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A Microarray Analysis of Temporal Gene Expression Profiles in Thermally Injured Human Skin

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Abstract

Partial-thickness burn injuries incite a multitude of responses which eventually culminate in cutaneous wound repair. We hypothesized that these events would evoke extensive alterations in gene expression thereby orchestrating the complexity of spatial and temporal events that characterize “normal” human wound healing. In the present study, gene expressions from partial-thickness areas at defined temporal periods (1-3 days, 4-6 days, and 7-18 days) after injury were compared to normal non-wounded skin. Gene alterations proved extensive (2,286 genes). Statistically significant alterations were noted among increased and decreased genes expressed in the 3 different temporal groupings. Our foundational data (based on samples from 45 individuals) provide a comprehensive molecular gene expression portrait of the cutaneous reparative responses that are initiated during the first 17 days after injury. Our efforts also represent an initial endeavor to move beyond the historically defined “morphological phases” of wound repair toward reporting molecular clues that define the temporal sequence of healing in human subjects. Further analysis of genes that are either modulated or remain non-modulated following injury to normal skin is expected to identify potential targets for therapeutic augmentation or silencing.

INTRODUCTION

Thermal injury to the skin can induce local and systemic perturbations that are costly in terms of human suffering as well as in strains on the health care system. While these unexpected cutaneous injuries are neither as prevalent as chronic wounds nor as well studied, these acute wounds are nevertheless substantial in terms of their numbers. According to the 2005 estimates by the American Burn Association, burn injuries in the United States exceeded 1.25 million. Approximately 600,000 burn patients per annum require emergency treatment while 50,000 victims sustain burn injuries severe enough to warrant admission to specialized burn centers. Deep partial thickness and full thickness skin damage that encompass large body surface areas create significant therapeutic challenges and measurably increase morbidity and mortality [1, 2]. Recently, a multi-centered, NIH funded microarray gene analysis was initiated to address the systemic inflammatory changes that occur after burn injury [3]. This top-down approach was designed to focus on data derived from blood samples and muscle biopsies while utilizing a diverse population to define simultaneous molecular derangements that occur in burn and trauma patients [3]. Molecular events within the cutaneous wound itself were not targeted for microarray

analysis and have remained unexplored, a situation we have sought to remedy in the present study.

Thermal injury to the skin evokes a cascade of events resulting in progressive deepening of the zone of injury during the first 24-48 hours after trauma [4-6]. In the days following injury, extensive gene expression alterations impart a host of derangements that can exert an overwhelmingly negative impact on the reparative capacity of human skin. While the literature is filled with postulated mediators of this progressive inflammation such as neuropeptides [7], pro-inflammatory interleukins-1, 6, & 8 [8-10], arachidonic acid pathway products [11], and tumor necrosis factors [12], no interventional therapies have emerged as standards of care to counteract the inevitable progression in the depth and extent of burn injury [5]. Deeper injuries usually require surgical excision with skin replacement through autografts, allografts, temporary dressings or permanent skin substitutes. Regardless of the treatment plan, most healing scenarios give way to extensive hypertrophic scarring and contracture, an undesirable scenario that develops in 30 to 60% of burn wounds [13, 14].

The initial genomic approach aimed to describe the local events within a burn model appeared in 2003 [15]. This early microarray study defined 35 over-expressed or under-expressed genes in hypertrophic scars and served as a valuable outcome study but was not designed to uncover evidence as to why burn wounds have the propensity to scar and undergo excessive fibrosis [16]. A potentially more gainful means to study hypertrophic scar lies in uncovering events occurring earlier during the acute wound phase that eventually lead to unsightly aesthetic results and functional impairments typical of hypertrophic scars [5].

To date, the more acute responses of human skin to injury and the sequential early events of human wound healing have not yet been examined through a functional genomic approach. The present study is based on our hypothesis that the perturbations within wounded skin during the acute period after injury and later during the subsequent processes of wound repair are best identified using a comprehensive method to analyze diverse patterns of genetic expression. To achieve our primary goal, a microarray experiment was devised to monitor modulation of gene expression within the target organ—injured skin from 45 burn patients as compared to normal skin from 15 healthy patients (Table 1). As a secondary goal, our study was designed to establish a foundational time-course aimed toward elucidating the sequential molecular events during wound healing that define the first seventeen days after injury. Our complex data set offers a screening approach that can contribute statistical precision in defining the temporal sequence of after cutaneous injury in previously normal skin that presumably has the capacity to heal. All parameters for this initial examination were deliberately designed to be broadly inclusive. The goal of our discovery process was to reflect universal molecular alterations (confirmed with statistical confidence) across a diverse population of patients with acute wounds.

MATERIAL AND METHODS

Study Design

A total of 60 patients were recruited for this study according to a protocol approved by the Institutional Review Board at Vanderbilt University. The control group consisted of normal skin specimens from 15 patients undergoing elective cosmetic surgery procedures in which excess skin was removed as a part of the operative plan (e.g. reduction mammoplasty, abdominoplasty, and blepharoplasty). Exclusion criteria included specimens that might have poor skin quality due to extensive stretch, sun exposure, or recurring skin lesions; medical comorbidity; or history of thwarted healing or recurrent skin infections. Exclusions were made based on findings of the investigators after a thorough history and physical exam was taken on each patient. Normal skin specimens in the control group were provided by a

predominance of female patients (13/15 patients) that ranged in age from 26-63 years. For these 15 control patients, the mean age was 39.5 years (median = 43).

The 45 patients who provided specimens for the burn group were identified by daily inspection of the Vanderbilt University Burn Center census. All human subjects in this study were in-patients with cutaneous injuries severe enough to warrant operative excision for deep partial-thickness or full-thickness burn. The demographics of this group were varied in regards to age (16-80 years), total body surface area burned (TBSA), depth of burn, and underlying comorbid conditions. Grossly infected specimens were excluded from this study. The mean age of the burn group was 39.7 years with a median of 37. Table 1 contains further demographic information. Vanderbilt's standards of care for burn victims such as standard wound management with topical antimicrobials and antibiotics and nutritional supplementation were consistently applied to this population that consented to supply injured skin tissues.

Skin specimens from burn patients were arbitrarily placed into three groups based on time from burn insult to operative excision (post burn day = PBD), which loosely corresponds to the phases of wound healing. We have previously published our unsupervised multivariate principal component analysis of protein expression patterns from similar burn wound samples that appropriately clustered dataset into their correct temporal healing periods [17]. This earlier study indicated a unique protein signature within each of 3 time periods after burn injury. Thus patient samples for microarray analysis were clustered similarly into early (PBD 0-3), middle (PBD 4-7), and late (PBD 7-17) time groupings.

