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Gene profiling of keloid fibroblasts shows altered expression in multiple fibrosis-associated pathways

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Abstract

Keloids are benign tumors of the dermis that form during a protracted wound healing process. Susceptibility to keloid formation occurs predominantly in people of African and Asian descent. The key alteration(s) responsible for keloid formation has not been identified and there is no satisfactory treatment for this disorder. The altered regulatory mechanism is limited to dermal wound healing, although several diseases characterized by an exaggerated response to injury are prevalent in individuals of African ancestry. We have observed a complex pattern of phenotypic differences in keloid fibroblasts grown in standard culture medium or induced by hydrocortisone. In this study Affymetrix-based microarray was performed on RNA obtained from fibroblasts cultured from normal scars and keloids grown in the absence and presence of hydrocortisone. We observed differential regulation of approximately 500 genes of the 38,000 represented on the Affymetrix chip. Of particular interest was increased expression of several IGF-binding and IGF-binding related proteins and decreased expression of a subset of Wnt pathway inhibitors and multiple IL-1-inducible genes. Increased expression of CTGF and IGFBP-3 was observed in keloid fibroblasts only in the presence of hydrocortisone. These findings support a role for multiple fibrosis-related pathways in the pathogenesis of keloids.

INTRODUCTION

Keloids are benign collagenous dermal tumors that form during a protracted wound healing process (Marneros *et al.*, 2004; Niessen *et al.*, 1999). The predisposition to form keloids is found predominantly in people of African and Asian descent. While this disfiguring disorder of wound healing significantly impairs the quality of life (Bock *et al.*, 2006), it is understudied relative to other chronic skin disorders. The key alteration(s) responsible for keloid formation

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CONFLICT OF INTEREST

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has not been identified, and there is no satisfactory treatment. 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. These diseases include hypertension (Dustan, 1995), nephrosclerosis (August and Suthanthiran, 2003), scleroderma (Mayes *et al.*, 2003), sarcoidosis (Rybicki *et al.*, 1998), allergic disease (Nickel *et al.*, 1999) and uterine fibroma (Flake *et al.*, 2003). We (Russell *et al.*, 1989) and others (August and Suthanthiran, 2003; Catherino *et al.*, 2004; Dustan, 1995) have suggested that a common etiopathology may operate in these diseases, and that common genetic factors may account for their unusual racial distribution.

A strong genetic component for keloid formation is supported by its different frequency of occurrence in different racial and ethnic populations. Keloids have been estimated to occur in ~1/30 African-Americans and ~1/625 of the overall US population (Barrett, 1973). While most cases occur sporadically, familial occurrence is observed. In a study of 14 pedigrees with familial keloids inheritance patterns were consistent with autosomal dominant inheritance with incomplete penetrance and variable expression (Marneros *et al.*, 2001). Locus heterogeneity is likely; in one family linkage to chromosome region 14q22-q23 was detected (Davis *et al.*, 2000), whereas in two other families linkage to regions 2q23 and 7p11 was shown (Marneros *et al.*, 2004). However, no major genes or susceptibility loci have been identified. Although some cases may be due to somatic mutation (Saed *et al.*, 1998), evidence for a multicellular origin of keloids (Chevray and Manson, 2004; Moulton-Levy *et al.*, 1984; Trupin *et al.*, 1977) argues against somatic mutation as the primary event, and suggests that an environmental factor present during wound healing triggers abnormal gene expression in genetically susceptible individuals.

By comparing fibroblast cultures from normal scars and keloids we have demonstrated differences in growth and matrix synthesis that may play a role in the prolonged activity of keloid fibroblasts *in vivo* (Russell et al., 1988). These include reduced serum dependence of low density keloid cultures, resistance of keloid fibroblasts to glucocorticoid down-regulation of collagen and elastin gene expression, altered growth response to glucocorticoids (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989) and resistance to growth inhibition by TGFβ(Russell *et al.*, 1988) and phorbol ester tumor promoters (Myles *et al.*, 1992).

The altered response to glucocorticoids is observed only in fibroblasts from the keloid nodule; fibroblasts cultured from the superficial dermis of keloids and from other dermal sites of keloid patients behave like normal skin and scar fibroblasts (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989). Fibroblasts from the keloid nodule, therefore, appear to be epigenetically distinct from other dermal fibroblasts in the affected individual.

Gene profiling studies using microarray technology have identified previously unsuspected genes and pathways of potential relevance to the pathogenesis of many diseases, including fibrotic disorders such as keloids (Naitoh *et al.*, 2005; Satish *et al.*, 2006; Tosa *et al.*, 2005), scleroderma (Whitfield *et al.*, 2003; Zhou *et al.*, 2001) and leiomyoma (Mangioni *et al.*, 2005; Tsibris *et al.*, 2002). Microarray studies have also identified common patterns of altered gene expression in autoimmune disorders (Aune *et al.*, 2003) and have focused attention on candidate genes in chromosomal regions linked to specific diseases (Aune *et al.*, 2004; Li *et al.*, 2003). To identify pathways critical to keloid pathogenesis and to define the altered program in lesional fibroblasts we have carried out genome-wide expression profiling on normal and keloid fibroblasts grown in the absence and presence of hydrocortisone (HC).

RESULTS

Normal and keloid fibroblasts differ in expression of a large number of genes

To define the altered gene expression pattern in keloid fibroblasts, RNA was isolated from confluent cultures from five keloids and five normal scars fed daily with standard culture medium or medium containing 1.5 μM HC. This concentration is in the physiological range, and it maximizes a differential effect on maximum cell density of normal and keloid fibroblasts (Russell *et al.*, 1978) and inhibits collagen and elastin synthesis in fibroblasts from normal scars but not in fibroblasts from the keloid lesion (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989). Biotinylated cRNA was prepared and hybridized to an Affymetrix GeneChip containing sequences from 38,500 human genes. Hybridized cRNA was visualized, and image data were used to generate gene expression values. The RNA from one normal culture, strain 58 grown with HC, was lost during processing. The remaining 19 samples were subjected to microarray analysis.

Preliminary analysis using a two-fold or greater change in gene expression identified many differences between the two cell types in control medium, with 175 defined genes expressed at higher levels in keloid fibroblasts and 559 at reduced levels. When HC was added, keloid cells showed higher expression of 221 genes and lower expression of 547 genes. Approximately 600 genes were down-regulated and 400 up-regulated at least two-fold by HC to a similar extent in both cell types. A small subset of genes was up- or down-regulated only in one cell type.

Unsupervised hierarchical clustering analysis (Figure 1) revealed that normal and keloid fibroblasts grown with and without HC comprise four distinguishable groups.

