Collagen Dysregulation in the Dermis of the *Sagg*/+ Mouse: A Loose Skin Model

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The *Sagg*/+ mouse is an ethylnitrosourea-derived mutant with a dermal phenotype similar to some of the subtypes of Ehlers–Danlos syndrome (EDS) and cutis laxa. The dermis of the *Sagg*/+ mouse has less dense and more disorganized collagen fibers compared to controls. The size of extracted Type I dermal collagen was the same as that observed in normal skin; however, more collagen could be extracted from *Sagg*/+ skin, which also showed decreased collagen content and decreased steady-state levels of α1(I), α2(I), α1(V), and α2(V) procollagen mRNAs. The biomechanical properties of *Sagg*/+ skin were significantly decreased relative to normal skin. However, there were no significant differences in the quantities of the major collagen cross-links, that is, dehydrohydroxylysinonorleucine and dehydrohistidinohydroxymerodesmosine between *Sagg*/+ and normal skin. Electron microscopic evaluation of *Sagg*/+ skin indicated that the mutation interferes with the proper formation of collagen fibrils and the data are consistent with a mutation in Type V collagen leading to haploinsufficiency with the formation of two sub-populations of collagen fibrils, one normal and one with irregular shape and a larger diameter. Further study of this novel mutation will allow the identification of new mechanisms involved in the regulation of normal and pathologic collagen gene expression.

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INTRODUCTION

Animal models have proven to be of great value in enhancing the understanding of biologically complex systems. They have facilitated the elucidation of the pathogenesis of numerous diseases, and have provided the means to test potentially useful therapeutic interventions. The *Sagg* mutation appeared in the offspring of a mouse that had been administered ethylnitrosourea and was named *Sagg* because of the saggy or loose skin observed in affected animals (Nolan *et al.*, 2000). The mutation is inherited in an autosomal dominant manner and is located on proximal chromosome 1 (Nolan *et al.*, 2000). No other studies have been reported on *Sagg*. The occurrence of a novel mutation that results in loose skin is of relevance to several connective tissue diseases such as Ehlers–Danlos syndrome (EDS) and cutis laxa.

EDS is a collection of diseases that affect approximately one in 5,000 live births, including men and women of all racial and ethnic groups. EDS comprises a heterogeneous group of hereditary connective tissue disorders, characterized by hyperelasticity of skin and hypermobility of joints to differing extents. The skin is easily injured and wound healing is delayed (Steinmann *et al.*, 2002). The molecular causes of this disorder are often, although not strictly, related to collagens and to the enzymes that process these proteins. The majority of EDS patients belong to EDS types I–III. Using the classification of Beighton *et al.* (1998), the classical forms of EDS, types I and II, can be caused by mutations in collagen V, whereas the cause of EDS type III (hypermobility type) is not known. The vascular type of EDS is caused by mutations in collagen type III (Pope *et al.*, 1977; Superti-Furga *et al.*, 1989); the kyphoscoliosis type of EDS is caused by mutations in lysyl hydroxylase-1 (Yeowell and Walker, 2000); the arthrochalasia type of EDS is caused by mutations in type I procollagen (Wirtz *et al.*, 1987); and the dermatosparaxis type of EDS is caused by mutations in the procollagen-*N*-peptidase (De Paepe and Malfait, 2004). Tenascin-X has also been demonstrated to be involved in some forms of EDS (Zweers *et al.*, 2004; Lindor and Bristow, 2005). Moreover, gene disruption of several other matrix molecules, thrombospondin, SPARC (secretory protein acidic and rich in cysteine), small leucine-rich proteoglycans, and others in mice, leads to phenotypes that mimic EDS, and these molecules have thus emerged as new players (Jepsen *et al.*, 2002; Fichard *et al.*, 2003).

Cutis laxa is a set of connective tissue diseases of the skin characterized by sagging skin, premature wrinkling, and reduced skin elasticity (Uitto and Shamban, 1987; Boulac

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Abbreviation: EDS, Ehlers–Danlos syndrome

