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Aberrant connective tissue differentiation towards cartilage and bone underlies human keloids in African Americans

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Abstract

Keloids are benign fibroproliferative tumors more frequently found among African Americans. Until now, keloid etiopathogenesis is not fully understood. To characterize keloids in African Americans, we performed transcriptional profiling of biopsies from large chronic keloids, adjacent nonlesional (NL) skin (n=3) and a newly formed keloid lesion using Affymetrix HGU133 2.0 plus arrays. Quantitative RT-PCR (qRT-PCR) and immunohistochemistry staining were done to confirm increased expression of relevant genes. We identified 1,202 up-regulated and 961 down-regulated differentially expressed genes (DEGs) between keloid and NL skin; 1,819 up- and 1,867 down-regulated DEGs between newly formed keloid and NL skin; and 492 up- and 775 down-regulated DEGs between chronic and newly formed keloid (Fold change >2, False discovery rate <0.05). Many of the top up-regulated DEGs between chronic keloid and NL skin, and between newly formed keloid and NL skin are involved in bone/cartilage formation including Fibrillin 2(FBN2), Collagen type X alpha1(COL10A1), Asporin(ASPN), Cadherin 11(CDH11), Bone morphogenic protein 1(BMP1), Secreted phosphoprotein 1(SPP1), and Runt-related transcription factor2(RUNX2). qRT-PCR confirmed significant ($p<0.05$) up-regulation of BMP1, RUNX2, CDH11 and FBN2 in chronic keloid compared to NL skin. Immunohistochemistry staining showed increased protein expression of ASPN, CDH11, BMP1 and RUNX2 on chronic and newly formed keloid compared to NL skin. Our study shows that large keloids in African Americans represent a dysplasia of cutaneous connective tissue towards immature cartilage or bone

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Supporting Information: Additional supporting data may be found in the supplementary information of this article.

differentiation. The phenotype is potentially regulated by overexpression of RUNX2. This knowledge may give insights to guide the development of better treatment for the disease in the future.

Keywords

Keloid; cartilage and bone formation; transcriptional profiling; connective tissue differentiation

Introduction

Keloids are benign fibroproliferative dermal tumors that are believed to result from a dysregulated wound-healing process. Lesions can be induced by trauma, surgery, or skin inflammation but can also develop spontaneously. Keloid growth can continue over months to years and may cause considerable pruritus, pain, restriction of mobility, and psychological impairment leading to decreased quality of life[1]. Keloids can be observed in all races, but are more common in native Africans, Hispanics, and Asians[2]. In the US, keloid occurs in ~1/30 of African Americans vs. ~1/625 of the overall US population[3]. Although keloid can occur at any age, it is most likely to occur between 10 and 30 years of age[4]. Keloids range in size from small lesions (<1 cm in diameter) to extensive, sometimes massive, tumors (>25cms in diameter)[5].

The etiopathogenesis of keloid is not fully understood. This is in part due to the fact that there are no validated animal models of keloid to study keloid formation or treatment. Many alternative hypotheses exist for keloid formation (all having different therapeutic implications), ranging from dysregulated synthesis of collagen by dermal fibroblasts, to insufficient degradation of deposited matrix, to altered growth factor signaling in disease[2]. Altered endothelial cells that bind or trap platelets, leading to increased release of PDGF-α or TGFβhas been proposed[6]. Altered cross-regulation of cell growth between epidermal and dermal compartments has also been suggested, since keratinocytes synthesize and release many growth factors such as PDGF, FGF, and VEGF that regulate underlying connective tissue structure and function[7].

Although there have been several gene expression profiling studies on keloid, most were done in cultured fibroblasts[8] with only a few in-vivo studies[9-11]. Interestingly, one study performed in Japanese subjects and with a limited gene array platform found several up-regulated genes associated with chondrogenic and osteogenic connective tissue differentiation[10]. However, studies done on other populations, with different array series, have failed to detect these alterations[11].

