The effect of skin tension on the formation of keloid scars

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Edna Suarez Pozos

School of Materials

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Abstract

Keloid scars (KS) are a type of abnormal scarring which is unique to humans. They extend beyond the confines of the original wound margins, do not regress over time and invade the surrounding unaffected skin. The mechanisms involved in the formation of KS remain largely unknown. Clinical observation has shown that in areas where increased tension occurs, such as the sternum, there is a greater propensity for developing KS. However, the precise relationship between skin tension and KS development is yet to be identified. In view of this, I hypothesize that skin tension plays a significant role in KS development by affecting tension-related biomarkers that may alter the phenotype of KS. Therefore, the objective of this research was to investigate the effect of skin tension in the formation of KS. To this end, the first aim was to identify possible targets among biomarkers that might contribute to the differentiation between KS and hypertrophic scars in tissue and cells obtained from diverse anatomical locations. The second aim was to investigate the effect of tension-related biomarkers on extracellular matrix (ECM) steady-state synthesis in keloid fibroblasts (KF) extracted from a highly tensioned body region (the sternum). The third aim was to develop a 3D *in-vitro* model to mimic *in-vivo* tension and to evaluate KF behaviour and ECM synthesis under tension. To achieve these aims 21 biomarkers were selected from published microarray and in-house microarray studies, the inclusion criteria was based on up-regulation of the genes in KS in relation to fibrosis, apoptosis and tension. For this purpose, samples from normal skin and KS were used to perform qRT-PCR screening in tissue and cells, as well as protein analysis by Western and In-cell Western blot. The siRNA knockdown technique was employed to evaluate the functional role of the tension-related markers in keloid fibroblasts. Finally, a photogrammetry technique was employed to evaluate skin tension *in-vivo*; the results from this evaluation were used in the development and design of a novel in-vitro 3D-model. The first biomarker screening in tissue showed convincing up-regulation of five tension-related targets (Hsp27, PAI-2 and α2β1-integrin, MMP-19 and CPRP). In addition, the expression of the above-mentioned targets was significantly higher in samples from the sternum compared to samples from other anatomical locations. To further validate these findings, the screening of the 21 biomarkers was assessed in KS and KF taken from the sternum. The results demonstrated over expression of 3 of the 5 tension-related targets (Hsp27, PAI-2 and α2β1-Integrin). It was also demonstrated that Hsp27, PAI-2 and α2β1-Integrin performed a functional role in terms of regulation of extracellular matrix production and deposition in KF when their expression was down-regulated by siRNA knockdown. Using the newly created 3D model, it was shown that mechanical tension significantly induced the expression of Hsp27, PAI-2 and α2β1-Integrin as well as ECM components such as Collagen I. Furthermore, the results showed that the knockdown of the expression of Hsp27, PAI-2 and α2β1-integrin in fibroblast populated collagen lattices subjected to tension influenced not only the ECM synthesis but also adhesion and spreading genes in keloid and normal fibroblasts. In summary, this research convincingly shows that skin tension alters keloid fibroblast behaviour, morphology, mechano-responsive gene expression and extracellular matrix production. The findings from my thesis offer insight into keloid pathobiology and provide options for targeted treatment of specific genes affected in keloids by biomechanical stress.

Author's Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright

List of abbreviations

α2β1-Integrin	Alpha-2-beta-1-integrin
α-SMA	Alpha-smooth muscle actin
BSA	Bovine serum albumin
CALCA, CGRP	Calcitonin-related polypeptide alpha
CO_2	Carbon dioxide
CFM	Cell force monitor
cDNA	Complementary deoxyribonucleic acid
CTGF	Connective tissue growth factor
Cyr61	Cysteine-rich, angiogenic inducer, 61
DEJ	Dermal-epidermal junction
DMEM	Dulbecco's Modified Eagle Medium
EGF	Epidermal growth factor
EP	Epidermis
ECM	Extracellular matrix
ERK	Extracellular signal-regulator kinases
FPCL	Fibroblasts populated collagen lattices
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FCS	Foetal calf serum
H&E	Haematoxylin and eosin
Hsp 27, HSPB1	Heat shock protein 27
HSPD1; Hsp 60	Heat shock protein 60
HSPA1A; Hsp 70	Heat shock protein 70

HSP90AA1; Hsp 90	Heat shock protein 90kDa alpha (cytosolic), class A member 1; Heat shock protein 90
Hsp's	Heat shock proteins
HS	Hypertrophic skin
ICW	In-Cell western blotting
IGF-I	Insulin-like growth factor-1
IL	Interleukin
KD	Keloid disease
KF	Keloid fibroblasts
KS	Keloid scars
K	Keratin layer
K	Keratin layer
KC	Keratinocytes
LHs	Linear hypertrophic scars
MMP-19	Matrix metallopeptidase 19
MMP-3	Matrix metallopeptidase 3; Stromelysin 1, progelatinase
MMP-13	Matrix metallopeptidase13 ;Collagenase 3
MMPs	Matrix metalloproteinases
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
MCPs	Monocyte specific chemokins
NHS	National Health Service
NPY	Neuropeptide Y
Nrp1	Neuropilin-1
NF	Normal fibroblasts

NO	Nitrous oxide
PD	Papillary dermis
p0	Passage 0
p3	Passage 3
p4	Passage 4
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide-3-kinase
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidenedifluoride membranes
qRT-PCR	Quantitative polymerase chain reaction
RD	Reticular dermis
RPL32	Ribosomal protein L32
RNA	Ribonucleic acid
SERPINB2; PAI-2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
Hsp 47, SERPINH1	Serpin peptidase inhibitor, clade H; Heat shock protein 47
SMADs	Sma and Mad related proteins
siRNA	Small interfering RNA
NaOH	Sodium hydroxide
TAC1, SP	Substance-P Tachykinin, precursor 1
TNXB	Tenascin XB
3D	Three dimensional
TGF-β1	Transforming growth factor, beta 1
TGF-β2	Transforming growth factor, beta 2

TGF-β3	Transforming growth factor, beta 3
TGF-β	Transforming growth factor-β
TBS	Tris-buffered saline
TNF-α	Tumour necrosis factor-alpha
P53	Tumour protein 53
VIP	Vasoactive intestinal peptide
VEGF	Vesicular endothelial growth factor
WHs	Widespread hypertrophic scars

Nomenclature

Cm	Centimetre
D	Confidence interval CI
F	Force
GPa	Giga Pascals
hr	Hours
DSC	Linear deformation
K	Linear stiffness
L	Litre
μmol	Micro mol
Mg	Micrograms
μL	Microlitre
Mm	Micrometre
Mg	Milligram
mL	Millilitre
Mm	Millimeter
mmolL-1	Millimol per Litre
mN	MilliNewton
min	Minutes
M	Molar
Ng	Nanogram
Nm	Nanometre
Ω	Ohm
Rpm	Revolutions per minute
N	Sample size
Sec	Seconds
Σ	Standard deviation
S.E.M.	Standard error of the mean
Zcrit	Standard normal deviate for a normal distribution
U	Unit
E	Young´s modulus

The Author

Ms Edna Suarez is currently a member of both, the Bio-engineering Group at the School of Materials and the Plastic and Reconstruction Surgery Research Group at the University of Manchester, UK. She graduated with a degree in Biopharmaceutical Chemistry from the Universidad Veracruzana, Mexico, before obtaining a Master´s degree in Molecular Biotechnology and Biomedicine from the National Polytechnic Institute/National School of Biologic Sciences of Mexico.

Publications resulting from this research work:

"Up-regulation of Tension Related Proteins in Keloids: Knock Down of Hsp27, α2β1-Integrin and PAI-2 Show Convincing Reduction of Extracellular Matrix Production". **Suarez E**, Syed F, Alonso-Rasgado T, Mandal P, Bayat A. Plast Reconstr Surg. (2013 Feb);131(2):158e-173e.

Submitted:

"Skin equivalent tensional force alters keloid fibroblast behaviour and phenotype". **Suarez E**, Syed F, Alonso-Rasgado T, Walmsley A, Mandal P, Bayat A. Wound Repair and Regeneration Journal (accepted for publication July, 2014).

"Expression profile characterization of Neuropeptides and Tension-related proteins in fibroproliferative scars". **Suarez E**, Syed F, Alonso-Rasgado T, Bayat A. Archives of Dermatological Research (February 28, 2014).

Poster presentations

2013 Mechanotransduction results in actin rearrangement/cytoskeletal reorganization in keloid fibroblasts grown in a 3D populated collagen gel. European Tissue Repair Society, Reims, France.

Oral presentations

2012 Up-regulation of tension-related proteins in keloids: knock down of Hsp27, α2β1-Integrin and PAI-2 show convincing reduction of ECM production. Wound Healing Society World Congress, Atlanta, USA.

2013 Design, development & optimization of novel *in-vitro* 3Dcollagen lattice model to assess the effect of mechano-responsive genes in keloid and normal skin fibroblasts. Wound Healing Society World Congress, Colorado, USA.

2014 Differential expression of tension-related biomarkers in scar fibroblasts & tissue helps with distinguishing hypertrophic from keloid scars. To be presented at Wound Healing Society World Congress, Florida, USA.

Awards

2013 Young Investigator Award winner for the The Wound Healing Society World Congress. "Design, development & optimization of novel *in-vitro* 3D collagen lattice model to assess the effect of mechano-responsive genes in keloid and normal skin fibroblasts"

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Chapter I: Introduction

Over the course of the previous decade mechanical tension has been proposed to influence the healing of traumatic wounds, promoting severe disruption in the regulation of cell proliferation and cell death ratios that characterise abnormal scar development, such as keloid scars[1-3].

Keloid scars (KS) are described as benign dermal fibroproliferative tumours of unknown origin that are unique to humans[4]. Keloids demonstrate site specific morphologies, such as the butterfly shaped scars frequently observed in the sternum[5]. Keloid disease (KD) is not a race-dependent disease, however, it is thought that there is a genetic predisposition in the Black, Hispanic and Asian races[6, 7]. In the United Kingdom, keloid incidence in Caucasian population is below 1%, which is low in comparison to the incidence reported for Blacks and Hispanics, which varies between 4.5 to 16%[8, 9]. KS usually appear in individuals aged between 10 and 30 years old and are rarely observed in elderly people[10]. KD presents with a higher incidence in women compared to men and has been found to be even more frequent during puberty or pregnancy[11]. Currently, the disease aetiology remains unknown. Despite several previously reported hypotheses, very limited evidence has been provided to support them. Some authors propose that keloids develop as result of excessive melanocyte-stimulating hormone production[12]. Whereas, other researchers suggest immunological dysregulation during the wound healing process may be a cause[13-15]. Fibroblast dysfunction has also been hypothesised[16]. Recent research into keloids has raised the hypothesis of viral implications[6] as well as mechanical tension of the skin and mechano-transduction[17, 18]. Despite this, keloid pathogenesis remains poorly understood and no effective treatment has been demonstrated. It has been observed that KS show a recurrence rate of nearly 45%[9]. A close relationship between KD development and skin tension has recently been proposed. However, the evidence provided to date remains insufficient to offer a satisfactory explanation for this postulation. The study of KD is limited by the lack of an appropriate animal model capable of mimicking progress of the pathology *in-vivo*. In this respect, the recent development of three-dimensional models as experimental tools has become increasingly important in this research field.

1.2 Research aims and specific questions to address

The aim of this research was to investigate the effect of skin tension on the formation of keloid scars. The research findings provide a better understanding of the role of skin mechano-transduction in keloid pathobiology and offers potential options to explore in the design of future keloid treatments. It is envisaged that RNA interference related therapies could be targeted towards tension-related proteins found in keloid tissue.

In order to achieve the proposed aim, this research was undertaken in three phases, which will be further detailed within the following chapters, briefly:

Chapter I provides a brief introduction to the research summarising the research aims.

Chapter II presents a literature review and a general overview of the keloid disease.

Chapter III includes a brief description of the general materials and methods employed in the realisation of the following research.

Chapter IV provides an understanding of the characterisation of the expression profiles of target biomarkers involved in the differentiation of hypertrophic from keloid scar samples obtained from different anatomical locations. The study was conducted with the aim of identifying target markers that may be responsible for the differences existing among keloid and hypertrophic scar pathogenesis. This chapter also provides further evidence about keloid heterogeneity in terms of phenotypic expression depending on the anatomical location.

Chapter V demonstrates the over expression of key tension-related biomarkers in keloid tissue and fibroblasts obtained from keloids from the sternum, a highly tensioned anatomical area. Moreover, chapter 5 provides further evidence of the existence of keloid mechano-regulation.

Chapter VI provides additional evidence of the effect that skin tension has during keloid scar development and behaviour with the use of 3D fibroblast-populated collagen gels. Moreover, chapter VI allows the establishment of the relationship between three tension-related target biomarkers and cell morphology, ECM

synthesis and proliferation in keloid fibroblasts, reinforcing the findings obtained in this research regarding keloid mechano-regulation.

Chapter VII includes a general discussion of the findings of this study and future perspectives.

1.3 Experimental design of the research

A brief summary of the experimental design performed in this research project is illustrated in Figure 1.1. A detailed version of the general methodology is provided in Chapter III of this document. In addition, where appropriate, chapter specific methods are provided following the introductions in subsequent chapters.

Figure 1.1 Experimental design summary.

1.4 Summary of original contributions of the thesis

A summary of the major research achievements and original contributions are presented as follows:

I. A panel of 21 tension-related biomarkers were selected from published microarray and in-house microarray studies, the inclusion criteria was based on up-regulation of the phenotype of the candidates in keloid scars and on the relationship with skin tension. These 21 biomarkers were evaluated in samples from normal skin, hypertrophic and keloid scars taken from different body locations using qRT-PCR and protein analysis by Western as well as In-cell Western blot. As result of the performance of the first biomarker screening in tissue and cells, five tension-related targets (Hsp27, PAI-2 and α2β1-integrin, MMP-19 and CPRP) were identified as potential targets in the differentiation between hypertrophic and keloid scars. In addition to these findings, the expression of the mentioned above targets displayed differences in the gene expression levels related to specific anatomical position where the sample was taken from and the amount of tension experienced.

II. The performance of a second screening of the 21 biomarkers assessed in keloid tissue and fibroblasts taken from the sternum showed over expression of 3 of the 5 tension-related biomarker targets (Hsp27, PAI-2 and α2β1-Integrin). It was also demonstrated that Hsp27, PAI-2 and α2β1-Integrin performed a functional role in terms of regulation of extracellular matrix production and deposition in keloid fibroblasts is steady-state. A novel 3D model to mimic *in-vivo* tension keloid fibroblasts was developed and validated *in-vitro* using keloid fibroblasts extracted from the sternum. Mechanical tension application using the newly created 3D model showed significantly induction of the expression of the target biomarkers (Hsp27, PAI-2 and α2β1-Integrin) as well as ECM components such as Collagen I in keloid fibroblasts from the sternum.

III. Finally, this research convincingly shows that mechanical tension of the skin alters keloid fibroblast behaviour, morphology, mechano-responsive gene expression and ECM production. This research demonstrated that regulation of the expression of the three tension related biomarkers, Hsp27, PAI-2 and α2β1-Integrin, in keloid samples by RNAi technology not only attenuates the expression of mRNA and proteins levels but also certain other ECM molecules.

The findings from this research offer further insight in understanding KD pathobiology and the potential options for future treatment strategy. It is envisaged that RNA interference could be targeted towards tension-related genes found in keloid tissue.

Chapter II: Literature review

Introduction

Raised dermal scars develop as a consequence of abnormal wound healing caused by a wide range of factors such as, anatomical location of the injury, severity of the lesion, tensional stress, injury depth and environmental factors[19]. Raised dermal scars can be classified as minor and major keloid scars, linear hypertrophic scars (LHs) and widespread hypertrophic scars (WHs)[20]. Keloids can be defined as neoplastic lesions characterised by excessive fibroproliferation associated with significant increases in collagen synthesis and deposition, gradual expansion beyond the boundaries of the original wound and lacking in spontaneous regression[21, 22]. In contrast, hypertrophic scars are considered fibrotic lesions that remain within the margins of the original lesion. Clinical observation shows that once hypertrophic scars have achieved a certain size, the lesions tend to undergo spontaneous resolution[23, 24]. These diseases produce pruritus, pain and contraction, which can affect patients' quality of life. Both types of aberrant scars present as flattened scars that rise above the skin level[25]. Although the pathogenesis of these two diseases is not well understood, it is considered that both result as a consequence of an abnormal wound healing process[26]. Thus, the aim of this research is to investigate the effect of skin tension on the formation of keloid scars.

2.1 Human skin

The skin is the largest organ in the human body and it is able to perform an outstanding list of functions. The skin is considered as a protective barrier against chemicals, light, heat and pathogenic microorganisms. The skin also controls the loss of essential body fluids, such as blood and water. This organ, also achieves regulation of the body temperature through perspiration. In addition the skin plays a key role as sensor of environmental stimulus and performs vitamin D synthesis[27, 28].

The skin is not uniform throughout the body. Differences between colour, texture and thickness are observed depending on the anatomical location. For example, the head presents a bigger amount of hair follicles than every other part of the body; the thinnest skin on the human body forms the eyelids and the most resistant is located in the palms of the hands and the soles of the feet[29, 30].

Every square inch of skin is made up of thousands of cells, hundreds of sweat and sebaceous glands, nerve endings and blood vessels arranged in three layers from the outermost layer to the innermost as follows: a) the epidermis (associated with appendages, sebaceous follicles and sweat glands), b) the dermis (separated from the epidermis by the dermal-epidermal junction) and finally c) the hypodermis (also known as subcutaneous layer), as shown in Figure 2.1[29].

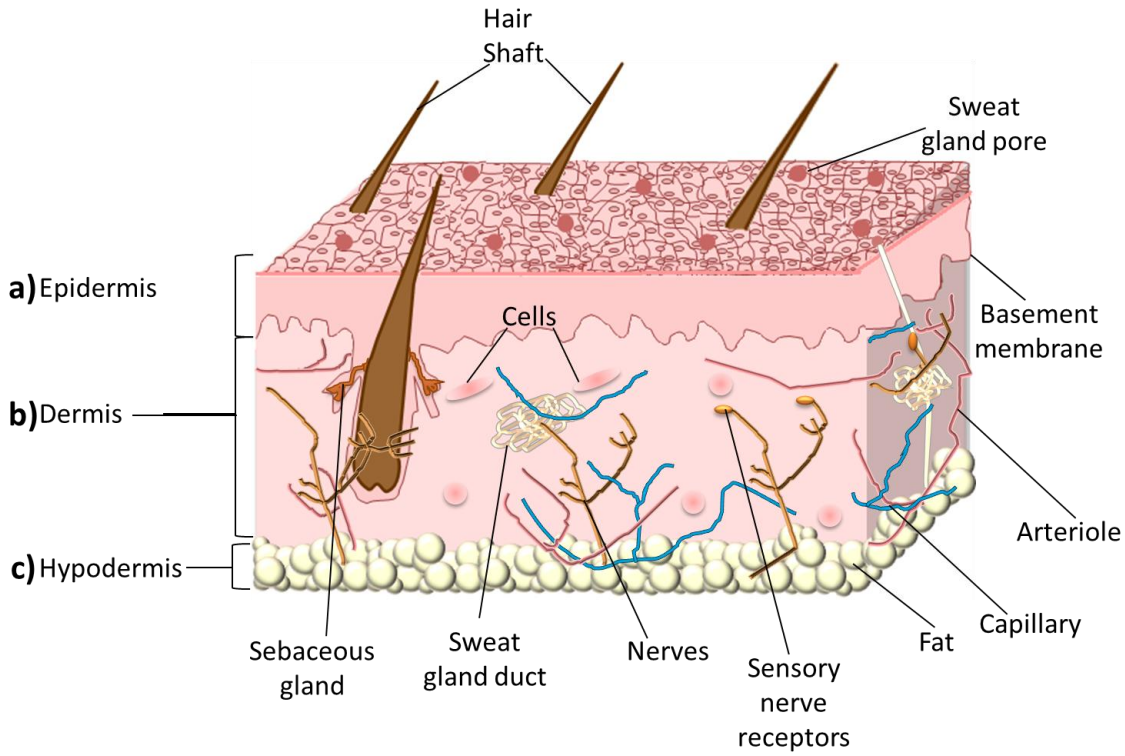


Figure 2.1 Skin layers and components.
(Modified from MacNeil S *et al.* 2007) [31].