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et al., 1999; Zlotogora, 1999). It can occur both as an acquired (Boulac *et al.*, 1999; Ozkan *et al.*, 1999; Braakenburg and Nicolai, 2000) and as a genetic disease (Damkier *et al.*, 1991; Imaizumi *et al.*, 1994; Rybojad *et al.*, 1999), and can be accompanied by marfanoid symptoms (Larregue *et al.*, 1990), pulmonary artery stenosis (Hayden *et al.*, 1968) skin webbing (O'Brien *et al.*, 1970), cardiac valve involvement (Baldwin *et al.*, 2001), osteoma cutis (Choi *et al.*, 2000), loss of psychomotor abilities (Shintaku *et al.*, 2000), and joint hypermobility (Robertson and Bankier, 1999). A specific X-linked form of cutis laxa also was classified as one of the rare EDS (Mentzel *et al.*, 1999). Molecular abnormalities identified to date include chromosome 16 abnormalities (Garson *et al.*, 1970) diminished elastin in the skin with an accompanying increase in the fibrillar components of the elastic fibers (Kitano *et al.*, 1989), increased elastase activity (Boulac *et al.*, 1999), and increased collagenase activity, leading to a decrease in collagen (Hatamochi *et al.*, 1991).

Although there are several genes that have been identified as causative for these diseases, only the wrinkly skin syndrome form of cutis laxa (Hurvitz *et al*, 1990; Zlotogora, 1999) and Ehlers–Danlos forms I, II, and IV (De Paepe and Malfait, 2004) have been linked to mutations in genes for which the mouse orthologs reside in the identified *Sagg* interval on the proximal arm of mouse chromosome 1 (Nolan *et al.*, 2000).

RESULTS

Gross appearance

The *Sagg*/+ mice had normal growth, development, and survival. The *Sagg*/+ mice were first recognizable at 2–3 months of age by the looseness of their skin in the interscapular region. When each mouse was picked up with forceps from the interscapular region of the back, the distance the *Sagg*/+ mice hang below the forceps is greater than that for normal mice (Figure 1). For the experiments reported here, only 3-month-old mice were investigated and both phenotypic and simple sequence length polymorphism (SSLP) analyses were performed on each mouse.

Histologic examination

Histologic examination of skin samples from 3-month-old normal mice (Figure 2a; ×100) compared to skin samples from *Sagg*/+ mice (Figure 2b; ×100) showed a thinning of the deep dermis near the panniculus carnosus. At ×400 magnification, the *Sagg*/+ dermis shows significant disorganization of the collagen fibers (Figure 2d) that are less tightly packed and have clearly evident spaces. The difference in the staining intensity with the Mason's trichrome stain is striking at this higher magnification (Figure 2c vs d). Sections of *Sagg*/+ dermis stained less intensely than the normal (not as dark blue) in over 20 different sections (10 *Sagg*/+ and 10 normal), suggesting that the fibers and fibrils are less densely packed.

Collagen content of skin

Hydroxyproline content of skin biopsy punches obtained from eight *Sagg*/+ and five age-matched normal mice was determined. On average, the hydroxyproline content of the dermis of *Sagg*/+ mice was approximately one-third that found in the dermis of normal mice, 23±9 vs 72±10 μg hydroxyproline per mg dry weight, $P<0.005$ (Table 1).

The quantitative or qualitative differences in the populations of the collagenous proteins of normal and *Sagg*/+ skin were measured by sequential extraction with neutral-salt buffer and 0.5 M acetic acid. The neutral-salt extract from *Sagg*/+ and control mice showed identical collagen species when electrophoresed by SDS-PAGE, indicating there were no qualitative differences (Figure 3). Similar results were observed for the acid extract (results not shown). Quantitative differences between extractable dermal collagen from *Sagg*/+ and control mice were determined using a constant ratio of extraction buffer to wet weight of the skin and

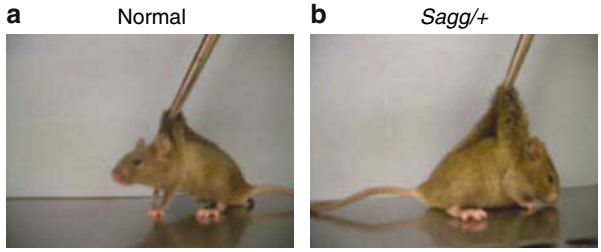

Figure 1. Photograph of a (a) normal and (b) *Sagg*/+ mouse showing the difference in the ability of the skin to be stretched. Notice that the normal mouse has been lifted off its front feet, whereas the *Sagg*/+ mouse has not, and even with the increased pull on the normal mouse, more skin can be drawn away from the *Sagg*/+ mouse.