To further characterize human keloids, we performed transcriptional profiling of biopsies from large chronic keloid lesions and adjacent non-lesional (NL) skin and a newly formed keloid in African Americans diagnosed with keloids. We studied the differentially expressed genes (DEGs) between chronic keloid and non-lesional skin of the same patient, and compared this to the DEGs of a newly formed keloid. We also performed immunohistochemistry and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) on relevant up-regulated genes to confirm increased expression on chronic

keloid and newly formed keloid compared to NL skin. Our microarray analysis revealed more than 2,000 DEGs and a molecular signal of dysplasia of connective tissue towards immature cartilage and bone differentiation in associated lesions.

Methods

Skin samples

Biopsies from non-lesional, chronic keloid lesion (n=3) and newly formed keloid skin were obtained from African American patients with severe keloids under the Rockefeller University IRB-approved protocols. Informed consent was obtained and the study was performed in adherence with the Principles of the Declaration of Helsinki. Patients who developed a new keloid from the non-lesional biopsy site were treated with intra-lesional steroid injection. The chronic keloid lesions were present on the patients for more than 20 years while the newly formed keloids on the non-lesional biopsy site developed about 6 weeks after the biopsy was done.

Gene Array and qRT-PCR

RNA was extracted from non-lesional, chronic keloid lesion and newly formed keloid biopsies using the RNeasy Mini KIT (Qiagen, Valencia, CA). RNA was amplified, labeled, and hybridized to Affymetrix HGU133 2.0 plus arrays. qRT-PCR was performed using EZ PCR core reagents, primers, and probes (Life Technologies, Grand Island, NY, U.S.A.) as previously published[12]. Sequences of primers and probes used in this study are found in Table S6a. The results were normalized to HARP housekeeping gene and transformed to base 2 logarithmic values before the analysis. The means and the differences between chronic keloid lesional and non-lesional tissue at each biomarker was assessed with a mixed-effect model (Fixed-factor: tissue, random intercept for each patient).

Immunohistochemistry and Immunofluorescence

Standard procedures were used for immunohistochemistry (IHC) and immunofluorescence (IF) as previously described[12]. For IHC, frozen tissue sections from non-lesional, chronic keloid and newly formed keloid biopsies were stained with Asporin (Abcam, Cambridge, MA), Cadherin 11 (Abcam, Cambridge, MA), Bone morphogenic protein 1 (BMP1) (Novus Biologics, Littleton, CO) and Runt-related transcription factor 2 (RUNX2) (Abcam, Cambridge, MA). Antibody clones and dilutions are shown in Table S6b. Biotin-labeled horse anti-mouse (Vector Laboratories, Burlingame, CA) and Biotin-labeled goat anti-rabbit (Vector Laboratories, Burlingame, CA) was used to detect the mouse monoclonal and rabbit polyclonal antibodies, respectively. The staining signal was amplified with avidin-biotin complex (Vector Laboratories, Burlingame, CA) and developed using chromogen 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO).

For IF, frozen tissue sections from nonlesional skin, chronic keloid and newly formed keloid biopsies were fixed with acetone and blocked in 10% normal chicken serum (Vector Laboratories) for 30 minutes. The tissue sections were incubated with RUNX2 mouse monoclonal antibody (Abcam, Cambridge, MA) and Collagen 10A1 affinity purified goat polyclonal antibody (Santa Cruz, Dallas, TX) overnight at 4°C. The next day, the same

sections were amplified with chicken anti-goat IgG Alexa Flour 594 (Invitrogen, Eugene, OR) for 30 minutes and then goat anti-mouse IgG2a Alexa Fluor 488 (Invitrogen, Eugene, OR) for another 30 minutes.