Statistical analysis of differences in gene expression between normal and keloid fibroblasts with and without HC supplementation

In the absence of HC 511 genes of known function were expressed at significantly different levels in keloid and normal cells when analyzed by nonparametric testing using a correction for multitesting. The criterion of up- or down-regulation of at least two-fold was not imposed here. Parametric testing revealed only 177 genes with statistically significant differences in expression. In the presence of HC nonparametric analysis revealed 515 differentially expressed genes, whereas parametric testing identified only 236 genes. Differences in number observed by these two methods of analysis are due to large variation between strains in magnitude of differences. As microarray data are often noisy and not normally distributed, nonparametric methods that do not assume a specific distribution of data are preferable (Troyanskaya *et al.*, 2002).

Nonparametric analysis revealed a subset of genes, many previously implicated in wound healing and fibrosis, that were expressed at higher (Table 1) or lower (Table 2) levels in keloid fibroblasts grown in standard culture medium. Tables 3 and 4 list subsets of genes that were differentially expressed in HC-supplemented medium. For genes that have multiple probes on the Affymetrix chip, the average difference in expression and number of informative probe sets are shown. Genes that also exhibited statistically significant differences in expression using a parametric test with correction for multitesting are marked with an asterisk. All genes whose expression differed significantly between normal and keloid strains using nonparametric analysis are listed in supplementary tables S1 (minus HC) and S2 (plus HC).

Most genes were differentially expressed to a similar extent in both the absence and presence of HC, although as presented in Tables 1–4 and as described below, some differences were seen only without or only with HC.

Expression of multiple homeotic (HOX) genes is reduced in keloid fibroblasts

In addition to altered expression of many individual genes we observed a significant reduction in expression of multiple genes in several clusters of HOX genes, important regulators of transcription, (Table 5) in the absence and presence of HC.

Multiple IGFBPs and IGFBP-related proteins are over-expressed in keloid fibroblasts

As seen in Tables 1 and 3, expression of several IGFBPs and IGFBP-related proteins was significantly increased. Table S3 compares the expression of additional genes in this superfamily, as well as STAT-1, a mediator of IGFBP-3 activity (Spagnoli *et al.*, 2002), in cultures grown with and without HC, using multiple probe sets on the Affymetrix chip. For some of these genes the difference in expression was more than two-fold but was not statistically significant, or as seen for IGFBP-3 differential expression was statistically significant only when a correction for multitesting was not used. IGFBP-2, -5, and -7 and STAT-1 were overexpressed in the absence and presence of HC. However, IGFBP-3 was only expressed at significantly higher levels (1.75-fold) in keloid cells grown with HC ($p<0.04$). As seen in Table S3 and in Table 3, the IGFBP-related protein, connective tissue growth factor (CTGF, IGFBP-8), was also only expressed at significantly higher levels in keloid fibroblasts when grown with HC ($p=0.0002$). The increased level of IGFBP-3 in keloid cells compared to normal cells grown in HC was due to a 54% reduction of expression in normal cells but only a 16% reduction in keloid cells. The increased expression of CTGF was due to a 46% reduction by HC in normal cells but a 40% increase in keloid cells. These findings extend the subset of fibrosis-associated genes that are glucocorticoid resistant in keloid cells.

Multiple genes in the Wnt signaling pathway are misexpressed in keloid fibroblasts

Expression of three inhibitors of Wnt signaling, DKK1, DKK3 and SFRP1 was decreased in keloid fibroblasts (Table 2), whereas expression of the Wnt stimulatory molecules FZD4 and DAAM1 was increased (Table 1). Moreover, expression of JAG1, a ligand of the Notch signaling pathway and a target of Wnt signaling (Katoh and Katoh, 2006), was increased approximately 10-fold in keloid cells (Table 1). Table S3 compares the expression of multiple genes in the Wnt pathway in cultures grown with and without HC, using multiple probe sets on the Affymetrix chip. In addition to the genes listed in Tables 1 and 2, we observed statistically significant differences in expression of several other Wnt pathway genes in cells grown in the absence of HC when expression of individual genes was analyzed using Student's *t*-test for unpaired data. These differences include a more than 5-fold reduction in expression of SFRP2 ($p<0.03$), a 1.7-fold increase in FZD2 expression ($p<0.04$) and a 1.8-fold increase in FZD7 expression ($p<0.02$) in keloid versus normal cells in standard culture medium. When cultures were grown with HC, differential expression of SFRP1 and SFRP2, as well as increases in FZD2, FZD4, and FZD7 were also observed, although the difference for SFRP1 was less pronounced. Decreased expression of DKK1 and DKK3 by keloid fibroblasts was not observed in the presence of HC. The differential regulation of DKK1, DKK3 and SFRP1 by HC further extends the subset of fibrosis-related genes that are glucocorticoid resistant in keloid cells.

Expression of some IL-1-responsive genes is decreased in keloid fibroblasts

As seen in Tables 2 and 4, expression of several genes regulated by IL-1 was significantly reduced in keloid fibroblasts. Table S3 presents expression values for these genes, some of which were lowered more than 2-fold, but statistical significance was not attained due to interstrain variation. In addition to a 20-fold decreased expression of CXCL-1 and a greater than 2-fold reduction in CXCL-12 seen in Table 2, a 99-fold reduction in CXCL-6 was observed in the absence of HC. In addition to the more than 20-fold reduction in expression of MMP-3 (Table 2), MMP-1 expression was reduced 4-fold ($p=0.06$). Reduced expression of these genes in keloid cells was also observed in the presence of HC.

Validation of microarray findings using QRT-PCR

To validate differences in expression of a selected subset of genes from Tables 1–4 and Table S3, RNA from the microarray studies was analyzed by QRT-PCR.

