Published in final edited form as:
*J Invest Dermatol*. 2010 October ; 130(10): 2489–2496. doi:10.1038/jid.2010.162.

# Epigenetically altered wound healing in keloid fibroblasts

**Shirley B. Russell**[1,2], **James D. Russell**[3], **Kathryn M. Trupin**[3], **Angela E. Gayden**[4], **Susan R. Opalenik**[5], **Lillian B. Nanney**[6], **Alan H. Broquist**[1], **Latha Raju**[7], and **Scott M. Williams**[8]

[1] Center for Human Genetics Research and Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[2] VA Tennessee Valley Healthcare System, Nashville, Tennessee, USA

[3] Meharry Medical College Nashville, Tennessee, USA (retired)

[4] Campbell Drive Middle School, Homestead, Florida, USA

[5] Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[6] Department of Plastic Surgery, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[7] Vanderbilt Microarray Shared Resource, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[8] Center for Human Genetics Research and Department of Molecular Physiology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

## Abstract

Keloids are benign dermal tumors that form during wound healing in genetically susceptible individuals. The mechanism(s) of keloid formation is unknown and there is no satisfactory treatment. We have reported differences between fibroblasts cultured from normal scars and keloids that include a pattern of glucocorticoid resistance and altered regulation of genes in several signaling pathways associated with fibrosis, including Wnt and IGF/IGF-binding protein 5 (IGFBP5). As previously reported for glucocorticoid resistance, decreased expression of the Wnt inhibitor secreted frizzled-related protein 1 (SFRP1), matrix metalloproteinase 3 (MMP3) and dermatopontin (DPT), and increased expression of IGFBP5 and jagged 1 (JAG1) are seen only in fibroblasts cultured from the keloid nodule. *In vivo*, decreased expression of SFRP1 and SFRP2 and increased expression of IGFBP5 are observed only in proliferative keloid tissue. There is no consistent difference in the replicative lifespan of normal and keloid fibroblasts, and the altered response to hydrocortisone (HC) and differential regulation of a subset of genes in standard culture medium are maintained throughout at least 80% of the culture lifetime. Preliminary studies using ChIP-chip analysis, Trichostatin A (TSA) and 5-aza-2′-deoxycytidine (5-aza-dC) further support an epigenetically altered program in keloid fibroblasts that includes an altered pattern of DNA methylation and histone acetylation.

## INTRODUCTION

Keloids are fibrotic tumors of the dermis that form during a protracted wound healing process. The predisposition to form keloids is found predominantly in people of African, Asian, and

Correspondence: Shirley B. Russell, Ph.D., Center for Human Genetics Research and Department of Medicine, Vanderbilt University School of Medicine, Address: 519 Light Hall, Telephone: 615-343-5853, Fax: 615-343-8619, shirley.b.russell@vanderbilt.edu.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

Hispanic descent (Butler *et al.*, 2008; Niessen *et al.*, 1999). Keloids occur in ~1/30 African Americans and ~1/625 of the overall US population (Barrett, 1973). While this disfiguring and sometimes disabling disorder of wound healing significantly impairs the quality of life, it is understudied relative to other chronic skin disorders (Bock *et al.*, 2006). The key alteration(s) responsible for the pathological process has not been identified and, as for other fibrotic disorders, there is no satisfactory treatment (Butler *et al.*, 2008; Lupher and Gallatin, 2006; Niessen *et al.*, 1999). Moreover, keloid formation is one of a group of fibroproliferative diseases characterized by an exaggerated response to injury that occur at higher frequency or with more severe manifestations in people of African ancestry (Smith *et al.*, 2008). We have reported differences in expression of a broad spectrum of wound healing-related genes between normal and keloid fibroblasts under standard culture conditions in medium containing 10% fetal bovine serum (Meyer *et al.*, 2000; Russell *et al.*, 1995; Smith *et al.*, 2008). We have also reported a pattern of differences in growth and synthesis of extracellular matrix induced by several regulators of wound healing. These include an altered growth response to hydrocortisone (HC) (Russell *et al.*, 1978) and resistance of keloid fibroblasts to HC downregulation of types I, III and V collagen, elastin, connective tissue growth factor (CTGF) and IGF-binding protein 3 (IGFBP3) gene expression (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989; Smith *et al.*, 2008). HC resistance is observed only in fibroblasts from the keloid nodule; fibroblasts cultured from superficial dermis of keloids and from unaffected dermis of keloid patients behave like normal skin and scar fibroblasts. These findings suggest that fibroblasts from the keloid nodule are distinct from other dermal fibroblasts in the affected individual. Although some cases of keloid formation may be due to somatic mutation (Saed *et al.*, 1998), multiple keloids in the same individual and evidence for a multicellular origin of keloids (Chevray and Manson, 2004) argue against somatic mutation as the primary event and suggest that an environmental factor present during wound healing triggers abnormal gene expression in genetically susceptible individuals. Various studies have implicated keratinocytes, Langerhans cells, mast cells, and T cells in skin fibrosis (Butler *et al.*, 2008; Niessen *et al.*, 1999; Phan *et al.*, 2003). Thus, differences in gene expression in keloid fibroblasts may be due either to expression of the abnormal gene(s) by the fibroblasts themselves, or expression by another cell type, causing selection of an epigenetically distinct subpopulation of fibroblasts in normal skin.

