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ORIGINAL PAPER

Identification of biomarkers involved in differential profiling of hypertrophic and keloid scars versus normal skin

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Abstract Among raised dermal scar types, keloid (KS) and hypertrophic scars (HS) are considered to present clinical similarities, but there are no known specific biomarkers that allow both scar types to be easily distinguished. Development and progression of raised dermal scars comprises the activation of several molecular pathways and cell defence mechanisms leading to elevated extracellular matrix component synthesis, delayed apoptosis, altered migration and differentiation. Therefore, the aim here was to identify biomarkers that may differentiate between KS and HS compared to normal skin (NS). To achieve this aim, NS ($n = 14$), KS ($n = 14$) and HS ($n = 14$) biopsies were evaluated using histology by H&E staining. Tissue biopsies and primary fibroblasts (passages 0–4) were employed to assess the gene expression levels of 21 biomarkers selected from our previous microarray studies using qRT-PCR. Finally, protein expression was evaluated using In-Cell Western Blotting in primary fibroblasts (p 0–4). Our results demonstrated that out of the 21 biomarkers screened at mRNA and protein levels, α2β1-integrin, Hsp27, PAI-2, MMP-19 and CGRP showed significantly higher expression ($p < 0.05$) in KS compared to NS and HS. Additionally, these five key biomarkers were found to be significantly higher ($p < 0.05$) at mRNA level in KS taken from the sternum, a region known to be subjected to high mechanical forces in the body during the performance of daily movements. In conclusion, our findings offer potential molecular targets in raised dermal scars differentiation. Future targeted research may allow provision of diagnostic and prognostic markers in keloid versus hypertrophic scars.

Keywords Raised dermal scars · Hypertrophic scars · Keloid scars · Biomarkers · Differential profiling

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Introduction

Cutaneous wound healing is a complicated, multistep process that involves the combination of several molecular, cellular, physiological, biochemical and mechanical factors [9, 13]. Abnormal wound healing occurs as a result of perturbation of the intricate balance during the process of repair. Environmental factors such as the nature of injury, severity, depth of injury, anatomical location, tensional stress, infection, and genetic factors including heritable predisposition to scarring, sex and hormone levels have been proposed to contribute to abnormal skin scar formation [8, 59]. This may result in the formation of raised dermal scars in the form of hypertrophic and keloid scars [28, 58, 59]. Hypertrophic scars (HS) are raised dermal scars that

remain within the boundary of the original injury, and can regress over time [8, 25]; whereas keloid scars (KS) are raised dermal lesions that spread beyond the boundaries of the original wound and invade the healthy surrounding tissue [7, 58].

To date, no mechanisms have been identified that clearly and comprehensively explain raised dermal scar formation and differentiation. It has been proposed that the development and progression of raised dermal scars comprises the activation of several molecular pathways and cell defence mechanisms that can lead to elevated extracellular matrix (ECM) component synthesis, delayed apoptosis, altered migration and differentiation [1, 25, 50, 51, 71]. The proteins and signalling pathways activated during pathologic scarring processes include neuropeptides, chaperones, cytokines and chemokines among other proteins [13, 25, 29, 31–34, 63].

The aim of the present study was to characterise the expression of target biomarkers involved in the differentiation of HS and KS. To this end, potential biomarkers (genes and proteins) were identified according to the following criteria. Firstly, a correlation between different microarray studies available to date was performed, and the up-regulated biomarkers in HS or KS with respect to normal tissue were selected and summarised in Tables 1 and 2, respectively; whereas Table 3 summarises all those genes that were found commonly up-regulated in both scar types after microarray comparisons performed by Shih et al. [60] and Huang et al. [32]. These three previously mentioned lists of biomarkers were compared with our own unpublished microarray data which was derived from KS tissue. The biomarkers that were highly statistically significant and which were shared among these datasets were included. The second criteria employed for the biomarker selection was based on previous studies on both scar types which highlighted genes, and molecular pathways (immunological dysregulation, genetic predisposition, neurogenic inflammation and mechano-transduction among others) [2, 21, 26, 35, 36, 43, 49, 55, 61] in HS or KS found in at least three independent studies. The final selection criteria employed was based on the biological relationship between potential target biomarkers identified from the above two approaches which were linked to both fibrosis and apoptosis. Candidate biomarkers ($n = 21$) were selected for evaluation in both tissue and cells extracted from HS and KS.

Table 1 List of biomarkers names and symbols

Gene symbol	Gene name	Different expression in study
COL5A2	Collagen, type V, alpha 2	Wu et al. [71], Tsou et al. [67]
COL6A2	Collagen, type VI, alpha 2	Paddock et al. [51], Tsou et al. [67]
HTRA1	HtrA serine peptidase 1	Wu et al. [71], Paddock et al. [51]
PTN	Pleiotrophin	Wu et al. [71], Paddock et al. [51], Tsou et al. [67]
QPRT	Quinolinate phosphoribosyltransferase	Dasu et al. [25]
PAI-2	Serine (or cysteine) proteinase inhibitor, clade B	Dasu et al. [25]
COL13A1	Collagen, type XIII α-1	Dasu et al. [25]
uPA	Plasminogen activator, urokinase	Dasu et al. [25]
NmU	Neuromedin	Dasu et al. [25]
LGALS3	Lectin, galactose binding protein 3	Dasu et al. [25]
NAGPA	*N*-Acetylglucosamine-I-phosphodiester-α-*N*-acetylglucosaminidase	Dasu et al. [25]
SECTM1	Secreted and transmembrane I	Dasu et al. [25]
BDKRB2	Bradykinin receptor B2	Dasu et al. [25]
HMOX1	HEME oxygenase (decycling) I	Dasu et al. [25]
IER3	Immediate early response 3	Dasu et al. [25]
FAS	Fatty acid synthase	Dasu et al. [25]
ACAN	Aggrecan	Paddock et al. [51]
COL1A1	Collagen type I alpha 1	Paddock et al. [51]
COMP	Cartilage oligomeric matrix protein	Paddock et al. [51]
TSP-4	Thrombospondin 4	Paddock et al. [51]
MEGF6	Epidermal growth factor-like-domain, multiple 3	Wu et al. [71]
COL11A1	Collagen, type XI, alpha 1	Wu et al. [71]
A2AAR	Adenosine A2a receptor	Wu et al. [71]
COL6A2	Collagen, type VI, alpha 2	Wu et al. [71]

Table 2 Demographic data of the tissue samples

Gene symbol	Gene name	Different expression in study
ACAN	Aggrecan	Seifert et al. [57], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
ANXA1	Annexin A1	Seifert et al. [57], Hu et al. [30], Huang et al. [32], Shih et al. [60]
C5ORF13	Chromosome 5 open reading frame	Smith et al. [62], Hu et al. [30], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
COL1A1	Collagen, type I, alpha	Seifert et al. [57], Naitoh et al. [45], Chen et al. [20], Huang et al. [32], Shih et al. [60]
COL4A1	Collagen, type IV, alpha	Seifert et al. [57], Satish et al. [54], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
COL5A2	Collagen, type V, alpha	Seifert et al. [57], Chen et al. [20], Huang et al. [32], Shih et al. [60]
COL11A1	Collagen, type XI, alpha	Hu et al. [30], Naitoh et al. [45], Chen et al. [20], Huang et al. [32], Shih et al. [60]
DCN	Decorin	Hu et al. [30], Chen et al. [20], Huang et al. [32], Shih et al. [60]
FAP	Fibroblast activation protein, alpha	Seifert et al. [57], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
FN1	Fibronectin	Seifert et al. [57], Satish et al. [54], Naitoh et al. [45], Chen et al. [20], Huang et al. [32], Shih et al. [60]
IGF2	Insulin-like growth factor 2 (somatomedin A)	Smith et al. [62], Hu et al. [30], Huang et al. [32], Shih et al. [60]
IGFBP7	Insulin-like growth factor binding protein	Smith et al. [62], Seifert et al. [57], Hu et al. [30], Huang et al. [32], Shih et al. [60]
JAG1	Jagged 1 (Alagille syndrome)	Smith et al. [62], Hu et al. [30]
NGF	Nerve growth factor (beta polypeptide)	Smith et al. [62], Chen et al. [20], Huang et al. [32], Shih et al. [60], Huang et al. [32], Shih et al. [60]
OGN	Osteoglycin	Smith et al. [62], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	Seifert et al. [57], Hu et al. [30], Huang et al. [32], Shih et al. [60]
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	Hu et al. [30], Naitoh et al. [45], Huang et al. [32], Shih et al. [60]
TGFB1	TGF, beta 1	Seifert et al. [57], Chen et al. [20], Huang et al. [32], Shih et al. [60]
VCAN	Versican	Seifert et al. [57], Naitoh et al. [45], Chen et al. [20], Huang et al. [32], Shih et al. [60]

Methods

The study was undertaken in three parts: (1) histological analysis of tissue specimens taken from normal skin (NS), KS and HS; (2) gene expression screening in NS, KS and HS tissue samples; and (3) gene and protein expression screening in primary fibroblast cultures established from NS, KS and HS biopsies. All samples employed in the study were obtained from biopsies taken from different anatomical locations. All HS and KS samples were confirmed to have clinical and pathological evidence of raised dermal scarring in the form of HS and KS as previously described by Syed et al. [65]. Haematoxylin and eosin staining was employed to enable histological comparison of the different skin scar tissue specimens. Gene expression was evaluated in the tissue samples using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Primary normal skin, keloid, hypertrophic fibroblast cultures form passages 0–4 were established from tissue samples collected and employed to evaluate gene and protein expressions by qRT-PCR and in-cell western blotting, respectively.

