



Therapeutic Targeting of TAZ and YAP by Dimethyl Fumarate in Systemic Sclerosis Fibrosis

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Systemic sclerosis (scleroderma, SSc) is a devastating fibrotic disease with few treatment options. Fumaric acid esters, including dimethyl fumarate (DMF, Tecfidera; Biogen, Cambridge, MA), have shown therapeutic effects in several disease models, prompting us to determine whether DMF is effective as a treatment for SSc dermal fibrosis. We found that DMF blocks the profibrotic effects of transforming growth factor- β (TGF β) in SSc skin fibroblasts. Mechanistically, we found that DMF treatment reduced nuclear localization of transcriptional coactivator with PDZ binding motif (TAZ) and Yes-associated protein (YAP) proteins via inhibition of the phosphatidylinositol 3 kinase/protein kinase B (Akt) pathway. In addition, DMF abrogated TGF β /Akt1 mediated inhibitory phosphorylation of glycogen kinase 3 β (GSK3 β) and a subsequent β -transducin repeat-containing proteins (β TRCP) mediated proteasomal degradation of TAZ, as well as a corresponding decrease of TAZ/YAP transcriptional targets. Depletion of TAZ/YAP recapitulated the antifibrotic effects of DMF. We also confirmed the increase of TAZ/YAP in skin biopsies from patients with diffuse SSc. We further showed that DMF significantly diminished nuclear TAZ/YAP localization in fibroblasts cultured on a stiff surface. Importantly, DMF prevented bleomycin-induced skin fibrosis in mice. Together, our work demonstrates a mechanism of the antifibrotic effect of DMF via inhibition of Akt1/GSK3 β /TAZ/YAP signaling and confirms a critical role of TAZ/YAP in mediating the profibrotic responses in dermal fibroblasts. This study supports the use of DMF as a treatment for SSc dermal fibrosis.

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INTRODUCTION

Scleroderma (systemic sclerosis [SSc]) is a complex devastating disease characterized by a triad of vascular, immune, and fibrotic changes in multiple organs. Despite significant therapeutic advancements, pulmonary and cardiac complications are associated with high mortality, and no effective therapy is currently available (Barnes and Mayes, 2012). Prominent skin and organ fibrosis is a hallmark feature of SSc and is accompanied by fibroproliferative vasculopathy and immune dysfunction (Allanore et al., 2015). Activated fibroblasts are the key effector cells in SSc responsible for the

excessive production of collagen and the subsequent development of fibrosis. Fibroblast recruitment and activation is regulated by a combination of autocrine and paracrine profibrotic mediators with additional cues derived from the stiffening extracellular matrix (Asano et al., 2005a, 2005b; Leask, 2015). Transforming growth factor- β (TGF β) plays a central role in regulating profibrotic gene expression; however, many additional factors including connective tissue growth factor (CTGF/CCN2) (Duncan et al., 1999; Igarashi et al., 1993), endothelin 1 (EDN1/ET-1) (Lagares et al., 2010; Shi-Wen et al., 2004), IL-6 (Giacomelli et al., 1996; Takemura et al., 1998), and others contribute significantly to this process. A highly complex and interconnected signaling cascade is involved in activation of the profibrotic gene program in SSc fibroblasts (Eckes et al., 2014; Kendall and Feghali-Bostwick, 2014; Lafyatis, 2014; Trojanowska, 2009).

Fumaric acid esters, including dimethyl fumarate (DMF), represent a class of molecules that augment the intrinsic cellular antioxidant response by enhancing the activity of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) signaling pathway (Scannevin et al., 2012). NRF2 activates genes encoding a number of phase II detoxifying and antioxidant enzymes (Kaspar et al., 2009). DMF has shown therapeutic effects in models of multiple sclerosis, cardioprotection, neuroprotection, neoangiogenesis, pulmonary hypertension, and renal fibrosis (Gold et al., 2012; Grzegorzewska et al., 2017; Oh et al., 2012, 2014; Scannevin et al., 2012).

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Abbreviations: Akt, protein kinase B; CTGF, connective tissue growth factor; DMF, dimethyl fumarate; EDN1, endothelin 1; GSK-3, glycogen kinase 3; HC, healthy control; MMP, matrix metalloproteinase; NRF2, nuclear factor (erythroid-derived 2)-like 2; PAAM, polyacrylamide; PI3K, phosphatidylinositol 3 kinase; SCF, SKP1-Cullin1-Fox protein; SSc, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; TGF β , transforming growth factor- β ; TRCP, transducin repeat-containing proteins; YAP, Yes-associated protein

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A mixture of various fumarate salts has been used for many years to treat inflammatory, autoimmune disease psoriasis (Brewer and Rogers, 2007). Recently, DMF (Tecfidera; Biogen, Cambridge, MA) was approved in the USA for the treatment of multiple sclerosis with a good safety profile (Miclea et al., 2016).

Here, we report that DMF has a potent antifibrotic effect in SSc and normal dermal fibroblasts. Mechanistically, we show that DMF targets the effectors of the Hippo signaling pathway, the transcriptional regulators TAZ (transcriptional coactivator with PDZ binding motif, WWTR1) and YAP (Yes-associated protein), by promoting their nuclear export and degradation. DMF was effective in preventing bleomycin-induced fibrosis in mice, a model of SSc dermal fibrosis. Together, the results of this study suggest that DMF could be beneficial for the treatment of SSc.

RESULTS

DMF blocks the TGF β -induced profibrotic response in healthy control and SSc dermal fibroblasts

To test the effects of DMF on the fibrogenic process, dermal fibroblasts were treated in culture with 2.5 ng/ml TGF β in the presence or absence of DMF ranging from 0 to 100 μ M for 4 hours (Supplementary Figure S1 online). This experiment indicated that 50 μ M DMF efficiently blocked the rapid induction of the profibrotic genes EDN1 and CCN2 in response to TGF β treatment, without affecting cell viability (Supplementary Figure S1). DMF (50 μ M) strongly suppressed TGF β -mediated COL1A1 gene expression and protein secretion both in healthy control (HC) and in fibroblasts that exhibit high basal COL1A1 expression, such as SSc fibroblasts (Figure 1a).