Since first described (Hayflick and Moorhead, 1961), it has been well established that human diploid fibroblasts display a limited proliferative lifespan followed by replicative senescence (Cristofalo *et al.*, 2004). Effects of cellular aging on gene expression have been reported, including increased expression of matrix metalloproteinase (MMP)-1 and -3, plasminogen activator inhibitor and cyclin-dependent kinase inhibitors, and both increased and decreased collagen synthesis (Cristofalo *et al.*, 2004; Ravelojaona *et al.*, 2008; Zeng *et al.*, 1996). Decreased responsiveness to HC during *in vitro* cellular aging has also been reported (Cristofalo and Rosner, 1979). Because fibroblasts in keloids may have undergone more population doublings during tumor formation than normal dermis or scar fibroblasts we investigated whether normal and keloid cultures differ in their *in vitro* proliferative capacity, and whether the different pattern of gene expression in normal and keloid fibroblasts is stable throughout the *in vitro* lifetime, or as normal fibroblasts age they adopt the gene expression pattern of keloid fibroblasts. A stable pattern over the *in vitro* lifetime and similar growth capacity of normal and keloid fibroblasts would refute the view that the altered program in keloid fibroblasts is due to increased cellular age and would support the hypothesis that keloid fibroblasts represent an epigenetically distinct population of fibroblasts selected during wound healing in individuals predisposed to form keloids.

# RESULTS

## Differential expression of a subset of fibrosis-associated genes in keloid fibroblasts is seen only in cultures from the keloid nodule (lesion)

Having previously observed that the pattern of glucocorticoid resistance of keloid fibroblasts to downregulation of elastin and collagen is confined to the keloid nodule, we determined whether differential expression of other genes in our gene expression profiling study was confined to fibroblasts from the lesion. We used quantitative real time (QRT)-PCR to measure levels of expression in cultures from an abdominal keloid, superficial dermis of the same lesion, and normal abdominal skin excised at the same time as the keloid (Russell *et al.*, 1978).

In the gene profiling studies we observed significantly increased expression of IGFBP5, jagged 1 (JAG1), and CTGF, and decreased expression of secreted frizzled-related protein 1 (SFRP1), MMP3, and dermatopontin (DPT) in keloid fibroblasts (Smith *et al.*, 2008). As seen in Table 1, altered expression is observed only in cultures from the lesion. Differences in expression in fibroblasts from the lesion compared to superficial dermis or nearby normal skin were similar to those previously seen when fibroblasts from keloid lesions were compared to normal scars from unaffected individuals. The only exception was that CTGF was expressed at higher levels in fibroblasts from the keloid lesion in the presence and absence of HC, although the increase was greater in its presence. Immunohistochemical examination revealed decreased SFRP1 and SFRP2 (Figure 1a–b) and increased IGFBP5 protein (Figure 1c) only in active areas of keloid tissue. Expression in inactive areas did not differ from that observed in normal dermis from unaffected individuals (data not shown).

## Do differences in cell age account for gene expression differences?

To determine whether differential patterns of gene expression reflect the greater age of keloid cells that may have undergone more population doublings during tumor formation, we assessed differences in proliferative potential and whether normal cells aged *in vitro* adopt an expression pattern similar to that of keloid cultures.

Included in our collection of fibroblasts are two strains each of normal (21 and 130) and keloid (33 and 50) fibroblasts that were cultured to senescence in the presence and absence of 1.5 μM HC and preserved in liquid nitrogen at different *in vitro* culture ages ranging from approximately 44 to 5 population doublings from senescence. These cultures would be roughly equivalent to passage numbers 1–22, assuming two doublings per passage. Because the number of generations undergone prior to establishing *in vitro* cultures can not be accurately assessed, culture age is expressed as generations from senescence or, where several strains are averaged, the approximate percentage of *in vitro* lifetime.

Proliferative histories of the four fibroblast strains are depicted in Figure 2(a–d). Age-related changes in growth characteristics were similar for all strains and were unaffected by continuous growth in HC (data not shown). Maximum cell density decreased in a roughly linear manner throughout the culture lifetime while population doubling time remained relatively constant until approximately 10 generations prior to senescence and then increased in an accelerating manner.

The total number of population doublings for each strain cultured with and without HC is summarized in Supplementary Table 1. Keloid strain 50 underwent fewer population doublings than the other strains; however, keloid strain 33 did not differ significantly in total population doublings from cells derived from normal skin or normal mature scar.

### Differential responses to HC and altered expression of fibrosis-associated genes in keloid fibroblasts grown in standard culture medium are maintained over the culture lifetime

We previously reported that growth of early passage fibroblasts in 1.5 μM HC increased the maximum cell density of normal fibroblasts but lowered or had no effect on the maximum density of fibroblasts from keloid lesions (Russell *et al.*, 1978). When the effect of HC was examined in normal strain 21 at different generations from senescence, the differential effect of HC persisted as maximum cell density decreased linearly over the culture lifetime (Figure 2e–f).

While we observed no differences in rate of collagen synthesis between multiple strains of normal and keloid fibroblasts grown in standard culture medium, we have reported a differential effect of 1.5 μM HC on percent collagen synthesis, mRNA levels for types I, III, and V collagen and prolyl hydroxylase activity (Russell *et al.*, 1978; Russell *et al.*, 1989; Trupin *et al.*, 1983).

To determine whether the rate of collagen synthesis changes and whether the differential effect of HC is maintained throughout the culture lifetime, percent collagen synthesis was estimated from rates of incorporation of $^{3}$H-proline into collagen and noncollagen protein (Russell *et al.*, 1978) at different generations from senescence. We observed little change in percent collagen synthesis as a function of cellular aging (Table 2), and the differential effect of HC on collagen synthesis in normal versus keloid fibroblasts was maintained throughout most of the culture lifetime.

To further examine the effect of cellular aging on the HC response, QRT-PCR was used to measure expression of α1(I) collagen, elastin, and CTGF genes in cell strains 21 and 33 grown with 0.28 mM ascorbic acid with and without HC. As seen in Table 2, HC downregulated the expression of all three genes in normal fibroblasts over many cell generations, but had little effect on collagen gene expression in keloid fibroblasts, and increased expression of elastin and CTGF.

