Matricellular Hevin Regulates Decorin Production and Collagen Assembly*†‡

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Matricellular proteins such as SPARC, thrombospondin 1 and 2, and tenasin C and X subserve important functions in extracellular matrix synthesis and cellular adhesion to extracellular matrix. By virtue of its reported interaction with collagen I and deadheesive activity on cells, we hypothesized that hevin, a member of the SPARC gene family, regulates dermal extracellular matrix and collagen fibril formation. We present evidence for an altered collagen matrix and levels of the proteoglycan decorin in the normal dermis and dermal wound bed of hevin-null mice. The dermal elastic modulus was also enhanced in hevin-null animals. The levels of decorin protein secreted by hevin-null dermal fibroblasts were increased by exogenous hevin in vitro, data indicating that hevin might regulate both decorin and collagen fibrillogenesis. We also report a decorin-independent function for hevin in collagen fibrillogenesis. In vitro fibrillogenesis assays indicated that hevin enhanced fibril formation kinetics. Furthermore, cell adhesion assays indicated that cells adhered differently to collagen fibrils formed in the presence of hevin. Our observations support the capacity of hevin to modulate the structure of dermal extracellular matrix, specifically by its regulation of decorin levels and collagen fibril assembly.

The structure of the extracellular matrix (ECM)† is an important determinant of cellular behavior, and, in turn, is regulated by signals from adjacent cells. Proteins termed “matricellular” represent a class of proteins that regulate both ECM synthesis by cells and cell-ECM communication. Matricellular proteins are non-structural, secreted glycoproteins thought to function in an adaptive fashion by virtue of their interactions with cell-surface receptors, ECM, growth factors, and/or proteases.

Hevin (also known as SC1 and SPARC-like 1) is a protein belonging to the SPARC (secreted protein acidic and rich in cysteine) family of matricellular proteins. Proteins in the SPARC family are modular and share homologous C-terminal domains, including an extracellular Ca²⁺-binding domain and a follistatin-like domain. Murine hevin exhibits 53% identity with murine SPARC at the amino acid level, but has a unique, acidic N-terminal domain. Hevin was first identified as synaptic cleft (SC)-1 from a screen of a rat brain expression library for synaptic junctional glycoproteins of the central nervous system (1). It was subsequently cloned as “hevin” from a human high endothelial venule cDNA library (2). Because the term “SC1” also has been assigned to an integral membrane adhesion protein structurally unrelated to hevin, we have chosen to use “hevin” (3).

Members of the SPARC family, including SPARC itself, regulate ECM production and/or assembly (4, 5). For example, SPARC-null mice have altered dermal collagen, including reduced overall collagen content and collagen I fibril diameters, but increased collagen VI at the expense of collagen I (5). In concert, these changes act to reduce significantly the tensile strength of SPARC-null mouse skin (5). Although hevin has been implicated in the regulation of ECM production due to its functional similarity to SPARC, its widespread expression in ECM, and its association with collagen I (6), we expect hevin and SPARC to have distinct effects on ECM structure. Hevin is expressed differentially from SPARC during development and in the adult and is found predominantly in neural tissues, whereas SPARC is found in connective tissue and bone. Hevin and SPARC are also expressed non-coincidently in the developing cochlea (7). Recent evidence indicates that hevin and SPARC have markedly different functions in the foreign body response (hevin deletion enhances inflammation, whereas SPARC deletion decreases fibrosis) (8). Thus, we expect that hevin will regulate an aspect of ECM synthesis/structure different from that of SPARC.

We hypothesized that hevin might influence ECM in one of several ways. The capacity of hevin to interact with collagen I...
Hevin Influences Dermal ECM Assembly

(6) and/or other ECM components might directly affect the stability or assembly of ECM. Alternatively, hevin might act by altering cell-ECM interactions, thus potentially affecting ECM synthesis or remodeling. Given the abundance of collagen I in the murine dermis, as well as the accessibility of dermal tissue regeneration models (e.g. dermal wound healing), we chose to investigate the role of the hevin in modulating normal and pathologic dermal ECM structure. A transgenic hevin-null mouse model was used to determine the effects of hevin deletion on the dermis. We found that collagen fibril structure was indeed altered by the deletion of hevin, indicative of a role for hevin in fibrillogenesis and/or fibril remodeling. The fiber morphology, however, was different from that previously described for SPARC-null dermis (5). Hevin did not affect the total quantity of collagen deposited. Instead, deletion of hevin decreased the levels of decorin protein in murine dermis. Given that decorin and other proteoglycans are known to modulate fibril formation and packing density (10–12), this result suggested that hevin was influencing collagen deposition/structure indirectly, at least in part, via decorin.