Sample Pooling

Since the goal of this study was to derive data that would be broadly representative of the general burn population, the burn patients recruited for this microarray analysis represented a wide demographic spectrum. In order to limit the possibility that the effects of any one patient's individual-specific genetic response to trauma would skew the results, equal quantities of RNA from five patients in each group were combined to create a pooled RNA sample for microarray hybridization [18-20]. The impact of individual genetic alterations that might represent extremes in comparison to the population is blunted when the RNA containing those "outlier" changes is combined with RNA corresponding to more expected (in terms of population-wide) responses to burn injury. Pooling of samples provided a cost-effective means to increase the power of the microarray study while minimizing the financial impact. For example, without pooling, the budget used for this study would have only allowed for the analysis of expression profiles of 12 patients as opposed to the 60 patients that were included in our study design. Pooling strategies have been validated and published previously [21] and approximately 15% of the data sets that are catalogued in the Gene Expression Omnibus Database involve RNA samples that have been pooled before hybridization [22]. For our study we used the sub-pooling approach recommended by Peng et al where subsets of samples were randomly selected and pooled onto one microarray but there were still multiple microarrays within each temporal grouping [23].

Specimen Collection

All specimens were obtained in the operating room within minutes of being removed from the patient. Specimens were placed in aluminum foil and immediately snap-frozen in liquid nitrogen to preserve molecular content. Specimens were taken from mid to deep partial-thickness areas since there was little point in trying to detect gene expression profiles in the areas of full-thickness injury where the skin was undergoing necrosis. The harvesting of partial-thickness tissue also allowed us to capture of viable cells from the multiple lineages important to the healing process and minimized the inclusion of non-viable cells destroyed

by full-thickness injury. The samples were stored at -80°C until RNA isolation. Adjacent samples were fixed and embedded in paraffin and stained with hematoxylin and eosin to confirm that the sample contained mid-to-deep partial thickness burn injury.

RNA Isolation

At the time of isolation, tissues were chopped into small pieces at -20°C . Samples were weighed and 1ml Trizol (Invitrogen, Carlsbad, CA) was added per 100mg of sample. RNA was extracted using Qiagen's Rnase-free DNase Set (Valencia, CA) supplied protocol with the following modification. RNA was precipitated with 0.25 volumes isopropanol (Fisher Scientific, Fairlawn, NJ) and 0.25 volumes of RNA precipitation solution (7% NaCl / 21% disodium citrate). DNase treatment was performed following the first Trizol isolation using Qiagen's Rnase-free DNase Set. Manufacturer's protocol was modified and 10 μl DNase in 70 μl Buffer RDD was added to every 100 μl of sample. Following DNase treatment the Trizol isolation was repeated once or occasionally twice to remove the DNase as well as any remaining fats or salts. RNA was re-suspended in RNase/DNase free H_2O . A small aliquot of each sample was sent to the Microarray Core for quantification and bioanalysis. If the sample met the Microarray Core's standards, samples were pooled equally by mass as to contain RNA from 5 tissue specimens for each array replicate. Pooled RNA samples were then resubmitted for bioanalysis quality assurance followed by microarray labeling and replicate hybridization.

RNA Quality Control

The Vanderbilt Microarray Shared Resource utilizes the RNA Integrity Number (RIN) as the primary determinant of the suitability of RNA samples for hybridization. The RIN is considered by some as superior to other single measures of quality (such as the 28s:18s ratio) because it considers the entire electropherogram, rather than just one or two points. To generate a RIN, each sample was quantified in 10mM Tris on a Nanodrop ND-1000 Spectrophotometer. In addition to concentration, this instrument reports the 260/280 absorption ratio, which can be an important indicator of hidden contaminants. The sample was then analyzed on an Agilent 2100 Bioanalyzer, which produced an electropherogram for each sample depicting the presence of certain contaminants or degradation products. The Agilent 2100 Bioanalyzer also calculated the 28s:18s ratio for each sample. The RINs generated from this process are expressed as a number ranging from 1—degraded to 10—intact. The RINs of the pooled RNA samples included in this experiment exceeded the recommended RIN of 7, with most samples in the 7.5 to 9 range.

Hybridization

In the normal skin group, quality confirmed RNA isolated from 5 patients was pooled together into 1 sample. The pooled RNA was rechecked for RNA integrity. The pool strategy was performed in a chronological (randomized) fashion such that the first 5 control specimens collected in the operating room were pooled, then the next 5, and so on. In a similar manner, 3 pooled, quality RNA samples of control “normal” skin were created.

Pooled samples, isolated from the thermally induced wounds of 45 burn patients were fashioned following an equivalent method. As collection proceeded, the first 5 acquired patient specimens in the same time group (early, middle, or late) were combined, confirmed for RNA integrity, and submitted for hybridization creating 9 pooled, quality RNA samples of burned “experimental” skin.

Following collection, pooling and quality assurance as outlined above, the RNA samples were prepared for microarray analysis using the standard Affymetrix protocol (Affymetrix Inc, Santa Clara, CA) and in accordance with standard techniques that have been previously

published [24, 25]. Briefly, a total of 5 μ g of total RNA was reverse transcribed to double-stranded (ds) cDNA using an oligo-dT primer coupled to a T7 promoter. *In vitro* transcription from the ds cDNA was then carried out using T7 polymerase and incorporating biotin-modified CTP and UTP ribonucleotides. The biotinylated cRNA (15 μ g) was fragmented and hybridized to an Affymetrix GeneChip Human Genome U133 Plus 2.0 Array containing 54,675 sets of 11 to 25-mer oligomers, representing 47,000 human transcripts including 38,000 well-characterized human genes. Hybridized cRNA was detected using streptavidin coupled to phycoerythrin and visualized using GeneChip Scanner 3000 7G. GeneChips were scanned using GeneChip Scanner 3000 7G and GeneChip Operating System (GCOS, Affymetrix, Santa Clara, CA). Default values were used to grid images (.DAT) and generate .CEL and .CHP files and to generate gene expression values and ratios of gene expression between the hybridized samples.