To determine the effect of cellular aging on several additional genes whose differential expression was seen only in fibroblasts from the keloid nodule, we compared normal strains 21 and 130 to keloid strains 33 and 50 at three different *in vitro* ages, approximately 16%, 59%, and 79% of the culture lifetime (Table 3). We observed that decreased expression of SFRP1 and MMP3, and increased expression of IGFBP5 and JAG1 in keloid fibroblasts were maintained for many cell generations.

### Silencing of SFRP1 is not due to hypermethylation in keloid fibroblasts; increased SFRP1 expression in response to Trichostatin A is consistent with a role for decreased histone acetylation in keloid fibroblasts

Epigenetic silencing of tumor promoters by hypermethylation and differential histone acetylation, including those of multiple SFRPs, often occurs in early stages of tumorigenesis (Jones and Baylin, 2007; Kawamoto *et al.*, 2008; Suzuki *et al.*, 2002). However, analysis of the SFRP1 promoter from 20 keloid and 10 normal strains using the Sequenom MassARRAY System failed to show detectable methylation in either cell type (data not shown). While preliminary genome-wide ChIP-chip assay of pooled DNA samples revealed differential methylation of multiple genes in keloid versus normal fibroblasts, it did not show differential methylation of the SFRP1 gene (Supplementary Figure 1). Hypermethylation of genes in the homeotic (HOX)A cluster correlated with decreased expression of HOXA9 and HOXA10 in keloid cells. Hypomethylation of the asporin, thrombin-like receptor and MMP3 promoters in the keloid sample also correlated with differential gene expression; asporin and the thrombin-like receptor are overexpressed whereas MMP3 is underexpressed (Smith *et al.*, 2008).

Preliminary experiments using 1-day treatment with 0.33 μM Trichostatin A (TSA), an inhibitor of histone deacetylation and 4-day treatment with 2μM 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation, revealed that expression of SFRP1 in keloid fibroblasts was increased almost 15-fold by TSA but not by 5-aza-dC (Table 4). TSA, but not 5-aza-dC, decreased expression of JAG1 whereas both TSA and 5-aza-dC decreased IGFBP5 expression more in keloid than in normal cells. HoxA10 expression was undetectable in keloid fibroblasts in the absence of inhibitors but was increased by both TSA and 5-aza-dC. Both inhibitors decreased expression of collagen and CTGF to a similar extent in normal and keloid fibroblasts. Experiments on two additional normal and keloid strains confirmed that TSA selectively increases expression of SFRP1 and decreases expression of IGFBP5 and JAG1 in keloid but not in normal cells (data not shown).

## DISCUSSION

We previously reported that an altered growth response to HC and resistance of keloid fibroblasts to HC downregulation of collagen and elastin are observed only in fibroblasts from the keloid nodule, findings that support the hypothesis that keloid fibroblasts are an epigenetically distinct subpopulation (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989). Here we provide further evidence for that hypothesis. The differential expression of several fibrosis-associated genes, including the Wnt inhibitor SFRP1, MMP3, DPT, JAG1, CTGF, and IGFBP5 is confined to fibroblasts cultured from the keloid nodule. Immunohistochemical measurements confirmed that decreased levels of SFRP1 and SFRP2 and increased levels of IGFBP5 are confined to active keloid tissue.

The hypothesis that differences between normal and keloid fibroblasts in culture are due to differences in *vivo* aging is not supported by our studies. While the number of *in vitro* population doublings may not accurately reflect the number of divisions undergone *in vivo* (Cristofalo *et al.*, 2004; Maier and Westendorp, 2009), detailed analysis of the replicative lifespan of two keloid and two normal strains aged in the presence or absence of HC revealed no consistent differences to support the hypothesis that fibroblasts cultured from keloids underwent more population doublings than fibroblasts from normal dermis or scar in the formation of the tumor. HC has been reported to extend (Cristofalo and Rosner, 1979) or have no effect (Didinsky and Rheinwald, 1981) on the replicative lifespan of normal fibroblasts. We observed little effect of HC on the lifespan of either normal or keloid fibroblasts.

It is routine to compare patterns of gene expression in cultured cells at low passage number to minimize loss of an *in vivo* phenotype (Feghali and Wright, 1999; Smith *et al.*, 2008; Tuan *et al.*, 2008). It has been reported that simply culturing cells results in loss of a difference in α1β1 integrin collagen receptor expression between fibroblasts from keloids and normal skin (Szulgit *et al.*, 2002). However, some characteristics of an altered program are retained for many generations in culture. An example is the persistent downregulation of Fli1, a suppressor of collagen transcription, in scleroderma fibroblasts *in vivo* and *in vitro* (Asano *et al.*, 2007; Wang *et al.*, 2006). We have found that the altered pattern of gene expression in keloid fibroblasts, including failure of HC to downregulate collagen, elastin, and CTGF, decreased expression of SFRP1 and MMP3, and increased expression of IGFBP5 and JAG1 in standard culture medium is not abolished for at least 80% of the replicative lifespan. While not identifying a mechanism, these findings support the hypothesis of an epigenetically regulated program of fibrosis. Furthermore, persistence of the stimulatory effect of HC on growth of normal fibroblasts throughout the culture lifetime contradicts the notion that as normal fibroblasts age they act like keloid fibroblasts.