We investigated the mechanism(s) underlying hevin function with dermal fibroblasts cultured from these mice and by in vitro fibrillogenesis assays. Hevin was found to stimulate the production of decorin protein by dermal fibroblasts in vitro, consistent with our findings in vivo. This effect did not appear to be dependent upon hevin-decorin binding. Hevin was also found to have a cell- and decorin-independent influence on collagen fibril formation in fibrillogenesis and cell adhesion assays. Taken together, our data indicate that hevin influences collagen structure by exercising classic matricellular functions: it regulates the accumulation of an ECM component, and it directly influences collagen fibril formation via interaction with collagen I.

MATERIALS AND METHODS

Reagents—10% neutral buffered formalin solution and paraformaldehyde were purchased from Sigma. 3,3’-Diaminobenzidine was purchased from Vector Laboratories (Burlingame, CA). Chondroitin ABC lyase was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Rat-tail collagen I was purchased from BD Biosciences. Alexa Fluor 488 phalloidin and Hoechst dye were purchased from Invitrogen. Fast Universal PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Chymotrypsin and elastase were purchased from Worthington Biochemical Corp. (Lakewood, NJ). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Recombinant murine hevin and rat IgG anti-hevin monoclonal antibody 12–51 were produced and purified according to established protocols (13). Recombinant human decorin core protein was from M. Höök (Center for Extracellular Matrix Biology and Department of Biochemistry and Biophysics, Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX). Its activity has been independently demonstrated by collagen binding and collagen fibrillogenesis assays.4 Rabbit IgG anti-mouse decorin polyclonal antisera LF113 was a gift from L. Fisher (Craniofacial and Skeletal Disease Branch, NIDCR, National Institutes of Health, Bethesda, MD) (14). LF113 was directed against a species-variable sequence near the N terminus of murine decorin that is not shared by any other small leucine-rich proteoglycans. All secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Cell culture reagents, including Dulbecco’s phosphate-buffered saline (DPBS), Dulbecco’s modified Eagle’s medium (DMEM), M199 medium, trypsin/EDTA, antibiotics, amphotericin B (fungizone), and fetal bovine serum (FBS) were purchased from Invitrogen.

Mice and Dermal Biopsies for Immunohistochemistry, Electron Microscopy, Tissue Extraction, Radioimmunoassay, Amino Acid Analysis, and Mechanical Testing—Hevin/SPARC-double-null mice of mixed background were obtained from L. Stetano-Coico (Weill Medical College of Cornell University, New York) and P. McKinnon (St. Jude Children’s Research Hospital, Memphis, TN). Wild-type (WT) and hevin-null colonies were subsequently developed on a pure 129 SVE background via extensive backcrossing (8). The animals were viable and fertile and maintained in a specific pathogen-free facility.

For collection of tissue sections, the dorsa of WT and hevin-null mice were shaved and rinsed with 70% ethanol. Shoulder skin sections for IHC and EM were dissected from animals and were fixed immediately (IHC: 10% formalin solution for 24 h followed by 70% ethanol; EM: Karnovsky’s fixative (15)). Samples for protein extraction, radioimmunoassay, and amino acid analysis were collected with a 5-mm punch biopsy and were frozen until use, whereas samples for mechanical tensile testing were collected with a dog bone-shaped (16) 2-cm long punch biopsy tool (oriented longitudinally along the animal) and were used immediately. IHC was performed according to standard protocols, with anti-hevin antibodies at 10 μg/ml and LF113 anti-decorin antisera at a dilution of 1:1000 where appropriate. Imaging was performed at ambient temperature on a Leica DMR microscope with a Leica PL FLUOTAR 40× objective, numerical aperture of 0.7 (Wetzlar, Germany). A SPOT RT Slider digital camera and the SPOT software package, version 3.02 (Sterling Heights, MI) were used for image acquisition. Photographs of decorin stains were overlaid with photographs of counterstains in Adobe Photoshop (San Jose, CA). For imaging of picrosirius red stain, samples were viewed and photographed under polarized light. All images were compiled into figures in Photoshop.

