Identification of TGF-Beta Gene Expression Profiles in Earlobe Keloids Using cDNA Microarrays

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Dedication

This dissertation is dedicated to Dr. George L. Adams (1941-2006), a tireless mentor and teacher with whom I had the honor and privilege to learn from. Dr. Adams dedicated over 39 years of his life to caring for head and neck cancer patients, as well as making significant contributions to the field. His passion for Otolaryngology and for life was endless. We are all better for have known him. Although he is no longer with us, his legacy continues on in the many medical students, residents, and fellows he taught throughout such a remarkable career. I am forever grateful for all he has done for me.

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INTRODUCTION

Keloids represent a pathological response to dermal wounding, resulting in raised, amorphous scars that grow beyond the boundaries of the original wound area. These lesions can cause devastating consequences for afflicted patients, including pruritis, pain and disfigurement. First described by French dermatologist Jean Louis Alibert as "canceroides," these lesions grossly resembled cancerous growths with crab claw-like extensions of the lesion into the surrounding skin [1]. Keloids are comprised of dense dermal connective tissue and fibroblasts, with an overabundance of randomly oriented fibers of collagen [2]. The pathogenesis of keloid formation remains elusive, but racial and familial predisposition, immunological, and possibly hormonal factors have been implicated. Although keloids can arise in all races, darker-pigmented individuals have a predisposition [2]. Skin wound tension is an important factor in the formation of keloids, as they commonly arise in high skin tension areas, such as upper arms, sternum, neck, shoulders, upper back, and the mandibular angle [3]. The earlobe and neck are the most common sites of keloid formation in the head and neck [3].

Treatment of keloids is challenging. Simple surgical excision can lead to recurrence in about 50-80% of individuals [2]. Other partially effective treatment approaches include radiation therapy, pharmacological management (intralesional injection with corticosteroids, retin, zinc oxide, and antineoplastic agents), laser excision, cryotherapy, silicone gel sheeting, and pressure devices [2]. The wide variety of treatment options reflects the lack of understanding the pathogenesis of keloids. Furthermore, optimal therapy for head and neck keloids is controversial.

Recent research into understanding the pathogenesis of keloids has largely involved the study of cellular and molecular mechanisms of tissue injury repair and wound healing. There is evidence that the expression of transforming growth factor beta (TGF-β) may be involved in the pathogenesis of keloids. There is increasing evidence to support the hypothesis that increased levels of TGF-β ligands and receptors found in keloids may result in increased signaling and a pivotal role for TGF-β related pathways in keloid formation [4]. Studies done on *in vitro* keloid fibroblasts have shown the persistent production of high levels of collagen, fibronectin, elastin, and proteoglycan compared to normal dermal fibroblasts, in response to metabolic modulators such as glucocorticoids, hydrocortisone, growth factors, and phorbol esters [5].

The multifunctional cytokine TGF-β regulates several physiological processes, including cellular growth and differentiation, apoptosis, embryonic development, and wound healing [6, 7]. TGF-β is found in human platelets, lymphocytes, fibroblasts, and keratinocytes [8]. The TGF-β family of growth factors includes three isotypes (β1, β2, and β3), activins, and bone morphogenetic proteins (BMPs) [8]. During the process of wound healing, studies have demonstrated that TGF-β1 is integral for the chemotaxis, activation and production of fibroblasts, the induction of extracellular matrix (ECM) deposition, as well as the production of collagens, fibronectin, and proteoglycans [1, 6]. Ultimately, the activity, expression, and cellular signaling of TGF-β must be tightly regulated. There is substantial evidence that dysregulation of TGF-β pathways is involved in other fibrotic disorders, dysfunctional wound healing, autoimmune disorders, cancer, and inflammatory diseases [7]. There is an increasing body of evidence to support that

isoforms of TGF-β are fundamental mediators of keloidogenesis, because its elevated production has been linked to many fibrotic disorders, including pulmonary fibrosis, liver cirrhosis, glomerulonephritis, hypertrophic scarring, and scleroderma [4]. TGF-β also has a pivotal role in wound repair and pathologic fibrosis by inducing fibroblast activation via paracrine signals and by maintaining and enhancing this effect by an autocrine mechanism [9]. Therefore, TGF-β is a potential drug target for scar reduction therapy.

Furthermore, transforming growth factor beta (TGF-β) plays an integral role in the process of fibroplasia and wound healing regulation of extracellular matrix (ECM) proteins, such as fibronectin and collagen. TGF-β upregulates the synthesis of the degrading enzymes plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinases 1 (TIMP-1) and downregulates expression of the proteases plasminogen activator (PA) and collagenases [5]. This interaction promotes the accumulation of ECM proteins induced by TGF-β and is the basis for fibrotic tissue formation due to the action of TGF-β. Interestingly, keloid fibroblast interaction with TGF-β results in further increase of an already augmented rate of collagen production, despite secreting TGF-β levels similar to normal fibroblasts. This has led to the idea that the aberrant response or unique sensitivity of keloid fibroblasts to TGF-β may represent an abnormality at the receptor level or postreceptor signaling [5]. In addition, researchers using an *in vitro* fibroplasias model have demonstrated that increased PAI-1 expression and concomitant decreased urokinase plasminogen activator (uPA) are unique to keloid fibroblasts [10]. It is hypothesized that decreased plasmin production due to increased PAI-1 activity may result in less plasmin-mediated collagenase activation and plasmin-

dependent collagen degradation by collagenases [5]. Thus, the excess collagen in keloids may result not only from increased collagen synthesis by fibroblasts, but also reduced collagen degradation and removal during scar remodeling.

Members of the TGF-β superfamily exert their effects by binding to serine/threonine kinase cell surface receptors expressed on virtually all cell types [11]. The binding of TGF-β ligand to the ubiquitous TGF-β receptor 2 results in heterodimerization with and activation of TGF-β receptor 1 and the phosphorylation of specific serine residues [4]. The signal is then propagated downstream through SMADs, a family of intracellular signaling molecules that transport information from the cell membrane to the nucleus [12]. The term "SMAD" is derived from a combination of genes characterized in *Caenorrhabditis elegans*, SMA, and *Drosophila malanogaster*, MAD [8]. The pathway-restricted SMADs or receptor-regulated SMADs (R-SMADs), 1, 2, 3, and 5, interact with and are phosphorylated by specific type I serine/threonine kinase receptors [8]. Upon receptor-induced phosphorylation and activation at the cell membrane, the receptor-SMADs form hetero-oligomeric complexes with the common SMAD (SMAD 4) before translocating into the nucleus, where, in association with other DNA binding factors or with transcriptional coactivators or coinhibitors, they modulate the transcription of TGF-β responsive genes [12].

