CELL INJURY, REPAIR, AGING, AND APOPTOSIS

FIZZ1-Induced Myofibroblast Transdifferentiation from Adipocytes and Its Potential Role in Dermal Fibrosis and Lipoatrophy

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Subcutaneous lipoatrophy characteristically accompanies dermal fibrosis with *de novo* emergence of myofibroblasts such as in systemic sclerosis or scleroderma. Recently dermal adipocytes were shown to have the capacity to differentiate to myofibroblasts in an animal model. Transforming growth factor β can induce this phenomenon *in vitro*; however its *in vivo* significance is unclear. Because found in inflammatory zone 1 (FIZZ1) is an inducer of myofibroblast differentiation but an inhibitor of adipocyte differentiation, we investigated its potential role in adipocyte transdifferentiation to myofibroblast in dermal fibrosis. FIZZ1 caused significant and rapid suppression of the expression of fatty acid binding protein 4 and peroxisome proliferator-activated receptor- γ in adipocytes, consistent with dedifferentiation with loss of lipid and Oil Red O staining. The suppression was accompanied subsequently with stimulation of α -smooth muscle actin and type I collagen expression, indicative of myofibroblast differentiation. *In vivo* FIZZ1 expression was significantly elevated in the murine bleomycin-induced dermal fibrosis model, which was associated with significant reduction in adipocyte marker gene expression and subcutaneous lipoatrophy. Finally, FIZZ1 knockout mice exhibited significantly reduced bleomycin-induced dermal fibrosis with greater preservation of the subcutaneous fat than wild-type mice. These findings suggested that the FIZZ1 induction of adipocyte transdifferentiation to myofibroblast might be a key pathogenic mechanism for the accumulation of myofibroblasts in dermal fibrosis. (*Am J Pathol* 2015, 185: 2768–2776; <http://dx.doi.org/10.1016/j.ajpath.2015.06.005>)

Developmentally the precise origin of dermal adipocytes is uncertain, although they are known to be derived from mesenchymal precursors that express platelet-derived growth factor receptor- α .¹ In adult skin, adipocyte precursors that express CD29, CD34, and Sca-1 have been reported.¹ In addition to its association with hair follicle cycling,² adipogenesis or adipocyte differentiation is activated in dermal wound healing, wherein it plays a role in fibroblast recruitment and extracellular matrix deposition.³ Moreover, suppression of adipogenesis results in marked reduction in myofibroblast numbers in the wound bed, suggesting that adipocytes may also be important in chronic fibrotic disorders wherein myofibroblasts are known to play a key role.⁴ However, other studies suggest that inhibition of adipocyte differentiation by profibrotic factors such as transforming growth factor (TGF)- β 1 is associated with dermal fibrosis in an animal model.⁵ The mechanism may be mediated by

TGF- β 1 suppression of peroxisome proliferator-activated receptor (PPAR)- γ expression a key indicator of adipocyte differentiation.⁶ Another profibrotic factor and inhibitor of adipocyte differentiation is found in inflammatory zone 1 (FIZZ1), also known as resistin-like molecule α (RELM α), a member of the newly described cysteine-rich secreted family of FIZZ/RELM.^{7–9}

The FIZZ family has a unique tissue expression pattern. FIZZ1, initially found in lung allergic inflammation, is expressed predominantly in white adipose tissue with low levels of expression noted in lung, heart, and mammary glands. FIZZ2 is specifically expressed by goblet cells and epithelial cells mainly in the gastrointestinal tract and was

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found in airway epithelium but not in white adipose tissue where FIZZ3 or resistin is exclusively expressed.⁸ FIZZ1 is dramatically induced in rodent bleomycin (BLM)-induced pulmonary fibrosis and chronic hypoxia model of pulmonary hypertension.^{9,10} FIZZ1 increases α -smooth muscle actin (α -SMA) expression through Jagged1/Notch1 signaling and thus promotes lung myofibroblast differentiation.¹¹ In humans, FIZZ2/RELM- β is induced in lung tissues of patients with idiopathic pulmonary fibrosis, scleroderma-associated pulmonary hypertension, and in serum of patients with systemic sclerosis (SSc).^{12–14} Interestingly, FIZZ1 is reported to inhibit adipocyte differentiation from preadipocytes *in vitro*. This inhibitory effect is reflected by a decrease in the mRNA levels of several adipocyte markers, including PPAR- γ , CCAAT/enhancer binding protein α (C/EBP- α), and adipocyte fatty acid binding protein 4 (FABP4).¹⁵ Thus, although inhibition of adipocyte differentiation in wound healing results in diminished myofibroblast differentiation, paradoxically profibrotic factors that promote myofibroblast differentiation also inhibit adipocyte differentiation.