EM sections were stained with lead citrate, uranyl acetate, and ruthenium red (17) prior to routine processing (15). Fibrils identified in electron micrographs were statistically sampled by the method of Gundersen and Jensen (18), and their diameters were measured with the Image J software package (Bethesda, MD). Imaging and image acquisition were performed at ambient temperature in air on a JEM-1200EX 11 (JEOL Ltd., Tokyo, Japan) microscope. Images were subsequently scanned and compiled in Adobe Photoshop.

Desmosine and hydroxyproline analyses on dermal biopsies were performed according to published protocols (19). Briefly, 50–200 mg of tissue was hydrolyzed in 6 N HCl, evaporated to dryness, and resuspended in water. Desmosine content was

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4 N. Seo and M. Höök, manuscript in preparation.
Hevin Influences Dermal ECM Assembly

determined by radioimmunoassay (20). Hydroxyproline content was determined by amino acid analysis.

For tensile analyses, 1.75 cm (width) × 2.5 cm (length) dog bone-shaped dorsal skin sections were biopsied in a longitudinal orientation (parallel to the animal’s midline), one biopsy on each side of the animal. The sections were stressed longitudinally at a constant rate of strain (0.1 mm/s) with a mechanical tensile tester (21), and the stress-strain profiles were recorded. Force and displacement data were converted to stress and strain using the gauge length (1.0 cm) and width (1.0 cm) of the specimens. The tensile modulus was defined as the slope of the stress-strain curve in the linear region. Ultimate tensile strength was the maximal value of the stress obtained from the samples.

**Collagen in Dermal Wounds**—Full-thickness excisional wounds were made on WT and hevin-null animals (22). Mice were anesthetized, shaved, and washed with topical antiseptic and wounded twice on opposing sides of their dorsa. For excisional wounding, a 5-mm diameter punch biopsy was used to remove skin sections. Healing was monitored over a time course of 2 weeks, or until wounds were fully closed. Collagen fibrils in the wound beds were evaluated by EM. For each condition, n = 4–10 wound beds were evaluated.

**Cell Culture and Cell Adhesion Assays**—Dermal fibroblasts were isolated from the skin of WT and hevin-null adult 129 SVE mice according to published protocols (5) and were maintained in DMEM supplemented with 15% FBS, 1% penicillin G/streptomycin sulfate, and 1 μg/ml fungizone in a humidified 37 °C incubator enriched with 5% CO_{2}. Cells were passaged two to three times prior to use; they were maintained until approximately passage 7.

Adhesion assays were performed a minimum of three times, each time in duplicate. 12-mm glass coverslips were incubated at 37 °C with a solution containing 2 μM rat-tail collagen I in 1× DPBS (containing Ca^{2+} and Mg^{2+}), pH 7.6, and, where appropriate, 2 μM hevin, decorin, and/or ovalbumin. Following gelation, gel-coated coverslips were washed in DPBS and incubated at 37 °C for 30 min in serum-free DMEM with or without 0.017 μg/μl chymotrypsin and 0.006 μg/μl elastase. Enzyme:substrate ratios were chosen to allow for complete digestion of gel-bound hevin, as analyzed by SDS-PAGE and Coomassie Blue staining of soluble hevin following digestion. The digestion was stopped by addition of 1 mm phenylmethylsulfonyl fluoride followed by extensive washing with DMEM containing 15% FBS. Coverslips were next incubated with 10° WT or hevin-null fibroblasts/coverlip in DMEM containing 1% FBS. 2 h after cell plating, coverslips were washed in DPBS, and the adherent cells were fixed in 3.7% paraformaldehyde and stained according to standard fluorescent cytochemistry protocols with Alexa Fluor 488 phalloidin and Hoechst dye. For immunofluorescent imaging of hevin on gel-coated coverslips, coverslips were treated as described, but were washed and fixed immediately following protease treatment and addition of phenylmethylsulfonyl fluoride. Gels were stained with anti-hevin antibodies at 10 μg/ml. Imaging was performed at ambient temperature on a Leica DMR microscope with a Leica PL FLUOTAR 100× objective, numerical aperture 1.3 (Wetzlar, Germany). A SPOT RT Slider digital camera and the SPOT software package, version 3.02 were used for image acquisition. Photographs of actin stains were overlaid with photographs of counterstains, and cell areas were measured in Adobe Photoshop (San Jose, CA).