To determine the full spectrum of genes regulated by DMF, HC fibroblasts from three different donors were treated with TGF β in the presence or absence of 50 μ M DMF for a duration of 24 hours and a microarray analysis of gene expression was performed (GEO accession number GSE83642). After normalization and gene filtering, we found 600 genes that were differentially expressed between TGF β and TGF β +DMF groups at a significance level of $P < 0.05$. The list of DMF upregulated genes ($2 \geq$ fold) is shown in Supplementary Table S1 online and the list of DMF downregulated genes ($2 \leq$ fold) in Supplementary Table S2 online. Consistent with the antifibrotic effect of DMF, gene expressions of several matrix metalloproteinases (MMPs), including MMP10 (3.0, $P < 0.05$), MMP14 (1.5, $P < 0.05$), MMP27 (3.0, nonsignificant [ns]), MMP8 (2.1, ns), MMP12 (2.8, ns), and MMP1 (2.7, ns), were upregulated, whereas those of various extracellular matrix proteins, including COMP, COL10A1, osteomodulin, and versican, were decreased. Notably, the analysis of the array revealed that many of the TGF β -upregulated genes that were suppressed by DMF are targets of the Hippo pathway effectors TAZ and YAP (TAZ/YAP), which are transcriptional regulators implicated in directing TGF β -induced transcription in various contexts including fibroblasts (Figure 1b) (Hiemer et al., 2014; Liu et al., 2015; Szeto et al., 2016; Varelas et al., 2008). We verified by qPCR that several genes identified in our microarray analysis were regulated by TAZ/YAP, as EDN1, IL-6, TEAD1, PAI-1, CCN2, and Cyr61 were all repressed after

TAZ/YAP knockdown (Figure 1c, left panel). Efficient TAZ/YAP knockout was confirmed at the mRNA and protein levels (Supplementary Figure S2 online). We next verified that DMF reduced the basal expression and TGF β -mediated upregulation of EDN1, IL-6, TEAD1, PAI-1, CCN2, and Cyr61 (Figure 1c, right panel). Our analyses of proteins known to be induced by TGF β showed that CCN2 protein levels were elevated in response to 6 and 24 hours of TGF β treatment and that DMF treatment or TAZ/YAP depletion abrogated this response (Supplementary Figure S3 online). Validation of additional TAZ/YAP target genes that were upregulated by DMF is shown in Supplementary Figure S4 online. These data therefore indicate that DMF inhibits the expression of TGF β -regulated profibrotic target genes, many of which are regulated by TAZ/YAP.

TAZ/YAP have been shown to impact TGF β -induced Smad activity downstream of Smad phosphorylation (Szeto et al., 2016; Varelas et al., 2008). Indeed, TAZ/YAP depletion in fibroblasts had no effect on TGF β -induced phosphorylation status of Smad2 (Figure 1d), but did inhibit the expression of the SMAD2/3-regulated 3TP-lux reporter in NIH3T3 fibroblasts treated with TGF β (Figure 1e). DMF also reduced TGF β -induced Smad1 phosphorylation in dermal fibroblasts and NIH3T3 cells in response to TGF β treatment (Figure 1d, middle and right panels), which is a modification that has been shown to contribute to TGF β -induced fibrotic response in SSc (Pannu et al., 2007, 2008). Notably, DMF also inhibited the activity of the TAZ/YAP-responsive 8XTGIIc-luciferase reporter (Figure 1e). Thus, collectively our data suggested that TAZ/YAP activity is inhibited by DMF, which prompted us to further explore this possibility.

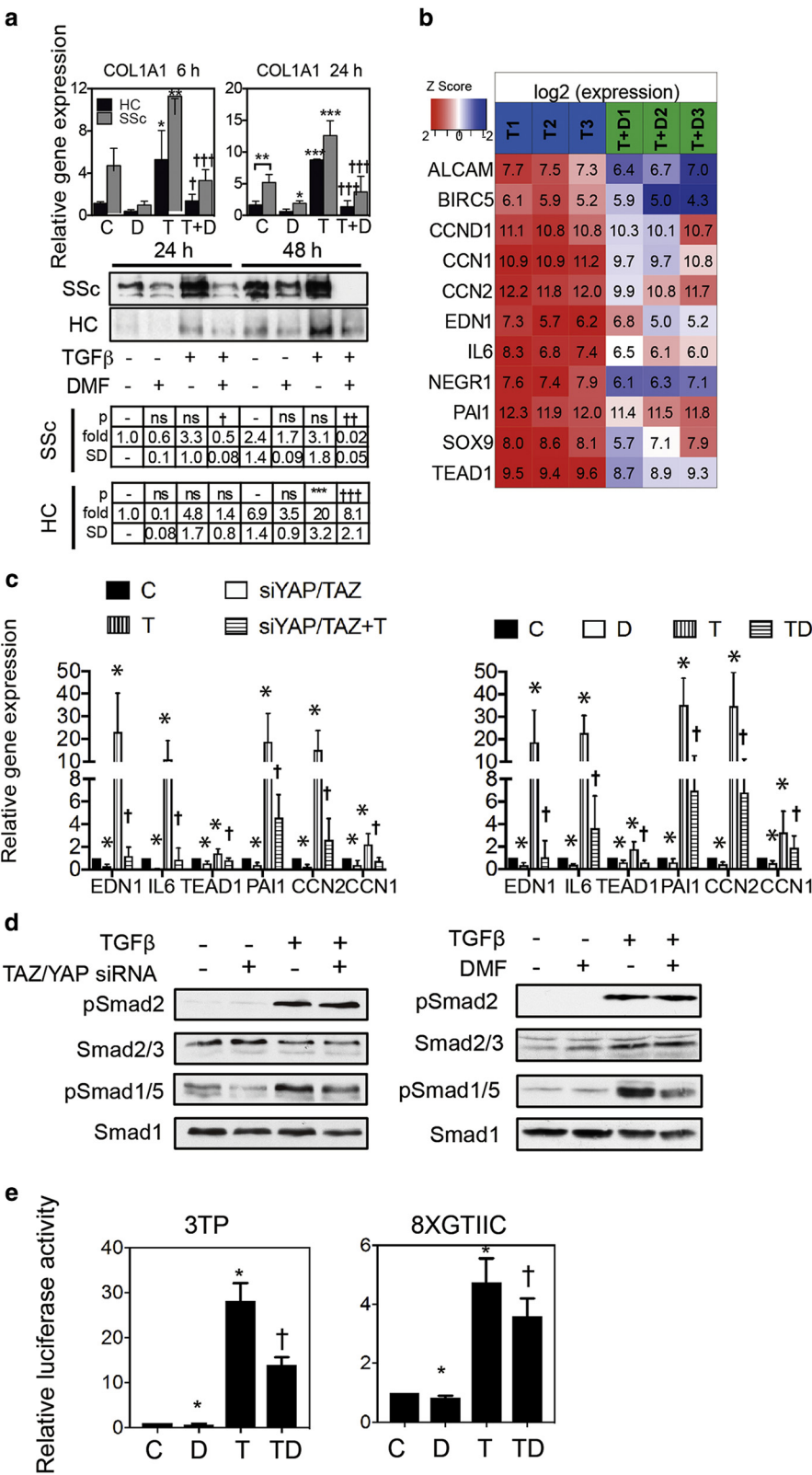
DMF and low substrate stiffness cause cytoskeletal changes and reduction of nuclear TAZ and YAP

TAZ/YAP have been implicated as key effectors of mechanotransduction signaling with their nuclear accumulation and activity increasing with matrix stiffness (Dupont et al., 2011). Therefore, to examine the effect of matrix stiffness on TAZ/YAP nuclear localization, HC and SSc fibroblasts were seeded on polyacrylamide (PAAm) gels with moduli of 600 or 6,000 Pa, representing the stiffness of healthy and sclerotic dermal tissue, respectively. At low stiffness, dermal fibroblasts possessed largely cytoplasmic TAZ/YAP as revealed by immunostaining (Figure 2a and b). Increasing PAAm substrate stiffness led to a pronounced increase in TAZ/YAP nuclear localization. In addition, higher substrate stiffness led to increases in the spread area of cells (Figure 2c and d) and the number and size of actin stress fibers (Figure 2a and b), indicative of a fibrotic transition in these cells. Importantly, DMF treatment reduced nuclear localization of both YAP and TAZ in fibroblasts on stiff PAAm gels (Figure 2e). DMF treatment additionally reduced the cell spread area and the number and size of stress fibers, with F-actin appearing more diffuse.