Images were acquired using the appropriate filters of a Zeiss Axioplan 2 widefield fluorescence microscope with a Plan Neofluar 20 x 0.7 numerical aperture lens and a Hamamatsu Orca ERcooled charge-coupled device camera, controlled by METAVUE software (MDS Analytical Technologies, Downington, PA). Alternatively, images were acquired using appropriate filters of an upright confocal microscope Zeiss Axioplan 2 (LSM 510) with C-Apochromat 40x/1.2 W objective using Argon/Krypton laser-488nm and 568nm. Images in each figure are presented both as single color stains (green and red) located above the merged image, so that localization of two markers on similar or different cells can be appreciated. Cells that co-express the two markers in a similar location are yellow in color. A white line denotes the junction between epidermis and the dermis. Some dermal collagen gave green auto-fluorescence, and antibodies conjugated with a fluorochrome often gave background epidermal fluorescence.

Statistical Analysis

Affymetrix (Santa Clara, CA) CEL files were scanned using software packages Harshlight[13] and array Quality Metrics from R/Bioconductor (www.bioconductor.org). Expression values (in log2-scale) were obtained using the GCRMA algorithm. Genes with expression higher than three in at least one sample and standard deviation >0.1 were included in the statistical analysis. Gene expression was modeled using mixed effect models with group (LS, NL, newly formed) as a fixed effect and a random intercept for each patient. Resultant p-values from the moderated paired t-test were adjusted for multiple hypotheses using the Benjamini–Hochberg procedure, which controls for the false discovery rate (FDR). The data discussed in this publication have been deposited in the Gene Expression Omnibus of the National Center for Biotechnology Information and are accessible through its series accession number GSEXXXXX.

Results

Clinical and histology images

Clinical photos of patients with extensive keloids who enrolled in the study are shown in Figure 1a. Representative histology images of chronic keloid lesion and background non-lesional skin are shown in Figure 1b-d. Non-lesional skin had a normal appearing epidermis and papillary dermis with clearly demarcated larger collagen bundles in the reticular dermis. Some vascular and stromal bundles were present, similar to the appearance of normal skin. The dermis of non-lesional skin extended to a depth of 4-5mm in all cases examined. No overt features of scars were present in non-lesional skin. In contrast, chronic keloid lesions were thicker than normal skin (6-7mm deep) and had a thickened epidermis with deep rete ridges (notable for the absence of dermo-epidermal junction flattening that often appears in scars). The dermis had two distinct zones of pathology. A superficial zone (~1 mm deep) showed staining of bright pink, homogenized collagen intermixed with stromal cells. Below this zone, a highly cellular infiltrate of spindle-shaped connective tissue cells with

appearance of fibroblasts or myofibroblasts organized in bundles or whorls were detected in some regions, while other regions continued markedly thickened, largely acellular collagen bundles. Areas of marked cellularity also contained pink-staining matrix and some smaller collagen bundles.

Microarray analysis showed significant upregulation of genes involved in cartilage and bone development in chronic and newly formed keloids

We observed a wide separation between chronic lesional and non-lesional skin in a PCA plot using the PC-1 axis (Figure S1a). A new keloid that formed at a biopsy site of non-lesional (NL) skin also clustered with the chronic keloid lesions. Using a conservative analysis approach (FCH>2, FDR<0.05) we detected >2000 DEGs between keloid and non-lesional skin; 1202 up-regulated and 961 down-regulated DEGs in chronic keloid tissue and 1819 up- and 1,867 down-regulated DEGs in new keloid tissue compared to NL skin (Figure S1b). The top 100 up-regulated DEGs are characterized by very large expression increases (20-fold to >50-fold increases from non-lesional skin) and also highly significant p-values ($p<10^{-4}$) after correction for multiplicity (FDR values), as shown in Table 1a/b and fully detailed in Tables S1 and S2. We also identified 1438 up- and 1066 down-regulated DEGs in chronic keloid and 2179 up- and 2360 down-regulated genes in new keloid lesion compared to normal skin; and 492 up- and 775 down-regulated DEGs between chronic keloid and newly formed keloid. Up and down-regulated DEGs between chronic keloid and newly formed keloid vs. normal skin, as well as the chronic vs. newly formed keloid tissue are shown in Tables S3 - S5.