Biomarkers (genes or proteins) were identified for inclusion in our study based on a number of criteria. Firstly, candidate biomarkers that had demonstrated high expression in at least three previous microarray studies in humans and provided detailed results of the gene expression evaluation, in addition to unpublished in-house microarray performed in KS were included [7, 15, 20, 25, 30, 40, 45, 51, 54, 57, 64, 67, 71]. Secondly, genes that had been identified as being associated with wound healing processes were chosen. Studies concerning key molecules and pathways investigated in both KS and HS were also included if evidence of up-regulation of the aforementioned molecules was found in more than three independent studies. Finally, candidate genes implicated in fibrotic and apoptotic processes were selected [5, 7, 15, 42, 63]. Use of the above strict criteria resulted in the generation of a list of 21 biomarkers which included neuropeptides, tension-related (Hsps, MMPs and MCPs), ECM-related and

Table 3 List of antibodies used in this study

Gene symbol	Gene name	Different expression in study
A2M	Alpha-2-macroglobulin	Huang et al. [32], Shih et al. [60]
ACAN	Aggrecan	Huang et al. [32], Shih et al. [60]
BMP6	Bone morphogenetic protein 6	Huang et al. [32]
C5ORF13	Chromosome 5 open reading frame	Huang et al. [32], Shih et al. [60]
CALD1	Caldesmon 1	Huang et al. [32]
CALU	Calumenin	Huang et al. [32]
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	Huang et al. [32]
COL10A1	Collagen, type X, alpha 1	Huang et al. [32]
COL1A1	Collagen, type I, alpha 1	Huang et al. [32], Shih et al. [60]
COL1A2	Collagen, type I, alpha 2	Huang et al. [32], Shih et al. [60]
COL3A1	Collagen, type III, alpha 1	Huang et al. [32]
COL4A5	Collagen, type IV, alpha 5	Huang et al. [32]
COL5A1	Collagen, type V, alpha 1	Huang et al. [32], Shih et al. [60]
COL5A2	Collagen, type V, alpha 2	Huang et al. [32], Shih et al. [60]
COL6A1	Collagen, type VI, alpha 1	
COMP	Cartilage oligomeric matrix protein	Huang et al. [32]
CTGF	Connective tissue growth factor	Huang et al. [32]
DCN	Decorin	Huang et al. [32]
FAM3C	Family with sequence similarity 3, member C	Huang et al. [32]
FN1	Fibronectin 1	Huang et al. [32], Shih et al. [60]
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Huang et al. [32], Shih et al. [60]
HTRA1	Htra serine peptidase 1	Huang et al. [32]
IFI16	Interferon, gamma-inducible protein 16	Huang et al. [32]
IFITM2	Interferon induced transmembrane protein 2 (1–8D)	Huang et al. [32]
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	Huang et al. [32]
INHBA	Inhibin, beta A	Huang et al. [32]
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Huang et al. [32]
ITGB5	Integrin, beta 5	Huang et al. [32]
LUM	Lumican	Huang et al. [32]
MARCKS	Myristoylated alanine-rich protein kinase C substrate	Huang et al. [32]
MGST3	Microsomal glutathione S-transferase 3	Huang et al. [32]
MMP14	Matrix metallopeptidase 14 (membrane-inserted)	Huang et al. [32]
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	Huang et al. [32]
POSTN	Periostin, osteoblast specific factor	Huang et al. [32], Shih et al. [60]
SEPTIN7	Septin 7	Huang et al. [32]
SFRP2	Secreted frizzled-related protein 2	Huang et al. [32]
SOX11	SRY (sex-determining region Y)-box 11	Huang et al. [32]
THBS4	Thrombospondin 4	Huang et al. [32]
TIMP1	TIMP metallopeptidase inhibitor 1	Huang et al. [32]
TMSB10	Thymosin beta 10	Huang et al. [32]
VCAN	Versican	Huang et al. [32], Shih et al. [60]
VEGFA	Vascular endothelial growth factor A	Huang et al. [32]
EGFR	Epidermal growth factor receptor	Shih et al. [60]
HDGF	Hepatoma-derived growth factor	Shih et al. [60]
SERPINF1	Serpin peptidase inhibitor, clade F	Shih et al. [60]
POSTN	Periostin, osteoblast specific factor	Shih et al. [60]
KRT19	Keratin 19	Shih et al. [60]

Table 4 Molecules strongly express in raised dermal scar (summary)

Gene symbol	Gene name
Neuropeptides	
CALCA, CGRP	Calcitonin-related polypeptide alpha
NPY	Neuropeptide Y
TAC1, SP	Substance-P tachykinin, precursor 1
VIP	Vasoactive intestinal peptide
Tension related	
Hsp 27, HSPB1	Heat shock protein 27
Hsp 47, SERPINH1	Serpin peptidase inhibitor, clade H; Heat shock protein 47
HSPD1; Hsp 60	Heat shock protein 60
HSPA1A; Hsp 70	Heat shock protein 70
HSP90AA1; Hsp 90	Heat shock protein 90 kDa alpha (cytosolic), class A member 1; Heat shock protein 90
MCP-3	Monocyte chemotactic protein-3
MCP-1	Monocyte chemotactic protein-1
MMP-13	Matrix metallopeptidase 13; collagenase 3
MMP-19	Matrix metallopeptidase 19
MMP-3	Matrix metallopeptidase 3; Stromelysin 1, progelatinase
SERPINB2; PAI-2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
ECM related	
ITGA2	α2β1-Integrin
Cyr61	Cysteine-rich, angiogenic inducer, 61
TNXB	Tenascin XB
Growth factor	
TGF-β1	Transforming growth factor, beta 1
TGF-β2	Transforming growth factor, beta 2
TGF-β3	Transforming growth factor, beta 3

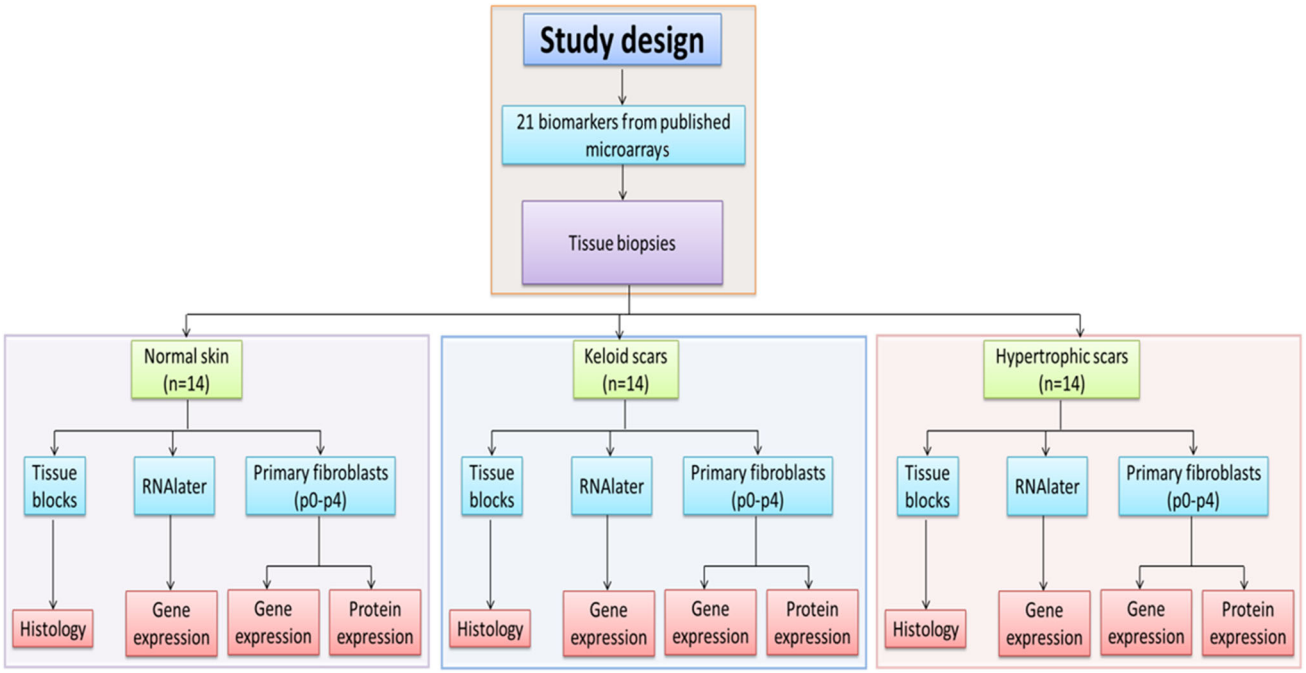

Fig. 1 Study design

cytokines genes. A list of the 21 biomarkers investigated is given in Table 4. In addition, the experimental design of the study is depicted in Fig. 1.

Patient data and tissue collection

Normal skin (NS) ($n = 14$), keloid (KS) ($n = 14$) and hypertrophic skin (HS) ($n = 14$) samples were employed in this study. Samples were taken from a number of different sites including the sternum, ear, pubis and scalp (see Table 5). Samples were obtained following informed consent from all patients (full ethical approval was obtained from the local hospital, University and regional NHS Ethics Committee in England, UK) prior to surgery. At the time of surgical excision, tissue biopsies from each lesional site were collected and processed as previously described by Syed et al. [65]. The samples were processed for cell culturing within 12 h.

Primary fibroblast culture establishment

The tissue was collected in Dulbecco's modified Eagle medium (Sigma-Aldrich, Dorset, UK) and fibroblast cultures were extracted using collagenase type I solution, 0.5 mg mL^{-1} (Roche Diagnostics, West Sussex, UK) and grown as described previously in Syed et al. [65] and Suarez et al. [63]. Fibroblasts from passages 0–4 were employed for the experiments.