This paradox is reflected in dermal fibrosis associated with SSc, wherein lipoatrophy accompanies the fibrosis. SSc is a chronic systemic disease characterized by autoimmunity, vascular lesions, and progressive fibrosis of the lungs and skin.^{16,17} The fibrotic component is dominant in SSc and is characterized by accumulation of collagens,¹⁶ which results in a loss of tissue function.¹⁸ There are currently no effective treatment, and 5-year mortality approaches approximately 30%.¹⁹ The specific physiologic and biochemical properties of the skin, the primary tissue affected by SSc, are based on increase of α -SMA expression, myofibroblast differentiation, and deposition of collagen.²⁰ In the BLM-induced animal model of SSc, myofibroblasts are observed in the dermis, and they gradually increase in parallel with the induction of dermal fibrosis and lipoatrophy.²¹

A potential resolution of this paradox is the hypothesis that adipocytes and/or adipocyte precursors can transdifferentiate to myofibroblasts under the influence of the profibrotic factor FIZZ1, an inhibitor of adipocyte differentiation¹⁵ but stimulator of myofibroblast differentiation⁹ and known to be essential for BLM-induced pulmonary fibrosis.²² To test this hypothesis the ability of FIZZ1 to suppress adipogenesis and to induce myofibroblast differentiation was examined in a preadipocyte cell line. The results showed that the FIZZ1 treatment caused significant repression of adipocyte FABP4 and PPAR- γ expression in fully differentiated adipocytes that induced from 3T3-L1 cells, which was accompanied subsequently with elevated levels of α -SMA and type I collagen expression, indicative of myofibroblast differentiation. The *in vivo* significance of this finding was affirmed by demonstration of reduced fibrosis and lipoatrophy in FIZZ1 knockout (KO) mice. Thus, FIZZ1 may be important in pathogenesis of dermal fibrosis by induction of adipocyte transdifferentiation to myofibroblasts.

Materials and Methods

Cell Culture and Adipocyte Differentiation

The murine preadipocyte cell line 3T3-L1 from ATCC (Manassas, VA) was grown in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) that contained 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and antibiotics (standard medium). To induce adipocyte differentiation, 3T3-L1 preadipocytes were grown to confluence in standard medium. Two days after the cells were confluent (day 0), the cells were induced to differentiate to adipocytes by supplementation with 15 μ g/mL insulin, 1 μ mol/L dexamethasone, 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich) known as differentiation medium (DM). After 48 hours (day 2) the DM was replaced with standard medium that contained 15 μ g/mL insulin only (after DM). The medium was changed every other day for an additional 5 days (day 7) when >90% of cells differentiated to mature adipocytes. In some experiments the mature adipocytes at day 7 were treated with FIZZ1 at the indicated doses and times. Untreated cells were kept in the same culture condition except in the absence of FIZZ1. Cell RNA and protein lysates were collected for quantitative RT-PCR (RT-qPCR) and Western blot analyses, respectively, after confirmation of differentiation by Oil Red O (Sigma-Aldrich) staining as previously described.²³

Animals and Induction of Dermal Fibrosis

C57BL/6 mice (8 to 10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). FIZZ1 KO mouse on C57BL/6 background was generated as previously described,²² and propagated at the University of Michigan. To induce dermal fibrosis, BLM (Blenoxane; Mead Johnson, Jersey City, NJ) was dissolved in sterile phosphate-buffered saline and subcutaneously injected (5 mg/kg) daily into the shaved upper backs of mice with the use of a 27-gauge needle, whereas control mice received the same volume of phosphate-buffered saline. At 21 days after BLM injection, the animals were sacrificed, and the injected skin samples were removed for mRNA and hydroxyproline analyses. When indicated, the skin tissues were fixed with 10% buffered formalin for hematoxylin and eosin, Masson trichrome staining, or immunohistochemistry.