2.2 Skin physiology

2.2.1 Epidermis

The epidermis is a stratified epithelium that renews itself constantly. Epidermis thickness varies considerably depending on the body area location. Thus, the skin in the eyelids is considered the thinnest at just 0.05 mm and the skin in the palms and soles is the thickest at 1.5mm[27]. Several cell types organise and arrange themselves to form the epidermal layer. The main cell types found in the epidermis are the

keratinocytes (KC). KC constitute between 90 to 95% of the total cell types in the epidermal layer. KC receive their name from their capability to produce keratin, a filamentary protein which has the primary function to protect cells in the epithelium from stressIngber DE [30. KC have an epithelial origin, since they are derived from the ectoderm through a differentiation process that results in flattened and enucleated cells (corneocytes) which emigrate from the skin surface. Between 5 and 10% of the remaining epithelial cells are non-keratinocytic cells, of which Langerhans cells, melanocytes and Merkel cells can be cited. The keratinocytes are arranged in four continuous layers or strata, from the most superficial to the innermost as follows: a) the stratum basal (5-10 sublayers), b) the stratum spinosum (5-15 sublayers), c) the stratum granulosum (1-3 sublayers), finally d) the stratum corneum (5-10 sublayers). Some parts of the body like the palms and soles present an additional, or fifth layer called the stratum lucidum, which is located between the stratum granulosum and the stratum corneum. Moreover, some researchers consider the basal and the spinosum layers as a single layer, which is called the stratum malphigi, Figure 2.2[31-35].

The KCs originate from the mitotic division of stem cells bound to the basal lamina. The immature cells migrate through the skin as they acquire morphological and biochemical differentiation (keratinization). Mature KC remain in the stratum corneum for a period of approximately 30 days until they are renewed. Two different types of functional units can be found in the dermis, the acrossyringium or superficial fraction of the sweat glands and the acrotrichium or hair follicles. Epithelial cells are also found in the dermis and maintain their biological properties depending on their particular histology[36].

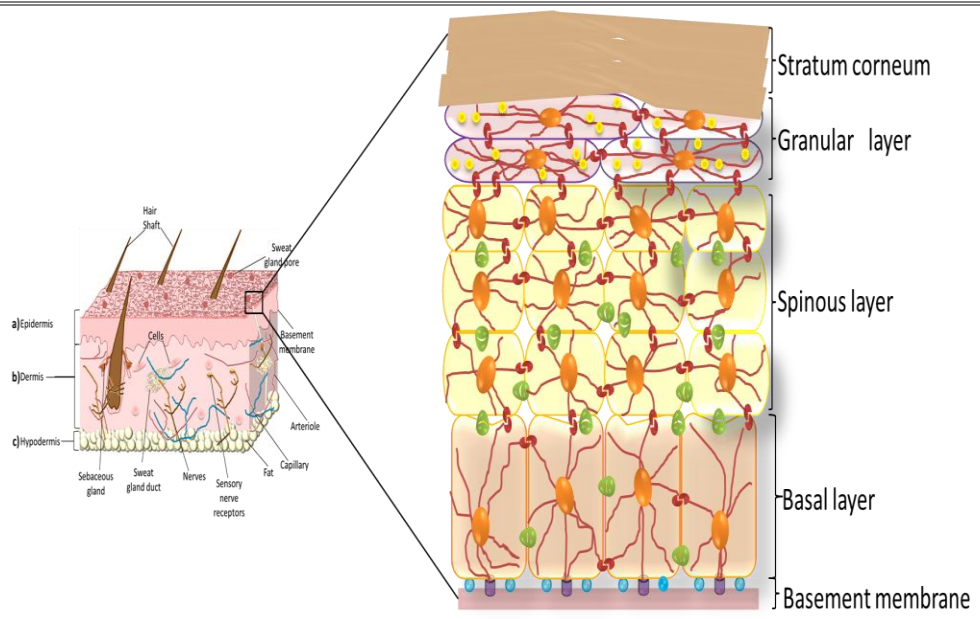


Figure 2.2 Schematic representation of the epidermal strata.
(Modified from MacNeil S *et al.* 2007 and Alonso L *et .al*.. 2003)[31, 32].

2.2.2 Dermal-epidermal junction (DEJ).

The dermal-epidermal junction (DEJ) is synthesized by basal KC and dermal fibroblasts. The DEJ plays a key role in the mechanical support of the adhesion of the epidermis to the dermis. The DEJ regulates the exchange of metabolic products between both compartments and serves as support for KC migration during wound healing. Several cell types go across the DEJ during inflammatory and immunologic processes to reach the damaged area[27].

The DEJ is composed of four regions: a) the cell membrane of basal KC, b) the lamina lucida crossed by filaments, c) the osmophilic lamina densely composed mainly by collagen type IV and d) the sub-basal lamina filamentous zone composed of anchored fibres[27].

2.2.3 Dermis

The dermis or corium is an elastic, connective and compressible tissue that serves as support and protects the epidermis and appendages. Vascular and nervous plexus run through the dermis, which is also composed of cells, fibres (interstitial collagen mainly type I and III, elaunin-oxytalan, elastina, fibrillin, and fibronectin) and support substances[37]. The dermis undergoes a continuous fracture governed by the mechanisms that regulate the synthesis and degradation of protein components. The

dermis thickness varies considerably depending on the anatomical location between 1 and 4 mm, being thicker on the back of the palms and soles and thinner on the eyelids. The dermis's fine structure varies within its depth and is named either superficial or papillary dermis, or, reticular or deep dermis[27].

The papillary dermis forms conical ascending projections, also named dermal papillae. Papillae are made of fibroblast-like cells, dermal dendrocytes and mast cells, as well as vessels and nerve endings. The papillary dermis is composed of collagen fibres arranged in bundles and thin elastic fibres that extend perpendicularly to the dermal-epidermal junction. In the toe area, the papillary presents tactile corpuscles, which are specialised nerve endings that serve as mechanoreceptors. The reticular lamina (deep dermis) is formed by thick collagen packages arranged in a parallel direction to the skin surface, Figure 2.3[37, 38].

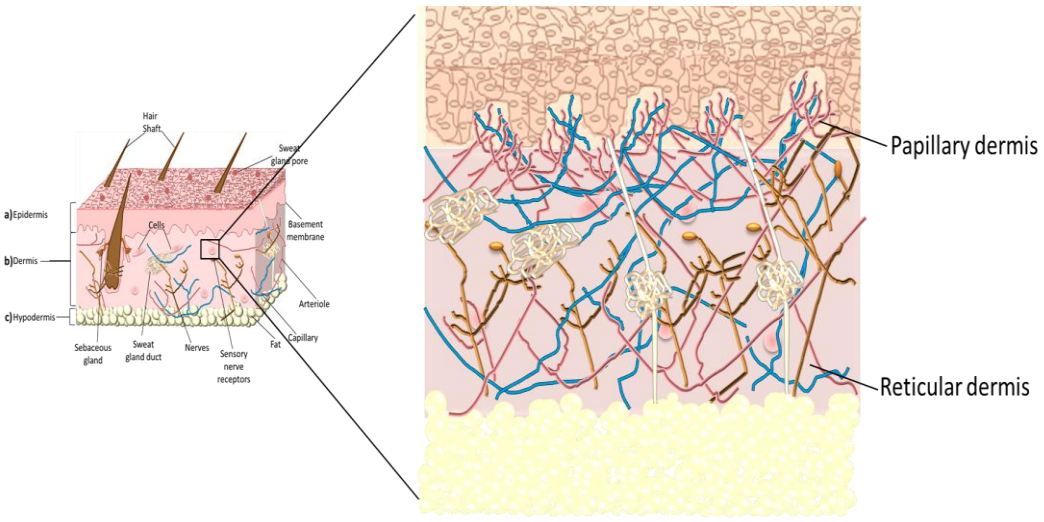


Figure 2.3 Dermal layer organisation.
(Modified from MacNeil S *et al.* 2007)[31].

2.2.4 Hypodermis

The hypodermis is the deepest and thickest skin layer. This layer plays an important role thermo-regulating, protecting and storing energy reserves in the skin.

The hypodermis layer is mainly composed of adipocytes, cells that are specialised in the accumulation and storage of fat. Adipocytes form clusters that are grouped

together in lobules separated by connective tissue. Adipocytes, also known as fat cells, vary in their morphology depending on the body region considered and the sex. Adipocytes coordinate the circulation of fat that takes place during the thermo-regulatory process. The hypodermal fat distribution over the body has a tendency to accumulate below the waist, around the thighs, hips and buttocks in women, whereas men tend to accumulate fat above the belt over the abdomen and shoulders[27].

2.3 Skin immunology

The main immunological role of skin is to serve as a first defence barrier, protecting the body against foreign bodies and organisms. The immune response is performed by a wide range of cell types that are responsible for the synthesis and metabolism of a large number of biological compounds that exert their effects in the skin. These biological compounds are also associated with immune or systemic responses[39].

Each skin layer performs specific functions that, when combined, provide immunological protection. Whilst the epidermis is directly related to the regulation of water retention, the stratum corneum is involved in pyrogenesis and neutrophilia control. The epidermis is closely related to the induction of acute phase responses and the regulation of allergic responses[39-43]. The dermis is a widely vascularised tissue that regulates inflammatory cell migration. The dermis also contributes to the regulation of allergic reactions and the maintenance of vascular integrity[44].

2.4 Skin bio-mechanical properties

The mechanical behaviour of the skin has been extensively studied. A sound knowledge and understanding of the biomechanical properties of the skin *in-vivo* is very important in clinical and cosmetic applications to improve the treatment of cutaneous defects.

The human skin is considered a complex organ. Hence, the study of the mechanical properties of the skin is a very complicated task. At the present time it is still

difficult to explain the contribution that each of the skin layers makes in modulating and coordinating the mechanical response of the skin[45].

The mechanical properties of the skin are determined by the correlation of its internal networks, which are mainly composed of collagen, elastic fibres, water, proteins and proteoglycans embedded in the extracellular matrix[46]. Approximately 70-77 % of the fat-free dry weight of the skin is composed of either collagen type I or III. When relaxed, the random arrangement of the collagen fibres is clearly appreciated, whereas under tension conditions these fibres appear aligned to each other. Moreover, collagen fibres provide high tensile strength and are considered the main structural scaffold of the skin. Collagen fibres are also described as stiff and non-extensible fibres. In addition, elastin fibres represent 4% of the skin's dry weight. Elastin fibres are described as very elastic fibres that maintain a close relationship with collagen fibres, thus allowing the formation of the skin's network. Once the forces that stretch the collagen fibres are removed, the collagen fibres recover their rest posture, this is a mechanism mediated by elastin. Elastin fibres are considered to be the main regulators of skin recoiling when mechanical forces are released[46,47-49].

The properties of the skin also vary with the strength and duration of applied force[49]. The aging process and daily environmental conditions modify the skin's properties, reducing the skin's elasticity and increasing the skin's breakability[33, 48]. *In-vivo*, every square inch of skin presents a complex attachment to all of the surrounding skin and contains each of the skin layers and components. Other causes of variations in the skin's properties could include disease, pathology exposure to solar radiation or injuries[50].

Nowadays, the skin's mechanical stretch, viscoelasticity, tensile elasticity and stiffness are the most common mechanical properties studied *in-vivo* and *in-vitro*[45, 46, 51].

The skin's stretching properties are highly related to the amount of fat, joint movements, anchorage and space arrangement of the collagen bundles. Mechanical forces are also involved in the distribution of the natural body fibres causing modifications in the stretching of the skin. The loss of the skin's stretching capability is frequently related to disease and pathological disorders that compromise the integrity of the connective tissue[46, 52, 53].

Several studies describe the skin as an anisotropic nonlinear-viscoelastic material that can be subjected to biaxial deformation. Modifications in the amount of strain applied promote time-dependent skin relaxation in order to achieve the recovery of the initial state of rest[51,35, 54]. The tension and elasticity of the skin varies depending on the morphological site. The differences among the bio-mechanical properties related to diverse anatomical sites are closely related to the collagen bundles and the arrangement of the elastic-fibres[55, 56]. Therefore, the skin is able to support deformation when subjected to tensional load, storing energy and using it later in order to restore the resting state[56, 57].

In the reticular dermis layer, Langer's lines correspond to the parallel orientation of the collagen bundles and elastic fibres in skin. These lines regulate elastic properties of the skin. Karl Langer first described the model of the distribution of tension lines in the body in 1861 after puncturing numerous holes at short distances from each other into a skin cadaver. Langer used a circular-shaped tip, similar to an ice pick, as a tool in order to create the holes. Langer observed the formation of ellipsoidal shapes after the punctures were produced in the skin and determined the directions of the lines from the longer axes of the ellipsoidal holes. *In-vivo,* these lines can change due to the natural movement of the body and the modification of the forces that act in a determinate moment and in a specific portion of the skin. Figure 2.4 shows the map representation of the Langer's line distribution in the human body[52, 58].

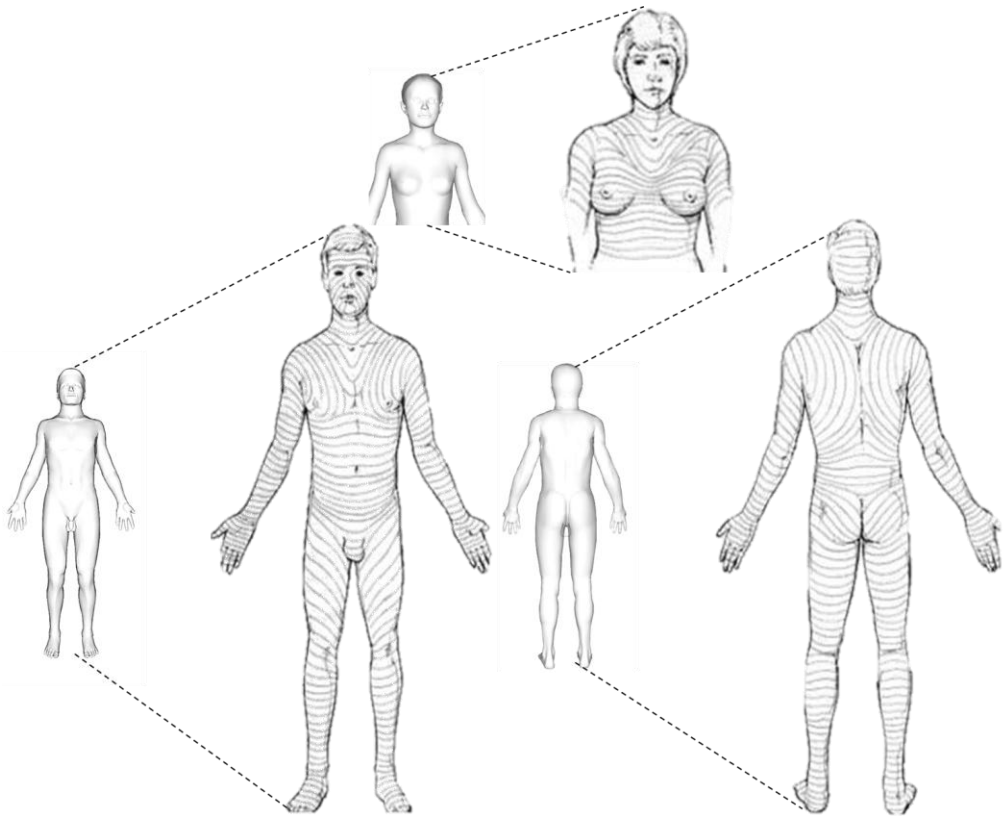
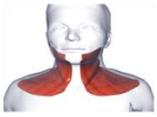
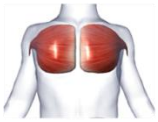
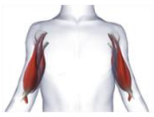
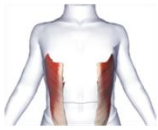
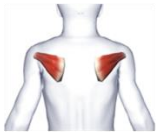
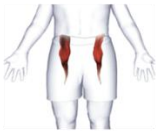
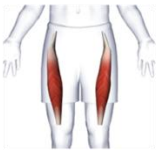
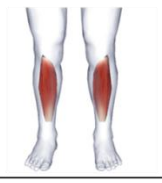


Figure 2.4 Schematic representations of the Langer's lines in human body.
(Modified from Pierard *et al.* 1987)[58].

Recent research conducted by Annaidh *et al., 2010* provided strong evidence that supports previous findings concerning the close relationship that exists between skin deformation characteristics, skin tension distribution and the orientation of tension lines. Recently, Ogawa *et al.* 2012 investigated the heterogenic stretching/contraction along nine different body parts measured in different directions *in-vivo* as shown in table 2.1. After analysing the published data, it was found that the upper back, shoulders, sternum and suprapubic regions are the highest tensioned areas in the body as shown in Figure 2.5, whereas the parietal region and the anterior lower leg were shown as the body areas with lower stretching/contraction values.

Table 2.1 Contraction/stretching ratio
(Modified from Ogawa et al. 2007)[59].