Haematoxylin and eosin staining (H&E)

Sections of 5 μm thickness from tissue specimens, which had previously been embedded in paraffin blocks and fixed in formaldehyde, were stained with haematoxylin and eosin (H&E) (Surgipath, Peterborough, UK) for histological evaluation. Tissue samples were first stained for nuclei with iron haematoxylin (Sigma-Aldrich, Dorset, UK) for 10 min. After incubation, three washes with top water were performed. A subsequent staining employing van Gieson (Sigma-Aldrich, Dorset, UK) was then performed for 10 min. Tissue sections were washed in 1 % acetic acid for 1 min and dehydrated in graded ethanol (95 and 100 %), treated with xylene for clearing and mounted. More details about the procedure can be found at Syed et al. [65].

RNA extraction, cDNA synthesis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was extracted from cells and tissue biopsies and cDNA synthesis and qRT-PCR were performed as previously described in Shih et al. [60] and Syed et al. [65]. The primer list employed can be found in Suarez et al. [63]. Each reaction was performed in triplicate. The gene expression levels were normalised with respect to a reference gene, the L32 ribosomal protein gene (RPL32).

Table 5 List of genes common up-regulated in hypertrophic scars from published microarray studies

Study ID	Gender	Ethnicity	Age (years)	Site	Age of scar (years)
KS 1	F	Black	30	Ear	3
KS 2	F	Black	37	Ear	2
KS 3	F	Asian	27	Sternum	4
KS 4	F	White	18	Sternum	15
KS 5	M	Black	45	Multiple	1
KS 6	M	Black	42	Scalp	5
KS 7	F	Black	20	Sternum, pubis	9
KS 8	M	Asian	74	Sternum	11
KS 9	F	Black	33	Pubis	2
KS 10	M	Black	42	Multiple	3
KS 11	F	White	29	Ear	10
KS 12	M	Black	36	Scalp	5
KS 13	F	Black	19	Ear	5
KS 14	M	Black	22	Ear	1
HS1	F	Black	16	Ear	1
HS2	F	White	35	Ear, sternum	1
HS3	F	White	42	Ear	1
HS4	M	White	38	Multiple	1
HS5	F	White	20	Multiple	1
HS6	F	White	25	Pubis	1
HS7	F	White	56	Sternum	1
HS8	F	Black	43	Multiple	1
HS9	M	Black	19	Ear	1
HS10	F	White	44	Sternum	1
HS11	M	Asian	25	Sternum	1
HS12	F	Black	42	Multiple	1
HS13	M	White	20	Ear	1
HS14	M	Black	36	Scalp	1
NS 1	F	White	17	Scalp	2
NS 2	M	White	33	Pubis	2
NS 3	F	White	47	Pubis	6
NS 4	F	White	39	Scalp	3
NS 5	F	White	35	Pubis	4
NS 6	F	Black	24	Sternum	2
NS 7	F	Black	61	Sternum	4
NS 8	F	Asian	29	Multiple	6
NS 9	F	White	34	Ear	5
NS 10	F	Black	25	Sternum	7
NS 11	F	Black	15	Scalp	2
NS 12	F	Black	30	Ear	2
NS 13	F	Black	37	Ear	3
NS 14	F	Asian	27	Sternum	9

Table 6 List of genes common up-regulated in keloid scars from published microarray studies

Antibody	Raised species	Isotype	Clone	Dilution	Product code	Source
Hsp 27	Mouse monoclonal	IgG1	–	1–500	mAb 2402	Cell signalling technology
α2β1 Integrin	Mouse monoclonal	IgG1	16B4	1–500	Ab30483	Abcam
MMP-19	Rabbit polyclonal	IgG	–	1–500	Ab39002	Abcam
PAI-2	Rabbit polyclonal	IgG	–	1–500	Ab47742	Abcam
Sustance P (SP)	Mouse monoclonal	IgG1	SP-DE4-21	1–500	Ab 14184	Abcam
Neurocakinin (NYP)	Rabbit polyclonal	IgG	–	1–250	Ab 48598	Abcam
vasoactive intestinal polypeptide (VIP)	Rabbit polyclonal	IgG	–	1–500	Ab22736	Abcam
B actin				1–200		
collagen I	Rabbit polyclonal	IgG	–	1–500	Ab59435	Abcam
Fibronectin	Rabbit polyclonal	IgG	–	1–200	ab2413	Abcam
PCNA	Mouse monoclonal	IgG2a	–	1–100	mAb 2586	Cell signalling technology
FAP-a				1–200		
NFK-B p65	Rabbit polyclonal	IgG	–	1–500	ab16502	Abcam
NFK-Bp100	Rabbit polyclonal			1–500		
CTGF	Rabbit polyclonal	IgG	–	1–250	Ab6992	Abcam
Vinculin	Mouse monoclonal	IgG1	hVIN-1	1–500	Ab11194	abcam
α-SMA	Mouse monoclonal	IgG2a	1A4	1–1,000	A5691	Sigma Aldrich
Tenesin	Mouse monoclonal	IgG1	BC-24	1–500	T2551	Sigma Aldrich
Goat anti-mouse secondary antibody	Mouse monoclonal	IgG		1–800	IRDye 800CW	LI-COR
Goat anti-rabbit secondary antibody				1–800	IRDye 800CW	LI-COR

In-cell western blotting (ICW)

p0–p3 fibroblasts were grown to 95–100 % confluence in T25 flasks. Samples were processed as described previously in Syed et al. [65]. The panel of antibodies used is given in Table 6. Data were acquired using the Odyssey software package 2.1 software (LI-COR Biosciences, Cambridge, UK), then exported and analysed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Statistical analysis

Data were expressed as the mean ± SEM of at least three independent experiments. To determinate statistical differences, a one-way nonparametric ANOVA analysis was performed followed by a Tukey multiple comparison post-test using GraphPad Prism 5 software (GraphPad Software, La Jolla, USA). The difference between the means for all conditions was considered statistically significant at $p < 0.05$.

Results

Histological comparison of normal skin with keloid and hypertrophic scars

Haematoxylin and eosin (H&E) stained tissue samples (Fig. 2) revealed that in the normal skin samples, the epidermal layer is well defined and relatively narrow and uniform in thickness compared to the scar tissue samples, which were found to have thicker, less well defined and non-uniform epidermal layers. Of the two scar tissue types analysed, the hypertrophic samples displayed the thickest epidermal layer. In normal skin, the characteristic random orientation and bundle formation of collagen fibres was observed (Fig. 2a). The KS samples exhibited normal epidermis thickness with regular basal cell organisation, an increased number of thick collagen fibres arranged in bundles in the reticular dermis region compared to both NS and HS samples. Collagen fibres were found horizontally arranged to the epidermal layer in most KS cases (Fig. 2b). The analysis of the hypertrophic tissue revealed disarray of basal epidermal cells, and thinner collagen fibres in the dermis. The collagen fibres were arranged randomly with respect to the epidermis and showed highly cellular zones in the reticular dermis (Fig. 2c) in HS tissue. The above are in keeping with similar findings reported previously in other studies [39, 68].

Gene expression screening in normal, keloid and hypertrophic scar tissue

For analysis purposes, target biomarkers were classified into the following four categories:

(a) Neuropeptides, CGRP (calcitonin-related polypeptide alpha), NPY (neuropeptide Y), SP (substance-P

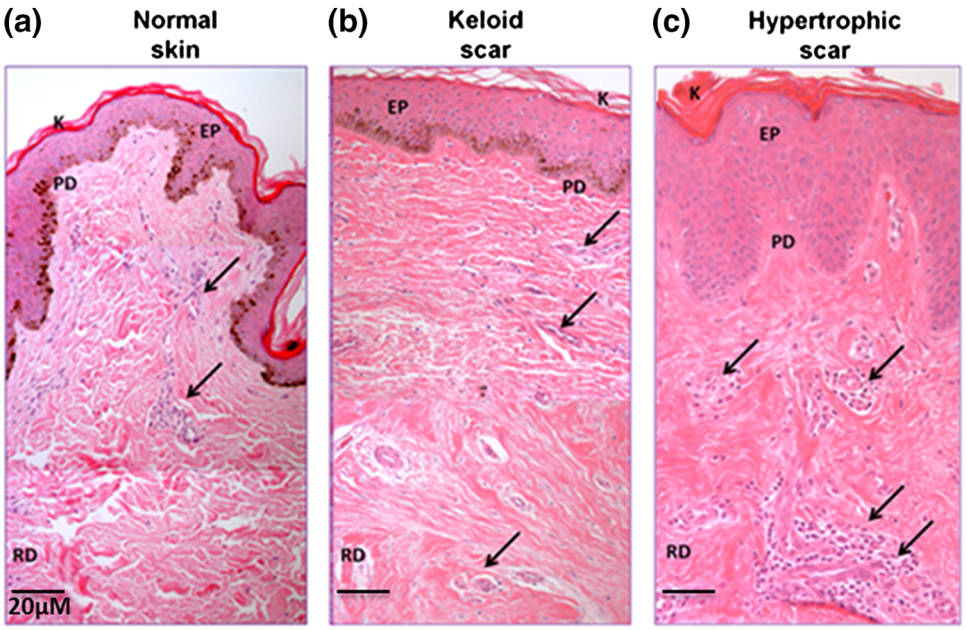

Fig. 2 Histology of normal skin sample compared hypertrophic and keloid scars. A histological comparison of **a** normal skin, **b** keloid scar and **c** hypertrophic scars biopsies employing haematoxylin and eosin (H&E) staining. Significant difference in the collagen fibres arrangement can be observed among the different tissue sections as well as highly cellular populated zones in both keloid and hypertrophic scars in the reticular dermis areas (*black arrows*). Normal skin presented random and relaxed collagen bundles arrangement whereas, keloid and hypertrophic tissue present more stretched and thicker collagen bundles. Abundant cellular islands were found in both the papillary and the reticular dermis of hypertrophic scar tissue. *K* keratin layer, *EP* epidermis, *PD* papillary dermis, *RD* reticular dermis

