Materials and Methods

Mice

Transgenic mouse strains: En-1^{Cre} (En1$^{tm2(cre)Wrst}$/J), En-1$^{Cre-ERT}$ (En1$^{tm7(cre/ESR1)Alj}$/J), R26^{mTmG} (Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J), Ai6 (B6.Cg-*Gt(ROSA)26Sor$^{tm6(CAG-ZsGreen1)Hze}$*/J), R26^{iDTR} (C57BL/6-*Gt(ROSA)26Sor$^{tm1(HBEGF)Awai}$*/J, and YAP$^{fl/fl}$ (YAP1$^{tm1.1Dupa}$/J).

Mice were bred and maintained at the Stanford University Comparative Medicine Pavilion in accordance with Stanford APLAC guidelines (APLAC-11048). Mice were housed and bred under the care of the Department of Comparative Medicine in the Veterinary Service Center (VSC). All transgenic mouse strains were obtained from Jackson Laboratories. *En1^{Cre}* and *En1$^{Cre-ERT}$* mice were crossed with Ai6 and mT/mG reporter mice to trace all EPFs and postnatal EPFs, respectively, as defined *in vivo* by their GFP positivity.

Transgenic mouse strains were validated by tissue collection and genotyping of each individual animal. The following primers were used: for En-1^{Cre} and En-1$^{Cre-ERT}$ mice (band size Cre: 102 bp, internal positive control: 74 bp) Cre forward 5'-GCG GTC TGG CAG TAA AAA CTA TC-3', Cre reverse 5'-GTG AAA CAG CAT TGC TGT CAC TT-3', IPC forward 5'-CAC GTG GGC TCC AGC ATT-3', IPC reverse 5'-TCA CCA GTC ATT TCT GCC TTT G-3'; for R26^{mTmG} (band size mutant: 140 bp, wt: 96 bp) mutant reverse 5'-GTT ATG TAA CGC GGA ACT CCA-3', wt reverse 5'-CAG GAC AAC GCC CAC ACA-3', common forward 5'-CTT CCC TCG TGA TCT GCA AC-3'; for Ai6 (band size mutant: 199 bp) mutant forward 5' -AAC CAG AAG TGG CAC CTG AC-3', mutant reverse 5' -GGC ATT AAA GCA GCG TAT CC-3' (note that the Ai6 primer sequences are highly sensitive to multiple freeze thaw cycles); for R26^{iDTR} (band size mutant: 300 bp) mutant 5' -GCG AAG AGT TTG TCC TCA ACC-3', common 5' -AAA GTC GCT CTG AGT TGT TAT-3', wt reverse 5' -GGA GCG GGA GAA ATG GAT ATG-3'; for YAP$^{fl/fl}$ (band size mutant: 270 bp, wt: 186 bp) forward 5' -AGG ACA GCC AGG ACT ACA CAG-3', reverse 5' -CAC CAG CCT TTA AAT TGA GAA C-3'. The PCR conditions were: 94℃ for 10 mins, 94℃ for 30 sec, 56℃ for 1:30 min, 72℃ for 1.5 min, repeat 35 cycles, 72℃ for 8 mins.

Harvesting Dermal Fibroblasts

Mice were euthanized by CO_2 narcosis and cervical dislocation, the dorsal fur was clipped, a depilatory cream was applied topically to the dorsum for 30 seconds. Next, the dorsal skin was harvested using dissecting scissors by separation along fascial planes, the subcutaneous fat was trimmed with a scalpel, and the skin was rinsed in betadine, followed by 5 rinses in cold PBS. To achieve a cell suspension, the harvested skin was finely minced using sharp scissors, enzymatically digested (Liberase DL, 0.5 mg/mL, 1 hour), and filtered through a 40 μm nylon mesh. ENFs and EPFs were isolated from En-1^{Cre};R26^{mTmG} mice (*En-1* lineage-negative cells, mTomato^{+}; *En-1* lineage-positive cells, GFP^{+}) via a previously reported FACS strategy. Briefly, a lineage gate (Lin) for hematopoietic (CD45, Ter-119), endothelial (CD31, Tie2), and epithelial (CD326, CD324) cell markers was used as a negative gate to isolate fibroblasts (Lin^{-}), which were sorted into ENFs (Tomato^{+} GFP^{-} Lin^{-}) and EPFs (Tomato^{-} GFP^{+} Lin^{-}). To isolate ENF subpopulations, dorsal skin cells were harvested from P1 *En-1^{Cre};Ai6* mice (*En-1* lineage-negative cells, no fluorescence; *En-1* lineage-positive cells, GFP^{+}) via mechanical and enzymatic digestion as described above. Cells were then stained for the aforementioned lineage markers, in addition to CD26, Dlk1, and Sca1 in order to derive ENFs of the papillary dermis (Lin^{-} CD26^{+} Dlk1^{-} Sca1^{-}), reticular dermis (Lin^{-}

CD26^{-} Dlk1^{+} Sca1^{-}), and hypodermis (Lin^{-} CD26^{-} Dlk1$^{+/-}$ Sca1^{+}). Cells were resuspended in FACS buffer and DAPI before FACS analysis.

