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Deregulation of keratinocyte differentiation and activation: a hallmark of venous ulcers

Olivera Stojadinovic [a], Irena Pastar [a], Sasa Vukelic [a], Mỹ G. Mahoney [c], Donna Brennan [c], Agata Krzyzanowska [a], Michael Golinko [b], Harold Brem [b], Marjana Tomic-Canic [a, d] *

[a] Hospital for Special Surgery of the Weill Cornell Medical College, Tissue Repair Lab, New York, NY, USA
[b] Columbia University College of Physicians and Surgeons, Department of Surgery, Wound Healing Program, New York, NY, USA
[c] Department of Dermatology and Cutaneous Biology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA, USA
[d] Department of Dermatology, Weill Cornell Medical College, New York, NY, USA

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Abstract

Epidermal morphology of chronic wounds differs from that of normal epidermis. Biopsies of non-healing edges obtained from patients with venous ulcers show thick and hyperproliferative epidermis with mitosis present in suprabasal layers. This epidermis is also hyperkeratotic and parakeratotic. This suggests incomplete activation and differentiation of keratinocytes. To identify molecular changes that lead to pathogenic alterations in keratinocyte activation and differentiation pathways we isolated mRNA from non-healing edges deriving from venous ulcers patients and determined transcriptional profiles using Affymetrix chips. Obtained transcriptional profiles were compared to those from healthy, unwounded skin. As previously indicated by histology, we found deregulation of differentiation and activation markers. We also found differential regulation of signalling molecules that regulate these two processes. Early differentiation markers, keratins K1/K10 and a subset of small proline-rich proteins, along with the late differentiation marker filaggrin were suppressed, whereas late differentiation markers involucrin, transgultaminase 1 and another subset of small proline-rich proteins were induced in ulcers when compared to healthy skin. Surprisingly, desomosomal and tight junction components were also deregulated. Keratinocyte activation markers keratins K6/K16/K17 were induced. We conclude that keratinocytes at the non-healing edges of venous ulcers do not execute either activation or differentiation pathway, resulting in thick callus-like formation at the edge of a venous ulcers.

Key words: venous ulcers • chronic wounds • keratinocyte differentiation • keratinocyte activation • desmosomes • wound healing • transcriptional profiling • microarrays

Introduction

Chronic wounds, such as venous ulcers, affect hundreds of thousands of patients each year. They represent a serious healthcare problem around theworld. Venous ulcers develop as a consequence of venous valvular incompetence causing venous reflux, obstruction and subsequently venous hypertension. Their chronicity, frequent relapses and associated complications heavily impact patient's quality of life and increase healthcare costs.

Venous ulcers are responsible for more than half of lower extremity ulcerations, with prevalence ranging from 0.06% to 2% [1]. A rough estimate shows that the prevalence of unhealed venous ulcers is about 0.3%, that is about 1 in 350 adults [2], or approximately 400,000–600,000 cases in the United States [3]. Treatment of these ulcers sometimes requires numerous hospitalizations and treatment modalities incorporating topical dressings, antibiotics, debridement, compression therapy and cellular therapy. After wound healing with non-operative methods the incidence rate of recurrent ulcerations after 3 and 5 years was reported to be 37% and 48%, respectively [3].The annual cost of

*Correspondence to: M. TOMIC-CANIC,
Hospital for Special Surgery, Weill Cornell Medical College, Tissue Repair Lab, Tissue Engineering, Regeneration and Repair Program, 535 E 70th Street, New York, NY, 10021.
Tel.: +(212)-774-7160
Fax: +(212)-249-2373
E-mail: tomicm@hss.edu

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the venous disease in the United States has been estimated between $1.9 billion and $2.5 billion [4], and in one retrospective cohort study the average total medical cost per patient was $9685 (median: $3036) [5]. Currently, only human skin equivalent is approved (for safety and efficacy) therapy in venous ulcers. [6]. Lack of understanding the biology at a cellular and molecular level is a barrier to creating further effective treatments.

Healthy epidermis provides a protective barrier that undergoes constant renewal. In this multi-layered, highly cellular tissue cell-to-cell connection occurs *via* desmosomes, adherens junctions, gap and tight junctions (TJs). TJs control paracellular permeability and maintain cell polarity, thus maintaining barrier function [7]. Basal keratinocytes divide and as they start differentiating and leaving the basal compartment they undergo changes in gene expression and commit to terminal differentiation resulting in formation of enucleated corneocytes. Once the barrier is broken, keratinocytes become activated in response to epidermal injury and start to proliferate, migrate and express a specific subset of keratin proteins suprabasally, keratins 6, 16 and 17 [8].

We have shown previously that skin deriving from the non-healing edge of chronic ulcers (*e.g.* venous, pressure and diabetic foot ulcers) exhibits distinct morphology. The epidermis becomes hyperproliferative, hyper- and parakeratotic with the presence of mitoticaly active cells in suprabasal layers [9, 10]. We have also shown that activation of c-myc and nuclearization of β-catenin in the epidermis of patients with chronic wound play a role in inhibition of keratinocyte migration and contribute to impairment of healing in chronic wounds [9].These changes suggest inadequate execution of either of the two major processes important for epidermal maintenance and homeostasis: activation and differentiation.

To test if indeed these two processes are impaired in venous ulcers, we utilized large-scale microarray analyses and biopsies of non-healing edges of three patients suffering from venous ulcers. Microarray technology has brought the ability to simultaneously analyse the expression patterns of tens of thousands of genes and thereby identify groups of differentially regulated genes involved in pathogenesis of many different diseases. It has been used successfully in gene expression analyses of various tumours [11, 12] and wound healing of different tissues [13–18]. To the best of our knowledge a focused, large-scale microarray analysis has not been performed for patients with chronic wounds.

In this study, we compared expression profiles of patients' biopsies from non-healing edges of venous ulcers to profiles obtained from biopsies of healthy skin. Among 1557 genes that are differentially regulated in a statistically significant manner ($P < 0.05$), we particularly focused on groups of genes that characterize regulation of main biological processes in keratinocytes: activation and differentiation. We found keratinocyte activation markers to be induced. Proliferation is a component of keratinocyte activation. Cell cycle related genes, both cell cycle activators and repressors, were differentially regulated suggesting the loss of cell cycle control. Furthermore, we found deregulation of early and late differentiation markers, as well as regulators of keratinocyte differentiation, suggesting improper execution of either the early or late phase of differentiation. Microarray data were evaluated and confirmed using quantitative real-time PCR and immunohistochemistry.

We conclude that keratinocytes at the non-healing edges of venous ulcers are caught in a loop of trying to execute either of two processes, proliferation and differentiation, thus resulting in a thick hyperproliferative, hyper- and parakeratotic epidermis.

Materials and methods

Skin specimens used in study

Institutional review board approval was obtained and skin biopsies deriving from non-healing edges of venous ulcers were collected from normally discarded tissue after surgical debridement procedures on three consented patients with venous reflux ulcers in accordance with the Declaration of Helsinki Principles. Three healthy skin specimens were obtained as discarded tissue from reduction mammoplasty. As previously shown, three biological replicates are sufficient to generate valuable microarray analyses [19]. A portion of biopsies were embedded in tissue-freezing media (Triangle Biomedical Sciences, NC, USA) and frozen on dry ice, whereas the rest of the samples were stored in RNAlater (Ambion, Foster City, CA, USA) for the immediate RNA isolation. Prior to RNA isolation from skin specimens, tissue morphology was evaluated histologically and mixed cell population was addressed as previously described [10].

Debridement techniques of venous ulcers

Venous ulcers biopsies were obtained from normally discarded tissue from patients. All patients were debrided in the operating room under monitored anaesthesia care or general anaesthesia and in all cases local lidocaine injection was used for local anaesthesia. After the wound was prepped in betadine, a No. 20 sterile surgical scalpel was used to debide the flat wound edges. No undermining was encountered. The non-healing edges used in this study were clinically identified by a single surgeon (Dr. Brem) as the edge of the most proximal skin edge to the ulcer bed, often times indicated by the presence of callus. Multiple biopsies were taken of each patient within 30 min. of the injection of local anaesthetic. Once the samples were brought to the laboratory, routine histolpathological analysis with hemotoxylin & eosin were performed to verify typical morphology of the non-healing edge as described by Stojadinovic *et al.* [9]. Briefly, acanthotic epidermis with hyperkeratosis and parakeratosis are indicative of a non-healing edge of the wound, in addition to presence of fibrosis. Furthermore, immunohistochemistry staining for makers of chronic wounds (c-myc and β-catenin) was performed and all samples stained positive. Only biopsies that fulfilled these two assays were used in the study.

Preparation and hybridization of probes

Samples were homogenized and total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Approximately 5 μg of total RNA was reverse transcribed, amplified and labelled as described [20]. Labelled cRNA was hybridized to HG-U133A-set Gene Chip probe arrays that contain probe sets representing approximately

22,000 genes (Affymetrix, Santa Clara, CA, USA). The arrays were washed and stained with avidin-biotin streptavidin-phycoerythin labelled antibody using Affymetrix fluidics station and then scanned using the Agilent GeneArray Scanner system (Hewlett-Packard, Palo Alto, CA, USA) as described by Affymetrix.