Inhibitory SMADs (I-SMADs), represented by SMAD6 and SMAD7, antagonize the activity of the receptor-regulated SMADs by blocking phosphorylation, thereby terminating the propagation of the TGF-β signal [8]. SMAD7 seems to be more specific for TGF-β signaling and functions in a pivotal negative feedback fashion by limiting the

amplitude and duration of cellular responses to TGF-β due to the rapid induction of its expression by TGF-β [12]. Therefore, SMAD7 induction serves as a critical intracellular "brake" on TGF-β-induced responses, serving as a SMAD7-mediated autoinhibitory feedback loop [12]. The SMAD pathway has a fundamental role in the regulation of collagen production and are key mediators of TGF-β-dependent stimulation [12]. TGF-β is implicated in initiating and sustaining fibroblast activation and SMADs serve as major intracellular effectors of TGF-β-induced profibrotic responses [12].

TGF-β signaling involves at least three different mammalian isoforms of transmembrane serine/threonine kinase receptors (types 1, 2, and 3) [4]. TGF-β receptors 1 and 2 are the main signaling receptors that are known to accelerate wound healing and to induce connective tissue deposition *in vivo* to promote fibrosis and scarring, whereas the type 3 receptor functions by presenting ligands to receptors 1 and 2 [4, 8, 13]. Chin et al found TGF-β1 and –β2 ligands to be expressed at higher levels in keloids fibroblasts compared with normal human dermal fibroblasts (NHDFs), suggesting a fibrosis-promoting role for TGF-β in the pathogenesis of keloids [4]. They also demonstrated greater expression of TGF-β receptors (types 1 and 2) and increased phosphorylation of SMAD 3 in keloid fibroblasts compared to NHDFs [4]. Exogenous administration of TGF-β1 and –β2 to adult wounds has been shown to result in increased collagen, proteoglycan, and inflammatory cell accumulation [14]. Furthermore, treatment of adult rat wounds with neutralizing antibody to TGF-β1 and –β2 diminishes scar formation. Also, the relative proportion of TGF-β isoforms, and not the absolute amount of one particular isoform, may influence wound repair. For example, researchers have found

TGF-β3 expression to be increased and TGF-β1 expression unchanged in scarless fetal wounds. Conversely, TGF-β1 expression is increased and TGF-β3 decreased in scarring fetal wounds [14].

The effects of exogenous TGF-β2 on keloids fibroblasts was studied by Smith et al, who found an increase in cell proliferation kinetics and DNA synthesis [15]. They went on to study fibroblasts from hypertrophic scars, keloids, and normal skin, observing that keloid-derived fibroblasts stimulated the most contraction of the three cell lines with the addition of exogenous TGF-β2 [15]. These results were substantiated when these effects were able to be reversed by adding polyclonal TGF-β antibodies [4]. Additional animal model studies have looked at the effects of exogenous TGF-β2 on keloid xenografts [4]. Polo et al applied explanted human proliferative scars in athymic nude rats and demonstrated elevated cell proliferation when TGF-β1 and 2 were systemically added [4]. Lee et al have shown elevated basal protein expression of TGF-β1 and β2 ligands in keloid-derived fibroblasts and which expressed sensitivity to TGF-β stimulation, translated by increased procollagen type I expression and collagen protein production [4]. Chin et al also demonstrated an increased expression of TGF-β receptors (types I and II) in keloids relative to normal human dermal fibroblasts (NHDFs) [4]. This led to the hypothesis that an increased number of receptors will accommodate the potentially elevated TGF-β ligands in keloids, thus leading to excessive TGF-β signaling [4]. Taken together, their data may substantiate the idea that TGF-β signaling mechanisms of keloid fibroblasts are upregulated, leading to enhanced activity of downstream intracellular molecules [4]. They conclude that these findings support the

model of an autocrine positive-feedback loop in keloids, as similarly suggested by Schmid et al in other skin disorders [4].

Several studies have shown an altered response of keloid fibroblasts to TGF-β, suggesting a change at the receptor level or intracellular signaling pathways [4]. Increased TGF-β type I and II receptor expression levels in granulation and hypertrophic scars have been studied by Schmid et al [4]. They concluded that excessive scarring was linked to a failure to regulate overactive fibroblasts during the tissue remodeling phase, leading to a prolonged autocrine, positive-feedback loop resulting in overproduction of ECM and ultimately fibrosis [4]. Overexpression of TGF-β signaling molecules in keloid tissue specimens compared to normal skin suggest that these activated downstream markers contribute to keloids pathogenesis [16].

Yu et al have found a decreased expression of inhibitory SMAD6 and 7 in keloids fibroblasts relative to normal scars and NHDFs [8]. Moreover, they demonstrated that TGF-β1 stimulation failed to increase SMAD7 expression levels. This has led to the conclusion that decreased expression of inhibitory SMADs may explain why TGF-β signaling is not terminated in keloids by an auto-negative feedback loop. Furthermore, other studies have shown that decreased SMAD expression is linked to cardiac fibrosis, TGF-β hyperresponsiveness in scleroderma, and contributes to pulmonary fibrosis [8].

TGF-β plays a central role in the pathogenesis of fibrosis as a major mediator of tissue remodeling by inducing and sustaining the activation of fibroblasts and is strongly implicated in the pathogenesis of scleroderma [12]. TGF-β1 has also been shown to function as a potent promoter of tissue remodeling in the prostate stroma by genetically

reprogramming fibroblasts into reactive myofibroblasts with elevated ECM production [17].

DNA microarrays have been successfully utilized to better understand molecular mechanisms and to identify candidate genes involved in ECM remodeling. DNA microarray analysis involves the identification of a consistently up-regulated or down-regulated gene from a group of patients with the same disease that may reveal an altered gene or biochemical pathway associated with a particular disease [18]. This advanced technique allows for the study of global gene profiles from normal and pathologic specimens, thus expediting the identification of differentially expressed genes [19]. Having identified the aberrant functional pathway or responsible gene(s), directed therapeutic strategies could then be developed. Using this knowledge, analysis of gene expression patterns across individuals with the same disease may show molecular-level differences that allow refinement of current disease classification, prognostication, and treatment selection [20]. Using a more comprehensive view of the molecular defects involved in the formation of keloids obtained through microarray analysis may produce unique biomarkers for utilization in the clinical setting.