Cell Engraftment

We used one-day old (P1) *En-1^{Cre};R26^{mTmG}* and *En-1^{Cre};Ai6* mice to isolate ENFs and EPFs for both engraftment and *in vitro* studies for the following three reasons. First, P1 mice are known to heal with a similar scarring outcome as older P60 mice. Second, neonatal mouse skin is more cellular than juvenile or adult mouse skin, so fewer mice can be sacrificed to derive the high cell numbers required for successful engraftment. Finally, we have observed that P1 cells retain higher viability after engraftment than P60 cells. Recipient mice (P60 C57BL/6 or R26^{mTmG}) were anesthetized (2% isofluorane), their dorsal hair was removed using depilatory cream, and their skin was prepped with alcohol wipes. Injection sites (6 mm circular regions at the level of the scapulae; either two per mouse, roughly 8 mm lateral to midline (ENF and EPF transplantation), or one, at midline (ENF subtype transplantation)) were marked with a skin marker, and fibroblasts were injected intradermally (100,000 cells per mouse; N = 3 mice each receiving ENFs, ENF subpopulations, or EPFs) around the border of each region. Cells were allowed to engraft for 48 h, after which separate, 6 mm full-thickness excisional wounds (see below) were made at each marked injection site, such that the engrafted cells were now located at the wound edge.

Dorsal Excisional Wounding

P30-60 *En-1^{Cre};R26^{mTmG}*, *En-1^{Cre};Ai6*, *En-1$^{Cre-ERT}$;Ai6*, *En-1$^{Cre-ERT}$;Ai6;R26^{iDTR}*, *En-1$^{Cre-ERT}$;R26^{mTmG};YAP$^{fl/+}$*, and *En-1$^{Cre-ERT}$;Ai6;YAP$^{fl/fl}$* mice were used for cutaneous wound healing experiments in accordance with well-established protocols. Briefly, mice were anesthetized (2% isofluorane), their dorsal hair was removed with depilatory cream, and the dorsal skin was prepped with alcohol wipes. Next, two 6 mm full-thickness circular wounds were placed through the panniculus carnosus on the dorsum of each animal at the same level, roughly 6 mm below the ears and 4 mm lateral to the midline. The wounds were then stented open by 12 mm diameter silicone rings secured around the wound perimeter with glue and 8 simple interrupted Ethilon 6-0 sutures (Ethicon). For mice receiving mechanotransduction inhibitor, 30 µL of Verteporfin (1 mg/mL) was injected locally into the wound base; PBS was injected into wounds for vehicle controls. Post-operative analgesia was accomplished with buprenorphine 0.05 mg/kg every four hours for three doses, and then as indicated. Dressings were changed every other day under anesthesia. All wounds were fully re-epithelialized by post-operative day (POD) 14, at which time the wound and surrounding skin (used as unwounded control) were harvested and processed for histology. Induction of *En-1$^{Cre-ERT}$;Ai6* mice (N = 4 mice) was achieved by 5 consecutive days of intraperitoneal tamoxifen injections (90% corn oil/ethanol v/v; 200 mg/kg body weight) prior to wounding. For genetic ablation of pEPFs, *En-1$^{Cre-ERT}$;Ai6;R26^{iDTR}* were systemically induced with tamoxifen and wounded. The wound base was then injected on POD 0, 2, and 4 with 200 ng of diphtheria toxin in 30 µL of PBS; control wounds were injected with PBS alone. In all experiments, a minimum of 3 mice with 2 wounds each was used for each treatment group.

Cell Culture

Fibroblasts were cultured in DMEM/F12 media supplemented with 10% fetal bovine serum and 0.1% penicillin/streptomycin at 37° C and 5% CO_2. Tissue culture polystyrene (TCPS) was coated with 0.1% gelatin prior to cell seeding. For three-dimensional culture, fibroblasts were resuspended in collagen hydrogels (EMD Millipore ECM675) and hydrogels were cured for 30

min at 37° C and 5% CO_2 before addition of media. For culture on substrates of variable stiffness, we analyzed N = 3 experimental replicates using P1 ENFs derived from separate litters. For ENF subtype culture, we similarly analyzed N = 3 experimental replicates using P1 ENFs derived from separate litters. For RNA-seq experiments, we analyzed N = 2 biological replicates per experimental group (pooled ENFs from 2 separate litters, 10 pups each).