Many of the upregulated genes that were highly expressed in chronic keloid lesions are involved in cartilage and bone development: Fibrillin2 (FBN2), Collagen type X alpha 1 (COL10A1), Asporin (ASPN), Cadherin11 (CDH11), Secreted phosphoprotein 1 (SPP1), Bone morphogenic protein 1 (BMP1) and Runt-related transcription factor 2 (RUNX2). SPP1, a gene for bone remodeling, and Neuropilin (NRP2), a nerve-associated gene, had increased expressions in new keloid lesion compared to NL and normal skin. Neurofilament, heavy polypeptide (NEFH), another nerve gene, also had an increased expression in chronic and new keloid lesion compared to NL and normal skin. Many unanticipated genes encoding cartilage and bone-related proteins were expressed at levels comparable to or higher than dermal collagen genes, e.g., collagen type I, alpha 1 (COL1A1) was up-regulated 31-fold and collagen type III, alpha 1 (COL3A1) was up-regulated 22-fold, but collagen type X, alpha 1 (COL10A1) was up-regulated by 48-fold (Table 1a).

qRT-PCR analysis confirmed significant upregulation of relevant genes in chronic keloid lesion compared to nonlesional skin

We performed quantitative RT-PCR to confirm upregulation of some DEGs namely BMP1, RUNX2, CDH11, FBN2, ASPN, SPP1, COL10A1, Collagen type X11 alpha 1 (COL12A1), ADAM metallopeptidase domain 12 (ADAM12) and Cartilage Intermediate Layer Protien 2 (CILP2). We found significant increases in BMP1, RUNX2, CDH11 and FBN2 ($p<0.05$) and COL12A1, ASPN, ADAM12, CILP2 ($p<0.1$) in chronic lesional keloid compared to NL skin (Figure 2).

Increased protein expression of chondrogenic genes in chronic and new keloid lesion

To confirm the protein expression of selected up-regulated genes associated with bone and cartilage formation, we performed immunohistochemistry staining of ASPN, CDH11, BMP1 and RUNX2 on non-lesional, chronic, and newly formed keloid tissue. We observed a strong expression of ASPN, CDH11, and BMP1 in the epidermis of non-lesional, chronic and newly formed keloid tissue (Figure 3). There was increased expression of Asporin and Cadherin in some dermal and endothelial cells of chronic and new keloid lesion compared to non-lesional skin (Figure 3a and 3b). BMP1 was also expressed strongly in some dermal cells of lesional skin compared to non-lesional and new keloid lesion (Figure 3c). RUNX2 showed moderate expression in the epidermis of non-lesional, chronic, and newly formed keloid tissue, but had increased expression of distinct dermal cells in chronic keloid and newly formed keloid compared to nonlesional skin (Figure 3d).

We also performed two-color immunofluorescence for RUNX2 and COL10A1 to determine co-expression of this transcription factor with the cells producing articular cartilage (Type 10A collagen). Our results showed many $COL10A1^+$ cells contained $RUNX2^+$ nuclei (white arrows) in the dermis of chronic keloid and new keloid lesion compared to non-lesional skin (Figure 3e).

Discussion

Keloids have previously been characterized by an abundance of disorganized type I and III collagen bundles in the extracellular matrix (ECM)[6]. Through proliferation and production of collagens I/III, dermal fibroblasts have been proposed as the main cellular element implicated in the pathogenesis of keloid[14,15]. Syed et al observed that keloid fibroblasts from the growing margin of keloid have elevated production of collagen I and III compared with other lesional sites[16]. Overexpression of TGF-β isoforms and TGF-β receptors has been detected in keloids and keloid-derived fibroblasts, which suggest that excess fibroplasia is regulated by TGF-β cytokines[2].