DMF decreases nuclear localization of TAZ and YAP via inhibition of the PI3K/Akt pathway

Given that DMF reduced nuclear localization of TAZ and YAP, we next wished to determine whether DMF targets signaling pathways involved in the regulation of this process.

Figure 1. DMF blocks the TGF β -induced profibrotic response and downregulates TAZ/YAP transcriptional targets. (a) Healthy control (HC) and SSc fibroblasts were treated with TGF β (T) and DMF (D). Gene expression was measured by qPCR, and type I collagen protein levels were measured by western blot. (b) A heatmap representation of TAZ/YAP transcriptional targets found in a microarray. (c) Gene expressions were determined by qPCR. Data shown as mean \pm standard deviation. $n = 3-6$ cell lines per group. * P versus control (C); $\dagger P$ versus TGF β . * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (d, left panel) HC dermal fibroblasts were transfected with TAZ/YAP siRNA or nonsilencing scrambled RNA, followed by 30-minute treatment with TGF β . (d, middle panel) HC fibroblasts (middle panel) or NIH3T3 cells (right panel) were pretreated for 1 hour with DMF, followed by 30-minute treatment with TGF β . Each experiment was repeated at least three times; representative blots are shown (e). The luciferase activities of 3TP-lux, ID1BRE-lux, and 8XGTIIC-lux are shown. DMF, dimethyl fumarate; siRNA, small interfering RNA; SSc, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; TGF β , transforming growth factor- β ; YAP, Yes-associated protein.



Recent studies have implicated the phosphatidylinositol 3 kinase (PI3K) pathway in promoting nuclear localization of YAP in epithelial cells (Elbediwy et al., 2016; Kim and Gumbiner, 2015). We, therefore, examined the effect of DMF on basal and TGF β -induced activity of protein kinase B

(Akt1) in HC and SSc fibroblasts. As shown in Figure 3a, DMF significantly reduced both basal and TGF β -induced Akt phosphorylation. Both SSc and HC fibroblasts responded in a similar manner. Because phosphorylation of YAP on Ser127 inhibits its nuclear localization, we next examined whether

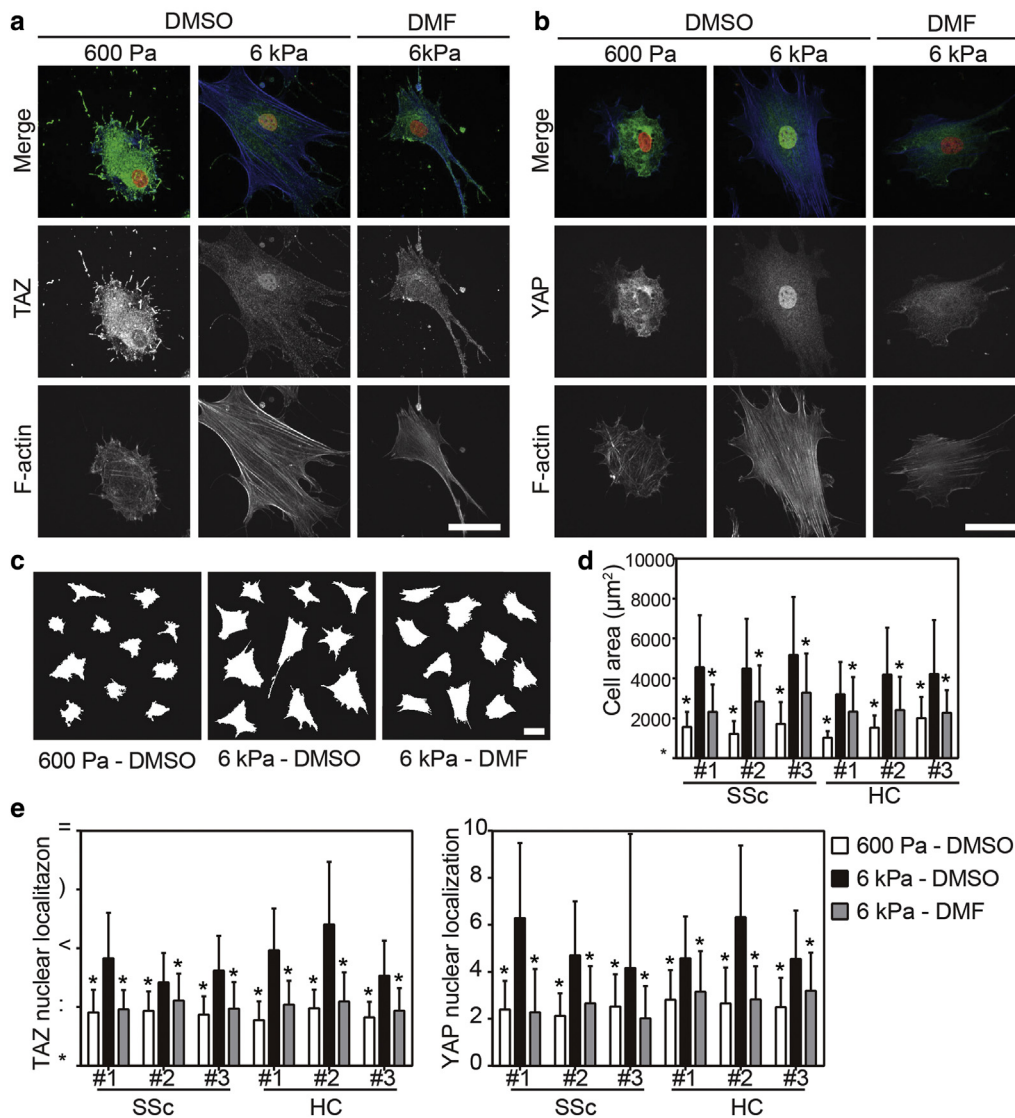


Figure 2. DMF attenuates cytoskeletal changes and nuclear translocation of TAZ and YAP caused by high substrate stiffness. (a, b) HC and SSc fibroblasts were seeded on gels with stiffness of 600 Pa or 6 kPa, and then treated with DMF or DMSO for 1 hour. Representative images of immunostaining with antibodies to (a) TAZ or (b) YAP in combination with F-actin antibody were shown. Images of SSc fibroblasts are shown. Scale bar = 25 μm. (c) Fluorescent images were segmented to identify the overall cell areas. (d) Quantification of cell area. (e) The ratio of average intensities between nuclear and cytoplasm regions was determined. n = 3 cell lines per group. Data shown as mean ± standard deviation. *P < 0.05 relative to cells on 6 kPa matrix treated with DMSO. DMF, dimethyl fumarate; HC, healthy control; SSc, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; YAP, Yes-associated protein.

