MATRIX PATHOBIOLOGY

Deficits in *Col5a2* Expression Result in Novel Skin and Adipose Abnormalities and Predisposition to Aortic Aneurysms and Dissections

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Accepted for publication
June 8, 2017.

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Classic Ehlers-Danlos syndrome (cEDS) is characterized by fragile, hyperextensible skin and hypermobile joints. cEDS can be caused by heterozygosity for missense mutations in genes *COL5A2* and *COL5A1*, which encode the $\alpha 2(V)$ and $\alpha 1(V)$ chains, respectively, of collagen V, and is most often caused by *COL5A1* null alleles. However, *COL5A2* null alleles have yet to be associated with cEDS or other human pathologies. We previously showed that mice homozygous null for the $\alpha 2(V)$ gene *Col5a2* are early embryonic lethal, whereas haploinsufficiency caused aberrancies of adult skin, but not a frank cEDS-like phenotype, as skin hyperextensibility at low strain and dermal cauliflower-contoured collagen fibril aggregates, two cEDS hallmarks, were absent. Herein, we show that ubiquitous postnatal *Col5a2* knockdown results in pathognomonic dermal cauliflower-contoured collagen fibril aggregates, but absence of skin hyperextensibility, demonstrating these cEDS hallmarks to arise separately from loss of collagen V roles in control of collagen fibril growth and nucleation events, respectively. *Col5a2* knockdown also led to loss of dermal white adipose tissue (WAT) and markedly decreased abdominal WAT that was characterized by miniadipocytes and increased collagen deposition, suggesting $\alpha 2(V)$ to be important to WAT development/maintenance. More important, *Col5a2* haploinsufficiency markedly increased the incidence and severity of abdominal aortic aneurysms, and caused aortic arch ruptures and dissections, indicating that $\alpha 2(V)$ chain deficits may play roles in these pathologies in humans. (*Am J Pathol* 2017; 187: 2300–2311; <http://dx.doi.org/10.1016/j.ajpath.2017.06.006>)

Type V collagen [col(V)] is a quantitatively minor fibril-forming collagen broadly expressed in vertebrate tissues as an $\alpha 1(V)_2\alpha 2(V)$ heterotrimer, comprising one $\alpha 2(V)$ and two $\alpha 1(V)$ chains.¹ In addition, $\alpha 1(V)_3$ homotrimers and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ heterotrimeric forms of col(V) also have been reported in tissues, although the significance and functions of such forms are only beginning to be explored.^{2–6} Early evidence suggested a pericellular or basement membrane-associated distribution for col(V) forms.^{7,8} However, subsequent studies have demonstrated that $\alpha 1(V)_2\alpha 2(V)$ heterotrimers are incorporated into the growing fibrils of the much more abundant fibrillar collagen I fibrils, and play roles in regulating the nucleation, size, and

shapes of the resulting collagen I/col(V) heterotypic fibrils.^{9–11}

Human patients with the heritable connective tissue disorder classic Ehlers-Danlos syndrome (cEDS) present with skin hyperextensibility, joint hypermobility, atrophic scarring, and easy bruising.¹² Most cEDS cases are due to heterozygosity for null alleles for the $\alpha 1(V)$ gene

Supported by the Department of Veterans Affairs (J.M.D.), the University of Wisconsin School of Medicine and Public Health (D.S.G.), and NIH grants AR044745 (D.E.B.), R01HL088447 (B.L.), and AR047746 and AI084853 (D.S.G.).

Disclosures: None declared.

COL5A1.^{12,13} Similarly, a null allele in the mouse $\alpha 1(V)$ gene *Col5a1* causes a cEDS-like phenotype in mice.^{14,15} In addition, a number of human cEDS cases have now been shown to be due to heterozygosity for missense mutations in *COL5A1*, or in the $\alpha 2(V)$ gene *COL5A2*.¹² Interestingly, however, no human cEDS case has yet been associated with heterozygosity for a *COL5A2* null allele, leading to the suggestion that haploinsufficiency for the $\alpha 2(V)$ chain may not lead to cEDS or, perhaps, to any clinically abnormal phenotype.¹²

To determine *in vivo* effects of reduced or absent $\alpha 2(V)$ chains, we recently generated and characterized mice with constitutively null *Col5a2* alleles.¹⁶ Homozygosity for the null *Col5a2* allele yielded embryonic lethality at approximately 12 days after conception,¹⁶ approximately 2 days later than the embryonic lethality previously described for homozygous null *Col5a1*^{-/-} mice,¹¹ although embryos of both genotypes had similar cardiovascular signs, suggesting deficits in early cardiovascular integrity. However, although *Col5a1*^{-/-} embryos lacked identifiable mesenchymal collagen fibrils,¹¹ *Col5a2*^{-/-} embryos had identifiable mesenchymal collagen fibrils, albeit with abnormally large diameters and abnormal configurations.¹⁶ Differences in collagen fibrils and survival time between the two types of homozygous null embryos suggested that aberrant $\alpha 1(V)_3$ homotrimers, which are formed by $\alpha 1(V)$ chains in the absence of $\alpha 2(V)$ chains,^{17,18} can functionally compensate, in part, for loss of $\alpha 1(V)_2\alpha 2(V)$ heterotrimers.