A microarray is an orderly arrangement of DNA strands or probes affixed to a solid surface. These probes are prepared from expressed DNA sequences or expressed sequence tags (EST) derived from comprehensive public databases. ESTs are putative genes created by forming a probe consisting of the complementary sequence of an mRNA sequence identified in a cell. A target sample containing labeled mRNA can be applied and hybridize to the microarray probes. With the knowledge of the precise DNA

probe sequence and location, the intensity and pattern of the labeled mRNA target allows quantification of expression for many genes in the target tissue sample.

There are two common methods of employing hybridization of labeled nucleic acid transcripts, the robotically spotted complementary DNA (cDNA) microarray and photolithographic oligonucleotide microarray (oligo chips). High-density oligonucleotide microarray GeneChips are manufactured by Affymetrix® (Santa Clara, CA) using photolithographic techniques [21]. Twenty-five base oligonucleotides are sequentially and simultaneously built individually on a glass surface. The chemical addition of nucleotides can be precisely controlled by masking specific strands and exposing others to a light source. A GeneChip contains thousands of probe sets, representing a single gene or EST. Each set contains a number of probe pairs for a single gene that specifically match different coding regions of that gene. To control for cross-hybridization, each probe pair contains a perfect match (PM) and mismatch (MM) sequence. Each probe pair has one oligonucleotide that is perfectly complementary to the target mRNA and the other oligonucleotide has an identical sequence with the exception of a switched base pair in the central position. Thus, the MM serves as an internal control and provides a measurement of baseline cross-hybridization. Furthermore, some probe sets contain oligonucleotides representing genes not present in the target genome and so serve as controls for the chip as a whole.

MATERIALS AND METHODS

Normal human dermal fibroblast (NHDF) cell lines (Cascade Biologics, Portland, OR, catalog #C-013-5C) isolated from adult skin were cultured according to

manufacturer's directions and passaged to keep the culture age as similar as possible at the time of analysis. Cells were cultured using Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose) supplemented with 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA), 500 U/ml penicillin, and 100 mcg/ml streptomycin at 37° C in a humidified CO_2 incubator. Once confluent, the primary cell lines were passed by detachment of cells from the culture plate using trypsin/EDTA solution (GIBCO BRL, Life Technologies Inc., Gaithersburg, MD). RNA was prepared from the specimens according to Affymetrix® protocols.

Informed consent from patients was obtained in accord with guidelines set forth by the Institutional Review Board of the Human Subjects Protection Committee at the University of Minnesota. The collection of keloids and normal tissue samples was performed as follows. Previously untreated, mature, full-thickness keloid specimens from the earlobe were obtained during surgical excision. After submitting a portion as a surgical pathology specimen for histological confirmation, each tissue sample was then placed in a glass vial and snap-frozen in liquid nitrogen and stored in a -80^0 freezer until extraction of mRNA. No significant medical comorbidities or patients having taken medications known to influence cellular transcriptional results were identified.

Normal control tissue was obtained from a tissue bank for scientific research purposes. The Tissue Procurement Facility through the University of Minnesota Cancer Center, using standardized procedures, obtained normal skin samples from surgical resection specimens from patients undergoing plastic surgery procedures. These samples

were immediately placed on ice and frozen in liquid nitrogen following removal of portions needed for pathological diagnosis.

Clinical keloid and normal tissues were harvested for RNA isolation using the StrataPrep® Total RNA Miniprep Kit (Stratagene) according to the manufacturer's instructions. Approximately 20-25 μg of total RNA from each keloid tissue was used for each microarray experiment. Subsequent RNA processing procedures were performed according to established protocols published in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA).

Each isolated RNA sample was subjected to Agarose gel electrophoresis as a quality control measure to confirm the presence of nondegraded RNA. The RNA was then stored in a -80^0 freezer until microarray experiments were performed.

Affymetrix® microarrays were performed as previously described [22]. Briefly, cDNA was prepared from 20 μg total RNA using a T7-dT_{24} primer. cRNA was synthesized from cDNA and biotinylated using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and hybridized with the Human GenomeU133 plus 2.0 Affymetrix® GeneChip microarray. Intensity of each gene expression in 2-3 batches of keloid tissues is presented as relative light unit (RLU, means±SD, n=2). Chip files were then analyzed and differentially expressed genes involved in the TGF-β signaling pathway were identified with the GeneSifter online program (http://www.genesifter.net/web/) following normalization of the raw data. Genes of interest met the criterion of greater than or equal to a 1.5-fold difference in mean expression between groups.

RESULTS

Extracted RNA from three NHDF cell cultures, two normal breast skin specimens, and two earlobe keloids was submitted for DNA microarray studies. Expressed genes involved many gene families and biological pathways. After gene expression analysis with the GeneSifter program, 104 genes were identified involving the TGF-beta signaling pathway (Fig.1). The green shading represents two-fold underexpression and the red shading represents two-fold overexpression. Black signifies the absence any differential expression. The genes identified in Fig. 1 represent those involved in a variety of cellular processes and molecular functions. Two genes were found with an average up- or down- regulation of at least 1.5-fold that have not been previously identified in human earlobe keloids. These genes include SMAD specific E3 ubiquitin-protein ligase 2 or *SM*AD *u*biquitination *r*egulatory *f*actor 2 (SMURF2) and *c*artilage *o*ligomeric *m*atrix *p*rotein (COMP).

The genes in normal skin specimens failed to demonstrate any differential expression compared to the keloid and NHDF groups (Fig.1). With this result, we are unable to draw any meaningful data about the gene profile differences unique to the normal skin specimens.

SMURF2 demonstrated at least a two-fold increased expression in NHDFs compared to the keloids (Fig.2). The average intensity level for NHDFs was 8.82, compared to an average intensity level of 4.14 for keloids (Table I). The SMURF2 gene is localized to chromosome 17q22 and is involved in TGF-β signaling regulation by way of ubiquitin-dependent proteasome-mediated degradation.

COMP showed at least a 1.5-fold decreased expression in NHDFs relative to the keloids (Fig.3). The average intensity level for NHDFs was 5.50, compared to 9.80 for the keloid group (Table II). The COMP gene is localized to chromosome 19p13.1 and is a pentameric extracellular glycoprotein and the fifth member of the thrombospondin gene family (TSP-5) [23].