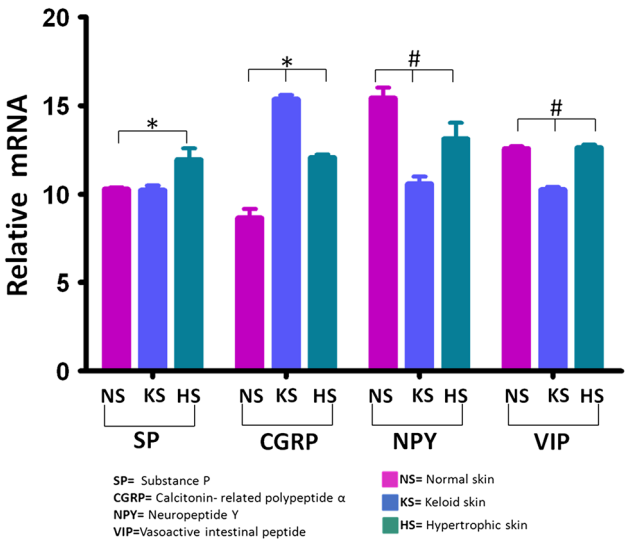

Fig. 3 Tissue mRNA expression of neuropeptides seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. The expression of neuropeptides including SP, CGRP, NPY and VIP was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from raised dermal scar biopsies versus normal skin

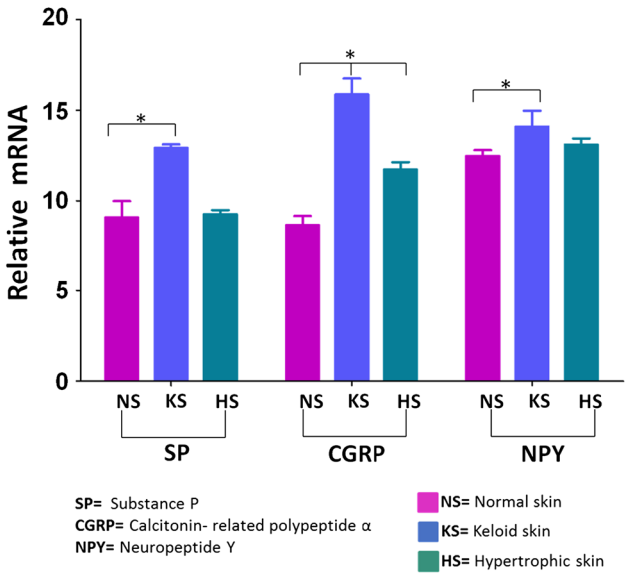

Fig. 4 Cellular mRNA expression of neuropeptides in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. Fibroblasts were cultured from passage 0 (p0) to passage 4 (p4). The expression of neuropeptides including SP, CGRP, NPY and VIP was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens

tachykinin, precursor 1), VIP (vasoactive intestinal peptide);

(b) Tension-related, PAI-2 [serpin peptidase inhibitor, clade B (ovalbumin), member 2]; Hsps, [Hsp27 (heat shock protein 27), Hsp47 (heat shock protein 47), Hsp60 (heat shock protein 60), Hsp70 (heat shock protein 70), Hsp90 (heat shock protein 90)]; MMPs, MMP-3 (matrix metallopeptidase 3), MMP-13 (matrix metallopeptidase 13), MMP19 (matrix metallopeptidase 19) and MCPs, MCP-1 (monocyte chemotactic protein-1), MCP-3 (monocyte chemotactic protein-3);

(c) ECM-related, ITGA2 (α2β1-Integrin), Cyr61 (cysteine-rich, angiogenic inducer, 61) and TNXB (tenascin XB) and

(d) Cytokines, TGF-β1 (transforming growth factor, beta 1), TGF-β2 (transforming growth factor, beta 2) and TGF-β3 (transforming growth factor, beta 3).

Neuropeptides gene expression in tissue biopsies and fibroblasts from keloid and hypertrophic scars

Total RNA was extracted from tissue biopsies and the expression of the neuropeptides SP, CGRP, NPY and VIP was evaluated using qRT-PCR. mRNA levels of neuropeptides detected were significantly different among the scar tissue types tested (Fig. 3). SP expression was significantly higher in HS compared to NS and KS ($p < 0.05$), whereas CGRP mRNA level was notably up-regulated in KS compared to NS and HS ($p < 0.05$). Moreover, mRNA levels were notably down-regulated for the NPY and VIP biomarkers in KS compared to NS and HS samples ($p < 0.05$). To evaluate the neuropeptide expression in fibroblasts, total mRNA was extracted from primary fibroblasts established from NS, KS and HS biopsies. qRT-PCR analysis of the samples (Fig. 4) revealed that of the four biomarkers tested, only CGRP was significantly up-regulated in KS and HS fibroblasts compared to NS

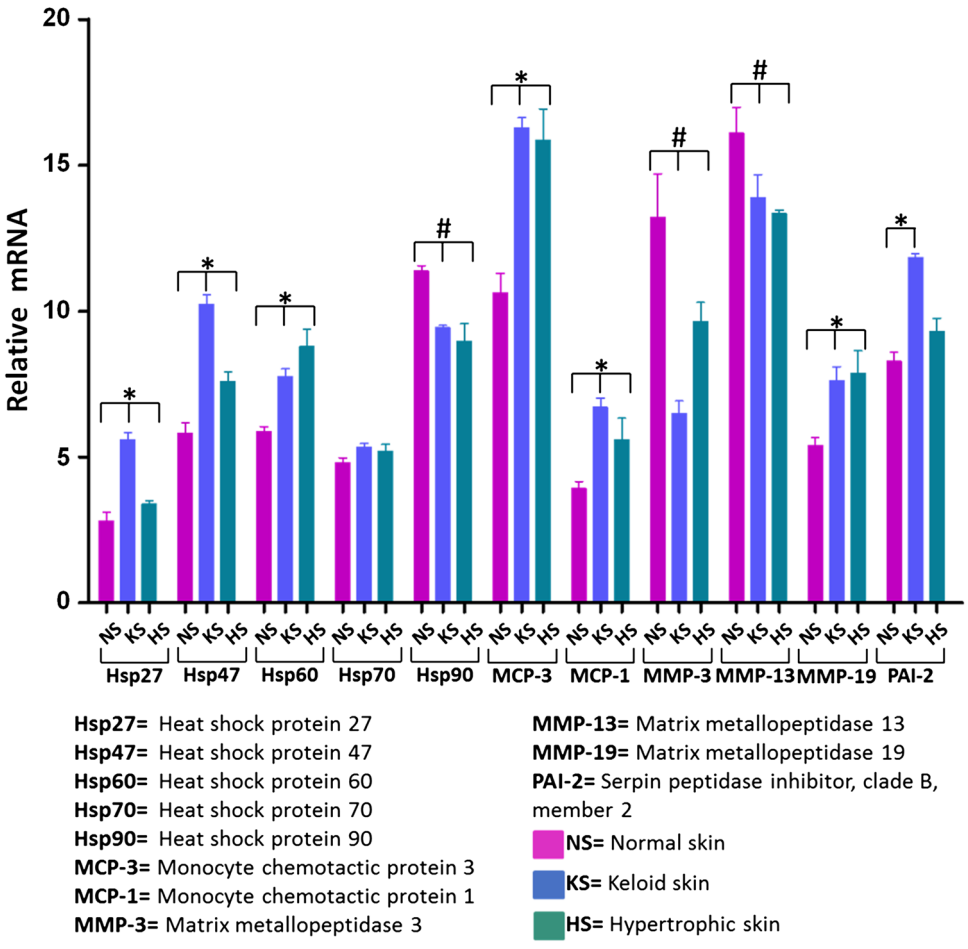

Fig. 5 Tissue expression of mRNA of tension-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. Total RNA extracted from tissue biopsies as described in "Methods" section was employed to evaluate the expression of tension-related proteins and normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

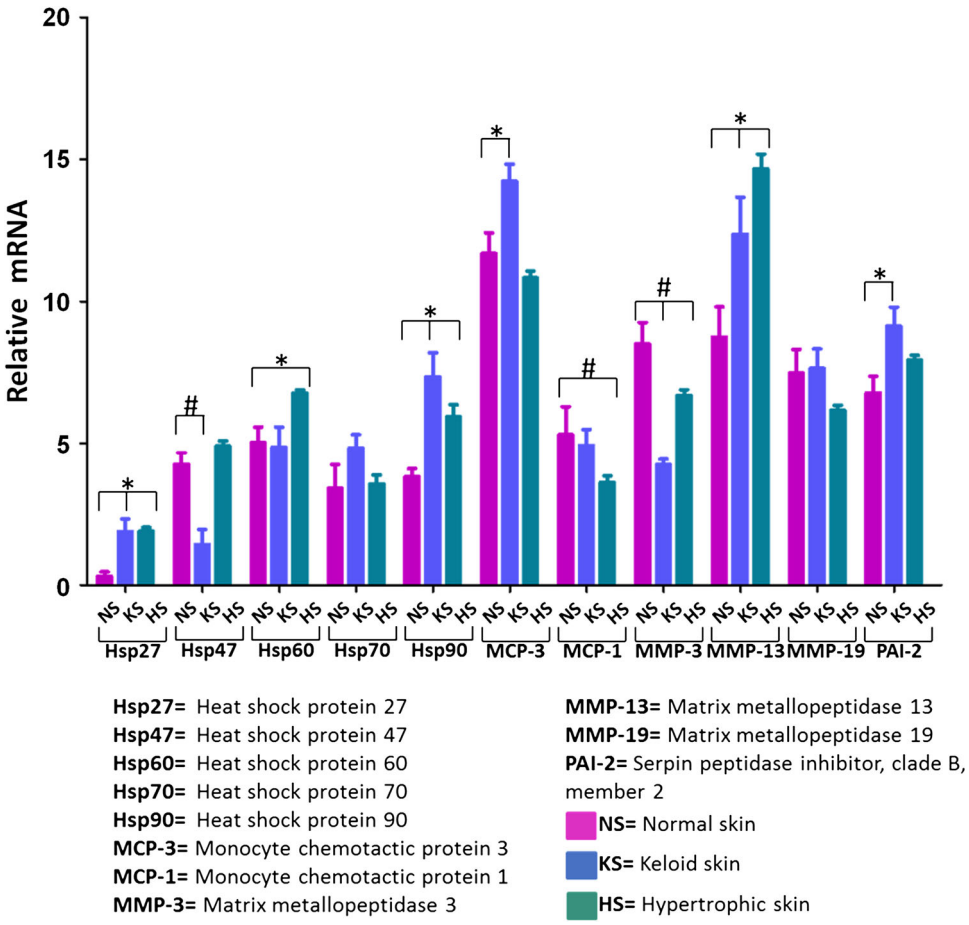

Fig. 6 Cellular mRNA expression for tension-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. Fibroblasts were cultured from p0 to p4. The expression of tension-related genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). *$p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