Anatomical location		Average contraction/stretching ratio (%)			
		Horizontal direction	Vertical direction	Oblique-left direction	Oblique-right direction
Forehead		1	1	0	1
Neck		10.3	2	4	9.3
Sternum		7	4	4	6.6
Upper arm		9.3	11	2.3	2.3
Dorsum		9	3	8.6	8.3
Scapular region		5.6	2.6	16	2
Suprapubic region		2.5	26.5	5	13
Lateral femur		5	5.3	11	12
Anterior lower leg		2.5	1.5	0	1

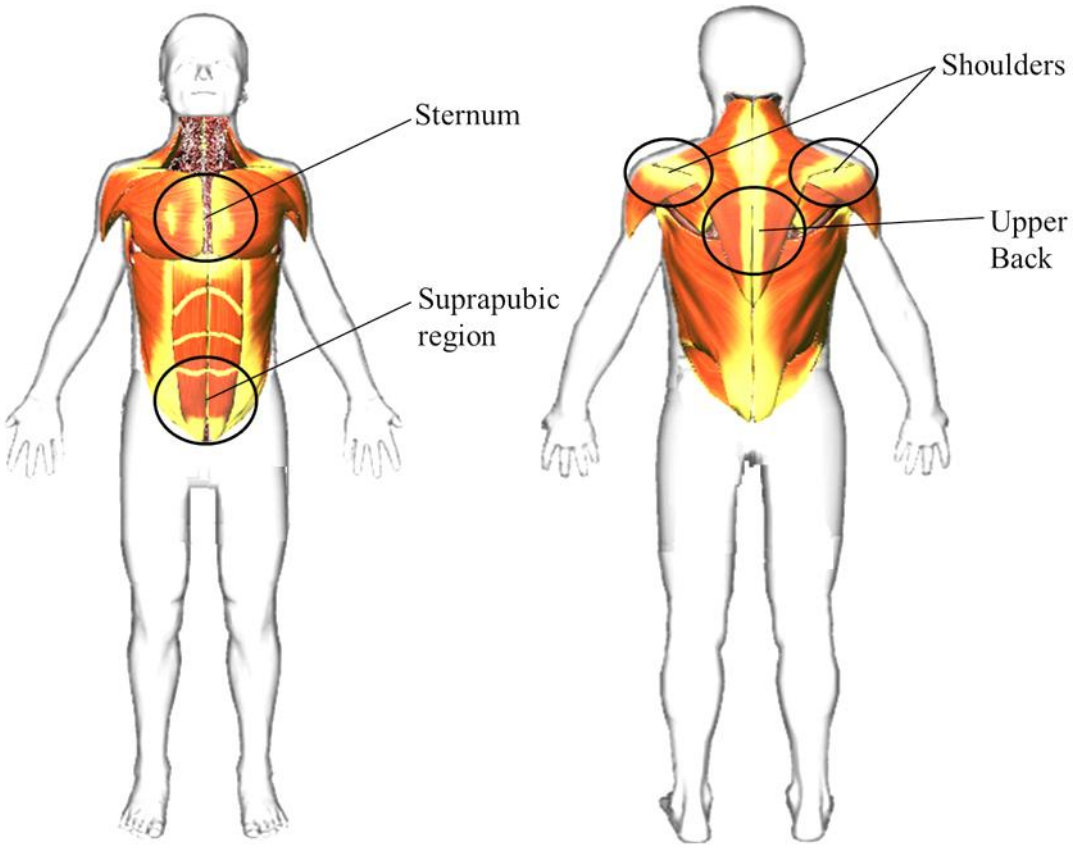


Figure 2.5 Human skin higher tensioned body regions.

The study of the biomechanical properties of the skin is very difficult to perform due to the great variety of factors that can affect the results. In this respect, the biomechanical properties of the skin have been evaluated over time using different and diverse methodologies, which can be classified into two main groups of tests, a) *in-vivo* tests and b) *in-vitro* tests[56].

2.4.1 In-vivo tests

The main objective of *in-vivo* tests is to provide useful information about the skin's behaviour when subjected to mechanical stimuli such as stretching, torsion, compression, indentation and shear by inducing as little disturbance as possible in the skin's normal conditions[60]. *In-vivo* methods have limitations since they only provide access to the epidermal skin layer, making the dermal layer inaccessible for further evaluation[57]. *In-vivo* tests are subdivided into two groups. The first group includes the tests known as statics, where the load is applied just once. These types

of methods are very useful for evaluating the skin's behaviour through various processes including ageing, wound healing, radiotherapy, fibrotic diseases, steroid application and others[61, 56, 62]. The second group of tests includes dynamic tests, in which the strain is applied recurrently and the modifications to the studied parameters are recorded[63, 64].

2.4.2 In-vitro tests

Compared to *in-vivo* tests all *in-vitro* tests are considered to be destructive and to this respect, the specimens can be analysed just once. *In-vitro* tests offer several advantages. Firstly, these tests are highly reproducible. Secondly, providing enough information to describe the property being investigated can control the parameters involved in these kinds of studies. One of the disadvantages of these methods is that, most of the time, the experimental conditions do not resemble the conditions that the specimen experiences *in-vivo*[45]. To perform *in-vitro* measurements, the skin is usually removed from the body and in most cases the fat tissue is also excised, thus influencing the outcome of the tests. In order to achieve better results all specimens are aligned in certain orientations, which influences the data obtained. The skin's characteristics of deformation vary with respect to the Langer's lines orientation and the direction of collagen fibres. It is also important to remark that once the skin specimen is removed from the body, the basal skin tension is modified as a result of the disturbance of the natural arrangement of the tension lines[60, 65]. To perform tensile tests of skin samples, the specimen is frequently cut into a dogbone shape, samples can be grouped according to the Langer's lines orientation: parallel, perpendicular or rotated 45° as can be appreciated in Figure 2.6.

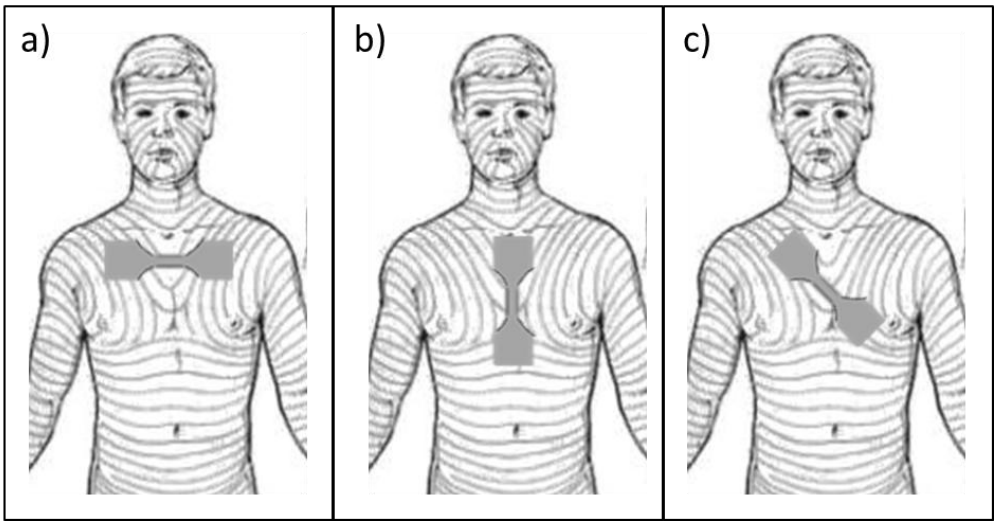


Figure 2.6 Orientation of skin samples according Langer's lines.

a) Parallel, b) Perpendicular and c) 45° rotation.

2.5 Dermal scarring

Dermal scarring is considered a highly dynamic and complex progression that comprises cell activation, proliferation, migration, differentiation, extracellular matrix production, tissue remodelling, neovascularisation and contraction among other strongly regulated and correlated processes. Such processes are summarised into three main phases that overlap over time as follows: inflammation, tissue formation and tissue remodelling[66]. A scar is produced as result of the wound healing process. Scarred tissue shows different biomechanical characteristics compared to undamaged tissue; for instance, scarred tissue is breakable and less functional that normal skin[33]. Despite the high recurrence of the scarring process during the human lifetime, there is no general definition able to describe scars so far. To this end, Ferguson *et al*., defined a scar as the macroscopic disturbance of the natural structure and function of the skin's architecture, resulting as the end product of a healed wound[67], whereas O'Kane *et al*. defined a scar as the natural process that the body possesses in order to regenerate damaged tissue after an injury[68].

While scar tissue appears as a breakable tissue that lacks elasticity, normal skin architecture is also modified. The amount of elastic fibres is lower and the amount of hyaluronic acid is higher than in normal skin[35, 68]. In addition, the collagen bundles in the dermal layer are thicker, align in parallel in the direction of the mechanical tension, and the basal membrane is flattened and without rete ridges, contrary to the appearance of normal skin[69]. Scar tissue appears devoid of hair follicles and sebaceous glands, while the skin's vascularity and nerve supplies are also compromised[68].

The progression of scar formation is influenced by age (among other factors). Clinical observation has shown that scar formation processes are more acute when presented in young people compared to old patients[70, 71]. The severity of scar formation can be evaluated based on two scales; the Vancouver scale and the Manchester scar scale[24]. These two scales are based on the evaluation and description of scar characteristics such as: anatomical position, number, size, margins, colour, surface and texture, assigning a score value to each of them. Scars are considered severe if high values are assigned following their evaluation[72, 73].

2.5.1 Wound healing phases

Three overlapping but biologically different wound healing phases have been described. These stages are comprised of a very coordinated and regulated series of events that promote closure of the injury as shown in Figure 2.7. These four phases are briefly described below.

a) Inflammatory phase

During the inflammatory phase, a large number of chemokines are released at the injury site in order to promote the activation and recruitment of platelet and immune cells, which are responsible for the formation of a provisional clot. Such a clot brings protection to the body against external aggressors, restores the homeostasis of the environment and functions as a provisional ECM, thus facilitating cell migration throughout the injured area. During this process, platelets promote the recovery of homeostasis and secrete great quantities of healing related molecules

such as thromboxane, epidermal and transforming growth factors as well as platelet-derived growth factor, among others[19, 67, 74].

Among the recruited cells during the inflammation phase, neutrophils play one of the most important roles since they are responsible for the deprivation of foreign particles and microorganisms, as well as processing and presenting antigens to macrophages. Activated macrophages release platelet-derived factor and vascular-endothelial growth factor, thus initiating the granulation tissue formation phase[75].

b) Re-epithelisation phase

The re-epithelisation stage begins a few hours after the damage occurs. The epidermal cells are responsible for the removal of the damaged tissue and the clotted blood at the injured zone[75, 76]. Simultaneously to this process, the intracellular junctions get dissolved, thus allowing increased cell migration[77]. The surface receptors of the epidermal cells allow the interaction between the cells and the ECM components. Such components mainly consist of fibronectin, which intertwines with collagen I and elastin fibres in the wound margin. Cell migration throughout the wound allows the separation of damaged tissue from viable tissue[76, 78].

c) Granulation tissue phase

The formation of granulation tissue takes place four days after initial skin damage occurs. During this phase, cell migration is still active macrophages, fibroblasts migrates together[78]. Throughout the tissue formation stage, the blood vessels provide oxygen and nutrients, which are elements necessary to maintain cellular metabolism. Macrophages promote the proliferation of fibroblasts and angiogenesis by producing growth factors. Concomitantly, fibroblasts synthesise ECM components that are used by the cells as a scaffold. Once fibroblasts migrate all the way through the healing space, the provisional ECM scaffold is gradually replaced by the newly formatted collagen fibres. Following collagen deposition in the wound area, collagen synthesis is stopped and the granulation tissue is replaced by scar hypocellular[68, 79].

In order to maintain the recently formed granulation tissue, the pre-existing blood vessels synthesise a great number of molecules including endothelial growth factors, transforming growth factor β, angiotropin, thrombosponsin, among others, in order to start the formation of blood vessels or angiogenesis[71, 80]. Once the damaged site is filled with granulation tissue, the angiogenesis ratio decreases and the new blood vessels undergo apoptosis, all this whilst the contraction takes place[81].

d) Wound contraction

The wound contraction phase requires cells, ECM components and cytokines. Firstly, fibroblasts differentiate into myofibroblasts[82, 83]. During wound contraction, fibroblasts attach to the collagen matrix through integrin receptors present in the membrane of the fibroblasts and using the cross-links formed between the collagen bundles. The contraction stage is characterised by the continuous synthesis and catabolism of collagen type III at low rates, which is controlled by the synthesis of proteolytic enzymes such as metalloproteinases secreted by macrophages, endothelial cells, epidermal cells and fibroblasts[84].

2.6 Effect of mechanical forces during cutaneous wound healing

The influence of mechanical forces during the skin repair process has been widely examined since the existence of skin's tension lines was reported by Langer in 1863. During the normal healing processes, the gaps in the skin are closed and a epidermal barrier is re-established[38, 45]. The wound healing overlapping stages are influenced by mechanical forces. The wound itself is contracted by forces generated by myofibroblasts surrounding the injured area[18]. Moreover, the wound is also influenced by extrinsic forces, including the natural tension of the skin. Thus, fibroblasts regulate the synthesis and degradation of collagen, fibronectin and ECM which results in rearrangement and remodelling of the three-dimensional ECM structures[85]. Subsequently, the binding of the cells to the ECM structure produce small forces in the cell cytoskeleton than can result in the deformation of the cell[35].

During the process of repair, the blood vessels permeability is increased promoting an increase in the volume of extracellular fluid. The extracellular fluid along with the ECM reorganization produces intrinsic forces such as tension, shear force, osmotic pressure and hydrostatic pressure. The mechanical forces contribute to the mechanism of cellular responses[17].

It has been demonstrated that during cutaneous wound healing mechanical tension is essential to allow fibroblasts to differentiate into myofibroblasts. The reduction of intra or extracellular tensile forces decreases fibroblasts rates of conversion which results in absence of contraction and the resultant wounds are likely to fail to mature[86, 87]. In addition to these findings, Ogawa, *et al.* proposed that incisions made across the Langer's lines are exposed to greater tension (from the orientation of collagen fibres or contraction of underlying muscles) and form quantitatively more scar tissue. This theory is substantiated clinically as wounds in high-mechanical-stress regions (such as the sternum) frequently develop in fibrotic lesions[17]. It has also been shown in animal models such as mouse and pigs, the manipulation of the scars by the increase of the mechanical stress loads tends to produce aberrant scarring[39, 41]. Whereas other studies demonstrated that cyclical stretching treatment on murine unclosed wounds promotes the activation of inflammatory cascades and promotes epidermal proliferation and angiogenesis[42]. Furthermore, the application of mechanical stress to pig skin explants in a bioreactor has shown to modulate collagen fibril thickness[43]. Other studies showed that the fibrosis produced *in-vivo* using pigs can be controlled by the manipulation of the mechanical forces that acts across the closed incisions[44]. It was demonstrated that those wounds closed under high tension exhibited greater scar formation when compared to those incisions that where closed under minimal tension conditions, which closely relates to scarring process in humans[17, 46]. These studies were complemented with the use of mathematical models based on finite element[44].

In order to understand the effect that mechanical forces play during wound healing mathematical and computational models had been developed, these models provide

further understanding of the combination of processes that takes place to achieve the successful healing of a wound[47]. These models aim to quantify the impact of the injury in the baseline of the system and to predict the effect of different therapeutic options. The characteristics and simplification of each model are different, making a challenge to select a unique model able to provide a satisfactory explanation. Nowadays it is becoming clear the most employed models are those that consider the wound healing as a multi-scale and multi-disciplinary system[53]. Thus systems-based mathematical modelling of wound healing have become key tools in personalized medicine. The state of the art of computational models in wound healing involves the integration of different cells types, nutrients and ECM components to restore the tissue integrity[47, 53].

Together these studies demonstrate that mechanical stress forces are key regulators of the wound healing process and suggest that any lack in the regulation of such forces may lead to the development of pathogenic mechanisms that result in abnormal wound healing.

2.7 Abnormal wound healing

Abnormal wound healing occurs upon the loss of balance during the wound healing stages. The anomaly produced on the healing outcome will depend on the kind of the aberrations presented and the current stage of the wound healing process. A wide range of wound healing abnormalities can be listed, such as ulcerations, fibrotic processes, chronic wounds and raised dermal scars.

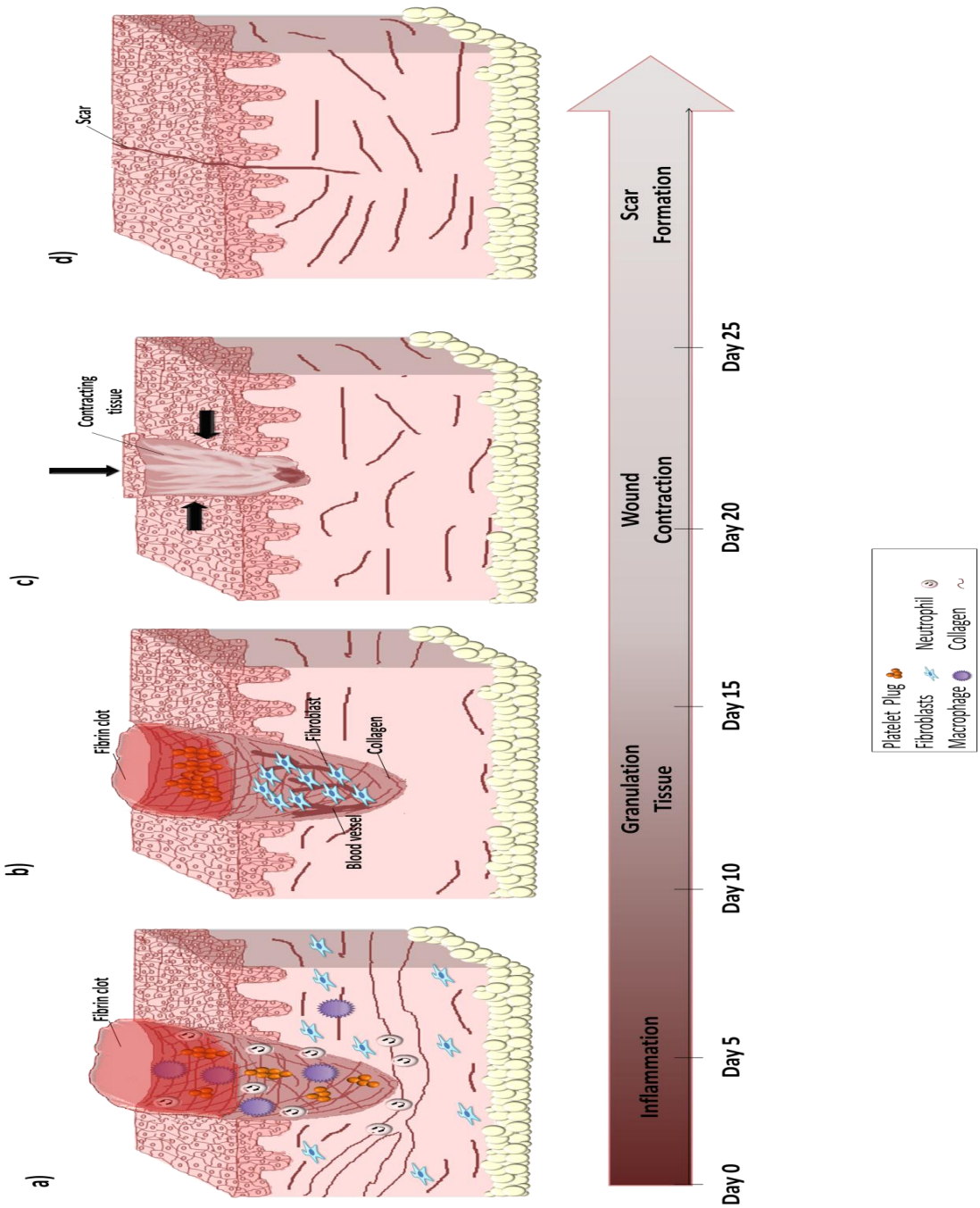


Figure 2.7 Wound healing phases.

