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TRPV4 ION Channel Is Associated with Scleroderma

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TO THE EDITOR

Scleroderma (SSc) is a multisystem idiopathic connective tissue disease with high morbidity and mortality. SSc is characterized by fibroblast activation, myofibroblast differentiation, and overproduction of matrix proteins such as collagens, which lead to unattenuated fibrosis of skin and internal organs (Castelino and Varga, 2014; Ho et al., 2014; Rubio-Rivas et al., 2014). Emerging data support a role for both a mechanical signal, for example, matrix stiffness, and a chemical signal, for example, transforming growth factor-β1, in fibroblast activation and differentiation (Hinz, 2009; Tschumperlin, 2013; Webster et al., 2014; Wong et al., 2012). Recently, we discovered that the transient receptor potential (TRP) channel of the vanilloid subfamily, TRPV4, a mechanosensitive, Ca^{2+}-permeable, plasma membrane channel, is required for transforming growth factor-β1/matrix stiffness-induced lung fibroblast differentiation and pulmonary fibrosis development in mice (Rahaman et al., 2014). TRPV4 is abundantly present in skin (Sokabe and Tominaga, 2010), and was shown to be activated by both chemical and physical stimuli in numerous cell types (Garcia-Elias et al., 2014). Various gain- and loss-of-function mutations in TRPV4 have been linked to human diseases (Everaerts et al., 2010; Garcia-Elias et al., 2014). Here, we provide evidence that increased numbers of TRPV4 positive myofibroblasts are present in skin tissues of patients with SSc. In addition, in a mouse model of SSc, we found that TRPV4-null mice are protected from bleomycin-induced skin fibrosis.

To assess the presence of TRPV4 proteins in human SSc skin tissues, paraffin-embedded sections of skin biopsies were obtained from patients with SSc (n = 5) and from age- and sex-matched healthy subjects (n = 5) at Boston University Medical Center. The study was approved by the institutional review board of the Boston University Medical Center, and

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CONFLICT OF INTEREST

The authors state no conflicts of interest.

all subjects signed informed consent. TRPV4 knock-out (TRPV4 KO) mice generated on a C57BL/6 background (Suzuki et al., 2003) were bred and maintained in the laboratory of Dr Zhang (Medical College of Wisconsin). Wild-type (WT) C57BL/6 mice were purchased from Charles River Laboratories (Massachusetts). All animal experiments were approved by the University of Maryland Animal Care Committee and followed Institutional Animal Care and Use Committee guidelines.

To determine the role of TRPV4 in our experimental mouse model of SSc, we analyzed the effect of bleomycin in TRPV4 KO mice and compared their response to congenic WT controls. Skin fibrosis was induced in 6-month-old WT and TRPV4 KO mice by bleomycin injection as previously described (Yamamoto et al., 1999). Because aging is a known risk factor for fibrosis and its progression, aged mice (6 months old) were studied for bleomycin-induced skin fibrosis. We see no discernible spontaneously developing skin fibrosis in TRPV4 KO mice up to 30 weeks of age. Equal volumes (100 μl) of bleomycin (10 mg/kg) or phosphate buffered saline (PBS) (control) were injected subcutaneously on the shaved backs of the mice (n = 5/group) once every other day for 28 days. Mice were euthanized 24 hours after the final injection, and skins were harvested and processed for histologic and immunostaining analyses. Skin samples were snap frozen in liquid nitrogen, embedded in optimal cutting temperature compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands), and stored at −80 °C. Cryostat sections (7 mm) were mounted on slides. Dermal and subcutaneous adipose layer thicknesses were determined by ImageJ software (NIH, Bethesda, MD) at five different sites from each mouse on Giemsa stained sections (Alfa Aesar, Ward Hill, MA). Skin sections were stained for visualization of collagen fibers using Masson's Trichrome staining kit (Sigma-Aldrich, Allentown, PA). Collagen accumulation was quantitated as percent blue-stained area. Total collagen content was determined with a biochemical assay (Sigma-Aldrich) based on hydroxyproline measurement (Rahaman et al., 2014). All quantitations were done using ImageJ software.

