Supplemental Materials and Methods

Animals: All animal investigations were performed in compliance with Northwestern University's institutional guidelines and under the National Research Council's criteria for humane care as outlined in the "Guide for the Care and Use of Laboratory Animals". Female New Zealand White rabbits (3-4kg) were purchased from Covance (Princeton, NJ) and were acclimated for a minimum of 7 days prior to enrollment in experiments. Anesthesia included intramuscular ketamine (45mg/kg) and xylasine (7mg/kg), or inhaled isofluorane (1-2%), as well as a local anesthetic (1% lidocaine HCl and 1:100,000 epinephrine). Post-operative analgesia was achieved by the administration of 0.05mg/kg buprenorphine every 6-12 hours for up to 3 days following surgery.

Skin and mucosal incisional grid model: The ventral surface of each ear and the vaginal introitus, perineum, and surrounding area was depilated and a surgical scrub was performed (N=8 per time point). On each ear, a 2cm x 2cm grid of shallow (30-50µm deep) perpendicular incisions was made with a maximum distance of 1mm between incisions. These wounds were covered with a semi-occlusive Tegaderm dressing (3M, St Paul, MN) until complete re-epithelialization occurred. In the vagina of the same animals, a series of shallow (30-50µm deep), parallel, longitudinal incisions was made from the introitus into the vagina (length $=$ 3cm). The incisions were made around the entire circumference of the vagina, with a maximum distance of 1mm between incisions. At defined intervals (12 hours, 24 hours, 3 days, 5 days) after injury, the animals were euthanized and samples of the cutaneous and mucosal wounds and uninjured tissue were taken. Each animal was used for one time point only.

Hypertrophic scar model: A reproducible model of cutaneous hypertrophic scar formation in the New Zealand White rabbit was utilized as previously described (Kloeters et al. 2008). Briefly, the ventral surface of each ear was depilated and a surgical scrub was performed. At each of six sites per ear, a 7mm diameter biopsy punch was used to score the skin to the depth of the cartilage, and the epidermis and dermis was removed, leaving the perichondrium intact. All wounds were dressed with Tegaderm dressing (3M, St Paul, MN), and the dressings were replaced as necessary until complete reepithelialization was observed on post-operative day 18. On post-operative days 20, 25, and 30, 7.5mg (50 (L)) of the interleukin-1 receptor antagonist Anakinra (Kineret TM) (Amgen, Thousand Oaks, CA), or vehicle control was injected subcutaneously at the site of each wound site (N=4 animals, 24-48 wounds per treatment). The dose of IL-1RA utilized was equivalent to the human daily therapeutic dose of 1-2mg/kg, but the treatment was administered at a reduced frequency due to the need to anesthetize the animals for each therapeutic treatment. All animals were euthanized at 35 days postsurgery and samples of wounded and control tissue were taken.

Histology: Following euthanasia, full thickness samples of all wounds and from normal skin and mucosa were taken and fixed overnight in 10% zinc formalin for histological analysis. All samples were embedded in paraffin (Paraplast X-tra, Fisher Scientific, Houston, TX) and were sectioned at $5/m$ thickness through the center of each wound. Sections were de-paraffinized and stained with hematoxylin and eosin, and were photographed under 10x or 20x magnification. The epithelial thickness (number of cells and absolute thickness in (m) was determined for all incisional wounds. The total scar area (scar elevation index, SEI) (Kloeters et al. 2008) of all full thickness punch wounds was determined using ImageJ software (NIH, Bethessa, MD). A SEI value of 1 indicates that the scar dermis is of an equivalent height to the dermis of the adjacent normal, unwounded skin.A SEI value of 2 indicates that the height of the scar dermis is twice that of unwounded skin.

RNA extraction: Following euthanasia, samples of all wounds were frozen in RNAlater (Ambion, Austin, TX) at -80oC for molecular analysis. The epithelium of all incisional grid samples was mechanically separated from the underlying mesenchymal tissue using a single-edged razor blade (Schierle et al. 2007). The cutaneous dermis was further separated from the underlying cartilage, and the cartilage was discarded. All full thickness punch wounds were separated from the underlying cartilage and were processed intact (epidermis and dermis together). Each tissue compartment (epithelium, connective tissue, or whole skin) was homogenized independently in RLT buffer (Qiagen, Valencia, CA) with 1% beta- mercaptoethanol using a PowerGen Model 500 Homogenizer (Fisher Scientific, Houston, TX). Total RNA was extracted and purified using the RNeasy kit (Qiagen, Valencia, CA) as previously described (Reno et al. 1997). RNA quality was determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA)

Microarray: A subset of paired rabbit skin and mucosal epithelial RNA samples (N=5-6) per time point) plus four common control (pooled) samples were chosen for microarray analysis based on the histologic analysis of each sample and on the RNA quality (A260/A280 ratio, 28s/18s RNA ratio, RNA integrity number). All 60 samples were labeled with the One-Color Quick-Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) and then were hybridized to an Agilent custom rabbit microarray containing 4 sets of 44,000 probes per slide (23679 unique probes, Amadid #017132) (Agilent Technologies, Santa Clara, CA). All labeling and hybridization was performed at the University of Florida Interdisciplinary Center for Biotechnology Research according to standard Agilent recommended protocols.