The keloids we profiled in this study were large and hard exophytic growths, corresponding to Stages II-IV in a recent classification scheme[5]. All lesions had a prominent dermal cellular component of spindle-shaped cells, but also acellular areas with enlarged collagen bundles. A small fraction of the spindle-shaped cells stained for Ki67, indicating ongoing proliferative activation, whereas $Ki67^+$ dermal cells were not common in non-lesional skin biopsies (data not shown). These lesions fit described diagnostic criteria for keloids by histopathology, but they are more cellular than many others described in the literature[2]. However, cells are not highly proliferative as might be seen in a fibrosarcoma. In molecular terms, our chronic keloids had mean up-regulation of collagen I by 31-fold and collagen III by 22-fold vs. non-lesional controls, along with an increase of TGFβ3 by 9-fold (Supplemental Table S1), fitting with the general concept of excess production of expected dermal collagens. We also detected a marked increase (44-fold) in IGF-2 (somatomedin a) mRNA and a 12-fold increase in IGF-1 (somatomedin c), which are key growth factors for connective tissue and epithelial cells, both acting through the IGF-1 receptor (Table 1a).

While these are impressive changes, the scope of gene alterations and the magnitude of change were even greater for connective tissue molecules not normally associated with dermal wound healing. These include: (1) Collagen 10A1, a short-chain minor collagen of cartilage formation[17] that is associated with events in the later stages of endochondral bone formation; (2) Asporin, also known as periodontal ligament-associated protein 1 (PLAP1) belongs to a family of leucine-rich repeat (LRR) proteins associated with the cartilage matrix[18]; (3) Cadherin 11, a cell surface glycoprotein that mediate Ca^{+2} dependent cell-cell adhesion. Expression of this gene in the osteoblastic cell line was up-regulated during differentiation, suggesting a specific function in bone cell differentiation and bone formation[19]; (4) Bone morphogenic protein 1 (BMP1), this locus encodes a protein that is capable of inducing formation of cartilage in vivo[20]; (5) Secreted phosphoprotein 1 (SPP1), which belongs to the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of secreted phosphoproteins, are involved in the anchoring of osteoclasts to the mineral of bone matrix[21], (6) Runt-related transcription factor 2 (RUNX2), which is expressed in adult bone marrow, thymus, and peripheral lymphoid organs[22], has a primary role in the differentiation of osteoblasts[23] and hypertrophy of cartilage at the growth plate, cell migration, and vascular invasion of bone. In chondrogenic and osteoblastic cell lines, RUNX2 has been demonstrated to transactivate Type 1 Collagen and Type X Collagen (Col10A1) [24]. Upstream activation of chondrogenic differentiation has been found to be mediated by RUNX2[25,26].

Thus the molecular fingerprint of cellular activation in keloids is more related to differentiation of cartilage and bone connective tissues than dermal connective tissue and may be mediated by RUNX2 overexpression. The association with IGF-1 and IGF-2 over-expression is intriguing, since the IGF-1 receptor mediates skeletal growth and development as a primary action (IGF-1 and IGF-2 mutations are responsible for growth restriction[27]). Previous work has identified over-expression of IGF-1 receptors on connective tissue cells in keloid lesions[28].

The gene expression changes we observe lead to excess production of bone and cartilage proteins in the extracellular matrix of keloids, as we show by IHC. However, no cartilage or bone differentiation is evident at the level of tissue histopathology. Hence, we consider that keloid lesions represent aberrant differentiation of cutaneous connective tissue towards bone and cartilage cellular lineages, but with incomplete differentiation of activated cells, i.e., dysplasia of immature connective tissue. A prior study of keloids in a Japanese population found over-expression of periostin, Cadherin 11 and COL10A1, among 32 upregulated DEGs[10], also pointing to aberrant chondrogenic differentiation. The high expression of RUNX2 is a likely key inducer of this aberrant differentiation program[24] and it might be a targetable axis for future therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

FBN2	Fibrillin 2
COL10A1	Collagen type X alpha 1, ASPN, Asporin
CDH11	Cadherin 11
BMP1	Bone morphogenic protein 1
SPP1	Secreted phosphoprotein 1
RUNX2	Runt-related transcription factor 2