Gene array data analysis

Microarray Suite 5.0 (Affymetrix) was used for data extraction and for further analysis, data mining tool 3.0 (Affymetrix, Santa Clara, CA, USA) and GeneSpring™ software 7.3.1 (Silicon Genetics, Redwood City, CA, USA) were used for normalization to the median and filtration on the Volcano plot for degree of change and *P*-value calculations. Samples were normalized per chip: to the 50th percentile and per gene to a median. Statistical comparisons of expression level between each condition were performed using ANOVA test. Only genes with a *P*-value less than 0.05 were considered to be statistically significant. Differential expressions of transcripts were determined by calculating the degree of change. Genes were considered regulated if the expression levels differed more than 2-fold relative to healthy skin. Clustering was performed based on individual gene expression profiles. We developed an extensive gene annotation table describing the molecular function and biological category of the genes present on the chip as previously described [10]. The genes were annotated according to this table.

Immunohistochemistry

Slides containing frozen five micrometer thick skin sections were fixed in acetone (–20°C) for 1 min. Sections were blocked in 5% bovine serum albumin (BSA) and following primary antibodies diluted in 5% BSA/Phosphate Buffered Saline (PBS) were used: antibody against Filaggrin [21] and K10 (gift from Dr. H Sun, New York University), Involucrin (NeoMarkers, Fremont, CA, USA) and K17 (Gift from Dr. P. Coulombe, Johns Hopkins University) [22]. For staining with Dsg3, Dsc2 and DP, sections were permeabilized with 1%Triton-X-100 in PBS for 5 min., washed and blocked in 1% BSA/0.1% Triton X-100 in PBS for 1 hr at room temperature (RT). Sections were incubated with antibodies against desmocollin 2 (7G6), desmoglein 3 (5H10) and desmoplakin (23F4) (kind gift from Dr. James K Wahl III, University of Nebraska) overnight at +4°C. Signal was visualized using Alexa-Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA) secondary antibody. Slides were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA). Staining was analysed using Nikon Eclipse E800 microscope and digital images were collected using SPOT-Camera Advanced program.

Quantitative real-time PCR analysis

For real-time PCR, 0.5 μg of total RNA from healthy skin and venous ulcer was reverse transcribed using a Omniscript Reverse Transcription kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed in triplicate using the iCycler iQ thermal cycler and detection system and an iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Relative expression was normalized for levels of HPRT1. The primer sequences used were: HPRT1, fw (5′-AAAGGACCCCACGAAGTGTT-3′) and rev primer (5′-TCAAGGGCATATCCTACAACAA-3′); DEFB4, fw (5′-GGTGGTATAGGCGATCCTGTT-3′) and rev primer (5′-AGGGCAAAAGACTGGATGACA-3′); KLK6, fw (5′-CATGGCGGACCCCTGCGACAAGAC-3′) and rev primer (5′-TGGATCACAGCCCGGACAACAGAA-3′); MMP11 fw (5′-AGATCTACTTCTTCCGAGGC) and rev primer (5′-TTCCAGAGCCTTCACCTTCA-3′); CCL27 fw (5′-TCCTGAGCCCAGACCCTAC-3′) and rev primer (5′-CAGTTCCACCTGGATGACCTT-3′); APOD fw (5′-AATCAAATCGAAGGTGAAGCCA) and rev primer (5′-ACGAGGGCATAGTTCTCATAGT-3′); S100A7 fw (5′-GGAGAACTTCCCCAACTTCC-3′) and rev primer (5′-ACATCGGCGAGGTAATTTGT-3′); BMP2 fw (5′-TCAAGCCAAACACAAACAGC-3′) and rev primer (5′-GTGGCAGTAAAAGGCGTGAT-3′); BMP7 fw (5′-AGGCCTGTAAGAAGCACGAG-3′) and rev primer (5′-GGTGGCGTTCATGTAGGAGT-3′); K17 fw (5′-GGTGGGTGGTGAGATCAATGT-3′) and rev (5′- CGCGGTTCAGTTCCTCTGTC). Statistical comparisons of expression levels were performed using Student's t-test.

Results

To determine genes that play a role in pathogenesis of venous ulcers we performed microarray analysis, utilizing biopsies from non-healing edges of venous ulcers from three patients with venous stasis ulcers and three healthy skin specimens. All three patients presented with an ulcer on a lower left leg, longer than an year in duration (Fig. 1). Proper diagnosis was made following previously published guidelines and protocols [3, 23]. Evaluation for ischaemia was performed either by non-invasive flow exams (ankle-brachial index of <0.9) or by arteriogram. All patients did not have evidence of ischaemia. In addition, all patients had a venous duplex exam and confirmed the presence of venous valvular incompetence and venous reflux. None of the patients had diabetes. All patients had haemosiderosis upon clinical exam.

To compare gene expression profiles from obtained skin specimens, we performed cluster analyses using GeneSpring™ 7.3.1 software. We generated a gene tree employing all genes present on the chip and visualized the expression profile of each sample (Fig. 2). As expected, skin specimens obtained from healthy, control skin were clustered together separate from cluster of skin specimens obtained from venous biopsies, thus confirming differential gene expression profiles derived from venous ulcers *versus* healthy skin.

Overall microarray data analyses

When the gene expression profile of skin derived from edges of non-healing wounds was compared to healthy skin, a differential expression of transcripts was determined by calculating the degree of change. Genes were considered regulated if the expression levels differed more than 2-fold relative to healthy skin. Duplicates were eliminated and genes of unknown function were excluded. Of approximately 22,000 genes presented on the chip, we found 1557 genes to be differentially regulated between non-healing edges of venous ulcers and healthy skin. Out of 1557 genes, 55% of genes were down-regulated and

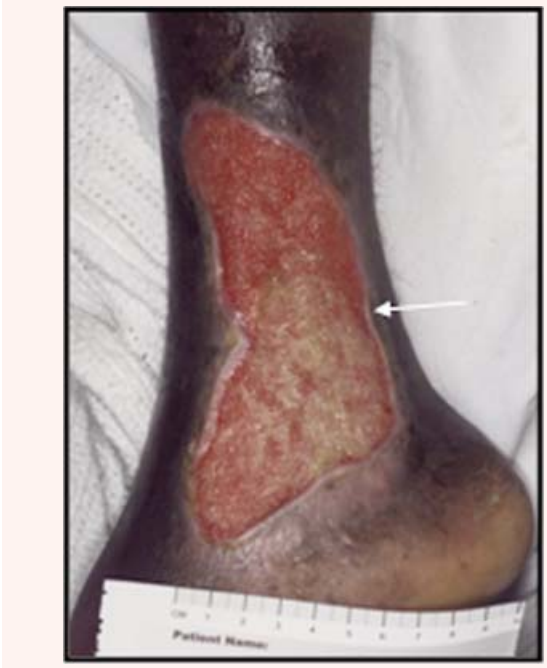

Fig. 1 An image of a venous ulcer used in this study. The arrow is pointing to the location of a non-healing edge from which biopsy for microarray analysis was obtained.

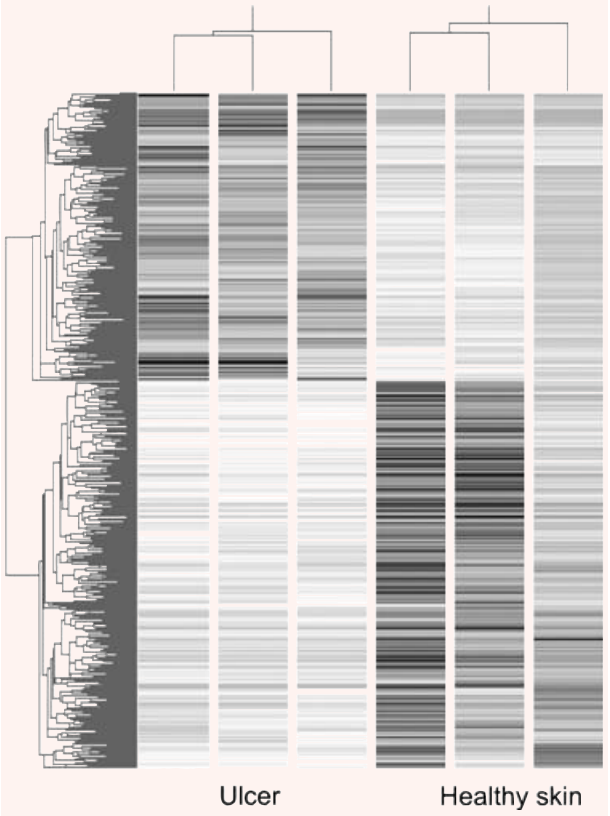

Fig. 2 Gene tree showing different gene expression patterns between venous ulcers (left) and healthy skin (right).