mRNA Analysis by Real-Time Quantitative PCR

For quantitative mRNA analysis, total RNA was isolated from cells or skin tissue samples. 6-Carboxyfluorescein-conjugated primers or probes were then purchased from Life Technologies. For each assay, 100 ng of total RNA was used as template. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control to normalize the amount of input RNA. One-step real time RT-qPCR was undertaken with TaqMan One Step RT-PCR Master Mix (Life Technologies)

Figure 1 FIZZ1 induction and induced adipocyte dedifferentiation. **A:** 3T3-L1 preadipocytes were grown to confluence in SM and then induced for adipocyte differentiation for 6 days without (DM) or with subsequent FIZZ1 treatment (DM + FIZZ1) for an additional 6 days. Oil Red O staining for intracellular lipids with hematoxyline (Thermo Scientific, Runcom, Cheshire, UK) for nuclei counterstaining are shown. **B** and **C:** FIZZ1 expression was analyzed in preadipocyte 3T3-L1, and fully differentiated adipocytes for mRNA levels by RT-qPCR, and the results were expressed as $2^{-\Delta\Delta C_t}$ with GAPDH as the reference and the corresponding untreated control as calibrator (**B**) or for protein by Western blot analysis with 50 μ g of cell lysates (**C**). GAPDH signal was used as an internal control. The preadipocytes were placed onto coverslips and induced to undergo adipocyte differentiation. **D:** Fully differentiated adipocytes were then subjected to double immunofluorescence microscopy for FABP4 and FIZZ1; nuclei were stained blue with DAPI (**D**). The single color staining for DAPI, FABP4, FIZZ1, and the merged image are shown. * $P < 0.05$. Original magnification: $\times 100$ (**A**, top row); $\times 400$ (**A**, bottom row, and **D**). DM, differentiation medium; FABP4, fatty acid binding protein 4; FIZZ1, found in inflammatory zone 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, quantitative RT-PCR; SM, standard medium.

with the use of a GeneAmp 7500 Sequence Detection System (Life Technologies). Results were expressed as $2^{-\Delta\Delta C_t}$.

Cell Transfection with *Notch1* siRNAs

Fully differentiated adipocytes from 3T3-L1 cells were transfected with 100 nmol/L *Notch1* or control siRNA by electroporation with the use of Nucleofector II (Amaxa Biosystem, Gaithersburg, MD). After 20 hours, recombinant mouse FIZZ1 was added, and incubation was continued for another 48 hours. *Notch1* siRNA (5'-UGGC-UUGCAGUAGCAAGGAAGCUAA-3') and control siRNA (5'-UGGACGUGAUGGAACGAAGCUCUAA-3') were purchased from Invitrogen (Carlsbad, CA).

Western Blot Analysis

Cell lysate was prepared with RIPA buffer (Thermo, Rockford, IL). Equal amount of protein was loaded onto SDS-PAGE gels for separation and transferred to nitrocellulose membrane for immunoblotting. The following antibodies were used: anti- α -SMA (Sigma-Aldrich), anti-collagen type I (Bioscience International, Saco, ME), anti-PPAR- γ (Cell Signaling Technology, Inc., Danvers, MA), anti-FABP4 (Cayman, Ann Arbor, MI), anti-FIZZ1 (R&D Systems, Minneapolis, MN), anti-Notch1 (Cell Signaling Technology, Inc.), and horseradish peroxidase-conjugated anti-glyceraldehyde-3-phosphate dehydrogenase (Sigma-Aldrich).

Immunohistochemical Analysis

For immunohistochemistry, the paraffin-embedded skin tissue sections were deparaffinized and rehydrated, followed by antigen retrieval in 10 mmol/L sodium citrate (pH 6.0) in the microwave for 20 minutes. Rat anti-FIZZ1/RELM α antibody (R&D Systems), cyanine 3 anti- α -SMA (Sigma-Aldrich), and anti-FABP4 (Cayman) were used as primary antibodies; 557-conjugated goat anti-rat IgG (R&D Systems), Alexa Fluor 488-conjugated anti-rabbit IgG were used as secondary antibodies for FIZZ1 and FABP4, respectively. Mounting medium that contained DAPI (Vector, Burlingame, CA) was applied, and the tissue sections were examined with the use of a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Hydroxyproline Assay