Recent inhibitor studies have provided evidence that epigenetic alterations occur during activation of wound healing and fibrosis. TSA blocks transforming growth factor β-mediated

myofibroblastic differentiation (Glenisson *et al.*, 2007) and induction of collagen gene expression (Ghosh *et al.*, 2007; Rombouts *et al.*, 2002) in human skin fibroblasts. TSA also prevents accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis (Huber *et al.*, 2007). TSA and 5-aza-dC have been reported to reverse epigenetic repression of the Fli1 gene and to decrease collagen expression in scleroderma fibroblasts (Wang *et al.*, 2006). Gene profiling studies have revealed no differences in expression of Fli1 in keloid fibroblasts; thus Fli1 does not appear to play a role in the keloid program of fibrosis.

Our findings support an altered program of DNA methylation and histone acetylation that could account for the stable pattern of differential gene expression in keloid fibroblasts in culture. These epigenetically distinct fibroblasts may have been produced or selected in the wound-healing environment of genetically predisposed individuals. While not irreversible, patterns of DNA methylation and histone modifications can be replicated over many cell generations *in vivo* and *in vitro* by complex albeit incompletely understood mechanisms involving chromatin architecture, long-range gene interactions and a complex network of trans-acting proteins and noncoding RNAs (Margueron and Reinberg, 2010). The observation that TSA-induced reversal of SFRP1 gene silencing is associated with decreased expression of profibrotic IGFBP5 and JAG1 supports a role for differential histone acetylation of the SFRP1 gene or of a gene(s) that regulates SFRP1 expression in keloids. SFRP1 is best known as an inhibitor of Wnt signaling and increased Wnt signaling has been reported to play a role in the pathogenesis of keloids (Sato, 2006) and several other fibrotic disorders including pulmonary and renal fibrosis (He *et al.*, 2009; Morrisey, 2003). SFRP1 and SFRP2 have recently been reported to inhibit bone morphogenetic protein signaling (Misra and Matise, 2010). Increased BMP signaling has been implicated in fibrotic disorders such as fibrodysplasia ossificans progressiva (Kaplan *et al.*, 2009). Therefore, silencing of SFRP1 may be important in the fibrosis signature displayed by keloid fibroblasts. While these inhibitor studies do not identify causal relationships between the expression of different genes, they provide additional evidence for an epigenetically altered program in keloid fibroblasts. Further elucidation of this program may be achieved by determining individual gene and genome-wide patterns of DNA methylation and histone modification. Manipulation of expression of specific epigenetically modified genes may identify causal relationships. Characterization of an epigenetically altered program in cultured fibroblasts may reveal mechanisms leading to keloid formation and suggest strategies to treat or prevent keloids and possibly other fibrotic disorders that disproportionately affect individuals of African ancestry.

## MATERIALS AND METHODS

### Cell culture

Methods of isolation and propagation of fibroblasts from keloids, normal dermis and scars have been described (Russell *et al.*, 1978; Smith *et al.*, 2008). Sources of cell strains, all of which were obtained from African American patients, have been presented (Russell *et al.*, 1978; Smith *et al.*, 2008; Trupin *et al.*, 1983). Cultures are grown at 37°C in an atmosphere of air and $CO_2$ adjusted to maintain a pH of 7.4 with 100% humidity. The culture medium consists of 90% F-10 (Sigma) and 10% fetal bovine serum (Invitrogen, Grand Island, NY)). No antibiotics or antimycotics are used so that culture dishes inadvertently subjected to bacterial or fungal contamination may be quickly detected, thus minimizing spread of infection to other dishes and avoiding antibiotic selection for mycoplasma. Cells are subcultured using 0.025% trypsin (Invitrogen) and 0.004% NaEDTA. Cell counts for initiating experimental cultures and for quantitating cell growth are done using a Coulter Counter Z1 (Beckman Coulter, Inc. Brea, CA). Cultures are preserved in liquid nitrogen using a programmable freezer (Planer Products, Sudbury on Thames, England). Institutional Review Boards at Vanderbilt University,

Tennessee Valley Health Care System and Meharry Medical College have approved all described studies. The study was conducted according to the Declaration of Helsinki Principles.

### Quantitative real-time PCR

Total RNA is isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed using a cDNA Archive kit (Applied Biosystems, Foster City, CA). Assays are performed using an iQ real-time PCR system (Biorad Laboratories, Hercules, CA). Specific gene expression is measured as described (Smith *et al.*, 2008). Fold-differences in expression of selected genes are estimated using the comparative $C_T$ method described in User Bulletin #2 ABI PRISM 7700 Sequence Detection System (Applied Biosystems 2001). Fold difference ranges are determined by evaluating the expression: $2^{-\Delta\Delta CT} \pm s$, where s is the standard deviation of $\Delta\Delta C_T$.

### Immunohistochemistry

Standard immunohistochemical procedures available in Vanderbilt's Immunohistochemistry Core Laboratory were modified where necessary for particular protein probes. For tissue sections, keloids were fixed in paraformaldehyde for 24 hours, embedded in paraffin, and sectioned at 5 microns. Sections were immunostained with rabbit polyclonal antibodies directed against IGFBP5 (Upstate Biotechnology, Lake Placid, NY), SFRP1 (Abcam, Cambridge, MA) and SFRP2 (Sigma). Antigen retrieval methods were used as needed.

### DNA Methylation analysis

Methylation was quantified using the Sequenom MassARRAY System (Sequenom, Newton, MA). Genomic DNA was isolated from fibroblasts and bisulfite treated to convert nonmethylated cytosines to uracils (C to T in PCR amplification products). These C/T variations appear as G/A variations in cleavage products generated from the reverse strand by base-specific cleavage. The G/A variations result in a mass difference of 16Da per CpG site, detectable by the Mass ARRAY system. Relative amount of methylation was calculated by comparing the signal intensity between the mass signals of methylated and nonmethylated templates. PCR primers, selected to hybridize with sequences that do not contain CpG, are designed to yield amplification products of between 200 and 600 bases. In the case of the SFRP1 CpG island, six amplicons were sufficient to provide overlapping coverage of the entire island.