45% were up-regulated. We sorted regulated genes according to their biological functions, *i.e.* process in which they participate (Fig. 3). Furthermore, we selected the top 50 up-regulated and the top 50 down-regulated genes based on the degree of change and *P*-values ($P < 0.05$) and grouped them by cellular functions and biological processes (Fig. 4). The most regulated genes fell into following biological processes: contact and motility, tissue remodelling, inflammation and immunity, proliferation, differentiation, cell death control, metabolism and signal transduction and transcription. To better understand molecular changes in non-healing edges of venous ulcers we focused on specific cellular functions and processes affected by venous ulcer environment.

Keratinocyte activation markers

Activation of keratinocytes is marked by the induction of a specific set of keratins: keratins 6, 16 and 17. We have shown previously induction of K6 at the non-healing edges of chronic wounds [9]. Microarray data confirmed induction of keratin 6 (K6). In addition, induction of keratin 16 (K16) was also found in venous ulcers. Interestingly, we could not detect keratin 17 (K17), another keratinocyte activation marker, among genes regulated more than 2-folds. We checked generated gene list of statistically regulated genes before we made the 2-folds cut-off, and found K17 among up-regulated genes. K17 expression was induced 1.86-fold. To confirm changes in K17 expression we performed real-time PCR and found K17 induced in non-healing edges of venous ulcers as compared to normal skin (Fig. 5A). Furthermore, we used K17-specific antibody to stain healthy skin and skin biopsies harvested form venous ulcers. As expected, healthy epidermis does not show K17 staining, whereas epidermis from non-healing edge of venous ulcers shows robust signal (Fig. 5B). Therefore, keratinocytes at the non-healing edge of venous ulcers express early activation markers.

Keratinocyte activation: proliferation

Keratinocyte proliferation is one of the cellular processes defining keratinocyte activation. Hyperproliferation of keratinocytes is characteristic of the epidermis of the non-healing edge. Interestingly, our microarray analyses show deregulation of cell cycle associated genes. In mammalian cells, a crucial checkpoint control for proliferation is provided by the pocket proteins of the retinoblastoma (Rb) family [24, 25]. All three pocket proteins of the Rb family, Rb, p107 and p130 were found down-regulated in

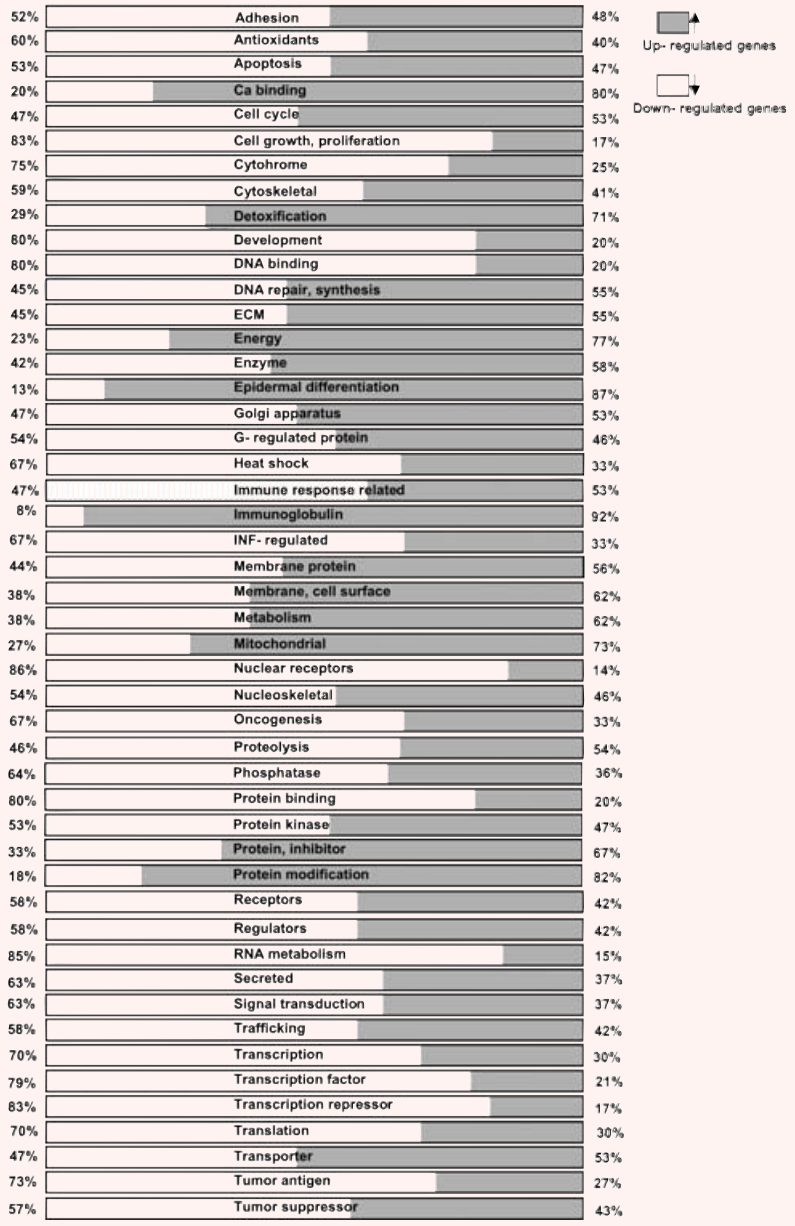

Fig. 3 Percentage of genes differentially regulated between non-healing edges of venous ulcers and healthy skin samples sorted according to biological functions.

our microarray data, suggesting loss of cell cycle control. Furthermore, cyclin B1, cyclin D2, cyclin A2, cyclin F and cyclin M4 were up-regulated along with cell division cycle 2 (CDC2) suggesting an increase of CDC2/cyclin B1 and CDC2/cyclin A2 complexes, promoting both cell cycle G1/S and G2/M transitions. In contrast, cyclin D1 and eukaryotic translation initiation factor 4E (EIF4E) which promotes the nuclear export of cyclin D1, were found down-regulated in non-healing edges. Microarray analyses further show a loss of cell cycle checkpoint regulation. Checkpoint suppressor 1 (CHES1) and Wee1-like protein kinase (WEE1) were down-regulated. WEE1 catalyses the inhibitory tyrosine phosphorylation of CDC2/cyclinB kinase and appears to coordinate the transition between DNA replication and mitosis by protecting the nucleus from cytoplasmically activated CDC2 kinase. Since both CDC2 and cyclin B1 were up-regulated coupled with loss of inhibitory phosphorylation by WEE1, we propose that this might account for the hyperproliferative phenotype in non-healing edges of venous ulcers. Two cyclin-dependent kinase inhibitors,