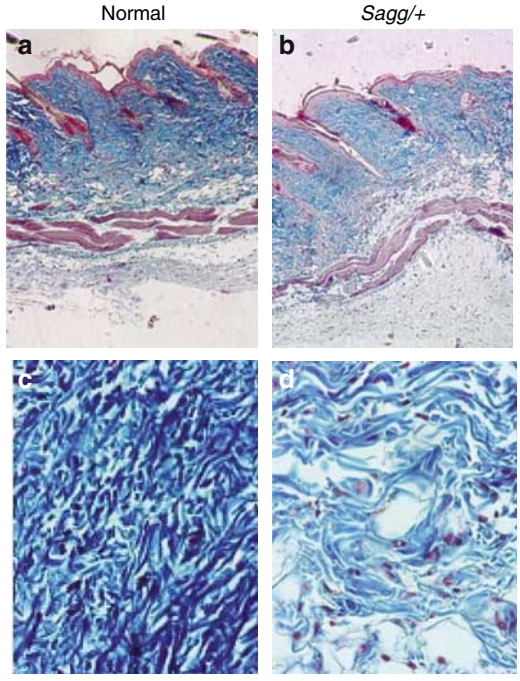

Figure 2. Light photomicrographs of skin sections from 3-month-old (a), (c) normal and (b), (d) *Sagg*/+ mice stained with Mason's trichrome. Note the marked decrease in blue-staining collagen fibers in the skin from *Sagg*/+ mice compared with normal mice. Top panels are at original magnification ×100 and bottom panels are at original magnification ×400.

Table 1. Hydroxyproline content of skin samples from *Sagg*/+ mice and normal littermates

	μg hydroxyproline/mg dry weight	
Mouse	Normal	*Sagg*/+
1	51.5	18.4
	61.4	18.9
2	69.7	18.9
	69.9	18.9
3	73.2	18.9
	75.4	18.9
4	77.0	19.0
	77.2	19.0
5	82.9	19.0
	83.0	19.0
6		19.1
		19.2
7		20.7
		35.7
8		41.7
		46.9
AVG	72.1	23.3
SD	9.7	9.2

Duplicate 4-mm-diameter biopsy samples were obtained from the interscapular region of each animal. Following lyophilization to determine their dry weight, samples were hydrolyzed and their hydroxyproline content determined by the method of Woessner (1961). The average (AVG) difference between the results of *Sagg*/+ skin and normal is significant to $P<0.005$ by the Student's *t*-test.

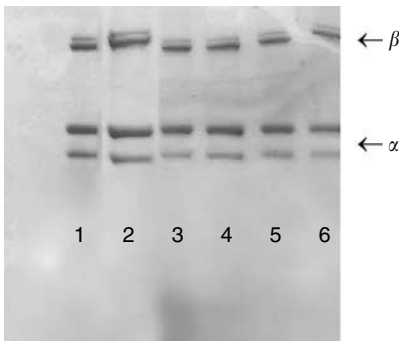

Figure 3. SDS-PAGE of Coomassie blue stained salt extracts from the skin of (lanes 1–3) normal and (lanes 4–6) *Sagg*/+ mice. Fifty micrograms of protein were electrophoresed in each lane. An arrow indicates the positions of the collagen α- and β-chains.

electrophoresing equal aliquots of extracts. The results demonstrate that the *Sagg*/+ dermis had more extractable collagen than the controls (Figure 4).

A densitometric comparison of the α1 and α2 bands indicated that approximately 8- and 2-fold more collagen

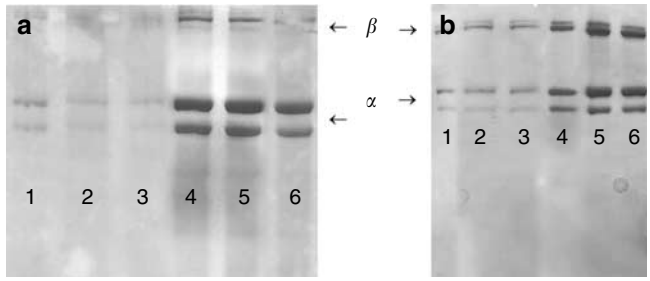

Figure 4. SDS-PAGE of Coomassie blue-stained salt extracts from the skin of (lanes 1–3) normal and (lanes 4–6) *Sagg*/+ mice. Ten microliters of extract were electrophoresed in each lane. (**a**) Salt extract and (**b**) acetic acid extract. Extractions were performed at a fixed ratio of 1 ml of extraction medium per 100 mg (wet weight) of skin. An arrow indicates the positions of the collagen α- and β-chains.

was extractable from *Sagg*/+ skin with neutral-salt buffer and acetic acid, respectively, compared to controls. The increased extractability of the collagen from the skin of *Sagg*/+ mice suggests possible differences in the collagen cross-linking.

