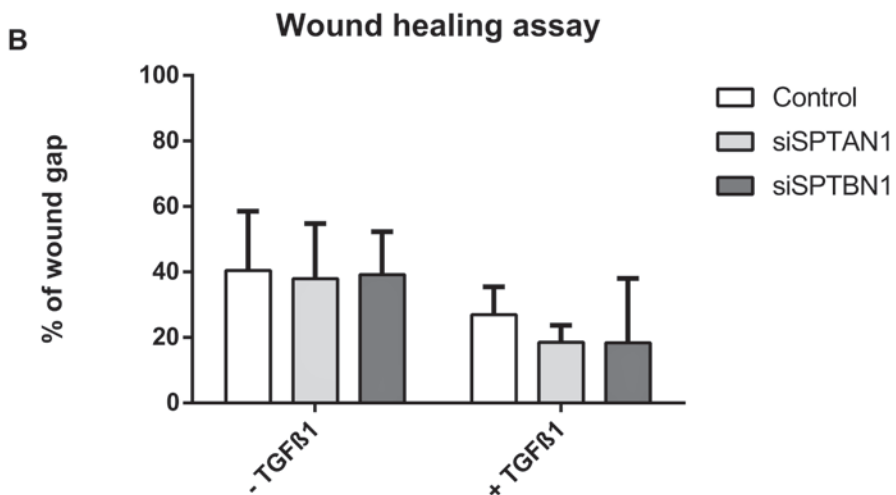
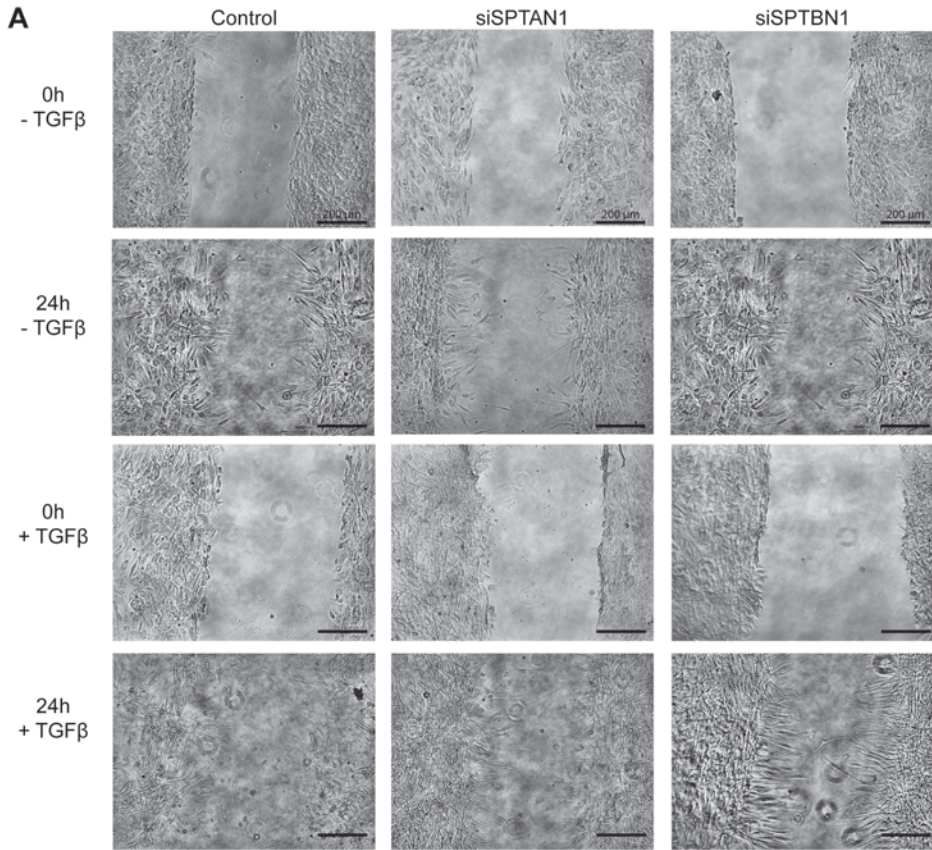


Figure 4. α II- and β II-spectrin do not influence wound gap closure. IBIDI Wound healing assay. Cells seeded at high density were left to repopulate the wound gap for 24 hours in presence or absence of TGF β 1 stimulation. Original magnification 100x. TGF, transforming growth factor.