DMF affects YAP phosphorylation. One-hour treatment of fibroblasts with DMF induced phosphorylation of YAP to the level comparable with that of PI3K inhibitor, LY294002, and Akt1/2/3 inhibitor, MK-2206 (Figure 3b). In contrast, ectopic expression of the constitutively active form of Akt significantly reduced the pYAP level (Figure 3c). To further confirm these findings, we used immunohistochemistry. Fibroblasts grown on plastic exhibited nuclear localization of YAP. Treatment with LY294002, MK-2206, and DMF significantly reduced nuclear localization of YAP (Figure 3d, left panel). We next examined TAZ cellular localization. In unstimulated fibroblasts, TAZ was partially localized to the nucleus and partially to the cytoplasm. Treatment with the inhibitors promoted cytoplasmic localization of TAZ (Figure 3d, right panel).

We next asked whether ectopic expression of the constitutively active Akt can rescue the effects of DMF on nuclear localization of TAZ/YAP. Treatment with adenovirus expressing myr-Akt had a negligible effect on the already nuclear basal localization of YAP, and slightly increased nuclear TAZ localization under basal conditions (Figure 3e).

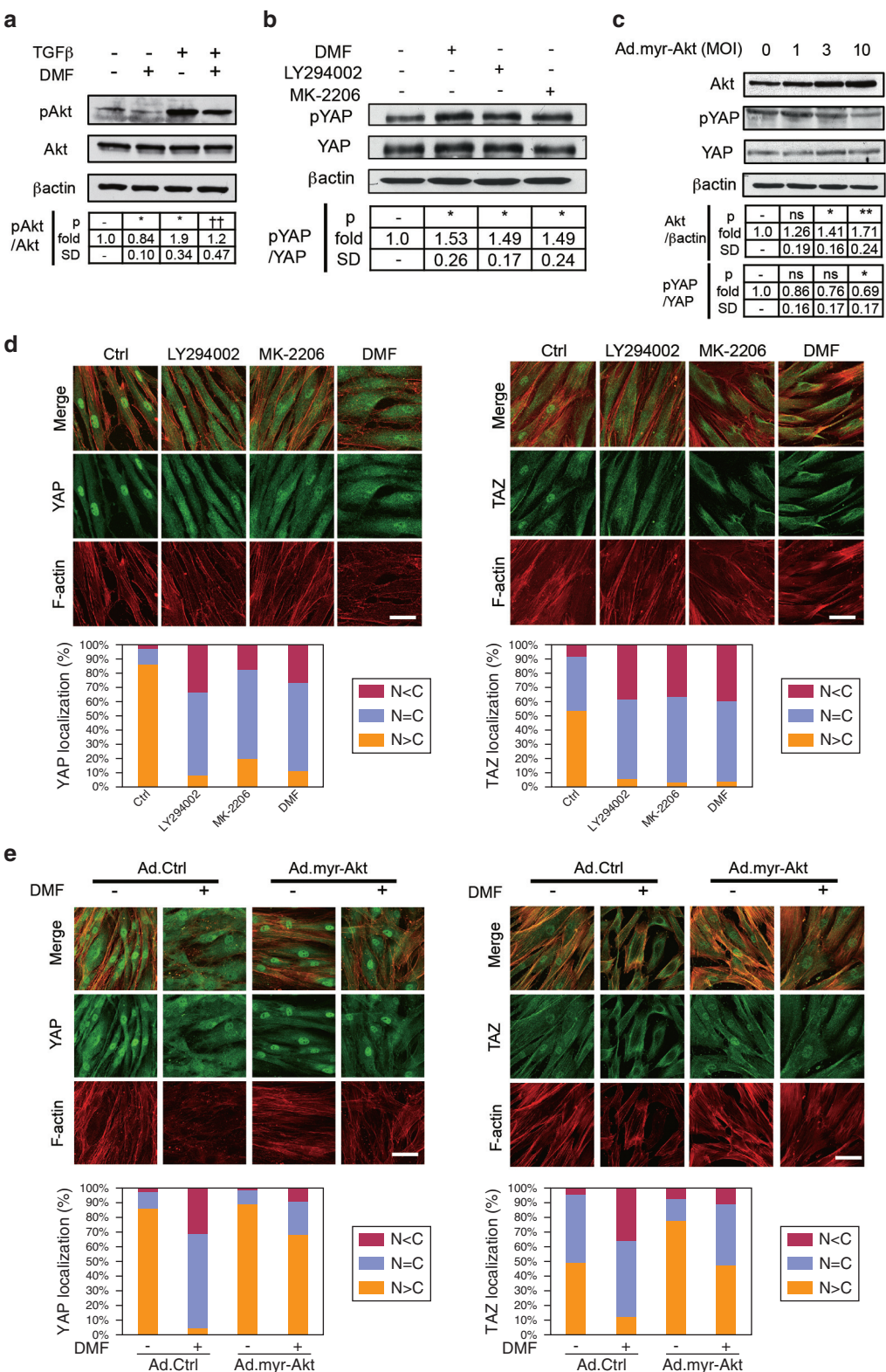
However, Adeno-Myr-Akt induced the pronounced nuclear localization of both TAZ and YAP in DMF-treated fibroblasts, thereby rescuing the effects of DMF on TAZ/YAP localization. Together, these results strongly suggest that DMF inhibits the activity of TAZ/YAP by promoting nuclear exclusion of TAZ/YAP.

DMF decreases protein levels of TAZ via proteasomal degradation

We next examined TAZ/YAP total protein levels in dermal SSc fibroblasts stimulated with TGFβ in the presence or absence of DMF. Treatment with DMF consistently led to a significant reduction of the basal and TGFβ-induced protein level of TAZ (Figure 4a). Moderate changes of YAP protein levels were observed only in some cell lines. Because TAZ and YAP mRNA expression were not affected by DMF (Supplementary Figure S5 online), we examined whether DMF-mediated TAZ/YAP downregulation is due to the 26S proteasome-mediated degradation. Fibroblasts were pre-incubated with 26S proteasome inhibitor MG132, followed by TGFβ and DMF treatments. Treatment with MG132

Figure 3. DMF decreases nuclear localization of TAZ and YAP through inhibition of PI3K/AKT signaling. (a) Fibroblasts were stimulated with TGFβ and DMF for 1 hour and analyzed for phospho- and total Akt by western blot. (b) Cells were treated with

indicated reagents for 3 hours. Phospho- and total YAP levels are shown. (c) Cells were infected with control adenovirus or adenovirus expressing myristoylated Akt1 (Ad.myr-Akt). Phospho- and total YAP levels are shown. (d) Cells were treated with indicated reagents for 3 hours. Representative fluorescent images of TAZ or YAP in combination with F-actin are shown. More than 50 cells from several random views were classified into three groups according to TAZ and YAP cellular localization. N > C indicates nuclear localization: N < C, cytoplasmic localization: N = C, even distribution. (e) Cells were treated with DMF and Ad.myr-Akt. Data shown as mean ± SD. n = 3–6 cell lines per group. *P versus control (C); †P versus TGFβ. *P < 0.05, **P < 0.01. Scale bar = 25 μm. Akt, protein kinase B; DMF, dimethyl fumarate; MOI, multiplicity of infection; PI3K, phosphatidylinositol 3 kinase; SD, standard deviation; SSC, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; TGFβ, transforming growth factor-β; YAP, Yes-associated protein.



significantly upregulated TAZ protein levels, whereas the effects of DMF and TGFβ were significantly diminished (Figure 4a). The effect of MG132 on YAP protein levels was less noticeable, likely due to the higher protein stability of YAP in comparison to TAZ. Consistent with our previous findings, the suppression of βTRCP by small interfering RNA

significantly upregulated protein levels of TAZ and YAP (Figure 4b) (Grzegorzewska et al., 2017).