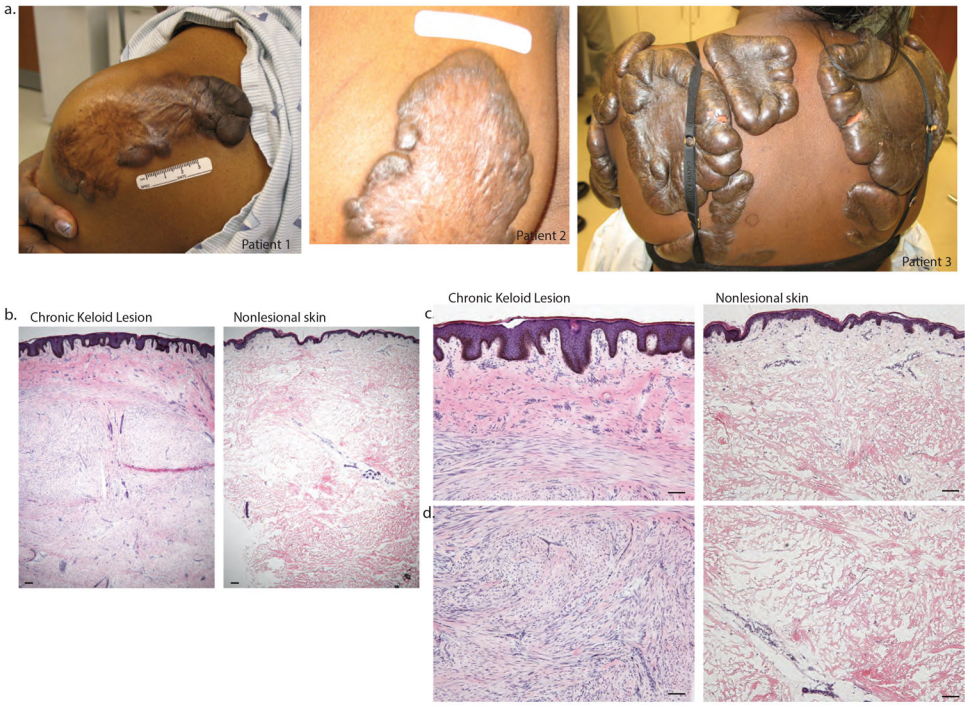

Figure 1.

Clinical photos of large keloids from patients who enrolled in the study are shown in 1a. The representative histology images of non-lesional and chronic keloid lesion are shown in (1b) lower magnification (4×), (1c) higher magnification (10×) and (1d) deeper dermis. Size bar = 100 um