As seen in Table 6 using comparisons of unpooled normal strains 21, 58, 116, 170 and keloid strains 50, 124, 145, 261 in control medium, and normal strains, 21, 116, 170 and keloid strains 50, 124, 145, 261 grown with HC, PCR findings validated those seen in microarray, and differences in expression were quantitatively similar. The more than two-fold difference in expression of several genes that did not reach significance in the microarray studies, including SFRP2, MMP-1 and IL-13Rα2, were also validated by QRT-PCR. As also seen in Table 6, using the same strains, QRT-PCR corroborated findings that the increased expression of elastin, IGFBP-3 and CTGF in keloid cells only in the presence of HC could be accounted for by a reduced sensitivity to HC down-regulation. The effect of HC on all genes listed in Table 6 is seen in Table S4. We had previously reported differential regulation of several collagen genes by HC (Russell *et al.*, 1989), but in the microarray studies HC failed to down-regulate collagen mRNA in either cell type. To mimic previous conditions we grew additional cultures of normal strains 21, 58, 131, 170 and keloid strains 33, 50, 124, 145, and 261 in media supplemented with 50 mM ascorbic acid. RNA samples from these strains were used to perform QRT-PCR on the COL1A1, elastin, CTGF, IGFBP-3, IGFBP-2, and IGFBP-5 genes. In the presence of ascorbic acid, the COL1A1 gene, as well as the elastin, CTGF and IGFBP-3 genes, were expressed at higher levels in keloid than in normal cells when grown with HC compared to cultures grown without added hormone (Table S5). As in the absence of ascorbate, this was attributable to a reduced sensitivity of keloid cells to HC down-regulation (Table S5). Down-regulation of collagen by HC in normal cells appears to require the presence of ascorbate. As in the absence of ascorbate, IGFBP-2 expression was increased by HC and IGFBP-5 was decreased to a similar extent in normal and keloid cells, emphasizing that differential regulation by HC is confined to a subset of fibrosis-related genes.

DISCUSSION

Microarray studies provide a broad spectrum of differentially regulated genes that may play roles in the pathogenesis of disease. However, it is not feasible to carry out independent measurements of RNA levels and functional studies for all abnormally expressed genes; therefore, we focused on the most likely candidates involved in fibrosis and keloid formation for validation and future study.

Our observation of increased expression in keloid cells of several IGFBPs, including IGFBP-3 and –5, and the IGFBP-related proteins IGFBP-7 and CTGF, is of particular interest. Roles for IGFBP-3 and -5 in fibrosis are supported by their increased expression in dermal fibroblasts from patients with systemic sclerosis (Feghali and Wright, 1999; Knauer *et al.*, 2001), idiopathic pulmonary fibrosis (Pilewski *et al.*, 2005; Yasuoka *et al.*, 2006b), and leiomyomas (Tsibris *et al.*, 2002), and by their stimulation of matrix synthesis in lung fibroblasts (Pilewski *et al.*, 2005). Increased CTGF expression has been observed in several fibrotic conditions (Ihn, 2002), including keloids (Igarashi *et al.*, 1996). Moreover, increased expression of IGFBP-3 has been reported in other studies of keloids (Satish *et al.*, 2006; Tosa *et al.*, 2005). Activation of the IGF-1 receptor has been associated with abnormal cell proliferation and differentiation in various diseases and with increased invasive activity of fibroblasts in keloids (Phan *et al.*, 2003; Yoshimoto *et al.*, 1999). In addition to binding to IGF-1, IGFBP-3 and -5 bind to extracellular matrix components, have nuclear localization signals and bind to putative receptors on the cell surface (Mohan and Baylink, 2002). Recently it was reported that injection of C57BL/6J mice with adenovirus expressing human IGFBP-5 induced skin fibrosis (Yasuoka *et al.*, 2006a). We observed increased expression of IGFBP-3 and CTGF in keloid cells only in the presence of HC.

HC alters transcription of many genes, up-regulating some and down-regulating others (Diamond *et al.*, 1990). Its effects vary with cell type and stage of development (Russell *et al.*, 1978; Russell *et al.*, 1988). Glucocorticoids generally impair wound healing (Meisler *et al.*, 1997) and inhibit collagen expression in normal fibroblasts (references cited in (Russell *et al.*, 1989). When used with TGFβ, glucocorticoids prevent stimulation of synthesis of collagen and tissue inhibitors of metalloproteinases, and reduce scarring (Meisler *et al.*, 1997; Slavin *et al.*, 1994; Song *et al.*, 1999). They are considered to be opposing physiological regulators of wound healing and fibrosis. While most genes are up- or down-regulated by HC to a similar extent in normal and keloid cells, the HC-refractory expression of a subset of TGFβ-inducible genes in keloid fibroblasts, including several collagen genes, elastin, IGFBP-3 and CTGF, suggests a role for glucocorticoid resistance in the pathogenesis of keloids. Antagonism between glucocorticoids and TGFβ in wound healing points toward an abnormality in their interaction that may confer susceptibility to fibrosis.

The highly conserved Wnt family of signaling proteins participates in multiple developmental events (Habas and Dawid, 2005; Moon, 2005) and in normal wound healing (Labus *et al.*, 1998; Okuse *et al.*, 2005). Overexpression of Wnt signaling molecules has been observed in pulmonary fibrosis (Morrisey, 2003), renal fibrosis (Surendran *et al.*, 2002), leiomyoma (Mangioni *et al.*, 2005) and most recently, keloids (Sato, 2006). Our observation of reduced expression of several inhibitors of Wnt signaling in keloid fibroblasts indicates a role for this pathway in the pathogenesis of keloids. Of particular note is the dramatic reduction of SFRP1 expression in keloid fibroblasts. Epigenetic silencing of multiple SFRPs, including SFRP1 and SFRP2, often occurs in early stages of the neoplastic process (Baylin and Ohm, 2006; Suzuki *et al.*, 2004), and has been seen in a variety of malignancies (Caldwell *et al.*, 2006). Epigenetic silencing of DKK1 has also been observed in colorectal cancer (Aguilera *et al.*, 2006). While over-expression of the canonical pathway in keloids was reported (Sato, 2006), increased expression of DAAM1 (Table 1), a mediator in the RhoA-activated noncanonical Wnt signaling pathway, suggests involvement of that pathway as well. Involvement of this pathway in fibrosis is supported by evidence that activation of pancreatic stellate cells is blocked by Rho kinase inhibitors (Masamune *et al.*, 2003). Furthermore, the DAAM1 gene is located at chromosomal region 14q22-q23, a region linked to keloid formation (Davis *et al.*, 2000). Increased Wnt signaling is consistent with our observation of a 10-fold increase in expression of JAG1 in keloid fibroblasts. JAG1, a ligand in the Notch signaling pathway, is a target of Wnt (Katoh and Katoh, 2006). However, increased JAG1 expression may also reflect an elevated TGFβ response. Functional interactions between TGFβ/Smad and Notch signaling in various tissues have been suggested, based on hierarchical activation of one pathway by the other, or by coordinate regulation of common target genes (Zavadil and Bottinger, 2005). A third possibility is that increased JAG1 expression is due to the combined effects of TGFβ and Wnt (Labbe *et al.*, 2007).

A statistically significant 2-fold reduction in expression of TGFβ receptor II (TGFβR2) combined with a small (1.4-fold) increase in expression of TGFβR1, was observed in keloid fibroblasts. This fits with the observation of an increased ratio of TGFβR1/TGFβR2 in keloid cells compared with hypertrophic scar and normal skin fibroblasts (Bock *et al.*, 2005), and with reports that an increased ratio of TGFβR1/TGFβR2 may promote fibrosis (Bock *et al.*, 2005; McCaffrey, 2000).