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in DMEM containing 0.5% FCS, 2 mM L-glutamine, 50 U/L penicillin/streptomycin and 0.17 mmol/L ascorbic acid (A8960; Sigma-Aldrich), and supplemented with or without TGF β 1 (10 ng/mL) for 72 h. For the wound healing assay, IBIDI inserts were removed after 48 h, leaving another 24 h for the cells to repopulate the wound area.

Fibronectin-functionalized polyacrylamide hydrogels

To determine the role of spectrins in cell adhesion and spreading, cells were seeded on fibronectin-functionalized polyacrylamide hydrogels with an elastic modulus of either 2 or 50 kPa. Polyacrylamide hydrogels were prepared as described previously³⁵. In brief, gels were prepared between a chemically modified glass plate and coverslip. The glass plate was cleaned by immersion in 99.9% ethanol for 15 minutes and treated with dichlorodimethylsilane to avoid polyacrylamide interactions. Glass coverslips were treated with 0.5% trimethoxypropylmethacrylate in 99.1% ethanol, which was activated using 0.3% glacial acetic acid to facilitate covalent adhesion of polyacrylamide hydrogels. Differences in stiffness (elastic modulus) were obtained by varying the ratio between acrylamide and bisacrylamide and the Young's modulus was validated by means of Atomic Force Microscopy (AFM). Hydrogel polymerization was initiated with TEMED and APS. To functionalize the surface of the hydrogels they were overlaid with 2 mg/ml L-DOPA (in 10 mM Tris), and incubated for 30 minutes. Next, L-DOPA was washed off and hydrogels were functionalized with 20 μ g/mL plasma fibronectin for 2 h at 37°C.

Table 1. Primer sequences

Gene name	Forward primer	Reverse Primer
ACTA	CTGTTCCAGCCATCCTTCAT	TCATGATGCTGTTGTAGGTGGT
COL1A1	GCCTCAAGGTATTGCTGGAC	ACCTTGTTGCCAGGTTAC
SPTAN1	AAGAAGCACGAAGACTTTGAGAA	TGGTTGCAAATTCATCTAATGC
SPTBN1	CCCAGCAGGACAACTCAAC	GGCATCCTTCTCCTGTCAA

RNA isolation, cDNA synthesis and qRT-PCR

To obtain total RNA, the FavorPrep Tissue Total RNA Purification Mini Kit (FATRK; Favorgen Biotech Corp., Taiwan) was used in accordance to the manufacturer's protocol. RNA concentration and purity were determined by UV spectrophotometry (NanoDrop Technologies, Wilmington, NC, USA). To assess gene expression, the RNA was reverse transcribed using the First Strand cDNA synthesis kit (Thermo Fisher Scientific) using random hexamer primers in accordance to the manufacturer's instructions. Gene expression quantification was performed using qRT-PCR analysis and SYBR Green Supermix (Roche, Basel, Switzerland). The thermal cycling conditions were 2 minutes at 95°C (enzyme activation), followed by 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C (40 cycles). All qPCRs were performed with a ViiA™ 7 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Melting curve analysis was performed to verify the absence of primer dimers. Analysis of the data was performed using ViiA 7™ Real-Time PCR System