### Methylated DNA immunoprecipitation (MeDIP) and microarray

Pooled DNA from normal and keloid strains used in gene profiling studies was digested with Mse1 to produce small fragments (200–1000 bp) while keeping CpG islands intact, denatured, and subjected to methylated DNA immunoprecipitation (MeDIP). Amplified ChIP samples were labeled with different fluorophores and co-hybridized to a Nimblegen CpG-Island Plus promoter array (Roche NimbleGen, Inc., Madison, WI). A computer program developed to analyze data from NimbleGen-tiled microarrays (ACME) was used to identify signals or "peaks" in the array data using a simple sliding window and threshold strategy. A probability value was assigned to each probe on the array (Scacheri *et al.*, 2006). Peak files (.gff) identifying regions of DNA methylation were generated from the p-value files, and peaks were mapped to the transcription start site of each gene and visualized using SignalMap, a software package provided by Nimblegen.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Joel Trupin for his contributions to every aspect of the keloid program since its inception. We acknowledge the outstanding contributions of the Molecular Genetics and Immunohistochemistry cores of the Vanderbilt Skin Diseases Research Center, the Vanderbilt Microarray Shared Resource and the DNA Resources Core of the Center for Human Genetics Research. We also thank Will Bush for his help in preparing figures. This work was supported by NIH grants CA-17229 (JDR), AG-02046 (JDR), P30AR041943 (SMW) F33AR052241 (SBR) 1UL1RR024975 (SBR), and by resources of the VA Tennessee Valley Health Care Center, Vanderbilt University School of Medicine and Meharry Medical College

## Abbreviations

| | |
|---|---|
| 5-aza-dC | 5-aza-2′-deoxycytidine |
| CTGF | connective tissue growth factor |
| DPT | dermatopontin |
| HOX | homeotic |
| HC | hydrocortisone |
| IGFBP | insulin-like growth factor binding protein |
| JAG1 | jagged 1 |
| MMP | matrix metalloproteinase |
| SFRP | secreted frizzled-related protein |
| QRT-PCR | quantitative real time PCR |
| TSA | Trichostatin A |

## References

Asano Y, Czuwara J, Trojanowska M. Transforming growth factor-beta regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. J Biol Chem 2007;282:34672–34683. [PubMed: 17884818]

Barrett, J. Keloid. In: Bergsma, D., editor. Birth Defect Compendium. Baltimore: Williams and Wilkens Company; 1973. p. 553

Bock O, Schmid-Ott G, Malewski P, Mrowietz U. Quality of life of patients with keloid and hypertrophic scarring. Arch Dermatol Res 2006;297:433–438. [PubMed: 16528552]

Butler PD, Longaker MT, Yang GP. Current progress in keloid research and treatment. J Am Coll Surg 2008;206:731–741. [PubMed: 18387480]

Chevray PM, Manson PN. Keloid scars are formed by polyclonal fibroblasts. Ann Plast Surg 2004;52:605–608. [PubMed: 15166997]

Cristofalo VJ, Lorenzini A, Allen RG, Torres C, Tresini M. Replicative senescence: a critical review. Mech Ageing Dev 2004;125:827–848. [PubMed: 15541776]

Cristofalo VJ, Rosner BA. Modulation of cell proliferation and senescence of WI-38 cells by hydrocortisone. Fed Proc 1979;38:1851–1856. [PubMed: 428564]

Didinsky JB, Rheinwald JG. Failure of hydrocortisone or growth factors to influence the senescence of fibroblasts in a new culture system for assessing replicative lifespan. J Cell Physiol 1981;109:171–179. [PubMed: 6460039]

Feghali CA, Wright TM. Identification of multiple, differentially expressed messenger RNAs in dermal fibroblasts from patients with systemic sclerosis. Arthritis Rheum 1999;42:1451–1457. [PubMed: 10403273]

Ghosh AK, Mori Y, Dowling E, Varga J. Trichostatin A blocks TGF-beta-induced collagen gene expression in skin fibroblasts: involvement of Sp1. Biochem Biophys Res Commun 2007;354:420–426. [PubMed: 17234156]

Glenisson W, Castronovo V, Waltregny D. Histone deacetylase 4 is required for TGFbeta1-induced myofibroblastic differentiation. Biochim Biophys Acta 2007;1773:1572–1582. [PubMed: 17610967]

Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Exp Cell Res 1961;25:585–621.

He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y. Wnt/beta-catenin signaling promotes renal interstitial fibrosis. J Am Soc Nephrol 2009;20:765–776. [PubMed: 19297557]

Huber LC, Distler JH, Moritz F, Hemmatazad H, Hauser T, Michel BA, et al. Trichostatin A prevents the accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis. Arthritis Rheum 2007;56:2755–2764. [PubMed: 17665426]

Jones PA, Baylin SB. The epigenomics of cancer. Cell 2007;128:683–692. [PubMed: 17320506]

Kaplan FS, Pignolo RJ, Shore EM. The FOP metamorphogene encodes a novel type I receptor that dysregulates BMP signaling. Cytokine Growth Factor Rev 2009;20:399–407. [PubMed: 19896889]

Kawamoto K, Hirata H, Kikuno N, Tanaka Y, Nakagawa M, Dahiya R. DNA methylation and histone modifications cause silencing of Wnt antagonist gene in human renal cell carcinoma cell lines. Int J Cancer 2008;123:535–542. [PubMed: 18404682]

Lupher ML Jr, Gallatin WM. Regulation of fibrosis by the immune system. Adv Immunol 2006;89:245–288. [PubMed: 16682276]

Maier AB, Westendorp RG. Relation between replicative senescence of human fibroblasts and life history characteristics. Ageing Res Rev 2009;8:237–243. [PubMed: 19491042]