Unlike heterozygous *Col5a1*^{+/-} adult mice, which exhibit hyperextensible skin at both high and low stress, and on electron microscopic examination display large-diameter dermal aggregates of collagen fibrils with cauliflower-like contours,¹⁴ cardinal features of cEDS,¹² adult heterozygous *Col5a2*^{+/-} mice exhibited hyperextensible skin at high, but not low, stress and dermal collagen fibrils with relatively mild abnormalities in contour and diameter.¹⁶ Together, these data suggested that human *COL5A2*^{+/-} heterozygotes, although likely more susceptible to skin tearing/wounding than normal, might not present with frank cEDS. This conclusion, together with the finding that aortas of *Col5a2*^{+/-} heterozygous mice have reduced tensile strength and increased elasticity, raised the question of the clinical ramifications of the *COL5A2*^{+/-} genotype in human populations.¹⁶

In the current report, we further elucidated the roles of $\alpha 2(V)$ collagen chain in adult tissues and pathology, in part by using a conditional-null mouse model with floxed *Col5a2* alleles that avoids embryonic lethality and allows study of the effects of postnatal *Col5a2* ablation. Data presented herein show the $\alpha 2(V)$ chain to play important roles in growth and that sufficiently low $\alpha 2(V)$ chain levels yield skin that, like that of cEDS patients,¹⁹ has delayed and aberrant wound healing, has greatly reduced breaking strength/tensile strength, and contains the large cauliflower-like collagen fibril aggregates pathognomic of cEDS.¹⁶ However, unlike skin of cEDS patients, this skin lacks hyperextensibility and had almost total absence of dermal white adipose tissue

(WAT). The latter, along with abdominal WAT depots of reduced size and fibrotic appearance, and in which adipocytes had a miniadipocyte appearance, suggests a role for $\alpha 2(V)$ in WAT development and/or maintenance. Key observations include the finding that $\alpha 2(V)$ chain haploinsufficiency yields markedly increased incidence, diameters, and pathology of abdominal aortic aneurysms (AAAs), and high susceptibility to fatal aortic arch ruptures and dissections in an angiotensin II-induced aneurysm mouse model. Thus, deficits in $\alpha 2(V)$ chain levels/function may play roles in these pathologies in humans.

Materials and Methods

Col5a2-Floxed Mice

The generation of *Col5a2*^{fl/fl} mice has been described previously.¹⁶

Induced Deletion of *Col5a2*

C57BL/6 *Col5a2*^{fl/fl} mice were mated with transgenic *Cre-ERT2* mice in which Cre recombinase expression was driven by the ubiquitin C (Ubc) promoter (JAX strain 008085),²⁰ to generate *Ubc Cre-ERT2*; *Col5a2*^{fl/fl} mice. Excision of *Col5a2* sequences was then induced with tamoxifen, administered into the peritoneum (i.p.) of recently weaned 4-week-old male mice (0.5 μ mol/L per gram body weight), weighing approximately 13 to 15 g, for a total of 10 days (two blocks of 5 consecutive days separated by 2 days without treatment). Littermates without the *Ubc Cre-ERT2* transgene were used as controls and were similarly treated with tamoxifen. Efficacy of tamoxifen-induced excision of *Col5a2* was assayed via PCR analysis of genomic DNA from ear samples digested in DirectPCR Lysis Reagent (Viagen Biotech Inc., Los Angeles, CA). Oligonucleotide forward (5'-GGTGATGGATGCTGACTTTG-3'/5'-AGCTTCTGTGCGTGCCCTGG-3') and reverse (5'-GGAGGGGAGGATAAAGAGCA-3') primers were used to detect the presence of floxed or excised alleles, represented by approximately 460- and approximately 510-bp bands, respectively, on resolution on 2.5% agarose gels.

Col5a2^{fl/fl} mice also were mated with transgenic *Cre-ERT2* mice, in which Cre expression is driven by the myosin heavy chain 11 promoter (JAX strain 019079),²¹ specific to smooth muscle cells, to generate *Myh11 Cre-ERT2*; *Col5a2*^{fl/fl} mice. Male mice, all of which must contain the Y chromosome-linked *Myh11 Cre-ERT2* transgene, were injected with either tamoxifen or vehicle (98% corn oil and 2% ethanol) using the same dosage and administration schedule as described above for *Ubc-Cre*; *Col5a2*^{fl/fl} mice.

All mice were housed and treated in accordance with NIH's *Guide for the Care and Use of Laboratory Animals*,²² using protocols approved by the Research Animal Resources Center of the University of Wisconsin—Madison.

Splinted Wound Healing

Splinted wound healing was performed on 8- to 10-week-old mice, as described previously.²³ Briefly, all wounding procedures were performed under isoflurane anesthesia. The backs of mice were shaved and sterilized. Two full-thickness wounds were generated on the midback using a 5-mm biopsy tool (Integra Miltex, York, PA). Circular, silicon splints, with a 6-mm inner diameter, were adhered around the edges of the wound using cyanoacrylate glue (Elmer's Products, Atlanta, GA) and fastened in place with four interrupted sutures. Wounds were covered by protective dressings for the duration of the experiment. Wound closure was analyzed using calipers by measuring two diameters per wound and using the average to estimate wound area. After sacrifice, the wounded tissue was carefully dissected for further analysis.

Dermal Fibroblast Culturing and Immunoblotting

Full-thickness skin was excised from the axillary region of 15-week-old adult mice. The tissue was then minced using a pair of scalpels and was digested overnight in 0.5 mg/mL type II collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, in a 5% CO₂ atmosphere at 37°C. The digest was pelleted at 1500 × *g* for 10 minutes, the supernatant was removed, and the pellet was subsequently resuspended and plated in Dulbecco's modified Eagle's medium and 20% fetal bovine serum growth medium.

For immunoblotting, cells were washed twice with phosphate-buffered saline to remove residual growth medium before being serum starved in Dulbecco's modified Eagle's medium supplemented with 75 µg/mL ascorbic acid and 40 µg/mL soybean trypsin inhibitor (Sigma, St. Louis, MO). The following day, conditioned media were collected and concentrated by centrifugation filters (Millipore, Billerica, MA). Samples were boiled in Laemmli sample buffer and 2.5% β-mercaptoethanol before loading onto a 6% acrylamide gel and SDS-PAGE. Primary antibodies specific for the mouse α1(V) proline/arginine-rich protein and variable domains or the α2(V) cysteine-rich domain were used, as previously described.¹⁶ Anti-procollagen C-proteinase enhancer 1 antibody (Sigma) was used at a 1:1000 dilution. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories Inc., Hercules, CA) was used at a 1:5000 dilution. Detection was with chemiluminescent substrate (Thermo Scientific, Waltham, MA).