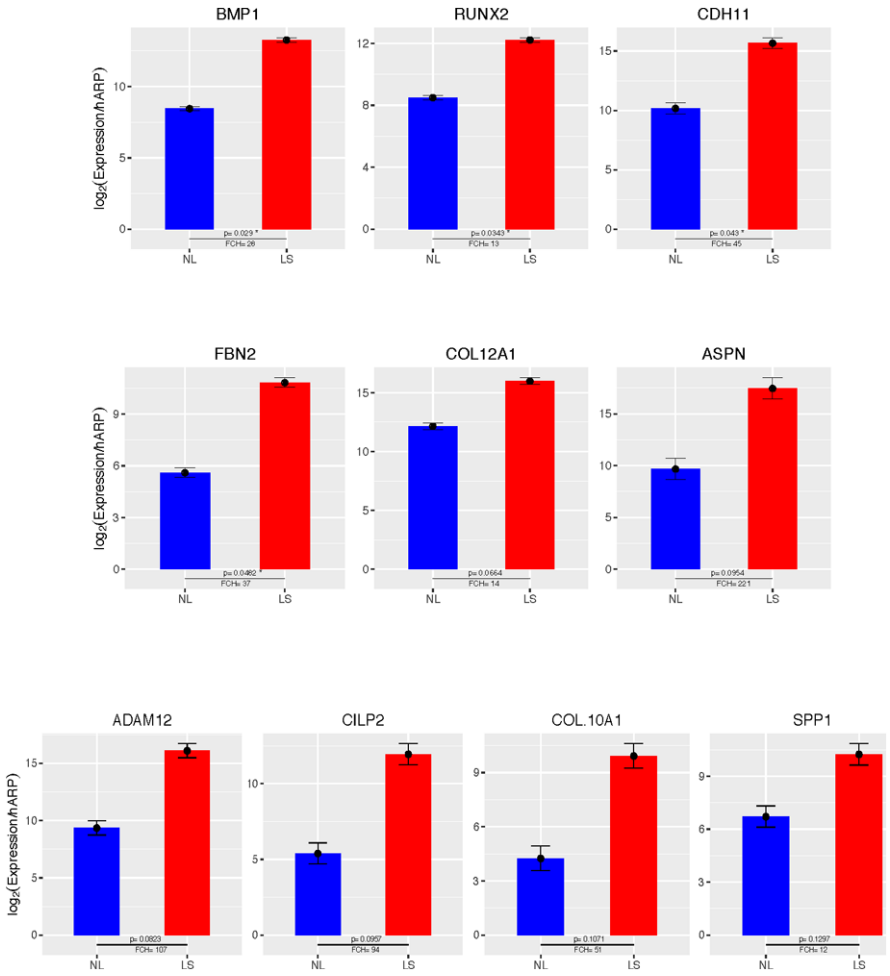

Figure 2.

qRT-PCR analysis confirmed significant upregulation of BMP1, RUNX2, CDH11, FBN2 ($p<0.05$) and COL12A1, ASPN, ADAM12, CILP2 ($p<0.1$) in chronic keloid lesion compared to non-lesional skin. Shown are average normalized Log expression values for non-lesional(NL) and lesional(LS) (n=3). Error bars represent standard errors of the mean and p values are as indicated.

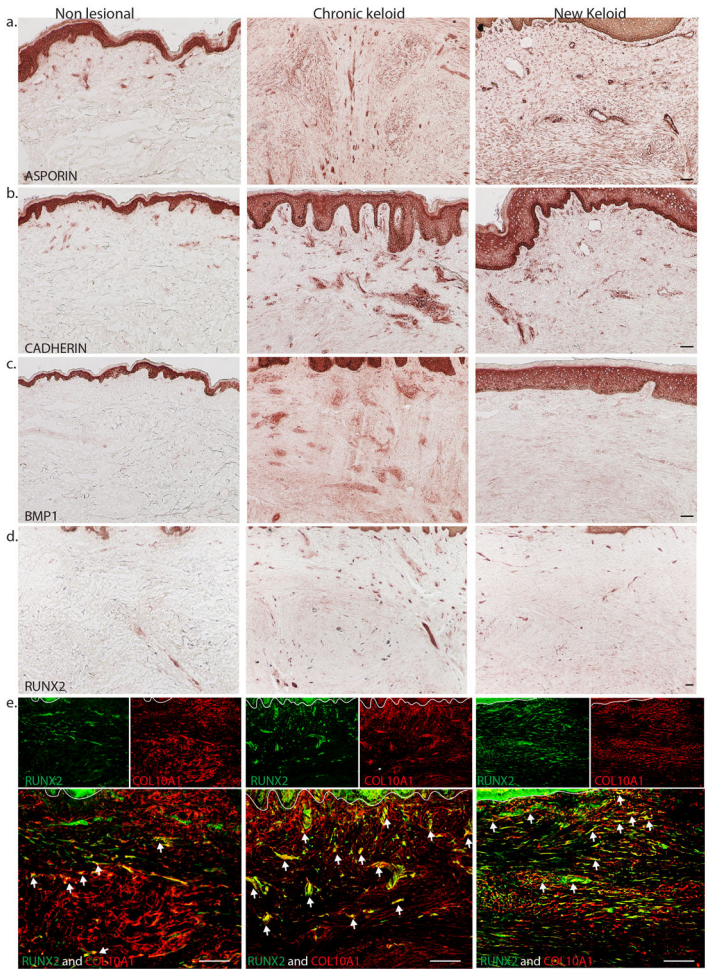

Figure 3.