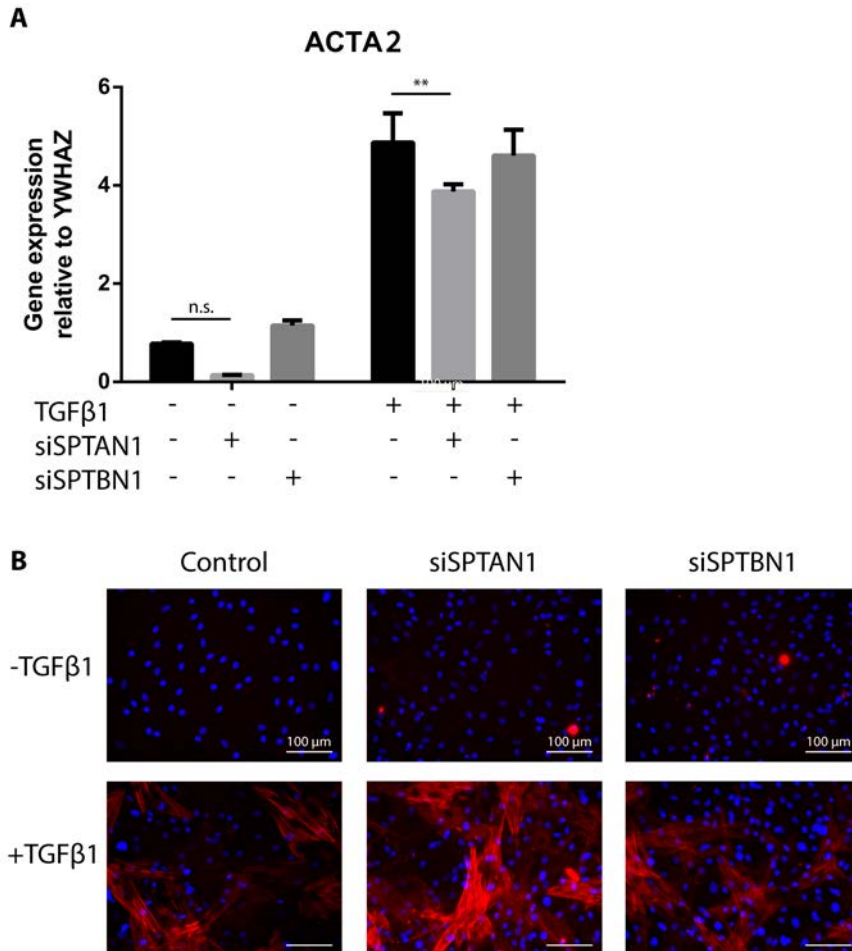


Figure 5. Spectrins do not regulate αSMA stress fiber formation. (A) mRNA expression of *ACTA2* (αSMA) after 4 days of stimulation with TGFβ1 on all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) KD cells. Two-way ANOVA; ** $p < 0.01$. (B) Representative immunofluorescent images of a smooth muscle actin stress fiber formation on all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) KD cells. Nuclei are stained with DAPI. Original magnification 200×. αSMA, a smooth muscle actin; DAPI, 4',6'-diamidino-2-phenylindole; TGF, transforming growth factor.