The stability of TAZ is regulated by glycogen kinase 3 (GSK3), which phosphorylates TAZ on two N-terminal phosphodegron sites, not conserved in YAP, resulting in the recruitment of the βTRCP E3 ligase complex and subsequent

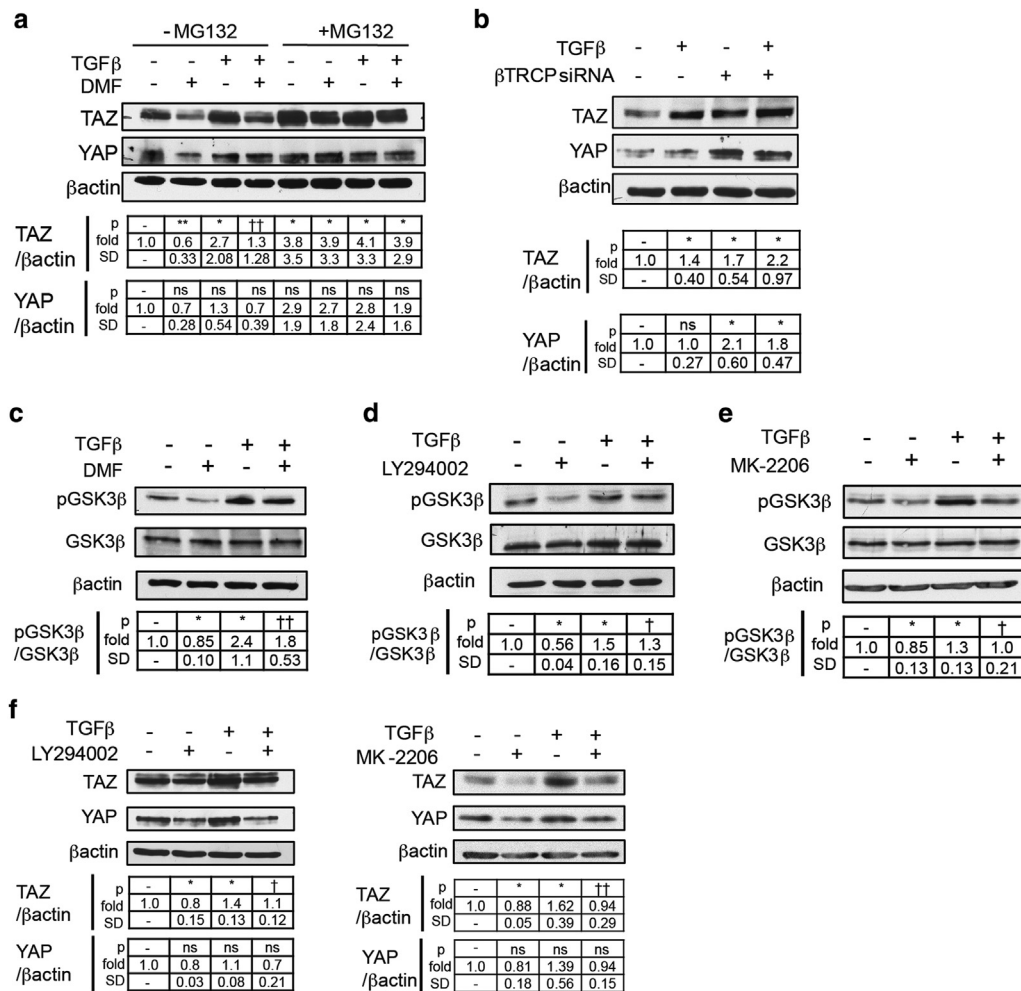


Figure 4. DMF decreases the TAZ protein level via proteasomal degradation through the PI3K/Akt/GSK3β pathway. (a) Healthy control and SSc fibroblasts were pretreated with 26S proteasome inhibitor MG132 (10 μM), and then treated with TGFβ and DMF. (b) Cells were transfected with siRNA specific to βTRCP gene or nonsilencing scrambled RNA. TAZ and YAP protein levels are shown. Cells were pretreated with (c) DMF, (d) LY294002, and (e) MK-2206 for 1 hour, and then stimulated with TGFβ for 1 hour. GSK3β phosphorylation status was analyzed. (f) Cells were pretreated with LY294002 or MK-2206, and treated with TGFβ for 6 hours. Data shown as mean ± SD. n = 3–6 cell lines per group. *P versus control (C); †P versus TGFβ. *P < 0.05, **P < 0.01. Akt, protein kinase B; DMF, dimethyl fumarate; GSK, glycogen kinase; PI3K, phosphatidylinositol 3 kinase; siRNA, small interfering RNA; SSc, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; SD, standard deviation; TGFβ, transforming growth factor-β; TRCP, transducin repeat-containing proteins; YAP, Yes-associated protein.

TAZ ubiquitination and degradation (Huang et al., 2012). On the other hand, GSK3β can be inhibited by Akt-dependent inhibitory phosphorylation (Cross et al., 1995; Frame et al., 2001), which would result in TAZ protein stabilization. We therefore examined the effect of TGFβ and DMF on the activity of GSK3β in SSc fibroblasts. TGFβ induced inhibitory phosphorylation of GSK3β, whereas DMF reversed these effects (Figure 4c). Likewise, a PI3K/Akt inhibitor, LY294002, and an Akt1/2/3 inhibitor, MK-2206, inhibited basal and TGFβ-induced phosphorylation of GSK3β (Figure 4d and e). Consistent with these results, LY294002 and MK-2206 reduced basal and TGFβ-induced protein levels of TAZ (Figure 4f). Together, these results indicate that DMF induces βTRCP-dependent proteasomal degradation of TAZ via activation of the PI3K/Akt1/GSK3β pathway.

DMF prevents skin fibrosis in a bleomycin mouse model

To test the efficacy of DMF in preventing the development of dermal fibrosis, we employed the widely used bleomycin mouse model. DMF injections normalized skin thickness and adipose layer loss induced by bleomycin (Figure 5a). Consistent with this finding, DMF treatment significantly reduced collagen content (Figure 5b). In addition, bleomycin-treated mice showed the increased number of

alpha smooth muscle actin (αSMA)- and TAZ/YAP-positive cells, which were localized predominantly in hypodermis, and DMF markedly reduced the number of those cells (Figure 5c and d). In addition, DMF treatment normalized mRNA levels of Col1α1, Ccn2, EDN1, and Il6 transcripts in bleomycin mice (Figure 5e).