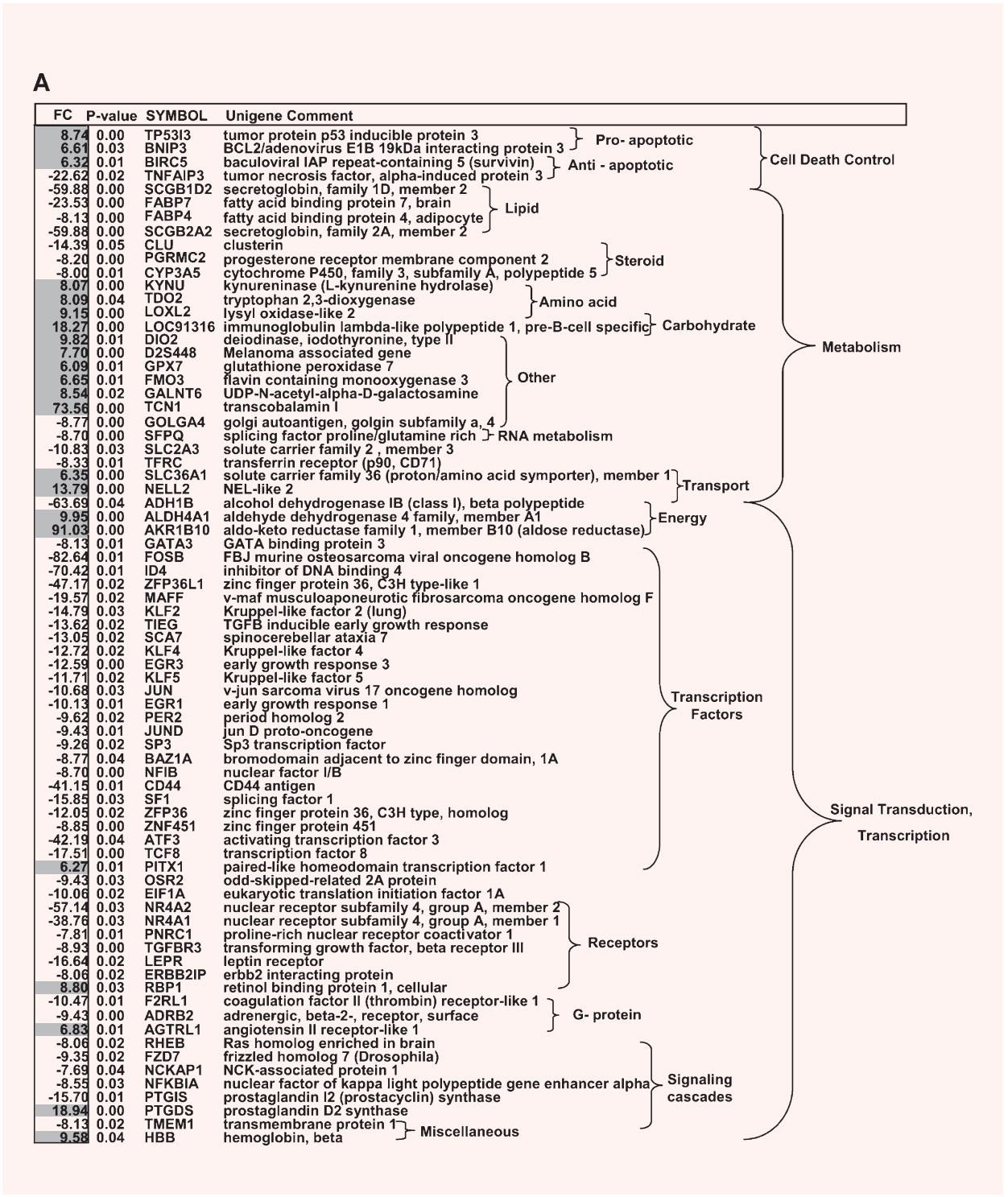

Fig. 4 A hundred top regulated genes. Fold changes are shown in left columns. *P*-values, gene symbols and unigene comments are given next. The genes are marked with brackets and grouped according to cellular functions and biological processes.

B

FC	P-value	SYMBOL	Unigene Comment		
-11.24	0.00	HAS1	hyaluronan synthase 1	Adhesion molecules, junctional	Contact and Motility
6.49	0.02	EVA1	epithelial V-like antigen 1		
6.59	0.04	TNC	tenascin C (hexabrachion)		
17.19	0.01	DSC2	desmocollin 2		
10.40	0.00	MCAM	melanoma cell adhesion molecule		
-11.30	0.03	PDZK3	PDZ domain containing 3		
-8.06	0.03	RABGGTB	Rab geranylgeranyltransferase, beta subunit	Cytoskeleton	
-9.71	0.00	DMN	desmuslin		
-10.00	0.01	ADD3	adducin 3 (gamma)		
23.94	0.00	KRT6A	keratin 6A		
37.66	0.01	KRT6B	keratin 6B		
40.20	0.04	KRT16	keratin 16		
6.47	0.00	GMFG	glia maturation factor, gamma		
-29.94	0.04	TNA	tetranectin	ECM	Tissue Remodeling
-21.32	0.02	TNXB	tenascin XB		
-11.20	0.01	MAGP2	microfibril-associated glycoprotein-2		
6.88	0.00	COL5A3	collagen, type V, alpha 3		
7.53	0.05	COL4A2	collagen, type IV, alpha 2		
8.48	0.00	COL5A1	collagen, type V, alpha 1		
19.25	0.02	COL4A1	collagen, type IV, alpha 1		
38.57	0.01	COL11A1	collagen, type XI, alpha 1		
-9.17	0.03	APP	amyloid beta (A4) precursor protein	Proteolysis	
7.95	0.05	WFDC1	WAP four-disulfide core domain 1		
18.41	0.02	KLK13	kallikrein 13		
25.63	0.02	MMP11	matrix metalloproteinase 11 (stromelysin 3)		
30.60	0.01	KLK6	kallikrein 6		
32.06	0.01	HAT	airway trypsin-like protease		
7.95	0.00	UBE2C	ubiquitin-conjugating enzyme E2C		
6.51	0.03	CSTB	cystatin B (stefin B)		
22.06	0.03	SERPINB13	serine (or cysteine) proteinase inhibitor, member 13	Proteolysis inhibitors	
94.96	0.00	SERPINB4	serine (or cysteine) proteinase inhibitor, member 4		
143.80	0.00	SERPINB3	serine (or cysteine) proteinase inhibitor, member 3		
146.50	0.00	PI3	protease inhibitor 3, skin-derived (SKALP)		
12.84	0.01	THY1	Thy-1 cell surface antigen	Immune response	Inflammation, Immunity
6.36	0.00	TRB	T cell receptor beta locus		
9.40	0.00	TRA	T cell receptor alpha locus		
-48.78	0.00	DAF	decay accelerating factor for complement		
-8.77	0.00	CD83	CD83 antigen		
6.55	0.01	IGLV3-10	immunoglobulin lambda variable 3-10	Immunoglobulin	
13.67	0.02	IGKV1OR2-108	immunoglobulin kappa variable 1/OR2-108		
21.86	0.00	IGKV1D-13	immunoglobulin kappa variable 1D-13		
37.27	0.01	IGKC	immunoglobulin kappa constant		
67.16	0.01	MGC27165	hypothetical protein MGC27165		
91.18	0.00	IGLJ3	immunoglobulin lambda joining 3		
96.56	0.00	IGL	immunoglobulin lambda locus		
126.70	0.00	IGHG3	immunoglobulin heavy constant gamma 3		
-12.06	0.04	IL6	interleukin 6	Secreted	
63.08	0.05	DEFB4	defensin, beta 4		
95.11	0.02	S100A8	S100 calcium binding protein A8 (calgranulin A)	S100 family	
22.78	0.00	S100A12	S100 calcium binding protein A12 (calgranulin C)		
-9.71	0.00	RAD21	RAD21 homolog	DNA repair, Synthesis	Proliferation
-7.87	0.01	STAG2	stromal antigen 2		
7.48	0.02	UPP1	uridine phosphorylase 1		
8.79	0.01	CDA	cytidine deaminase		
13.66	0.00	CHN1	chimerin (chimaerin) 1		
14.99	0.00	RRM2	ribonucleotide reductase M2 polypeptide	Cell cycle	
-10.80	0.01	WEE1	WEE1 homolog		
-8.77	0.01	INSIG1	insulin induced gene 1		
6.50	0.00	CDC20	CDC20 cell division cycle 20 homolog		
10.33	0.01	EGFL6	EGF-like-domain, multiple 6	Cell growth	
7.07	0.02	NDRG4	NDRG family member 4		
-124.38	0.00	IGFBP5	insulin-like growth factor binding protein 5	Secreted	
-30.96	0.05	APOD	apolipoprotein D		
-25.77	0.00	CCL27	chemokine (C-C motif) ligand 27		
-14.31	0.04	CXCL2	chemokine (C-X-C motif) ligand 2		
6.39	0.00	BMP1	bone morphogenetic protein 1		
-22.83	0.00	THH	trichohyalin		Differentiation
-8.70	0.03	FLG	filaggrin		
8.10	0.00	IVL	involucrin		
15.72	0.00	SPRR1B	small proline-rich protein 1B (cornifin)		
130.70	0.01	SPRR3	small proline-rich protein 3		
33.35	0.00	SPRR1A	small proline-rich protein 1A		
118.00	0.00	S100A9	S100 calcium binding protein A9 (calgranulin B)	S100 family	
105.80	0.01	S100A7	S100 calcium binding protein A7 (psoriasin 1)		
10.96	0.02	CALML3	calmodulin-like 3		

Fig. 4 Continued.

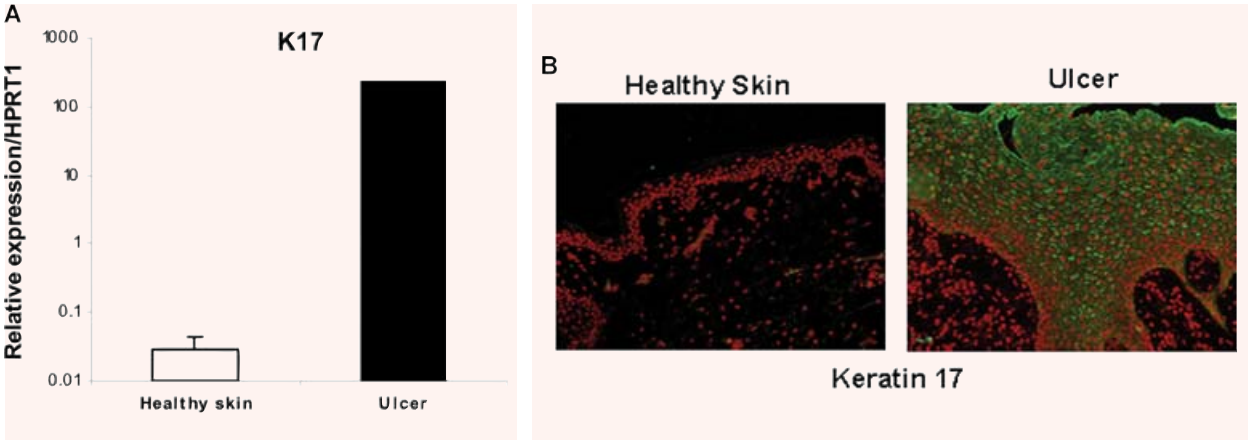

Fig. 5 K17 is up-regulated in non-healing edges of venous ulcers. (**A**) Real-time PCR confirms K17 up-regulation in ulcers. K17 expression was normalized to expression level of HPRT. (**B**) Immunofluorescence with K17-specific antibody shows induction of K17 in non-healing edge of venous ulcer. Scale bar 50 μm.

cyclin-dependent kinase inhibitor 2B (CDKN2B) and cyclin-dependent kinase inhibitor 3 (CDKN3) were up-regulated. Deregulation of cell cycle associated genes suggest a loss of cell cycle control, which may account for the hyperproliferative potential of the keratinocytes present in the epidermis of venous ulcers.