In an earlier study to test the hypothesis that altered production or response to Th2 cytokines plays a role in the pathogenesis of keloids, RNase protection was used to determine mRNA levels for IL-13, IL-4 and their receptors (Smith, 2005). These Th2 cytokine mRNAs were not expressed at detectable levels in normal or keloid fibroblasts, and IL-4Rα and IL-13Rα1 mRNAs were expressed at similar levels in both cell types. However, expression of the decoy receptor IL-13Rα2 (Chiaramonte *et al.*, 2003), which was low in normal cells, was virtually

absent in keloid cells. These results were validated in our microarray studies, although the more than 20-fold-reduction in average expression was not statistically significant because of low and variable expression in normal fibroblasts. These findings and their validation by QRT-PCR support a role for increased IL-13 activity in keloid formation.

Reduced mRNA levels for the IL-1 receptor and for multiple chemokine (CXC type) ligands induced by IL-1 support the view that an alteration in the inflammatory response plays a role in keloid formation (McCauley *et al.*, 1992; Niessen *et al.*, 1999; Nirodi *et al.*, 2000). The reduction in expression of the IL-1 regulated genes MMP-1 and MMP-3 may account, at least in part, for the abnormal turnover of matrix components. Increased levels of collagenase inhibitors in keloid tissue and reduced degradation of collagen polypeptides in keloid cell cultures has been observed (Niessen *et al.*, 1999). IL-13 increased collagen synthesis in cultured normal and keloid fibroblasts and inhibited the production of IL-1β-induced MMP-1 and MMP-3 (Oriente *et al.*, 2000). These findings are in agreement with our observation of reduced expression of IL-1-inducible genes and of the decoy receptor IL-13Rα2.

Reduced expression of multiple HOX genes, highly conserved master control genes (Chopra and Mishra, 2006), may also contribute to the altered epigenetic program of keloids. While HOX genes play major roles in anterior-posterior development in the embryo, some regulate gene expression in adult differentiated cells, including human dermal fibroblasts (Chang *et al.*, 2002). Silencing of specific HOXA and HOXD genes has been observed in human lung adenocarcinomas and in some normal lung tissue of cancer patients (Shiraishi *et al.*, 2002). Differential expression of HOX genes may be a component of the tumorigenic phenotype of keloids. Alternatively, it may be related to different anatomic sites from which cultures were isolated (Chang *et al.*, 2002). As seen in Table S6, normal and keloid cultures used in this study were not site-matched because tissues were excised only for medical reasons due to the high risk of keloid formation even in African Americans with no history of keloid formation. The possibility that anatomic location may account for differential gene expression is contraindicated by earlier reports that differential regulation of several genes and cell growth by HC is confined to cells from the keloid lesion. Cultures from the superficial dermis of the same keloids act like cultures from normal scars and skin (Russell *et al.*, 1978; Russell *et al.*, 1995; Russell *et al.*, 1989). Also, as seen in Figure 1, keloid strain 145 from a patient's back clustered with strains from earlobes, all of the normal strains clustered together, and the complete data set (available at GSE7890 at geo@ncbi.nlm.nih.gov) shows that for most genes whose expression differed between normal and keloid cultures, including all HOX genes except B6, B7, and C6, expression in cell strain 145 was similar to that of other keloid strains. Studies on fibroblasts from additional keloids and normal scars and from perilesional and lesional cultures from the same keloids should further clarify this issue.

Some differentially regulated genes in keloid fibroblasts, including IGFBP-3 and dermatopontin, were misexpressed in other gene profiling studies (Catherino *et al.*, 2004; Satish *et al.*, 2006; Tosa *et al.*, 2005), whereas the reported over-expression of IL-6–related genes in keloid fibroblasts (Ghazizadeh *et al.*, 2007; Tosa *et al.*, 2005) was not seen here. These and other differences in gene expression reported from different laboratories may be due to differences in origin of cultures, culture conditions or the microarray platform.

Relevant to the potential of microarray analysis to identify candidate genes and susceptibility loci for keloid formation is our observation that several misregulated genes are located at 14q22-q23, a region linked to keloid formation (Davis *et al.*, 2000). Potential relevance is evident for some of these misexpressed genes. SIX1 is overexpressed in multiple tumors and abrogates growth control (OMIM 601205); DAAM1 is a mediator of a noncanonical Wnt signaling pathway (OMIM 606626); DLG7 is a mammalian homologue of a family of Drosophila tumor suppressors (OMIM 601014); and cyclin-dependent kinase inhibitor-3 is a

growth inhibitor (OMIM.123832). Other misregulated genes, including IGFBP-2, IGFBP-5, FZD7, HOXD, STAT1, IGFBP-3 and HOXA, are located on chromosomal arms 2q or 7p, reported to be linked to keloid formation (Marneros *et al.*, 2004).

In conclusion, these studies support involvement of genes in a limited number of fibrosis-associated pathways, including the IGF-1, Wnt, IL-1 and IL-13 signaling pathways, provide preliminary evidence for epigenetic silencing of a subset of genes in the altered program of keloid fibroblasts, further support a role for glucocorticoid resistance in the pathogenesis of keloids, and suggest candidate genes for keloid formation. Reports that some differentially regulated genes in these pathways are also differentially expressed in scleroderma and leiomyoma, that decreased dermatopontin expression has been reported in keloid tissue (Catherino *et al.*, 2004), leiomyoma (Catherino *et al.*, 2004; Tsibris *et al.*, 2002), systemic sclerosis, and hypertrophic scars (Kuroda *et al.*, 1999) and that increased expression of mesoderm specific transcript (MEST) has also been observed in leiomyoma (Tsibris *et al.*, 2002) support the view that common patterns of misregulation are present in these fibrotic disorders that occur with disproportionately high frequency in individuals of African origin, and suggest common susceptibilities and therapeutic strategies.

MATERIALS AND METHODS

Cell culture

Methods of isolation and propagation of fibroblasts from keloids and normal scars have been described (Russell *et al.*, 1978). Briefly, cultures were initiated by mincing tissue and immobilizing the pieces under a glass coverslip. Fibroblasts that migrate from the pieces were subcultured twice and aliquots were frozen in liquid nitrogen, using a programmable freezer (Planer Products, Sudbury on Thames, England). Sources of cell strains, all of which were obtained from African American patients, are described in earlier publications (Meyer *et al.*, 2000; Russell *et al.*, 1978; Russell *et al.*, 1989; Trupin *et al.*, 1983). Sources of strains used in this study are provided in Table S6. The medical ethical committees of Vanderbilt University, Tennessee Valley Health Care System, and Meharry Medical College have approved all described studies. The study was conducted according to Declaration of Helsinki Principles.

