SUPPLEMENTARY MATERIAL, Mack et al.

ADDITIONAL MATERIALS AND METHODS

Histology and Immunohistochemistry

Histochoice-fixed or 4% paraformaldehyde-fixed, 5 μm paraffin sections were stained with hematoxylin and eosin using standard methods. For collagen visualization, the Masson Trichrome staining kit (Thermo Fisher Scientific, Pittsburgh, PA) was used according to the manufacturer's protocol. Sections were rehydrated and HA visualized by immunofluorescence using a biotinylated HA-binding probe (bHABP) and streptavidin-Cy3 (Passi *et al.*, 2004). For standard immunohistochemistry, sections were incubated overnight at 4 °C with either a biotinylated rat monoclonal antibody against CD44 (1:1000 dilution, IM7, gift of Katalin Mikecz, Rush Medical College, Chicago, IL), goat polyclonal antibodies to K10 or K14 (1:1000 dilution, Covance, Princeton, NJ), rabbit anti-mouse antibody to Ki67 (1:1000, Neomarkers, Fremont, CA), rat anti-mouse RB6-85c for neutrophils (Fukuzawa *et al.*, 2009) (1:100 dilution; gift from Rob Fairchild, Cleveland Clinic), rat anti-mouse F4/80 for macrophages (1:50, Serotec, Raleigh, NC), or rabbit anti-mouse antibody to α-smooth muscle actin (α-SMA, 1:200, Abcam, Cambridge, MA). These were then visualized with secondary horseradish peroxidase (HRP) labeled antibodies and 3,3'-Diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA) or streptavidin-Cy3 (for CD44). Specimens stained with anti-Ki67 required initial antigen retrieval with sodium citrate. The ABC staining system (Santa Cruz Biotechnologies, Santa Cruz, CA) was used when visualizing specimens with Ki67, RB6-85c, F4/80, or α-SMA.

Quantitation of Hyaluronan by an Enzyme-Linked Immunosorbant Assay (ELISA)-like Assay

For the TPA studies, the epidermis was separated from the dermis with dispase (Invitrogen, Carlsbad, CA; 20 mg/ml at 37 °C for 1 h), under a dissecting microscope. The tissue was dried in ice cold acetone, weighed, and digested with papain (400 μg/ml; Sigma) in 5

mM EDTA/5 mM cysteine at 60°C for 24-48 h; then boiled 10 min to inactivate the enzyme (Pasonen-Seppanen *et al.*, 2003). Solubilized tissues were used in a competitive ELISA-like assay for HA, modified from the report by Fosang (Fosang *et al.*, 1990). Briefly, HA (25 µg/ml; from human umbilical cord; Sigma) in PBS/0.05% Tween20 (PBS-T) was plated onto 96 well plates overnight. Plates were washed with PBS-T, blocked in 1% BSA/PBS-T for 90 min at 37°C. Then HA standards and samples (diluted in 100 µl PBS-T) were added, and bHABP (100 µl, 1 µg/ml) in PBS-T was added and left at room temperature overnight. After further washing, HRP-streptavidin (100 µl, 1 µg/ml) was added in 1% BSA/PBS-T for 30 min at 37°C. For detection, 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonic acid (ABTS) was added for 10 min, and absorbance read at 405 nm. Assays were done in triplicate.

RNA Isolation and qPCR Analysis

RNA was prepared in Trizol (Invitrogen, Carlsbad, CA) from total skin adjacent to and including full-thickness incisional wounds, or from dispase-separated epidermis after TPA treatment using the provided protocol, and was then reversed transcribed using the Superscript III Reverse Transcription Kit (Invitrogen). The RNA was treated with DNAse 1 (Invitrogen) prior to reverse transcription. For real time quantitative PCR (qPCR), assay reagents and predeveloped TaqMan gene expression probes for Has1, Has2, Has3, and 18S rRNA were from Applied Biosystems (Foster City, CA) and used according to the manufacturer's protocol. The 18S ribosomal RNA (rRNA) probe was used as an endogenous control. For each gene of interest, mRNA expression levels were measured in triplicate in control and experimental tissues, and calculated using the 2-ΔΔCt method (Schmittgen and Livak, 2008). The mRNA levels were expressed as a fold difference relative to vehicle controls.