Validation by qRT-PCR

Aliquots of each pooled RNA sample from the microarray experimentation were also used to produce cDNA for validation by qPCR [26, 27]. Briefly, a total of 3 μ g of total RNA was reverse transcribed to double-stranded (ds) cDNA using random primers, SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and the supplied protocol. Following reverse transcriptase, each 20 μ l reaction was diluted with 25 μ l of DNase/Rnase-free D.H₂O. Quantitative PCR was then performed using 1 μ l of cDNA per reaction well. Seven experimental genes and one control gene were assayed by qPCR. All reaction were carried out at the following thermocycling conditions: Step 1: 10mins at 95°C, Step 2: 40 cycles of 15secs at 95°C and 1min at 60°C. The following five gene specific assays were purchased from Applied Biosystems (Foster City, CA) as 20X reaction mixtures containing primers and TaqMan probes: TNFRSF10B (Assay ID: Hs00366272_m1), THBS1 (Assay ID: Hs00170236_m1), IL8 (Assay ID: Hs00174103_m1), SPP1 (Assay ID: Hs00959010_m1), and IL6 (Assay ID: Hs00174131_m1). The following primers and probe sequences and concentrations were used for human Beta-actin control detection: 5' Primer: GCCACCCCACTTCTCTCTAAGG at 50nM, 3' Primer: GGGCACGAAGGCTCATCATTC at 300nM, and TaqMan Probe: VIC-CCCAGTCCTCTCCCAAGTCCACACAGG-TAMRA at 50nM.

Analysis and Statistical Methods

Relative quantitation and standard deviation calculations were performed by the Comparative C_T method described in Applied Biosystem's "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR". Briefly, a dilution series was assayed for each gene, and the efficiencies of the assays were determined to be approximately equal. The 12 cDNA samples were then assayed in triplicate for each gene of interest and the control. The average C_T and the standard deviation were calculated for each sample for each gene. For each sample and each gene, the beta-actin control average C_T was subtracted from the gene average C_T. The resulting value is the ΔC_T . The standard deviation for the ΔC_T is calculated by taking the square root of the sum of the control and gene standard deviations. For comparison to normal, the average ΔC_T for the normals was subtracted from the average ΔC_T of the early, middle, and late burn samples. This value is known as the $\Delta\Delta C_T$. Fold changes compared to normal are given by applying the expression $2^{-\Delta\Delta C_T}$, and $\Delta\Delta C_T \pm$ standard deviation applied to this expression gives the range.

Statistical Methods

In total, 12 pooled samples were hybridized. All analysis was done using GeneSpring v7.3 software (Agilent Technologies, Foster City, CA). Probe-level analysis was performed using the Robust Multichip Analysis (RMA) method [28]. Signal intensities from each

independent replicate, pooled burn sample from 0-3 days, 4-7 days, or >7 days were compared to the average signal intensity of the 3 pooled, normal skin samples. Filtering was done to isolate those probe sets showing at least two-fold up-regulation (or down-regulation) across 2 of the 3 replicates in each group. This narrowing of focus to only those probe sets with consistent, higher fold changes was done due to the vast number of differentially expressed genes in each time point when compared to normal samples.

Nonparametric analyses (Welch t-Tests) were performed on the data with a Benjamini-Hochberg correction for multi-testing to identify gene sets differentially expressed between normal and burn samples with a false discovery rate (FDR) of less than 0.05. Analysis of variance (ANOVA) methods were used to examine differences between mean expression levels among three groups of three replicates each (also with Benjamini-Hochberg multi-testing correction). The expansive nature of array experiments necessitates the application of the correct level of stringency in analytical testing as legitimate alterations in expression may be excluded solely by the incorporation of an analytical tool that is too strict [29, 30]. For this reason, both t-test and ANOVA were utilized. Our microarray data were posted on GEO repository and made public on 06/03/07 with the accession record #GSE8056.

RESULTS AND DISCUSSION

Over 54,000 probe sets, representing approximately 38,000 known genes in the human genome were analyzed using the Affymetrix GeneChip Human Genome U133 Plus 2.0 (Affymetrix Inc, Santa Clara, CA). Data normalization and various applications of statistical methods, described further in the materials and methods section, yielded evidence of gene up-regulation and down-regulation in the cutaneous burn samples that carried a level of statistical significance with corrected p-values < 0.05.

Thermally Injured Skin in Early, Middle, and Late Time Groups versus Normal Skin

Tissue specimens of partial-thickness injury obtained from the wound margins of 45 burn patients were subdivided into groups according to the elapsed time from thermal injury (Table 1). We have previously reported our rationale and validation for this particular temporal grouping pattern using principal component analysis and 2-D DIGE analysis of protein signature pattern isolated from similar burn margins [17]. This broad, all encompassing analytical strategy yielded a total of 2,139 genes whose expression profiles were significantly changed compared to steady-state conditions existing in the normal skin samples. While the use of a 2-fold criteria may seem arbitrary, we were most interested in genes that were differentially expressed among the 3 differing temporal periods. Previous work analyzing the natural variation in human gene expression found a median variance ratio of 1.5 to 2.5 [17, 31, 32]. To be inclusive, we utilized a ratio of 2.0 in this analysis. Our expansive analysis scheme yielded a probable dataset of 1,136 up-regulated genes and 1,003 down-regulated genes during the 1-17 day period after burn trauma. The extensive number of gene expression changes within each of the time groups (early, middle, and late burn periods), as well as the overlap among these temporal groups are represented in Figure 1. The pie charts in Figure 2A & B demonstrate a breakdown of the number of upregulated and down-regulated genes in each time group and among the overlapping time group subsets.

Statistical evaluation using a t-testing strategy identified changes for individual genes compared to their expression levels in the control replicates. An analysis of variance was also performed to comparatively examine the comparative expression levels of individual genes across all time groups (early, middle, and late) and the control (skin from non-burned patients). This alternative type of analysis was executed in order to identify genes that have similar expression to the control for any given time point, but also to identify genes that exhibit a significant change in expression relative to the other time groups. The ANOVA

confirmed an additional 147 genes that met this condition. We next combined the ANOVA derived list of significantly changed genes with the significantly changed genes identified by t-test analysis. By casting this broad statistical net, we note that 2,286 individual genes comprised the list of noteworthy expression alterations specific to thermally injured skin. By using analytic tools, we are able to illustrate with mathematical strength that several thousand gene expression changes are occurring in cells in partial-thickness healing skin wounds when compared to normal human skin.