Representative immunohistochemistry images show increased expression of (a) Asporin and (b) Cadherin 11 in dermal cells of chronic and new keloid lesion compared to non-lesional skin. There is also increased expression of (c) BMP1 and (d) RUNX2 in dermal cells of chronic and new keloid compared to non-lesional skin. (e) Representative two-color immunofluorescence images show many $COL10A1^{+}$ cells contained $RUNX2^{+}$ cells (yellow) in chronic and new keloid lesion and a few in non-lesional skin. Size bar = 100 um

Table Ia

Selected Upregulated DEGs between Chronic keloid Lesion and NL skin

GENE	FCH
FBN2 – fibrillin 2	51.12
COL10A1- collagen, type X, alpha 1	48.63
COL12A1 - collagen, type XII, alpha 1	47.91
ADAM12 - ADAM metallopeptidase domain 12	46.48
IGF2 - insulin-like growth factor 2 (somatomedin A) | INS-IGF2 readthrough	44.06
ASPN - asporin	41.39
COL14A1 - collagen, type XIV, alpha 1	37.62
CILP2 - cartilage intermediate layer protein 2	37.19
LAMP5 - lysosomal-associated membrane protein family, member 5	36.22
VCAN - versican	31.97
COL1A1 - collagen, type I, alpha 1	31.42
CDH11 - cadherin 11, type 2, OB-cadherin (osteoblast)	28.96
ACAN - aggrecan	28.68
COMP – cartilage oligomeric matrix protein	27.52
COL6A1 - collagen, type VI, alpha 1	26.74
BGN - biglycan	26.41
BMP1 - bone morphogenetic protein 1	25.37
COL11A1 - collagen, type XI, alpha 1	25.01
COL3A1 - collagen, type III, alpha 1	22.02
PCDH17 – protocadherin 17	21.10
RUNX2 - runt-related transcription factor 2	21.06
NEFH - neurofilament, heavy polypeptide	20.37

Table Ib

Selected Upregulated DEGs between New keloid lesion and NL skin

GENE	FCH
COL12A1 - collagen, type XII, alpha 1	77.48
COL10A1 - collagen, type X, alpha 1	68.49
ADAM12 - ADAM metallopeptidase domain 12	60.18
COL11A1 - collagen, type XI, alpha 1	56.3
S100A7A- S100 calcium binding protein A7A	54.88
SPP1 - secreted phosphoprotein 1	54.05
WT1- Wilms tumor 1	48.7
FBN2-fibrillin 2	48.53
ASPN - asporin	45.66
MMP16 - matrix metallopeptidase 16 (membrane-inserted)	42.6
CDH11 - cadherin 11, type 2, OB-cadherin (osteoblast)	41.82
RUNX2 - runt-related transcription factor 2	39.13
COL6A1 - collagen, type VI, alpha 1	38.08
VCAN - versican	38.01
FN1 - fibronectin 1	38.07
COL14A1 - collagen, type XIV, alpha 1	36.49
SPON1 - spondin 1, extracellular matrix protein	36.38
NRP2 - neuropilin 2	35.19
COL1A1 - collagen, type I, alpha 1	33.99
PCDH17 - protocadherin 17	31.47
PLOD2 - procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	30.55
EDNRA - endothelin receptor type A	29.8
NEFH - neurofilament, heavy polypeptide	29.36
TLL2 - tolloid-like 2	29.04
BMP1 - bone morphogenetic protein 1	28.01
MMP14 - matrix metallopeptidase 14 (membrane-inserted)	27.28
TNFRSF9 - tumor necrosis factor receptor superfamily, member 9	25.05