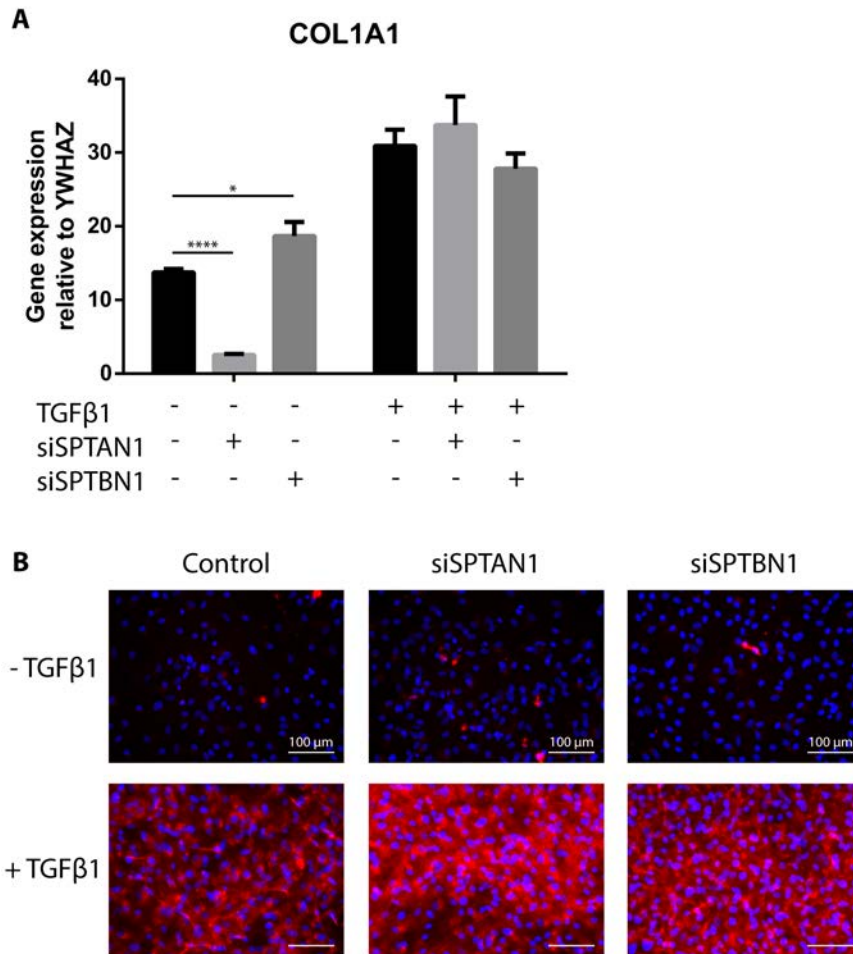


Figure 6. spectrins do not regulate collagen type I synthesis (A) mRNA expression of *COL1A1* after 4 days of stimulation with TGFβ1 on all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) KD cells. Two-way ANOVA; * $p < 0.05$, *** $p < 0.001$. (B) Representative immunofluorescent images of collagen type I deposition on all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) KD cells. Nuclei are stained with DAPI. Original magnification 200x. ANOVA, analysis of variance; DAPI, 4',6'-diamidino-2-phenylindole; TGF, transforming growth factor.

Software v1.2.4 (Applied Biosystems). Primer sequences are provided in **Table 1**.

Immunofluorescence

For spectrin immunofluorescence, PFA-fixated cells cultured for 7 days were incubated with 10% goat serum for 1 h. Primary antibodies were incubated in PBS + 2.2% BSA at room temperature (RT) for 2 h. For YAP immunofluorescence, cells were permeabilized with 0.5% Triton X-100, and subsequently incubated with PBS 10% goat serum RT for 1 h. Primary antibodies were incubated in PBS + 0.1% Triton X-100 and 2.2% BSA at

4°C for 16 h. For α -smooth muscle actin and collagen immunofluorescence, methanol/acetone (1:1) fixed cells were incubated with 10% goat serum for 1 h, and primary antibodies were incubated in PBS + 2.2% BSA at RT for 2 h. For all immunofluorescence, secondary antibodies were diluted in PBS + 2.2% BSA at RT for 1 h, and subsequently incubated with Alexa647-labeled streptavidin in PBS containing 4',6-diamidino-2-phenylindole (DAPI, 1:5000, 10236276001; Roche) for 30 min. Actin was visualized by incubation with TRITC labeled-Phalloidin in PBS for 30 minutes. Between incubations, cells were washed thrice with PBS containing 0.5% Tween 20. Slides were mounted in Citifluor (Agar Scientific, Stansted, UK) and used for immunofluorescence microscopy.

Statistics

All data are represented as means \pm SD of at least three independent experiments and were analyzed by GraphPad Prism Version 6.01 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) by either one-way or two-way ANOVA followed by Bonferroni post hoc analysis.

RESULTS

all- and β II-spectrins do not influence cell adhesion

In order to elucidate the role of spectrins on fibroblast behavior, we performed siRNA mediated knockdown. We found that both all and β II-spectrin have a long half-life; we only observed >90% gene expression knockdown 168 h (7 days) after transfection (**Figure 1A**), and also observed knockdown at the protein level (**Figure 1B**). Interestingly, β II-spectrin siRNA knockdown also decreased the expression of all-spectrin about two-fold. Next, we investigated whether all and β II-spectrin have an effect on cell adhesion (**Figure 2A**) by seeding cells on either 2 kPa or 50 kPa fibronectin-coated substrates of different stiffness. Cell adhesion did not differ between 2 kPa and 50 kPa in either the control cells or the all-spectrin and β II-spectrin deficient cells.