Skin tissue samples were homogenized for hydroxyproline assay as previously described.²⁴

Results

FIZZ1 Inhibits Adipocyte Differentiation in 3T3-L1 Cells

As a secreted protein, FIZZ1 is expressed in white adipose tissue. To evaluate the effects of FIZZ1 on the fate of cultured adipocytes, we cultured 3T3-L1 preadipocytes in DM to induce adipocyte differentiation. FIZZ1 was added at day 6 after differentiation induction when the cells were fully differentiated with approximately 90% of cells positively stained by Oil Red O (Figure 1A) in contrast to the mostly Oil Red O⁻ cells in undifferentiated preadipocytes (Figure 1A). In cells further treated with FIZZ1, the number of lipid droplets that contained adipocytes induced by DM was reduced by FIZZ1 treatment (Figure 1A). These alterations in structural characteristics suggested that FIZZ1 might have a potential role in adipocyte dedifferentiation. Interestingly, FIZZ1 expression was markedly induced when 3T3-L1 cells were fully differentiated into adipocytes, which was almost undetectable in undifferentiated cells at both mRNA

Figure 2 FIZZ1 inhibits adipocyte gene expression. Fully differentiated 3T3-L1 adipocytes were treated with 25 ng/mL FIZZ1 for 3 days. The indicated adipocyte specific markers were analyzed by RT-qPCR. The resulting mRNA levels were expressed as $2^{-\Delta\Delta C_t}$ with GAPDH as the reference and the corresponding untreated control as calibrator (**A**) or by Western blot analysis with 20 μ g of cell lysates (**B**). GAPDH signal was used as an internal control. * $P < 0.05$. C/EBP- α , CCAAT/enhancer binding protein α ; FABP4, fatty acid binding protein 4; FIZZ1, found in inflammatory zone 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPAR γ , peroxisome proliferator-activated receptor γ ; RT-qPCR, quantitative RT-PCR.

(Figure 1B) and protein (Figure 1C) levels, suggesting the differentiated adipocyte as a possible cellular source of FIZZ1 *in vivo*. FIZZ1 was expressed in virtually all FABP4⁺ adipocytes in culture as shown by double immunofluorescence (Figure 1D).

To confirm the FIZZ1 dedifferentiation effect, FIZZ1-treated fully differentiated adipocytes were analyzed for adipocyte-specific gene expression. Three days after FIZZ1 treatment, PPAR- γ , FABP4, and C/EBP- α were significantly suppressed at both mRNA (Figure 2A) and protein (Figure 2B) levels. Such suppression remained up to 7 days after FIZZ1 treatment (Figure 3C). This FIZZ1 suppression of adipogenesis was accompanied subsequently with increased levels of type I procollagen and α -SMA mRNA, two key markers for myofibroblast differentiation (Figure 3A). Western blot analysis confirmed the FIZZ1-induced increase in α -SMA at the protein level in a dose-dependent manner.

Significant induction by TGF- β was observed as expected (Figure 3B). Kinetic analysis of α -SMA, FABP4, and PPAR- γ expression as a function of FIZZ1 treatment duration indicated prompt reduction in FABP4 and PPAR- γ expression, which was essentially maximal (>50% inhibition by FIZZ1) by day 1 (Figure 3C). However, FIZZ1-induced up-regulation of α -SMA expression was delayed with maximal effect achieved only on day 7. Double immunofluorescence staining for FABP4 and α -SMA was also performed to confirm such

differentiation. Only a few weak α -SMA signals were observed in untreated cells, whereas significant increase in α -SMA staining including those within cytoskeletal fibers was induced after 7 days of FIZZ1 treatment (Supplemental Figure S1). In contrast, FIZZ1 failed to significantly alter the mRNA levels of myofibroblast markers α -SMA and procollagen I in 3T3-L1 preadipocytes (Supplemental Figure S2). Thus, FIZZ1 caused rapid adipocyte dedifferentiation, which