fibroblasts ($p < 0.001$). No expression of the gene VIP was detected among the samples. Interestingly, the expression of SP and NPY was significantly higher in KS fibroblasts compared to NS ($p < 0.05$).

Characterisation of the mRNA levels of tension-related biomarkers in keloid and hypertrophic scar tissue biopsies and fibroblasts

Figure 5 shows the results of the tension-related gene expression characterisation of tissue biopsies for 11 of the previously identified 21 genes. Of the 11 biomarkers tested in this group, 5 showed significant over-expression at mRNA levels ($p < 0.05$) in both KS and HS when compared to NS samples, these were Hsp47, Hsp60, MCP-3, MCP-1 and MMP-19. In contrast, three genes, Hsp90, MMP-3 and MMP-13, demonstrated lower expression at mRNA levels in both scar types compared to NS. No significant difference was detected in Hsp70 expression among the samples. Hsp27 and PAI-2 were found to be over-expressed in KS ($p < 0.05$). The expression of tension-related biomarkers was also evaluated in primary fibroblast cultures; the results are shown in Fig. 6. At

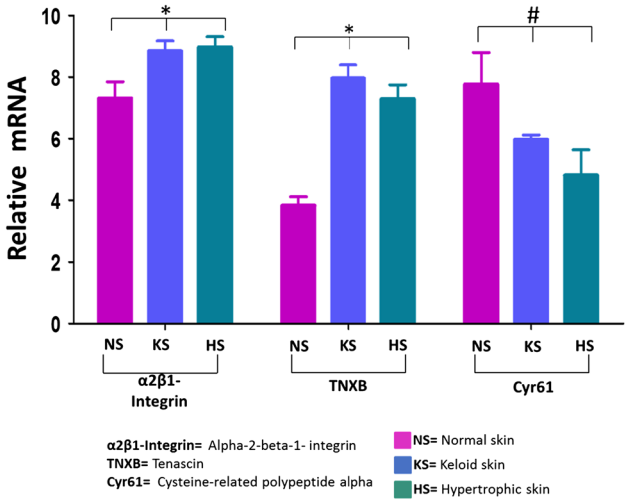

Fig. 7 Tissue mRNA expression of ECM-related seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. The expression of neuropeptides including SP, CGRP, NPY and VIP was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). *$p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

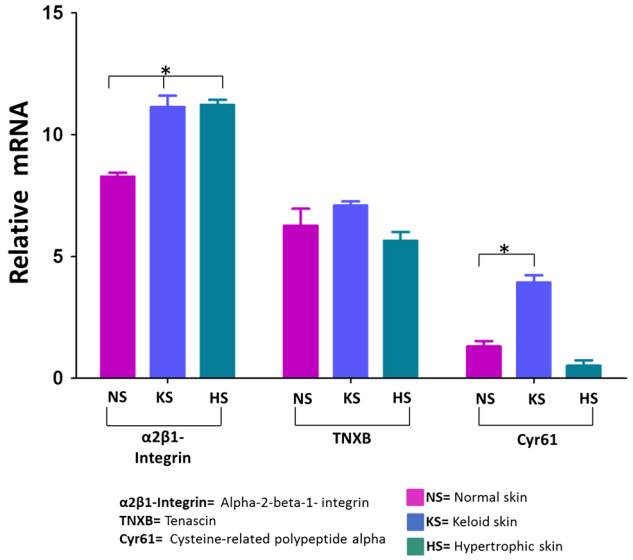

Fig. 8 Cellular mRNA expression for ECM-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. Fibroblasts were cultured from p0 to p4. The expression of tension-related genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

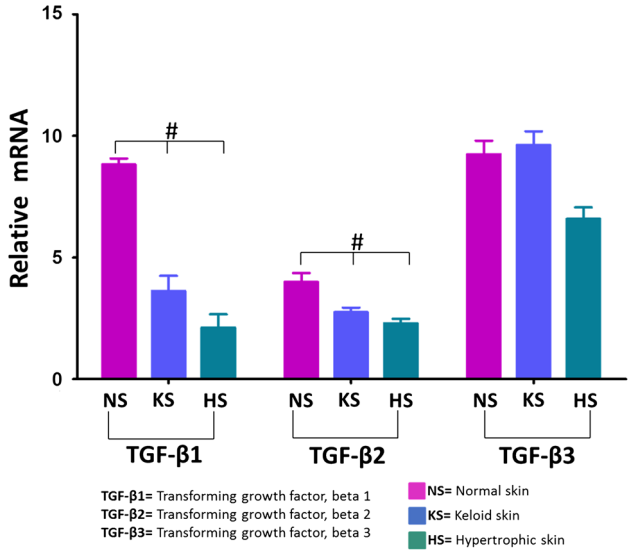

Fig. 9 Tissue mRNA expression of cytokines seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. The expression of neuropeptides including SP, CGRP, NPY and VIP was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

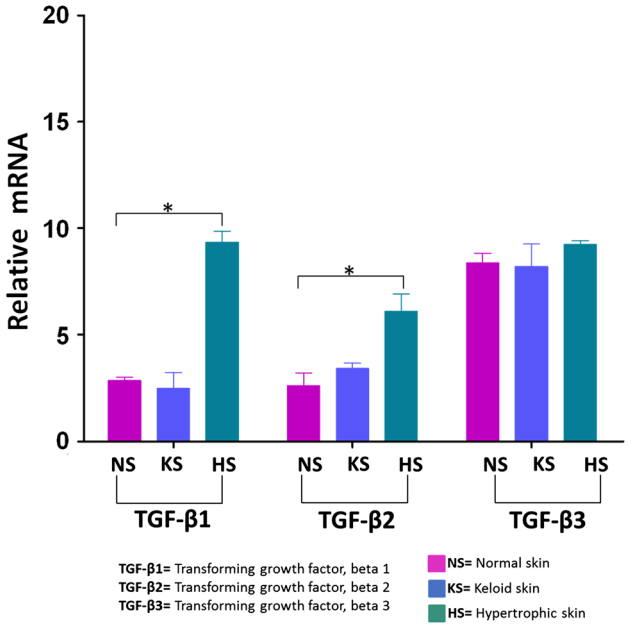

Fig. 10 Cellular mRNA expression for ECM-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR. Fibroblasts were cultured from p0 to p4. The expression of tension-related genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^{*}p < 0.05$ indicates a significantly increased difference between the mRNA expression in tissue biopsy specimens from normal skin ($n = 14$); whereas $^{\#}p > 0.05$ expresses significant reduction on the mRNA expression extracted from dermal scar biopsies versus normal skin

mRNA level, significant difference was found between the raised dermal scar cell samples and NS fibroblasts for the Hsp27, Hsp90, MMP-13 and MMP-3 biomarkers ($p < 0.05$), whereas no significant difference was found among the samples for the Hsp70 and MMP-19 genes. The expression of the MCP-3 and PAI-2 was significantly higher in KS fibroblasts compared to the other samples ($p < 0.05$ and $p < 0.01$, respectively), but the Hsp47 gene exhibited a lower expression in KS fibroblasts. In HS fibroblasts samples the expression of Hsp60 was significantly higher compared to NS fibroblasts ($p < 0.05$), but the expression was lower for the MCP-1 gene.

Expression profile of ECM-related biomarkers in keloid and hypertrophic scar tissue and fibroblasts

The following ECM-related biomarkers, tenascin, α2β1-integrin and Cyr61, were evaluated for their association with KS and HS. The analysis of the mRNA expression of these biomarkers was performed using NS, KS and HS samples. The qRT-PCR results presented in Fig. 7 show a clear over-expression of tenascin and α2β1-integrin in both KS and HS compared to NS ($p < 0.05$), whereas Cyr61 showed lower mRNA levels in both raised dermal scar

types ($p < 0.05$).When we assessed the expression of ECM-related biomarkers in fibroblasts by qRT-PCR, the results showed significantly higher expression levels of α2β1-integrin in both KS and HS fibroblasts ($p < 0.01$) (Fig. 8), whereas no significant difference was found among the samples when tenascin where evaluated. The expression of Cyr61 was found to be significantly up-regulated in KS compared to NS fibroblasts ($p < 0.05$).