Data preprocessing was performed using the Bioconductor Agi4x44PreProcess package. The Agilent Feature Extraction (AFE) algorithm was used for image analysis and background correction. Quantile normalization was then used to correct for the nonbiological variations across arrays. Probes were filtered based on the criteria that at least 5 of the 60 samples must have a signal level above background and negative controls. Duplicated probes were combined using their median intensities. As the gene expression level of unwounded skin and mucosal epithelium were quite different, in order to detect gene expression changes over time in response to injury, the data were normalized against the unwounded tissue by subtracting the respective tissue T=0 sample mean (in log2 scale) from each value.To identify differentially expressed genes, we applied routines implemented in the Bioconductor Limma package to fit linear models to the normalized expression values (Smyth 2005). The variance used in the t-score calculation was corrected by an empirical Bayesian method for better estimation under small sample size.

To control for the effects of multiple testing and to minimize false positives, a subset of statistically significant transcripts was selected for further analysis based on the following filtering criteria: 1) absolute fold change >2 , 2) p value <0.01, and 3) Benjamini &

Hochberg FDR < 0.05 . The data were analyzed to identify 1) temporal changes in gene expression over time in each tissue, and 2) genes whose expression differed between the two tissues. The latter utilized an F test, which identified a total of 1075 genes which had a significantly different fold change (normalized to unwounded tissue) between skin and mucosa at at least one time point. The gene profiles were then scaled to a standard deviation of 1 without shifting the mean of the profile, thereby keeping the direction of the fold-change unchanged. Next, hierarchical clustering was applied to get an overview of the cluster distribution. A cutoff threshold was visually selected to define initial clusters. Based on these initial clusters, K-Means clustering was performed. Five clusters of genes with different temporal expression patterns were identified. Functional analysis was performed on the genes which were differentially expressed over time in each tissue and on the genes that were located in the five clusters. First, the outlier genes which are were located far away from the cluster centers were filtered out. Then the overrepresented functionalities of the genes were identified by using the Hypergeometric test based on the Gene Ontology database (Ashburner et al. 2000).

Raw and processed microarray data can be accessed at the Gene Expression Omnibus accession number (http://www.ncbi.nlm.nih.gov/geo/ , GSE25261).

Quantitative RT-PCR: Reverse transcription was performed using one microgram of total RNA and M-MLV Reverse Transcriptase (Promega, Madison, WI) as per the manufacturer's instructions. Quantitative real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each PCR reaction was run in triplicate and contained $0.03/g$ cDNA template along with 900nM primers and 250nM probe and Taq Man Universal Master Mix (Applied Biosystems, Foster City, CA) in a final reaction volume of $25/L$. Cycling parameters were: 50°C for 2 minutes, 95°C for 10 minutes to activate DNA polymerase, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. No-template controls were run in parallel to evaluate for amplification of genomic DNA. After thermal cycling, the relative mRNA expression of each amplicon was calculated by normalizing its Ct value relative to a housekeeping molecule (18s) and then expressing the value as a proportion of baseline levels (unwounded skin)(2-∆∆Ct). All parameters were assessed using a one-sided ANOVA or using a paired t-test, with a p value of <0.05 indicating significance.

Referecnces:

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Supplemental Figure 1: Microarray analysis of injured epithelium: Major differences in gene expression between wounded skin and mucosa

Microarray analysis of cutaneous and mucosal epithelium at various times post-wounding (unwounded, 12 hours, 1 day, 3 days, 5 days, $N=5$) indicates that there are significant differences in how the two tissues respond to injury. Principal component analysis of 14647 probes that met the preprocessing criteria (A) indicates that the two biggest sources of variability in the data are tissue type (principal component 1), and time postinjury (principal component 2). A heatmap of the top 50 probes in the first principal component (B) indicates that gene expression in the skin and mucosal epithelium is significantly different prior to injury, and that many of those differences persist over time. A heatmap of the top 50 probes in the second principal component (C) indicates that injury results in a change of expression of a number of molecules involved in epithelial proliferation and differentiation, inflammation, and extracellular matrix synthesis and reorganization. Furthermore, it is evident that the cutaneous epithelium responds more strongly to injury, and that injury-associated gene expression changes persist for a longer period of time in the skin.

Supplemental Figure 2: Functional analysis: Inflammation, response to stress, and anabolic processes (structure development) pathways are differentially upregulated in skin and mucosal epithelium following injury

Functional analysis of the genes which are differentially expressed over time in each tissue (A) and which are identified in each cluster (B) was performed using the Gene Ontology database. Gene functionalities, which are significantly overexpressed in healing skin as compared to mucosa, include response to wounding, response to stress, inflammation, structure development. These functionalities are more highly enriched in the skin samples, and are overexpressed for a longer following injury. All numbers in red indicate the top 10 GO categories for each sample group. The intensity of the background color corresponds to the p-value of enrichment tests.

Supplemental Figure 3: Alignment of Human and Rabbit IL-1RA sequences

In order to determine if a human interleukin-1 receptor antagonist would maintain its biological function if used in a rabbit, a multiple sequence alignment was performed between the human and rabbit IL-1RA sequence. Based on the work of Schreuder (Schreuder, Tardif et al. 1997), it is clear that all but one of the 17 amino acids that are responsible for IL-1RA binding to the IL-1 receptor are identical between human and rabbit.

Mismatch

Consensus symbols: ! is anyone of IV \$ is anyone of LM % is anyone of FY # is anyone of NDQEBZ

Supplemental Table 1: Genes identified in each cluster