2.7.1 Scar classification

Abnormal scars can be classified by taking into consideration the dynamic balance during the scar formation and the regeneration of the tissue. Additional factors such as the nature of injury, severity, depth, anatomical location, tensional stress,

infection, environmental factors, genetic predisposition, sex and hormone levels also produce the following scar type spectrum[24]:

a) **Widespread scars or stretched scars:** Scars that appear after a surgical procedure when the thin lines composing the scar eventually become stretched and widened. Stretched scars commonly develop three or four weeks after surgery. These scars are flattened, pale, soft and appear frequently on shoulders and knees. Stretch marks are considered a variation of the widespread scars in which the epidermal layer is not damaged, but the dermis is cracked[24, 88].

b) **Atrophic scars:** Scars that are flattened and depressed, surrounded by healthy skin. Atrophic scars are shown as small and circular shapes with inverted centres that appear as a consequence of acne or smallpox[89].

c) **Scar contractures:** Scar contracture appears in each part of the body that is subjected to contraction, like joints or skin creases. These scars can be observed when the scars are still immature. Scar contractures commonly develop into hypertrophic scars. Scar contractures developed usually after burn injury and tend to be disabling for the patients[24].

d) **Raised dermal scars:** Raised scars are formed as a result of weak scarring processes due to a wide range of factors. These scars are bulky, itchy and sometimes pruritic and painful. Raised scars grow within the wound boundaries and can be classified in two types: hypertrophic and keloid scars[24].

e) **Hypertrophic scars:** Scars that are raised, erythematous, pruritic and fibrous that remain within the boundaries of the original lesion and may undergo spontaneous resolution. These type of scars commonly include dermal layer damage[23].

f) Keloids: Keloids are abnormal raised scars unique to humans, characterised by the excessive deposition of collagen in the dermis and subcutaneous tissues secondary to traumatic or surgical injures. Clinical keloids are defined as dermal fibroproliferative tumours that grow beyond the confines of original wounds and rarely regress over time[24, 90, 91].

2.8 Keloid scars

Keloid scars (KS) are fibrous raised scars characterised by uncontrolled collagen synthesis and deposition that expands beyond the margins of the original wound boundaries. These scars invade the adjacent healthy skin and do not present spontaneous remission over time[16, 21]. Keloids were described as a dermal disease for the first time in 1802 by Alibert, who described them as crab claw-like lesions, introducing the name cheloid which derives from the Greek "Chele" (crab's claw) and the suffix "oid" (like)[9]. Over time, KS compromise the patient's quality of life. Keloids affect features, such as the biomechanical properties of the site, which can be disabling or modify normal movement[2, 17, 92]. Aesthetic modifications are also frequent in keloid patients, the modification of the natural elasticity of the skin results in a scanty glassy skin, devoid of hair follicles[4, 5]. Finally, psychological and social implications can compromise the patients' lifestyle by disrupting the performance of their daily activities, these frequently include depression, anxiety, post-traumatic stress reactions, sleep disturbance, disability, loss of self-esteem and stigmatisation[90, 93, 94].

2.8.1 Epidemiology

Keloid disease is not a race-dependent disease; however it is thought that there is a genetic predisposition in the Black, Hispanic and Asians races[6]. In the United Kingdom, keloid incidence in the Caucasian population is less than 1% compared to the incidence reported for Blacks and Hispanics which varies from 4.5 to 16%[8, 9]. KS usually appear in individuals aged between 10 to 30 years old and are rarely observed in the elderly[10]. Keloid disease presents a higher incidence in women compared to males, and is more likely to occur during puberty or pregnancy[11].

2.8.2 Histopathology

Keloid histology is characterised by a thicker epidermal layer than normal skin[95, 96], abundant vasculature, thicker dermis and a higher amount of inflammatory cells compared to normal or undamaged skin. Collagen fibres appear relaxed and randomly arranged in intact skin[97], however collagen fibres are thicker and more abundant in keloid tissue[98]. Moreover, keloids frequently present an excess of proteoglycan deposition in the ECM. The reticular dermis is characterised by the deposition of great amounts of fibrous bands[24, 77, 90, 94]. Although, the mechanism that triggers the formation of KS is not yet well described, it has been proposed that KS result from the combination of several deregulations occurring during the normal healing process[6, 99, 100]. It is also proposed that aspects such as environmental factors, ethnic variations, anatomical site, skin tension, hormonal factors, genetic predisposition, and prolonged healing phases, among other causes, may compromise the normal healing of scars and result in keloid formation[93]. One of the particularities of KS is the heterogeneity observed among the different anatomical sites[5, 101]. KS are more likely to develop and raise in more aggressive and recurrent manners on those sites where skin tension is higher[5,17, 59, 102-104]. It is strongly suggested that keloids are the result of an incapability to regulate and stop the wound healing process promoted by over activation of the fibroblasts, endorsed by the skin tension generated during the execution of natural body movements[17, 105]. It is proposed that skin tension promotes aberrant cell signalling transduction during keloid development and progression[102, 106].

2.8.3 Normal wound healing phases vs. abnormal wound healing (keloid development)

Several factors have been proposed to explain the development of keloid scars, among these, one of the most frequently stated is the inability of the body to maintain the balance between the healing phases and being able to stop the process[9, 15]. Several researchers have proposed that keloid formation is mainly the result of a larger granulation stage whereas other groups of researchers suggest that keloids arise as a result of abnormal regulation of the inflammatory phase. However to date,

no conclusive evidence has been provided that gives a satisfactory explanation for the disease[19,9, 107].

A great number of molecules are released during the inflammation phase of wound healing, such as growth factors, cytokines, and ECM components. It has been suggested that these contribute to a loss of regulation during the inflammatory stage and trigger the formation of KS by promoting cellular proliferation and excessive ECM deposition[9]. Shih *et al*. 2009, after performing an extensive literature review, provided a list of molecules that show significant differences in their expression when comparing normal wound healing and KS[19, 21, 108]. Among these, the cytokines TGF-β1, TGF-β2, TNF-α and IL-6 showed higher expressions in keloids. Growth factors such as EGF, PDGF, PDGF-receptors, VEGF, CTGF, IGF-I-receptor were also found to be up-regulated in keloid samples. Moreover, molecules including histamine, p53, PAI-1, metalloproteinases (MMP-1,2,3,13 and 19) showed increased expression in keloids in comparison to normal scars. Signalling pathways involving the molecules mentioned above result in the modulation of cellular function such as ECM synthesis and deposition; cell recruitment, proliferation and migration; re-epithelialisation and a delay in granulation tissue formation[8, 19, 90, 91, 107, 109, 110].

During granulation tissue formation, the expression of growth factors such as VEGF, PDGF-α and CTGF appear to be up-regulated in keloids[111-113]. The expression of these factors has been reported to be closely related to the expression of the TGF-β family[114-117]. The combination of these factors together regulates Collagen I and III synthesis, neovascularisation and cell migration amongst others. An increase in the concentration of collagen I and III is frequently reported in KS when compared to normal scars[118,22, 94, 119,19, 26, 93].

Finally, it has been shown that during the last wound healing stage there exists a well regulated production and degradation of ECM components, which allows scar maturation, whereas the equilibrium is lost in keloids. Keloid fibroblasts demonstrate reduced ability to degrade collagen fibres. Apoptosis is also very

important during the final healing stage, producing scar tissue deprived of cells and vascularity, whereas keloid disease appears to present low apoptotic ratios, a factor that may contribute to create the imbalance between the ECM synthesis and degradation. Figure 2.8 represents a summary of the aberrations that can occur during wound healing which may result in the generation of keloid pathogenesis[3, 19, 26, 120, 121].

2.8.4 Skin mechano-transduction, wound healing and keloid development

The skin protective barrier plays a key role adapting and responding to the mechanical forces throughout one's lifetime. The bio-mechanical properties of skin vary accordingly in different regions of the body and directions. Skin adapts itself according to the rate of force applied. The aging process reduces the skin's ability to support environmental conditions and recover its original shape[54, 122-124].

Factors such as diseases, pathologies, exposure to solar radiation or injures have been reported as causes that may modify the skin bio-mechanical properties[60]. Daily movements such as changing the body position produce a great amount of force that acts upon the skin. These forces alter the skin structure promoting rearrangement of the skin's elastic fibres in order to support the distension of the skin area compromised. When the force is removed, the elasticity of the skin allows the tissue to return to its natural shape. The correct performance of the skin involves rheological functions of the tissue and the equilibrium of the tensile properties (flexibility and elasticity). The ideal conditions of equilibrium in the skin are difficult to maintain due to the existence of biological and physical variables that influence the modulation of tensile properties. The activation of several cell mechano-signals also contributes to the maintenance of the equilibrium of the skin's tensile properties[125, 126].

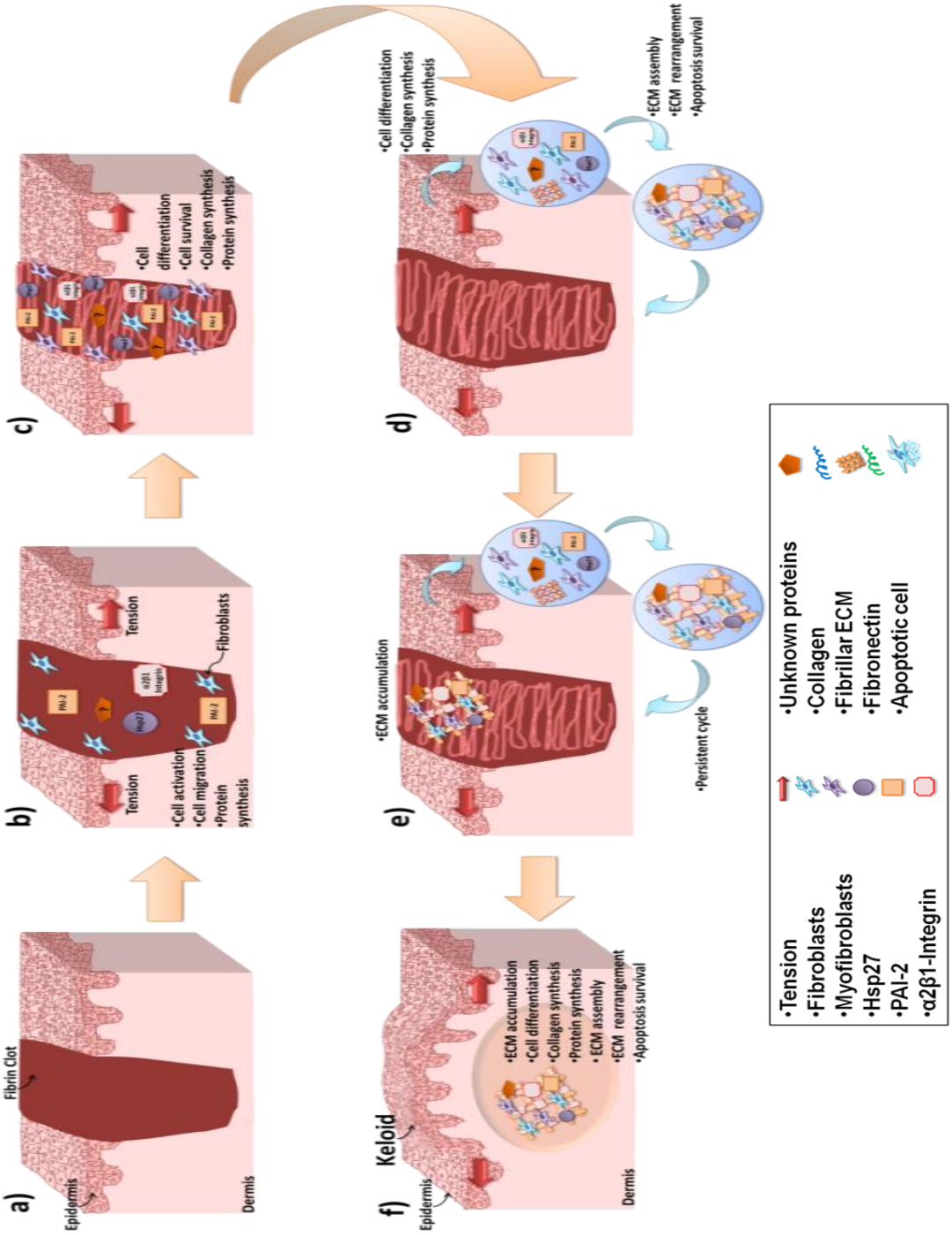


Figure 2.8 Wound healing aberrations that result in keloid development.

Several studies have shown that the application of high-tension forces in a specific body area generates local deformation; this phenomenon occurs frequently while wound healing takes place[45, 48, 103]. Clinical observation suggests that better scars are

produced when wounds are generated parallel to the tension lines, also known as Langer's lines, in contrast to the ones produced across such lines[48, 127].

If skin integrity is compromised by disease or injury, the biomechanical properties are modified, reducing the skin's elasticity, stiffness and viscoelasticity amongst other properties[51]. Once the mechanical stimuli is sensed by the skin's sensory nerves receptors, activation of intracellular pathways takes place and the physical stimuli received is transformed into biomechanical signals that involve the activation of several cells and molecules. This process is called mechano-transduction[17, 126]. Nowadays, it has become clear the needed to understand and analyse how mechanical forces affect the biochemical activities of living cells individually and as part of a more complex structure such as a tissue[32]. Mechano-transduction has several implications on the regulation of cell behaviour, including proliferation, spreading, differentiation, migration, apoptosis, among others[34].

Mechano-transduction processes can be classified into two categories: direct mechano-transduction which involves changes in the cytoskeleton conformation to transmit the information received from the extracellular environment to the cell nucleus in the form of mechanical signals[34]. Whereas the indirect mechano-transduction employs chemical signals to pass the information to the nucleus through diverse signalling pathways[34]. In this context, several molecules, cellular components, and extracellular structures (including ECM, cell-ECM and cell-cell adhesion, membrane components, cytoskeletal filaments and nuclear structures) have been considered mediators of cellular mechano-transduction[36]. To better explain how cells perceive and transform tension loads from the extracellular environment into biomechanical signals several models have been proposed, including the mechanical and tensegrity models[30]. Mechanical models fail to provide a satisfactory explanation of mechano-transduction due to they consider the cell as an elastic membrane or cortex surrounding a homogeneous cytoplasm that is viscous, viscoelastic or elastic provided of a nucleus in the centre of the structure and ignore the cell internal microstructure[30]. Whereas the tensegrity model, considers that cell's microfilaments (actin) and microtubules (tubulin) form a

tensegrity structure in the cell (tensegrity can be defined as a design principle that explains how structures develop stability in their shapes)[36]. In other words, the cell tensegrity model proposes that the cytoskeleton mechanically stabilises the cell as a tensed tensegrity structure that stabilised its shape by constant tension rather than compression [30]. Tensional forces are transmitted by the cell actin microfilaments and tubulin microtubules, and then these forces are stabilised by the connection of structural elements (internal microtubules and ECM adhesions) that resist compression. It is also suggested that individual filaments can perform a dual role allowing tension and compression at different size scales depending on the structural context[32, 36]. Therefore, the tensegrity model differs from the mathematical models in considering the application of local stress on the cell surface can be related to the direct deformation of structures, which depends on the molecular connectivity across the cell membrane and through the cytosol. Cell tensegrity proposes that cell-surface adhesion receptors such as integrins play a key role during the regulation of the translation of mechanical signals into biochemical responses in the cell. Integrins acts as link between the cell cytoskeleton and the ECM. Mechanical stimuli applied to integrin-binding sites results in local recruitment of focal adhesion molecules which results in an increase in cytoskeleton tension and the activation of signalling cascades. The application of higher loads to integrin-mediated focal adhesion contacts results in physical alteration of the cell membrane, filaments realignment and molecular rearrangement[30, 32, 34].

Having in consideration the mechano-transduction process and cell tensegrity model, several studies conducted on keloids have hypothesised a very close relationship between keloid development and skin mechano-transduction[100, 128-131]. These studies show that there is an increased incidence of keloid growth in those body areas that are highly tensioned. This hypothesis is also supported by the marked tendency of keloids to adopt specific shapes depending on the anatomical location, which appears to be related to the mechanical force distribution and the direction of the skin tension fibres[17]. It has also been proposed that the stress developed within the tissue surrounding the keloid lesion combined with the natural body movement plays an important role in directing the formation of the irregular shapes observed in keloid scars[2, 59, 131, 132].

Studies based on finite element method had also been developed in order to better explain the role that mechano-transduction play during keloid development. Akaishi, *et al.* demonstrated that high tensioned areas at the edges of the keloid can be appreciated after simply stretching the keloid model. Moreover, it is proposed that low tension in the centre of the keloid may be related to better healing, this is frequently observed in the centre of large keloids. This study also shows that the application of vertical stretching to the model disperses the tension on the edges of the keloid. This model also provides evidence of the correlation existing between the skin stiffness and the tension generated in the keloid area, showing that an increase in the skin stiffness results in a significant increment in the tension applied to the keloid. This suggests that the keloid growth in the superior abdominal region may be explained by tension[2, 48]. Same results have been shown in a study conducted by Nagasao, et al. who analysed keloid samples from the sternum[55].

Results demonstrated a significant reduction in scars redness, flattening and shine[132] by the use of adhesive bans that were positioned in such a way that reduces or nullifies the tension exerted in the wound region post keloid surgery removal or in keloid lesion[56, 57, 132]. Moreover, it was also observed that after keloid surgical removal all those patients that were treated with adhesive bands did not present new evidence of keloid development once the bands were removed. Taking together this results, it is suggested a relationship between skin tension and the triggering of keloid development[56].

2.8.5 Mechano-signalling: Normal wound healing vs. keloid

Mechano-signalling plays a key role controlling the wound healing process by the regulation of biomechanical signals closely related to cell survival, recruitment, differentiation, migration, proliferation and apoptosis, as well as, phenotypic and protein expression[17, 105, 133]. Regulation of these pathways is performed by the interaction of several and complex elements such as focal adhesion components that are employed to help the cells to attach to the ECM[85]. Focal adhesion complexes transport the load signals from the extracellular region to the cells interior, allowing the establishment of communication between cells and the environment creating a

balance between them[134]. It has been proposed that a loss in cell-ECM balance and signal transduction promotes the development of skin diseases and fibrosis[17, 85, 134].

To date, the study of the scarring mechano-signals has provided a wide list of interconnected pathways mainly activated in dermal cells (fibroblasts and myofibroblast), considered to be key regulators of ECM production and scar contraction[135]. To this end, pathways such as the transforming growth factor-β-SMADs, focal adhesion-integrin and calcium ion-channel mediated are the main pathways involved in scar mechano-transduction as shown in Figure 2.9. Furthermore, signalling mediated by mitogen activated protein kinases (MAPK)-G protein, focal adhesion kinase (FAK), tumour necrosis factor-α (TNF-α), Rho GTPases, phosphoinositol-3-kinase (PI3K) and interleukin (IL) pathways have also been reported to be key during wound healing, mechano-regulation and signalling[17, 85, 134, 135].