Margueron R, Reinberg D. Chromatin structure and the inheritance of epigenetic information. Nat Rev Genet 2010;11:285–296. [PubMed: 20300089]

Meyer LJ, Russell SB, Russell JD, Trupin JS, Egbert BM, Shuster S, et al. Reduced hyaluronan in keloid tissue and cultured keloid fibroblasts. J Invest Dermatol 2000;114:953–959. [PubMed: 10771477]

Misra K, Matise MP. A critical role for sFRP proteins in maintaining caudal neural tube closure in mice via inhibition of BMP signaling. Dev Biol 2010;337:74–83. [PubMed: 19850029]

Morrisey EE. Wnt signaling and pulmonary fibrosis. Am J Pathol 2003;162:1393–1397. [PubMed: 12707021]

Niessen FB, Spauwen PH, Schalkwijk J, Kon M. On the nature of hypertrophic scars and keloids: a review. Plast Reconstr Surg 1999;104:1435–1458. [PubMed: 10513931]

Phan TT, Lim IJ, Bay BH, Qi R, Longaker MT, Lee ST, et al. Role of IGF system of mitogens in the induction of fibroblast proliferation by keloid-derived keratinocytes in vitro. Am J Physiol Cell Physiol 2003;284:C860–869. [PubMed: 12620890]

Ravelojaona V, Robert L, Robert AM. Effect of cellular aging on collagen biosynthesis: II. Collagen synthesis and deposition by a human skin fibroblast strain over 25 passages. Arch Gerontol Geriatr 2008;47:368–376. [PubMed: 17953997]

Rombouts K, Niki T, Greenwel P, Vandermonde A, Wielant A, Hellemans K, et al. Trichostatin A, a histone deacetylase inhibitor, suppresses collagen synthesis and prevents TGF-beta(1)-induced fibrogenesis in skin fibroblasts. Exp Cell Res 2002;278:184–197. [PubMed: 12169274]

Russell JD, Russell SB, Trupin KM. Differential effects of hydrocortisone on both growth and collagen metabolism of human fibroblasts from normal and keloid tissue. J Cell Physiol 1978;97:221–229. [PubMed: 701387]

Russell SB, Trupin JS, Kennedy RZ, Russell JD, Davidson JM. Glucocorticoid regulation of elastin synthesis in human fibroblasts: down-regulation in fibroblasts from normal dermis but not from keloids. J Invest Dermatol 1995;104:241–245. [PubMed: 7829880]

Russell SB, Trupin JS, Myers JC, Broquist AH, Smith JC, Myles ME, et al. Differential glucocorticoid regulation of collagen mRNAs in human dermal fibroblasts. Keloid-derived and fetal fibroblasts are refractory to down-regulation. J Biol Chem 1989;264:13730–13735. [PubMed: 2760040]

Saed GM, Ladin D, Olson J, Han X, Hou Z, Fivenson D. Analysis of p53 gene mutations in keloids using polymerase chain reaction-based single-strand conformational polymorphism and DNA sequencing. Arch Dermatol 1998;134:963–967. [PubMed: 9722726]

Sato M. Upregulation of the Wnt/beta-catenin Pathway Induced by Transforming Growth Factor-beta in Hypertrophic Scars and Keloids. Acta Derm Venereol 2006;86:300–307. [PubMed: 16874413]

Scacheri PC, Crawford GE, Davis S. Statistics for ChIP-chip and DNase hypersensitivity experiments on NimbleGen arrays. Methods Enzymol 2006;411:270–282. [PubMed: 16939795]

Smith JC, Boone BE, Opalenik SR, Williams SM, Russell SB. Gene profiling of keloid fibroblasts shows altered expression in multiple fibrosis-associated pathways. J Invest Dermatol 2008;128:1298–1310. [PubMed: 17989729]

Suzuki H, Gabrielson E, Chen W, Anbazhagan R, van Engeland M, Weijenberg MP, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–149. [PubMed: 11992124]

Szulgit G, Rudolph R, Wandel A, Tenenhaus M, Panos R, Gardner H. Alterations in fibroblast alpha1beta1 integrin collagen receptor expression in keloids and hypertrophic scars. J Invest Dermatol 2002;118:409–415. [PubMed: 11874478]

Trupin JS, Russell SB, Russell JD. Variation in prolyl hydroxylase activity of keloid-derived and normal human fibroblasts in response to hydrocortisone and ascorbic acid. Coll Relat Res 1983;3:13–23. [PubMed: 6301749]

Tuan TL, Hwu P, Ho W, Yiu P, Chang R, Wysocki A, et al. Adenoviral overexpression and small interfering RNA suppression demonstrate that plasminogen activator inhibitor-1 produces elevated collagen accumulation in normal and keloid fibroblasts. Am J Pathol 2008;173:1311–1325. [PubMed: 18832570]

Wang Y, Fan PS, Kahaleh B. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. Arthritis Rheum 2006;54:2271–2279. [PubMed: 16802366]

Zeng G, McCue HM, Mastrangelo L, Millis AJ. Endogenous TGF-beta activity is modified during cellular aging: effects on metalloproteinase and TIMP-1 expression. Exp Cell Res 1996;228:271–276. [PubMed: 8912720]