Figure 3 FIZZ1 induces myofibroblast differentiation. **A:** Fully differentiated 3T3-L1 adipocytes were treated with 25 ng/mL FIZZ1 for 7 days. The indicated genes were analyzed by RT-qPCR. **B:** α -SMA protein induction with indicated doses of FIZZ1 was analyzed by Western blot analysis. GAPDH signal was used as an internal control. **C:** The cells were treated with 25 ng/mL FIZZ1 for the indicated days, and kinetic analysis of gene expressions of α -SMA, FABP4, and PPAR- γ by RT-qPCR is shown. The horizontal line indicated the control level at 100%; thus, values above or below this line indicated stimulation or inhibition by FIZZ1, respectively. * $P < 0.05$. FABP4, fatty acid binding protein 4; FIZZ1, found in inflammatory zone 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PPAR- γ , peroxisome proliferator-activated receptor γ ; RT-qPCR, quantitative RT-PCR; TGF- β , transforming growth factor- β ; α -SMA, α -smooth muscle actin.

Figure 4 FIZZ1 expression and localization in BLM-induced dermal fibrosis model. **A:** Twenty-one days after daily BLM subcutaneous injections, skin tissue RNA was analyzed for α -SMA and type I procollagen by RT-qPCR for fibrogenic gene expression. **B:** FIZZ1 mRNA was also determined at day 10 and 21 after BLM injection. **C:** FIZZ1 and FABP4 immunofluorescence microscopy was performed on paraffin-embedded skin tissue sections. FIZZ1 signals are shown as red color, FABP as green, and the nuclei are stained with DAPI. Merged images shown. The images from the section of PBS control or BLM-dermal fibrotic skin are shown. **D:** mRNA levels of the indicated adipocyte marker gene (PPAR γ and FABP4). **E:** After 21 days of daily BLM injection, skin tissue samples from the respective indicated murine strain were analyzed for hydroxyproline content, and the results (in μ g) were normalized per gram of wet weight of the skin sample. **F:** Histopathology and collagen deposition were examined by H&E- and Masson trichrome-stained skin tissue sections. **G:** The lung tissues from BLM- or PBS-treated WT or FIZZ1 KO mice were homogenized and lysed in RIPA buffer. The lung tissue α -SMA and FIZZ1 proteins were analyzed by Western blot analysis. GAPDH signal was used as an internal control. * $P < 0.05$. Original magnification: $\times 400$ (C); $\times 100$ (F). B or BLM, bleomycin; ColI, type I collagen; FABP4, fatty acid binding protein 4; FIZZ1, found in inflammatory zone 1; H&E, hematoxylin and eosin; KO, knockout; P or PBS, phosphate-buffered saline; PPAR- γ , peroxisome proliferator-activated receptor γ ; RT-qPCR, quantitative RT-PCR; WT, wild-type; α -SMA, α -smooth muscle actin.

preceded myofibroblast differentiation, suggesting a sequential process of transdifferentiation.

FIZZ1 Expression and Role in Dermal Fibrosis of BLM-Induced Mouse Model of SSC

To evaluate the possible *in vivo* role of FIZZ1 in inducing transdifferentiation of adipocyte to myofibroblast, thus contributing to lipatrophy and emergence of α -SMA-expressing myofibroblasts, we used the murine BLM-induced model of dermal fibrosis. C57Bl/6 mice were injected intradermally and daily with BLM at the upper back. Twenty-one days later, skin tissue samples were collected for gene expression analysis by real-time quantitative PCR. The data showed that, although skin type I procollagen and α -SMA gene expression increased as expected (Figure 4A), FIZZ1 expression was also significantly induced. The FIZZ1 expression was induced by >10-fold 10 days after BLM injection and remained >sixfold

elevated at day 21 (Figure 4B). Thus, induced FIZZ1 expression was associated with dermal fibrosis.

A potential cellular source for its expression in skin was evaluated with the adipocyte marker FABP4 because FIZZ1 is expressed in fully differentiated adipocytes in cell culture studies and predominately in adipose tissue. Dual immunofluorescence for FIZZ1 and FABP4 revealed that in normal skin tissue FIZZ1 expression was detectable, mainly localized to the intradermal adipose tissue layer, and colocalized with FABP4 signals (Figure 4C). Scattered signals were also observed in the reticular dermis and in hair follicle epithelial cells (data not shown). The FIZZ1 signals were readily apparent and more abundant in the fibrotic skin tissue 21 days after BLM injection (Figure 4C). The FIZZ1 staining was mainly localized in FABP4⁺ cells, indicating predominant expression in non-adipocytes unlike in normal skin wherein colocalization of FIZZ1 and FABP4 staining was extensively encountered. These manifestations of increased FIZZ1 expression and dermal fibrosis were accompanied by significant reductions in mRNA levels of adipocyte-specific markers (PPAR- γ and