Keratinocyte activation: regulators

Among secreted molecules that participate in keratinocyte activation, insulin-like growth factor binding protein 5 (IGFBP5) was among top 50 down-regulated genes. Furthermore, bone morphogenetic proteins (BMP) were differentially regulated. The expression of BMP-2 and BMP-7 was down-regulated (Fig. 6A), whereas the expression of BMP1 was up-regulated. Furthermore, we found that the leptin receptor was down-regulated; however, there was no change in leptin expression. Angiogenesis factors, vascular endothelial growth factor (VEGF), epiregulin (EREG) and angiopoetin-like 6 (ANGPTL6) were markedly down-regulated. Previous data have shown that ANGPTL6 promotes epidermal proliferation, remodelling and regeneration [26]. Conversely, we detected induction of additional pro-angiogenic growth factors and receptors, such as platelet-derived endothelial cell growth factor (ECGF1), receptor neuropilin (NRP1) and stromal cell-derived factor 1-α (CXCL12/SDF-1α). We have found profound down-regulation of apolipoprotein D (APOD) and up-regulation of defensin B4 (DEFB4) (Fig. 6B). Up-regulation of DEFB4 has been shown to be associated with benign hyperplasia in skin [27].

Chemokines which mediate T cell chemotaxis were found suppressed. The expression of cutaneous T-cell attracting chemokine (CCL27), was almost abolished (Fig. 6C), along with IL-7, which is essential for T-cell memory generation, while the expression of IL7R was induced. Surprisingly, the expression of platelet-derived growth factors α and β (PDGFA and PDGFB) was increased, but the expression of transforming growth factor-β_2 (TGFβ_2), transforming growth factor β receptor III (TGF-βR3), fibroblast growth factor 13 (FGF13) and interleukin 6 (IL-6) was decreased. Expression of the stromelysin-3 gene (MMP-11) was up-regulated (Fig. 6C), consistent with previously published data [28].

In summary, microarray data show changes consistent with morphology, suggesting deregulation and incomplete activation of keratinocytes at the non-healing edge in epidermis of venous ulcers.

Keratinocyte differentiation: markers, cornified envelope and barrier formation

In intact skin, the major role of keratinocyte terminal differentiation is to form a physical barrier that acts as permeability barrier against water loss, foreign microbes and toxins. The cornified cell envelope and TJs [7] are two important components of skin barrier function.

Microarray data obtained from this study confirm epidermal morphology of non-healing edges. Early markers of differentiating keratinocytes, K1 and K10, were down-regulated as well as the late markers, filaggrin (FLG) and thrichohyalin (THH), which are molecules that associate with the keratin cytoskeleton during terminal differentiation (Fig. 7). Interestingly, additional components of the cornified envelope, including involucrin (IVL) and small proline-rich proteins (SPRR1A, SPRR1B, SPRR2B, SPRR3) were up-regulated, whereas transglutaminase 1 (TGM1), an enzyme responsible for cross-linking SPRR's and IVL into the cornified envelope, was up-regulated. To confirm microarray data at the protein level, we used immunohistochemistry and stained patients' biopsy specimens with K10, IVL and FLG-specific antibodies. We found robust increase of IVL, whereas K10 and FLG were barely detected in specimens derived from venous ulcers

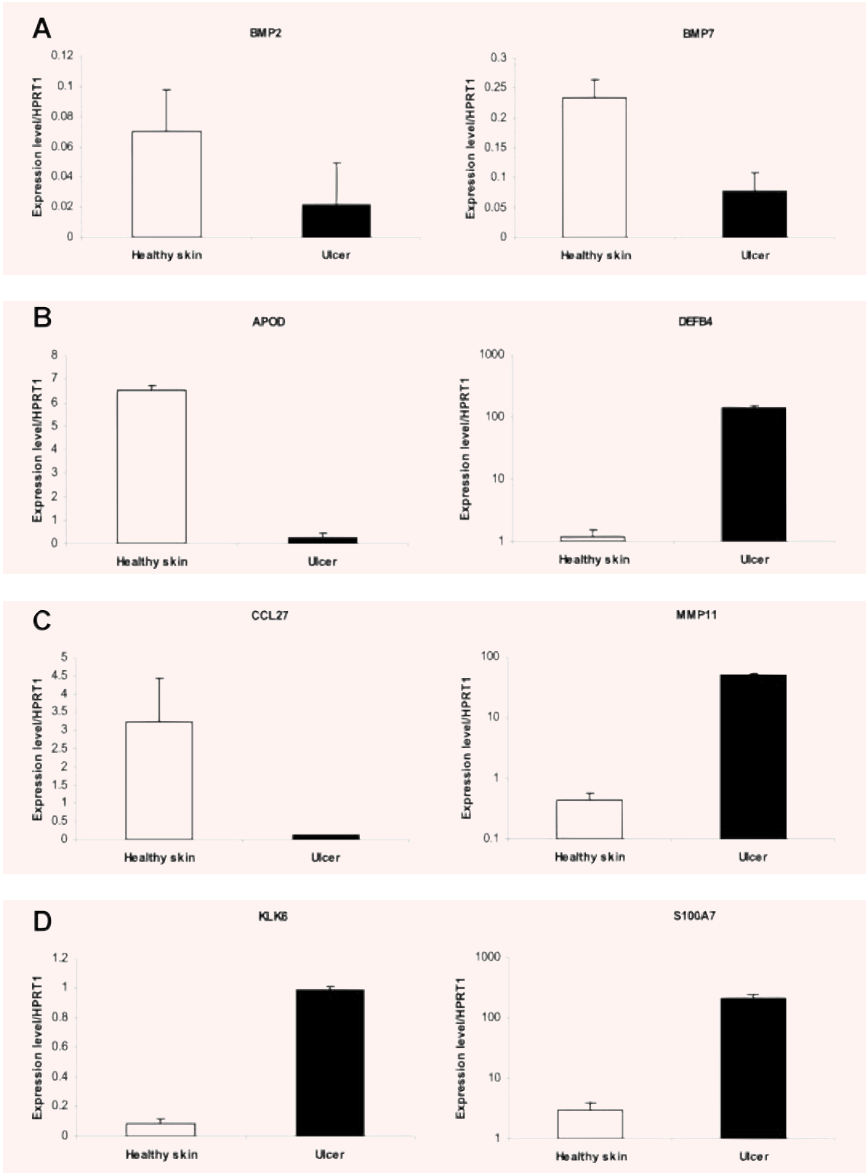

Fig. 6 Real-time PCR confirmation of selected genes differentially expressed in healthy skin and venous ulcers. Quantitative real-time PCR for the expression of BMP2 and BMP7 (**A**), APOD and DEFB4 (**B**), CCL27 and MMP11 (**C**), S100A7 and KLK6 (**D**). Mean values of expression levels were represented after normalization to expression level of HPRT1.