Cultures were grown at 37°C in an atmosphere of air and CO_2 adjusted to maintain a pH of 7.4 with 100% relative humidity. The standard culture medium consisted of 90% F-10 (Sigma, St. Louis, MO) and 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY). No antibiotics or antimycotics were used. Cells were subcultured using 0.025% trypsin (Invitrogen) and 0.004% NaEDTA.

Microarray

Strains at low passage number were thawed from liquid nitrogen storage, subcultured in 60-mm culture dishes and grown to confluency (10 days) under standard culture conditions with daily feeding with or without 1.5 μM HC (Sigma). Experimental cultures were plated at 5×10^3 cells/cm^2, using a Coulter Counter to determine the appropriate dilution of cell suspensions. Total RNA was isolated from pooled triplicate cultures using a standard TRIZOL (Invitrogen) method with DNase treatment followed by a second TRIZOL extraction. Pooling three cultures of each strain helps ensure that variation between strains is due to a biological difference rather than a technical artifact introduced during culture or subsequent manipulation.

After verifying the high quality of the RNA, biotinylated cRNA (15μg) was prepared for Affymetrix microarray analysis in the Vanderbilt Microarray Shared Resource. Biotinylated cRNA (15μg) was fragmented and hybridized to an Affymetrix GeneChip (U133 Plus 2.0 Array) containing sets of 11 different 25-mer oligonucleotides, each representing one of 47,000

human transcripts and variants from 38,500 human genes. Hybridized cRNA was detected using streptavidin coupled to phycoerythrin and visualized using a laser scanner. Image data were quantitated to generate gene expression values and ratios of gene expression between various combinations of samples. Each RNA sample was analyzed on a single GeneChip. The resulting data set was appropriate for statistical analysis of differences between strains of the same type as well as for comparisons of gene expression patterns between keloid and normal strains.

Analysis of microarrays

Following data collection, arrays were analyzed using both the MAS5.0 algorithm (default Affymetrix approach; Affymetrix Users Guide, www.affymetrix.com) and the Robust Multichip Analysis (RMA) approach (Bolstad *et al.*, 2003). The MAS5.0 algorithm is the most widely used analysis method for GeneChips. The RMA algorithm, an alternative analysis procedure that is more robust than MAS5.0 (Bolstad *et al.*, 2003) for data with normal errors or long-tailed symmetric errors (robust fitting) (Cleveland, 1981), was used in the statistical analyses.

Following normalization, average gene expression values for normal strains were used as the baseline comparison for all arrays. Using the average baseline, genes that were differentially expressed in keloid strains were identified using parametric and nonparametric (Wilcoxon rank sum test) techniques with corrections for multitesting, in this case the Benjamini-Hochberg method. Version 7.2 of Genespring was used for statistical analyses. This version uses an F distribution to calculate the *p*-value after the nonparametric *t*-test. Following data analysis, all microarray data sets were annotated based on MIAME standards (www.mged.org), and were made publicly available according to those standards using the series record number GSE7890 at geo@ncbi.nlm.nih.gov

Quantitative real-time PCR

Additional aliquots of RNA prepared for microarray were used to validate microarray findings by QRT-PCR. RNA was reverse-transcribed using a cDNA Archive kit (Applied Biosystems, Foster City, CA). Assays were performed in the Molecular Genetics Core Laboratory of the Skin Diseases Research Center at Vanderbilt University Medical Center, using an iQ real-time PCR system (Biorad Laboratories, Hercules, CA). Measurements of specific gene expression were obtained using pre-designed gene-specific fluorescently labeled 5′nuclease probe-based assays (Applied Biosystems), as per manufacturer's protocols. Each predesigned assay is tested and standardized for reproducible expression analysis by the manufacturer. Primers, probes and cDNA (50 ng equivalents) were added to iQ supermix (Biorad Laboratories) in a final volume of 20μl. Each reaction was run in triplicate for the gene of interest and a housekeeping gene, 18s ribosomal RNA. Each assay was validated using 10-fold serial dilutions of human cDNA to examine its specific dynamic range. Because of the high abundance of 18s ribosomal RNA, cDNA samples were diluted 1/100 to permit examination within the dynamic range of the 18s rRNA 5′ nuclease assay. Universal thermal cycling parameters were used (3 min activation at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C).

Fold-differences in expression of selected genes between normal and keloid strains were estimated using the comparative C_T method described in User Bulletin #2 ABI PRISM 7700 Sequence Detection System (Applied Biosystems 2001). The ranges for fold differences were determined by evaluating the expression: $2^{-\Delta\Delta CT} \pm s$, where s is the standard deviation of $\Delta\Delta C_T$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CTGF	connective tissue growth factor
CXCL	chemokine (CXC type) ligand
DKK	Dickkopf
HOX	homeotic
HC	hydrocortisone
IGFBP	insulin-like growth factor binding protein
JAG1	jagged 1
MMP	matrix metalloproteinase
OMIM	Online Mendelian Inheritance in Man
SFRP	secreted frizzled related protein
TGFβ	transforming growth factor-β