Collagen cross-linking

A cross-link analysis was performed on the skins of each type of mouse. The major collagen cross-links of both Sagg/+ and control mice were reducible cross-links, dehydrohydroxylysinonorleucine and dehydrohistidinohydroxymerodesmosine. The non-reducible, mature cross-links such as pyridinoline or histidino-hydroxylysinonorleucine (Yamauchi *et al.*, 1987) were under detectable levels. The results demonstrate that there were no significant differences in the collagen cross-linking between *Sagg*/+ and control (+/+) mice (Table 2).

Steady-state levels of α1(I) procollagen mRNA in *Sagg*/+ skin

The steady-state levels of α1(I) procollagen mRNA in skin samples of *Sagg*/+ and normal littermate controls were compared by Northern hybridizations or real-time PCR. The Northern analyses employed a species-specific cDNA. The levels of α1(I) procollagen transcripts were substantially lower in *Sagg*/+ skin than in normal mouse skin (Figure 5). The decrease in α1(I) procollagen mRNA in *Sagg*/+ mouse skin was consistent in repeated analyses of RNA obtained from the same mouse; however, there was some variability in the level of decrease between individual mice (Figure 5).

The results of a semiquantitative analysis of the Northern blots by densitometry followed by correction for loading and transfer of RNA by hybridization to a rat glyceraldehyde-3-phosphate dehydrogenase cDNA indicated that the steady-state level of α1(I) procollagen mRNA transcripts in *Sagg*/+ dermal fibroblasts was 23±14% of that found in normal dermal fibroblasts.

Additional experiments were performed with real-time PCR to determine the message levels for *Col1a2*, *Col3a1*, *Col5a1*, and *Col5a2*. The results were normalized to β-actin and are expressed as a percentage of the message levels

Table 2. Quantitation of cross-links in *Sagg*/+ and normal mouse skin

Mouse	deH-HLNL	Mouse	deH-HLNL
+/+	0.060	*Sagg*/+	0.045
+/+	0.037	*Sagg*/+	0.043
+/+	0.059	*Sagg*/+	0.052
+/+	0.043	*Sagg*/+	0.047
+/+	0.042		
AVG	0.048	AVG	0.047
SD	0.011	SD	0.004

Mouse	deH-HHMD	Mouse	deH-HHMD
+/+	0.403	*Sagg*/+	0.276
+/+	0.334	*Sagg*/+	0.252
+/+	0.122	*Sagg*/+	0.205
+/+	0.187	*Sagg*/+	0.252
+/+	0.151		
AVG	0.240		0.246
SD	0.122		0.030

Skin samples weighing approximately 2 mg were analyzed from five control (+/+) and four *Sagg*/+ mice to determine the amount of the collagen cross-links: dehydrohydroxylysinonorleucine (deH-HLNL) and dehydrohistidinohydroxymerodesmosine (deH-HHMD). The quantities of the cross-links are expressed as mol/mol of collagen using a value of 300 residues of hydroxyproline per collagen molecule. The average (AVG) values and standard deviations (SD) for the amount of each type of cross-link for each of the two groups of mice are shown. There was no statistically significant difference between +/+ and *Sagg*/+ skin for either type of cross-link.

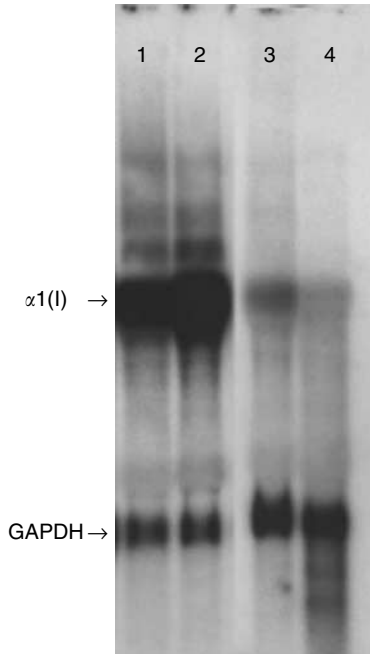

Figure 5. Northern hybridization analysis of total RNA extracted from the skin of (lanes 1 and 2) normal and (lanes 3 and 4) *Sagg*/+ mice. The blot was hybridized with a rat α1(I) procollagen cDNA and with a cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The positions of the α1(I) procollagen and GAPDH transcripts are shown.