Deciphering the Significance of Alterations in Global Gene Expression

Fundamental aims of this study were to a) demonstrate the extent of gene expression changes that occur in human skin in response to acute injury and b) define the comprehensive set of gene expression changes that occur across a broad population of patients with a capacity to heal (as opposed to patients with an impaired ability to heal and who develop chronic wounds). Our findings derived from 45 burned tissue specimens from a highly diverse population of burn patients validate this claim. Although our hypothesis seemed intuitive, this circumstance has not been demonstrated in the literature in microarray studies nor by small scale RNA approaches. Moreover, a comparison of the amount and intricacy of expression changes observed in the acute burn wound with published findings for the older, more maturing burn wound model displays a true polarization when grouped by temporal sequence. At a much later time-point after burn injury, microarray profiles from hypertrophic scars detected only 31 upregulated genes and 4 down-regulated genes [15]. Our data, encompassing the 1-17 day period following thermal injury, identified 50-60 fold set of gene alterations based on more stringent analytical methods. These data collected from a broad randomly injured segment of the population (burn victims) show that an exceptionally large number of complex disturbances are set in motion that are actually more comprehensive than the disturbances reported for diseases such as cancer [33, 34]. Our data-set included more than adequate numbers of tissue specimens and demographic variations such that reliable patterns in the levels of gene expression along various cellular processes and pathways became evident (Table 1). Inspection of the up-regulated data generated by analysis of injured skin compared to normal skin yielded gene pathways that represented the inflammatory/immune response, cell cycling, regulation of apoptosis, cell adhesion, and collagen metabolism—well known functions within the wound healing milieu (Table 2). As an example, genes encoding for pro-inflammatory cytokines IL-6, IL-1, and IL-8 were continually and significantly up-regulated throughout all time periods in the present study. The present results substantiate the notion of ongoing cellular turmoil and a complexity of reparative processes in the partial-thickness skin wounds.

Concordance and Confirmation using qRT-PCR

Given the magnitude of statistically significant alterations in gene expression after injury to the skin that precluded an immediate confirmation by qRT-PCR of all 2,286 genes that showed evidence of regulation in response to cutaneous injury and early wound repair, we used a spot checking strategy to confirm the validity of the temporal data discovered during the microarray analysis. Gene selection for subsequent verification by qRT-PCR was admittedly restricted and is an obvious limitation of this and any microarray analysis. Our selection of genes was based on several factors. We elected to examine sample genes based on a spectrum of expression levels; i.e., highly upregulated genes, modestly upregulated genes and several genes that remained at a steady state or showed a lack of modulation in healing skin (Table 3). In addition, all the genes we selected for confirmatory qRT-PCR have previously been implicated in the literature as playing roles in burn injury and/or during wound repair.

Selection of the SPP1 gene (also known as osteopontin) was based on its high magnitude of change in this microarray work and our subsequent analysis through Ingenuity software (data not shown) that also indicated that it was a highly upregulated gene in the first 17 days after injury. The temporal window for increased mRNA expression levels in the present study mirrored the appearance of osteopontin in the red Duroc pig that is noted for its remarkable scarring that resembles human hypertrophic scarring [35]. Other authors have used a knockdown scheme and have shown that osteopontin hinders the rate of wound repair and that osteopontin secreting fibroblasts in the granulation bed contribute to wound fibrosis [36]. Moreover, they suggested that this extracellular matrix molecule may be a logical target for therapeutic modulation. Our human data sets allow us to confirm for the first time using both microarray and qRT-PCR data that this molecule is greatly upregulated in wound repair. Osteopontin is also known as secreted integrin-binding protein. Identification of osteopontin upregulation with more advanced stages of wound repair (Table 3) is a curious discovery that mirrors an earlier theme that we have noted previously while performing proteomic profiling of acute burn wounds; markers previously considered as tumor markers are also prevalent in the acute wound healing circumstance [17]. A previous expression profiling study identified osteopontin as a lead marker in colon cancer progression [21]. Our finding that osteopontin is expressed in major levels in healing wounds suggests that it has broader functions as possibly a non-specific marker of highly proliferative cells and/or extracellular environments that are undergoing rapid remodeling. Our data are supportive of the value of future studies aimed at using osteopontin as a therapeutic modality.

When the microarray data in the present study showed an elevation in IL-8, we selected this molecule as another example of a factor that was highly expressed in the two earliest time groupings. This molecule was also included in our confirmatory panel of markers since we have previously reported the modulation of the IL-8 receptor in the epithelium during the inflammatory and proliferative stages of human burn wound healing [37]. Others have shown concordant IL-8 data at 1-3 days and 4-6 days after injury [38]. Rennekampff et al, 2000 also suggested that IL-8 enhanced re-epithelialization *in vivo* and reduced wound contraction while serving as an early attractant for neutrophils [39]. Our qRT-PCR data subsequently confirmed that this molecule peaked in the middle time grouping (Table 3). Based on *in vitro* responses of keratinocytes and fibroblasts, other authors have suggested that IL-8 may be the contributing factor that retards the healing of burn injuries [40].

Inspection of the later burn replicates in our microarray data detected statistically significant up-regulation of several interleukins (IL) 1, 6, & 8 as well as genes encoding for their receptors and chemokine ligands (CXCL) 1, 3, & 5. We decided to focus some attention on the chemokine IL-6 which has been implicated as a serum marker of inflammation in burn victims. IL-6 was thus used as a confirmatory molecule since it showed a modest upregulation in the early two burn periods on the microarray. Our qRT-PCR data confirmed a similar pattern in the same RNA (Table 3). The chemokine may have specific significance since presence in burn victims correlates with the development of the Systemic Inflammatory Response Syndrome (SIRS) and Multi-Organ Dysfunction Syndrome (MODS) [41]. In the present study, the differential expression of IL-6 in the skin suggests that an ongoing inflammatory response at the wound bed might also contribute to systemic effects in addition to its role in local wound response.

Thrombospondin-1 has been implicated in wound repair in many different settings for the past decade [42-44]. It has been proposed as a matricellular molecule that serves to modulate cell functions and cell-matrix interactions. Thus this was another molecule that was selected for a further confirmatory assay by qRT-PCR. The congruency of the data between our microarray and the qRT-PCR data was not a perfect match for a thrombospondin-1 gene expression. The microarray data indicated peak upregulation in the middle time grouping at

4-6 days while the qRT-PCR data indicated the peak levels at the larger healing period of 7-17 days (Table 3). This was not surprising since the microarray data are calculated and normalized based on numerous sequences on the microchip while qRT-PCR in our study are based on a single sequence normalized against β -actin. Nevertheless, our data unequivocally indicate that thrombospondin-1 is a gene that is regulated in response to human injury and during the early phases of cutaneous repair.

Finally, a molecule from the category of little or no significant modulation in gene expression was also selected for the additional confirmatory work by qRT-PCR. Tumor necrosis factor receptor 10B generally showed marginal shifts during the three different burn periods in the microarray but did show some selective modulation in the later wound healing grouping (Table 3). Again, we believe that this is due to the normalization based on many sequences for this molecule in the microarray while the qRT-PCR readout is based on a sole oligonucleotide sequence.