**Western Blots and Quantitative Reverse Transcription-PCR for Detection of Decorin**—Dermal skin sections were minced and extracted overnight at 4 °C in 8 M urea, and the total extracted protein was determined with the Pierce BCA Protein Assay Kit. The proteoglycan fractions from equal amounts of WT and hevin-null extracts were purified according to Kinsella et al. (23). Briefly, extracts were concentrated and partially purified on diethylaminoethyl-Sephacel™ columns. Proteoglycans were further purified by ethanol precipitation, and the glycosaminoglycan chains were removed by digestion with chondroitin ABC lyase. Digests were subsequently boiled for 10 min under reducing conditions and were resolved on 10% polyacrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories), and Western blots were performed with LF113 anti-decorin antiserum diluted 1:5000. For analysis of decorin protein levels in cultured cells, equal numbers of WT or hevin-null dermal fibroblasts were plated onto plastic culture dishes at ~90% confluence and were allowed to adhere for ~4 h in DMEM containing 15% FBS. Fibroblasts were washed and cultured for 1–24 h at near confluence in serum-free DMEM containing 250 nm hevin or bovine serum albumin (BSA) where indicated. This concentration was chosen because it induced detectable deadhesion activity by dermal fibroblasts when introduced into culture media (data not shown). Conditioned media and cell extracts for Western blotting were processed according to Kinsella et al. (23). Conditioned media were collected, and cell monolayers were scraped into 8 M urea solution. Both the media and extracts were subsequently concentrated, purified, and analyzed by SDS-PAGE and Western blotting. Experiments were performed a minimum of three times.

For isolation of RNA, cells were treated with trypsin and were pelleted following hevin/BSA stimulation. Cell pellets were lysed, and mRNA was collected and purified by use of an RNaseasy Mini Kit (Qiagen, Valencia, CA). First-stranded cDNA was generated from mRNA with the Qiagen Omniscript RT kit. Specific cDNA fragments were amplified by standard quantitative PCR protocols using the Applied Biosystems 7900HT Fast Real-Time PCR system with a 96-well fast block and TaqMan gene expression assays Mm00514535_m1 (murine decorin) and Mm99999915_g1 (murine glyceraldehyde-3-phosphate dehydrogenase). PCR data analysis was performed with the relative standard curve method and the SDS 2.2.1 software package from Applied Biosystems. Experiments were performed twice in triplicate.

**Collagen Fibrillogenesis Assays**—*In vitro* fibrillogenesis assays were performed a minimum of three times in triplicate. 2 μM rat-tail collagen I solutions were formulated on ice at pH 7.6 in 1× DPBS (containing Ca^{2+} and Mg^{2+}). 0.2–0.2 μM hevin, 0.2–0.2 μM ovalbumin, or 0.2–0.5 μM decorin was added where appropriate. These collagen and decorin concentrations were chosen to be comparable to those previously described (24–26); hevin:collagen molar ratios were chosen based on the range of SPARC concentrations known to influence collagen fibrillo-
Fibrillogenesis was initiated by warming the solutions to 37 °C in 96-well plates loaded into an OPTImax tunable microplate reader (Molecular Devices, Sunnyvale, CA). Gelation was detected by monitoring turbidity at 400 nm in 30-s intervals, and the resulting data were exported to Microsoft Excel (Redmond, WA) for analysis.

The lag time, rate of fibrillogenesis, and absorbance plateau were calculated from raw data plots. Lag time was calculated as the amount of time elapsed before the absorbance at 400 nm reached 10% of its final plateau value. The rate of fibrillogenesis was defined as the slope of the linear region following the lag phase and was calculated by least-squares fit of a line to the points in this region for which the slope remained constant within 10% of its value. The absorbance plateau was defined as the average value of the absorbance at 400 nm between time = 45 min and time = 55 min, between which all samples had reached their maximal turbidity.

Collagen Gel Contraction Assays—WT and hevin-null primary dermal fibroblasts were plated in 24-well plates at 100,000 cells/ml into 0.5 mg/ml rat-tail collagen I solutions buffered by M199 medium in the presence of 15% FBS. After gelation, gels were separated from the walls of the wells with a sterile needle and were allowed to contract for 5 h. Gels were fixed in 10% formalin solutions for 10 min, washed, and removed from