Cell spreading on soft and stiff substrates is independent of all- and β II-spectrin

The morphological and cytoskeletal changes of fibroblasts are well documented for cells cultured on fibronectin-coated surfaces with stiffness ranging from 2 to 55 kPa. When grown in sparse culture with no cell-cell contacts, fibroblasts show an abrupt change in spread area that occurs at a stiffness range above 3 kPa³⁶. We indeed observed major differences in cell size (spreading) between 2 kPa and 50 kPa gels: cells cultured on 2 kPa were markedly smaller than cells cultured on 50 kPa (**Figure 2B-C**). This was the case both for control cells as for all-spectrin or β II-spectrin deficient cells, but we observed no significant differences in cell size between the spectrin-deficient cells and the control group. These data suggest that all- and β II-spectrins do not affect the stiffness-dependent changes in cell size of dermal fibroblasts.

all- and β II-spectrin do not regulate YAP localization

YAP is a mechanosensitive transcriptional co-activator that has been shown to govern the phenotypical switch to myofibroblasts and accumulates in the nucleus on increased stiffness of the ECM^{31,37}. One of the mechanisms of YAP nuclear accumulation involves

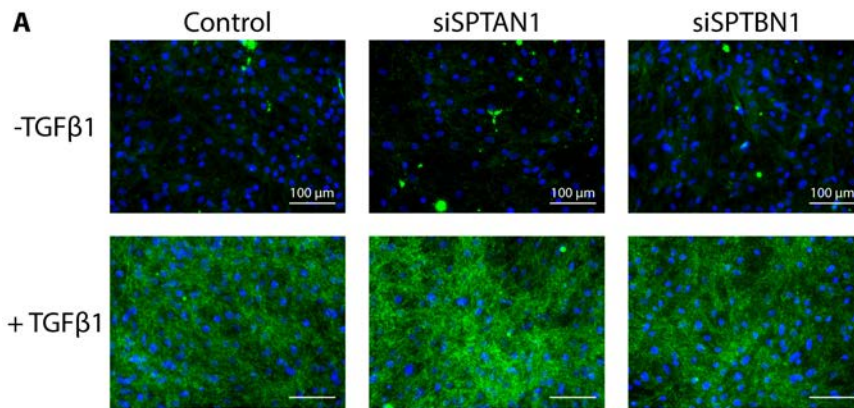


Figure 7. α - and β II-spectrin do not control vinculin adhesions. Representative immunofluorescent images of vinculin after 4 days of stimulation with TGF β 1 on all-spectrin (*SPTAN1*) and β II-spectrin (*SPTBN1*) KD cells. Original magnification 200 \times . DAPI, 4',6'-diamidino-2-phenylindole; TGF, transforming growth factor.

polymerization of actin monomers into stress fibers^{33,38,39}. Recently, α - and β II-spectrin were shown to regulate cytoplasmic retention of YAP in stretched epithelial cells, by interacting with and activating Hippo signaling at the plasma membrane²⁸. Because fibroblasts and myofibroblasts rely heavily on their contractile cytoskeleton and are known for their ability to spread over great distances, we investigated the effects of substrate stiffness and presence of α - and β II-spectrin on YAP localization. We observed major differences in YAP localization between 2 and 50 kPa hydrogels (**Figure 3**): on 2 kPa all cells displayed cytoplasmic retention of YAP, while on 50 kPa all cells showed both nuclear and cytoplasmic localization of YAP. However, we observed no differences in YAP localization between spectrin-deficient cells and the control cells, suggesting that spectrins are not primarily involved in fibroblast stiffness-dependent YAP localization.

α - and β II-spectrin do not regulate fibroblast migration and wound healing

Others showed β _H-spectrin to be involved in epithelial cell migration in *Sophophora*⁴⁰. Therefore, we asked whether spectrins are necessary for fibroblast wound closure in vitro. We mimicked wound closure by means of IBIDI inserts, and found that no differences in wound repopulation in fibroblasts stimulated with or without TGF β 1 (**Figure 4**). Moreover, knockdown of spectrins did not affect the population rate of the wound area (**Figure 4**).