Our study has acknowledged limitations that are inherent in a human tissue collection study. In our burn center, the majority of burn victims are male. In our vicinity, the elective surgical population with excess normal skin is nearly exclusively female. In the final analysis, we note a mismatch in tissues collected from males and females. We are unsure how to assess the impact of this mismatch in gender composition. The current literature reveals that sexually dimorphic gene expression in somatic tissues (such as the skin) is poorly understood. A few studies do indicate that differences are tissue specific with liver showing large numbers of sex induced gene based differences while other organs such as brain show little difference [45]. The gender affected gene expression changes have largely been noted in the average range of a 1.2 fold difference, a fold change that was below the 2.0 threshold level that was used as a selection criterion in the present study. Thus it is likely that few of the differentially regulated genes that we report in our study can be attributed to sexually dimorphic gene expression.

An acknowledged limitation in our study was the need to balance the fiscal constraints of expensive microarray analysis with the desire to obtain a dataset that was broad and represented the general burn population. Since the literature suggests that a pooling strategy using sub-pools of independent samples within each temporal grouping is a statistically valid approach, we took this course of analysis [22, 23, 31]. Individualized microarrays are the ideal but we recognized that it would take hundreds of these to get age matched, gender matched, race matched, TBSA matched and comorbidity matched samples for detailed statistical analysis. Instead, we focused on initiating a class comparison (normal and 3 temporal points), class discovery of the transcriptome that provides investigators with a novel foundational portrait of differential gene expression following burn injury [46]. We believe that our findings will be broadly applicable to the burn victim population and provide a starting basis to begin the process of defining the temporal sequencing of molecular events during wound repair.

The qRT-PCR data provide additional confidence to our evidence that the observed gene expression changes in the microarray setting are reproducible in the setting of cutaneous injury and wound repair. Admittedly, the confirmations were only performed on a limited subset of genes. We will leave the more comprehensive analysis to a host of future burn investigators.

CONCLUSIONS

Through global gene microarray analysis of cutaneous burn wounds, we have demonstrated considerable alterations in gene expression following injury and during the subsequent

events of acute wound repair. In the field of cancer biology, microarray applications have been geared toward translating microarray data into clinical relevance by using expression signatures as diagnostic and prognostic tools [47, 48]. We believe the data within this report may begin to establish a similar foundation. The wound signature derived from the “non-impaired” wound healing circumstance found in accidental burns generated a temporal expression pattern for genes that are modulated during the response to injury and the early sequelae of cutaneous repair.

Our current global examination suggests that the transcriptional activities driving the acute wounding response are diverse as well as extensive. We assert that a better understanding of wound pathophysiology can be accomplished through further mining of the data with the goals of clustering significantly altered genes within like cellular pathways and identifying novel candidate genes involved in the eventual healing and or scarring/contracture outcomes in burn wounds. We previously conducted a limited proteomic profiling with similar acute wound material using 2D-difference gel electrophoresis (2D-DIGE) [17]. We did not find a one-to-one correlation between the genes expressed in the present study and the proteins translated in the 2D-DIGE study. This was not unexpected. Gene expression can be modified by microRNAs that prevent translation into proteins. Also, the 2D-DIGE technique we used was limited to detection of larger proteins in the 20-200 kDa range. Many of the overexpressed genes in this study were smaller molecules such as Il-6 or Il-8 below the detection range of the 2D-DIGE. The 2D-DIGE study was designed to detect temporal phases of repair on few samples. The present microarray study was more expansive, and the data provide the first bench-side transcriptional evidence from acute human burn wounds. These data in concert with previous reports derived from animal studies and the hypertrophic scarring phases of healing human wounds may assist in moving the wound healing field forward through identification of potential therapeutic targets that may allow for better management of the events that define acute wound healing.

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FIGURE 1.

Number of significantly altered genes broken down by time group determined by Welch's t-test with a Benjamini-Hochberg multiple corrections testing yielding p-values < 0.05 as significant. The large circle top-left represents the early time group (0-3 days), top-right represents the middle time group (4-7 days), and bottom-center represents the late time group (>7 days). Overlapping sections of the Venn diagram demonstrate individual genes whose expression levels are significantly altered in more than one time group.

FIGURE 2.

A: Schematic representing the numerical breakdown of upregulated genes across all three time groups. Percentage of total number of upregulated genes (N=1136) also shown.

B: Schematic representing the numerical breakdown of down-regulated genes across all three time groups. Percentage of total number of down-regulated genes (N=1003) also shown

TABLE 1

Demographics for patients who provided skin specimens included in this work. TBSA = Total Body Surface Area (burned); PBD = Post Burn Day; NP = Normal Pool; EP = Early Pool; MP = Middle Pool; LP = Late Pool. TBSA and PBD do not apply to patients included in the normal pools (NP1, NP2, and NP3).

SAMPLE	AGE	SEX	RACE	PROCEDURE	TBSA %	TBSA % MEAN (SD)	PBD	MEAN AGE	MEDIA N AGE	MALE to FEMALE RATIO
NP1.1	55	F	C	Blepharoplasty		N/A		41	43	0:5
NP1.2	43	F	C	Reduction Mammoplasty						
NP1.3	22	F	C	Reduction Mammoplasty						
NP1.4	22	F	C	Reduction Mammoplasty						
NP1.5	63	F	C	Blepharoplasty						
NP2.1	35	F	C	Reduction Mammoplasty		N/A		28.8	26	0:5
NP2.2	26	F	C	Reduction Mammoplasty						
NP2.3	43	F	C	Blepharoplasty						
NP2.4	20	F	C	Reduction Mammoplasty						
NP2.5	20	F	C	Excision, back						
NP3.1	32	F	AA	Reduction Mammoplasty		N/A		45.4	44	0:5
NP3.2	43	F	AA	Reduction Mammoplasty						
NP3.3	52	F	C	Reduction Mammoplasty						
NP3.4	56	F	C	Reduction Mammoplasty						
NP3.5	44	F	C	Thigh Lift						
EP1.1	17	F	C	Excision, abdomen	30	23 (± 14)	3	42.4	39	3:2
EP1.2	52	F	C	Excision, upper extremity	40		3			
EP1.3	80	M	AA	Excision, lower extremities	23		3			
EP1.4	39	M	C	Excision, back	20		3			
EP1.5	24	M	C	Excision, hand	2		3			
EP2.1	51	F	C	Excision, abdomen	80	24 (± 32)	1	48	51	3:2
EP2.2	36	M	C	Excision, lower extremity	5		2			
EP2.3	29	M	C	Excision, abdomen	15		3			
EP2.4	68	F	C	Excision, foot	3		3			