DISCUSSION

The role of TGF-β in wound healing is becoming better elucidated and there is increasing evidence that TGF-β is an important mediator in the pathogenesis of keloids. The complex functions of TGF-β rely on tightly regulated interactions between receptors, SMADs, and DNA-binding proteins. In normal fibroblasts, SMADs are essential cytoplasmic effectors for TGF-β-induced stimulation of the synthesis of collagen, connective tissue growth factor, and of TGF-β itself (autoinduction) [12]. Therefore, signal transduction from the ligand to the target gene requires SMAD interactions with a variety of intracellular molecules [24]. Dysregulated pathways have been implicated in some forms of human cancer and developmental disorders. For instance, cancer cells have been observed to have a loss of inhibitory responses to TGF-β, and increased TGF-β activity is linked to fibrotic diseases in various organs [4]. During wound healing, collagen production must be tightly regulated, otherwise an enhanced or prolonged profibrotic response can result in pathologic fibrosis [25]. Also, aberrant SMAD7 regulation appears to be involved in cardiac and hepatic fibrosis [8]. Therefore, it seems logical that investigating the TGF-β signal transduction pathway in keloids can enhance our understanding of its pathogenesis.

TGF-β signaling regulation also occurs by way of a ubiquitin-dependent proteasome-mediated degradation mechanism, or ubiquitination, of SMAD proteins [26]. Ubiquitination is a unique process for covalently attaching ubiquitin to proteins for targeting their degradation by proteasomes [24]. This evolutionarily conserved process of protein ubiquitination is achieved via an enzyme cascade consisting of E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin-protein ligases) [24]. Moreover, E3 ubiquitin-protein ligases play a pivotal role in defining substrate specificity and specifying which proteins are targeted for degradation by the 26S proteosomes [27]. The two classes of E3 ubiquitin-protein ligases consist of the RING (*r*eally *in*teresting *g*ene) domain class and the HECT (*h*omology to *E*6AP *c*arboxyl *t*erminus) domain class [28]. This mechanism of regulated degradation has been linked to other cellular responses, including the heat shock response, cell cycle degradation, DNA repair, signal transduction, and transcription [27]. There is clear evidence that ubiquitin-dependent protein degradation is involved in signal transduction, cell cycle events, and transcriptional regulation [11].

Ubiquitination is facilitated by SMURF2, which targets R-SMADs for degradation [26]. SMURF2 was found to be underexpressed in the keloid samples in comparison to NHDFs. SMURF2 is a HECT domain ubiquitin-protein E3 ligase that targets TGF-β-activated SMADs and mediates its ubiquitination and subsequent proteasome-dependent degradation, thereby reducing its transcriptional activity in TGF-β signaling [24]. SMAD degradation by SMURF2 occurs independently of receptor activation, thus regulating SMAD levels and controlling the sensitivity of a cell's

response to TGF-β signaling [27]. Kavsak et al have demonstrated that SMAD7functions as an adaptor protein that recruits SMURF2 to the TGF-β receptor complex to promote its degradation via a ubiquitin-mediated process, thereby downregulating and terminating TGF-β signaling [28, 29]. This suggests that SMURF2 acts to enhance the inhibitory activity of SMAD7, thus SMURF2 and SMAD7 may be mutually dependent on each other for exerting complete inhibitory activity on TGF-β [28, 29]. Ohashi et al have shown that both SMURF2 mRNA and protein are increased by TGF-β stimulation as well as TGF-β induced SMURF2 promoter activity elevation [30]. This suggests that TGF-β activates SMURF2 transcription by way of TGF-β receptors in a SMAD-independent manner [30].

The observation in our study of elevated SMURF2 gene expression in NHDFs relative to keloid tissue is consistent with the understanding that SMURF2 functions to regulate TGF-β signaling. Underexpression of SMURF2 in keloid samples may be a mechanism controlling keloid formation by not being able to serve as a critical “brake” on TGF-β effects, such as hyperproliferation of fibroblasts and collagen overproduction.

A second interesting gene identified is COMP, which was found to be overexpressed in the keloid specimens in comparison to NHDFs. The human COMP gene is located on chromosome 19p13.1 and the nucleotide sequence is 2460 base pairs long [31]. It is predominantly found in cartilage and tendon ECM, ligament, and bone [23]. COMP plays an important role in ECM assembly and is known to bind ECM proteins, including fibronectin, and is involved with the stabilization of collagen networks by interacting with collagens I, II, and IX [23]. The pentameric structure of COMP contains

a central core that can bind hydrophobic ligands, such as retinoids and vitamin D3 [23]. Studies have shown that vitamin D can synergize with TGF-β by inducing the phosphorylation of SMAD3 [23]. Furthermore, the topical administration of a vitamin D3 analogue to hairless mice results in in vivo collagen expression and skin thickness [23].

Serum COMP levels in rheumatoid and osteoarthritis patients have been demonstrated to correlate with disease activity [23]. The importance of COMP for skeletal development and growth is illustrated by the identification of mutations in the COMP gene resulting in pseudoachondroplasia and multiple epiphyseal dysplasia, whereby patients develop short bones and early onset arthritis, and the intracellular accumulation of COMP in chondrocytes [23, 32, 33]. Genetic testing to identify these COMP mutations have already shown clinical utility to aid reproductive decision-making and to facilitate testing for at-risk family members [34].

Farina et al have shown that COMP protein accumulates in the skin of systemic sclerosis (SSc) patients [23]. They also demonstrated that TGF-β induced a significant increase in COMP mRNA and protein in SSc fibroblasts. In addition, increased levels of fibronectin and collagen I in ECM resulted from murine fibroblasts engineered to overexpress human COMP. These investigators concluded that COMP is overexpressed in SSc skin and cultured fibroblasts presumably due to autocrine TGF-β activation, and that COMP overexpression can stimulate excess ECM deposition.

Neidhart et al have demonstrated the up regulation of galectin-3 in rheumatoid arthritis synovial fibroblasts following cell adhesion to COMP through a integrin-dependent mechanism [35]. Furthermore, increased galectin-3 expression has been

implicated in the development of tumors and inflammation because of its role as an anti-apoptotic molecule. Therefore, some researchers speculate that its up regulation after cellular adhesion might induce a state of resistance to apoptosis. Ladin et al have also suggested that keloidogenesis may share characteristics with other types of neoplasia after they demonstrated p53 tumor suppressor gene dysregulation in association with upregulation of bcl-2 expression and delayed apoptosis in keloid formation [36]. Furthermore, keloid fibroblasts displayed enhanced apoptosis rates following treatment with hydrocortisone, gamma-interferon, and hypoxia in comparison with NHDFs.