Among the signalling pathways mentioned above, proteins and genes regulated by the activation of signals such as the integrin and MAPK/ERK mediated pathways, including heat shock proteins, metalloproteinases, and cytokines, have been reported to be closely related to keloid scar development, promoting cell survival, and excess ECM production and deposition. More studies are required to elucidate the role that mechanical tension plays in the development of keloid scars, as well as to elucidate the pathology progression mechanism[2, 3, 136-138].

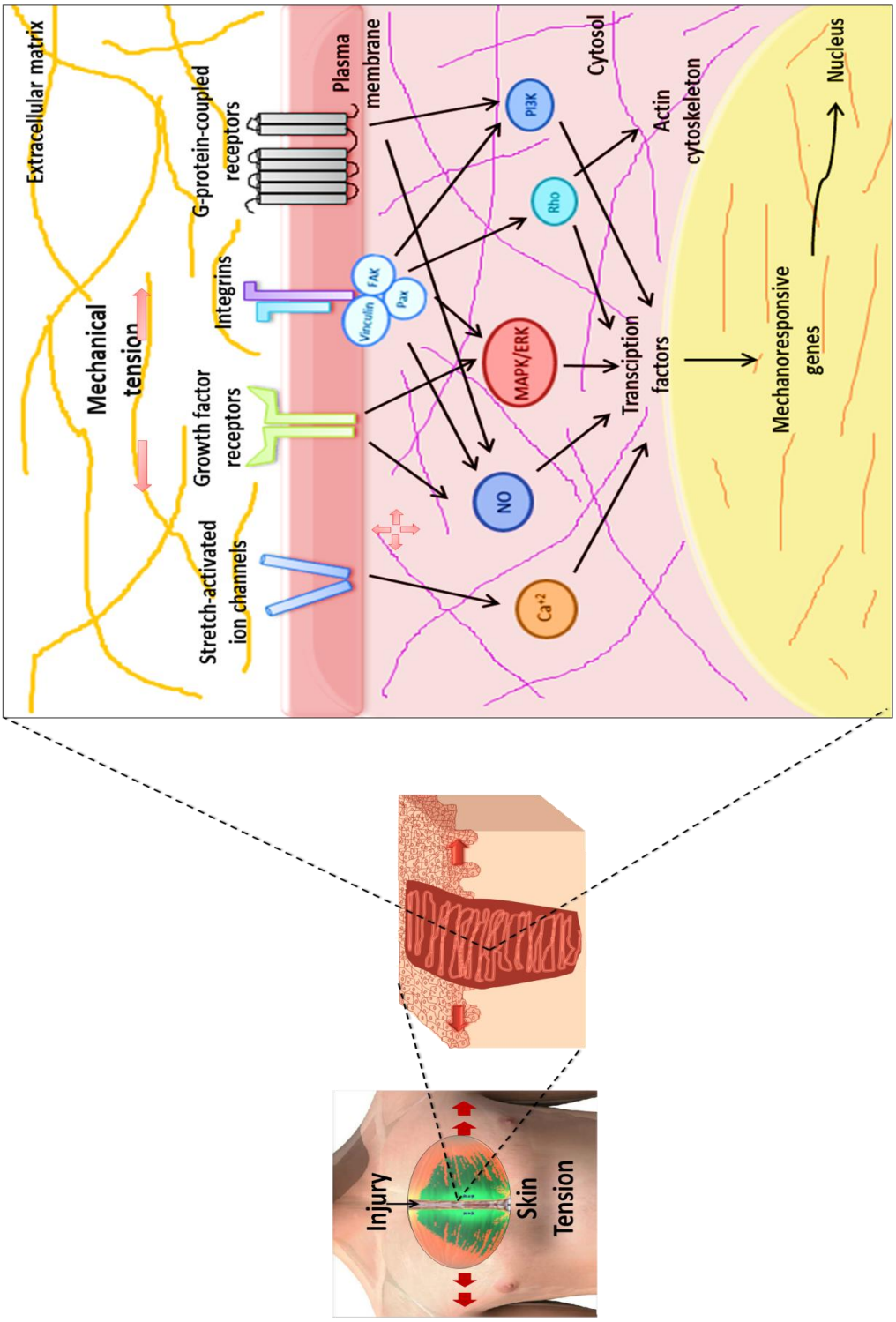


Figure 2.9 Wound healing mechano-transduction.
(Modified from Wong *et al*., 2011)[17].

Chapter III: Materials and methods

3.1 Introduction

The present chapter includes a description of the general materials, consumables and methods employed in the realisation of this research. Particular methodologies or modification to specific methods are described at material and methods section in the required chapter.

3.2 General consumables

All general reagents were obtained from Sigma-Aldrich (Dorset, UK), whereas general laboratory consumables were obtained from Scientific Laboratory Supplies Ltd. (Nottingham, UK).

3.2.1 Primary cell culture reagents and media

General cell culture reagents, supplements and media were obtained from PAA Cell Culture Company (Cölbe, Germany), Gibco-Invitrogen (Paisley, UK) or Sigma-Aldrich (Dorset, UK)

3.2.2 Solutions and cell culture media

Collagen gel pH 7.0	Minimum Essential Media (MEM)	20%
	Rat Tail collagen type I	80%
	NaOH (Neutralizing solution)	1M
Primary fibroblast culture medium	Dulbecco's Modified Eagle Medium (DMEM)	
	Foetal bovine serum (FBS)	10% (v/v)
	Penicilin/streptomycin	100µg ml^{-1}
	L-glutamine	10µM
Permealising Buffer	Phosphate buffered saline pH 7.4	
	Triton X-100	1%
Washing Buffer	Phosphate buffered saline pH 7.4	
	Tween-20	0.10%

3.3 Patient data and tissue collection

Tissue biopsies from normal skin and KS were obtained after acquiring informed consent from all patients before surgery (full ethical approval was obtained from the local hospital, University and regional NHS Ethics Committee). All keloid patients were confirmed to have clinical and pathological evidence of keloid disease under the following selection criteria. Patients with typical clinical aspect of keloid scaring and confirmed by histological diagnostic of keloid were included into the study. An abnormal scar was considered as a typical keloid if the lesion presented significant growth beyond the margins of the original injury, was present for 1 or more years, was not responding to any conventional treatment or recurred after surgery or therapy. Normal skin tissue samples were collected from tissue adjacent to the lesion in the same anatomical location following the criteria previously described by Syed, *et al*., 2010. [22] At the time of surgical excision, the central area of the keloids was obtained and divided in two sections, one of the portions was kept in 10% formalin buffer solution (Sigma-Aldrich, Poole, U.K), and the other collected in Dullbecco´s modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Cölbe, Germany). The samples in DMEM were processed for cell culturing within 12 hr; whereas, tissue samples embedded in formalin were processed within 24 hr for histological purpose.

3.4 Bio-molecular techniques

3.4.1 Histology

a) Tissue paraffin embedding

Tissue samples embedded in formalin were processed for paraffin-wax blocks preparation using a Leica tissue processing 1020 (Leica Biosystems, Milton Keynes, UK) under the following series of conditions: tissue samples were treated first in 70% ethanol for 2 hr, followed by 2 hr immersion in 90% ethanol and 5 further steps in 100% ethanol solution for 2 hr each. Tissue biopsies were then treated with xylene (3 steps 1hr each) and finally, 2 additional immersion steps in wax, 2 hr each, were performed. Tissue samples were embedded in paraffin blocks. Sections of 5µm thickness were cut and stored at room temperature for further experiments.

b) Haematoxylin and eosin staining

Sections of 5μm thickness from tissue specimens previously embedded in paraffin blocks and fixed in formaldehyde were stained with haematoxylin and eosin (H&E) (Surgipath, Peterborough, U.K.) 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 tap water were performed. A subsequent staining employing van Gieson (Sigma-Aldrich) was performed for 10 min. Tissue sections were washed in 1% acetic acid for 1 min and dehydrated in graded ethanol (95% and 100%), then treated with xylene for clearing and mounted.

c) Immunohistochemistry (IHC) protocol

Tissue biopsies were embedded in 4% v/v formalin, cut into 5 μm slides, fixed on glass slides and dried at room temperature overnight. After been deparaffinised the slides were treated with primary antibodies overnight at 4°C. The list of primary antibodies employed and their respective concentration is shown in the appendix section Tables A1 and A2. Following incubation each slide was thoroughly rinsed three times and incubated for 1 hr at room temperature with the secondary antibody (in TBS with 1% BSA). The primary antibodies specific binding sites were visualized by the respective chromogen addition. All sections were examined by using a Zeiss MC-100 microscope.

3.4.2 Primary keloid and normal skin fibroblast culture establishment

The tissue was collected in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK) and fibroblasts were extracted using collagenase type I solution, 0.5 mg mL^{-1} (Roche diagnostics, UK). The fibroblast extraction protocol is described step by step as follows: Tissue biopsies freshly extracted were placed in DMEM supplemented with 10% of heat-inactivated foetal bovine serum (FBS), 100 U mL^{-1} penicillin and 100 uUmL^{-1} streptomycin, 10% heat-inactivated FCS, 2 mmol L^{-1} $_{L}$-glutamine (PAA Laboratories) and kept at 4°C until use. The epidermis and hypodermis skin layers were removed and disposed of following the health and safety dispositions. All tissue biopsies were chopped, the tissue digestion was carried out using collagenase type I solution (Firstlink, Wolverhampton,UK)

incubated for 2 hr at 37°C in a water bath, followed by a further incubation at 4°C overnight to achieve a more accurate cell extraction. The obtained tissue suspension was transferred into T25 culturing binding flasks (Sigma-Aldrich) and fresh culture medium was added. Freshly extracted cells were maintained at 37°C under standard conditions (5% CO_2 and 95% humidity) (Figure 3.1).

3.4.3 RNA extraction protocol in tissue and primary cell cultures

A total of 800µl of TRIzol solution was employed for every 10mg of tissue sample or every 5-10x10^6 cells. Samples were incubated for 5 min at room temperature. Tissue biopsies were disrupted using a tissuelyser II (Qiagen, Manchester, UK) machine for 10 min at 30rpm/min, whereas all cellular samples were lysated by pipetting until a homogeneous lysate was obtained. Following disruption, 350µl of chloroform was added. Samples were mixed by shaking by hand until a milky red colour appearance was observed. The homogenates were centrifuged for 15 min at room temperature at 14500rpm. The aqueous layer was collected and transferred into a new eppendorf tube. RNA was precipitated by the addition of 350µl of 70% ethanol to the respective aqueous layers and homogenised by gentwele pipetting. A volume of 700µl of sample was transferred to the RNeasy spin column placed in a collection tube and centrifuged for 30 sec at 14000rpm. The flow through was discarded and 600µl of RW1 buffer was added to the mini column. The spin column was centrifuged for 30 sec at 14500rpm. The flow through was discarded, the spin column was transferred into a new collector tube and 500µl RPE buffer was added. The column was centrifuged for 30 sec at 14500rpm. The flow through was discarded. After the washing steps, the spin columns were dried out by 60 sec centrifugation at maximum speed. The columns were transferred into a new collector tube and a total amount of 35µl of RNase free was added directly to the spin column membrane. After 3 min incubation at room temperature, all samples were centrifuged for 1 min at 14500rpm to achieve RNA elution. RNA samples were quantified using a NanoDrop ND-1000 UV-visible spectrophotometer (Thermo scientific, UK).

3.4.4 cDNA synthesis

The total RNA concentration was estimated and normalised for all tissue samples to 1µg and to 100ng for all cell samples for cDNA synthesis. qScriptTM, cDNA SuperMix (Quanta Biosciences, UK) was employed for cDNA synthesis according to the manufacturer's protocol. Briefly, 4µl of qScriptTM, cDNA SuperMix was added per every 20µl cDNA synthesis reaction, followed by the normalised amount of RNA to achieve the previously described RNA concentrations. Tubes were centrifuged for 30 sec and incubated for 5 min at room temperature. Further incubations of 1 hr at 42°C, 5 min at 85°C were carried out afterwards. Finally samples were cooled and stored at 4°C.

3.4.5 Quantitative polymerase chain reaction (qRT-PCR)

All quantitative reverse transcription-PCR was performed in a final reaction volume of 10µl using a LightCycler® 480 II platform (Roche Diagnostics) as follows: 5µl of Light Cycler® 480 probes master mix (Roche Diagnostics) was mixed with 4µl of cDNA template, 0.2µmol/L of the respective forward and reverse primers (Sigma Aldrich), 1µl of Universal Probe Library (Roche Diagnostics) and 0.7µl of nuclease-free water (Ambion, UK). Each reaction was carried out in triplicate. All experiments were performed using 96-well plates (Roche Diagnostics). An initial cycle of 95°C for 10min was required to initiate the reaction (Hot Start Taq polymerase activation), followed by 40 amplification cycles of 10s at 95°C (denaturation) and of 30 sec at 60°C (annealing and elongation), finally a cooling step of 30 sec at 40°C was carried out. The normalization of the gene expression levels was performed to the expression of the internal reference gene RPL-32.

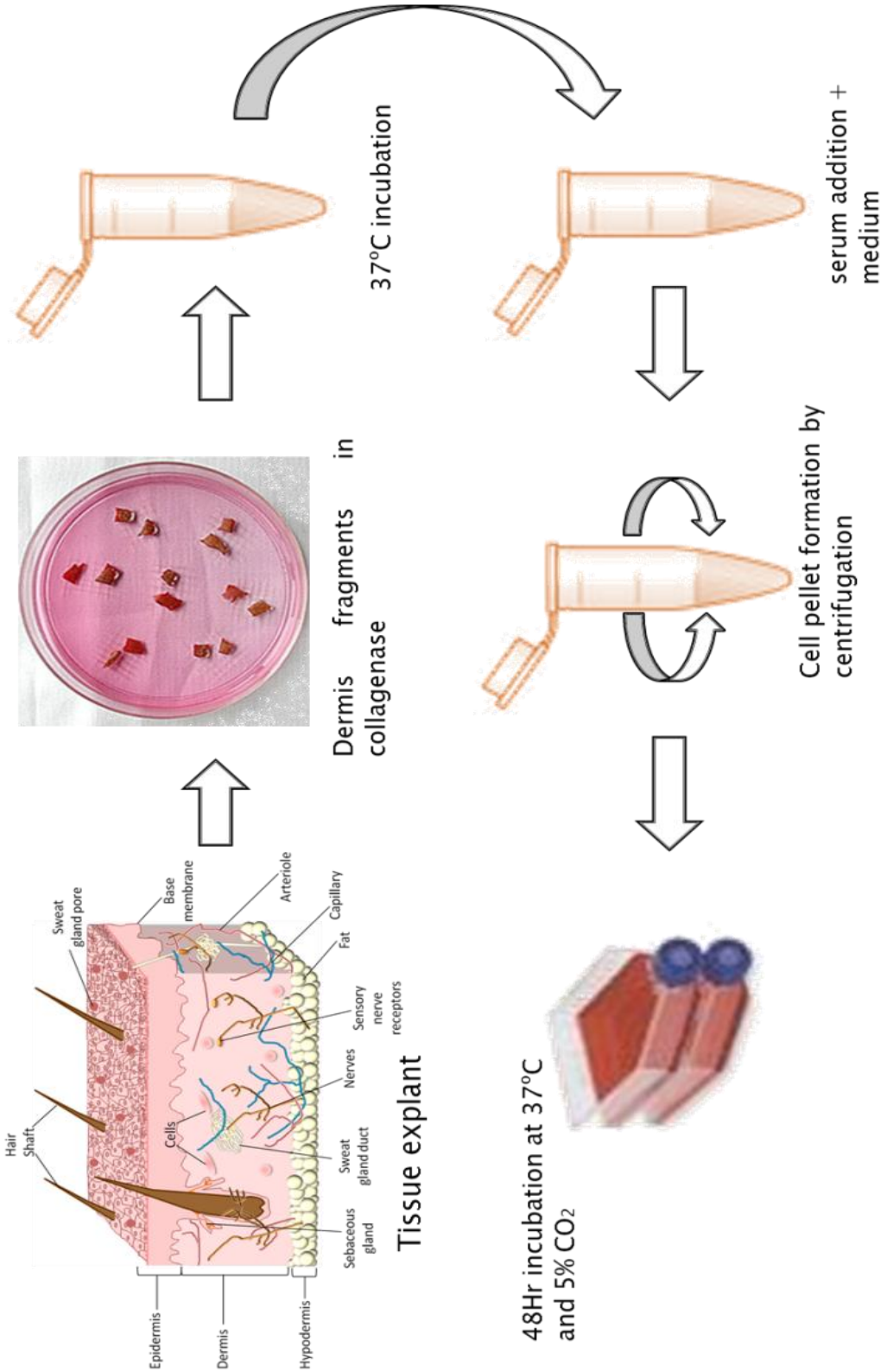


Figure 3.1 Primary fibroblast establishment.

3.4.6 High-Throughput In-Cell Western Blotting and quantification

Eighty to 90 per-cent confluent primary keloid fibroblasts and fibroblasts from normal skin (passage 0-3) were starved in 0.2% serum Dulbecco's Modified Eagle Medium for 24 hr. Cells were then trypsinized and counted using fluorescence-activated cell sorting (Accuri C6 Flow Cytometer System; BD, Oxford,United Kingdom). 1 x 10^4 cells were inoculated into each well of a 96-well plate. The cells were then grown to confluence for 24 hr in Dulbecco's Modified Eagle Medium. After 24 hr, the cells were washed once with phosphate-buffered saline (PBS) and fixed using 4% formaldehyde for 1 hr at room temperature. In-cell Western blotting was carried out using the standard protocol. Briefly, once fixed the cells were washed 3 times with PBS and made permeable with PBS-1%Triton X-100 for 15min at room temperature. Cells were blocked in Odyssey blocking buffer (LI-COR) for 1.5 hr at room temperature and incubated with 50µl of primary antibody/well in Odyssey buffer blocking buffer-PBS (1:1 volume) overnight at 4°C. Following antibody incubation cells, 3 washing steps were performed with PBS-0.1% Tween-20 and one additional washing step with PBS. Secondary antibodies were prepared in Odyssey buffer blocking buffer-PBS (1:1 volume) and incubated for 1 hr at room temperature, light cover. The wells were washed with PBS-0.1% Tween-20 three times and one more time with PBS. Washing solution was removed and the wells were left to dry under light cover. The well plates were imaged using an Odyssey infrared scanner (LI-COR) set with sensitivity 7 in 800nm wavelength channel. The data normalisation was carried out employing data obtained from those wells treated only with secondary antibody. β-Actin was used as a loading control; the panels of primary and secondary antibodies used in this study are listed in the appendix tables 1A and 2A.