SAMPLE	AGE	SEX	RACE	PROCEDURE	TBSA %	TBSA % MEAN (SD)	PB D	MEAN AGE	MEDIA N AGE	MALE to FEMALE RATIO
EP2.5	56	M	AA	Excision, upper extremity	15		3			
EP3.1	42	F	C	Excision, hand	2	14 (± 20)	1	44.2	42	2:3
EP3.2	39	M	C	Excision, back	10		2			
EP3.3	28	F	AA	Excision, upper extremity	3		3			
EP3.4	50	M	C	Excision, back	50		3			
EP3.5	62	F	AA	Excision, thigh	4		3			
MP1.1	8	M	C	Excision, upper extremity	6	9 (± 4)	5	19.8	19	3:2
MP1.2	19	M	C	Excision, upper extremity	8		4			
MP1.3	30	M	C	Excision, flank	10		4			
MP1.4	11	F	AA	Excision, chest	5		5			
MP1.5	31	F	C	Excision, chest	15		5			
MP2.1	26	M	AA	Excision, trunk	30	16 (± 11)	4	30.4	33	3:2
MP2.2	33	M	C	Excision, upper extremity	5		5			
MP2.3	37	F	C	Excision, lower extremity	4		6			
MP2.4	38	F	C	Excision, trunk	20		7			
MP2.5	18	M	C	Excision, back	20		7			
MP3.1	51	M	C	Excision, abdomen	41	19 (± 15)	4	42.2	42	5:0
MP3.2	25	M	C	Excision, upper extremity	10		5			
MP3.3	42	M	C	Excision, abdomen	27		5			
MP3.4	28	M	C	Excision, back	5		7			
MP3.5	65	M	C	Excision, upper extremity	10		6			
LP1.1	54	M	C	Excision, abdomen	20	20 (± 8)	10	29	30	5:0
LP1.2	30	M	C	Excision, back	15		11			
LP1.3	5	M	AA	Excision, chest	20		13			
LP1.4	55	M	C	Excision, lower extremity	12		14			
LP1.5	1	M	C	Excision, upper extremity	33		17			
LP2.1	33	M	AA	Excision, upper extremity	20	17 (± 17)	8	35.4	33	4:1
LP2.2	30	M	H	Excision, chest	10		11			

SAMPLE	AGE	SEX	RACE	PROCEDURE	TBSA %	TBSA % MEAN (SD)	PB D	MEAN AGE	MEDIA N AGE	MALE to FEMALE RATIO
LP2.3	55	F	AA	Excision, lower extremity	5		14			
LP2.4	11	M	AA	Excision, foot	3		15			
LP2.5	48	M	C	Excision, hand	45		16			
LP3.1	40	M	C	Excision, lower extremity	60	34 (± 25)	8	46	40	5:0
LP3.2	77	M	C	Excision, foot	10		9			
LP3.3	50	M	C	Excision, abdomen	50		9			
LP3.4	40	M	C	Excision, foot	5		9			
LP3.5	23	M	C	Excision, upper extremity	45		11			

TABLE 2

Selected genes whose expression is significantly up-regulated in burned tissue specimens compared to control.

PROBE SET	p VALUE	ABBREVIATION	GENE NAME	BIOLOGICAL PROCESS
1552553_a_at	0.0144	CARD12	caspase recruitment domain family, member 12	Apoptosis
1552701_a_at	0.0144	COP	CARD only protein	Apoptosis
1555758_a_at	0.0144	CDKN3	cyclin-dependent kinase inhibitor 3	Apoptosis
227345_at	0.0144	TNFRSF10D	tumor necrosis factor receptor superfamily 10d	Apoptosis
227458_at	0.0144	PDCD1LG1	programmed cell death 1 ligand 1	Apoptosis
202695_s_at	0.0218	STK17A	serine/threonine kinase 17a (Apoptosis-inducing)	Apoptosis
201505_at	0.0341	LAMB1	laminin, beta 1	cell adhesion
201645_at	0.0144	TNC	tenascin C (hexabrachion)	cell adhesion
203477_at	0.0144	COL15A1	collagen, type XV, alpha 1	cell adhesion
226609_at	0.0144	DCBLD1	discoidin, CUB and LCCL domain containing 1	cell adhesion
216005_at	0.0144	TNC	tenascin C (hexabrachion)	cell adhesion
235075_at	0.0144	DSG3	desmoglein 3 (pemphigus vulgaris antigen)	cell adhesion
204750_s_at	0.0144	DSC2	desmocollin 2	cell adhesion
227048_at	0.0144	LAMA1	laminin, alpha 1	cell adhesion
204751_x_at	0.0218	DSC2	desmocollin 2	cell adhesion
205534_at	0.0144	PCDH7	BH-protocadherin (brain-heart)	cell adhesion
205595_at	0.0144	DSG3	desmoglein 3 (pemphigus vulgaris antigen)	cell adhesion
205656_at	0.0144	PCDH17	protocadherin 17	cell adhesion
203256_at	0.0144	CDH3	cadherin 3, type 1, P-cadherin (placental)	cell adhesion
202803_s_at	0.0144	ITGB2	integrin, beta 2 (antigen CD18 (p95))	cell adhesion
210184_at	0.0144	ITGAX	integrin, alpha X (antigen CD11C (p150), alpha polypeptide)	cell adhesion
204105_s_at	0.0144	NRCAM	neuronal cell adhesion molecule	cell adhesion
205828_at	0.0144	MMP3	matrix metalloproteinase 3	collagen catabolism
203936_s_at	0.0144	MMP9	matrix metalloproteinase 9	collagen metabolism
204475_at	0.0144	MMP1	matrix metalloproteinase 1	collagen metabolism
211966_at	0.0144	COL4A2	collagen, type IV, alpha 2	collagen metabolism
204575_s_at	0.0144	MMP19	matrix metalloproteinase 19	collagen metabolism
211980_at	0.0144	COL4A1	collagen, type IV, alpha 1	collagen metabolism
209125_at	0.0144	KRT6A	keratin 6A	epidermal differentiation
209126_x_at	0.0144	KRT6B	keratin 6B	epidermal differentiation
205157_s_at	0.0144	KRT17	keratin 17	epidermal differentiation
205916_at	0.0341	S100A7	S100 calcium binding protein A7 (psoriasin 1)	epidermal differentiation
212236_x_at	0.0144	KRT17	cytokeratin 17	epidermal differentiation
209800_at	0.0144	KRT16	keratin 16	epidermal differentiation
206300_s_at	0.0144	PTH1H	parathyroid hormone-like hormone	epidermal differentiation
1552487_a_at	0.0144	BNC	basonuclin	epidermal differentiation
213680_at	0.0144	KRT6B	keratin 6B	epidermal differentiation
214580_x_at	0.0144	KRT6A	keratin 6A	epidermal differentiation
206569_at	0.0144	IL24	interleukin 24	Inflammatory/Immune Response