Histologic Analyses

Hair was removed from subscapular skin samples, and WAT depots were isolated; samples were then fixed overnight in 10% formalin, followed by embedding in paraffin, and dividing into sections (5 µm thick). For hematoxylin and eosin staining, sections were fixed for 5 minutes in 10% formalin, then rinsed with water, and stained in modified

Harris hematoxylin solution (Newcomer Supply, Inc., Middleton, WI) for 4 minutes. Samples were dehydrated in 1% acid alcohol, and the nuclei were blued in 1% ammonia water for 30 seconds. Cytoplasmic staining was achieved by immersing the slides in 1% alcoholic eosin Y solution (Newcomer Supply, Inc.). Slides were then dehydrated through 95% and 100% ethanol, cleared in xylene, and mounted with Cytoseal 60 (Thermo Fisher).

For trichrome staining, sections were fixed for 5 minutes in 10% formalin and placed in Bouin's solution for 1 hour at 60°C. Slides were washed until tissue sections were clear. Tissue nuclei were stained with working Wiegert's Iron Hematoxylin solution (Newcomer Supply, Inc.) for 10 minutes. Slides were then stained for collagen with Gomori's one-step trichrome solution (Newcomer Supply, Inc.) for 20 minutes. Last, slides were rinsed with 1% glacial acetic acid to enhance contrast, dehydrated through 95% and 100% ethanol, cleared in xylene, and mounted with Cytoseal 60. Bright-field images were captured using a Zeiss Axiophot 2 microscope (Zeiss, Thornwood, NY) with an attached charge-coupled device camera.

Transmission Electron Microscopy

Mouse subscapular skin was fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, with 8.0 mmol/L CaCl₂ for 2 to 4 hours and post-fixed with 1% osmium tetroxide. Further sample preparation and imaging was as previously described.¹⁶

Biomechanical Analysis of Skin

Tensiometric properties of moist skin strips (20 × 30 mm, trimmed to generate a 5-mm waist that provided a uniform breaking pattern) from the dorsal midline were evaluated using an Instron Tensiometer (model 5542; Instron, Canton, MA). Mechanical properties (breaking energy and Young's modulus) were calculated with Bluehill software version 2.32 (Instron, Norwood, MA).

Elastase and Angiotensin II Infusion Models for Aortic Aneurysms

For the elastase model, 12- to 15-week-old adult mice were anesthetized and underwent sterile preparation. A laparotomy was performed to expose the abdominal aorta. The aorta was temporarily ligated at two ends, and an aortotomy was performed between the ligations. Through the aortotomy, the aorta was perfused with 0.414 U/mL type I porcine pancreatic elastase (Sigma) in saline for 5 minutes at a pressure of 100 mmHg. After perfusion, the aortotomy was closed and the ligations were removed. Preperfusion measurements of aorta diameters were recorded using digital calipers, and measurements were taken again on sacrifice 2 weeks after surgery. The percentage change in diameter was calculated by comparing these two values.

Figure 1 Tamoxifen-induced *Col5a2* ablation. **A:** Schematic shows placement of *loxP* and *frt* sequences, represented in black and gray, respectively. *LoxP* sequences flank exon 1 (open box) and adjoining promoter and enhancer sequences. On tamoxifen treatment, site-specific excision by induced Cre recombinase deletes *LoxP*-flanked sequences to produce the excised (ablated) allele. **B:** PCR genotyping of genomic DNA from *Col5a2^{fl/fl}* and *Ubc-Cre; Col5a2^{fl/fl}* mice treated with tamoxifen shows genomic excision of exon 1 only in mice (*Ubc-Cre; Col5a2^{fl/fl}*) harboring the *Ubc-Cre* transgene. **C:** Immunoblotting of conditioned media from primary dermal fibroblasts isolated from *Ubc-Cre; Col5a2^{fl/fl}* mice shows a striking deficit in $\alpha 2(V)$ chain secretion, compared with *Col5a2^{fl/fl}* fibroblasts. Both types of fibroblasts are seen to secrete similar levels of $\alpha 1(V)$ chains. Procollagen C-proteinase enhancer 1 (PCOLCE1) was used as a loading control. B, BamHI; K, KpnI; M, MfeI; N, NcoI; X, XbaI.

For the angiotensin II (Ang II) infusion model, constitutively heterozygous null *Col5a2^{+/-}* mice¹⁶ were crossed with *Ldlr^{-/-}* mice (Jackson Laboratory, Bar Harbor, ME), and *Col5a2^{+/-};Ldlr^{-/-}* and *Col5a2^{+/+};Ldlr^{-/-}* control male mice at the age of 6 to 8 months were subjected to a 28-day infusion of Ang II. Alzet model 2004 osmotic minipumps (Durect Corp., Cupertino, CA), implanted s.c., were used to deliver Ang II (1000 ng/kg per minute; Sigma-Aldrich, St. Louis, MO) or vehicle (normal saline). Mice were anesthetized with isoflurane and administered buprenorphine (0.05 to 0.10 mg/kg) via i.p. injection after the procedure. At the completion of Ang II infusion, mice were perfused with normal saline, followed by 4% paraformaldehyde, at 100 mmHg for 5 minutes. Periadventitial tissues were carefully removed from the aortic wall, and a digital caliper (Absolute Digimatic; Mitutoyo, Aurora, IL) was used to measure maximal external diameters of suprarenal abdominal aortas. For this study, AAA was defined as $\geq 50\%$ enlargement of maximal abdominal aorta diameter. Characterization of the severity of AAA formation was as previously described²⁴: type I, dilated lumen in the suprarenal aorta with no thrombus; type II, remodeled tissue in the suprarenal region that frequently contains thrombus; type III, a pronounced bulbous form of type II that contains thrombus; and type IV, multiple aneurysms containing thrombus, some overlapping, in the suprarenal area of the aorta. Necropsy was performed as soon as possible on animals that expired before completion of the study. These animals were not included in the histologic or AAA quantification analyses, but only in the mortality data.

Statistical Analysis

Data are expressed as means $\pm$ SEM. To compare incidence of AAA formation between the two groups, χ^2 test and two-tailed Fisher's exact probability test were used. A two-tailed

t-test for normally distributed data and a Mann-Whitney *U* nonparametric test for skewed data that deviate from normality were used to compare two conditions. Differences with $P < 0.05$ were considered significant.

