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Tight skin 2 mice exhibit a novel time line of events leading to increased extracellular matrix deposition and dermal fibrosis

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ABSTRACT

The tight skin 2 (Tsk2) mouse model of systemic sclerosis (SSc) has many features of the human disease including tight skin, fibrosis, extracellular matrix abnormalities, and reported antinuclear antibodies (ANA). Here we report that Tsk2/+ mice develop excess dermal fibrosis with age, as skin is not significantly fibrotic until 10 weeks, a full eight weeks after the development of the physical tight skin phenotype. Concomitantly with the tight skin phenotype at two weeks of age, Tsk2/+ mice demonstrate increased levels of total transforming growth factor beta 1 (TGF-β1) and excessive accumulation of dermal elastic fibers. The increase in elastic fibers is not responsible for tight skin, however, because Tsk2/+ mice genetically engineered to lack skin elastic fibers nevertheless have tight skin and fibrosis. Finally, about two months after the first measurable increases of total collagen, a portion of Tsk2/+ mice produce ANAs, but at a similar level to wild-type littermates. The timeline of disease development in the Tsk2/+ mouse shows that fibrosis is progressive, with elastic fiber alterations and TGF-β1 over-production occurring at least two months before *bona fide* fibrosis, that is not dependent on ANA production.

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disorder of unknown origin, characterized by excess accumulation of the extracellular matrix (ECM), primarily collagen, in skin and other internal organs, vascular alterations, and production of antinuclear antibodies (ANAs) (LeRoy et al., 1988; Okano, 1996). Recently, classification of SSc has been redefined. Patients presenting with "skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints" fulfills the new major criterion, and alone is enough for a diagnosis. If absent, a score is calculated based on seven additional criteria, and if patients score at or above the threshold (a score of 9), they are classified as having SSc (van den Hoogen et al., 2013). SSc is divided into two major subsets based on total skin involvement. Limited cutaneous SSc involves dermal thickening of skin below the elbows and knees, with or without facial involvement, while diffuse cutaneous SSc manifests as dermal thickening of skin in the extremities, face, and trunk, as well as involvement of at least one internal organ, including the lungs, kidneys, heart, esophagus, and gastrointestinal tract (LeRoy et al., 1988). Survival rates vary significantly between and within subsets of disease, and are usually determined by the severity of the internal organ involvement (Ferri et al., 2002; Scussel-Lonzetti et al., 2002; Steen, 2005). Currently, there are no effective treatments for this disease.

There are several animal models of disease, but while mouse models have proved useful in studying various clinical features, no one mouse model exhibits all the signs of SSc (Artlett, 2010; Yamamoto, 2010). Two main genetic mouse models of disease are the tight skin (Tsk) 1 (Green et al., 1976) mouse and the Tsk2 (Peters and Ball, 1986). Each mouse strain bears a different homozygous lethal mutation that requires mice to be bred and evaluated as heterozygotes (e.g., Tsk1/+ and Tsk2/+). The cause of disease in Tsk1/+ mice is a mutation in the fibrillin 1 gene on chromosome 2 (Siracusa et al., 1996), whereas the cause of scleroderma-like signs in Tsk2/+ mice is a mutation in the collagen type III, alpha I gene (*Col3a1*) on chromosome 1 (Long, unpublished observations). The Tsk1/+ model, while valuable, has several disease signs that differ from SSc, including hypodermal collagen accumulation (Baxter et al., 2005) and an emphysema-like lung pathology (Szapiel et al., 1981).

By contrast, Tsk2/+ mice have increased collagen and ECM changes in the dermis. However, the timing of these changes is controversial. Studies by Christner et al. demonstrate a marked increase in collagen accumulation in the dermis of 10 day old Tsk2/+ mice, which was still present at seven to eight months of age compared to wild-type (WT) littermates. In addition, they observed a mononuclear inflammatory cell

Abbreviations: ANA, antinuclear antibody; αSMA, alpha smooth muscle actin; B6, C57Bl/6J; Col1a1, collagen, type 1, alpha 1; Col3a1, collagen, type 3, alpha 1; Fbln5, fibulin 5; SSc, systemic sclerosis; TGF-β1, transforming growth factor beta-1; Tsk, tight skin; WT, wild-type.

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infiltrate (Christner et al., 1995). However, a subsequent report failed to confirm these findings (Barisic-Dujmovic et al., 2008). Christner et al. showed increased transcription of *Col1a1* and *Col3a1* and up-regulation of collagen production in fibroblasts isolated from Tsk2/+ mice of unstated sex and age (Christner et al., 1998). In agreement, Barisic-Dujmovic et al. (2008) showed that cultured Tsk2/+ fibroblasts had increased expression of a collagen I-promoter-driven GFP reporter compared to control WT fibroblasts, but together these findings do not address the timing of up-regulation of collagen in vivo. This group also noted that Tsk2/+ mice have decreased dermal adipose tissue, a significant reduction in body size and mass, yet normal lung morphology at two to three months of age (Barisic-Dujmovic et al., 2008). Finally, studies by Gentiletti et al. (2005) reported that aged Tsk2/+ mice, on a C3HxC57Bl/6J background, produce numerous antinuclear antibodies (ANAs) that are also observed in SSc.