3.4.7 Western Blot

Primary keloid fibroblasts (KF) from passage 0 to 3 were seeded in 24 well plates (1x10^5cells/well). Cells were incubated for 24 hr at 37°C. 24 hr post-seeding a wash step was performed using 500µl of PBS. Cells were harvested using RIPA buffer (Sigma-Aldrich, UK) supplemented with phosphatase inhibitors cocktail (Protease Inhibitor Cocktail Set III, EDTA-Free, Calbiochem, UK (1:200 dilution)) and lysed by pipetting. The total amount of protein was quantified with BSA protein assay kit

(Thermo scientific, UK). A total amount of 30µg of protein was employed to perform all the assays. All proteins were denatured at 100°C per 5 min using thermo-block. After denatured all samples were kept on ice until used. Equal amounts of protein were resolved on 4-12% Nupage Bis-Tris Mini gels and electrophoresis was performed according to the manufacturer's instructions (running conditions 200V and 135 mA). Proteins were blotted on polyvinylidenedifluoride membranes (PVDF) from polyacrylamide gels using iBlot® Dry Blotting System (Life technologies, Paisley, UK). The blocking step was carried out at room temperature for 1 hr using Odyssey blocking buffer (LI-COR, Cambridge, UK). After blocking, membranes were incubated overnight at 4°C with the respective primary antibodies (antibody dilutions were prepared in Odyssey blocking buffer (1:2 dilution with PBS)). Two washing steps were performed using buffer PBS-0.1% Tween, 10 min each. A final washing step was performed using PBS (10 min). Secondary antibody dilutions were prepared using Odyssey blocking buffer (1:2 dilution, PBS) then incubated for 1 hr at room temperature on gentle shaking. This was followed by two washing steps using buffer PBS-0.1% Tween, 10 min each. A final washing step was performed using PBS (10 min). The PVDF protein membranes were developed using Amersham™ ECL™ prime western blotting detection kit (GE Healthcare, UK). Membranes were exposed to Kodak X-OMAT X-ray film (Sigma-Aldrich, UK). Densitometry was performed with Image-J software (Maryland, USA)**.** Data was analysed with the Prism GraphPad Software (San Diego, CA, USA). The optical protein densities were normalised to the densities of loading control α-tubulin levels.

3.4.8 Cytotoxicity detection (LDH)

Cytotoxicity ratios were measured employing LDH assay (Roche Diagnostics) as follows: supernatant DMEM was collected from the respective samples and a dilution 1:2 was prepared with PBS. 100µl of the supernatant solution was added into 96 well plates and mixed with 100µl of LDH working reaction. All well plates were incubated for 30 min at room temperature in darkness. All samples were prepared in triplicate. Net absorbance (A450–A690) was calculated.

3.4.9 3D human fibroblast populated collagen lattice protocol

To perform the tensional testing human fibroblasts were seeded in three dimensional (3D) collagen gels as described by Eastwood *et al*., 1994[128], briefly: to prepare a total volume of 8mL of working collagen gel suspension 5 mL of rat tail collagen type I in acetic acid (First link) was added into a 15 mL falcon sterile falcon tube (protein concentration of 2.28mg/mL) followed by 1.7 mL of DMEM medium (Gibco BRL). Collagen type I suspension was mixed by tube inversion until complete homogenisation (yellow colour, pH 2). Collagen gel mix reaction was neutralised by the adhesion of 1M NAOH until colour changed from yellow to citrus pink (optimal pH 7.5). The pH determination was performed by the use of pH indicator strips. Following the neutralisation step, 1.3 mL of primary fibroblast previously re-suspended in DMEM was added to give a concentration of 10^6 cells/mL. Finally, the working reaction was mixed by tube inversion and cast between two cell attachment units into a rectangular plastic module (4.9 x 1.9 x 0.9cm) and incubated for 15 min at 37ºC in a 5% CO_2 humidified incubator. Once set, the collagen gel was physically detached from the chamber and floated in 10mL of DMEM (Figure 3.2).

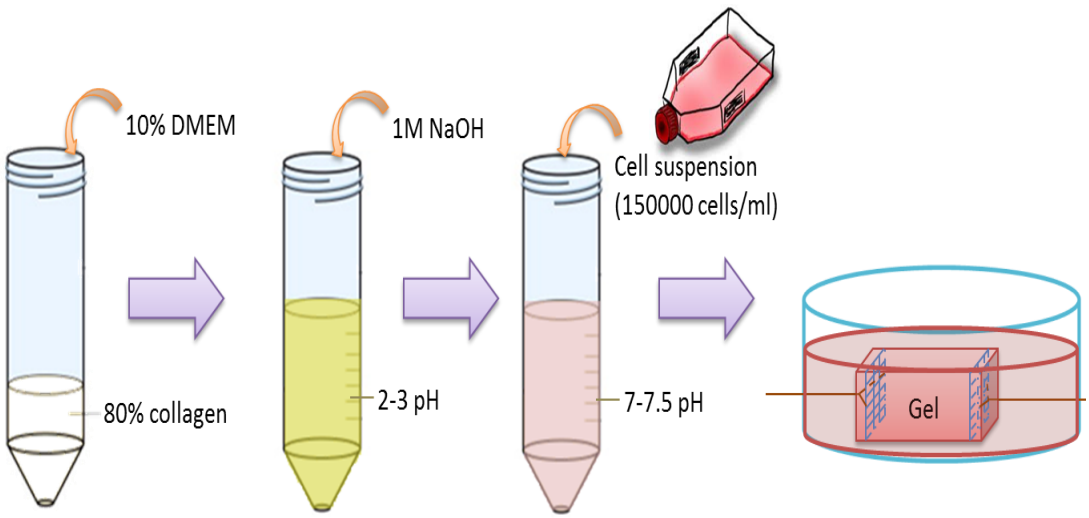


Figure 3.2 Collagen gel preparation.

3.4.10 Statistics

All data were expressed as the mean ±S.E.M. (standard error of the mean) of at least three independent experiments. To determine statistical differences, a one-way analysis of variance and non-parametric analysis were performed with Tukey multiple comparison post-tests. The difference between the means for all conditions were considered statistically significant at $p<0.05$, for which the GraphPad Prism 4 software was used.

Chapter IV: Biomarkers involved in differential profiling of hypertrophic versus keloid scars

4.1 Introduction

Cutaneous wound healing is a complicated, multistep process that involves the combination of several molecular, cellular, physiological, biochemical and mechanical factors[80, 139]. 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 as contributing to abnormal skin scar formation[21, 24]. This may result in the formation of raised dermal scars in the form of hypertrophic and KS[9, 21, 25]. Hypertrophic scars (HS) are raised dermal scars that remain within the boundary of the original injury, and can regress over time[24, 140], whereas, KS are raised dermal lesions that spread beyond the boundaries of the original wound and invade the healthy surrounding tissue[9, 141]. It had been extensively discussed the keloid's growth patterns due to this kind of scars present a highly variable and unpredictable pattern. However, it is widely accepted that keloid scars progress growing over the time if the scars remain untreated[28, 29, 33].

Nowadays, there is no a single effective therapeutic regimen capable to achieve a total remission for the keloid disease. However, there are a great variety of medical procedures used as keloid treatments including occlusive dressings, cryosurgery, surgical excisions, radio therapy, and pharmacological treatments[90]. All surgical wounds in keloid-prone patients should be closed with minimal tension and the incisions should follow skin creases when possible[142, 143]. Currently a great amount of drugs had been tested in order to provide a better resolution to keloid scars including corticosteroids injections[139, 144, 146], calcium channel blockers[144, 145], antiproliferative agents[146-148], interferon therapies[142, 144], immune modulators[149] and onion extracts [150, 151]. Further clinical managements had been employed in combination with pharmacological therapies including surgery[142, 147, 153, 154], laser therapy[142, 147, 159-162], silicon therapy[142, 147, 159-162], pressure therapy[142, 147, 155-158] and tension release therapies (adhesive bands and skin expanders)[90]. Unfortunately none of these treatments had shown total keloid reminiscence.

The first surgical treatment of keloids was described by Druit in 1844. Scar revision requires planning and preoperative counselling due to its high rate of recurrence. In patients that presents hypertrophic scars a simple excision is needed to achieve good results. Whilst keloid scars require a total excision; but in most of the cases this therapy is not successful due to the fact that this kind of excisions promotes the collagen synthesis. For this reason an intramarginal surgical excision is more appropriated to reduce as much as possible the additional collagen synthesis. However, the recurrence rate is reported between 45 and 100%. Nowadays, the surgical procedures are combined with other further treatments such as pressure, corticosteroids, and radiotherapy. Surgical procedures are considered as a second-line therapy for patients showing poor response to other procedures[142, 144, 152, 153].

The aim of the present chapter 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 as shown in appendix 9A and 10A respectively. In addition, appendix 11A summarises all those genes that were found to be commonly up-regulated in both scar types after the microarray comparisons performed by Shih *et al*. 2010 and Huang *et al*. 2013. These three previously mentioned lists of biomarkers were compared with my own unpublished microarray data which was derived from KS tissue. The biomarkers that were highly significant and 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)[3, 6, 10-12, 14, 16, 100, 131] 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 with both fibrosis and apoptosis. Candidate biomarkers (n=21) were selected for evaluation in both tissue and cells extracted from HS and KS.

4.2 Materials and 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 abnormal raise dermal scarring from HS and KS as previously described by Syed *et al.*[22]. 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 from passages 0 to 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 my study based on a number of 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, all those genes that were found commonly up-regulated in both scar types after microarray comparisons were selected (a gene was considered up regulated if its expression was found at least 1 fold higher express in raised scars compared to normal skin samples). The selected biomarkers were then compared with the lab's own unpublished microarray data which was derived from KS tissue. The biomarkers that were statistically higher expressed (at least 1 fold) and which were shared among the generated 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) in HS or KS found in at least three independent studies also were included. The final selection criteria employed was based on the biological relationship between potential target biomarkers identified from the above two approaches which were linked with both fibrosis and apoptosis[4, 90, 102, 141, 154].. 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 cytokines genes. A list of the 21 biomarkers investigated is given in Table 4.1. The design of the study is depicted in Figure 4.1.

4.2.1 Patient data and tissue collection

Normal skin (n=14), keloid (n=14) and hypertrophic skin (n=14) samples were employed in this study (Table A.3). Samples were taken from a number of different sites including the sternum, ear, pubis and scalp (see Table A3). At the time of surgical excision, tissue biopsies from each lesion site were collected and processed as previously described in chapter III section 3.3) The samples were processed for cell culturing within 12 hr and the tissue was processed within 24 hr.

4.2.2 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 chapter III (See section 3.4.2). Fibroblasts from passages 0 to 3 were employed for the experiments.

Table 4.1 List of gene names and symbols

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 90kDa 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 metallopeptidase13; 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

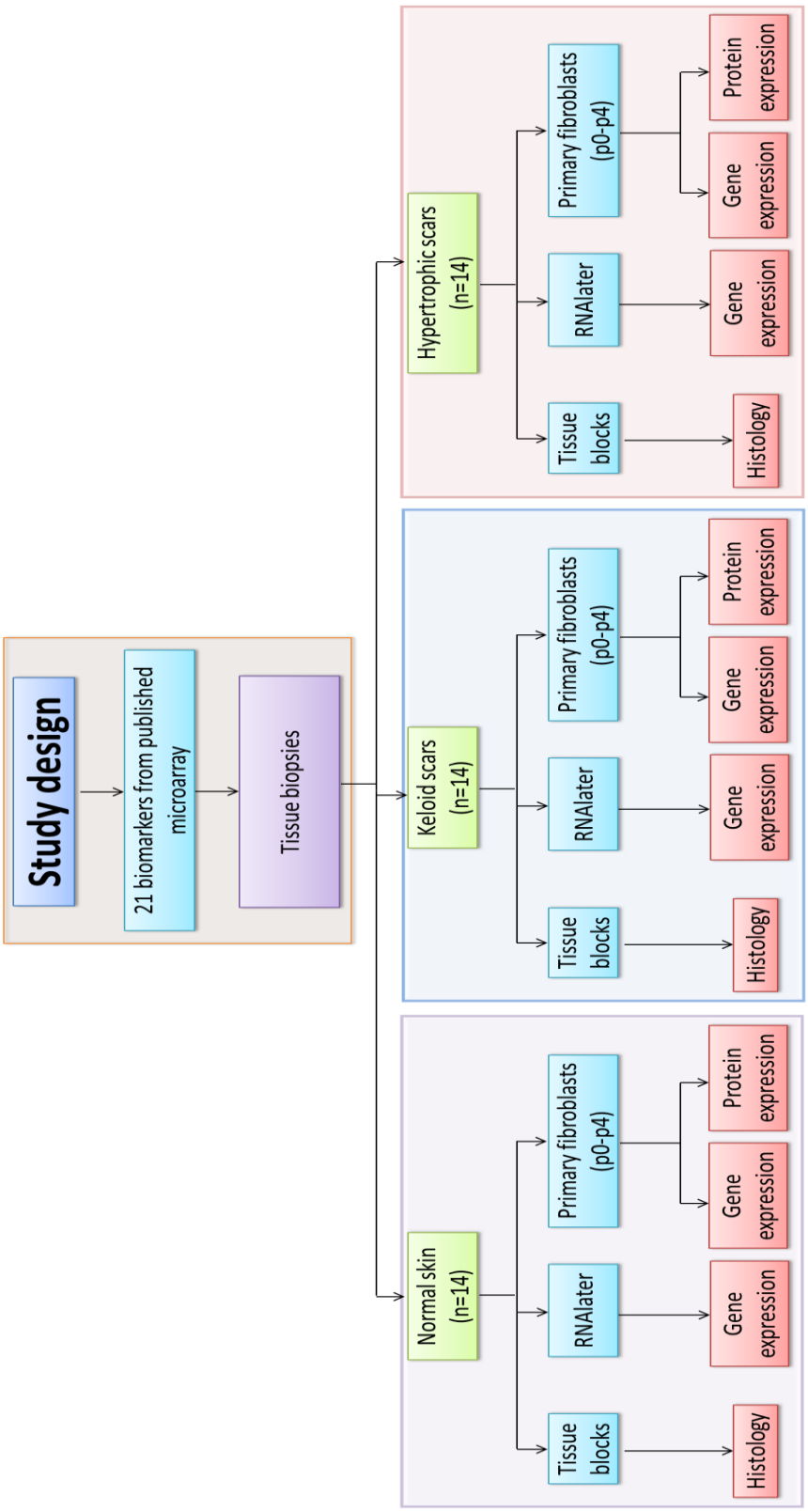


Figure 4.1 Study design.

4.2.3 Haematoxylin and Eosin staining (H&E)

Sections of 5μm thickness from Normal skin (n=14), keloid (n=14) and hypertrophic skin (n=14) samples were employed in the study (Table A.3), these were processed and embedded in paraffin blocks for H&E staining. Tissue sections were fixed and mounted as previously described in chapter III (sections 3.4.1).

4.2.4 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 reactions were performed as previously described in chapter III (Sections 3.4.3-3.4.5). The primer list employed can be found in Table A4. Each reaction was performed in triplicate. The gene expression levels were normalized with respect to a reference gene, the L32 ribosomal protein gene (RPL32).

4.2.5 In-Cell western blotting (ICW)

P0-p3 fibroblasts were grown to 95-100% confluence in T25 flasks. Samples were processed as described previously in chapter III (Sections 3.4.6). The panel of antibodies used is given in Tables A1 and A2. 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).

4.2.6 Statistical analysis

The statistical analysis was performed as described previously in chapter III (Section 3.4.10).

4.3 Results

4.3.1 Histological comparison of normal skin with keloid and hypertrophic scars

A histological comparison of **a)** normal skin, **b)** keloid and **c)** hypertrophic scars biopsies was undertaken employing haematoxylin and eosin (H&E) staining (Figure 4.2). Tissue biopsies were fixed for 24 hr and embedded in paraffin blocks. Five micrometer sections were cut and stained, using the standard protocol as described in materials and methods (chapter III, sections 3.4.1). Among the different tissue sections significant difference in the collagen fibres arrangement can be observed. Highly cellular populated zones were appreciated in the abnormal scars reticular dermis areas (black arrows) (Figures 4.2b and 4.2c). Haematoxylin and eosin (H&E) stained tissue samples (Figure 4.2) 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 (Figure 4.2a). The KS samples exhibited normal epidermis thickness with regular basal cell organization, but 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 arranged horizontally to the epidermal layer in most KS cases (Figure 4.2b). 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 (Figure 4.2c) in HS tissue. The above are in keeping with similar findings reported previously in other studies[96, 155].

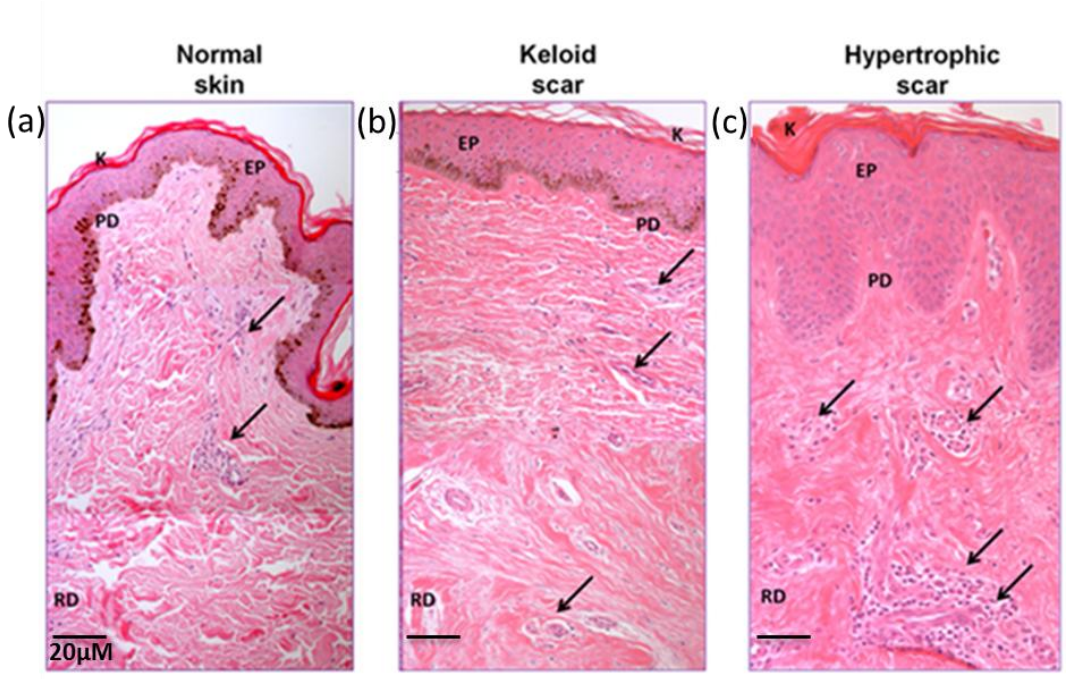


Figure 4.2 Histology of normal skin sample compared hypertrophic and keloid scars (n=14).
K, keratin layer; EP, epidermis; PD, papillary dermis; RD, reticular dermis. Original magnification x 200.

Having established histological differences between the tissue samples, which were to be employed in further experiments, an evaluation of potential differences existing among the samples at the molecular level was carried out.