Results

Validation of *Col5a2* Conditional Knockout

To determine the effects of ablating *Col5a2* function in juvenile and adult mice, the embryonic demise associated with constitutive loss of *Col5a2* function¹⁶ was avoided via conditional *Col5a2* knockdown. Toward this end, mice were generated in which exon 1 and adjoining promoter and enhancer regions were flanked with *flox* (*loxP*) sites. Crossing with transgenic mice in which Cre expression is driven by the ubiquitin C promoter generated *Ubc-Cre; Col5a2^{fl/fl}* mice, in which Cre activation by administration of tamoxifen excises *Col5a2* sequences flanked by the *loxP* sites (Figure 1A). Genomic DNA extracted from ear punch samples after tamoxifen administration showed an upward mobility shift of approximately 50 bp from *Ubc-Cre; Col5a2^{fl/fl}* DNA, indicating genomic excision by Cre, unlike *Col5a2^{fl/fl}* littermate controls, which were also administered tamoxifen, but lacked the *Ubc-Cre* transgene (Figure 1B). Moreover, neither secreted pro- $\alpha 2(V)$ chains nor mature $\alpha 2(V)$ chains were detected in cultures of dermal fibroblasts isolated from 15-week-old tamoxifen-treated adult *Ubc-Cre; Col5a2^{fl/fl}* mice (Figure 1C).

Ubc-Cre; Col5a2^{fl/fl} Mice Are Runt and Have Abnormalities of Skin and WAT

Ubc-Cre; Col5a2^{fl/fl} mice failed to gain weight subsequent to tamoxifen treatment and had significantly decreased weight, compared with tamoxifen-treated *Col5a2^{fl/fl}*

Figure 2 Morphologic appearance and features of skin and white adipose tissue (WAT) depots of *Col5a2* adult mutant mice. **A–C:** Conditional loss of *Col5a2* function results in markedly reduced mass (**A**) and length (**B**), and severe kypholordosis (**C**). The white outlined object above the head of each mouse in **C** is the ear tag. **D:** Hematoxylin and eosin (H&E) and Masson's trichrome staining of subscapular skin samples. Vertical bracket and arrow denote dermal WAT in *Ubc-Cre; Col5a2^{fl/fl}* and *Col5a2^{fl/fl}* littermate control skin, respectively. **E:** Masses of inguinal fat depots, normalized to the body weights of the mice from which they were recovered, were compared for *Col5a2^{fl/fl}* and *Ubc-Cre; Col5a2^{fl/fl}* mice. **F:** Representative H&E-stained sections are shown of *Col5a2^{fl/fl}* and *Ubc-Cre; Col5a2^{fl/fl}* inguinal fat depots. Masson's trichrome staining of a section of *Ubc-Cre; Col5a2^{fl/fl}* inguinal fat depot is shown, evidencing a characteristic collagenous seam. Although only data for inguinal fat depots are shown in **E** and **F**, results were similar for gonadal fat depots. **G** and **H:** Biomechanical measurement shows *Ubc-Cre; Col5a2^{fl/fl}* skin to have markedly decreased breaking strength ($P < 0.0001$; **G**), but no significant difference ($P < 0.638$; **H**) in extensibility (Young's modulus), compared with control skin. Data are presented as means $\pm$ SEM (**A**, **B**, **E**, **G**, and **H**). ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bar = 50 μ m (**F**).

controls, as early as 7 weeks of age (approximately 2 weeks after the final tamoxifen treatment). Weight differences further increased until weights of *Ubc-Cre; Col5a2^{fl/fl}* mice were only approximately 60% those of *Col5a2^{fl/fl}* controls at adulthood (Figure 2A). *Ubc-Cre; Col5a2^{fl/fl}* mice were also of shorter length than *Col5a2^{fl/fl}* controls (Figure 2B) and exhibited severe kypholordosis (Figure 2C).

Skin abnormalities are a hallmark of cEDS. We, therefore, subjected subscapular skin samples of *Ubc-Cre; Col5a2^{fl/fl}* and control mice to histologic analysis. Hematoxylin and eosin staining showed *Ubc-Cre; Col5a2^{fl/fl}* dermis to be markedly thinner, and seemingly more compact than control skin (Figure 2D). Masson's trichrome staining of serial sections confirmed a thinner, more compact, dermis and also suggested *Ubc-Cre; Col5a2^{fl/fl}* dermis to be more collagen dense than control dermis (Figure 2D). *Ubc-Cre;*

Col5a2^{fl/fl} skin also apparently lacked dermal WAT, with an absence of morphologically identifiable adipocytes (Figure 2D). Consistent with these histologic results, on handling of mice, a thinning of the skin was readily apparent, and most evident while grasping mice by the scruff of the neck or other areas of dorsal skin.

To determine whether loss of WAT was limited to *Ubc-Cre; Col5a2^{fl/fl}* skin, inguinal and gonadal fat depots were isolated from *Ubc-Cre; Col5a2^{fl/fl}* and *Col5a2^{fl/fl}* control mice and their weights were compared. Although *Ubc-Cre; Col5a2^{fl/fl}* mice are approximately 60% the weight and approximately 60% the length of *Col5a2^{fl/fl}* controls (Figure 2, A and B), *Ubc-Cre; Col5a2^{fl/fl}* inguinal and gonadal fat depots were only 18% and 15% the mass of the corresponding *Col5a2^{fl/fl}* depots, respectively; and were approximately 30% and approximately 25%, respectively,

when normalized to the body weights of the mice from which they were recovered (Figure 2E). As *Ubc-Cre; Col5a2^{fl/fl}* mice had food intake approximately 60% that of *Col5a2^{fl/fl}* mice (data not shown), and thus proportional to their reduced size, the aggregate data from the much reduced WAT of skin and abdominal depots suggest that the $\alpha 2(V)$ chain plays a role in WAT development and/or maintenance. Consistent with this possibility, the adipocytes in *Ubc-Cre; Col5a2^{fl/fl}* inguinal and gonadal WAT depots were miniadipocytes, of an abnormally small size (Figure 2F). *Ubc-Cre; Col5a2^{fl/fl}* inguinal and gonadal WAT depots were also abnormal in having seams of dense collagenous matrix not found in control inguinal or gonadal WAT depots (Figure 2F). These fibrotic seams in abdominal WAT depots were reminiscent of the dense collagenous matrix in *Ubc-Cre; Col5a2^{fl/fl}* skin (Figure 2D), and are evidence of an abnormal collagenous matrix in both tissues.