(Fig. 8). Among newly identified potential markers of keratinocyte terminal differentiation [29], we found skin-derived protease inhibitor 3 (SKALP/PI3), oxysterol-binding protein-like 8 (OSBPL8), adducing 3 (ADD3), early growth response 3 (EGR3), inhibitor of DNA binding 4 (ID4), occluding (OCLN) and decay-accelerating factor for complement (DAF) down-regulated, whereas septin 8 (SEPT8), serine/threonine kinase 10 (STK10) and serine/cysteine proteinase inhibitor, clade B, member 3 (SERPINB3) were up-regulated (Fig. 7). In addition, several other molecules that participate in epidermal differentiation were found to be regulated. S100A7, a member of human epidermal differentiation complex (EDC), S100A8, S100A9, S100A12 and kalikrein 6 (KLK6) were among the top up-regulated genes. We used real-time PCR to confirm these findings (Fig. 6D). TJ in skin are complex structures localized in granular layer. These junctions are composed of transmembrane (claudin 1–20, ocludin) and plaque (symplekin (SYMPK) and ZO 1–3) proteins [30, 31]. We found that different structural components of TJs, such as TJ protein 3 (ZO3, TJP3), spectrin 1(SPTBN1), InaD-like protein (MUPP1, INADL), occludin (OCLN), claudin 5 (CLDN5), claudin 8 (CLDN8) down-regulated in the non-healing edge of venous ulcers. Interestingly, only SYMPK was found up-regulated. Formation of TJs in epidermis, during differentiation, is a precisely regulated spatiotemporal process. An important component of this regulation is polarity complex Par3, Par6, atypical PKC-iota (PARD) and CDC42 [32]. We found that different components of polarity

Fold Change	P-value	Biological Function	SYMBOL	Unigene Comment
17.19	0.01	Adhesion	DSC2	desmocollin 2
-2.71	0.02	Adhesion	DSC3	desmocollin 3
-5.24	0.01	Adhesion	DSG2	desmoglein 2
-2.17	0.03	Adhesion	TJP3	tight junction protein 3 (zona occludens 3)
-3.19	0.01	Adhesion	INADL	InaD-like protein
-2.05	0.02	Adhesion	OCLN	occludin
-2.87	0.02	Adhesion	CLDN5	claudin 5
-6.17	0.00	Adhesion	CLDN8	claudin 8
2.12	0.01	Adhesion	SYMPK	symplekin
5.25	0.04	Adhesion	DSG3	desmoglein 3
-3.57	0.05	Adhesion	DSP	desmoplakin
-2.10	0.00	Adhesion	PKP2	plakophilin 2
3.43	0.01	Cell cycle	SEPT	septin 8
-2.31	0.05	Cell cycle	PARD3	par-3 partitioning defective 3 homolog
2.76	0.00	Cell cycle	CCNA2	cyclin A2
4.82	0.00	Cell cycle	CCNB1	cyclin B1
2.01	0.02	Cell cycle	CCND2	cyclin D2
2.87	0.04	Cell cycle	CCNF	cyclin F
3.45	0.02	Cell cycle	CNNM4	cyclin M4
-3.70	0.03	Cell cycle	CCND1	cyclin D1 (PRAD1)
-3.86	0.02	Cell cycle	CHES1	checkpoint suppressor 1
-10.80	0.01	Cell cycle	WEE1	WEE1 homolog)
3.49	0.02	Cell cycle	CDKN3	cyclin-dependent kinase inhibitor 3
2.68	0.00	Cell cycle	CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)
-22.83	0.00	Cytoskeletal	THH	trichohyalin
-4.88	0.00	Cytoskeletal	SPTBN1	spectrin, beta, non-erythrocytic 1
-3.69	0.02	Cytoskeletal	KRT1	keratin 1
-4.90	0.01	Cytoskeletal	KRT10	keratin 10
40.20	0.04	Cytoskeletal	KRT16	keratin 16
37.66	0.01	Cytoskeletal	KRT6B	keratin 6B
-10.00	0.01	Cytoskeletal	ADD3	adducin 3
-2.97	0.05	Cytoskeletal	CDC42	cell division cycle 42
-8.70	0.03	Epidermal differentiation	FLG	filaggrin
8.10	0.00	Epidermal differentiation	IVL	involucrin
33.35	0.00	Epidermal differentiation	SPRR1A	small proline-rich protein 1A
15.72	0.00	Epidermal differentiation	SPRR1B	small proline-rich protein 1B (cornifin)
23.02	0.01	Epidermal differentiation	SPRR2B	small proline-rich protein 2B
130.70	0.01	Epidermal differentiation	SPRR3	small proline-rich protein 3
5.37	0.01	Epidermal differentiation	TGM1	transglutaminase 1
105.80	0.01	Epidermal differentiation	S100A7	S100 calcium binding protein A7 (psoriasin 1)
95.11	0.02	Epidermal differentiation	S100A8	S100 calcium binding protein A8 (calgranulin A)
118.00	0.00	Epidermal differentiation	S100A9	S100 calcium binding protein A9 (calgranulin B)
22.78	0.00	Epidermal differentiation	S100A12	S100 calcium binding protein A12 (calgranulin C)
-48.78	0.00	Immune response	DAF	decay accelerating factor for complement
-5.41	0.02	Metabolism	OSBPL8	oxysterol binding protein-like 8
2.23	0.02	Protein kinase	STK10	serine/threonine kinase 10
146.50	0.00	Proteolysis inhibitor	PI3	protease inhibitor 3, skin-derived (SKALP)
143.80	0.00	Proteolysis inhibitor	SERPINB3	serine (or cysteine) proteinase inhibitor,member 3
30.60	0.01	Proteolysis	KLK6	kallikrein 6 (neurosin, zyme)
25.63	0.02	Proteolysis	MMP11	matrix metalloproteinase 11 (stromelysin 3)
5.99	0.00	Receptor, cyto-, chemokine	IL7R	interleukin 7 receptor
2.74	0.03	Receptor, growt factor	NRP1	neuropilin 1
-8.93	0.00	Receptor, growt factor	TGFBR3	transforming growth factor, beta receptor III
-2.10	0.01	Regulator	PLD1	phospholipase D1, phophatidylcholine-specific
2.82	0.02	Regulator	PLD2	phospholipase D2
2.49	0.04	Regulator, Notch pathway	MFNG	manic fringe homolog
-2.17	0.00	Regulator, Notch pathway	NOTCH2	Notch homolog 2
-2.11	0.00	Regulator, Rb pathway	RBL1	retinoblastoma-like 1 (p107)
-5.18	0.02	Regulator, Rb pathway	RBL2	retinoblastoma-like 2 (p130)
-124.38	0.00	Secreted	IGFBP5	insulin-like growth factor binding protein 5
6.39	0.00	Secreted	BMP1	bone morphogenetic protein 1
-3.95	0.01	Secreted	BMP2	bone morphogenetic protein 2
-2.40	0.02	Secreted	BMP7	bone morphogenetic protein 7
-2.39	0.03	Secreted	VEGF	vascular endothelial growth factor
-7.69	0.01	Secreted	EREG	epiregulin
-3.19	0.05	Secreted	ANGPTL6	angiopoietin-like 6
5.51	0.03	Secreted	ECGF1	endothelial cell growth factor 1
4.26	0.04	Secreted	CXCL12	chemokine ligand 12 (stromal cell-derived factor 1)
-30.96	0.05	Secreted	APOD	apolipoprotein D
63.08	0.05	Secreted	DEFB4	defensin, beta 4
-25.77	0.00	Secreted	CCL27	chemokine (C-C motif) ligand 27
-3.79	0.02	Secreted	IL7	interleukin 7
2.41	0.02	Secreted	PDGFA	platelet-derived growth factor alpha polypeptide
2.12	0.00	Secreted	PDGFB	platelet-derived growth factor beta polypeptide

Fig. 7 Activation and differentiation related genes. Fold changes, *P*-values, biological functions, gene symbols and unigene comments for genes implicated in keratinocyte activation and differentiation are shown.

complex, PARD and CDC42 are down-regulated in chronic wounds when compared to healthy skin.

Taken together, data obtained from microarray analysis indicate deregulation of early and late differentiation markers, aberrant cornified envelope assembly and deregulation of TJ molecules. This suggests a possible loss of barrier function in the epidermis of venous ulcers.

Keratinocyte differentiation: regulators

Microarray analyses revealed down-regulation of kuppel-like factor (KLF4), transcription factor, important for the establishment of skin barrier function as well as cross-linking of cornified envelope proteins [7, 33]. Furthermore, up-regulation of phospholipase D2

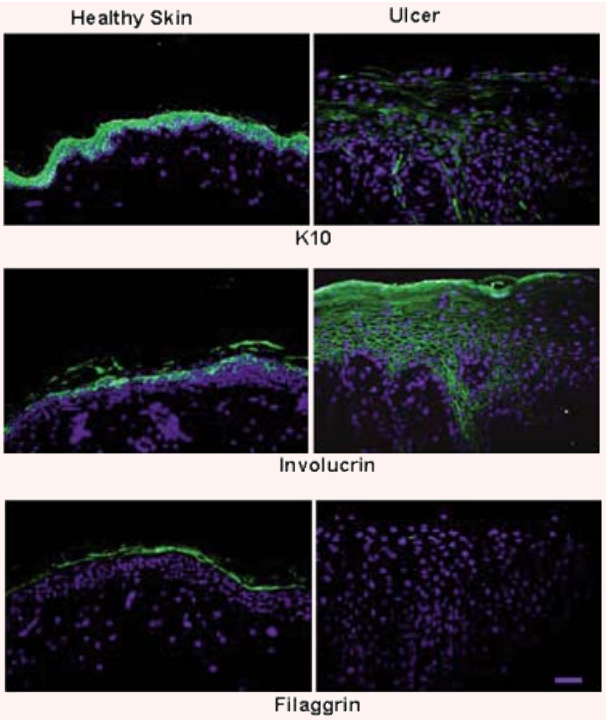

Fig. 8 Keratinocyte differentiation markers are deregulated in venous ulcers. Immunofluorescence staining of skin specimens deriving from venous ulcers with K10, involucrin and filaggrin-specific antibodies show increased involucrin expression while K10 and filaggrin are barely detected. Scale bar 50 μm.