Table S1. Time course of appearance of inflammatory cells and markers of fibroplasia during cutaneous wound healing in wildtype (WT) and *Has1/3* null mice.

(A) INFLAMMATORY CELL INFILTRATES

	Neutrophils (PMN) *		Macrophages (MP) **	
TIME (days) after wounding	WT	*Has 1/3* Null	WT	*Has 1/3* Null
0 (**)	0/2	0/2	0/2	0/2
1	Scattered areas of PMN. In 1/3 mice, a few small intravascular aggregates of PMN are seen (*See* Supplem Fig. 1)	Many areas with abundant PMN (*See* Fig. 4i). In 3/3 mice, large PMN aggregates are seen within vessels (*See* Supplem Fig. 1)	not done	not done
3	2/2 Abundant PMN, mainly in fibrin clot	2/2 Abundant PMN, mainly in fibrin clot	1/2 Few MP	1/2 Few MP
5	2/2 Abundant PMN are present.	2/2 PMN still present, but fewer than in WT	2/2 Abundant MP	2/2 Abundant MP
10	not done	not done	2/2 Abundant MP	2/2 Abundant MP

Time course after creation of 5-mm diameter excisional wounds on the backs of mice.
Data are number of mice positive for staining/ number of mice examined.

* Polymorphonuclear cells (PMN) were stained with antibody RB6/85c, followed by peroxidase/ DAB technique and visualization by light microscopy.

** Macrophages were stained with antibody F4/80, followed by peroxidase/ DAB and visualization by light microscopy.

*** Unwounded skin

(B) MYOFIBROBLASTS AND COLLAGEN DEPOSITION

	α-Smooth muscle actin #		Masson-Trichrome stain ##	
TIME (days) after wounding	WT	*Has 1/3* Null	WT	*Has 1/3* Null
0	0/2	0/2	0/2	0/2
1	0/2	0/2	0/2	0/2
3	0/2	0/2	0/2	0/2
5	1/3 Weak staining at the wound edge	3/3 Strong staining at the wound edge (*See* Supplem Fig 1d')	0/3 Few spindle-shaped cells apparent	3/3 Abundant spindle-shaped cells, aligned in parallel within a collagen matrix, at wound edge and beneath wound bed (*See* Supplem Fig 1e')
10	2/2 Strong staining at the wound edge and beneath wound bed	2/2 Strong staining at the wound edge and beneath wound bed	2/2 Abundant spindle-shaped cells within neodermis	2/2 Abundant spindle-shaped cells within neodermis

Immunohistochemical marker for myofibroblasts.

Masson Trichrome is a histological stain useful for visualizing collagen and fibroblasts.