Ubc-Cre; Col5a2^{fl/fl} Skin Is Markedly Fragile, But Not Hyperextensible

To determine whether these differences translated into differences in mechanical strength, skin samples were examined for tensile strength, the amount of force required for the skin to stretch and break. Mechanical testing, and analysis that included correction for the thinner dorsal skin of *Ubc-Cre; Col5a2^{fl/fl}* skin specimens, showed the latter to have markedly reduced breaking energy (toughness), with the breaking energy of *Ubc-Cre; Col5a2^{fl/fl}* skin reduced by 49% compared with control skin ($P < 0.0001$) (Figure 2G). Surprisingly, however, the elastic modulus of *Ubc-Cre; Col5a2^{fl/fl}* skin showed no significant difference with that of *Col5a2^{fl/fl}* littermate controls (Figure 2H), indicating lack of the hyperextensibility characteristic of cEDS,¹² and of the *Col5a1^{+/-}* cEDS mouse model,¹⁴ and even of constitutively *Col5a2^{+/-}* heterozygous null adult mice.¹⁶

Ultrastructural Analysis Reveals *Ubc-Cre; Col5a2^{fl/fl}* Mice to Have cEDS-Like Dermal Collagen Fibrillar Aggregates

cEDS is characterized, in part, by large-diameter aggregates of collagen fibrils with cross-sectional cauliflower-like contours.¹² However, as null *COL5A2* alleles have yet to be identified in cEDS patients,¹² and as we previously found *Col5a2^{+/-}* mice to have dermal collagen fibrils with relatively mild abnormalities in contour and diameter,¹⁶ we sought to determine the extent to which *Ubc-Cre; Col5a2^{fl/fl}* mice would have changes in dermal collagen fibril size and geometry. To this end, transmission electron microscopy was used to examine skin ultrastructure. *Ubc-Cre; Col5a2^{fl/fl}* and *Col5a2^{fl/fl}* mice were both injected with tamoxifen and sacrificed at 15 weeks of age, at which time subscapular skin was collected. Analysis using transmission electron microscopy demonstrated that *Ubc-Cre; Col5a2^{fl/fl}* skin had readily apparent large-diameter collagen fibril aggregates with cross-sectional cauliflower-like contours (Figure 3B),

highly similar to the similar fibril aggregates that are a cardinal feature in the skin of cEDS patients.¹⁶ This feature was absent from control *Col5a2^{fl/fl}* skin (Figure 3A). Examination of longitudinal collagen fibril profiles further emphasized the overall fibril disorganization and larger fibril diameters of *Ubc-Cre; Col5a2^{fl/fl}* skin (Figure 3, C and D). Quantitative analyses of fibril cross sections also showed *Ubc-Cre; Col5a2^{fl/fl}* skin to have a lower density of fibrils, compared to *Col5a2^{fl/fl}* controls ($P = 0.027$) (Figure 3E), likely due, at least in part, to the presence of fibril aggregates and larger-diameter fibril diameters, such that there are fewer fibrils per unit space. Indeed, quantitative examination showed *Ubc-Cre; Col5a2^{fl/fl}* skin to have a rightward shift in the distribution of collagen fibril diameters, with an extended right tail of atypically large-diameter sizes, ranging from 240 to 480 nm. The data thus indicate both an overall increase in collagen fibril diameters as well as a subpopulation of fibril aggregates of abnormally large diameters (Figure 3F).

Ubc-Cre; Col5a2^{fl/fl} Mice Have Impaired Wound Healing

Because cEDS patients have delayed and aberrant wound healing,¹⁹ we used a wound-healing assay to determine whether *Ubc-Cre; Col5a2^{fl/fl}* mice might also have deficits in wound healing. Toward this end, 5-mm-diameter full skin thickness punch biopsy specimens were excised from *Ubc-Cre; Col5a2^{fl/fl}* and *Col5a2^{fl/fl}* control littermate mice. Wounds were sutured open to prevent contraction and to allow healing primarily by reepithelialization, extracellular matrix (ECM) deposition, and granulation tissue formation, the predominant mechanisms of wound healing in humans. Wounds were measured every other day for 2 weeks until the mice were sacrificed. Examination of wounds at 14 days revealed significantly delayed healing of *Ubc-Cre; Col5a2^{fl/fl}* wounds, for which healing was always incomplete, as evidenced by readily apparent nonconjoined wound edges (Figure 4A), whereas wounds of *Col5a2^{fl/fl}* controls reproducibly showed complete or nearly complete healing (Figure 4A). By 14 days after wounding, *Ubc-Cre; Col5a2^{fl/fl}* wounds remained approximately 60% open, whereas *Col5a2^{fl/fl}* wounds were essentially closed (Figure 4, B and C).