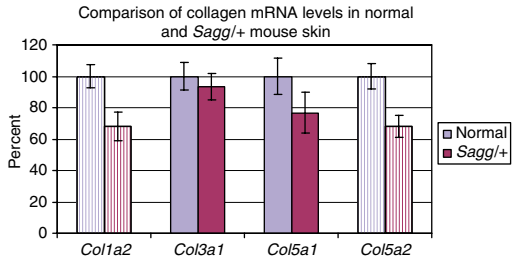

Figure 6. Results of a real-time PCR, which amplified *Col1a2*, *Col3a1*, *Col5a1*, and *Col5a2*. The relative expression of mRNA extracted from the skin of normal and *Sagg*/+ age- and sex-matched mice was normalized to β-actin and then expressed as a percent of the value obtained for normal skin. Error bars indicate ±1 standard deviation. The differences between normal and *Sagg*/+ message levels for *Col1a2* and *Col5a2* were significant at $P<0.05$ and are highlighted as striped bars.

detected in the normal skin of age- and sex-matched mice (Figure 6). The results show that the message levels for each of the above four genes in the skin of *Sagg*/+ mice were 67, 94, 78, and 68%, respectively, of the levels observed in normal control mouse skin. The differences in the values for *Col1a2* and *Col5a2* between normal and *Sagg*/+ skin were significant at $P<0.05$ and are highlighted in Figure 6 as striped bars.

Electron microscopic examination of *Sagg*/+ skin

Electron microscopic studies were performed on the dermis of *Sagg*/+ mice and normal age-matched controls. The dermis of the control animals was composed of a relatively homogeneous population of cylindrical collagen fibrils (Figure 7a). In contrast, the dermis of the *Sagg*/+ mice contained a population of cylindrical fibrils that were comparable to those seen in the dermis of control mice and a second population of structurally abnormal fibrils (Figure 7b). These fibrils were considerably larger than the fibrils with circular cross-sectional profiles and had irregular contours (Figure 7b, arrows).

Morphometric differences between *Sagg*/+ skin and normal skin

In an attempt to quantify this difference observed between *Sagg*/+ and normal skin at the electron microscopic level, the number of fibrils present in twenty 1,200 by 1,200 nm squares was counted from five normal and five *Sagg*/+ mice. The average of the total number of fibrils in the normal skin was 72, whereas the number observed in the *Sagg*/+ skin was 35. The *Sagg*/+ mouse skin had approximately one-half the number of fibrils per unit area compared to the normal skin. In addition, the normal fibrils never exceeded 150 nm in diameter, whereas there were at least 10 abnormal *Sagg*/+

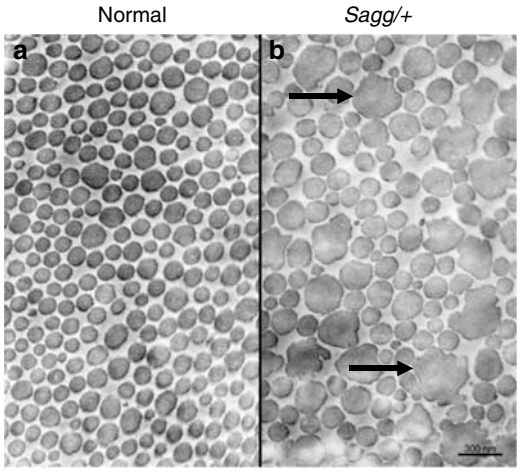

Figure 7. Electron micrograph of the dermis from a (a) normal and (b) a *Sagg*/+ mouse in cross-section. The collagen fibrils are clearly evident. Several of the strikingly irregular fibrils from the *Sagg*/+ dermis (**b**) are marked with an arrow.

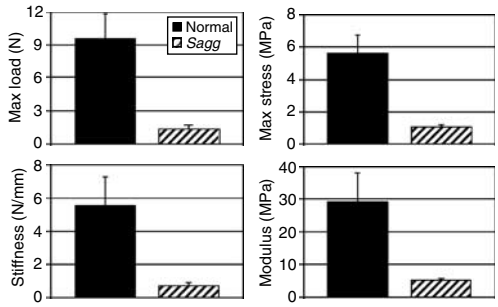

Figure 8. Mechanical properties of *Sagg*/+ and normal mouse skin. Significant differences were found between normal and *Sagg*/+ mice for maximum load ($P=0.0001$), maximum stress ($P<0.0001$), stiffness ($P<0.001$), and modulus ($P<0.001$).

fibrils (Figure 7b) with a diameter exceeding 300 nm and one fibril that exceeded 450 nm in diameter.