FIGURE 1. Hevin-null mice exhibit altered dermal ECM. A–L, sections of dermis from WT (A, B, E, F, I, and J) and hevin-null (C, D, G, H, K, and L) animals were stained for hevin (brown with blue hematoxylin counterstain, A and C); Hart’s stain (B and D); Movat’s pentachrome stain (E–H); decorin (green fluorescein isothiocyanate with blue Hoechst counterstain, I–L). A minimum of three age- and sex-matched adult (3–8 month) animals per genotype was examined; representative images from two animals are shown to illustrate variations in staining within a given genotype. Orientation (shown in A): f = fat, d = dermis, e = epidermis. Scale bar (shown in A) = 100 μm. M, protein was extracted from sections of WT and hevin-null dermis, and the proteoglycan fractions were purified, digested with chondroitin ABC lyase, and analyzed by SDS-PAGE and Western blotting. The intensity of the decorin band in the hevin-null lane (black bar) is shown relative to the intensity of the band in the WT lane (white bar) on the adjacent graph. Error bars represent one standard deviation from the mean for a sample population of n = 3. Extractions were performed on three animals per genotype; representative experiments are shown. +/+, WT; −/−, hevin-null.

5 R. Vernon, M. Gooden, and E. H. Sage, unpublished observations.
24-well plates, and their diameters and thicknesses were measured with calipers. Gel volumes were calculated from the thicknesses and diameters of the gels with the assumption that the gels were perfect cylinders. Experiments were performed three times, in triplicate.

RESULTS

Given the widespread distribution of hevin in ECM, as well as the known effects of its family member SPARC on ECM structure (5), we hypothesized that hevin deletion would lead to alterations in the dermal ECM. The expression and distribution of hevin and various ECM components were examined in sex-matched and age-matched adult (3–8 months) WT and hevin-null mice by IHC (Fig. 1). Hevin was present within the dermis and was also observed along the basement membrane of the dermal-epidermal junction and around hair follicles (Fig. 1A, arrows). Hevin did not appear to affect the integrity of the dermal elastic matrix, as revealed by Hart’s stain (purple fibers, Fig. 1, B, E, and F). In contrast, staining with Movat’s pentachrome revealed distinct differences in overall dermal architecture between WT and hevin-null mice (Fig. 1, G–H). Hevin-null animals displayed an altered connective tissue staining pattern (red) that was coincident with patterns revealed by stains typically used for collagenous proteins (e.g., Fig. 2, A–H) and was suggestive of alterations in the distribution or structure of connective tissue protein. Furthermore, overall staining for proteoglycan (blue) was reduced in hevin-null animals, in comparison with WT. Specific detection of murine decorin (green, Fig. 1, I–N) was performed by EM (with uranyl acetate, lead citrate, and ruthenium red staining), and fibrils in the dermis were photographed in cross-section. I and J, dermis, adjacent to the dermal-epidermal junction. K and L, mid-dermis. M and N, deeper dermis. Scale bar (shown in I) = 100 nm. O, fibril diameters in the upper (adjacent to the dermal-epidermal junction) dermis (e.g., I and J) from photographs of WT (white bars) and hevin-null (black bars) animals were sampled and analyzed digitally. A minimum of three animals per genotype was examined.
higher density of collagen I fibers in the hevin-null dermis in comparison with those of WT dermis (Fig. 2, A–D). EM revealed that these macroscopic changes were consistent with nanoscale differences in collagen fibril structure and arrangement, with thinner, more uniform, and more densely packed fibrils in hevin-null dermis (Fig. 2, I–O). In 5-month-old animals, average cross-sectional fibril diameters were 54.6 ± 12.7 nm in WT dermis versus 43.7 ± 11.1 nm in hevin-null dermis (p < 0.05) (Fig. 2O), and average packing densities (percent of cross-sectional area covered by fibrils) were 61 ± 5% versus 73 ± 4%, respectively (p < 0.05). Collagen fibril maturity was further examined with picrosirius red (Fig. 2, E–H), which stains thicker, more cross-linked fibrils red, and thinner, less cross-linked fibrils yellow-green, when viewed under polarized light (27). Despite the differences in average fibril thickness between the genotypes, differences in picrosirius red staining were minimal, when the variability of staining within a given genotype was considered (e.g. Fig. 2E versus Fig. 2F). This result indicated that the picrosirius red stain was not sufficiently sensitive to detect the differences in fibril thickness or that other contributions to staining color were masking these differences (e.g. fibril cross-linking).