TAZ and YAP proteins are localized to the nucleus of fibroblasts in adult normal and SSc skin

Protein expression of TAZ and YAP was evaluated in skin biopsies from seven HCs and six patients with SSc, including three limited and three patients with diffuse SSc. Tissue sections were coimmunostained for platelet-derived growth factor receptor β, a fibroblast marker (Bonner, 2004; Hewitt et al., 2012; Ohlund et al., 2017), along with either TAZ or YAP using immunofluorescence. The number of double-positive cells was elevated in SSc biopsy sections, in which nuclear localization of TAZ and YAP was observed (Figure 6a). The increased number of YAP/TAZ-positive cells was also confirmed by immunohistochemistry (Figure 6b). No significant differences between limited and diffuse SSc skin specimens were observed.

DISCUSSION

Despite significant progress in understanding the mechanisms of fibrosis, effective therapy for fibrotic diseases, including SSc,

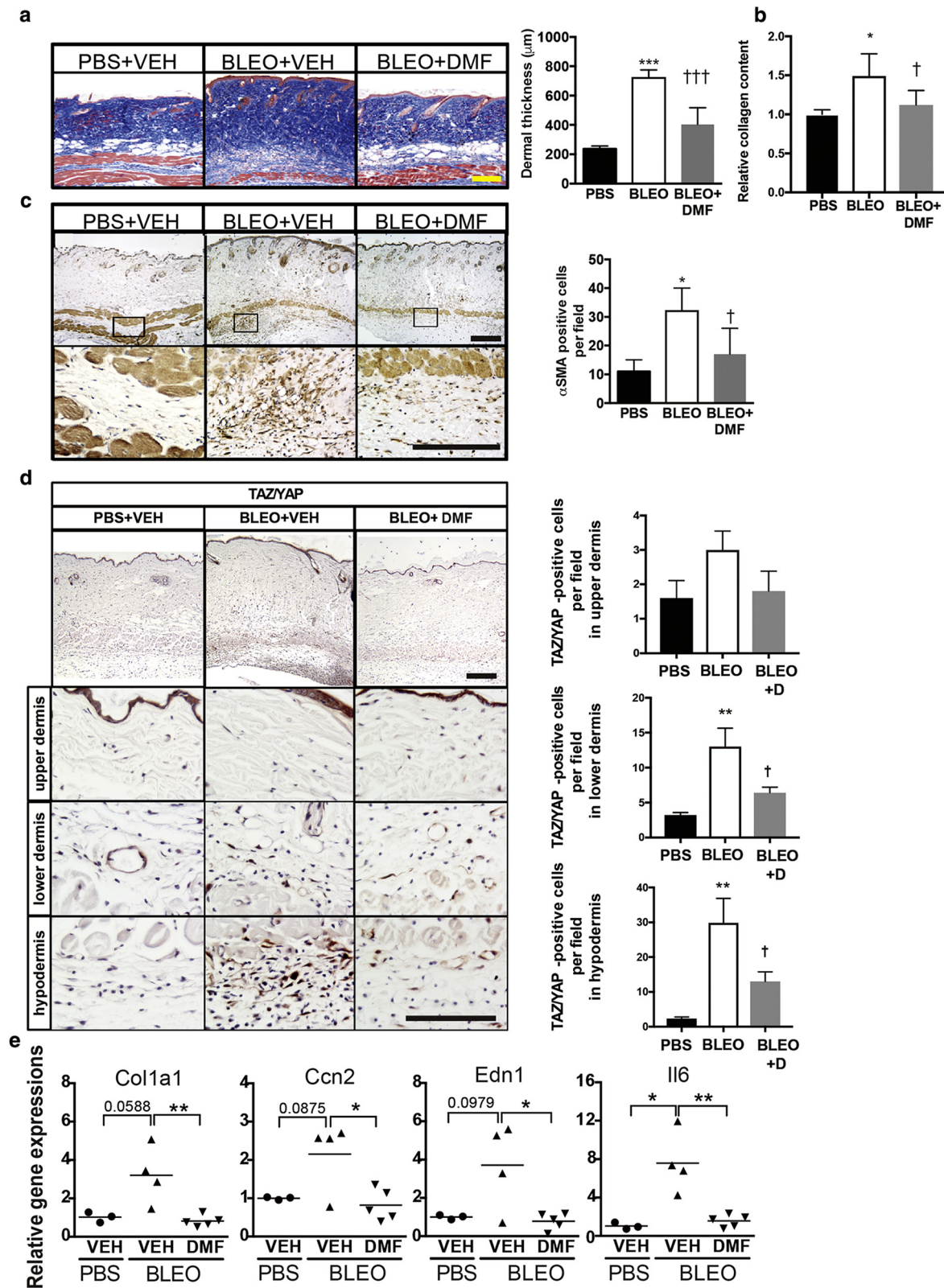


Figure 5. DMF alleviates skin fibrosis and abnormally elevated TAZ/YAP expression in the skin lesion of bleomycin-induced SSC model mice. (a) Trichrome staining of skin sections of bleomycin-administered mice treated with vehicle (BLEO+VEH) or DMF (BLEO+DMF) and PBS-administered mice (PBS+VEH). (b) Collagen content was measured by hydroxyproline assay. (c) α SMA expression was evaluated by immunohistochemistry of mouse skin tissue sections. (d) Immunostaining for TAZ/YAP was performed. TAZ/YAP-positive cells were counted in each part of the skin sections and summarized as graphs, respectively. Data shown as mean \pm standard deviation. $n = 4-6$ mice per group. * P versus PBS; † P versus BLEO+VEH. (e) Col1a1, Ccn2, Edn1, and Il6 mRNA levels in the lesional skin of mice were measured by quantitative real-time reverse transcription-PCR. $n = 4-6$ mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bar = 200 μ m. α SMA, alpha smooth muscle actin; BLEO, bleomycin; DMF, dimethyl fumarate; PBS, phosphate buffered saline; SSC, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; VEH, vehicle; YAP, Yes-associated protein.