Dermal fibroblasts have been shown to produce COMP in vitro [23]. COMP protein has also been identified in skin wound tissue, but absent from normal skin [23]. Thus, COMP may serve as a biomarker of interstitial fibroblast injury or activation [23]. COMP expression has been linked to pancreatic fibrosis in the development of chronic pancreatitis [23]. COMP has also been found in equine skin scars and granulation tissue associated with tissue injury and TGF-β release [23]. This data has lead to the notion that COMP expression outside of cartilage is closely associated with fibrotic conditions involving tissue exposure to TGF-β [23]. Therefore, it is plausible that by directly binding to ECM components, COMP induces the deposition of collagen and other matrix proteins, thereby exacerbating the process of dermal fibrosis [23]. This is the first known report of upregulated COMP gene in earlobe keloid tissue.

It is useful to analyze the fibrosis model found in other disease processes, such as scleroderma/systemic sclerosis, which involves the overproduction of collagen and other ECM components, in order to gain a better understanding of keloid pathogenesis [37].

Scleroderma and other related fibrotic conditions share the fundamental process of TGF-β mediated fibroblast activation [25]. Dysregulation of SMAD signaling is thought to be the fundamental problem in pathologic lung, liver, and kidney fibrosis [25]. Scleroderma may arise as a result of altered signaling downstream of the TGF-β receptor through SMAD transcription factors. A deficiency in SMAD7 levels in scleroderma fibroblasts may explain the elevation of TGF-β expression and resistance to apoptosis [12]. Constitutive SMAD activation may contribute to the profibrotic phenotype of scleroderma fibroblasts, but the exact mechanisms remain unknown [12]. One proposed explanation is autocrine stimulation by endogenous TGF-β, which is supported by the observation that increased collagen synthesis can be reduced when endogenous TGF-β signaling is disrupted [12]. Aberrant SMAD 2/3 signaling has also been implicated in the pathogenesis of cystic fibrosis, chronic inflammatory bowel disease, and arthritis [26].

The enigmatic biology of keloids is slowly becoming better understood, but we are only skimming the surface of this problematic condition. Based on our findings, disabling the intracellular TGF-β and SMAD signaling pathway may be an effective approach to preventing fibrosis and the subsequent development of keloids [25]. This could be done by targeting SMURF2 to upregulate its expression or enhance its effects in individuals prone to developing keloids, thereby limiting the pro-fibrotic effects of TGF-β signaling. Alternatively, it may be possible to develop mechanisms to limit COMP production or its interactions with signaling molecules in keloids. This could be accomplished by selectively inhibiting COMP with a blocking antibody, thereby regulating the formation of keloids. Thus, a better understanding of TGF-β and SMAD

mechanisms underlying keloidogenesis may provide opportunities to predict disease behavior and possibly direct more effective treatment plans. This possibility is already starting to become realized, and clinical benefits have recently been reported with the application of 5-fluorouracil, which has been identified as a potent inhibitor of TGF-β/SMAD signaling in keloids and hypertrophic scars [38].

Recent studies have explored keloid treatments aimed at the molecular level. For example, Berman and Villa have reported initial success with topically applied imiquimod 5% cream following keloid excision [39]. This treatment is based on the ability of interferon (IFN)-α2b to reduce keloid recurrence, via local induction of IFN-α.

The identification of accurate and reliable biomarkers in the form of gene expression signatures may serve as prognostic indicators of keloid biology, such as severity of the disease. These genetic biomarkers may also provide data to predict which patients predisposed to keloid formation may be at greatest risk for recurrence. Appropriate treatment plans could then be designed to target these individuals.

Limitations of this study include the small sample sizes yielding limited results. However, the genetic profiling of earlobe keloids, to our knowledge, has not been done before. Therefore, this study also serves as validation for proof of concept and as a pilot study for future investigations. Also, the lack of validation studies to confirm our findings greatly limit the impact of our results. Because this is a preliminary report of ongoing investigations, we are currently in the process of performing validation experiments with real time polymerase chain reaction (RT-PCR) technology, which will hopefully substantiate our results.

Extraction of keloid RNA proved quite challenging and will require closer scrutiny of our methods. The most difficult challenge was mechanically breaking down the keloid tissue by way of homogenization. Keloid specimens are hard and fibrotic, which prolonged the time and energy needed to breakdown the tissue, increasing the susceptibility of unstable RNA. Also, the genes in normal skin specimens failed to demonstrate differential expression (Fig.1). It is possible that the unstable RNA was of poor quality despite quality control measures, and no useful data could be extracted in order draw meaningful conclusions. These specimens came from a tissue bank, which made it impossible to know the exact methods utilized in their procurement. For example, an extended time delay before freezing may have altered the RNA. In addition, it is noted that our analysis is based on excised pathologic specimens. There may be genetic differences in the central portion of keloids relative to the periphery, where mechanisms leading to keloid formation may be most active. The potential existence of such differences certainly could alter our findings.

High throughput gene expression profiling techniques offer a specialized mechanism for investigating transcriptome-wide levels of gene expression. DNA microarrays are novel and powerful techniques that allow global gene expression patterns of samples taken from normal and diseased patients. However, this technology has potential limitations. For instance, there can be technical problems with the analysis of expression profiles derived from heterogenetic tissue samples composed of different cell types [40]. Such heterogeneity of expression in subpopulations of cells can lead to over-interpretation or misleading results. A heterogeneous sampling can be investigated

further by using laser capture microdissection techniques coupled with newer technology to amplify RNA from limited tissue quantities. Also, DNA microarrays can generate enormous amounts of data that require rapid and reliable strategies of validation. It should be noted that many methods exist to extract data from microarray studies, while newer analytical tools are continuously being developed. Furthermore, no standardized approach exists. Therefore, only a small amount of information is presented from such an enormous amount of data that is generated from microarray experiments. To this end, we chose to focus only on the unique genes involved in the TGF-β signaling pathway to better understand mechanisms in keloidogenesis.