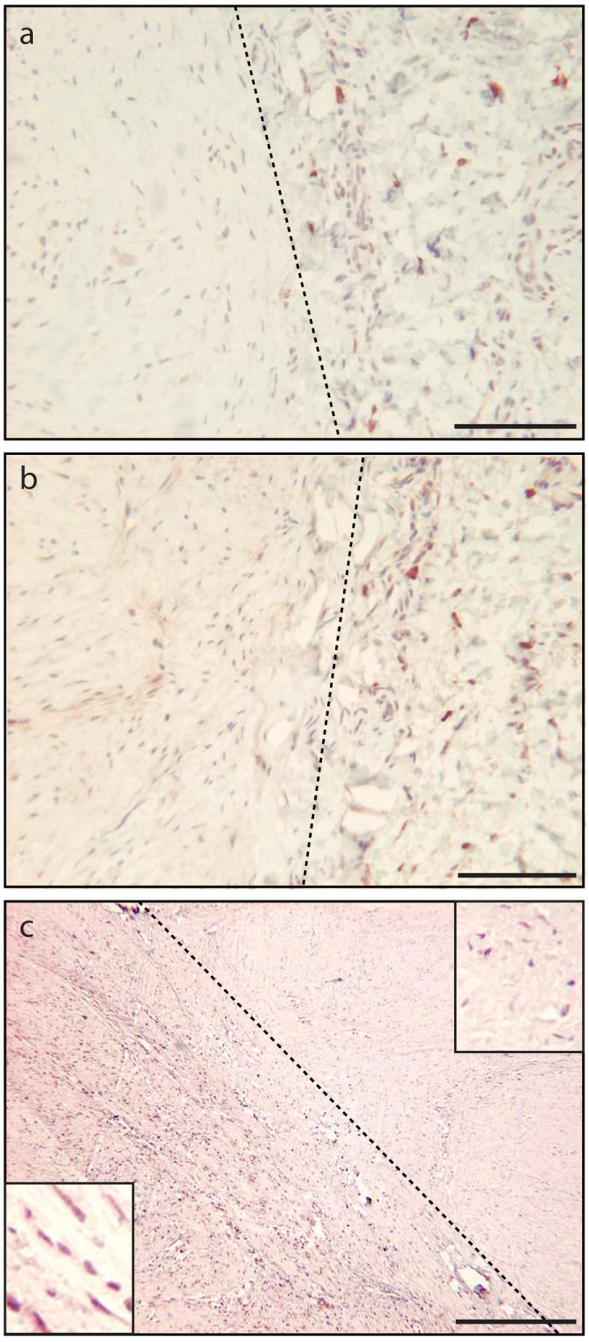


**Figure 1. Distribution of SFRP1, SFRP2, and IGFBP5 immunostaining in keloid tissue**

(**a**) Active fibroblasts (left of dotted line) show no SFRP1 staining; inactive fibroblasts (right of dotted line) show positive staining; (**b**) Active fibroblasts (left of dotted line) show minimal SFRP2 staining; inactive fibroblasts (right of dotted line) show positive staining. Only keloid tissue left of dotted lines in **a** and **b** stains for type 1 procollagen, a marker of activated fibroblasts (data not shown); (**c**) Robust IGFBP5 immunoreactivity is seen only in area left of dotted line, which contains numerous elongated fibroblasts that appear to be actively growing and migrating. Weakly stained fibroblasts right of dotted line exhibit a rounded more mature phenotype and are sparsely distributed in matrix. Insets on lower left and upper right show high

magnification field of each area. Scale bars for (**a**) and (**b**) = 100μm. Scale bar for (**c**) = 500μm.

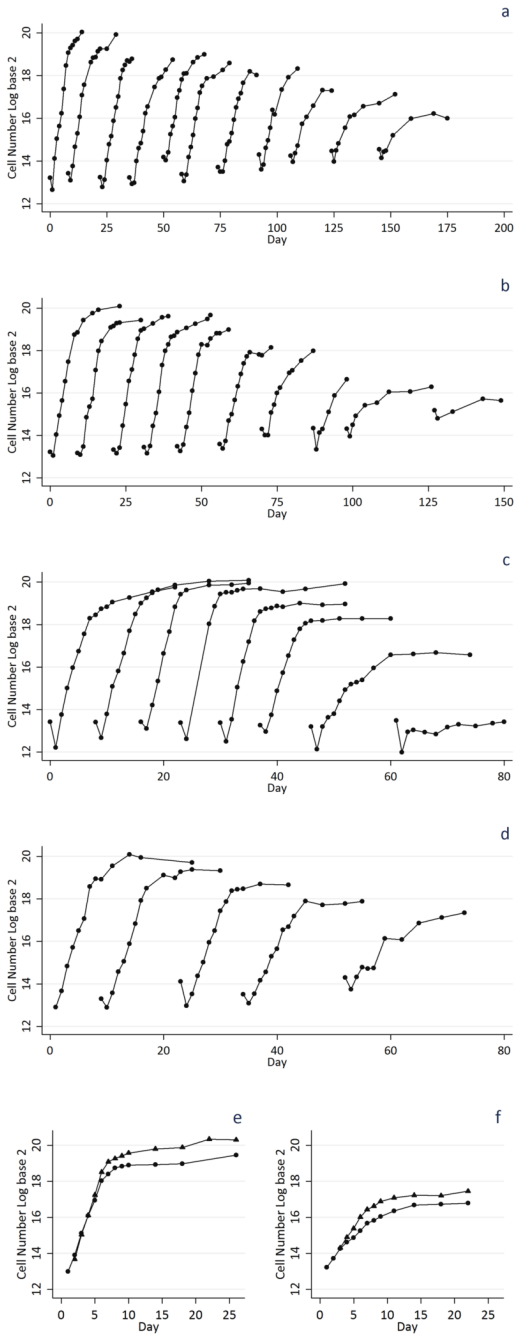


**Figure 2. Proliferative history of two normal and two keloid strains and effect of *in vitro* aging on HC-mediated growth stimulation of normal fibroblasts**

Each cell culture was initiated from the previous cycle on day indicated. Cell counts are averages of duplicate cultures and expressed as $\log_2$ of cell number so that each unit equals one population doubling. (**a**) normal strain 130; (**b**) normal strain 21; (**c**) keloid strain 33; (**d**) keloid strain 50; (**e**) effect of HC on maximum cell density of strain 21 during the 4$^{th}$ culture cycle prior to a change in growth rate; (**f**) effect of HC on maximum cell density of strain 21 during the 7$^{th}$ culture cycle after the growth rate change. Control (circles); HC (triangles).