4.4 Gene expression screening in normal, keloid and hypertrophic scar tissue

Abnormal scars result as a consequence of aberrations produced during the wound healing process and are characterised by excessive ECM production and deposition frequently combined with pruritus and pain. A number of factors have been implicated in the promotion of abnormal scar development, including anatomical location, lesion severity, tensional stress, injury deepness and environmental factors.

Among the catalogue of scar types keloid and hypertrophic scars are considered to present clinical similarities but little is known about the physiologic responses produced during the healing process to promote their differentiation. Recently, it has been proposed that skin tension related genes could be implicated in the promotion of abnormalities during the wound healing process. Therefore, the aim of

this study is to identify tension-related target genes that are responsible for the differentiation of keloid and hypertrophic scars. 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 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).

4.4.1 Neuropeptide gene expression in tissue biopsies from keloid and hypertrophic scar types

Previous findings have suggested that mechanical tension in the skin promotes the expression and release of neuropeptides during wound healing[156, 157]. In order to investigate this further, total RNA was extracted from tissue biopsies (n=14) and the expression of the neuropeptides SP, CGRP, NPY and VIP was evaluated using qRT-PCR. The expression of target genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of

independent experiments (n = 14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin (n=14); whereas #p >0.05 represents a significant reduction in the mRNA expression extracted from abnormal scar biopsies from normal skin. It can be clearly seen upon inspection of the results of the analysis, shown in Figure 4.3 that the neuropeptide mRNA levels detected were significantly different among the scar tissue types tested. SP expression was significantly higher in hypertrophic scars compared to normal and keloids, whereas CGRP mRNA was notably up-regulated in keloid tissue compared with normal skin ($p < 0.05$). Moreover, mRNA levels were notably down-regulated for the NPY and VIP genes in keloid tissue compared with the normal skin samples ($p < 0.05$).

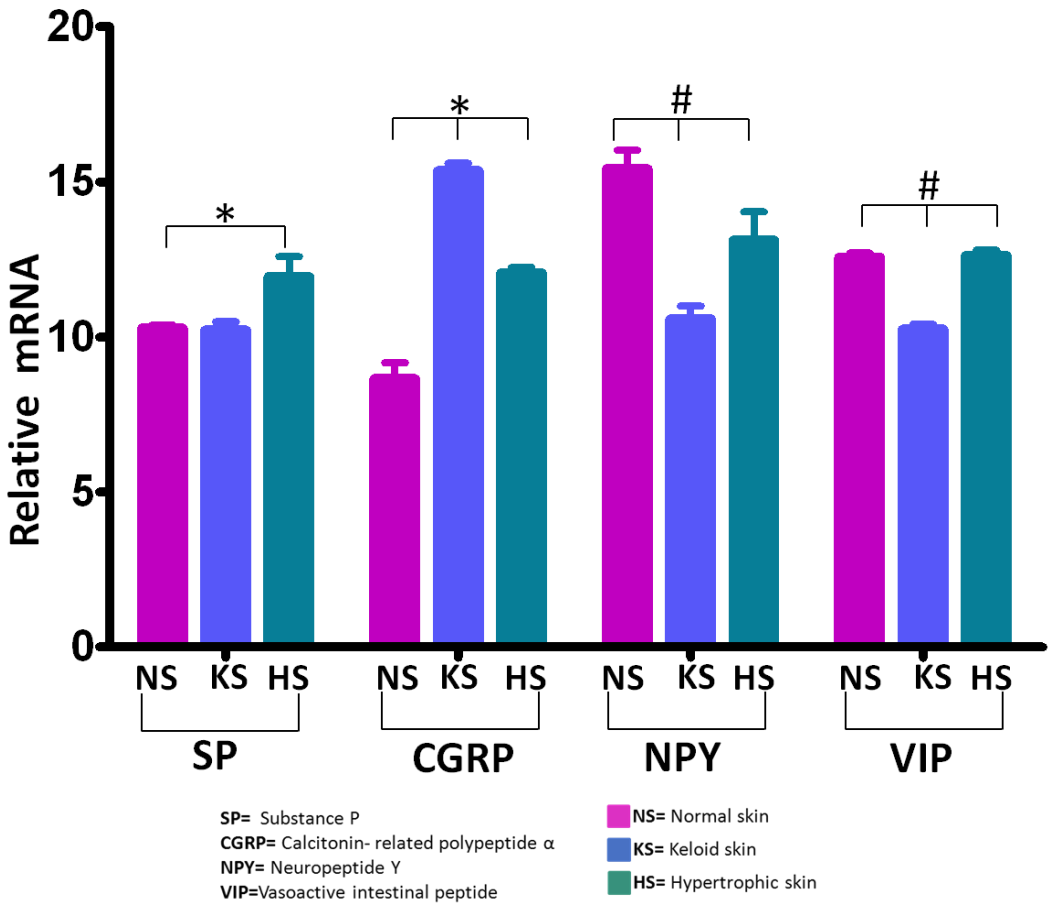


Figure 4.3 Tissue mRNA expression of neuropeptides seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

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

Figure 4.4 shows the results of the tension related gene expression characterisation (Hsps, MMPs and MCPs)[105, 110, 118]of tissue biopsies for 11 of the previously identified 21 genes. Total RNA extracted from tissue biopsies as described in chapter III (Sections 3.4.3-3.4.5, pages) was employed to evaluate the expression of the genes included in this group and normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments (n = 14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin (n=14); whereas #p >0.05 represents a significant reduction in the mRNA expression extracted from abnormal scar biopsies from normal skin. Of the 11 genes tested in this group, 5 showed significant over-expression at mRNA levels ($p<0.05$) in both keloid and hypertrophic scars when compared to normal tissue samples, these were Hsp47, Hsp60, MCP-3, MCP-1 and MMP-19. In contrast, three genes, Hsp90, MMP-3 and MMP-13, demonstrated a lower expression at mRNA levels in both scar types compared to normal skin. No significant difference was detected in Hsp70 expression among the samples. Hsp27 and PAI-2 were found to be over expressed in the keloid tissue samples.

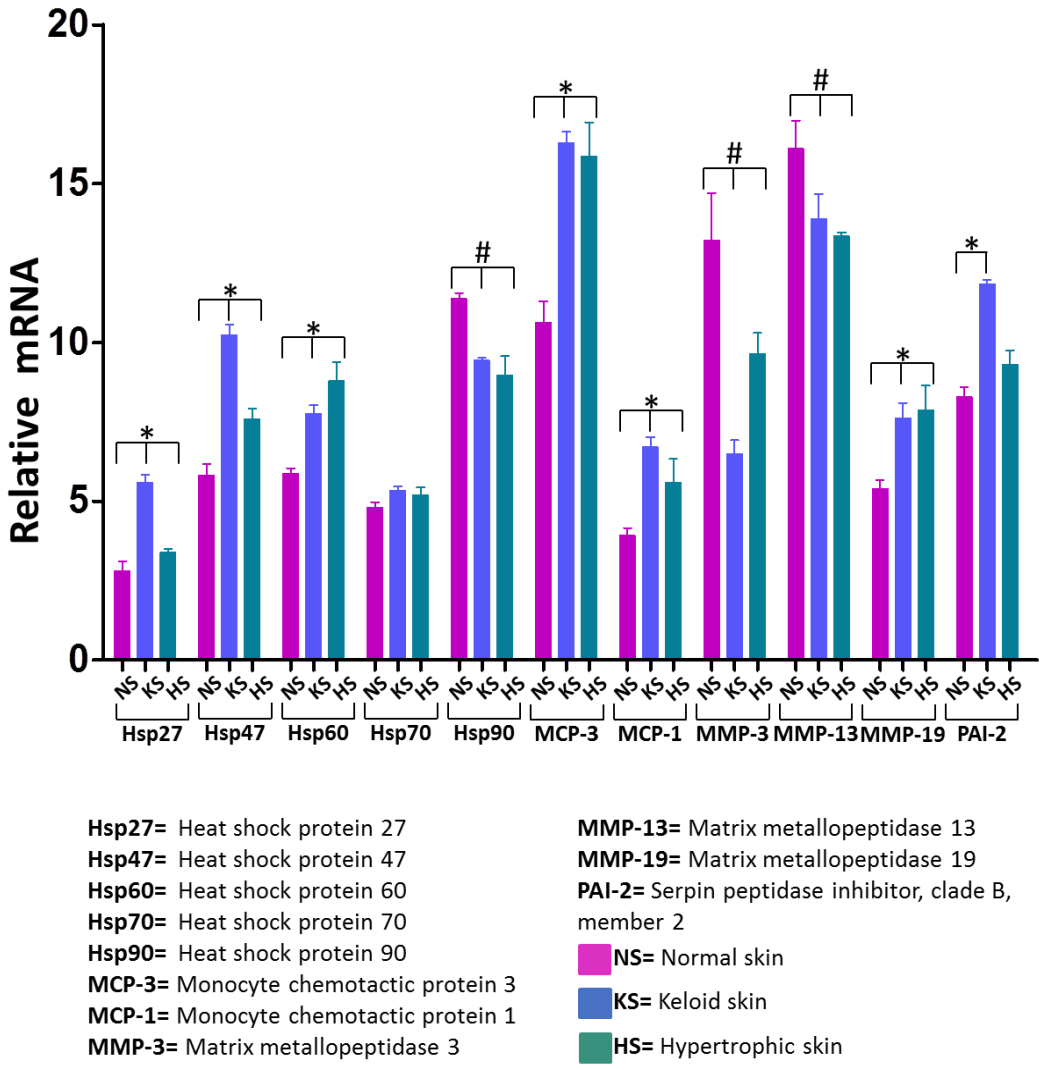


Figure 4.4 Cellular mRNA expression of neuropeptides in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

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

Several reports have suggested differences in the expression of genes involved in cell adhesion, communication, spreading, remodelling and ECM production when evaluated among different scar types [158, 159]. To further investigate these claims, a group of 3 genes, tenascin, α2β1-integrin and Cyr61, considered to be related to the aforementioned processes, which have been reported as being up regulated under tension conditions were assessed. The analysis of the mRNA expression of this

group of genes was performed using normal, keloid and hypertrophic scar samples (n=14). Total RNA extracted from tissue biopsies as described in chapter III (Sections 3.4.3-3.4.5) was employed to evaluate the expression of ECM related proteins and normalized to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments. *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin; whereas #p >0.05 signifies a significant reduction in the mRNA expression extracted from abnormal scar biopsies from normal skin. The qRT-PCR results presented in Figure 4.5 show a clear over expression of tenascin and α2β1-integrin in both keloid and hypertrophic scars compared to normal skin, whereas Cyr61 is down regulated in both abnormal scar types (p<0.05).

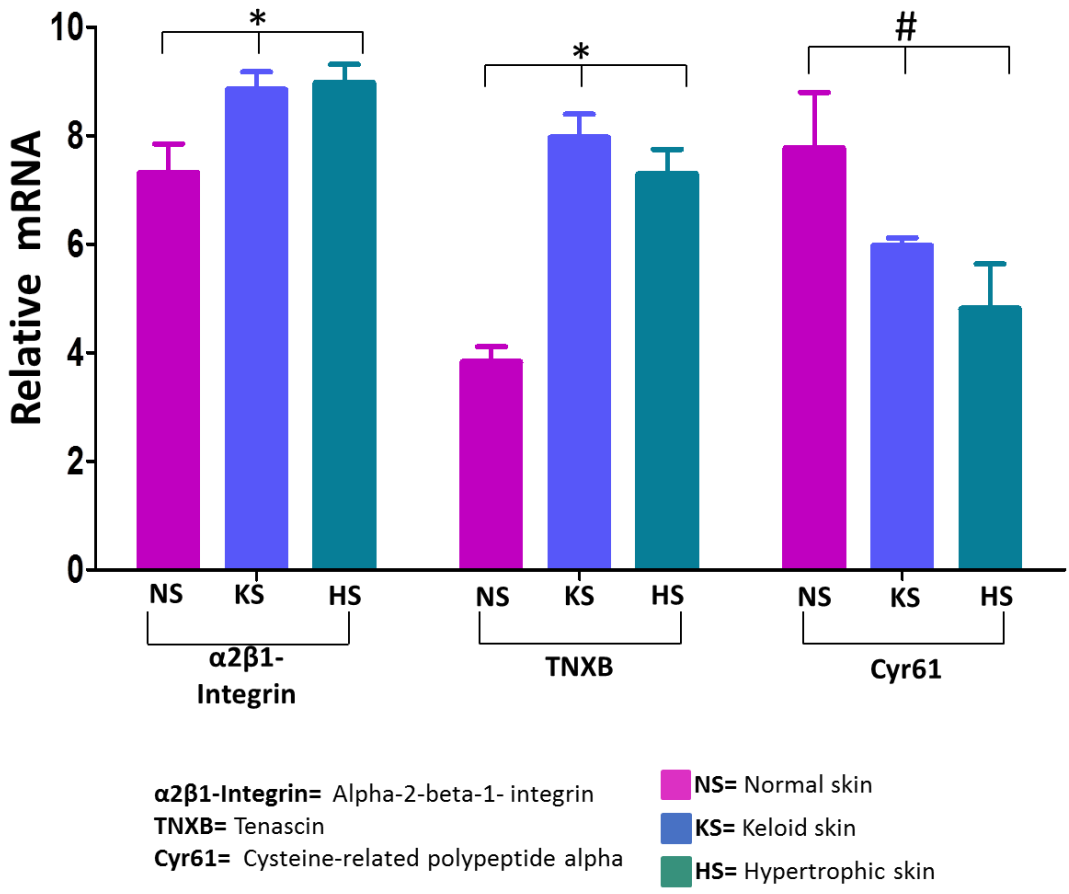


Figure 4.5 Tissue expression of mRNA of tension-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

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

The transcription factor expression TGF-β has been closely related to the action of physical forces in the body [117]. Therefore, I evaluated the expression of 3 TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3 using qRT-PCR. Total RNA extracted from tissue biopsies as described in the methods section chapter III (Sections 3.4.3-3.4.5) was employed to evaluate the expression of TGF-β transcription factor family genes and normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments (n = 14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin (n=14); whereas #p >0.05 represents a significant reduction in the mRNA expression extracted from abnormal scar biopsies from normal skin.

The results of the analysis are shown in Figure 4.6 where it can be seen that that the expression of TGF-β1 and TGF-β2 is significantly lower in keloid and hypertrophic scar tissue compared to normal skin samples (p<0.05). However, no statistical difference was found in the expression of TGF-β3 among the study samples.

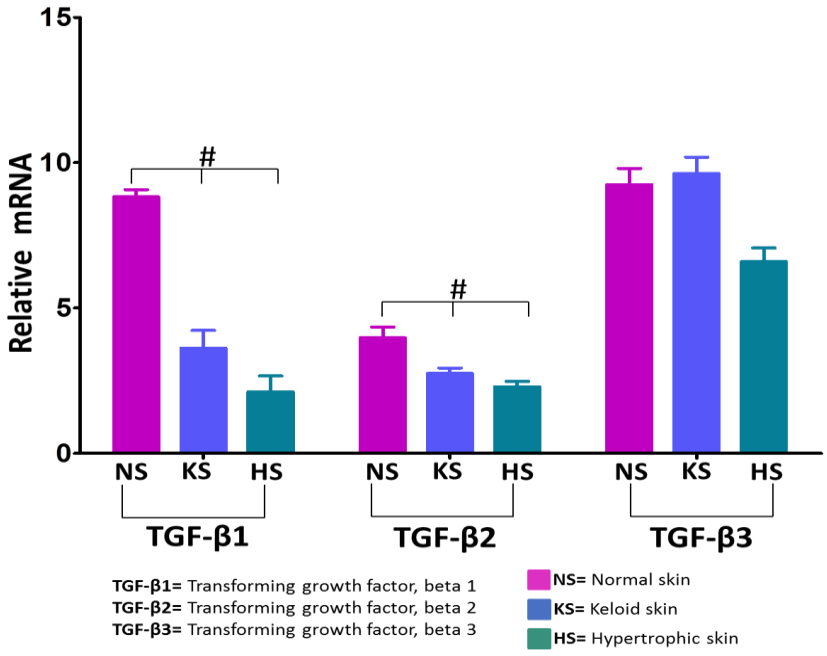


Figure 4.6 Tussue mRNA expression for tension-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

4.5 Gene expression screening in primary fibroblast cultures established from normal, keloid and hypertrophic scar tissue

To further confirm the results obtained from tissue samples, the expression of the tension markers classified into the four groups mentioned in the above section (a) neuropeptides, b) tension related (Hsps, MMPs and MCPs), c) ECM related and d) growth factor, were evaluated in primary fibroblast cultures established from the study samples. Fibroblasts from passage 0 (p0) to passage 3 (p3) were used for all experiments carried out in this study to avoid possible phenotypic alterations that could result from fibroblasts ageing through multiple passsages [160], [22, 90]. Thus, in order to minimise variability and have optimal consistency in data, only cells up to P3 were utilised in this study.

4.5.1 Neuropeptide gene expression in primary fibroblasts from keloid and hypertrophic scar types

To evaluate the neuropeptide expression in fibroblasts, total mRNA was extracted from primary fibroblasts established from normal, keloid and hypertrophic skin tissue biopsies from passage 0 (p0) to passage 4 (p4) as described in the methods section chapter III (Sections 3.4.3-3.4.5). The expression of neuropeptides including SP, CGRP, NPY and VIP was normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments (n = 14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin. qRT-PCR analysis of the samples (Figure 4.7) revealed that of the four genes tested, only CGRP was up regulated in keloid and hypertrophic cells compared to normal skin fibroblasts. No expression of the VIP gene was detected among the samples. Interestingly, the expression of SP and NPY was significantly higher in keloid fibroblasts compared to normal skin ($p<0.05$).