(PLD2) but not phospholipase D1 (PLD1) was found. The activation of these regulators has been implicated in late keratinocyte differentiation [34]. The Notch signalling pathway has been shown to play a role in defining different steps of keratinocyte differentiation [35, 36]. We have found Manic Fringe (MFNG) to be up-regulated and NOTCH2 to be down-regulated. Taken together, these data demonstrate deregulation of molecules that govern both early and terminal stages of differentiation.

Adhesion molecules of differentiating keratinocytes

In addition to changes in TJ expression, we observed a deregulation of several genes coding for proteins of the desmosomal complex in venous ulcers. Among desmosomal cadherins, desmocollin 2 (DSC2) and desmoglein 3 (DSG3) were up-regulated whereas desmocollin 3 (DSC3) and desmoglein 2 (DSG2) were down-regulated. Two other desmosomal molecules, desmoplakin (DP) and plakophilin 2 (PKP2) were also down-regulated in venous ulcers. To confirm the microarray data we stained healthy skin and skin specimens derived from venous ulcers with DSG2, DSG3 and DP-specific antibodies. Staining with DSG3 and DSC2 showed

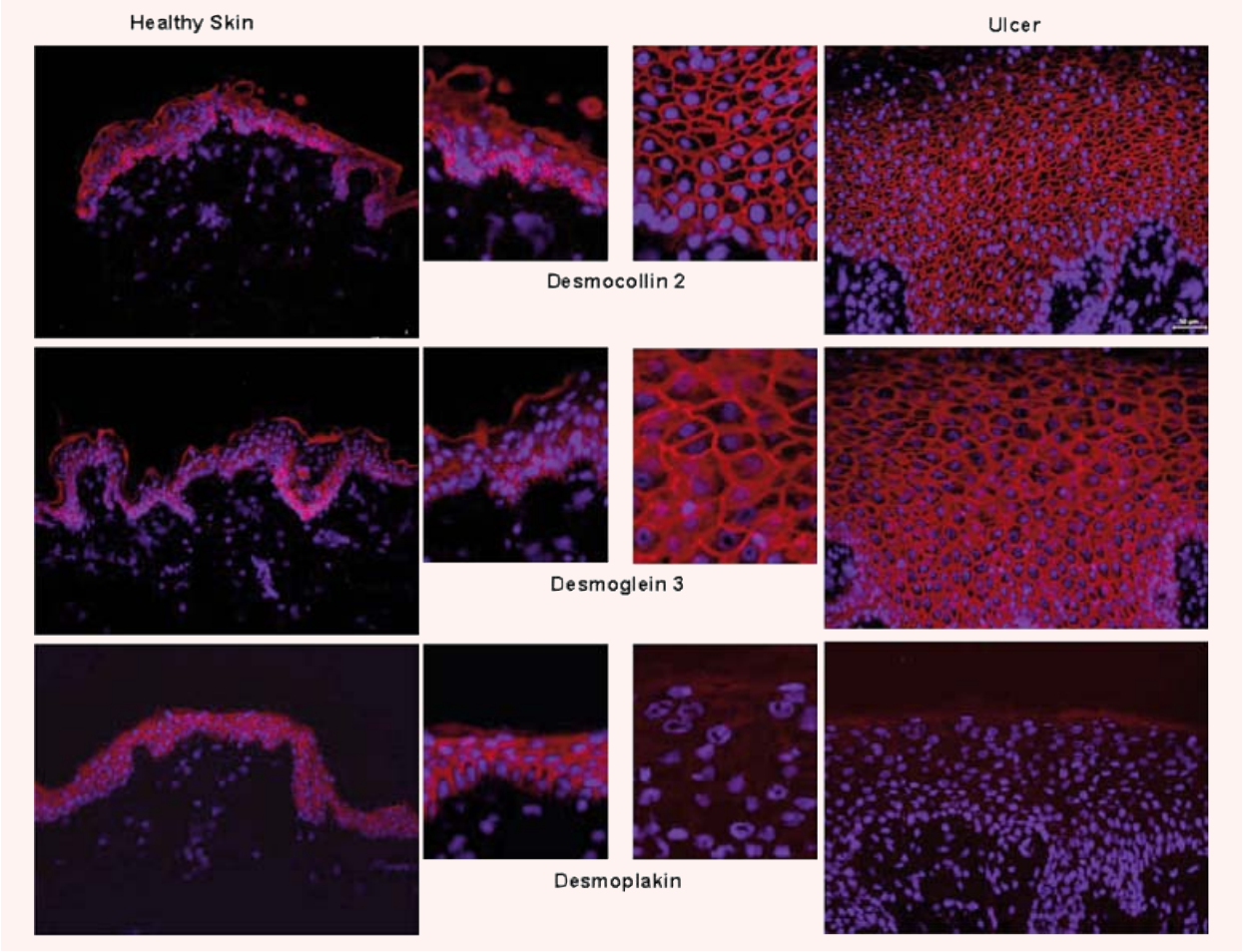

Fig. 9 Desmosomal components are missexpressed and deregulated in venous ulcers. Immunofluorescence staining with DSC2- and DSG3-specific antibodies showed increased signal throughout the epidermis of venous ulcers while staining signal of DP was decreased. Scale bar 50 μm.

increased signal throughout the epidermis of venous ulcers while signal of DP was decreased thus confirming deregulation of desmosomal proteins (Fig. 9).

Discussion

In this paper we present comprehensive analyses of the pathogenesis of non-healing edges of venous ulcers using transcriptional profiling, real-time PCR and immunohistochemistry. We found that hyperproliferation of keratinocytes in the epidermis of non-healing edges is, in part, due to a loss of a cell cycle control and incomplete activation. Furthermore, we found deregulation of early and late differentiation markers, as well as regulators of this processes, coupled with aberrant cornified envelope assembly and deregulation of TJ and desmosomal molecules. Taken together, data obtained from this study suggest that keratinocytes at the non-healing edges of venous ulcers are not capable of accomplishing either of two main processes in skin homeostasis: proliferation and differentiation.

In healthy skin, basal epidermal keratinocytes proliferate and, as they exit the basal cell compartment, they commit to a differentiation program. Keratinocyte differentiation requires DNA degradation, nuclear destruction and substantial proteolytic activity and leads to cell death resulting in the formation of a cornified layer and subsequently desquamation of dead cells. Our previous findings show that epidermis of chronic wounds is hyperproliferative with mitotically active cells present in suprabasal cell layers. These findings, coupled with the presence of the nuclei in a very thick cornified layer, suggest aberrant activation and incomplete differentiation of keratinocytes in chronic wounds.

When a wound occurs, keratinocytes at the wound edge become activated and start to express activation markers, keratins

6, 16 and 17. Keratinocytes from the non-healing edge of venous ulcers show a similar expression pattern. Although, the expression of K17 on microarrays showed less than a two fold increase, real-time PCR and immunohistochemistry confirmed robust induction. Keratins belong to the small group of genes that are highly expressed, thus exceeding a linear range of hybridization, leading to variable microarray readings [37–39]. Nevertheless, K17 was induced along with keratins K6 and K16, suggesting that initial activation process takes place in venous ulcers. In addition, it has been shown that pro-inflmmatory signals, including tumour necrosis factor-α (TNF-α), IL-1 and interferon-γ (IFN-γ) induce expression of these genes [40–43]. Thus, their induction is consistent with prolonged inflammation found in chronic wounds [44, 45].

We have shown previously that non-healing edge keratinocytes are marked by induction of c-myc and nuclear presence of β-catenin, leading to increased proliferation and inhibition of migration [9]. Data collected in this study further expands and confirms previous findings. Promotion of G1/S and G2/M cell cycle transitions in venous ulcers environment and loss of cell cycle control coupled with c-myc induction and keratinocytes activation may lead to a hyperproliferative phenotype. However, further conformation would require evaluation at the protein level, including post-translation modifications and phosphorylation in particular. On the other hand, the loss of cell cycle control is not complete and some factors, like cyclin D1 and EIF4E, usually overexpressed during tumourigenesis [46] were down-regulated in venous ulcers. MFNG expression is restricted to proliferative basal layer during embryonic epidermal stratification [36], and up-regulation of MFNG in non-healing edges coupled with the presence of mitotically active cells in suprabasal layers may suggest its possible role in the induction of keratinocyte proliferation. We found down-regulation of IL-6 expression in non-healing edges of venous ulcers, which is consistent with findings in IL-deficient mice (interleukin-6 knockout (IL-6KO)) that display delayed wound closure [47]. SDF-1α from keratinocytes and myofibroblasts has been shown to participate in homing of endothelial progenitor cells to the wound site. The db/db mouse wound model exhibit decreased expression of SDF-1α, suggesting possible decrease in diabetic foot ulcers [48, 49]. Interestingly, we found SDF1α up-regulated in venous ulcers. This may indicate that changes in SDF-1α expression may be associated specifically with diabetes rather than with impaired healing in general.