PROBE SET	p VALUE	ABBREVIATION	GENE NAME	BIOLOGICAL PROCESS
211163_s_at	0.0144	TNFRSF10C	tumor necrosis factor receptor 10c	Inflammatory/Immune Response
210405_x_at	0.0144	TNFRSF10B	tumor necrosis factor receptor 10b	Inflammatory/Immune Response
220088_at	0.0144	C5R1	complement component 5 receptor 1 (C5a ligand)	Inflammatory/Immune Response
205119_s_at	0.0144	FPR1	formyl peptide receptor 1	Inflammatory/Immune Response
208130_s_at	0.0144	TBXAS1	thromboxane A synthase 1	Inflammatory/Immune Response
201110_s_at	0.0144	THBS1	thrombospondin 1	Inflammatory/Immune Response
206026_s_at	0.0144	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	Inflammatory/Immune Response
202859_x_at	0.0144	IL8	interleukin 8	Inflammatory/Immune Response
201888_s_at	0.0144	IL13RA1	interleukin 13 receptor, alpha 1	Inflammatory/Immune Response
204232_at	0.0144	FCER1G	Fc fragment of IgE	Inflammatory/Immune Response
206420_at	0.0144	IGSF6	immunoglobulin superfamily, member 6	Inflammatory/Immune Response
207072_at	0.0144	IL18RAP	interleukin 18 receptor accessory protein	Inflammatory/Immune Response
204268_at	0.0144	S100A2	S100 calcium binding protein A2	Inflammatory/Immune Response
203535_at	0.0144	S100A9	S100 calcium binding protein A9 (calgranulin B)	Inflammatory/Immune Response
208075_s_at	0.0144	CCL7	chemokine (C-C motif) ligand 7	Inflammatory/Immune Response
205476_at	0.0144	CCL20	chemokine (C-C motif) ligand 20	Inflammatory/Immune Response
206336_at	0.0144	CXCL6	chemokine (C-X-C motif) ligand 6	Inflammatory/Immune Response
209906_at	0.0341	C3AR1	complement component 3a receptor 1	Inflammatory/Immune Response
205099_s_at	0.0144	CCR1	chemokine (C-C motif) receptor 1	Inflammatory/Immune Response
204563_at	0.0144	SELL	selectin L (lymphocyte adhesion molecule 1)	Inflammatory/Immune Response
206211_at	0.0144	SELE	selectin E (endothelial adhesion molecule 1)	Inflammatory/Immune Response
1553297_a_at	0.0144	CSF3R	colony stimulating factor 3 receptor (granulocyte)	Inflammatory/Immune Response
218368_s_at	0.0144	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	Inflammatory/Immune Response
226218_at	0.0144	IL7R	interleukin 7 receptor	Inflammatory/Immune Response
238542_at	0.0144	ULBP2	UL 16 binding protein 2	Inflammatory/Immune Response
202877_s_at	0.0144	C1QR1	complement component 1, q subcomponent, receptor 1	Inflammatory/Immune Response
229560_at	0.0218	TLR8	toll-like receptor 8	Inflammatory/Immune Response
203922_s_at	0.0144	CYBB	cytochrome b-245, beta polypeptide	Inflammatory/Immune Response
214511_x_at	0.0144	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	Inflammatory/Immune Response
214974_x_at	0.0144	CXCL5	chemokine (C-X-C motif) ligand 5	Inflammatory/Immune Response
215101_s_at	0.0144	CXCL5	chemokine (C-X-C motif) ligand 5	Inflammatory/Immune Response
227266_s_at	0.0144	FYB	FYN binding protein (FYB-120/130)	Inflammatory/Immune Response
204007_at	0.0144	FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	Inflammatory/Immune Response
215223_s_at	0.0144	SOD2	superoxide dismutase 2, mitochondrial	Inflammatory/Immune Response
221477_s_at	0.0144	SOD2	superoxide dismutase 2, mitochondrial	Inflammatory/Immune Response
211612_s_at	0.0144		IL-13 receptor	Inflammatory/Immune Response
209277_at	0.0144	TFPI2	tissue factor pathway inhibitor 2	Inflammatory/Immune Response
236345_at	0.0144	TBXAS1	thromboxane A synthase 1	Inflammatory/Immune Response
217497_at	0.0144	ECGF1	endothelial cell growth factor 1 (platelet-derived)	Inflammatory/Immune Response