In the present study, we examined disease pathology and progression over time in Tsk2/+ male and female mice bred to a standard C57Bl/6J (B6) background. We report a novel timeline of events in Tsk2/+ disease development and show that the signs of fibrotic disease are progressive, starting from two weeks of age. Our study also demonstrates that Tsk2/+ mice have excessive elastic fiber accumulation at two weeks; however, analyses of fibulin-5 knockout mice (with a defect in skin elastic fiber formation) demonstrate that elastic fibers are not responsible for the tight skin phenotype or later fibrosis. Also at this age, increased levels of TGF-β1 were observed, and eight weeks later measurable increases in total collagen protein levels were detected, suggesting that the slow accumulation of collagen is TGF-β1 dependent. In addition, we demonstrate that ANAs are present in Tsk2/+ mice only after disease is well established.

2. Results

2.1. Footpad thickness predicts the tight skin phenotype in Tsk2/+ mice

The tight skin phenotype in Tsk2/+ mice is physically evident and felt upon pinching the interscapular skin of young mice as early as two weeks of age (Christner et al., 1995), but this assessment is variable and subjective (Baxter et al., 2005). To provide a more quantitative assay for the Tsk2/+ trait, we assessed the thickness of mouse footpads corrected for body weight. Body weight must be factored due to changes in weight that characterize normal mouse development, weight differences between male and female mice, or between members of different litters. For all ages, weight differences were observed between male and female mice ($p < 0.0001$), as well as between same-sex Tsk2/+ vs. WT mice ($p < 0.001$); mice carrying the *Tsk2* mutation were noted to weigh significantly less than their WT littermates (Fig. 1A) (Barisic-Dujmovic et al., 2008). Footpad thicknesses were highly significantly associated with inheritance of the *Tsk2* gene, with low variance and excellent reproducibility (Fig. 1B and C). At four to six weeks of age, Tsk2/+ male and female mice have significantly thicker footpads than WT mice

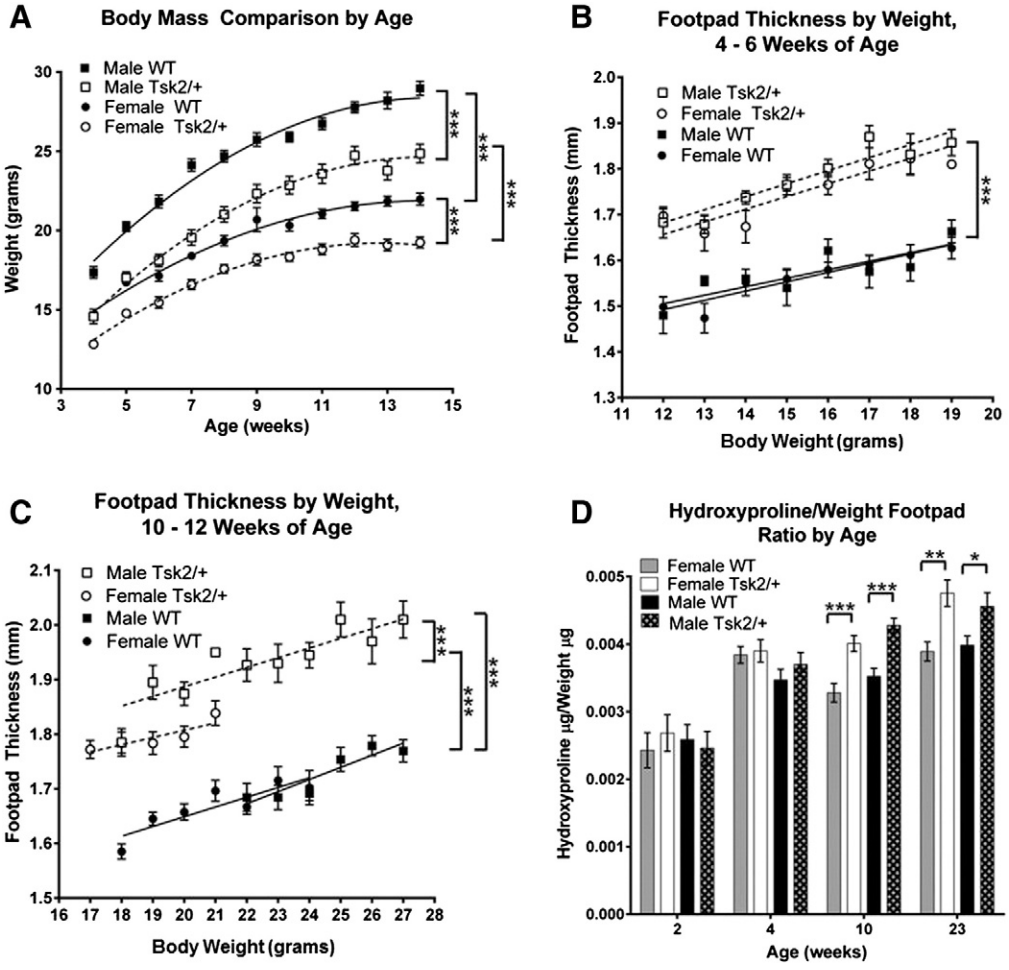

Fig. 1. Tsk2/+ mice have smaller bodies but thicker footpads. Body mass and footpad thickness comparison by age between Tsk2/+ and WT littermates (A–C). Mice were weighed and footpad thickness was measured weekly. A, Body mass was compared directly over time. B and C, Footpad thickness was binned by weight and compared by ages four to six weeks (B) and 10 to 12 weeks (C). Hydroxyproline content of the footpad by age between Tsk2/+ and WT littermates. D, Hydroxyproline (μg)/total weight (μg) was determined from footpads. A–C, n > 32 mice per group; D, n = 9–12 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.