Differences in mechanical properties of *Sagg*/+ and normal skin

Tensile tests were carried out to determine if the differences observed in the amount of collagen (Figures 1 and 4) as well as the differences observed in the fibril architecture (Figure 7) had an effect on the material and structural properties of *Sagg*/+ skin. Normal mouse skin was found to be consistently and significantly higher than *Sagg*/+ skin for maximum load ($P=0.0001$), maximum stress ($P<0.0001$), stiffness ($P<0.001$), and modulus ($P<0.001$) (see Figure 8).

DISCUSSION

The work reported here is the first step in characterizing the *Sagg*/+ mouse phenotype with the eventual goal of identifying the responsible mutation and understanding the mechanisms whereby it leads to decreased collagen accumulation as well as aberrant collagen fibril size and shape. A wider consequence of these studies is that they will lead to a better understanding of the complex regulatory mechanisms controlling the expression of the collagen gene(s) under both normal and altered pathologic conditions.

Histologic examination of skin samples from *Sagg*/+ mice showed marked thinning of the dermis and depleted deposition of collagen compared to controls. Patients with cutis laxa have been reported to have similar changes in their collagen fibers; however, when present, they are believed to be a secondary result caused by the abnormalities in the elastic fiber (Uitto and Pulkkinen, 2002). There are also reports of increased collagen mRNA (Jung *et al.*, 1996), and increased collagen synthesis (Taieb *et al.*, 1987), decreased size of the collagen α-chains (Tsukahara *et al.*, 1988) and increased collagenase (Hatamochi *et al.*, 1991). These different findings are most likely due to the fact that cutis laxa is comprised of several connective tissue diseases (Hayden *et al.*, 1968; O'Brien *et al.*, 1970; Larregue *et al.*, 1990; Robertson and Bankier, 1999; Mentzel *et al.*, 1999; Choi *et al.*, 2000; Shintaku *et al.*, 2000; Baldwin *et al.*, 2001). Wrinkly skin syndrome, a type of cutis laxa (Zlotogora, 1999) has been localized to chromosome 2q32 (Kreutz and Wittwer, 1993), which contains the genes *COL3A1* and *COL5A2* and is syntenic to the region of chromosome 1 where these collagen genes and *Sagg* are located in the mouse (Christner *et al.*, 1996; Nolan *et al.*, 2000). *COL3A1* and *COL5A2* have been suggested as candidate genes for wrinkly skin syndrome (Kreutz and Wittwer, 1993) and a mutation in either of the above collagen genes may be the cause of wrinkly skin syndrome. The work of Nolan *et al.* (2000) that mapped the *Sagg* mutation to proximal chromosome 1 makes *Col3a1* and *Col5a2* both reasonable candidate genes for *Sagg*. However, *Col3a1* seems less likely to be the candidate gene, because mutations in COL3A1 have been reported to cause vascular involvement (Giunta and Steinmann, 2000), which is absent in *Sagg*/+ mice. The *Sagg*/+ mouse did not show any visceral changes by histological examination (data not shown). In addition, we also found decreased collagen content and lowered steady-state levels of α1(I), α2(I), α1(V), and α2(V), procollagen mRNA in the *Sagg*/+ mouse skin compared to that found in the skin from normal mice, but no significant decrease in α1(III) message levels.

The biomechanical properties of the *Sagg*/+ skin were markedly different from those of the normal skin, indicating that the difference in collagen content and architecture does indeed affect the strength and elasticity of the *Sagg*/+ skin. Electron microscopic analyses demonstrated the presence of numerous irregular oversized collagen fibrils, suggesting the possibility that the collagen of these fibrils is abnormal or that another extracellular matrix component protein associated with the collagen fibrils might be abnormal. This conclusion is supported by the fact that the collagen in the *Sagg*/+ dermis is more easily extracted with both salt and acid. Although the above findings seen in *Sagg*/+ mice, that is, impaired mechanical properties and increased extractability of skin collagen, would indicate a defective cross-linking profile in these mice, there are no significant differences in

collagen cross-links between *Sagg* and control mice (Table 2). These data indicate that neither the gene expression nor the activity of lysyl oxidase is significantly altered in *Sagg*/+ mice. A likely explanation for these data is that the saggy skin phenotype and the hyperextractability of the collagen from the skin are due to the aberrant type I collagen fibrillogenesis, which is clearly seen in Figure 7.