## Table 1

Differential expression of a subset of fibrosis-related genes is observed only in fibroblasts cultured from the keloid nodule

| | − hydrocortisone | | +hydrocortisone | |
|---|---|---|---|---|
| **Gene Symbol** | **nodule/superficial dermis** | **nodule/normal dermis** | **nodule/superficial dermis** | **nodule/normal dermis** |
| SFRP1 | 0.0003 | 0.0004 | 0.009 | 0.001 |
| MMP3 | 0.0002 | 0.0009 | 0.009 | 0.023 |
| DPT | 0.002 | 0.001 | 0.006 | 0.002 |
| IGFBP5 | 6.32 | 11.79 | 70.03 | 43.41 |
| JAG1 | 55.71 | 36.76 | 17.39 | 8.94 |
| CTGF | 4.41 | 4.41 | 10.06 | 7.26 |

## Table 2

Resistance of keloid fibroblasts to HC downregulation of a subset of matrix and matrix-related genes is maintained during *in vitro* aging

**Differential effect of HC on percent collagen synthesis**

| Cell strain | Generations from senescence | Percent collagen synthesis | | |
|---|---|---|---|---|
| | | Control | +HC | HC/Control |
| **Normal 21** | 39 | 15 | 3.6 | 0.24 |
| | 34 | 9.5 | 2 | 0.21 |
| | 29 | 11.4 | 3.2 | 0.28 |
| | 24 | 10.5 | 3.2 | 0.30 |
| | 17 | 11.8 | 3 | 0.25 |
| | 14 | 11.4 | 2.3 | 0.20 |
| | 10 | 9.5 | 3.6 | 0.38 |
| | 6 | 6.8 | 7.7 | 1.13 |
| **Normal 130** | 48 | 8 | 2 | 0.25 |
| | 40 | 8.2 | 2.7 | 0.33 |
| | 31 | 8.2 | 3 | 0.37 |
| | 25 | 8.9 | 3.9 | 0.44 |
| | 16 | 9 | 5 | 0.56 |
| **Keloid 33** | 38 | 9.1 | 5 | 0.55 |
| | 27 | 7.3 | 4.5 | 0.62 |
| | 24 | 8.6 | 6 | 0.70 |
| | 20 | 8.6 | 7 | 0.81 |
| | 13 | 10.9 | 7 | 0.64 |
| | 7 | 10.7 | 8.9 | 0.83 |
| **Keloid 50** | 25 | 17.7 | 16.4 | 0.93 |
| | 18 | 20 | 17.3 | 0.87 |
| | 11 | 20 | 18.4 | 0.92 |
| | 7 | 22 | 19.5 | 0.89 |

**Differential effect of HC on expression of type I collagen, elastin and CTGF genes**

| Cell strain | Generations from senescence | Type I Collagen HC/Control | Elastin HC/Control | CTGF HC/Control |
|---|---|---|---|---|
| **Normal 21** | 38 | 0.23 | 0.09 | 0.31 |
| | 26 | 0.19 | 0.08 | 0.54 |
| | 15 | 0.25 | 0.04 | 0.26 |
| **Keloid 33** | 37 | 0.76 | 2.09 | 5.79 |
| | 25 | 0.93 | 2.70 | 3.17 |
| | 11 | 1.05 | 1.52 | 1.87 |

## Table 3

Altered expression of a subset of fibrosis-related genes is maintained in keloid fibroblasts during *in vitro* aging

| | | **Keloid/Normal (range) n=2, n=2** | **Keloid/Normal (range) n=2, n=2** |
|---|---|---|---|
| **Average percent *in vitro* lifetime ± SD** | **16 ± 5** | **59 ± 6** | **79 ± 9** |
| SFRP1 | 0.009 (0.001–0.067) | 0.005 (0.001–0.025) | 0.003 (0.002–0.003) |
| MMP3 | 0.05 (0.02–0.10) | 0.013 (0.003–0.050) | 0.009 (0.008–0.009) |
| IGFBP5 | 10.06 (8.03–12.62) | 12.40 (12.19–12-60) | 8.07 (5.55–11.76) |
| JAG1 | 7.64 (1.56–37.18) | 63.27 (30.35–131.60) | 9.50 (0.81–112.19) |

**Table 4**

Effect of Trichostatin A and 5-aza-2′-deoxycytidine on gene expression in keloid and normal fibroblasts

| Gene symbol | Normal TSA/control | Keloid TSA/control | Normal 5-aza-dC/control | Keloid 5-Azad/control |
|---|---|---|---|---|
| SFRP1 | 1.26 | 14.59 | 0.42 | 0.57 |
| SFRP2 | 0.33 | 0.25 | 0.06 | 0.34 |
| DKK3 | 0.83 | 0.41 | 0.23 | 0.14 |
| HOXA10 | 0.59 | ↑↑ | 0.13 | ↑↑ |
| MMP3 | 0.64 | 0.87 | 0.56 | 0.52 |
| IGFBP5 | 0.60 | 0.08 | 0.74 | 0.09 |
| JAG1 | 0.16 | 0.03 | 0.17 | 0.16 |
| COL1A1+HC | 0.44 | 0.41 | 0.11 | 0.23 |
| CTGF+HC | 0.43 | 0.55 | 0.17 | 0.23 |