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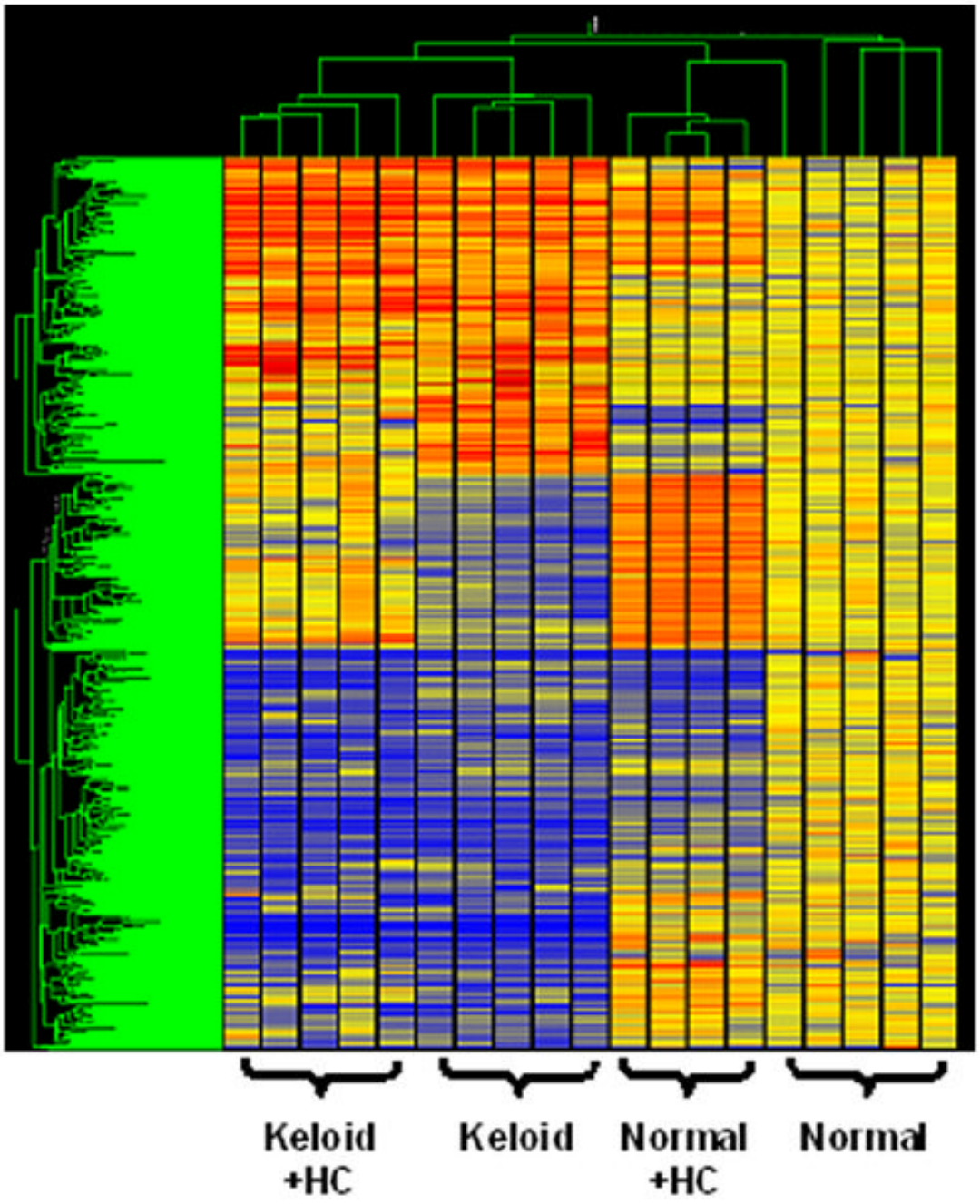

Figure 1.

Unsupervised hierarchical clustering was performed on 19 samples of normal and keloid fibroblasts grown with and without hydrocortisone, using a subset of genes (n = 7303) whose expression varied by at least two-fold relative to the average normal strain in at least two of the 19 samples. Gene expression of cell strains grown in medium with and without (control) 1.5 μM hydrocortisone supplementation fell into four distinguishable groups relative to gene expression in the average normal strain grown in control medium. From right to left are 5 normal strains in control medium, 4 normal strains with hydrocortisone (one strain lost in processing), 5 keloid strains in control medium, five keloid strains with hydrocortisone. Red

indicates increased expression, blue indicates reduced expression, and yellow indicates no difference in expression.

Table 1

Subset of Genes with Increased Expression in Keloid Fibroblasts in the Absence of Hydrocortisone[1]

Gene name (Gene symbol)	Keloid/Normal (number probe sets)	Biological function	Chromosomal location
Asporin (ASPN)	34.77 (2)	interaction with TGFβ and collagen	9q22.31
Coagulation factor II (thrombin) receptor (F2R)*	8.17 (1)	blood coagulation response to wounding	5q13
Coagulation factor II (thrombin-like) receptor (F2RL2)	19.89 (1)	blood coagulation response to wounding	5q13
Cyclin D2 (CD2)	11.13 (1)	regulation of cell cycle promotion	12p13
Dishevelled-associated activator of morphogenesis 1 (DAAM1)	1.77 (2))	cell organization and biogenesis	14q22-q23
Frizzled homolog of, 4 (FZD4)	7.92 (1)	Wnt pathway receptor	11q14.1
Four and a half LIM domains 1 (FHL1)*	3.01 (5)	cell differentiation	Xq27
IGF-binding protein 2 (IGFBP2)	40.18 (1)	regulation of cell growth	2q33-34
IGF-binding protein 5 (IGFBP5)*	7.03 (6)	regulation of cell growth	2q33-34
IGF-binding protein 7(IGFBP7)*	4.49 (3)	regulation of cell growth	4q12
Jagged 1 (JAG1)*	9.89 (3)	notch signaling pathway	20p12
Mesoderm specific transcript (MEST)*	36.68 (1)	mesoderm development	7q32
Nerve growth factor beta (NGFβ)	2.93 (1)	cell-cell signaling, development	1p13
Netrin 4 (NTN4)*	12.21 (1)	basement membrane/extracellular matrix	12q23
Nuclear factor 1/B (NF1B) *	3.85 (4)	transcription factor activity	9p24
Nuclear receptor subfamily 2 group F2 (NR2F2)	2.66 (3)	regulation of transcription	15q26
piggyBac transposable element derived 3 (PGBD3)*	3.90 (3)	DNA repair	10q11.23
RAB3B*	3.64 (3)	small GTPase-mediated signal transduction	1p32
Sine oculis 1 (SIX1)	3.03 (1)	regulation of transcription	14q23
Signal transducer and activator of transcription 1 (STAT-1) *	2.22 (2)	regulation of transcription	2q32

Footnote 1

* statistically significant by parametric as well as nonparametric analysis

Table 2

Subset of Genes with Decreased Expression in Keloid Fibroblasts in the Absence of Hydrocortisone [2]