Type V collagen is known to mediate the initiation and formation of collagen fibrils and is a key determinant in the assembly of tissue-specific matrices (Birk *et al.*, 1988; Birk *et al.*, 1990; Marchant *et al.*, 1996; Wenstrup *et al.*, 2004a, b; Birk, 2001). A mutation in this molecule would explain the phenotype reported here for *Sagg*/+ mice. Wenstrup *et al.* (2004a) reported that *Col5a1*+/− mice had a reduction in both fibril content and dermal collagen content. These findings are similar to what we report for the *Sagg*/+ mice. In addition, the electron micrographs shown by Wenstrup *et al.* (2004a) are almost identical to those we show for *Sagg*/+ mice. Both the *Col5a1* heterozygote and the *Sagg*/+ mouse have numerous irregular fibrils, the irregular shape is similar in both mutants, and they are similarly increased in size. *Sagg* cannot be a mutation in *Col5a1* as that gene lies on another chromosome. However, *Col5a2* is located within the *Sagg* interval on chromosome 1 (Nolan *et al.*, 2000). We favor the hypothesis that *Sagg* is a mutation in the *Col5a2* gene, because the collagen type I fibril abnormalities observed in *Sagg*/+ mice are similar to that reported by Wenstrup *et al.* (2004a, b) and because the *Sagg*/+ mouse shares some of the features of a complex mouse model reported by Andrikopoulos *et al.* (1995) and Chanut-Delalande *et al.* (2004) with a mutation in *Col5a2*. Studies are currently underway to determine whether *Sagg* is the result of a mutation in *Col5a2*.

MATERIALS AND METHODS

Animals

Breeding pairs of 3-month-old *Sagg*/+ mice from the MRC Mammalian Genetics Unit (Harwell, UK) were housed in a fully accredited AALAC (American Association for Accreditation of Laboratory Animal Care) facility and the Thomas Jefferson University's institutional animal care and use committee approved all experiments. The mutation arose on the BALB/cJ background and was received on hybrid (C3H/HeJ × BALB/cJ) F1 mice. Animals used for the experiment had been crossed at least five generations onto C3H/HeJ (Jackson Laboratories, Bar Harbor, ME). *Sagg*/+ and matched normal littermates $3 \pm \frac{1}{4}$ months of age were used. All mice were classified as either *Sagg* or normal by SSLP analyses of *D1Mit18* and by the phenotypic procedure described in the Results section. All mice described in these experiments were male, because the level of penetrance for the *Sagg* phenotype was more consistent in males than in females, although equal numbers of males and females were affected.

Histology

For histopathologic studies, full-thickness samples of skin, including subcutaneous tissue and deep fascia, were obtained from 10 *Sagg*/+ mice and 10 age-matched +/+ mice at 3 months of age. Samples were processed and stained with hematoxylin–eosin and Masson's trichrome (Fisher, Philadelphia, PA) as described previously (Jiménez *et al.*, 1984).

Transmission electron microscopy

Subscapular skin from male animals was used in these experiments. Transmission electron microscopy was carried out as described previously (Birk and Trelstad, 1984; Birk *et al.*, 1997). Sections were examined and photographed at 75 kV using a Hitachi 7000 transmission electron microscope (Schaumburg, IL).

Quantitative collagen assays

To measure the collagen content of skin, equal diameter skin samples were obtained from the interscapular region of each mouse with a 4 mm disposable biopsy punch (Chester A Baker Laboratories, Miami, FL) following depilation with Nair (Shop-Rite, Philadelphia, PA). Hydroxyproline determination was performed according to the procedure of Woessner (1961). Total protein content of the hydrolysate was determined by either a Ninhydrin assay (Jiménez and Bashey, 1978) or a bicinchoninic acid assay (Smith *et al.*, 1985).

Collagen extraction and gel electrophoresis

For extraction of skin collagen, approximately equal samples of interscapular skin from normal and *Sagg*/+ mice were obtained after depilation with Nair. The samples were extracted with 1.0 M NaCl at 4°C with shaking for 5 days. The ratio of skin (wet weight) to extraction buffer was 100 mg/ml of extract. After 5 days, the skin samples were removed from the salt extract and further extracted with the same volume of 0.5 M acetic acid at 4°C for an additional 5 days. The extracted collagen from the skin of normal and *Sagg*/+ mice was analyzed by polyacrylamide slab gel electrophoresis according to the method of Laemmli (1970). Sample buffer contained 1% SDS, 1% β-mercaptoethanol, and electrophoresis was performed using 10% gels, which were stained with Coomassie blue (Fisher, Philadelphia, PA).