Mechanical Loading of Wounds

20 mm-long linear wounds were produced on the dorsum of P60 *En-1*$^{Cre\text{-}ERT}$*;Ai6* mice and then closed with sutures. On POD 4, a loading device (constructed from 22 mm expansion screws and Luhr plate supports) was secured over each wound with adhesive and simple interrupted sutures. For mice receiving mechanotransduction signaling inhibitor, 30 μL of Verteporfin (1 mg/mL) was injected along the suture line both at POD 0 and POD 4; PBS was injected into wounds for vehicle controls. Device tension was increased by distracting expansion 2 mm every 2 days for 10 days total. Mice with unexpanded devices served as sham surgery controls. On POD 14, wounds were harvested and processed for histology to characterize the effects of increased wound tension on activation of ENFs to scarring pEPFs (N = 4-5 mice/condition).

Histology and Immunofluorescent Staining

Tissues were fixed in 2% paraformaldehyde for 16 h at 4°C. Samples were prepared for embedding by soaking in 30% sucrose in PBS for 1 week at 4°C. Samples were then removed from the sucrose solution, and tissue blocks were prepared by embedding in Tissue Tek O.C.T. (Sakura Finetek) under dry ice to achieve rapid freezing. Frozen blocks were mounted on a Thermo Scientific CryoStar NX70 cryostat, and 10 μm-thick sections were transferred to Superfrost/Plus adhesive slides (Fisher). For hematoxylin and eosin staining, standard protocols were used with no modifications. For immunofluorescent staining, slides were blocked for 1 hr with Power Block (Biogenex) prior to addition of the following primary antibodies: Abcam ab34710 (anti-collagen type I), Abcam ab2413 (anti-fibronectin), Abcam ab28340 (anti-CD26), Invitrogen MA5-15915 (anti-Dlk1), Abcam ab51317 (anti-Sca1), Abcam ab5694 (anti-α-SMA), Santa Cruz Biotechnology sc-101199 (anti-YAP), Abcam ab7800 (anti-CK14), and Abcam ab52625 (anti-CK19). Slides were then incubated for 1 h with Alexa Fluor 568 or Alexa Fluor 647-conjugated anti-rabbit, anti-rat, or anti-mouse antibodies (Invitrogen). Finally, slides were mounted in Fluoromount-G mounting solution with DAPI (Thermo Fisher). Brightfield images were acquired with a Leica DMI4000B microscope, while fluorescent images were acquired with a Leica DM6000 SP5 upright confocal microscope.

Pixel Co-localization Analyses and 3D Image Reconstruction

For collagen-I co-localization quantification, confocal z-stacks were analyzed using Imaris 8.1.2 software (Bitplane). The surfaces of collagen-I immunofluorescence and of the transplanted ENFs or pEPFs were first reconstructed in three-dimensions. Next, the percent of surface contact between collagen I and the transplanted fibroblasts was determined by the colocalization module. Each dot in Figure 1D represents the average contact calculated from the immunofluorescence histology of one wound. For YAP nuclear localization quantification, confocal z-stacks were analyzed using the EzColocalization package in ImageJ to quantify colocalization of nuclei and YAP (N = 4-5 mice/condition).(*41*)

Bulk RNA Sequencing

Total RNA was harvested by lysing cells in Trizol reagent (Invitrogen). For RNA-seq of skin and wound fibroblast subtypes (pEPFs, eEPFs, and ENFs), N = 2 biological replicates were sequenced per experimental group (24 excisional wound scars and 6 unwounded skin pieces from 6 mice pooled into 2 groups each). For short hairpin RNA knockdown of *Engrailed-1* expression, cultured cells were treated with polybrene in media (16 hrs, 1 μg/mL) and 5 μL of control or *En-1* shRNA lentiviral particles (Santa Cruz Biotechnology, sc-45653-V) were spiked into the corresponding well (multiplicity of infection = 2). After 24 hours, media was replaced and the cells were incubated overnight. Cells were then selected in puromycin-containing media (1 μg/mL), with uninfected wells to confirm cell killing. RNA extraction and library preparation was performed by the Stanford Functional Genomics facility using standard Qiagen kits and protocols. Directional RNA-Seq libraries were analyzed with an Agilent Bioanalyzer to ensure successful library creation, and then sequenced with the Illumina HiSeq 4000 System (2x75 bp, 150 cycles). Paired-end reads were mapped to the mouse genome reference sequence mm10 using the STAR aligner. Differential gene transcription analysis was achieved in Matlab 2019a using a negative binomial model. It is common practice to normalize read counts by the total number of reads and the length of each transcript, yielding reads per kilobase mapped (RPKM) values. However, such analysis may be skewed towards a few highly expressed genes that dominate the total lane count. Thus, we opted to instead normalize counts by a size factor, calculated by taking the median of the ratios of observed counts to those of a pseudo-reference sample (whose counts are the geometric means of each gene across all samples). For hypothesis testing of differential gene transcription, the read counts were modeled according to a negative binomial distribution, with the variance considered as the sum of the shot noise term and a locally regressed non-parametric smooth function of the mean. P values were then adjusted by the Benjamini-Hochberg statistical method to account for multiple testing, and counts were considered significantly different at a threshold of 0.00005 for *in vitro* studies and 0.01 for *in vivo* studies. RNA-seq data can be accessed at the following Github repository: https://github.com/shamikmascharak/Mascharak-et-al-ENF