Because of the apparent differences in dermal collagenous structure and the alterations in proteoglycan levels, both of which are known to influence collagen fibrillogenesis under non-pathologic conditions (10–12, 28), we next investigated the structure of newly synthesized collagen in a dermal repair model. Full-thickness excisional dorsal wounds were made with a 5-mm circular punch and the wound bed was monitored by EM during healing. Representative EM images of collagen in 14-day wound beds, shown in Fig. 3, underscored differences in newly synthesized ECM. Collagen fibrils in WT animals appeared normal within the wound bed (Fig. 3, A–F). In contrast, fibrils in null animals were irregular in cross-sectional shape and appeared hollow upon routine staining with lead citrate and uranyl acetate (Fig. 3, G–L). Ruthenium red staining of proteoglycans (or other anionic connective tissue components) around WT fibrils (Fig. 3, B and E, arrows) was reduced around null fibrils (Fig. 3, H and K, arrowheads), results consistent with...
We therefore examined decorin production by cultured WT and hevin-null primary dermal fibroblasts. After 24 h in serum-free DMEM, purified proteoglycan fractions from the conditioned media and cell lysates of dermal fibroblasts were analyzed by Western blotting (Fig. 5A, left panel). Decorin protein levels were ~50% lower in both the conditioned media and the cell lysates of hevin-null cells. Furthermore, we could rescue decorin production levels in hevin-null dermal fibroblasts by addition of recombinant hevin (Fig. 5A, right panel). We also examined decorin mRNA levels in hevin-null fibroblasts incubated with hevin or a negative control (BSA). mRNAs collected from cell lysates were analyzed by quantitative reverse transcription-PCR (Fig. 5B). That decorin mRNA levels were unchanged by the addition of hevin 1–24 h after stimulation indicated that hevin was affecting neither the production nor the stability of decorin mRNA.

We postulated that hevin might be influencing the levels of decorin protein by a direct protein-protein interaction. However, we were unable to co-immunoprecipitate hevin and decorin from dermal fibroblast cell lysates; we detected only a weak hevin-decorin interaction based on enzyme-linked immunosorbent assay (data not shown). We therefore concluded that hevin-decorin binding likely did not account for the increased levels of decorin in the presence of hevin.

In addition to its regulation of collagen structure via decorin, we expected that hevin might also influence fibrillogenesis by interacting with collagen I (6). We examined collagen I fibril formation in vitro in the presence and absence of hevin, decorin, and a negative control (ovalbumin) by monitoring the solution turbidity at 400 nm (24, 29, 30). As expected, the addition of ovalbumin did not measurably affect fibril formation (data not shown). In contrast, inclusion of hevin shortened the lag phase preceding fibrillogenesis and enhanced the rate of fibril formation. Inclusion of decorin decreased the average final fibril diameter, as indicated by the lower plateau in final gel turbidity (Fig. 6 and Table 1). Addition of hevin and decorin together produced a decrease in the lag time and the final absorbance plateau, consistent with the separate addition of hevin or decorin, respectively. However, the rate of fibrillogenesis was not significantly different from that of collagen alone (Fig. 6 and Table 1). This result indicated that the influence of hevin on fibril formation kinetics was sensitive to the presence of decorin, and that hevin and decorin might have both distinct and concerted functions regarding fibril formation.

To evaluate further the direct influence of hevin on collagen fibrillogenesis, we investigated the adhesion of WT and hevin-null primary dermal fibroblasts, i.e. the tensile modulus of hevin-null skin was higher than that of WT skin. Given the correlation between cross-linking and tensile strength, we hypothesized that fibril morphologies might be altered. Because data in Fig. 1, and with the proposal that the lack of hevin might indirectly affect fibrillogenesis by influencing the deposition of decorin or other proteoglycans.

To test for other differences in dermal collagen structure between mice of the two genotypes, we examined the mechanical properties of their skin. In particular, the ultimate tensile strength of a tissue should reflect the total amount, thickness, and arrangement of its fibrillar collagens and other fibrillar proteins. The tissue tensile modulus should be determined by the structure and cross-linking of its fibrillar proteins. These properties were therefore measured in skin biopsied from WT and hevin-null animals by the use of a mechanical tensile tester (Fig. 4). No difference was observed between the genotypes in ultimate tensile strength; in contrast, differences in tensile modulus between the genotypes were observed, i.e. the tensile modulus of hevin-null skin was higher than that of WT skin. The tissue tensile modulus should be determined by the structure and cross-linking of its fibrillar proteins. These properties were therefore measured in skin biopsied from WT and hevin-null animals by the use of a mechanical tensile tester (Fig. 4).

To evaluate further the direct influence of hevin on collagen fibrillogenesis, we investigated the adhesion of WT and hevin-null primary dermal fibroblasts, i.e. the tensile modulus of hevin-null skin was higher than that of WT skin. Given the correlation between cross-linking and tensile modulus, these results supported differential fibril cross-linking between the genotypes.