Gene name (Gene symbol)	Keloid/Normal (number probe sets)	Biological function	Chromosomal location
Angiopoietin 1 (ANGPT)	0.13 (2)	angiogenesis, signal transduction, development	8q22-q23
Chemokine (CXC) ligand 1 (CXCL1)*	0.05 (1)	chemotaxis, inflammatory response, cell proliferation	4q21
Chemokine (CXC) ligand 12 (CXCL12)	0.42 (2)	inflammatory response, immune response, cell adhesion, cell-cell signaling	10q11.1
Chemokine (CXC) ligand 14 (CXCL14)	0.12 (1)	Inflammatory response, immune response	
Collagen Type XVIII alpha 1(COL18A1) *	0.23 (2)	cell adhesion, cell proliferation, organ morphogenesis	21q22.3
Cyclin-dependent kinase inhibitor 1C (CDKN1C)	0.34 (4)	negative regulation of cell proliferation	11p15.5
Cyclin-dependent kinase inhibitor 3 (CDKN3)	0.36 (2)	negative regulation of cell proliferation	14q22
Dermatopontin (DPT)	0.15 (3)	cell adhesion	1q12-q23
Dickkopf homolog 1 (DKK1)	0.45 (1)	negative regulation of Wnt signaling pathway	10q11.2
Dickkopf homolog 3 (DKK3)	0.31 (3)	negative regulation of Wnt signaling pathway	11p15.2
Discs large homolog 7 (DLG7)*	0.25 (1)	cell-cell signaling, cell proliferation	14q22.2
Interleukin 1 receptor, type 1 (ILR1)	0.42 (1)	signal transducer activity	2q12
Interleukin 7 (IL7)*	0.06 (1)	immune response, cell-cell signaling	8q12-q13
Interleukin 8 (IL8) *	0.03 (1)	angiogenesis, cell motility, chemotaxis, cell proliferation, cell adhesion	4q13-q21
Matrix metalloproteinase 3 (MMP3) *	0.03 (1)	collagen catabolism	11q22.3
Membrane metallo-endopeptidase (MME)	0.08 (2)	proteolysis and peptidolysis	3q25.1-q25.2
Pleiotrophin (heparin binding growth factor) (PTN)	0.10 (3)	cell cycle regulation	7q33-q34
Secreted frizzled related protein 1 (SFRP1)	0.02 (3)	negative regulation Wnt signaling pathway	8p12-p11.1
Stathmin-like 2 (STMN2) *	0.01 (2)	intracellular signaling	8q21.11-q21.12
Syndecan (SDC1)*	0.26 (2)	cytoskeletal protein binding	2p24.1
Tenascin C (TNC)	0.52 (1)	cell adhesion	9q33
Thyroid hormone receptor, beta (THRB)*	0.04 (2)	transcriptional regulation	3p24.1
Tissue factor pathway inhibitor 2 (TFPI2)*	0.04 (2)	blood coagulation, endopeptidase inhibitor	7q21.3
Transforming growth factor beta receptor II (TGFBR2)	0.52 (2)	cell proliferation, extracellular matrix production	3p22

Footnote 2

* statistically significant by parametric as well as nonparametric analysis

Table 3

Subset of Genes with Increased Expression in Keloid Fibroblasts in the Presence of Hydrocortisone [3]

Gene name (Gene symbol)	Keloid/Normal (number probe sets)	Biological function	Chromosomal location
Asporin (ASPN)*	49.21 (2)	interaction with other extracellular matrix proteins and TGFβ	9q22.31
Coagulation factor II (thrombin) receptor (F2R)*	16.19 (1)	blood coagulation response to wounding	5q13
Coagulation factor II (thrombin-like) receptor (F2RL2)*	31.98 (1)	blood coagulation response to wounding	5q13
Connective Tissue Growth Factor (CTGF)*	2.51 (1)	cell growth, response to wounding	6q23
Elastin (ELN)	4.15 (1)	extracellular matrix component, organ morphogenesis	7q11.23
Four and a half LIM domains 1 (FHL1)*	2.79 (5)	cell differentiation	Xq27
Frizzled homolog of, 4 (FZD4)*	4.51 (1)	Wnt pathway receptor	11q14.1
IGF binding protein 5 (IGFBP5)	15.22 (2)	regulation of cell growth	2q33-q34
IGF binding protein 7 (IGFBP7)*	6.56 (3)	regulation of cell growth	4q12
Jagged 1 (JAG1)*	7.04 (4)	notch signaling pathway	20p12
Mesoderm specific transcript (MEST)	9.21 (1)	mesoderm development	7q32
Nerve growth factor beta (NGFB)*	7.91 (1)	cell-cell signaling, development	1p13
Netrin 4 (NTN4)*	17.10 (1)	basement membrane/extracellular matrix	12q23
Nuclear receptor subfamily 2 group F2 (NR2F2)*	2.08 (2)	transcriptional regulation	15q26
Osteomodulin (OMD)	4.34 (2)	cell adhesion	9q22.31
piggyBac transposable element derived 3 (PGBD)*	4.44 (3)	DNA repair	10q11.23
RAB3B*	3.81 (4)	small GTPase-mediated signal transduction	1p32
Sine oculis 1 (SIX1)	3.97 (1)	transcriptional regulation	14q23

Footnote 3

* statistically significant by parametric as well as nonparametric analysis

Table 4

Subset of Genes with Decreased Expression in Keloid Fibroblasts in the Presence of Hydrocortisone [4]

Gene name (Gene symbol)	Keloid/Normal (number probe sets)	Biological function	Chromosomal location
Angiopoietin 1 (ANGPT)	0.28 (2)	angiogenesis, signal transduction, development	8q22-q23
Chemokine (CXC) ligand 1 (CXCL1) *	0.11 (1)	chemotaxis, inflammatory response, cell proliferation	4q21
Chemokine (CXC) ligand 12 (CXCL12)	0.12 (1)	inflammatory response, immune response, cell adhesion, cell-cell signaling	10q11.1
Collagen Type XVIII alpha 1(COL18A1)	0.38 (2)	cell adhesion, cell proliferation, organ morphogenesis	21q22.3
Cyclin-dependent kinase inhibitor 3 (CDKN3)*	0.27 (2)	negative regulation of cell proliferation	14q22
Dermatopontin (DPT)	0.10 (3)	cell adhesion	1q12-q23
Dickkopf homolog 2 (DKK2)	0.12 (1)	negative regulation of Wnt signaling pathway	4q25
Discs large homolog 7 (DLG7)*	0.23 (1)	cell-cell signaling, cell proliferation	14q22.2
Interleukin 1 receptor, type 1 (ILR1)*	0.30 (1)	signal transducer activity	2q12
Interleukin 7 (IL7)*	0.06 (1)	immune response, cell-cell signaling	8q12-q13
Matrix metalloproteinase 3 (MMP3) *	0.02 (1)	collagen catabolism	11q22.3
Matrix metalloproteinase 7 (MMP7)*	0.02 (1)	collagen catabolism	11q21-q22
Membrane metallo-endopeptidase (MME)*	0.12 (2)	proteolysis and peptidolysis	3q25.1-q25.2
Pleiotrophin (heparin binding growth factor) (PTN)	0.26 (1)	regulation of cell cycle	7q33-q34
Stathmin-like 2 (STMN2) *	0.02 (2)	intracellular signaling	8q21.11-q21.12
Thyroid hormone receptor, beta (THRB)*	0.05 (2)	transcriptional regulation	3p24.1
Tissue factor pathway inhibitor 2 (TFPI2)*	0.09 (2)	blood coagulation, endopeptidase inhibitor	7q21.3
Transforming growth factor beta receptor II (TGFBR2)	0.55 (1)	cell proliferation, extracellular matrix production	3p22
Transforming growth factor beta receptor III (TGFBR3)	0.45 (1)	cell proliferation, extracellular matrix production	1p33-p32