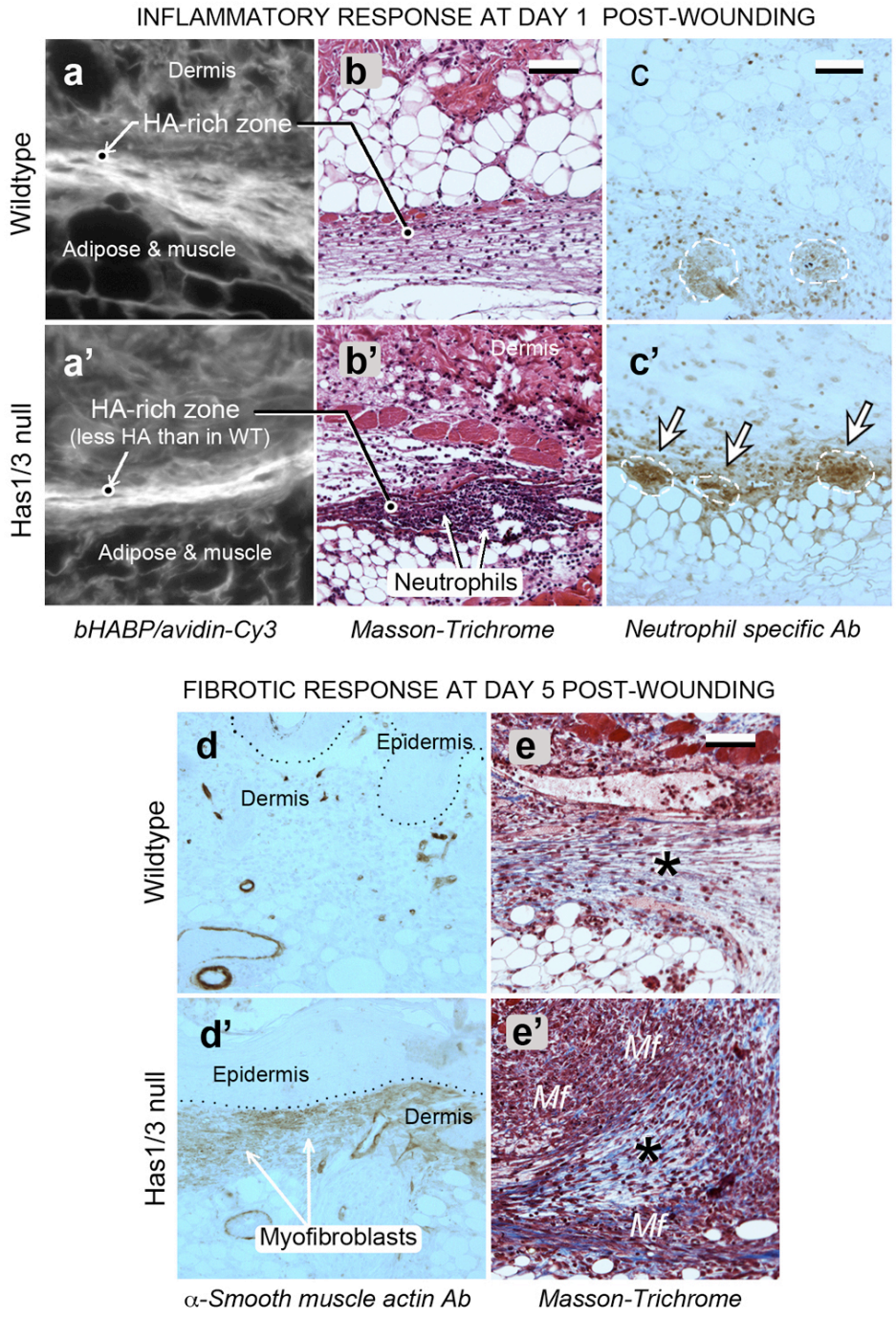

Figure S1. **Reductions in HA in the subdermal region in Has1/3 null mice correlate with enhanced neutrophil extravasation and early appearance of myofibroblasts at the wound edge**.

(***a, a'***) The abundant levels of HA staining normally seen in the subcutaneous region that contains the subdermal vascular plexus (SVP) of wildtype skin (***a***), is reduced in Has1/3 null mouse skin (***a'***). HA was visualized in unwounded skin by staining with biotinylated HA binding protein (bHABP) followed by streptavidin-Cy3. A similar HA reduction in *Has1/3* null skin is observed at 1 day after wounding; see Fig. 4 of

the manuscript. (***b, b'***) Abundant neutrophil extravasation appears to correspond to an increased efflux of neutrophils from vessels in *Has1/3* null wounds in the HA-expressing SVP region, shown here at 24 h *(*Masson-Trichrome stain). (***c, c'***) Immunoperoxidase staining (RB6/85c neutrophil-specific antibody) shows dense aggregates of neutrophils (*arrows*) within vessels (*dotted lines*) at 24 h post-wounding, compared to wildtype. (***d, d'***) Immunoperoxidase staining for alpha-smooth muscle actin reveals abundant myofibroblasts in 5-day old wounds from *Has1/3* null mice (***d'***). In contrast, myofibroblasts are still undetectable at 5 days in wildtype mice (***d***). (**e, e'**) Myofiboblasts can be appreciated by morphological criteria (spindle-shaped cells embedded in a "young" collagen matrix that is light blue in color, by MT staining) in 5-day old wounds in *Has1/3* null mice (***e'***) but not in 5-day old wounds from wildtype mice (**e**). Scale bars, 50 μm.