We performed double immunofluorescence staining of skin sections from patients with SSc or healthy subjects, and from bleomycin- or PBS-treated WT mice to identify α-smooth muscle actin (α-SMA)-positive myofibroblasts expressing TRPV4 proteins (Rajkumar et al., 2005). After deparaffinize and rehydrating, sections were labeled with anti-α-SMA (1:50; Sigma-Aldrich), anti-TRPV4 (1:50;Alomone Labs, Israel), or isotype control IgG (Thermofisher, Waltham, MA). Immunofluorescence intensity was quantitated, and results were expressed as integrated density.

TRPV4 activation is known to promote α-SMA expression, a hallmark of myofibroblast differentiation (Rahaman et al., 2014). On the basis of this evidence, we hypothesized that TRPV4 was the driver of myofibroblast differentiation in SSc, and would be expressed in the skin of patients with SSc and the skin of SSc mice. Therefore, we examined the presence of TRPV4 in myofibroblasts in skin tissues of patients with SSc (Figure 1a and b) and in the SSc mouse model (Figure 1c and d) by immunofluorescence staining. We found an increased presence of α-SMA positive (α-SMA$^+$) myofibroblasts in the skin of patients with SSc that was consistent with previous findings (Rajkumar et al., 2005). In addition, as we hypothesized, α-SMA$^+$ myofibroblasts were also stained positive for TRPV4, indicating coexpression of TRPV4 and α-SMA in myofibroblasts in the skin of patients with SSc. We

detected 3.5-fold increased expression of TRPV4 proteins in the skin of patients with SSc compared with healthy controls (Figure 1b). Similarly, bleomycin injection induced a 4-fold increase in α-SMA$^+$ cells expressing TRPV4 in the skin of WT mice (Figure 1c and d) compared with PBS-treated mice. These results suggest a potential role for TRPV4 in the pathogenesis of SSc. We showed clinical parameters of the patients with SSc in Figure 1e. The specificity of the TRPV4 antibody (for mouse and human tissue) is shown by a western blot (Figure 1f) and immunofluorescence staining (Figure 1g), demonstrating that TRPV4 signal was completely abolished by preincubation of the TRPV4 antibody with its control peptide antigen before immunoblotting or immunofluorescence assays.

We next determined whether TRPV4 was required for skin fibrosis development by assessing the effect of bleomycin or PBS in TRPV4 KO mice and WT controls. In WT mice and to a lesser extent in TRPV4 KO mice, bleomycin administration effectively caused histologic (dermal thickness and subcutaneous fat), cellular (α-SMA$^+$ myofibroblasts), and molecular (collagen content) changes in the skin of treated mice versus PBS controls, as expected (Figure 2a-h). However, distinct histologic differences were seen in the skin of WT and TRPV4 KO mice after bleomycin treatment. Dermal thickness in bleomycin-treated WT mice was 50% greater than in bleomycin-treated TRPV4 KO mice (Figure 2b); subcutaneous fat accumulation was unchanged in bleomycin-treated versus PBS-treated TRPV4 KO mice, but was roughly 25% greater than in bleomycin-treated WT mice (Figure 2c); collagen deposition was roughly 3-fold greater in bleomycin-treated WT mice than in bleomycin-treated TRPV4 KO mice (Figure 2e and f); and myofibroblast abundance was roughly 4-fold greater in bleomycin-treated WT mice than in bleomycin-treated TRPV4 KO mice (Figure 2h). These results suggest that TRPV4 contributes to the development of skin fibrosis in the bleomycin-induced SSc mouse model.

Bleomycin-induced skin fibrosis is a well-recognized model of SSc and other fibrotic skin diseases (Castelino and Varga, 2014; Ho et al., 2014; Rubio-Rivas et al., 2014). Our data provide two key observations suggesting that TRPV4 may be associated with skin fibrosis development in SSc: (i) we found an increased presence of TRPV4-positive myofibroblasts in skin tissues of patients with SSc compared with normal controls; (ii) we found that genetic loss of TRPV4 in mice was protective against bleomycin-induced skin fibrosis. This latter finding is consistent with our recent report showing that TRPV4 is required for transforming growth factor-β1/matrix stiffness-induced lung fibroblast differentiation and pulmonary fibrosis development in mice (Rahaman et al., 2014). Our current data, although preliminary, identify a role of TRPV4 in skin diseases such as SSc. Fibroblasts may not be the only source of myofibroblasts in fibrosis. There is reasonable evidence suggesting that other cell types including epithelial cells and pericytes may differentiate into myofibroblasts during fibrosis (Rajkumar et al., 2005; Tanjore et al., 2009). Further studies are warranted to determine the mechanisms by which TRPV4 regulates fibrosis, and to evaluate the possibility of targeting TRPV4 therapeutically to ameliorate SSc and other fibrotic diseases.