Gene Set Enrichment Analysis

Gene Ontology (GO) analyses of significantly up- or down-regulated genes in Fig. 3C and Fig. S13E were performed using g.Profiler (https://biit.cs.ut.ee/gprofiler/gost) with a p value cutoff of 0.05. Ranked whole genome enrichment analyses in Supplementary Figures 4, 5, 6, and 13 were performed using GSEA software developed by the Broad Institute with nominal p value and false discovery rate (FDR) cutoffs of 0.01 and 0.25, respectively. All g.Profiler and GSEA results are available in the following Github repository: https://github.com/shamikmascharak/Mascharak-et-al-ENF

Quantitative Analysis of Collagen Ultrastructure

For analysis of Picrosirius Red-stained histologic sections, scars and surrounding normal skin from three biologic replicates/condition were randomly imaged at 5 to 10 separate locations each, for a minimum of 20 images per experimental condition. Next, we performed color deconvolution of Picrosirius Red images in ImageJ using the algorithm previously described by Ruifrok et al.,(*42*) wherein each pure stain is characterized by absorbances within three RGB channels (Color 1 = [1 0 0], Color 2 = [0 1 0], Color 3 = [1 1 1]). Ortho-normal transformation of the histology images produced individual images corresponding to each color's individual contribution to the image. Applied to birefringent Picrosirius Red images (green to red color under polarized light depending on packing of fiber bundles), this technique produced deconvoluted red and green images

corresponding to mature and immature connective tissue fibers, which were then analyzed independently. Analysis was thus performed purely using extracellular matrix fibers, with no cellular elements included. Noise reduction of deconvoluted fibers was achieved using an adaptive Wiener filter in Matlab 2019a (*wiener2* function), which tailors itself to the local image variance within a pre-specified neighborhood (3-by-3 pixels in our application). The filter preferentially smooths regions with low variance, thereby preserving sharp edges of fibers. Smooth images were then binarized using the *im2bw* command and processed through erosion and dilation filters with both linear and diamond-shaped structuring elements to select for fiber-shaped objects. Finally, the fiber network was "skeletonized" using the *bwmorph* command and various parameters of the digitized map (fiber length, width, persistence, alignment, etc.) were measured using the *regionprops* command. Dimensionality reduction of quantified fiber network properties by t-distributed stochastic neighbor embedding (t-SNE) was achieved using the default *tsne* (distance metric specified as Euclidian distance) command in Matlab (each point on t-SNE represents a single image). A Matlab script containing our fiber quantification pipeline is available at the following Github repository: https://github.com/shamikmascharak/Mascharak-et-al-ENF

Tensile Strength Testing

Tensile strength tests for unwounded skin (N = 7) and PBS (N = 5) or Verteporfin-treated (N = 4) wounds in P60 C57BL/6 mice were conducted at POD 30 using an Instron 5565 equipped with a 100 N load cell. Dorsal skin was harvested and cut into 4mm-by-15 mm strips. Tissue strips were then secured using custom grips with the scar positioned equidistant to each grip edge and preloaded to a force of 0.02 N to remove slack before the length of the tissue was measured using digital calipers; the width and thickness of the strips were also re-measured to confirm accurate dimensions. Finally, the skin was subjected to an extension test to failure, defined by a sharp decrease in stress with increasing strain, at a rate of 1%/s. The wound breaking force, or yield force, was determined at the maximal force before the tissue entered plastic deformation and eventual failure. True strain was calculated as the change in length divided by the original gauge length, and true stress was calculated as the force divided by the original cross-sectional area. The Young's Modulus was calculated by taking a least-squares regression of the slope during the linear, elastic portion of the stress-strain curve ($R^2 > 0.99$). Points plotted represent individual mice.

Statistical Testing

Unlike otherwise stated, two-group comparisons were made using unpaired, two-tailed Student's t-test. For multi-group comparisons, one-way ANOVA with Bonferroni's *post hoc* correction was run ($p = 0.05$ significance level) prior to t-test comparisons.