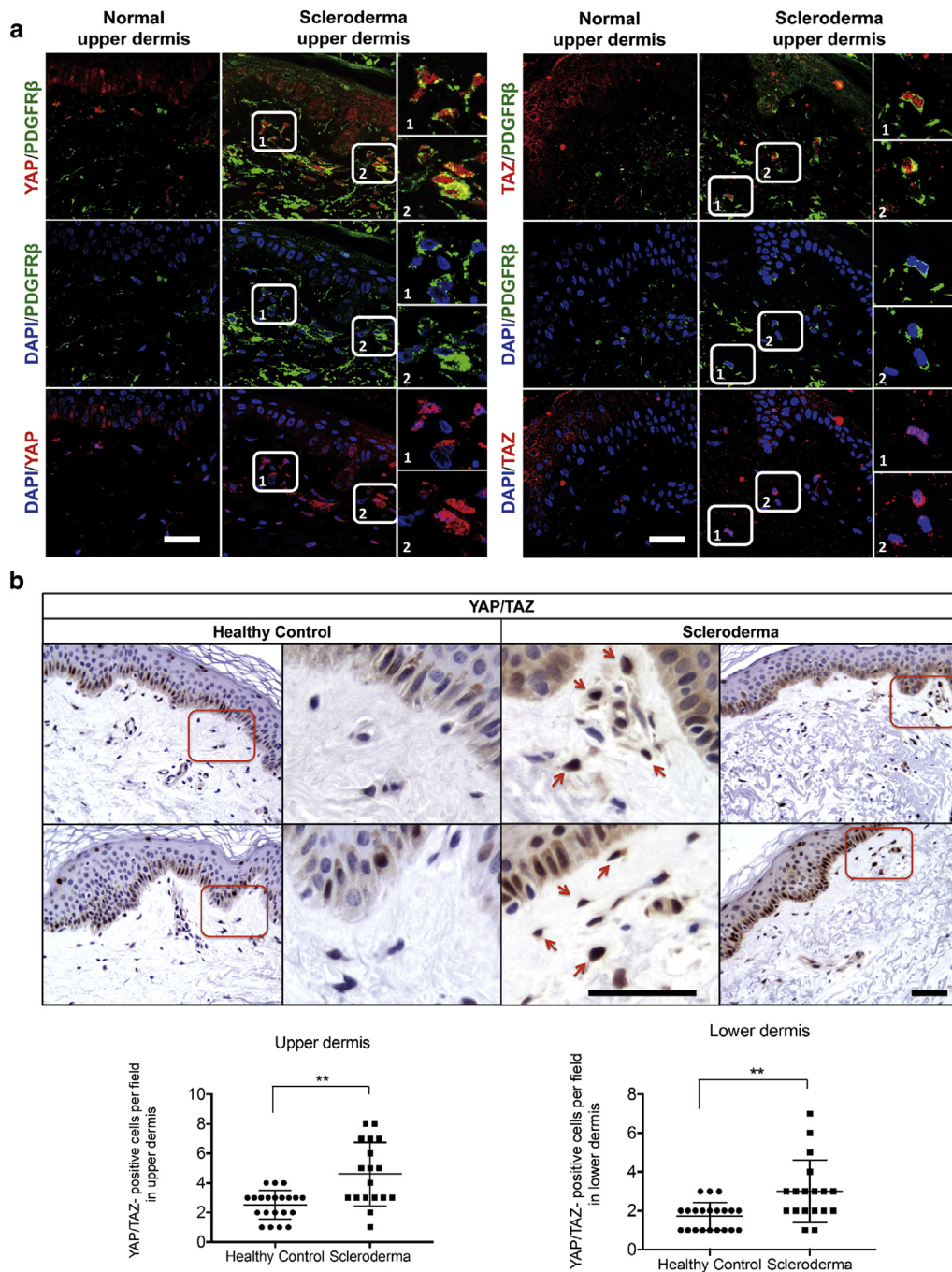


Figure 6. TAZ and YAP proteins are localized to the nucleus of fibroblasts in adult normal and SSc skin.

(a) Immunofluorescent staining was performed on skin tissue sections from scleroderma dorsal forearm biopsies ($n = 6$) and healthy control biopsies ($n = 7$) using antibodies to TAZ (red), YAP (red), and PDGFR β (green) and counterstained with DAPI (blue). Scale bar = 50 μ m. (b) Immunohistochemical staining for TAZ/YAP was performed on sections from the same biopsies. Scale bar = 100 μ m. (c) YAP/TAZ-positive cells were counted in three random views from each skin section. Data shown as mean \pm standard deviation. $**P < 0.01$. PDGFR, platelet-derived growth factor receptor; SSc, systemic sclerosis; TAZ, transcriptional coactivator with PDZ binding motif; YAP, Yes-associated protein.

remains elusive. Progress in therapeutic advancement is hindered in part by the complexity of the fibrotic process that involves not only activation of numerous signaling pathways in fibroblasts but also dysregulation of immune cells, as well as changes in the mechanical properties of the extracellular matrix. In an attempt to find a potential treatment for SSc, we focused on DMF, because of its distinct anti-inflammatory and antioxidative activities (Cross et al., 2011). The current study evaluated the effects of DMF on profibrotic responses in dermal fibroblasts. DMF attenuated basal and TGF β -induced production of several mediators of fibrosis, including CCN2, EDN1, and IL-6, in cultured SSc and healthy skin dermal fibroblasts. Interestingly, the antifibrotic effects of DMF did not

impact Smad2/3 phosphorylation, but were, at least in part, mediated by targeting the Hippo pathway effectors TAZ and YAP. This was confirmed by the knockdown of TAZ and YAP in HC and SSc fibroblasts that mimicked the effects of DMF on the expression of CCN2, EDN1, and IL-6. The antifibrotic potential of DMF was further confirmed in vivo using a bleomycin-induced model of dermal fibrosis. DMF prevented the development of fibrosis, which correlated with decreased accumulation of TAZ/YAP-positive spindle cells in the dermis. The therapeutic effects of DMF in vivo are likely mediated through several mechanisms, including anti-inflammatory and antioxidative activity via induction of the NRF2 signaling pathway (Wei et al., 2017).

The evolutionarily conserved Hippo pathway regulates proliferation, apoptosis, cell fate, and differentiation (Varelas, 2014). TAZ and YAP function as transcriptional coactivators by interacting with their primary partner transcription factor TEAD, as well as a number of other transcription factors, including Smads, Egr1, and Runx, to regulate specific subsets of genes (Mauviel et al., 2012). The nuclear accumulation of TAZ and YAP is regulated by diverse stimuli, including the Hippo signaling cascade, G protein coupled receptors, and the mechanical properties of the extracellular matrix.

We report herein that DMF exerted a strong inhibitory effect on TAZ/YAP signaling via the dual mechanism: (i) by inducing rapid nuclear exclusion of YAP and TAZ and (ii) by promoting degradation of TAZ and, to a lesser degree, YAP proteins. Central to these effects of DMF was inhibition of basal and TGF β -induced activity of Akt1/GSK3 β . Notably, GSK3 is known to play an important role in regulating actin cytoskeleton dynamics and cell migration (Sun et al., 2009; Wu et al., 2011; Xu et al., 2014). Thus, the effect of DMF on cellular localization of TAZ/YAP could be explained by its effects on the activity of GSK3 β and the corresponding changes in actin fiber formation. Furthermore, the inhibitory effect of DMF on Akt1 phosphorylation was consistent with other reports in human neutrophils stimulated with IL-8 and tumor necrosis factor- α (Muller et al., 2016) and human glioblastoma cells (Booth et al., 2014). Previous studies in epithelial cells have shown that different upstream negative regulators of the Hippo pathway, including G protein-coupled receptors, integrin/focal adhesive kinase/Src, and tyrosine kinase receptors, converge on the PI3-K pathway (Kim and Gumbiner, 2015). We now confirm that the PI3-K/Akt pathway, which is targeted by DMF, also plays a key role in regulating activity of TAZ/YAP in HC and SSc fibroblasts. Consistent with the earlier studies (Liu et al., 2010; Zhao et al., 2010), we confirmed the involvement of the β TRCP SCF ubiquitin ligase in mediating TAZ degradation. DMF exerted a stronger effect on TAZ protein in comparison to YAP under our experimental condition, likely because YAP protein is relatively stable, whereas TAZ protein undergoes a rapid turnover in the cell (Finch-Edmondson et al., 2015). We further showed that the stimulatory effects of TGF β and the inhibitory effects of DMF on TAZ protein stability were mediated through the Akt1/GSK3 β pathway.