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Abbreviations:

KO	knock-out
PBS	phosphate buffered saline
α-SMA	α-smooth muscle actin
SSc	scleroderma
TRP	transient receptor potential
TRPV4	transient receptor potential cation channel subfamily V member 4
WT	wild type

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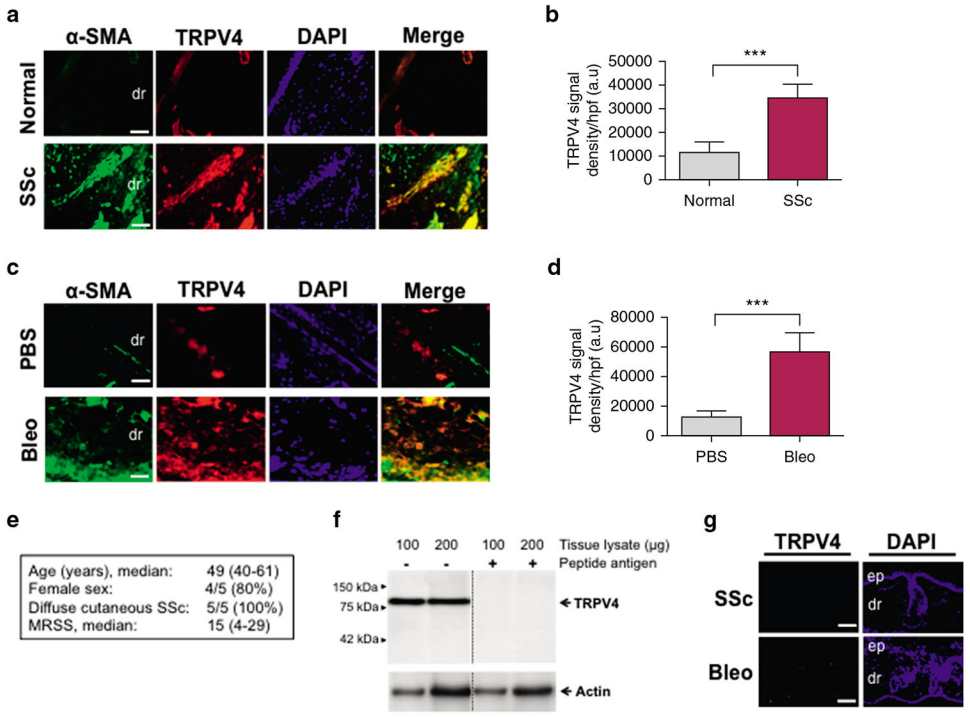

Figure 1. Overexpression and colocalization of TRPV4 and α-SMA in SSc surgical skin biopsies and in skin tissues in bleomycin-treated mice.

(**a**) Representative dual immunofluorescence staining of SSc and control normal skin sections for TRPV4 (red) and α-SMA (green); colocalized staining in merged images (yellow). Nuclei were stained with DAPI (blue). (**b**) Quantitation of TRPV4 staining results from SSc and normal samples in (**a**). Results shown are mean ± SEM (***$P < 0.001$, n = 5, *t*-test). (**c**) Immunofluorescence images of skin sections from WT mice treated subcutaneously with bleomycin or PBS for 28 days. Sections were costained with α-SMA (green) and TRPV4 (red) IgG as above, visualized with Alexa Fluor-conjugated secondary IgG, and nuclei were stained with DAPI. (**d**) Quantitation of TRPV4 staining results from (**c**). Results shown are mean ± SEM (***$P < 0.001$, n = 5, *t*-test). Scale bar = 100 μm. (**e**) Demographic and clinical characteristics of patients with SSc (n = 5). (**f**) TRPV4 signal in fibrotic mouse skin tissue lysates was completely abolished by preincubation of the TRPV4 antibody with its control peptide antigen before immunoblotting, whereas control actin signal was unaffected. (**g**) TRPV4 immunofluorescence signal was totally blocked in human SSc skin tissue sections immunostained with TRPV4 antibody that had been preincubated with its control peptide antigen. Epidermis (ep), and dermis (dr). hpf, high-power field; MRSS, modified Rodnan skin score; PBS, phosphate buffered saline; SEM, standard error of the mean; α-SMA, α-smooth muscle actin; SSc, scleroderma; TRPV4, transient receptor potential cation channel subfamily V member 4; WT, wild type.