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APPENDIX A

Condition	Target	Int.	Quality
normal	JLin_1_U133_plus_2	5.1895	0 (A)
	JLin_2_U133_plus_2	4.9536	0 (A)
skin fibroblast	JLin_3_U133_plus_2	8.6102	0 (A)
	JLin_4_U133_plus_2	9.0093	0 (A)
	JLin_5_U133_plus_2	8.8367	0 (A)
keloid	JLin_6_U133_plus_2	4.3447	0 (A)
	JLin_7_U133_plus_2	3.9310	0 (A)

Table I: SMURF2

Condition	Target	Int.	Quality
normal	JLin_1_U133_plus_2	8.0730	0 (A)
	JLin_2_U133_plus_2	8.1361	0 (A)
skin fibroblast	JLin_3_U133_plus_2	5.7396	0 (A)
	JLin_4_U133_plus_2	5.5404	0 (A)
	JLin_5_U133_plus_2	5.2199	0 (A)
keloid	JLin_6_U133_plus_2	9.1481	0 (A)
	JLin_7_U133_plus_2	10.4486	0 (A)

Table II: COMP

APPENDIX B


keloid :: TGF
pathway :: TGF−beta
signaling pathwa
−2
0
2
normal
skin fibroblast
keloid
1 thrombospondin 1 /FL=gb:NM_003246.1
2 thrombospondin 1 /FL=gb:NM_003246.1
3 Homo sapiens E3 ubiquitin ligase Smurf2 mRNA, complete cds. /PROD=E3 ubiquitin l
4 Homo sapiens cDNA FLJ11157 fis, clone PLACE1006951
5 thrombospondin 1 /FL=gb:NM_003246.1
6 Homo sapiens thrombospondin 1 (THBS1), mRNA. /PROD=thrombospondin 1 /FL=gb:NM_00
7 Homo sapiens transforming growth factor, beta receptor II (70−80kD) (TGFBR2), mR
8 E3 ubiquitin ligase SMURF2
9 Homo sapiens mitogen−activated protein kinase 1 (MAPK1), mRNA. /PROD=mitogen−act
10 Homo sapiens mitogen−activated protein kinase 1 (MAPK1), transcript variant 2, m
11 mitogen−activated protein kinase 1
12 Homo sapiens follistatin (FST), transcript variant FST344, mRNA. /PROD=follistat
13 Homo sapiens transcription factor Dp−1 (TFDP1), mRNA. /PROD=transcription factor
14 Homo sapiens PAC clone RP4−808A1 from 7q21.1−q31.1
15 E2F transcription factor 5, p130−binding
16 Human TGF−b superfamily receptor type I mRNA, complete cds. /PROD=TGF−b superfam
17 Homo sapiens small GTP binding protein RhoA (ARHA) mRNA, complete cds. /PROD=sma
18 ESTs
19 Homo sapiens tumor necrosis factor (TNF superfamily, member 2) (TNF), mRNA. /PRO
20 Homo sapiens, Similar to bone morphogenetic protein 7 (osteogenic protein 1), cl
21 ESTs
22 MAD (mothers against decapentaplegic, Drosophila) homolog 5
23 ESTs
24 Homo sapiens, Similar to bone morphogenetic protein 7 (osteogenic protein 1), cl
25 Homo sapiens decorin (DCN), mRNA. /PROD=decorin /FL=gb:NM_001920.1 gb:AF138300.1
26 Rho−associated, coiled−coil containing protein kinase 1
27 ESTs
28 Human transforming growth factor−beta−2 mRNA, complete cds. /PROD=transforming g
29 Homo sapiens cDNA FLJ90021 fis, clone HEMBA1000875.
30 Homo sapiens bone morphogenetic protein 8 (osteogenic protein 2) (BMP8), mRNA. /
31 ESTs
32 MAD (mothers against decapentaplegic, Drosophila) homolog 3 /FL=gb:U58019.1 gb:U
33 Homo sapiens Smad5 mRNA, complete cds. /PROD=Smad5 /FL=gb:AF035528.1 gb:AF043640
34 ESTs
35 Homo sapiens ribosomal protein S6 kinase, 70kD, polypeptide 2 (RPS6KB2), mRNA. /
36 Homo sapiens cDNA: FLJ23077 fis, clone LNG05840
37 Homo sapiens, ras homolog gene family, member A, clone MGC:2330, mRNA, complete
38 ESTs
39 decorin /FL=gb:BC005322.1
40 E3 ubiquitin ligase SMURF1
41 Homo sapiens inhibin, beta A (activin A, activin AB alpha polypeptide) (INHBA),
42 Human ERK1 mRNA for protein serinethreonine kinase. /PROD=protein serinethreonin
43 Homo sapiens mitogen−activated protein kinase 1 (MAPK1), transcript variant 2, m
44 ESTs
45 Human transcription factor E2F−5 mRNA, complete cds. /PROD=E2F−5 /FL=gb:U15642.1
46 ESTs
47 Homo sapiens mRNA for bone morphogenetic protein type IB receptor, complete cds.
48 Homo sapiens activin A receptor, type I (ACVR1), mRNA. /PROD=activin A type I re
49 Homo sapiens bone morphogenetic protein 5 (BMP5), mRNA. /PROD=bone morphogenetic
50 Homo sapiens transforming growth factor, beta receptor I (activin A receptor typ
51 Human placenta mRNA for bone morphogenetic protein 4 (BMP−4), complete cds. /PRO
52 ESTs
53 Homo sapiens decorin variant A mRNA, complete cds. /PROD=decorin variant A /FL=g
54 Human mad homolog JV15−2 mRNA, complete cds. /PROD=JV15−2 /FL=gb:U58019.1 gb:U75
55 Homo sapiens MAD (mothers against decapentaplegic, Drosophila) homolog 2 (MADH2)
56 Human MAD−related protein 2 (MADR2) mRNA, complete cds. /PROD=MAD−related protei
57 hypothetical protein FLJ13576
58 Homo sapiens SMAD5 (Smad5) mRNA, complete cds. /PROD=SMAD5 /FL=gb:U59913.1 gb:U7
59 Homo sapiens cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyse
60 Human type II serinethreonine kinase receptor mRNA, complete cds. /PROD=type II
61 Homo sapiens cDNA FLJ10198 fis, clone HEMBA1004805
62 Homo sapiens E3 ubiquitin ligase SMURF1 mRNA, partial cds. /PROD=E3 ubiquitin li
63 Homo sapiens mitogen−activated protein kinase 5 (MAPK5), mRNA. /PROD=mitogen−act
64 Homo sapiens follistatin (FST), transcript variant FST317, mRNA. /PROD=follistat
65 Rho−associated, coiled−coil containing protein kinase 1 /FL=gb:U43195.1 gb:NM_00
66 ESTs
67 Homo sapiens hypothetical protein BC012008 (LOC144467), mRNA. /FL=gb:NM_138473.1
68 Human transforming growth factor−beta (tgf−beta) mRNA, complete cds. /PROD=trans
69 Homo sapiens mRNA; cDNA DKFZp434M245 (from clone DKFZp434M245).
70 Homo sapiens activin A receptor, type II (ACVR2), mRNA. /PROD=activin A type II
71 ESTs
72 Homo sapiens activin receptor−like kinase 7 (ALK7), mRNA. /PROD=activin receptor
73 Homo sapiens growth differentiation factor 5 (cartilage−derived morphogenetic pr
74 Homo sapiens decorin variant C mRNA, complete cds. /PROD=decorin variant C /FL=g
75 Human thrombospondin−1 gene, partial cds
76 ESTs
77 ESTs
78 Homo sapiens KIAA0305 gene product (KIAA0305), mRNA. /PROD=KIAA0305 gene product
79 Homo sapiens bone morphogenetic protein receptor, type IA (BMPR1A), mRNA. /PROD=
80 retinoblastoma−like 2 (p130) /FL=gb:NM_005611.1
81 ESTs
82 Homo sapiens CREB binding protein (Rubinstein−Taybi syndrome) (CREBBP), mRNA. /P
83 Homo sapiens mRNA; cDNA DKFZp547C244 (from clone DKFZp547C244)
84 Homo sapiens decorin D mRNA, complete cds, alternatively spliced. /PROD=decorin
85 ESTs
86 Homo sapiens MAD (mothers against decapentaplegic, Drosophila) homolog 7 (MADH7)
87 Homo sapiens v−myc avian myelocytomatosis viral oncogene homolog (MYC), mRNA. /P
88 Homo sapiens mRNA; cDNA DKFZp547C244 (from clone DKFZp547C244)
89 inhibitor of DNA binding 2, dominant negative helix−loop−helix protein
90 Human Id−4H protein mRNA, complete cds. /PROD=Id−4H protein /FL=gb:NM_001546.1 g
91 E1A binding protein p300
92 Homo sapiens DKFZP586M0622 protein (DKFZP586M0622), mRNA. /PROD=DKFZP586M0622 pr
93 Homo sapiens, activin A receptor, type IB, clone MGC:2177, mRNA, complete cds. /
94 Homo sapiens cDNA FLJ11812 fis, clone HEMBA1006364
95 Homo sapiens inhibitor of DNA binding 3, dominant negative helix−loop−helix prot
96 Human mRNA for Id−2H, complete cds. /PROD=Id−2H /FL=gb:NM_002166.1 gb:M97796.1 g
97 ESTs
98 ESTs
99 Human mad−related protein MADR1 mRNA, complete cds. /PROD=mad−related protein MA
100 ESTs
101 inhibitor of DNA binding 4, dominant negative helix−loop−helix protein
102 Homo sapiens inhibitor of DNA binding 2, dominant negative helix−loop−helix prot
103 Human mRNA for Id−1H, complete cds. /PROD=Id−1H /FL=gb:NM_002165.1 gb:BC000613.1
104 inhibitor of DNA binding 4, dominant negative helix−loop−helix protein /FL=gb:NM