α - and β II-spectrin do not affect the myofibroblast phenotype

Myofibroblasts play an important role in both regular wound healing as well as dysregulated wound healing, the latter resulting in fibrosis. α SMA stress fiber formation is an important hallmark of the myofibroblast phenotype, which can be induced by TGF β 1. We indeed observed an increase in *ACTA2* mRNA levels (**Figure 5A**). This was reflected in the differences in the amount of α SMA-positive

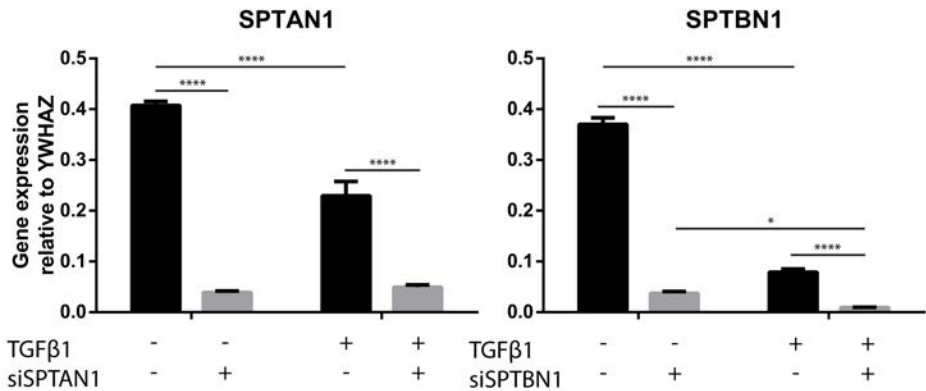


Figure 8. TGFβ1 stimulation decreases all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) gene expression. mRNA expression of all-spectrin (*SPTAN1*) and βII-spectrin (*SPTBN1*) after TGFβ1 stimulation. Two-way ANOVA; * p<0.05, **** p<0.0001. ANOVA, analysis of variance; TGF, transforming growth factor.

cells between fibroblasts cultured with or without TGFβ1 (**Figure 5B**). However, we observed no differences in the percentage of αSMA-positive cells between spectrin-deficient and control cells, indicating that spectrins are not involved in the TGFβ1-induced development of the cytoskeleton. This was also reflected in the mRNA levels of *ACTA2* between spectrin-deficient and control cells, although knockdown of βII-spectrin resulted in slightly lower *ACTA2* mRNA levels in TGFβ1-stimulated cells. Interestingly, knockdown of all-spectrin had a significant effect on *ACTA2* expression in non-stimulated cells: the mRNA level was markedly lower (**Figure 5A**).

Collagen deposition: the second hallmark of fibrosis

To determine if another hallmark function of myofibroblasts, namely the increased synthesis of collagen type I, is regulated by spectrins, we determined mRNA levels and collagen deposition. We indeed observed large differences between cells stimulated with or without TGFβ1. The increase in *COL1A1* mRNA levels (**Figure 6A**) was accompanied by an increase in collagen deposition (**Figure 6B**). However, all- and βII-spectrin knockdown did not affect mRNA levels of *COL1A1* in TGFβ1-stimulated cells (**Figure 6A**) or the deposition of collagen type I (**Figure 6B**). Interestingly, knockdown of *SPTAN1* had a major effect on *COL1A1* expression in non-stimulated cells: the mRNA level was markedly lower.

all- and βII-spectrin are not necessary for focal adhesion assembly

We determined the formation of focal adhesions by means of vinculin staining as a function of TGFβ1. As expected, an increase in focal adhesions was observed under the influence TGFβ1 (**Figure 7**). However, we did not observe any differences in focal adhesion formation between spectrin deficient and control cells.

TGFβ1 attenuates SPTAN1 and SPTBN1 expression

Since we did not observe differences in myofibroblast parameters between control and cells KD for spectrins, we wondered what happens with endogenous *SPTAN1* and *SPTBN1* mRNA levels when cells are stimulated with TGFβ1. Interestingly, TGFβ1 stimulation had a direct negative effect on *SPTAN1* and *SPTBN1* gene expression, as incubation with TGFβ1 resulted in significantly lower mRNA levels of *SPTAN1* and *SPTBN1* (**Figure 8**). The effect of TGFβ1 on *SPTBN1* was more pronounced than for *SPTAN1*.

DISCUSSION

Although much is known about the function of spectrins in erythrocytes, less detailed information is available regarding the function of spectrins in non-erythroid cells. In fact, hardly any information is available on the role of spectrins in fibroblasts. Since spectrins regulate cell morphology and are potential mechanosensors, we investigated whether α - and β -spectrin are required for the phenotypic properties of adult human dermal (myo)fibroblasts.