Based on our findings in vivo, we expected that hevin would influence collagen fibril structure at least in part by regulating the levels of decorin (Fig. 1, E–I; Fig. 3, B, E, H, and K, arrows). We therefore examined decorin production by cultured WT and hevin-null primary dermal fibroblasts. After 24 h in serum-free DMEM, purified proteoglycan fractions from the conditioned media and cell lysates of dermal fibroblasts were analyzed by Western blotting (Fig. 5A, left panel). Decorin protein levels were ~50% lower in both the conditioned media and the cell lysates of hevin-null cells. Furthermore, we could rescue decorin production levels in hevin-null dermal fibroblasts by addition of recombinant hevin (Fig. 5A, right panel). We also examined decorin mRNA levels in hevin-null fibroblasts incubated with hevin or a negative control (BSA). mRNAs collected from cell lysates were analyzed by quantitative reverse transcription-PCR (Fig. 5B). That decorin mRNA levels were unchanged by the addition of hevin 1–24 h after stimulation indicated that hevin was affecting neither the production nor the stability of decorin mRNA.

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To evaluate further the direct influence of hevin on collagen fibrillogenesis, we investigated the adhesion of WT and hevin-null dermal fibroblasts (Fig. 7) to fibrillar collagen substrates formed with and without the addition of hevin, decorin, and/or ovalbumin. Two hours after plating, fibroblasts were observed to attach and spread onto gels formed from collagen alone or collagen in the presence of ovalbumin (Fig. 7, A, B, and K). In contrast, cell spreading and actin network formation were significantly retarded on gels formed in the presence of hevin and/or decorin (Fig. 7, C–E and K), results indicating that fibril morphologies might be altered. Because...
substrate-bound hevin is known to be deadhesive for many cell types, including the dermal fibroblast\textsuperscript{6} (13, 31, 32), we reasoned that fibroblasts might be responding to hevin bound to collagen fibrils, rather than to the fibrils themselves. Both matrix-bound and free decorin are also known to be deadhesive (33–40). We therefore investigated whether removal of hevin and decorin bound to the fibrils would negate the deadhesive response of the fibroblasts. Collagen gels were incubated with a mixture of chymotrypsin and elastase and were subsequently washed extensively. Cleavage and removal of hevin and decorin (data not shown) were evaluated by SDS-PAGE (Fig. 7M) and fluorescence microscopy (Fig. 7, N and O); collagen gel integrity was confirmed microscopically (data not shown). Based on fluorescent staining of F-actin, fibroblasts were found to spread similarly on protease-treated gels and on untreated gels formed from collagen alone or collagen containing ovalbumin or hevin (Fig. 7, A–C, F–H, K, and L). This result indicated that the deadhesive behavior of the cells on gels formed in the presence of hevin was due to alterations in fibril morphologies caused by hevin, as opposed to a direct interaction of the cells with hevin. We also have evidence from collagen gel contraction assays that cell interactions with collagen are altered by hevin (supplemental Fig. S1), because WT dermal fibroblasts contracted collagen gels less efficiently than hevin-null fibroblasts. Cell spreading on gels formed from collagen and decorin was moderately more efficient following protease digestion and removal of substrate-bound decorin (Fig. 7, D, I, K, and L). This result indicated that the deadhesive behavior of cells on gels formed in the presence of decorin was due to both a direct interaction with decorin and to alterations in fibril morphologies resulting from the presence of decorin.

**DISCUSSION**

Hevin, a member of the SPARC family of matricellular proteins, has been implicated in the regulation of cell adhesion, migration, and proliferation. These functions have been relatively well studied in the context of endothelium and the nervous system; in contrast, the function of hevin during ECM synthesis is relatively understudied despite its widespread matrix-associated expression. This work represents the first extensive examination, to our knowledge, of the phenotype of the hevin-null animal. The structural similarities between hevin and SPARC are significant and indicate that hevin could play an important role in directing connective tissue synthesis/
Hevin Influences Dermal ECM Assembly

The observations that decorin levels were reduced in hevin-null animals and that hevin could regulate decorin protein production or stability in vitro suggested decorin as a potential target for association with hevin. Although our analyses of hevin-decorin binding were not supportive of a strong interaction between the two proteins, they did not rule out the possibility of a ternary link among hevin, decorin, and collagen I, or an interaction between hevin and its precursor, prodecorin (Fig. 8). The hevin-collagen I interaction might influence fibrillogenesis by affecting the binding of decorin to collagen I (Fig. 8A). This perturbation could result in both stabilization and/or modulation of the decorin-collagen I interaction, as well as protection of the decorin protein from degradation. Such protection would result in the observed regulation of decorin protein levels.