Col5a2 Ablation Predisposes to Increased Abdominal Aortic Aneurysm Diameters and to Aortic Arch Dissection and Rupture in Mouse Aortic Aneurysm Models

It has been reported that patients with cEDS have a higher incidence of dilated aortic roots.²⁵ Therefore, the tensile strength of aortas of heterozygous *Col5a1^{+/-}*¹⁴ and *Col5a2^{+/-}*¹⁶ mice were previously examined *ex vivo* and were shown to have decreased aortic tensile strength and stiffness. Thus, we previously suggested that deficits in col(V) content might predispose to aortic aneurysms.¹⁶ To test this, however, *Ubc-Cre; Col5a2^{fl/fl}* mice, which had relatively small body mass (Figure 2, A and B),

Figure 3 Transmission electron microscopic analysis of skin from 15-week-old *Col5a2^{fl/fl}* and *Ubc-Cre; Col5a2^{fl/fl}* mice. *Ubc-Cre; Col5a2^{fl/fl}* mice (**B**) have large aggregates of collagen fibrils, known as collagen cauliflowers¹² (asterisks), with cross-sectional cauliflower-like profiles not seen in *Col5a2^{fl/fl}* controls (**A**). This alteration in fibril structure is highlighted in *Ubc-Cre; Col5a2^{fl/fl}* (**D**) and *Col5a2^{fl/fl}* (**C**) longitudinal sections. Mutant mice (red) also have decreased fibril density (**E**) and a corresponding distribution skewed toward larger fibril sizes (**F**) compared with controls (blue). Data are presented as means ± SEM (**E**). Scale bar = 200 nm (**A–D**).

also had large decreases in the sizes of their aortas, which were, therefore, not appropriate for comparison to controls in aorta induction experiments. It was also anticipated that *Ubc-Cre; Col5a2^{fl/fl}* mice might have poor survivability in such studies, because of a general failure to thrive. Thus, instead, *Col5a2^{fl/fl}* mice were crossed with transgenic *Myh11-CreERT2* mice²¹ to produce *Myh11-Cre; Col5a2^{fl/fl}* mice, in which tamoxifen induction of Cre activity induces *Col5a2* excision only in gastrointestinal smooth muscle cells (SMCs) and in vascular SMCs,²⁶ with the latter a major source of secreted collagens in vessel walls. The *Myh11-Cre* transgene is Y linked, and all male littermates in these crosses contain the *Myh11-Cre* transgene. Some of these mice were treated with tamoxifen, for *Col5a2* excision, whereas control littermates, with the same genotype,

were treated with vehicle. Subsequent to tamoxifen/vehicle treatment, there were no differences in weight, nor were other gross external differences noted between the two groups of mice (data not shown). In a model in which elastase perfusion of the abdominal aorta induces AAAs,²⁷ AAAs were induced in both groups of mice approximately 8 weeks after tamoxifen/vehicle treatment. Interestingly, tamoxifen-treated mice had larger aneurysms than the vehicle-treated mice (158.8% change ± 16.66% versus 110.0% change ± 10.75%; $P < 0.033$), indicating that *Col5a2* ablation in aortic SMCs was sufficient to render the aorta more prone to dilation (**Figure 5**, A and B).

The fact that SMCs represent only a fraction of cells of which the aortic wall is composed complicated our ability to

Figure 4 Delayed wound healing because of *Col5a2* ablation. **A:** Representative images are shown of hematoxylin and eosin–stained histologic sections of *Col5a2*^{fl/fl} control and *Ubc-Cre; Col5a2*^{fl/fl} wounds at 14 days after wounding. **Dotted lines** denote wound margins; **arrows** denote edge of migrating epithelial tongues in a *Ubc-Cre; Col5a2*^{fl/fl} wound, whereas a *Col5a2*^{fl/fl} control wound is completely reepithelialized. **B:** Representative images at wounding and 14 days after injury. **C:** Quantification of open wound areas shows wound closure to occur significantly more slowly in *Ubc-Cre; Col5a2*^{fl/fl} than in *Col5a2*^{fl/fl} control wounds. Data are expressed as means $\pm$ SEM (**C**). $n = 9$ (**C**, *Ubc-Cre; Col5a2*^{fl/fl} wounds); $n = 10$ (**C**, *Col5a2*^{fl/fl} wounds). * $P < 0.05$, *** $P < 0.001$.

ascertain the efficiency with which *Col5a2* sequences were excised using the *Myh11-Cre* transgene (described above). In addition, although *Myh11-Cre* should excise *Col5a2* sequences from mature SMCs, it would not excise *Col5a2* sequences from progenitor/stem cells involved in SMC turnover and replacement, as such SMC-lineage cells do not yet express *Myh11*.²⁸ Thus, SMCs from which *Col5a2* had been excised could be replaced by progenitors that retained ability to express *Col5a2*. For these reasons, we thought that the *Myh11-Cre; Col5a2*^{fl/fl} studies described above might underrepresent *Col5a2* roles in aortic aneurysm pathogenesis. Thus, we sought to bolster such experiments by examining aortic aneurysm formation in *Col5a2*^{+/-} heterozygotes, in which all aortic cells would be *Col5a2* haploinsufficient. For this study, *Col5a2*^{+/-} mice were crossed onto an *Ldlr*^{-/-} background, for use in the model system in which Ang II is infused into dyslipidemic mice to induce AAAs with various pathological features of human AAAs.^{29,30}

In the Ang II infusion model, *Col5a2*^{+/-} mice had marked increases in the incidence (Figure 6A), diameters (Figure 6B), and severity of vascular pathology (Figure 6C) of induced AAAs, compared with *Col5a2*^{+/+} controls. Representative

images of *Col5a2*^{+/-} and *Col5a2*^{+/+} Ang II–induced AAAs are shown in Figure 6D. Surprisingly, in addition to effects of Ang II infusion on AAA size, 6 of the 13 *Col5a2*^{+/-} mice subjected to Ang II infusion died within the first 7 days of the start of Ang II treatment (Figure 7, A and B) of aortic arch rupture and dissection (Figure 7, C and D).