Keratinocyte early differentiation markers K1/K10 were down-regulated in our experiment, which is consistent with previous findings and further validates our microarray analyses [50]. Up-regulation of TGM1, an enzyme involved in the initial cross linking during cornification process along with IVL, which is a major early cross-linked component of cornified envelope as well as S100A7 and SPRR1A, SPRR1B, SPRR2B, SPRR3, indicates improper cornified envelope assembly [51]. FLG, one of the key proteins that facilitate terminal differentiation of the epidermis and formation of the skin barrier, was found down-regulated. It has been shown that once keratinocytes process pro-FLG into FLG they undergo specific changes that mark terminal differentiation [52], and that perhaps this process demarcates the 'line of regenerative potency' among the population of differentiating keratinocytes [53]. Down-regulation of FLG in venous ulcer coupled with deregulation of other keratinocyte differentiation markers suggests that keratinocytes at the edge of non-healing venous ulcers start to differentiate but are not capable of completing the process. In support of this finding, we also detected down-regulation of PLD1 in venous ulcers. It has been shown that during keratinocyte differentiation PLD1 mRNA levels increase [54]. Furthermore, the highest levels of PLD1 expression were found in the more differentiated layers of the epidermis, with negligible expression in basal keratinocytes [55]. Down-regulation of PLD1 expression in venous ulcers may indicate some form of de-differentiation and supports the notion that these keratinocytes do not execute the process to its completion. Similarly, we also found APOD (recently associated with differentiating keratinocytes) to be down-regulated in our venous ulcers [29].

Similar to findings of deregulation of differentiation markers and molecules that participate in this process and consistent with morphology of epidermis in venous ulcers, we also found deregulation of signalling molecules that control the process of differentiation. The overall findings are that the regulatory signals are sending 'mixed messages' to the cells leading to deregulation of the process. In differentiating epidermal keratinocytes, BMPs stimulate expression of K1, IVL and distal-less homeobox 3 (Dlx3) transcription factor, suggesting that these molecules may be targets for BMP signalling [56, 57]. It's been shown that BMP2 inhibits keratinocyte proliferation and promotes their terminal differentiation [58]. Thus, down-regulation of BMP2 in venous ulcers may account for keratinocyte hyperproliferation and deregulation of terminal differentiation. In addition, down- regulation of KLF4, a transcription factor highly expressed in differentiating layers of epidermis important for the establishment of skin barrier function and expression/cross-linking of cornified envelope proteins, further confirms improper regulation of differentiation process in venous ulcers [7, 33].

Another interesting observation is the down-regulation of IκBα expression in venous ulcers, which supports previous findings of prolonged inflammation. It has been shown that keratinocytes in IκBα-deficient mice are highly proliferative with deregulated epidermal differentiation [59], thus exhibiting phenotype similar to that of a non-healing epidermis of venous ulcers. Changes in expression of genes participating in regulation of keratinocyte differentiation program contribute to abnormal keratinocyte differentiation.

Adhesion molecules exert a dynamic role in epidermal differentiation/stratification. Down-regulation of TJ proteins, particularly claudins suggest a loss of permeability barrier function of the epidermis in our venous ulcer samples. This is corroborated by the inability of claudins to prevent TJ formation in KO mice, as well as a complete alteration in their function [60, 61]. Recent papers suggest that activity of complex Par3, Par6, atypical PKC-iota and CDC42 in granular layer of epidermis is necessary for TJ formation and keratinocyte differentiation [62]. Furthermore during calcium-induced differentiation of keratinocytes, atypical PARD was necessary for establishment of barrier formation. This complex

had characteristic re-distribution during wound healing and it may also be an endogenous regulator of asymmetric cell division of basal keratinocytes. Asymmetric cell division, on other hand, promotes stratification and wound healing in the skin by keeping balance between proliferation and differentiation [30, 63]. Down-regulation of atypical PARD and CDC42 suggests loss of cell polarity further suggesting a loss of balance between keratinocyte proliferation and differentiation and deregulation of TJ formation. Further investigation is necessary to analyse the exact localization of the polarity complex as well as TJ in hyperproliferative epidermis of venous ulcers.

We also observed deregulation of desmosomal components in the non–healing edges of venous ulcers. In the normal epidermis, DSC2 and DSG2 are found highly expressed in the proliferative basal layers of the epidermis. We recently showed that DSG2 plays a role in keratinocyte cell proliferation and survival [64]. Therefore a suppression of DSG2 expression may alter keratinocyte proliferation in venous ulcers. DSG3 expression, on the other hand, extends from the basal to spinous layers with intensity decreasing with differentiation. In venous ulcers, DSG3 was up-regulated and expressed throughout hyperproliferative epidermis, which is in agreement with previously publish data obtained from murine models, suggesting that differential expression of desmosomal proteins within the epidermis participate in the regulation of the tissue proliferation and differentiation [64–68]. When DSG3 was overexpressed under the control of K1 promoter in the suprabasal epidermis of transgenic mice, histological analysis of the skin revealed a hyperproliferative epidermis with hyper- and parakeratosis, along with abnormal epidermal differentiation [65]. Interestingly, despite the high expression of DSG3 and DSC2 in venous ulcers, expression of their associated plaque protein DP was greatly suppressed. This apparent lack of association between DSG3 and DP has been shown in DP RNAi-treated cells [69]. Similar to that observed here in venous ulcers, reduction of DP expression in a mild form of striate palmoplantar keratoderma also results in hyperproliferation and hyperkeratosis [70] further supporting a role for DP in regulating keratinocyte proliferation and differentiation. These data suggest that deregulation and atypical expression of desmosomal components may play a role in altered keratinocyte differentiation.

In the future, several different comparative genomics studies would prove very useful in delineating molecular pathogenesis of chronic wounds. For example, comparing transcriptional profiles of non-healing edges of chronic to acute wounds in different stages would identify specific wound-healing genes which contribute to non-healing phenotype. In addition, comparing transcriptional profiles of non-healing edges from venous to diabetic foot ulcers or pressure ulcers will identify common pathways that lead to non-healing phenotype in majority of chronic wounds. Proposed experiments underscore technical challenges in this field. Specifically, selection of controls is fundamental to the outcome of the data. Our laboratory has worked over the years to establish how variable human samples actually show consistent expression profiles [37] and, further, how to standardize the wound biopsy from patient to patient [10]. To address the issue of mixed cell populations in our skin samples, mRNA levels of abundantly expressed genes specific for two major cell types: perlecan and vimentin (fibroblasts) and stratifin and junctional plakoglobin (keratinocytes) were compared using microarrays. The signal intensity levels correlate to the hybridization signal intensity of vimentin, perlecan, stratifin and junctional plakoglobin and are a direct measure of the mRNA levels in each biopsy. All biopsies show consistent levels of expression for perlecan, stratifin and junctional plakoglobin suggesting that both the keratinocytes and fibroblasts are present at the consistent numbers in our venous ulcer biopsies [10]. Evaluations of microarray findings were performed on biopsies from venous ulcers of subsequent set of patients, which further documents the validity of the data obtained. In addition, technical aspects of harvesting the tissue, such as lidocaine injection, presence of infection, could be perceived as factors that may influence the data outcome. There is evidence in literature that lidocaine decreases proliferation of cultured human keratinocytes [71]. However, samples for this study were harvested within 30 min. after the lidocaine injection and immediately processed for RNA purification. Therefore, the lidocaine did not affect the outcome. In addition, we have established that specimens harvested using this procedure exhibit marked hyperproliferation [9, 10] which was further confirmed by microarrays presented in this manuscript.

Our data indicate that keratinocytes in venous ulcers initiate differentiation, carry out atypical cornified envelope assembly, and proceed towards terminal differentiation, but fail to completely execute the terminal process of differentiation and barrier formation. Most of the current understanding of the pathophysiology of venous ulcers comes from the tissue level [72], but our data expands on previous work to examine why a venous wound might not heal on a cellular level. For a physician, the implications of this research provide a rational biologic basis for debridement of venous ulcers to remove the skin cells that do not have the ability to migrate properly, leaving those that do.

Taken together, the data collected from this microarray study suggest that, on the one hand, keratinocytes at the non-healing edges of venous ulcers express activation markers, show deregulation of a cell cycle associated genes, and loss of cell cycle control. On the other hand, they show deregulation of early and late differentiation markers, TJ and desmosomal proteins as well as regulators of differentiation and perform aberrant cornified envelope assembly. Thus, keratinocytes at the non-healing edges of venous ulcers do not successfully complete either of the two possible pathways in a proper manner: proliferation or differentiation. Instead, keratinocytes are constantly trying, without success, to achieve either of the two processes.

Acknowledgements

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