Figure 1: TGF-B Gene Expression Profile. Genes number 4 (SMURF2) and 59 (COMP) Displayed Differential Expression. Green designates under-expression and red signifies over-expression.

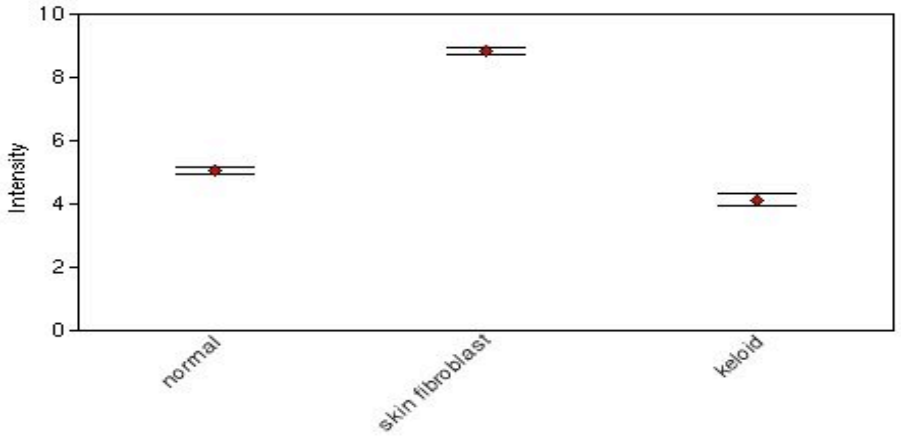

Figure 2: SMURF2 Relative Expression Measured as Intensity.