FABP4) (Figure 4D), consistent with the dermal lipoatrophy noted in tissue sections (see next paragraph). Of note, the number of α -SMA-expressing skin cells was increased, some of which were found to be positive for adipocyte marker FABP4 by double immunohistochemical staining in dermal fibrosis after BLM injection (Supplemental Figure S3), but such colocalization was not seen in phosphate-buffered saline-treated control skin (data not shown).

To further evaluate the *in vivo* role of FIZZ1 in this BLM model of SSc, the effects of FIZZ1 deficiency on dermal fibrosis and subcutaneous lipoatrophy were analyzed with FIZZ1 KO mice. Although dermal fibrosis in wild-type (WT) mice was confirmed by increased hydroxyproline content 21 days after BLM injection, it was significantly reduced in FIZZ1 KO mice (Figure 4E). The reduced dermal fibrosis in FIZZ1-deficient mice was also reflected in the skin tissue histopathology. Compared with the substantially thickened fibrotic dermis with lipoatrophy (loss of subcutaneous fat layer) in BLM-treated WT mice, FIZZ1 KO mice exhibited reduced dermal fibrosis, thickness, and less dense extracellular matrix deposition with greater preservation of subcutaneous fat (Figure 4F). Reduced collagen content in FIZZ1 KO skin was confirmed by Masson trichrome staining. The reduction of fibrosis in FIZZ1 KO mice was accompanied by the reduced protein levels of α -SMA, an indicator of decreased myofibroblast differentiation, and FIZZ1, thus confirming FIZZ1 deficiency (Figure 4G). Collectively, these results indicated that the activation of FIZZ1 signaling plays vital roles in the dermal fibrotic processes caused by BLM.

Notch1 Potentially Mediates FIZZ1 Effects on Adipocyte Transdifferentiation

To seek the signaling pathway that possibly mediated the FIZZ1 effect on adipocyte transdifferentiation, the role of Notch1 was examined because Notch1 signaling activation is reported as a potential mechanism by which FIZZ1 induces lung myofibroblast differentiation.¹¹ FIZZ1 induced Notch1 mRNA expression in differentiated adipocytes *in vitro* (Figure 5A), which was associated with increased myofibroblast differentiation and inhibited adipocyte differentiation (Figures 2 and 3).

The effect of Notch1 knockdown in these adipocytes was analyzed to see if Notch1 was essential for FIZZ1 induction of myofibroblast transdifferentiation from adipocytes. FIZZ1 stimulated α -SMA and inhibited FABP4 expression as expected in cells treated with control siRNA, but both these effects were markedly blocked when Notch1 was essentially knocked down on *Notch1* siRNA transfection (Figure 5B). Of note, the BLM-induced Notch1 expression observed in dermal fibrosis was reduced in FIZZ1 KO mice (Figure 5C), indicating the potential association with adipocyte transdifferentiation *in vivo*.

Discussion

Intradermal adipose tissue is now considered to play important roles in diverse homeostatic and disease processes on the basis

Figure 5 Notch1 mediates FIZZ1 effect on adipocyte transdifferentiation. Fully differentiated adipocytes were treated with 25 ng/mL FIZZ1 for 24 hours. **A:** Notch1 mRNA was analyzed by RT-qPCR. The adipocytes were transfected with 100 nmol/L *Notch1* or control siRNA by electroporation, and the cells were treated with FIZZ1 as indicated. **B:** NIC, α -SMA, and FABP4 protein levels were analyzed by Western blot analysis. **C:** NIC protein was also analyzed in WT or FIZZ1 KO mice after PBS or BLM treatment. GAPDH signals were used as internal controls. * $P < 0.05$. B or BLM, bleomycin; FABP4, fatty acid binding protein 4; FIZZ1, found in inflammatory zone 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KO, knockout; NIC, Notch1 intracellular domain; P or PBS, phosphate-buffered saline; RT-qPCR, quantitative RT-PCR; WT, wild-type; α -SMA, α -smooth muscle actin.