PROBE SET	p VALUE	ABBREVIATION	GENE NAME	BIOLOGICAL PROCESS
214146_s_at	0.0144	PPBP	pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	Inflammatory/Immune Response
211434_s_at	0.0144	CCRL2	chemokine (C-C motif) receptor-like 2	Inflammatory/Immune Response
207850_at	0.0144	CXCL3	chemokine (C-X-C motif) ligand 3	Inflammatory/Immune Response
209774_x_at	0.0144	CXCL2	chemokine (C-X-C motif) ligand 2	Inflammatory/Immune Response
205114_s_at	0.0144	CCL3	chemokine (C-C motif) ligand 3	Inflammatory/Immune Response
207008_at	0.0144	IL8RB	interleukin 8 receptor, beta	Inflammatory/Immune Response
219434_at	0.0144	TREM1	triggering receptor expressed on myeloid cells 1	Inflammatory/Immune Response
201422_at	0.0144	IFI30	interferon, gamma-inducible protein 30	Inflammatory/Immune Response
203561_at	0.0144	FCGR2A	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	Inflammatory/Immune Response
204006_s_at	0.0144	FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	Inflammatory/Immune Response
207674_at	0.0144	FCAR	Fc fragment of IgA, receptor for	Inflammatory/Immune Response
207697_x_at	0.0144	LILRB2	leukocyte immunoglobulin-like receptor, subfamily B member 2	Inflammatory/Immune Response
207857_at	0.0144	LILRB1	leukocyte immunoglobulin-like receptor, subfamily B member 1	Inflammatory/Immune Response
210146_x_at	0.0144	LILRB2	leukocyte immunoglobulin-like receptor, subfamily B member 2	Inflammatory/Immune Response
212013_at	0.0144	D2S448	Melanoma associated gene	Inflammatory/Immune Response
210176_at	0.0218	TLR1	toll-like receptor 1	Inflammatory/Immune Response
205483_s_at	0.0144	GIP2	interferon, alpha-inducible protein (clone IFI-15K)	Inflammatory/Immune Response
204924_at	0.0144	TLR2	toll-like receptor 2	Inflammatory/Immune Response
203233_at	0.0144	IL4R	interleukin 4 receptor	Inflammatory/Immune Response
210644_s_at	0.0144	LAIR1	leukocyte-associated Ig-like receptor 1	Inflammatory/Immune Response
202086_at	0.0218	MX1	myxovirus (influenza virus) resistance 1	Inflammatory/Immune Response
202917_s_at	0.0218	S100A8	S100 calcium binding protein A8 (calgranulin A)	Inflammatory/Immune Response
206157_at	0.0144	PTX3	pentaxin-related gene, rapidly induced by IL-1 beta	Inflammatory/Immune Response
210118_s_at	0.0218	IL1A	interleukin 1, alpha	Inflammatory/Immune Response
205067_at	0.0144	IL1B	interleukin 1, beta	Inflammatory/Immune Response
205207_at	0.0144	IL6	interleukin 6 (interferon, beta 2)	Inflammatory/Immune Response
205237_at	0.0144	FCN1	ficolin (collagen/fibrinogen domain containing) 1	Inflammatory/Immune Response
216950_s_at	0.0144	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor for (CD64)	Inflammatory/Immune Response
201743_at	0.0144	CD14	CD14 antigen	Inflammatory/Immune Response
202878_s_at	0.0144	C1QR1	complement component 1, q subcomponent, receptor 1	Inflammatory/Immune Response
204748_at	0.0144	PTGS2	prostaglandin-endoperoxide synthase 2	Inflammatory/Immune Response
207442_at	0.0144	CSF3	colony stimulating factor 3 (granulocyte)	Inflammatory/Immune Response
210895_s_at	0.0144	CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	Inflammatory/Immune Response
223502_s_at	0.0218	TNFSF13B	tumor necrosis factor (ligand) superfamily, member 13b	Inflammatory/Immune Response
210423_s_at	0.0144	SLC11A1	solute carrier family 11	Inflammatory/Immune Response
208581_x_at	0.0144	MT1X	metallothionein 1X	Inflammatory/Immune Response
202831_at	0.0144	GPX2	glutathione peroxidase 2 (gastrointestinal)	Inflammatory/Immune Response

PROBE SET	p VALUE	ABBREVIATION	GENE NAME	BIOLOGICAL PROCESS
216841_s_at	0.0144	SOD2	superoxide dismutase 2, mitochondrial	Inflammatory/Immune Response
216598_s_at	0.0144	CCL2	chemokine (C-C motif) ligand 2	Inflammatory/Immune Response
207356_at	0.0218	DEFB4	defensin, beta 4	Inflammatory/Immune Response
200989_at	0.0144	HIF1A	hypoxia-inducible factor 1	Inflammatory/Immune Response
208438_s_at	0.0144	FGR	Gardner-Rasheed feline sarcoma viral oncogene homolog	Inflammatory/Immune Response
204103_at	0.0218	CCL4	chemokine (C-C motif) ligand 4	Inflammatory/Immune Response
207526_s_at	0.0144	IL1RL1	interleukin 1 receptor-like 1	Inflammatory/Immune Response
209949_at	0.0144	NCF2	neutrophil cytosolic factor 2	Inflammatory/Immune Response
205863_at	0.0144	S100A12	S100 calcium binding protein A12 (calgranulin C)	Inflammatory/Immune Response
203645_s_at	0.0144	CD163	CD163 antigen	Inflammatory/Immune Response
204351_at	0.0144	S100P	S100 calcium binding protein P	Inflammatory/Immune Response
206172_at	0.0144	IL13RA2	interleukin 13 receptor, alpha 2	Inflammatory/Immune Response
213418_at	0.0144	HSPA6	heat shock 70kDa protein 6 (HSP70B')	Inflammatory/Immune Response
204170_s_at	0.0144	CKS2	CDC28 protein kinase regulatory subunit 2	regulation of transcription
213524_s_at	0.0144	G0S2	putative lymphocyte G0/G1 switch gene	regulation of transcription
205687_at	0.0144	UBPH	similar to ubiquitin binding protein	regulation of transcription
211981_at	0.0144	COL4A1	collagen, type IV, alpha 1	regulation of transcription
218384_at	0.0144	CARHSP1	calcium regulated heat stable protein 1, 24kDa	regulation of transcription
225655_at	0.0144	UHRF1	ubiquitin-like, containing PHD and RING finger domains, 1	regulation of transcription
204959_at	0.0218	MNDA	myeloid cell nuclear differentiation antigen	regulation of transcription
211964_at	0.0144	COL4A2	collagen, type IV, alpha 2	regulation of transcription
203574_at	0.0144	NFIL3	nuclear factor, interleukin 3 regulated	regulation of transcription
202957_at	0.0144	HCLS1	hematopoietic cell-specific Lyn substrate 1	regulation of transcription
202580_x_at	0.0144	FOXM1	forkhead box M1	regulation of transcription
206700_s_at	0.0218	SMCY	Smcy homolog, Y-linked (mouse)	regulation of transcription
202391_at	0.0144	BASP1	brain abundant, membrane attached signal protein 1	regulation of transcription

Table 3

Gene expression by qRT-PCR normalized to Beta-actin

Gene	Microarray			qRT-PCR Normalized to Beta-actin		
	E vs N	M vs N	L vs N	E vs N	M vs N	L vs N
TNFRSF10B	2.27	2.2	1.99	3.65 (2.83-4.72)	5.25 (4.28-6.43)	9.21 (6.99-12.13)
THBS1	4.8	8.9	6.43	15.82 (12.28-20.37)	8.22 (6.44-10.49)	36.06 (27.97-46.48)
IL6	19.39	24.56	12.8	8.93 (6.75-11.81)	15.02 (11.34-19.89)	5.50 (4.22-7.15)
IL8	121.72	146.85	66.31	58.58 (44.65-76.84)	62.78 (47.24-83.42)	33.38 (26.05-42.78)
SPP1	22.06	78.38	72.05	33.77 (23.12-49.33)	106.40 (73.71-153.59)	145.91 (88.89-239.49)

Note: E = Early Burn Period (0-3 days), M = Middle burn period (4-7 days), L = Late Burn Period (8-17 days), N = Normal Unwounded, TNFRSF10B = tumor necrosis factor receptor superfamily 10B, THBS1 = thrombospondin 1, IL6 = interleukin 6, IL8 = interleukin 8, SPP1 = secreted phosphoprotein 1 or osteopontin