Hevin might also influence the availability of decorin by interacting with/stabilizing prodecorin (Fig. 8B). The increased persistence of prodecorin would in turn be expected to result in elevated levels of mature decorin, such as we observed. Increased levels of decorin would be expected to affect fibrillogenesis directly, given the known influence of decorin on controlling collagen fibril structure (11). Decorin-null animals have lax, fragile skin with overall dermal thinning and loose connective tissue. Individual collagen fibrils are loosely packed and irregularly shaped, with a distribution of diameters, presumably due to poorly controlled lateral fusion of fibrils (11). Other models have also illustrated the importance of proteoglycans in the control of fibril fusion (10).

Finally, hevin might have a decorin-independent influence on collagen fibril structure (Fig. 8C). We therefore performed fibrillogenesis assays, under cell-free conditions, with and without decorin. The capacity of hevin alone to enhance the initiation and rate of fibrillogenesis was suggestive that it indeed could alter direct associations between collagen monomers and/or fibrils. The influence of decorin alone was consistent with the literature for the most part. Several groups have shown that decorin increases the lag time prior to fibril formation, decreases the rate of fibrillogenesis, and/or decreases the final size of the fibrils formed (e.g. the solution turbidity) (24–26, 29). Although our data did not support a significant increase in lag time or decrease in fibril formation rate in the presence of a similar concentration of decorin alone, we expect that different preparations of collagen and decorin and/or minor differences in buffer composition would influence the kinetics of fibril formation. In support of this assertion, the kinetics of fibrillogenesis differ significantly within the literature and do not all demonstrate the 3-fold effect of decorin claimed by Rada et al. (24–26, 29, 30). Furthermore, our data describing the combined influences of hevin and decorin infer that decorin slows the rate of fibrillogenesis; the added presence of decorin negated the increases caused by the inclusion of hevin.

Our cell adhesion assays were confirmation that hevin had an independent influence on fibril formation and, furthermore,
Hevin Influences Dermal ECM Assembly

provided a more sensitive probe for the final morphologies of the collagen fibrils formed. We expected that any alterations in fibril structure caused by the presence of hevin might affect collagen-integrin interactions. For example, fibroblasts have been shown to recognize different collagen sequences as a function of the topographical organization of the substrate (41).

Other ECM-integrin interactions are also known to be influenced by ECM topography/organization. Cell interactions with fibronectin are influenced both by stretching of the fibronectin, which is believed to expose different integrin recognition sites...
Figure 8. Mechanisms by which hevin might influence fibril formation. A, a ternary interaction between hevin, decorin, and collagen I results both in alterations in fibril formation and enhanced decorin stability. B, an intracellular hevin-prodecorin interaction enhances the stability/persistence of prodecorin and decorin. Increased levels of decorin alter fibrillogenesis. C, a hevin-collagen I interaction directly influences fibril formation.

7 M. M. Sullivan, P. A. Puolakkainen, T. H. Barker, S. E. Funk, and E. H. Sage, manuscript in preparation.

Hevin Influences Dermal ECM Assembly

Hevin could influence ECM generation/regeneration by a number of different mechanisms, but it clearly plays a role distinct from those of its closest functional relatives. For example, we have demonstrated that hevin and SPARC have opposite effects on fibril packing, influence a fundamentally different dermal mechanical characteristic (hevin deletion increases elastic modulus whereas SPARC deletion reduces tensile strength), and have distinct effects on collagen I deposition (5). We have also identified a unique regulatory function for hevin based on its capacity to alter decorin protein levels. Given its fundamental role as an intermediary between cells and their ECM, we expect that more functions for hevin in connective tissue synthesis will be identified. Enhanced numbers of macrophages observed in hevin-null dermal wound beds and in the foreign body response indicate that hevin might influence inflammatory cell recruitment (8). Hevin has also been linked to angiogenesis due to its high levels of expression in tumor endothelia (48). A critical component of tissue regeneration, neovascularization is postulated to be dependent upon endothelial cell migration (9). Elucidating the function of hevin as a modulator of adhesive interactions and ECM assembly will thus provide insights into multiple stages of the tissue generation/regeneration process.

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