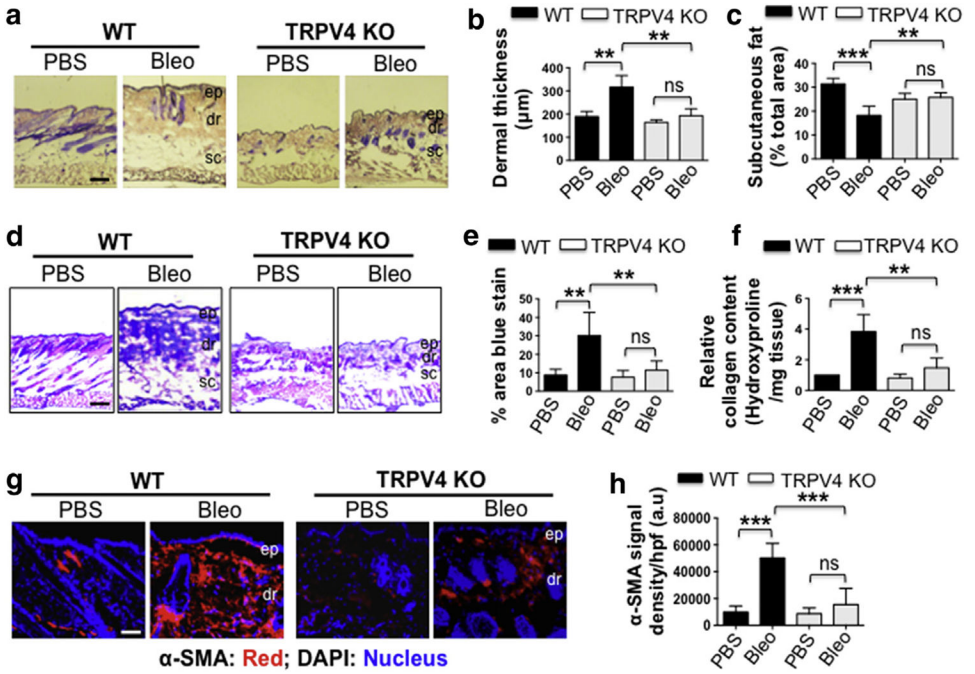

Figure 2. TRPV4 KO mice are protected from bleomycin-induced skin fibrosis in vivo.

Representative images of sections of skin from WT and TRPV4 KO mice treated with bleomycin or PBS (subcutaneously) for 28 days. Sections were stained with Giemsa (**a**), and dermal thickness (**b**) and percent subcutaneous fat area (**c**) were quantified. Sections were stained with Masson's trichrome (**d**), and deposition of collagen (blue) was quantified (**e**). Relative collagen content of skin tissues was determined by hydroxyproline assay (**f**). Sections were stained with α-SMA antibody and visualized with Alexa Fluor 594 conjugated secondary IgG (red), and nuclei were stained with DAPI (blue) (**g**), and myofibroblast accumulation (α-SMA$^+$ cells) was quantified (**h**). Scale bar = 100 μm, n = 5 mice/group, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, *t*-test. Epidermis (ep), dermis (dr), and subcutaneous tissue (sc). KO, knock-out; ns, nonsignificant; PBS, phosphate buffered saline; α-SMA, α-smooth muscle actin; TRPV4, transient receptor potential cation channel subfamily V member 4; WT, wild type.