of its ability to elaborate multiple paracrine factors and to serve as a precursor pool for mesenchymal cells.^{25,26} Constituent adipocyte differentiation from known preadipocyte precursors involves an intricate transcriptional program that involves C/EBPs and PPAR- γ , which occurs in wound healing and certain stages of hair follicle cycling.^{2,3} Moreover, this adipocyte differentiation is required for optimal wound healing with requisite myofibroblast differentiation and matrix deposition, which is substantially impaired in transgenic mice devoid of white adipose tissue.^{3,26,27} However, loss of adipocytes is characteristic of dermal fibrosis such as in SSc and the BLM model.²⁸ To resolve this apparent paradox, we evaluated the role of FIZZ1, an inhibitor of adipocyte differentiation but an inducer of myofibroblast differentiation, in adipocyte-to-myofibroblast transdifferentiation. Such a possibility would explain how loss of adipocytes and emergence of myofibroblasts are directly linked to account for the simultaneous importance of the adipocytes and myofibroblasts in dermal fibrosis.

Adipocyte Dedifferentiation and Myofibroblast Differentiation

The present study found that FIZZ1 significantly suppressed adipocyte differentiation in differentiated 3T3-L1 cells as shown by decreased adipocyte-specific gene expression, including PPAR- γ , FABP4, and C/EBP α , consistent with a previous study showing that FIZZ1 had an inhibitory effect on the conversion of preadipocyte into adipocyte when subjected to a differentiation program.¹⁵ Our present findings indicated that the expression levels of myofibroblast differentiation markers α -SMA and type I collagen were elevated, whereas the adipocyte markers were suppressed by FIZZ1 treatment, and preadipocytes were not affected by FIZZ1 treatment. Kinetic analysis indicated rapid suppression of adipocyte marker gene expression by FIZZ1, which preceded the increase in myofibroblast marker gene expression by 1 to 2 days. Thus, FIZZ1 caused prompt dedifferentiation of adipocytes that is essentially maximal by day 1, followed by gradual increase in myofibroblast differentiation that became maximal only on day 7. Therefore, FIZZ1 could cause dedifferentiation of adipocytes followed by differentiation into myofibroblasts *in vitro*, consistent with a role in adipocyte transdifferentiation to the myofibroblast, a key cell in pathogenesis of chronic fibrosis.^{4,29} However, because FIZZ1 caused incomplete (<60%) inhibition of adipocyte gene marker expression, direct adipocyte-to-myofibroblast transdifferentiation without going through a dedifferentiated state cannot be ruled out.

Role of FIZZ1 in Dermal Fibrosis and Lipoatrophy

To evaluate the potential *in vivo* significance of this FIZZ1 *in vitro* on adipocyte-to-myofibroblast transdifferentiation, we studied the effects of FIZZ1 deficiency on BLM-induced dermal fibrosis that models the myofibroblast accumulation, fibrosis, and lipoatrophy characteristic of SSc skin lesions. Our results showed that BLM-induced dermal fibrosis and lipoatrophy in WT mice was accompanied with induction of FIZZ1 expression. Moreover, in FIZZ1 KO mice the fibrosis, myofibroblast differentiation, and lipoatrophy were all significantly reduced relative to that seen in WT mice. The subcutaneous fat preservation was evident in tissue sections from BLM-injected skin of FIZZ1 KO mice, especially compared with the striking lipoatrophy in similarly treated skin tissue samples from WT mice as confirmed also by reduction in adipocyte marker gene expression. FIZZ1 is dramatically induced in rodent BLM-induced pulmonary fibrosis, and its deficiency also impaired fibrosis and myofibroblast differentiation in this model.^{9,22} In the lung primary sources of induced FIZZ1 expression appear to be macrophages and epithelial and endothelial cells.^{9,30,31} In the case of the BLM skin model, the main sources appear to be adipocytes, macrophages, and hair follicle epithelial cells. In humans, FIZZ2 is highly induced in the lungs of patients with idiopathic pulmonary

fibrosis¹² and scleroderma-associated pulmonary hypertension¹³ and in the serum of patients with SSc.¹⁴