Footnote 4

*statistically significant by parametric as well as nonparametric analysis

Table 5

Expression of Genes in Several Homeotic Gene Clusters in Keloid versus Normal Fibroblasts

Gene symbol	Keloid/Normal Control	Keloid/Normal Hydrocortisone	Chromosome location
HOXA3	0.21	0.28	7p14-p15
HOXA5	0.19	0.08	7p14-p15
HOXA9	0.03	0.02	7p14-p15
HOXA10	0.09	0.08	7p14-p15
HOXA11	0.09	0.10	7p14-p15
HOXB2	0.51	0.58	17q21.3
HOXB5	0.42	0.50	17q21.3
HOXB6	0.09	0.15	17q21.3
HOXB7	0.22	0.31	17q21.3
HOXC6	0.23	0.16	12q13.3
HOXC9	0.05	0.07	12q13.3
HOXC10	0.02	0.01	12q13.3
HOXD4	0.42	0.49	2q31.1

Table 6

QRT-PCR Validation of Differences in Gene Expression Seen in Microarray

Gene expression in keloid relative to normal fibroblasts in control versus hydrocortisone-supplemented medium				
Gene symbol	**Normal/Normal (range) Control n=4**	**Keloid/Normal (range) Control n=4, n=4**	**Normal/Normal (range) Hydrocortisone n=3**	**Keloid/Normal (range) Hydrocortisone n=4, n=3**
CDKN3	1.00 (0.55–1.82)	0.44 (0.22–0.90)	1.00 (0.71–1.42)	0.98 (0.38–2.50)
COL1A1	1.00 (0.75–1.32)	1.46 (1.01–2.09)	1.00 (0.78–1.28)	1.16 (0.73–1.85)
CTGF	1.00 (0.66–1.51)	1.37 (0.65–2.88)	1.00 (0.71–1.41)	6.28 (3.40–11.60)
DKK1	1.00 (0.80–1.25)	0.35 (0.16–0.76)	1.00 (0.87–1.14)	0.67 (0.28–1.57)
DKK3	1.00 (0.61–1.64)	0.17 (0.08–0.39)	1.00 (0.62–1.61)	0.46 (0.20–1.05)
DLG7	1.00 (0.49–2.03)	0.41 (0.22–0.76)	1.00 (0.63–1.59)	0.31 (0.15–0.63)
DPT	1.00 (0.60–1.67)	0.04 (0.01–0.36)	1.00 (0.59–1.70)	0.02 (0.00–0.14)
ELN	1.00 (0.52–1.93)	0.66 (0.38–1.16)	1.00 (0.66–1.52)	3.04 (1.19–7.75)
F2R	1.00 (0.20–5.11)	13.88 (7.45–25.90)	1.00 (0.24–4.11)	9.63 (1.10–84.08)
FZD4	1.00 (0.57–1.74)	3.06 (1.92–4.87)	1.00 (0.14–7.22)	1.48 (0.63–3.52)
IGFBP2	1.00 (0.39–2.59)	56.1 (10.85–290.15)	1.00 (0.77–1.29)	6.36 (1.73–23.46)
IGFBP3	1.00 (0.36–2.81)	0.57 (0.18–1.81)	1.00 (0.36–2.81)	3.88 (1.31–11.45)
IGFBP5	1.00 (0.36–2.77)	9.63 (2.98–31.12)	1.00 (0.54–1.85)	7.50 (0.89–63.38)
IGFBP7	1.00 (0.53–1.88)	6.66 (3.84–11.56)	1.00 (0.57–1.74)	5.77 (2.61–12,76)
IL13Ra1	1.00 (0.91–1.10)	0.89 (0.65–1.22)	1.00 (0.38–2.61)	2.19 (1.10–4.35)
IL13Ra2	1.00 (0.57–1.75)	0.02 (0.01–0.04)	1.00 (0.35–2.88)	Not detectable in keloid
IL8	1.00 (0.26–3.91)	0.02 (0.01–0.06)	1.00 (0.52–1.91)	0.28 (0.04–1.93)
JAG1	1.00 (0.52–1.91)	9.70 (4.50–20.89)	1.00 (0.55–1.80)	8.09 (3.37–19.42)
MEST	1.00 (0.64–1.56)	51.63 (19.20–138.83)	1.00 (0.40–2.47)	25.83 (6.40–104.15)
MMP1	1.00 (0.35–2.88)	0.17 (0.02–1.36)	1.00 (0.30–3.38)	0.19 (0.04–0.88))
MMP3	1.00 (0.24–4.13)	0.01 (0.001–0.07)	1.00 (0.54–1.87)	0.02 (0.01–0.05)
SFRP1	1.00 (0.16–6.34)	0.04 (0.03–0.04)	1.00 (0.15–6.56)	0.17 (0.08–0.37)
SFRP2	1.00 (0.34–2.97)	0.11 (0.02–0.58)	1.00 (0.32–3.11)	0.06 (0.01–0.27)
TGFBR1	1.00 (0.79–1.27)	1.76 (0.92–3.35)	1.00 (0.37–2.71)	1.40 (1.01–1.93)
TGFBR2	1.00 (0.55–1.83)	0.53 (0.30–0.95)	1.00 (0.50–1.99)	0.73 (0.33–1.60)
TGFBR3	1.00 (0.72–1.39)	3.60 (1.49–8.84)	1.00 (0.46–2.19)	0.19 (0.36–2.13)

Effect of hydrocortisone on CTGF, elastin, and IGFBP-3 gene expression in normal versus keloid fibroblasts

Gene symbol	Normal Control/Normal Control (range) n=4	Normal Hydrocortisone/Normal Control (range) n=3	Keloid Control/Keloid Control (range) n=4	Keloid Hydrocortisone/Keloid Control (range) n=4
CTGF	1.00 (0.61–1.64)	0.22 (0.15–0.31)	1.00 (0.48–2.10)	1.03 (0.56–1.90)
ELN	1.00 (0.45–2.23)	0.28 (0.19–0.43)	1.00 (0.57–1.76)	1.30 (0.45–3.74)
IGFBP3	1.00 (0.85–1.18)	0.11 (0.04–0.32)	1.00 (0.31–3.20)	0.65 (0.22–1.91)