In summary, this study provided an insight into the anti-fibrotic effects of DMF and identified TAZ and YAP as DMF targets in dermal fibroblasts. The results of this study support the use of DMF as treatment for SSc dermal fibrosis.

MATERIALS AND METHODS

Cell culture

On written informed consent and in compliance with the Institutional Review Board for Human Studies, fibroblasts were obtained by skin biopsy from patients with diffuse cutaneous SSc (three females and three males; median age 45 years, range 30–62 years). All patients fulfilled the criteria of the American College of Rheumatology for SSc and had not undergone any treatment for SSc at the time of biopsy. Control fibroblasts were obtained by skin biopsy of a healthy donor; these were matched with each patient with SSc for

age, race, gender, and biopsy site and were processed in parallel. Dermal fibroblasts were cultured from the biopsy specimens as described previously (Pannu et al., 2006).

LY294002 (PI3K inhibitor; EMD Millipore, Billerica, MA), MK-2296 (Akt inhibitor; Cayman Chemical, Ann Arbor, MI), and MG-132 (26s proteasome inhibitor; Cayman Chemical) were used to treat dermal fibroblasts at the concentration of 30, 5, and 10 μ M, respectively.

Real-time PCR

Gene expression levels were determined by quantitative real-time reverse transcription PCR, as described previously (Markiewicz et al., 2013).

Western blot analysis

Cells were lysed and processed as described previously (Pannu et al., 2006), using anti-type I collagen (SouthernBiotech, Birmingham, AL), anti-phospho-Smad1/5 (Ser463/465) (41D10; Cell Signaling Technology, Danvers, MA), anti-Smad1 (Cell Signaling Technology), anti-phospho-Smad2 (Ser465/467) (Cell Signaling Technology), anti-Smad2/3 (Cell Signaling Technology), anti-CTGF (L-20; Santa Cruz Biotechnology, Dallas, TX), anti-YAP/TAZ (D24E4; Cell Signaling Technology), anti-phospho-GSK3 β (Ser9) (119A11; Cell Signaling Technology), anti-GSK3 β (H-76; Santa Cruz Biotechnology), anti-phospho-Akt (Ser473) (193H12; Cell Signaling Technology), anti-phospho-YAP (S127) (D9W21; Cell Signaling Technology), and anti-Akt (C67E7; Cell Signaling Technology) antibodies. Densitometric quantification was done using Image J.

Promoter assay

NIH3T3 fibroblasts were transfected with p3TP-lux and 8XGT10C-lux plasmids together with pCMV- β -galactosidase plasmid. Promoter activity was determined using the Luciferase Assay System (Promega, Madison, WI), and standardized by β -galactosidase activity.

Microarray analysis

Refer to [Supplementary Materials](#) online.

Small interfering RNA-mediated depletion

Cells were transfected with either small interfering RNA specific to both human TAZ and YAP, human β TRCP (ON-TARGETplus SMART pool; ThermoFisher, Waltham, MA) or negative control small interfering RNA at the concentration of 10 nM using a Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher) according to the manufacturer's protocol.

Polyacrylamide hydrogels

The preparation of PAAm hydrogels was adapted from a previously described protocol (Yeung et al., 2005) with minor modifications. Refer to [Supplementary Materials](#) for details.

Variable substrate stiffness gels

After fixation and permeabilization, cell-seeded PAAm substrates were incubated with 1:200 mouse monoclonal anti-YAP (Santa Cruz Biotechnology) or 1:200 anti-TAZ (BD Pharmingen, Franklin Lakes, NJ), and then with 1:1,000 Alexa Fluor 555 goat anti-mouse IgG (H+L) (ThermoFisher Scientific) consecutively for 1 hour each at room temperature. Samples were counterstained for F-actin with Alexa Fluor 488-conjugated phalloidin. Cell area and YAP/TAZ nuclear localization were quantified with custom Matlab scripts.

Adenoviral construct and transduction

Replication-incompetent adenoviral vectors expressing mouse full-length Akt1 fused at N terminus by c-Src myristoylation sequence,

which is a constitutively active mutant of Akt1, were purchased from Vector Biolabs (Malvern, PA). Control, empty vector adenovirus was described previously (Pannu et al., 2007). The dose of 10 multiplicities of infection of the adenovirus was used for immunofluorescence experiments. Cells were treated with indicated doses of multiplicities of infection of the adenovirus for 48 hours.

Bleomycin-induced skin fibrosis

Male C57BL/6 mice were purchased from The Charles River. All of the experiments were performed under the guidelines of the Boston University Institutional Animal Care and Use Committee (protocol AN-15037). Dimethyl fumarate (Sigma Aldrich, St. Louis, MO) intraperitoneal injections were performed daily with a dose of 90 mg/kg in vehicle: 10% (2-hydroxypropyl)- β -cyclodextrin (Sigma Aldrich) in sterile phosphate buffered saline. Bleomycin (Hospira, Lake Forest, IL) or phosphate buffered saline was delivered in Alzet osmotic miniature pumps (model 1007D) at a dose of 1.8 unit/mouse and mice were killed after 4 weeks. Total mouse skin RNA was isolated by using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA).

Tissue collection and histology

For histology, 5- μ m sections of mouse skin biopsies were stained with Gomori trichrome (Chromaview, Dublin, OH). For immunohistochemistry, slides were processed and stained as described before (Stawski et al., 2012). Primary antibodies used are anti- α SMA (1:100, Novus Biologicals) and anti-YAP/TAZ (1:100, D24E4; Cell Signaling Technology).

Immunohistochemistry and immunofluorescence

Formalin-fixed, paraffin-embedded 8- μ m skin tissue sections were processed as described previously (Stawski et al., 2012). Primary antibodies used are 1:100 anti-platelet-derived growth factor receptor β (Cell Signaling), 1:100 anti-YAP (Santa Cruz Biotechnology), 1:100 mouse anti-TAZ (BD Pharmingen), and 1:200 Alexa Fluor-conjugated secondary antibodies (ThermoFisher Scientific). Samples were visualized by an Olympus and Leica confocal microscope.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

The experiments were performed by TT, APL, PH, BMB, ADS, and LS; the experimental design was made by MT, TT, APL, and XV; the manuscript was written by MT, TT, APL, and XV; all authors contributed to the discussion and preparation of the final manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2017.08.024>.

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