Differential expression of cytokines in keloid and hypertrophic scar tissue samples and fibroblasts

The expression of the 3 TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) were evaluated in raised dermal raised scars at mRNA level, using qRT-PCR. The results of the analysis are shown in Fig. 9 where it can be seen that that the expression of TGF-β1 and TGF-β2 is significantly lower in KS and HS when compared to NS ($p < 0.05$). However, no statistical difference was found in the expression of TGF-β3 among the study samples, whereas, the expressions of the TGF-β1 and TGF-β2 isoforms were found to be significantly up-regulated in HS scars when compared to NS fibroblasts ($p < 0.05$) (Fig. 10). No significant difference was found in the expression of TGF-β3 among the samples considered.

Characterisation of neuropeptide protein expression in keloid and hypertrophic scar fibroblasts

To further investigate our aim of identifying target biomarkers involved in the regulation of raised dermal scar development and differentiation, the protein expression of the four neuropeptides, SP, CGRP, NPY and VIP, was evaluated in primary fibroblast cultures by In-Cell Western Blotting. The results of the analysis are shown in Fig. 11, where upon inspection, it can be seen that SP and CGRP were significantly expressed higher in KS fibroblasts compared to NS fibroblasts ($p < 0.05$), whereas the NYP protein expression was significantly higher in HS compared to NS fibroblast ($p < 0.05$). In addition, the protein levels of VIP were significantly lower in HS fibroblasts ($p < 0.05$).

Characterisation of the protein expression of tension-related biomarkers in keloid and hypertrophic scar fibroblasts

Based on the results of our qRT-PCR analysis in keloid and hypertrophic fibroblasts, 4 of the 11 biomarkers included in this group, Hsp27, Hsp47, MMP-19 and PAI-2,

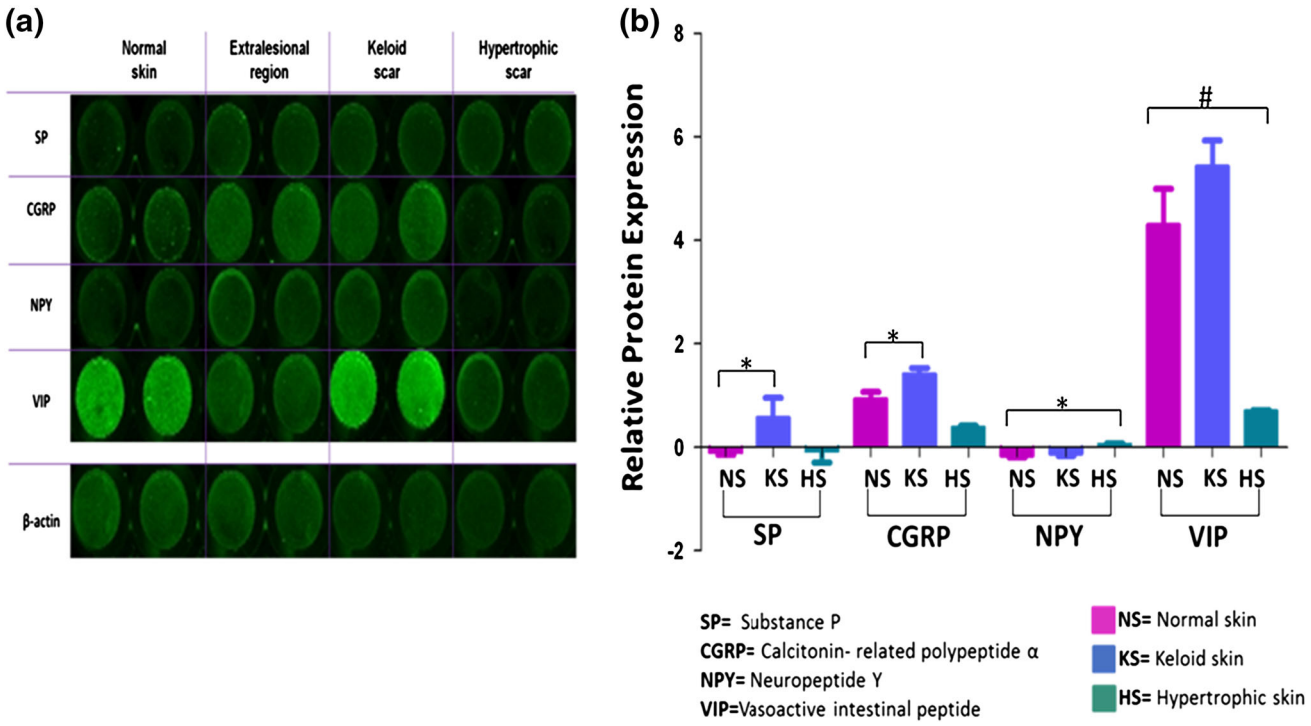

Fig. 11 Cellular protein levels of neuropeptides in keloid and hypertrophic scar compared to normal skin evaluated by in-cell western blotting. Fibroblasts were cultured from p0 to p4. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (*green*) from 96-well plates is shown in **a**. **b** The *bar graphs* represent the quantification of the expression of the analysed proteins normalised to the β-actin loading control; the result is expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ indicates significant up-regulation of the neuropeptides expression when compared to normal skin fibroblasts, while $^{\#}p < 0.05$ can be appreciated as significant down-regulation of the neuropeptides expression from dermal scar cells versus normal skin fibroblasts ($n = 14$)

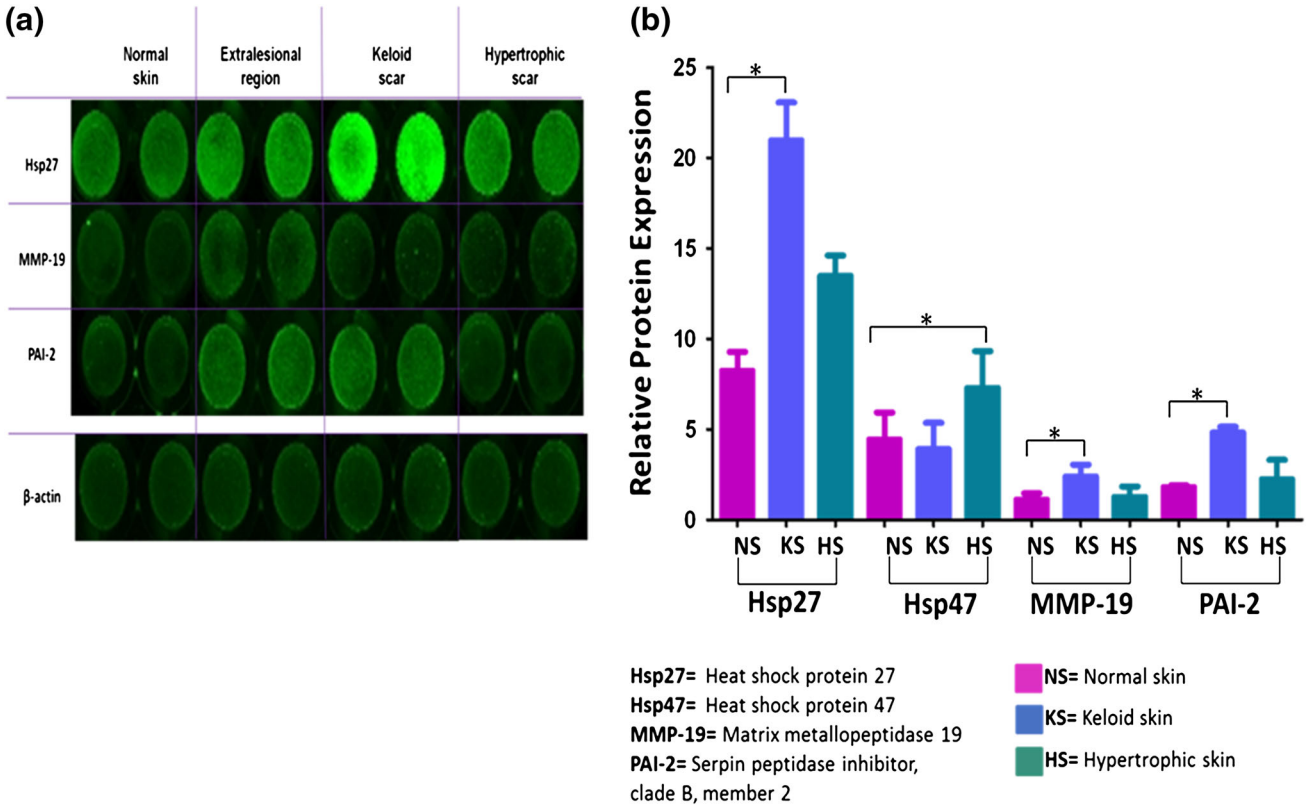

Fig. 12 Cellular protein levels of tension-related in keloid and hypertrophic scar compared to normal skin evaluated by in-cell western blotting. Fibroblasts were cultured from p0 to p4. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (*green*) from 96-well plates is shown in **a**. **b** The *bar graphs* represent the quantification of the expression of the analysed proteins normalised to the β-actin loading control; the result is expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ indicates significant up-regulation of the tension-related biomarkers expression when compared to normal skin fibroblasts, while $^{\#}p < 0.05$ can be appreciated as significant down-regulation of the tension-related biomarkers from dermal scar cells versus normal skin fibroblasts ($n = 14$)

representative of the range of results reported previously, were chosen for protein expression evaluation in primary fibroblast cultures. The results are shown in Fig. 12 and indicate that Hsp27, MMP-19 and PAI-2 protein levels were significantly higher in KS compared to NS and HS fibroblasts ($p < 0.05$), whereas Hsp47 protein levels were significantly higher in HS fibroblasts compared to NS fibroblasts ($p < 0.05$).

Protein expression of ECM-related proteins in primary fibroblast from keloid and hypertrophic scars

Figure 13 shows the differences in the protein levels among the cell samples. Fibronectin, α-SMA and vinculin protein expression levels were found to be significantly higher in both raised dermal scar cell types ($p < 0.05$). α2β1-integrin and collagen I were strongly expressed in KS fibroblasts compared to NS cells ($p < 0.05$).