We first determined the effect α - and β -spectrin on cell adhesion and cell spreading on 2 kPa and 50 kPa gels, and noticed that α - and β -spectrin do not regulate the adhesion or spreading of adult dermal fibroblasts, nor did we find morphological differences. This is of interest, as knockdown of spectrins results in major changes in shell shape in a variety of cell types³⁰. Mouse embryonic fibroblasts devoid of *Sptbn1* obtained at E14.5 showed an impaired cell spreading, and had a more rounded and spiky appearance. In addition, a reduction in cell proliferation was observed⁵. Unfortunately, *Sptbn1* null mice are embryonic lethal, so the functions of β -spectrin in adult fibroblasts are not known. The discrepancy between our data obtained with adult cells compared with the above mentioned embryonic cells points toward the direction that there could be age-related differences regarding the role of spectrins in cell shape of a specific cell type. This is also substantiated by the observation that no changes in cell shape or morphology were observed in embryonic epithelial cells of *Sptbn1* knockdown mice⁵, whereas major cell shape differences were observed in adult epithelial cells of humans^{41,41}

Next we determined whether α - and β -spectrin have an effect on the translocation of YAP as a function of stiffness and cell spreading. We mimicked cell spreading by sparsely culturing the fibroblasts on 2 and 50 kPa gels. As expected, YAP was largely localized in the cytoplasm in cells cultured at 2 kPa, and was abundantly localized in the nucleus in cells cultured at 50 kPa. Deficiency of α - or β -spectrin did not change the translocation pattern of YAP. It has been shown that α -spectrin and β -spectrin have a mechanosensory function in the Hippo pathway in epithelial cells²⁸. This pathway is activated in densely confluent epithelial cell cultures and inactivated when cell density is sparse allowing cells to spread across the substrate. In these situations the transcriptional activator YAP is mainly located in the cytoplasm or nucleus, respectively^{38,42}. Knockdown of α -spectrin and β -spectrin prevents retention of YAP in the cytoplasm in high density cultures²⁸. Since knockdown of α - and β -spectrin did

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not have an effect on the localization of YAP under our conditions (sparse cell density on a soft or stiff substrate), we postulate that under these conditions the localization of YAP is mainly regulated by Hippo-independent mechanisms, including actin polymerization and Smad3 shuttling^{33,42}. Our data indicate that spectrins are, in contrast to their crucial role in the Hippo pathway to regulate YAP, not required to regulate YAP in the mechanotransduction pathway that acts parallel to the Hippo pathway.

Fibroblasts, and more specifically myofibroblasts, are at the heart of fibrosis⁴³. Fibroblasts undergo major morphological changes when they are activated into myofibroblasts by e.g. TGF β , and changes occur in tissue stiffening during the fibrotic process^{44,45}. We therefore wondered whether spectrins play a role in the myofibroblast phenotypical switch. We found that knockdown of spectrins did not affect myofibroblast formation, nor did we observe changes in the organization of α SMA stress fibers. Additionally, we found that focal adhesion assembly was unaffected by spectrin deficiency. The finding that the function of myofibroblasts without α - and β II-spectrin seems unchanged, is illustrated by the observation that collagen type I mRNA expression and protein deposition are unaffected, together with unaffected wound closure. These results were unexpected, because it has been shown that knockdown of β II-spectrin leads to the disruption of TGF β -signaling as mediated by SMAD proteins^{26,46-48}. However, these studies primarily focused on the epithelial lineage and mainly in the context of embryonic mouse development. Since we were puzzled by our observations, we also investigated endogenous gene expression of *SPTBN1* when fibroblasts were stimulated with TGF β 1, and noted a four-fold reduction in *SPTBN1* mRNA levels. This suggests that in adult human myofibroblasts, β II-spectrin does not interfere with Smad-mediated gene expression, which is confirmed by our siRNA data, where *SPTBN1* levels are reduced more than 20-fold in combination with TGF β without seeing an effect on collagen production or α SMA formation.

In conclusion, α - and β II-spectrin do not regulate cell adhesion, cell size and YAP localization in human dermal fibroblasts, and are not required for the myofibroblast phenotypical switch. Taken together, despite the diverse roles of spectrins in a variety of other cells, they do not seem to be suitable candidates to interfere in fibrotic processes.

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