The findings with the use of the BLM-induced dermal fibrosis model found loss of adipogenic gene expression that was accompanied with increased myofibroblast differentiation and collagen deposition, consistent with the *in vitro* effects of FIZZ1 on adipocytes. Notch1 expression was also noted to be up-regulated simultaneously with elevation of FIZZ1 gene expression in WT but was essentially abolished in FIZZ1 KO skin. Jagged/Notch signaling plays a critical role in cell fate control during development and in mature cells by regulating the implementation of differentiation, proliferation, and apoptotic programs of a wide range of cells. FIZZ1-activated Jagged/Notch signaling mediates induction of myofibroblast differentiation by increasing CBF1/Suppressor of Hairless/LAG-1-dependent transcription of α -SMA.³² Although the role of Notch signaling in adipogenesis is somewhat controversial,^{33–35} evidence is mounting that Notch signaling inhibits adipocyte differentiation via hairy and enhancer of split-1, which functions as a transcriptional repressor in preadipocytes.^{34,35} The present study suggested that FIZZ1 induction of adipocyte transdifferentiation to myofibroblast depended on Notch signaling. The increase of Notch in BLM-induced dermal fibrosis depended on FIZZ1 and possibly mediated FIZZ1-induced dermal myofibroblast differentiation and adipocyte dedifferentiation. Its precise role, however, will need to be fully elucidated in future studies.

Adipocyte Transdifferentiation to Myofibroblast as a Link between Lipoatrophy and Fibrosis

SSc causes abnormal collagen deposition with cellular infiltration in the affected skin, resulting in the thickened dermis, which is associated with subcutaneous fat atrophy.^{17,36} Myofibroblasts are observed in the extensive thickened dermis and gradually increase in parallel with the induction of dermal sclerosis. The origin of extracellular matrix-producing myofibroblasts is controversial.^{21,37,38} They may arise from the *in situ* activation of quiescent resident fibroblasts in response to extracellular stimuli. Alternatively, myofibroblasts may also arise from other cell types, including endothelial cells and pericytes.^{39,40} Both myofibroblasts and pericytes are associated with vascular remodeling and injury. Pericytes may provide the pathogenetic link between vascular injury and fibrosis of scleroderma.³⁹ Despite their different origins, myofibroblasts are all characterized by specific properties such as high contractibility, *de novo* expression of α -SMA, and production of extracellular matrix.³⁹ During dermal fibrosis the intradermal adipose layer shows striking atrophy and is virtually replaced by densely packed connective tissue.^{17,36} The deficient expression and/or function of adipogenesis marker PPAR- γ and the adipokine adiponectin are noted in SSc patients and BLM-induced dermal fibrosis. These observations suggest that the expansion of dermal fibrous tissue may be closely linked with intradermal adipose atrophy.

The basis for the loss of adipose tissue subjacent to dermal fibrosis is unclear. One potential mechanism might be attributed to the loss of PPAR- γ and related adipogenic factors with consequent failure of adipogenic differentiation. Down-regulation of PPAR- γ expression will cause lipoatrophy and de-repression of collagen I and α -SMA expression, leading to myofibroblast differentiation.^{41–44} Indeed, TGF- β abrogates PPAR- γ ligand-driven adipogenic differentiation of fibroblasts and induces adipocyte dedifferentiation. In contrast, PPAR- γ ligand rosiglitazone administration into mice causes substantial expansion of the intradermal adipose layer with accumulation of intracellular lipids. When delivered together with BLM, it partially preserves this adipose layer and attenuates BLM-induced dermal fibrosis.^{6,43} This is further supported by the development of subcutaneous lipoatrophy and concomitant accumulation of fibrous connective tissue at the same location in the mice with adipocyte-specific deletion of PPAR- γ .^{41,42} A recent cell fate-mapping study that used transgenic mice with adiponectin promoter-driven Cre recombinase to specifically label mature adipocytes also found such a novel link between intradermal adipose loss and dermal fibrosis by showing that adiponectin⁺ intradermal precursors give rise to dermal myofibroblasts.²⁹

In this study we report a potential role for FIZZ1 in regulating dermal fibrosis in SSc by targeting the dermal adipocyte and/or its progenitor cell, a suggestion that is supported by *in vivo* observations of up-regulation of FIZZ2 expression in SSc.¹⁴

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.06.005>.

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