Discussion

The known molecular bases of cEDS include heterozygosity for *COL5A1* null alleles or for missense *COL5A2* mutations.¹² However, there have been no described cEDS patients with *COL5A2* null alleles. In a previous study of the phenotypic consequences of null alleles for $\alpha 2(V)$ -encoding genes, we demonstrated¹⁶ that mice heterozygous for *Col5a2* constitutively null alleles had cEDS-related skin features that included increased extensibility and decreased tensile strength at high stress. However, a frank cEDS phenotype was lacking, as skin lacked hyperextensibility at low stress and large-diameter cauliflower-shaped aggregates of collagen fibrils, both of which are pathognomonic for

Figure 5 *Col5a2* knockdown in vascular SMCs increases the diameters of elastase-induced AAAs. **A:** Image of representative AAAs, 2 weeks after elastase treatment, of *Myh11-Cre; Col5a2^{fl/fl}* mice treated with tamoxifen or with vehicle. **Arrowheads** indicate borders of aortic dilation. **B:** Quantification of AAA maximal external infrarenal aortic diameters. $n = 9$ (**A**, tamoxifen treatment); $n = 8$ (**A**, vehicle treatment). $*P < 0.05$.

cEDS. We were interested in whether reducing *Col5a2* expression to levels lower than those found in haploinsufficiency might produce a phenotype more typical of cEDS. Because complete loss of *Col5a2* function in mice is embryonic lethal,¹⁶ the present study used the first described conditional knockdown for *Col5a2*. Ubiquitous ablation at weaning allowed assessment of $\alpha 2(V)$ chain roles in late perinatal development, in growth/maturation and in mature tissues.

Initial gross histologic examination of *Ubc-Cre; Col5a2^{fl/fl}* (*Col5a2* knockdown) skin showed it to be extremely thin, with a compact, collagen-dense dermis in which dermal WAT was reduced to levels that were not readily detectable. This finding, along with our finding that abdominal fat depots were also much reduced in the *Col5a2* knockdown mice, even when normalized to body weight, suggests an important role for the $\alpha 2(V)$ chain in WAT development and/or

maintenance, rather than a skin-specific effect [although the effect of $\alpha 2(V)$ loss was most dramatic in skin].

Interestingly, the extreme thinness of *Col5a2* knockdown dermis and lack of dermal WAT are not characteristic of cEDS. In contrast, transmission electron microscopy analysis showed *Col5a2* knockdown skin to have the large-diameter cauliflower-contoured collagen fibril aggregates pathognomonic of cEDS. Thus, precipitous postnatal loss of $\alpha 2(V)$ chain expression produced a phenotype overlapping, but not identical with, cEDS.

Col5a2 knockdown skin had a 49% loss of breaking energy (toughness), compared with controls, similar to the extent of reduction of tensile strength in *Col5a1^{+/-}* skin in a cEDS mouse model.¹⁴ However, *Col5a2* knockdown skin differs from cEDS skin, and even from the skin of *Col5a2^{+/-}* heterozygotes,¹⁶ in lacking hyperextensibility, even under high stress. Thus, the presence of large

Figure 6 *Col5a2* deficiency also exacerbates Ang II-induced AAA formation in mice. **A:** AAA incidence. **B:** Quantification of AAA maximal external suprarenal aortic diameter on day 28. An AAA is defined as percentage increase in aortic diameter of $\geq 50\%$ (red dashed line). **C:** AAA classification in *Col5a2^{+/+}* versus *Col5a2^{+/-}* mice after 28 days of Ang II infusion. **D:** Representative images of aortas. **Arrowheads** demarcate the area of the abdominal aorta treated with elastase. For comparing AAA incidence, Fisher's exact probability test was used. For comparison of aortic diameter differences, Mann-Whitney *U* test was used. $n = 3$ (**A**, both genotypes of saline controls); $n = 9$ (**A** and **C**, *Col5a2^{+/+}* Ang II group); $n = 7$ (**A** and **C**, *Col5a2^{+/-}* Ang II group). $**P < 0.01$.

Figure 7 *Col5a2* deficiency predisposes mice to aortic dissection and ruptures after Ang II infusion. **A:** Mortality due to aortic dissection and rupture within the first 7 days of Ang II treatment. Fisher's exact probability test was used to compare *Col5a2^{+/+}* versus *Col5a2^{+/-}*. Of the deaths, 50% were because of DeBakey type I dissection, 16% were because of type II dissection, and 33% were because of type III dissection. **B:** Kaplan-Meier survival curve ($P = 0.03$, log-rank test). **C:** Representative image of dissection. **D:** Hematoxylin and eosin-stained section of thoracic dissection and rupture (arrow). $n = 9$ (**A**, *Col5a2^{+/+}*); $n = 13$ (**A**, *Col5a2^{+/-}*); $n = 3$ (**A**, type I dissection); $n = 1$ (**A**, type II dissection); $n = 2$ (**A**, type III dissection). * $P < 0.05$. Scale bar = 200 μ m (**D**).

cauliflower-shaped collagen fibril aggregates, but lack of hyperextensibility, in *Col5a2* knockdown skin unlinks these two cardinal cEDS features, and shows that the cEDS skin hyperextensibility does not rely on the presence of large cauliflower-shaped collagen fibril aggregates. We speculate that, although the cauliflower-shaped collagen fibril aggregates form in *Col5a2* knockdown skin because of $\alpha 2(V)$ levels that, on tamoxifen-induced *Col5a2* ablation, are lower than those found in haploinsufficiency, absence of hyperextensibility in *Col5a2* knockdown skin derives from the persistence of some level of normal ECM deposited before tamoxifen treatment.

A seed-and-feed process has been proposed for the formation of fibrillar collagenous ECM,³¹ in at least some tissues. The seed phase of the process involves the extrusion of uniformly small-diameter collagen fibrils by stromal cells to form the fibrillar collagenous architecture of tissues in early prenatal development, whereas the feed phase occurs later in development and in homeostasis, and involves growth of collagen fibrils via fusion of small-diameter fibrils and accretion of collagen monomers onto fibril surfaces. Col(V) appears to play an important nucleation role in the seed phase of collagen fibrillogenesis, as collagen fibrils do not form in early embryonic tissues in the absence of col(V).¹¹ Our current findings, in which collagen cauliflower-shaped aggregates are caused by postnatal *Col5a2* knockdown, indicate that aberrantly low levels of normal $\alpha 1(V)_2\alpha 2(V)$ heterotrimers during the feed phase are sufficient for formation of such aggregates. In contrast, the previous observation that constitutive *Col5a2* haploinsufficiency results in hyperextensibility,¹⁶ together with

the present observation that even lower levels of postnatal *Col5a2* expression do not, suggests that insufficient $\alpha 1(V)_2\alpha 2(V)$ levels during the seed phase of fibrillar collagenous ECM may be primarily responsible for the skin hyperextensibility of cEDS.