Characterisation of cytokines protein expression in keloid and hypertrophic scar fibroblasts

As can be seen from the results of the in-cell western blotting assay shown in Fig. 14, TGF-β2 protein levels were significantly higher in both KS and HS fibroblasts compared to NS cells ($p < 0.05$). Furthermore, TGF-β1 was up-regulated in KS fibroblasts compared to the rest of the samples ($p < 0.05$), whereas TGF-β3 expression was lower in HS fibroblasts ($p < 0.05$).

Gene expression in keloid scar tissue samples and fibroblasts analysed by anatomical location

Figure 15 shows the gene expression for five molecules, α2β1-integrin, Hsp27, PAI-2, MMP-19 and CGRP, which our analysis showed were significantly up-regulated in KS tissue and fibroblasts. The results are presented based on the location from which the scar tissue samples and cells

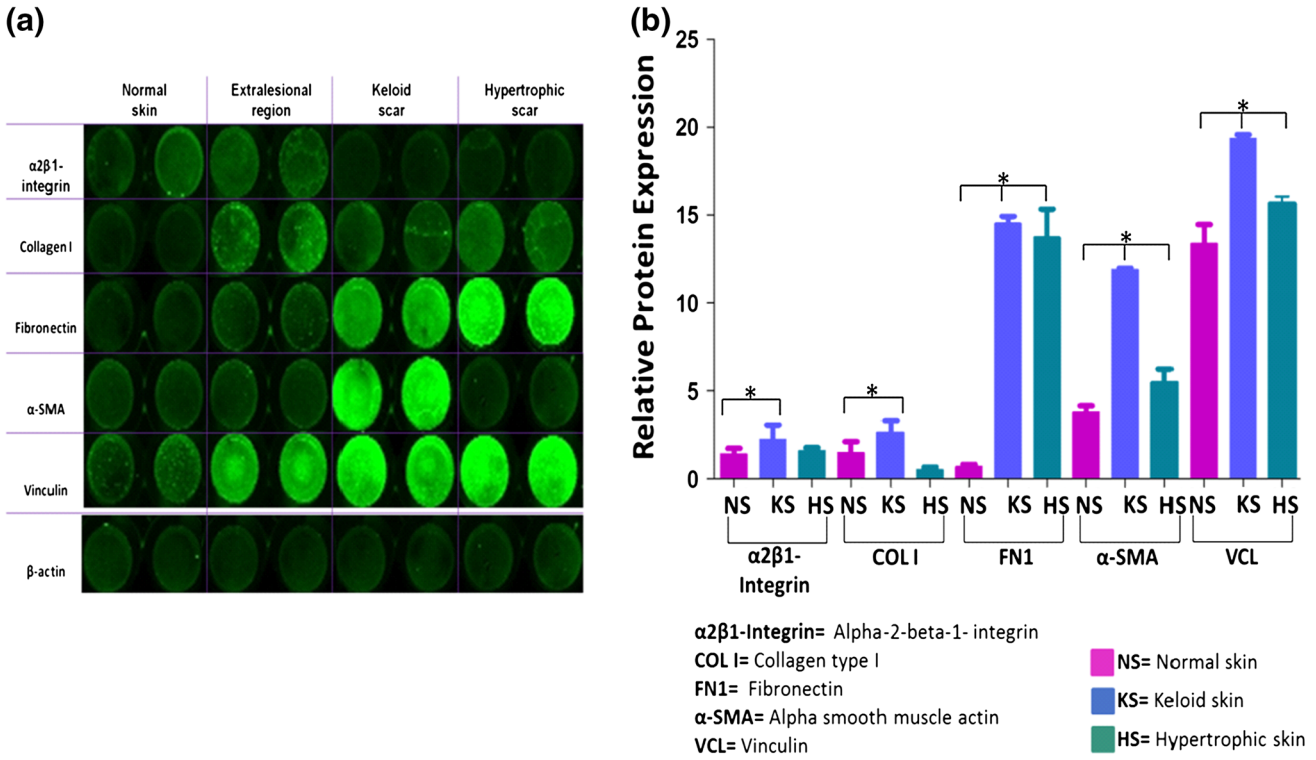

Fig. 13 Cellular protein levels of ECM-related in keloid and hypertrophic scar compared to normal skin evaluated by in-cell western blotting. Fibroblasts were cultured from p0 to p4. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (*green*) from 96-well plates is shown in **a**. **b** The *bar graphs* represent the quantification of the expression of the analysed proteins normalised to the β-actin loading control; the result is expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ indicates significant up-regulation of the ECM-related biomarkers expression when compared to normal skin fibroblasts, while $^{\#}p < 0.05$ can be appreciated as significant down-regulation of the ECM-related biomarkers from dermal scar cells versus normal skin fibroblasts ($n = 14$)

were collected. It can be seen upon inspection of Fig. 15 that the highest expression for the five biomarkers shown in both tissue and cells was found to be in the samples taken from the sternum ($p < 0.05$).

Discussion

In the present study, a list of 21 candidate biomarkers was selected following an extensive literature review in addition to using our own unpublished microarray data. We analysed for the first time, mRNA levels in tissue and primary fibroblasts as well as protein levels in primary fibroblasts obtained from KS and HS. The findings, summarised in Table 7, provide a list of candidate biomarkers that may be involved in the development and differentiation of HS and KS.

We identified five potential biomarkers including CGRP, Hsp27, MMP-19, PAI-2 and α2β1-integrin that were selected on the basis of consistent up-regulation at both mRNA (tissue and cells) and protein (cells) levels in KS compared to HS and NS. However, there was a lack of consistency in expression levels (mRNA and protein) in tissue and cells from hypertrophic scar tissue samples. Several discrepancies in the patterns of expression of proposed biomarkers were found among the samples used in this study, although this was not totally unexpected as this phenomenon had been identified and discussed by other investigators [6, 49, 69, 70]. Indeed, Shih et al. [60], demonstrated that transcriptomic data obtained from primary fibroblast cultures do not always correlate exactly to their respective tissue biopsy sample data. It has also been suggested that culturing conditions exert a profound impact on gene expression levels [24]; and the passage number during culturing could significantly affect gene and protein expression levels in primary fibroblast cultures [10, 62, 65]. The five biomarkers found to be consistently up-regulated in KS are strongly associated with mechanical tension [2, 11, 12, 22].

In addition, our results obtained at mRNA level in both tissue and fibroblasts from KS were analysed by scar anatomical location. This was carried out in order to better define gene expression differences seen in specific anatomical sites. The expression of the five target biomarkers

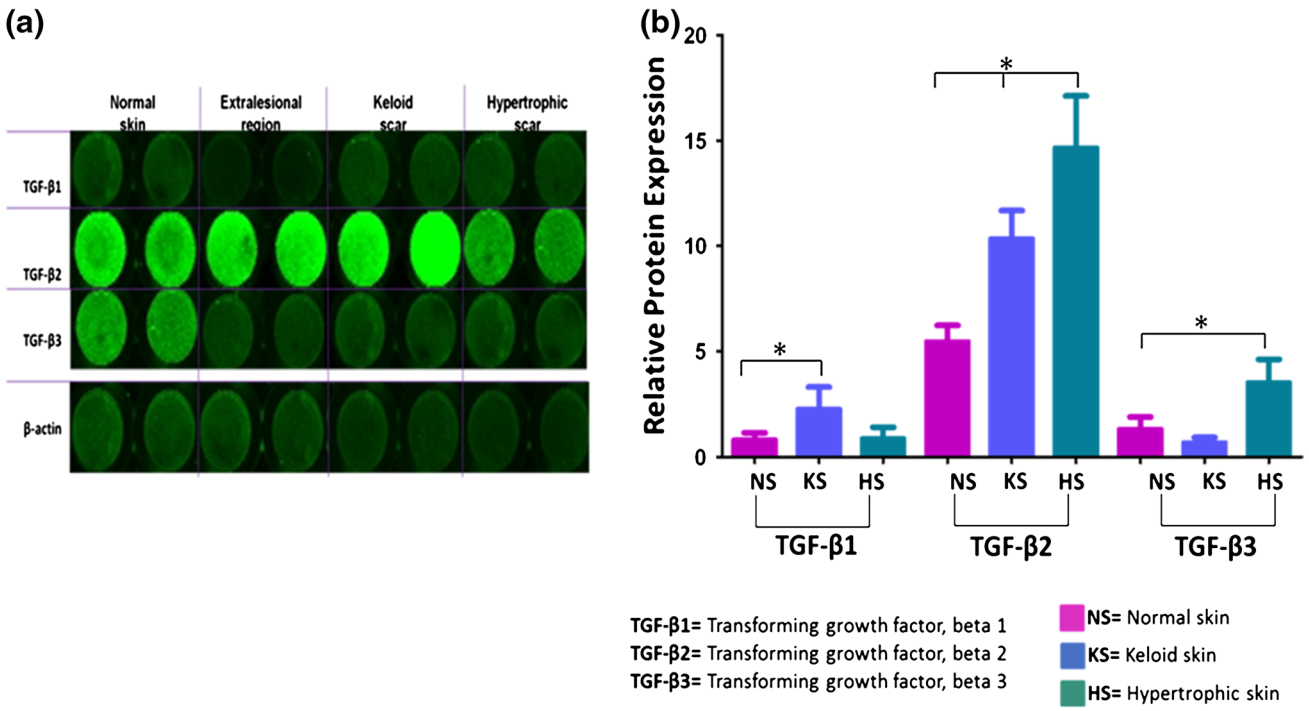

Fig. 14 Cellular protein levels of cytokines in keloid and hypertrophic scar compared to normal skin evaluated by in-cell western blotting. Fibroblasts were cultured from p0 to p4. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (*green*) from 96-well plates is shown in **a**. **b** The *bar graphs* represent the quantification of the expression of the analysed proteins normalised to the β-actin loading control; the result is expressed as mean ± SEM of three independent experiments. $^{*}p < 0.05$ indicates significant up-regulation of the cytokines expression when compared to normal skin fibroblasts, while $^{\#}p < 0.05$ can be appreciated as significant down-regulation of the cytokines from dermal scar cells versus normal skin fibroblasts ($n = 14$)

was highest in the samples taken from the sternum, which, correlates with other studies previously undertaken in relation to anatomical site and mechanical tension [1, 6, 47, 48].