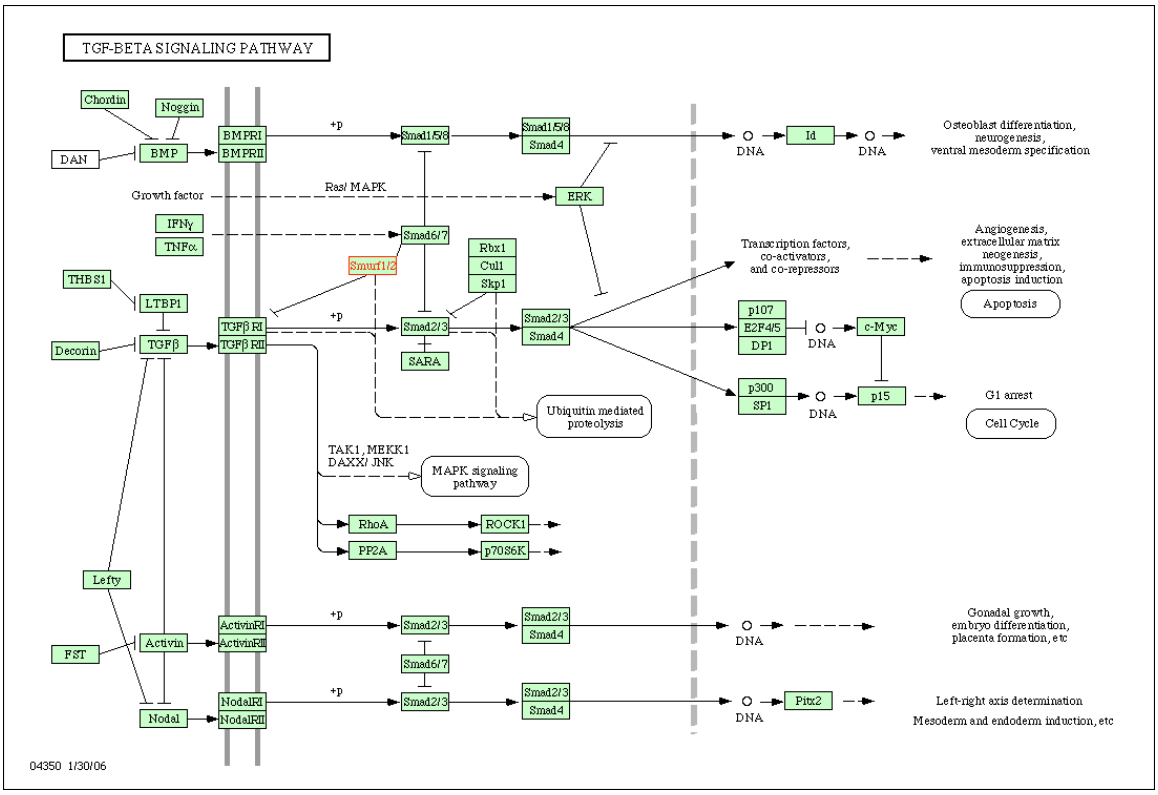

Figure 3: TGF-B Signaling Pathway and SMURF2. Note that SMURF2 is highlighted in red.

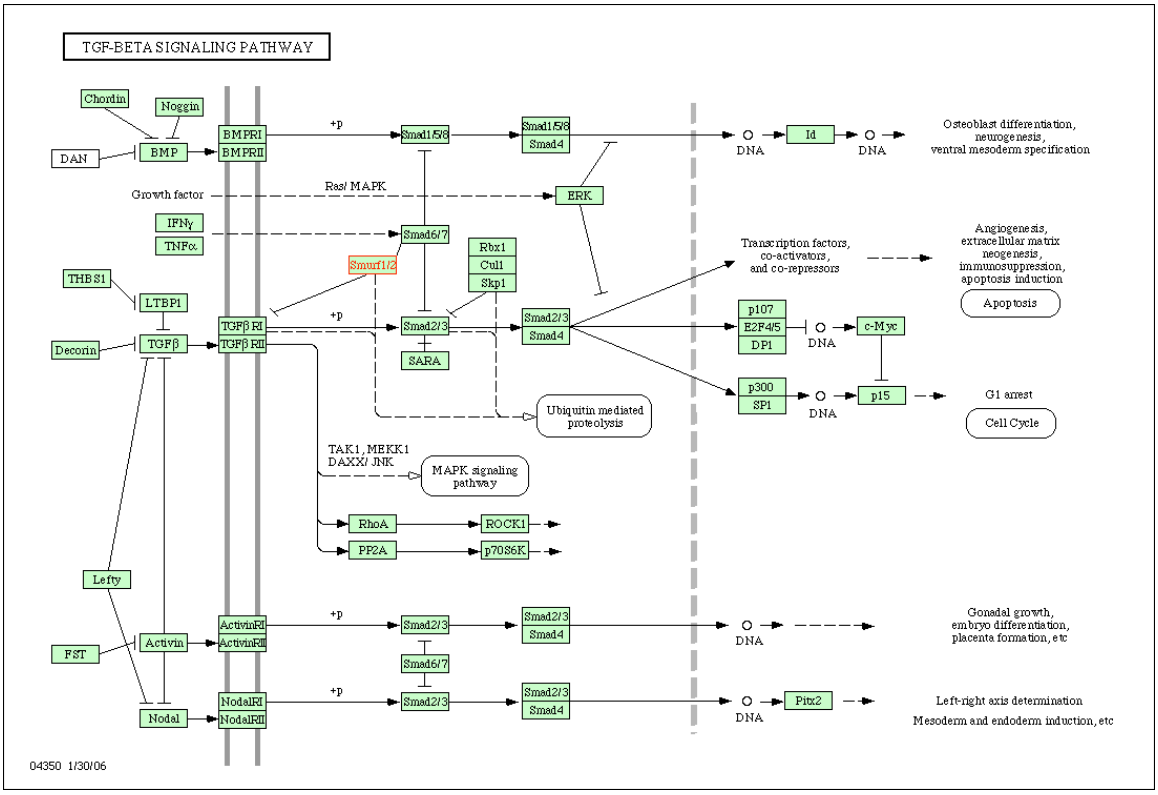

Figure 4: TGF-B Signaling Pathway and COMP. Note that COMP is represented in red and is designated THB (thrombospondin).

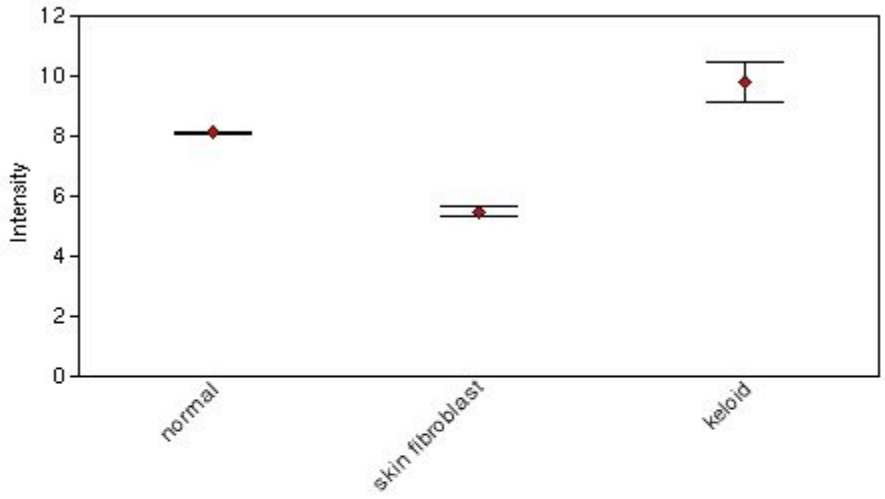

Figure 5: COMP Relative Expression Measured as Intensity.