RNA extraction

Total RNA from skin was obtained using the acid–guanidium-thiocyanate–phenol–chloroform method of Chomcyznski and Sacchi (1987). Briefly, tissues were homogenized with a Tekmar polytron (Tekmar-Dohrmann, Mason, OH) and particulate material removed by centrifugation. RNA purity was assessed by the 260/280 nm absorbance ratio and the amount of RNA was calculated using the conversion factor of 40 μg RNA per optical density unit at 260 nm.

Northern hybridizations

Northern hybridizations of total RNA were performed according to the method of Sambrook *et al.* (1989). Samples of total RNA obtained from normal and *Sagg*/+ mouse skins were hybridized with ^{32}P-labeled murine α1(I) procollagen cDNA (French *et al.*, 1985) or glyceraldehyde-3-phosphate dehydrogenase cDNA (Fort *et al.*, 1985). After hybridizations, the filters were washed, air-dried, exposed to X-ray film with an intensifying screen, and scanned on a Joyce-Loebl densitometer (Gateshead, UK).

Real-time PCR

RNA was prepared as above and then used as template to produce cDNA with SuperScript III First Strand kit (Invitrogen, Carlsbad, CA). Probes were designed using Primer Express 2.0v software (Applied

Bioscience, Foster City, CA) for *Col1a2*, *Col3a1*, *Col5a1*, and *Col5a2*. Specificity of each probe was checked using the NCBI BLAST website. The PCR was carried out in a MyiQ Real-time PCR machine (BioRad Laboratories, Hercules, CA) with cyber green (Cyber Green PCR Master Mix; Applied Biosystems, Foster City, CA) as the reporter. MyiQ Optical System software 1.0v was used (BioRad Laboratories, Hercules, CA) to analyze the data. All results were normalized to the amount of β-actin in the sample. Values of P were obtained using an unpaired non-parametric two-tailed *t*-test (software program Instat, version 3.05; Graph Pad, San Diego, CA).

Cross-link analysis

Skin samples were dissected from the back of normal ($n=5$) and *Sagg*/+ ($n=4$) mice. After hair was shaved, the samples were cut into small pieces with a razor blade, pulverized in liquid nitrogen using a Spex Freezer Mill (Spex Industries Inc., Edison, NJ), washed with distilled water, and lyophilized. Approximately 2 mg of each sample was then suspended in 0.15 M *N*-trismethyl-2-aminoethane sulfonic acid, pH 7.4, reduced with standardized NaB_3H_4, hydrolyzed with 6 N HCl, and subjected to quantitative collagen cross-link analysis as described (Yamamoto and Yamauchi, 1999). The quantities of the cross-links were expressed as mol/mol of collagen using a value of 300 residues of hydroxyproline per collagen molecule (Yamauchi and Shiiba, 2002).

Skin biomechanics

Skin was dissected posteriorly from normal ($n=5$) and *Sagg*/+ ($n=4$) mice. A razorblade stamp was used to create two (superior and inferior) dogbone-shaped skin specimens from each mouse. The resulting specimens were oriented normal to the saggital plane and the right side of each was marked to ensure consistency of direction. Cross-sectional area was calculated based on measurements of specimen geometry (thickness and width) using established protocols (Soslowsky *et al.*, 1994, 2000). Each specimen was marked with lines demarcating the gage length for use in optical strain measurements, as described previously (Derwin *et al.*, 1994). Each sample was clamped in custom test fixtures to facilitate mechanical testing in an Instron 5,543 materials testing system (Instron Corp., Canton, MA). All specimens were subjected to the following testing protocol, while submerged in 37°C phosphate-buffered saline: preload to 0.03 N, hold for 120 seconds, and constant ramp to failure at a rate of 1.67%/second. Stress was calculated from the load response divided by the measured cross-sectional area. Modulus was calculated as the slope of the stress–strain curve within the elastic region of the ramp to failure. The pairs of resulting properties for each mouse were averaged to obtain a single value for each property. Differences in means between normal and *Sagg*/+ mice were compared using unpaired *t*-tests with significance set at $P \leqslant 0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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