The mechanisms leading to the development of both KS and HS are thought to involve the interaction of many biomarkers, several signalling pathways as well as environmental influences [4, 14]. The aim of this study was to identify potential biomarkers that could be used to distinguish between KS and HS. For analysis purposes, target biomarkers were classified into the following four categories: (a) neuropeptides, (b) tension-related, (c) ECM-related and (d) cytokines. To this end, we included neuropeptides in the study, in order to evaluate the potential role of constant stimulation of the sensory skin nerve fibres produced by mechanical stress that may affect the transmission of signals from the sensory nerves resulting in release of specific neuropeptides in the skin [23, 56]. Neuropeptides binding to their respective receptors located on skin cells' surface could induce vasodilatation and vessel permeabilisation evoking inflammatory responses [2, 56, 72]. This may then lead to the development of neurogenic inflammation followed by an exaggerated immune response triggering the release of proteins, such as Hsps, MMPs and MCPs [1, 23, 27, 37, 44, 50, 57, 70]. We also decided to study the proposed correlation existing between the expression of several Hsps and the increased synthesis of collagen I, as well as Hsps participation as mediators in keratinocyte proliferation and differentiation [11]. Hsps also regulate the proliferative phase during wound healing and promote new tissue formation. Hsps act as cellular chaperones that modulate cell death signals such as the FAS-mediated apoptotic pathway, allowing to cells adapt to gradual changes in their environment and to survive in otherwise lethal conditions [16, 18, 38, 44, 52]. In addition, MMPs have been implicated in angiogenesis, scar resorption, inflammation, re-epithelialisation and remodelling phases of wound healing [63, 66]. MCPs, transcription factors and cytokines are also linked with the regulation of inflammatory processes and cell recruitment in normal wound healing [27].

Interestingly, a correlation between neuropeptides, HSPs, MMPs and MCPs and the skin mechanical tension has been proposed in that this may promote the development and progression of raised dermal scars such as KS and HS [70], as they are prone to develop frequently at highly tensioned anatomical locations [28]. Several researchers have suggested that the site of injury has been

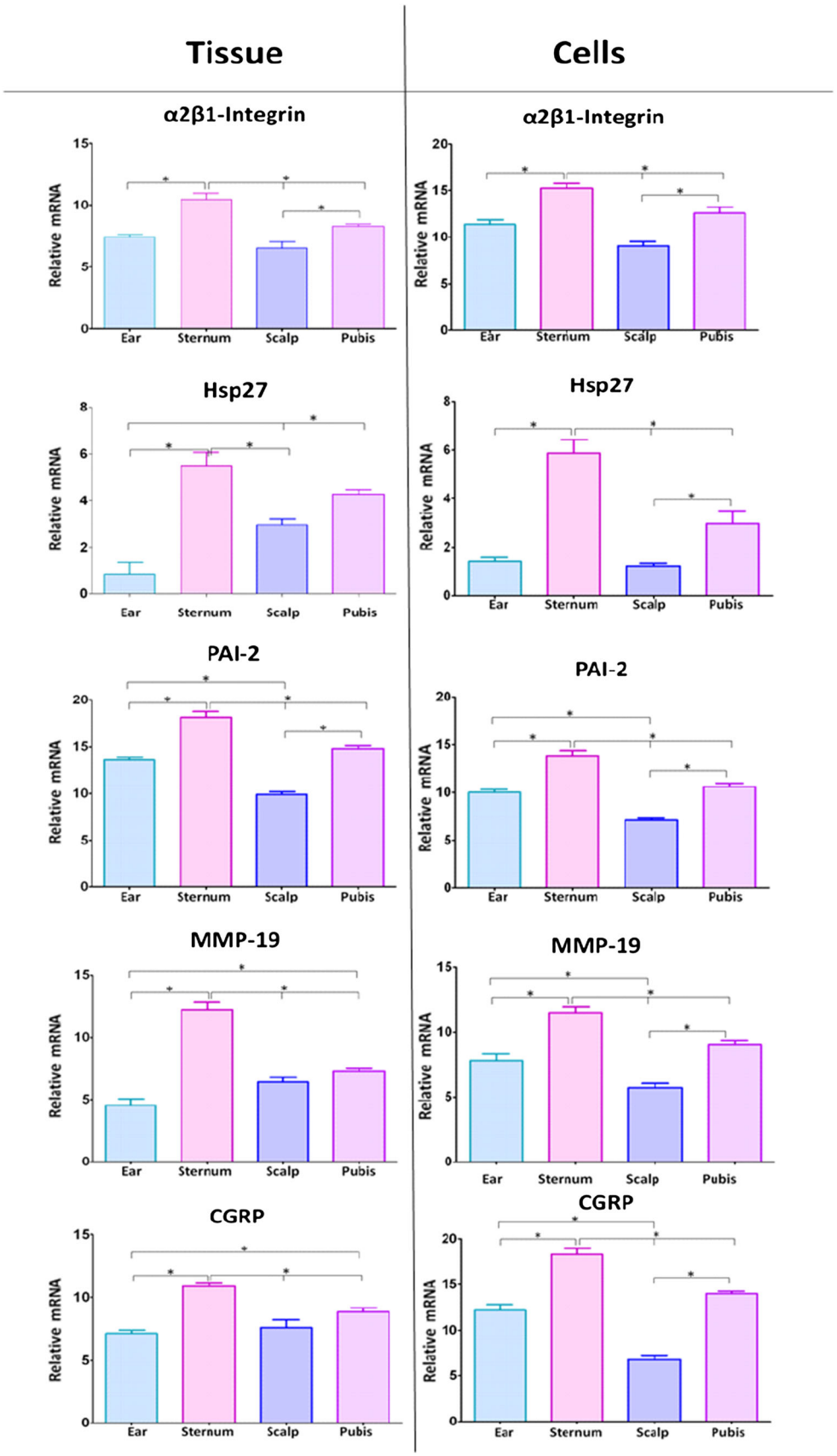

Fig. 15 Tissue and cellular mRNA expression for the top 5 up-regulated biomarkers in keloid scar compared to normal skin evaluated by qRT-PCR. The *bar graphs* represent the quantification of the total mRNA expression of the top 5 up-regulated biomarkers extracted from tissue biopsies and fibroblasts from keloid samples and normalised to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates of independent experiments ($n = 14$). $^*p < 0.05$ indicates a significantly increased difference between the mRNA expression among the scars collected from specific anatomical locations ($n = 14$)

Table 7 List of genes common up-regulated in hypertrophic and keloid scars from published microarray studies

Keloid			Hypertrophic		
mRNA tissue	mRNA fibroblasts	Protein fibroblasts	mRNA tissue	mRNA fibroblasts	Protein fibroblasts
–	SP	SP	SP	–	–
CGRP	CGRP	CGRP	CGRP	CGRP	–
–	NYP	–	–	–	NYP
Hsp27	Hsp27	Hsp27	Hsp27	Hsp27	–
Hsp47	–	–	Hsp47	–	Hsp47
Hsp60	–	–	Hsp60	Hsp60	–
	Hsp90	–	–	Hsp90	–
MCP-3	MCP-3	–	MCP-3	–	–
MCP-1	–		MCP-1	–	–
MMP-19	MMP-19	MMP-19	MMP-19	MMP-19	–
PAI-2	PAI-2	PAI-2	–	–	–
α2β1-Integrin	α2β1-Integrin	α2β1-Integrin	α2β1-Integrin	α2β1-Integrin	–
Tenascin	–	–	Tenascin	–	–
–	Cry61	–	–	–	–
–	–	TGF-β1	–	TGF-β1	–
–	–	TGF-β2	–	TGF-β2	TGF-β2
–	–	–	–	–	TGF-β3

found to be substantially influenced by scar formation and wound closure [50] with raised dermal scars tending to occur more frequently in body areas subjected to greater mechanical forces [2, 46]. In this context, the sternum, shoulders and suprapubic region are body zones considered as strong candidates for the development of raised dermal scarring [2, 13, 29, 53, 60]. Furthermore, the development of pathologic healing processes have been linked to mechano-signal transduction [47], whereby mechanical stress signals are transduced into biomechanical signals resulting in cellular responses that may promote raised dermal scar development [31, 33, 63]. Despite this knowledge, target molecules that promote and differentiate abnormal scar types have not been clearly identified to date.

Based on our results, we propose that the regulation of both raised dermal scaring processes are closely related to pathways that regulate mechanisms including proliferation and migration, angiogenesis, ECM degradation, inflammation, communication and cell survival among others [2, 3, 17, 19, 23, 41, 46] and also, that the expression of these target molecules is closely related to highly tensioned body areas. The limitations of this study include sample size as well as the lack of an in-house microarray dataset in HS samples. Despite this, we were able to identify five potential biomarkers that may be used in evaluation of HS and KS but would certainly require further validation in larger studies with different cohort of samples including both varieties of raised dermal scars. These potential biomarkers may be used in the diagnostic and prognostic evaluation of both scar types. In addition to repeat validation, further studies are required to fully explore the mechanisms involving these biomarkers in KS and HS pathogenesis.

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