As noted above, the extreme thinness of *Ubc-Cre; Col5a2^{fl/fl}* knockdown skin, deriving in large part from almost complete lack of dermal WAT, is not reminiscent of cEDS in humans, nor has it been reported for the *Col5a1^{+/-}* cEDS mouse model.¹⁴ This difference in phenotypes may lie in the fact that decreased $\alpha 1(V)$ chain levels result in only normal col(V), albeit at decreased levels, whereas decreased $\alpha 2(V)$ chain levels result not only in lower levels of col(V), but also in the generation of an aberrant form of col(V). This is because, in the absence of sufficient $\alpha 1(V)$ levels, excess $\alpha 2(V)$ chains are unstable and are degraded, such that only normal $\alpha 1(V)_2\alpha 2(V)$ heterotrimers are deposited in tissues; however, in the absence of sufficient $\alpha 2(V)$ chain levels, $\alpha 1(V)$ chains can form stable $\alpha 1(V)_3$ homotrimers.^{17,18,32} The result is that tissues in which $\alpha 2(V)$ chains are at low levels would be affected not only by reduced deposition of $\alpha 1(V)_2\alpha 2(V)$ heterotrimers, but also by deposition of aberrant levels of $\alpha 1(V)_3$ homotrimers. Thus, we speculate that aberrant ECM with lowered $\alpha 1(V)_2\alpha 2(V)$ content and elevated $\alpha 1(V)_3$ content presents a microenvironment not conducive to adipogenesis and/or adipocyte maintenance. That the $\alpha 2(V)$ chain may well play key roles in microenvironments that inform adipogenesis and/or adipocyte maintenance is supported by a study in which mice expressing a certain type of $\alpha 2(V)$ chain with truncated N-terminal non-triple helical

sequences had dermal WAT up to sixfold thicker than wild type.³³ Consistent with possible key roles for col(V) in WAT biology is the finding that adiponectin overexpression leads to reduced expression of col(V) genes, accompanying a suppression of fat accumulation,³⁴ and our previous finding that knockout of the gene for the $\alpha 3(V)$ chain of col(V) leads to subtle reductions in the thickness of dermal WAT and substantial changes to WAT function.⁶ Interestingly, the appearance of abnormally small miniadipocytes in the reduced mass of *Col5a2* knockdown abdominal WAT depots is reminiscent of miniadipocytes reported in knockout mouse models in which ECM is rendered relatively rigid and not easily remodeled by extracellular proteases.³⁵ Such ECM is thought to impair the hypertrophic response necessary to complete adipocytic maturation,³⁵ suggesting that the *Col5a2* knockdown ECM found in WAT may be similarly abnormally rigid and resistant to remodeling.

We demonstrate *Ubc-Cre; Col5a2^{fl/fl}* mice, like cEDS patients¹⁹ and *Col5a1^{+/-}* mice,¹⁵ to have deficits in wound healing. However, we speculate that, in the case of the *Col5a2* knockdown mice, the paucity of dermal WAT may to some degree contribute to the severity in the deficits in wound healing, as both adipose-derived stem cells and mature adipocytes have been suggested to be important to the reepithelialization, revascularization, and fibroblast repopulation necessary to skin repair.³⁶

As cEDS patients have a higher incidence of dilated aortic roots than normal²⁵ and mice haploinsufficient for *Col5a1*¹⁴ or *Col5a2*¹⁶ have aortas with diminished tensile strength, we sought to determine the extent to which lowered levels of $\alpha 2(V)$ chains might affect the pathogenesis of aortic aneurysms. On treatment with tamoxifen, conditional *Col5a2* knockdown in aortic smooth muscle cells was sufficient to increase the diameters of elastase-induced AAAs. Moreover, as tamoxifen has been shown to be protective against elastase-induced aneurysms in rodent models, in which it reduced aneurysm sizes by approximately 50%,³⁷ the effect of deficits in $\alpha 2(V)$ levels on aneurysm progression may well have been underestimated in our study. However, although it seems unlikely, we cannot formally dismiss the possibility that tamoxifen may have had differential effects on *Ubc-Cre; Col5a2^{fl/fl}* and *Col5a2^{fl/fl}* control mice.

In a follow-up study of *Col5a2^{+/-}* heterozygotes, in which Ang II infusion induced AAAs with pathological features similar to those of human AAAs,^{29,30} not only did *Col5a2^{+/-}* mice have marked increases in the incidence, diameter, and severity of AAAs, compared with controls, but more than half died of aortic arch dissection and rupture. These data are exciting, in that they suggest that *COL5A2* dysfunction in the general human population may predispose to aortic dilations, dissection, and rupture. That deficits in col(V) function can underlie loss of arterial integrity in humans is supported by recent reports of predisposition to rupture and dissection in the major arteries of a few cEDS

patients with certain *COL5A1* missense mutations,³⁸ the occurrence of an aortic aneurysm in a cEDS patient with a *COL5A1* splice site mutation,³⁹ and *COL5A1* or *COL5A2* gene variants of unknown significance in two nonsyndromic cases of aortic root aneurysms.³⁹

In sum, a previous study of *Col5a2^{+/-}* mice¹⁶ together with the present study of conditional *Col5a2* knockdown provide evidence indicating that insufficiency for $\alpha 2(V)$ chains is unlikely to produce a frank cEDS-like phenotype, thus helping explain the absence of cEDS patients with *COL5A2* null alleles.¹² Moreover, the lack of an obvious phenotype in the absence of stress in *Col5a2^{+/-}* mice suggests that *COL5A2^{+/-}* individuals probably exist in the general human population, but would be unlikely, on routine clinical examination, to be diagnosed not only with cEDS, but perhaps with other clinical abnormalities as well. However, our findings that insufficiency for $\alpha 2(V)$ chains can exacerbate AAA formation and predispose to dissecting and ruptured aneurysms of the aortic arch suggest *COL5A2* as an important and novel candidate marker/target for the identification and treatment of nonsyndromic individuals prone to aortic aneurysm and dissection.

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