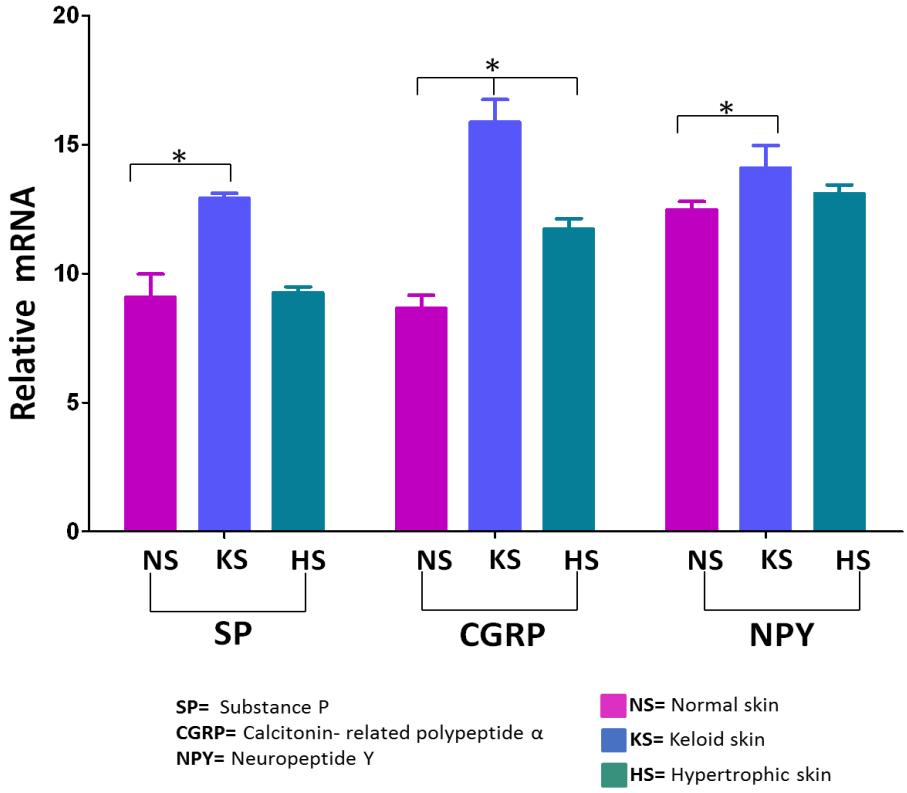


Figure 4.7 Tissue mRNA expression of ECM-related seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

4.5.2 Characterisation of the mRNA levels of tension-related biomarkers in primary fibroblasts from keloid and hypertrophic scar

The expression of tension related genes were also evaluated in primary fibroblast cultures (n=14). Fibroblasts were cultured from p0 to p4. Total RNA was extracted from primary fibroblast cultures from normal and abnormal scar samples as described in methods section chapter III (Sections 3.4.3-3.4.5). The expression of tension related genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments (n=14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin (n=14). Whereas #p >0.05 signifies a significant reduction in the mRNA expression extracted from abnormal scar fibroblasts from normal skin. The results are shown in Figure 4.8. At the mRNA level, where upon inspection it can be seen that a significant difference was found between the abnormal scar cell samples and normal fibroblasts for the Hsp27,

Hsp90, MMP-13 and MMP-3 genes, 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 genes was significantly higher in keloid fibroblasts compared to the other samples, but the Hsp47 gene exhibited a lower expression in keloid cells. In hypertrophic cell samples the expression of Hsp60 was significantly higher compared to normal fibroblasts ($p<0.05$) but the expression was lower for the MCP-1 gene.

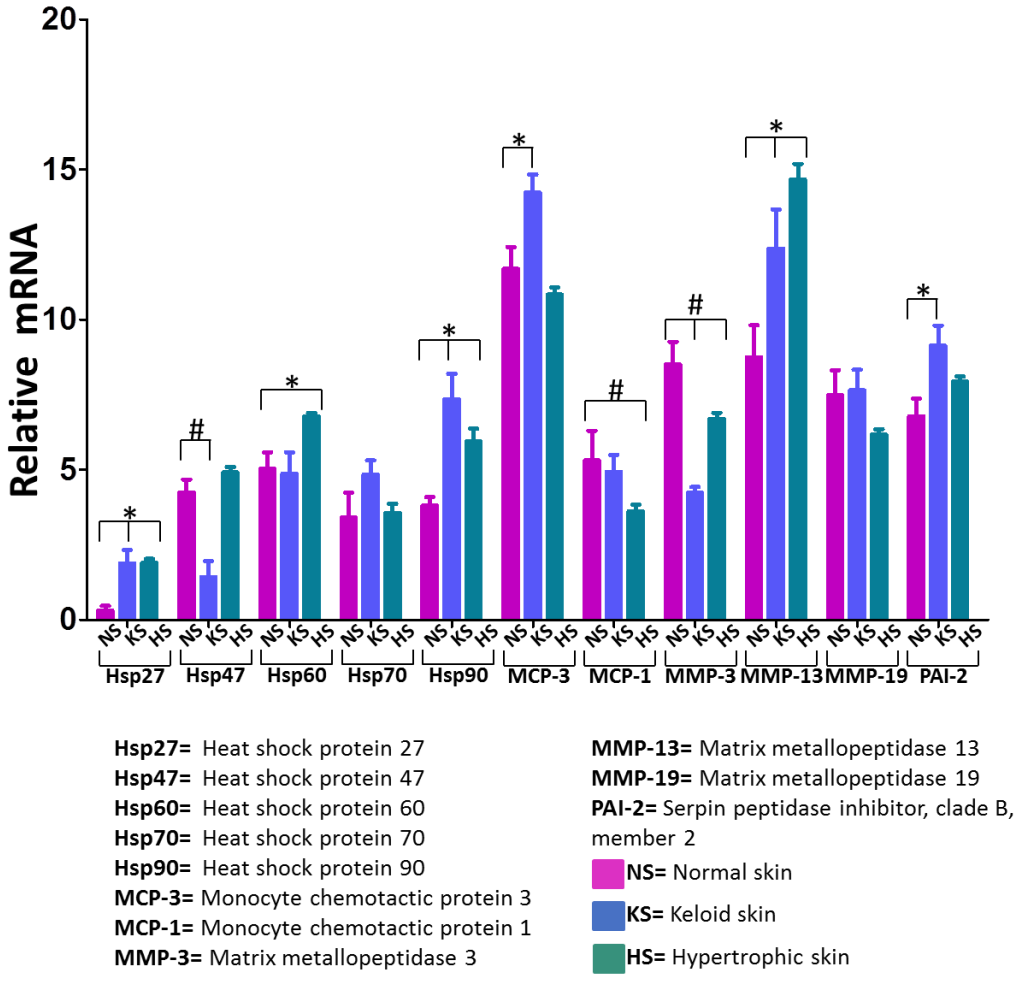


Figure 4.8 Cellular mRNA expression for ECM-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

4.5.3 Expression profile of ECM-related biomarkers in primary fibroblasts from keloid and hypertrophic scar

Total RNA extracted from primary fibroblast cultures from normal and abnormal scar samples (n=14) was employed to assess the expression of the adhesion, communication, spreading, remodelling and ECM related genes, which was normalised to an internal reference gene (RPL32). The results were expressed as mean ± S.E.M. of triplicates of independent experiments (n=14). *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin. qRT-PCR analysis showed significantly higher expression levels of α2β1-integrin in both keloid and hypertrophic scars (Figure 4.9), whereas no significant difference was found among the samples when tenascin was evaluated. The expression of Cyr61 was found to be significantly up-regulated in keloid cells compared to normal fibroblasts (p<0.05).

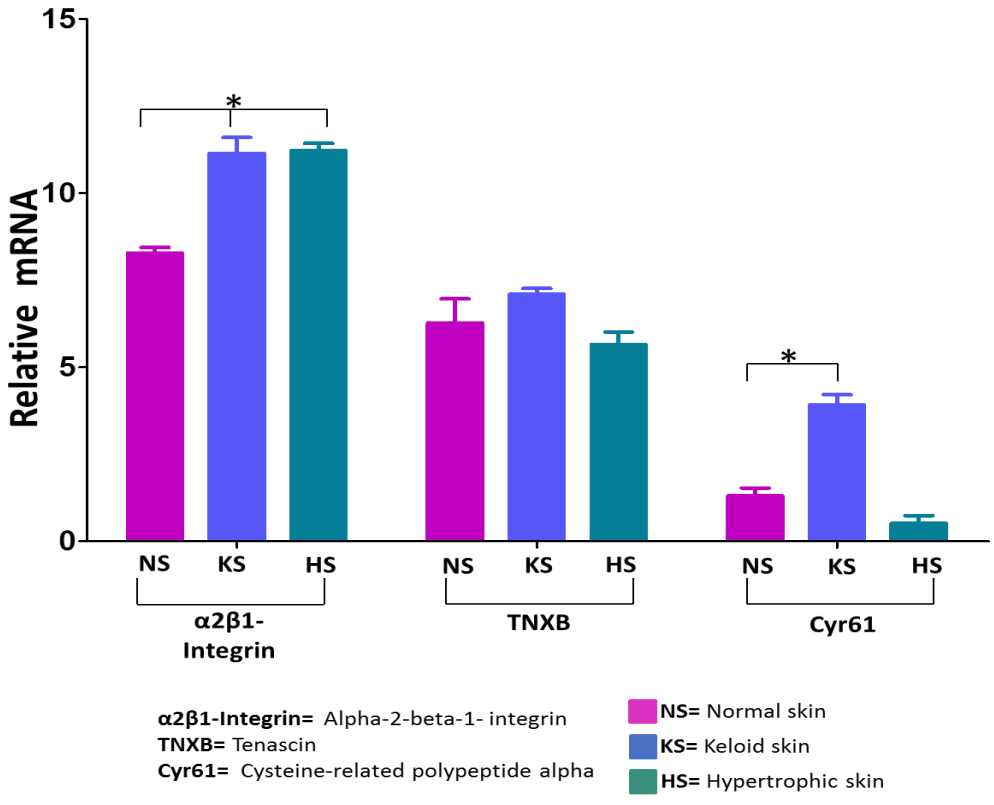


Figure 4.9 Tissue mRNA expression of cytokines seen in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

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

Finally, total RNA extracted from primary fibroblast cultures from normal and abnormal scar samples (n=14) as described in chapter III (Sections 3.4.3-3.4.5) was employed to evaluate the expression of TGF-β transcription factor family genes and normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments. *p <0.05 indicates a significant increased difference between the mRNA expression in tissue biopsy specimens from normal skin. The expressions of the TGF-β1 and TGF-β2 isoforms were found to be up-regulated in hypertrophic scars when compared to normal skin fibroblasts (Figure 4.10). No significant difference was found in the expression of TGF-β3 among the samples considered (p<0.05).

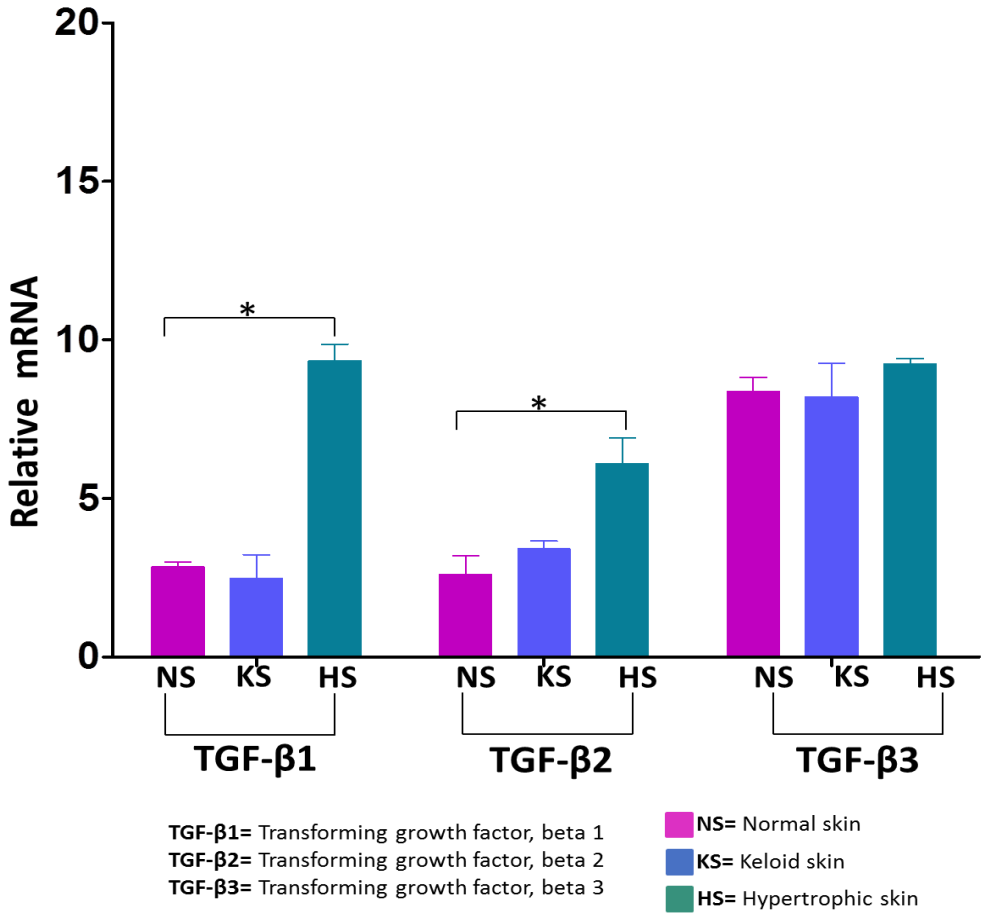


Figure 4.10 Cellular mRNA expression for ECM-related biomarkers in keloid and hypertrophic scar compared to normal skin evaluated by qRT-PCR (n=14).

4.6 Protein expression screening in primary fibroblast cultures established from normal, keloid and hypertrophic scar tissue

To further my aim of identifying strong target genes involved in the regulation of abnormal scar development, the relative protein expression of the 4 tension markers classified into the four groups mentioned above: a) neuropeptides, b) tension related, c) ECM and d) growth factors was evaluated in primary fibroblast cultures by in-cell western blotting.

4.6.1 Characterisation of neuropeptide protein expression in keloid and hypertrophic fibroblasts

To further investigate my aim of identifying target biomarkers involved in the regulation of raised dermal scar development and differentiation, the protein expression of the 4 neuropeptides, SP, CGRP, NPY and VIP, was evaluated in primary fibroblast cultures form keloid and hypertrophic scars by in-cell western blotting A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (green) from 96-well plates is shown in Figure 4.11, panel **a) ;** in panel **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 ± S.E.M. of three independent experiments. *$p < 0.05$ indicates significant up-regulation of the neuropeptides expression when compared to normal skin fibroblasts, while $^{\#}p < 0.05$ signifies significant down-regulation of the neuropeptides expression when compared to their respective control (normal skin fibroblasts) (n=14). The results of the analysis are shown in Figure 4.11, where upon inspection, it can be seen that SP and CGRP were significantly higher expressed in keloid fibroblasts compared to normal skin fibroblasts, whereas the NYP protein expression was significantly higher in hypertrophic samples compared to normal cells. In addition, the protein levels of VIP were significantly lower in hypertrophic cells.

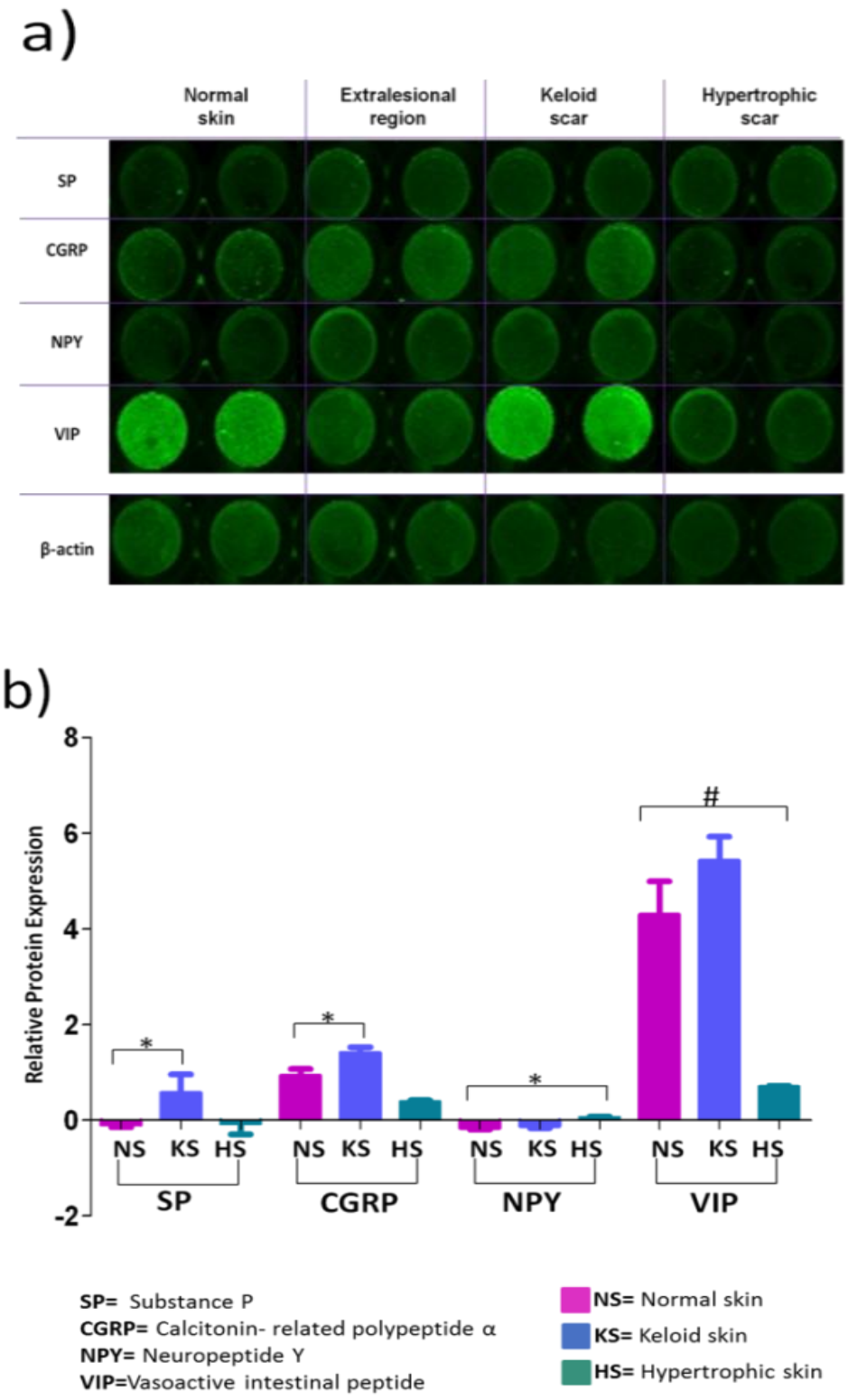


Figure 4.11 Cellular protein levels of neuropeptides in keloid and hypertrophic scar compared to normal skin evaluated by In-cell western blotting (n=14).

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

Based on the results of my qRT-PCR analysis in keloid and hypertrophic fibroblasts, 4 of the 11 genes included in this group, Hsp27, Hsp47, MMP-19 and PAI-2, representative of the range of results reported previously, were chosen for protein expression evaluation in primary fibroblast cultures. Serum-starved keloid and hypertrophic fibroblasts (n=14) were seeded in 96-well plates (1×10^4 cells/well) and grown up to 24 hr. The cells were fixed with 4% formaldehyde /phosphate-buffered saline. After blocking, the tension related proteins, were revealed by incubation with their respective antibodies, followed by a second incubation with IRDye 800-labelled secondary antibodies. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (green) from 96-well plates is shown in Figure 4.12 panel **a)**; in panel **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 ± S.E.M. of three independent experiments. *p <0.05 indicates significant up-regulation of the tension related proteins expression when compared to normal skin fibroblasts (n=14). The results are shown in Figure 4.12 and indicate that Hsp27, MMP-19 and PAI-2 protein levels were significantly higher in keloid cells, whereas Hsp47 protein levels were significantly higher in hypertrophic fibroblasts compared to normal fibroblasts ($p<0.05$).

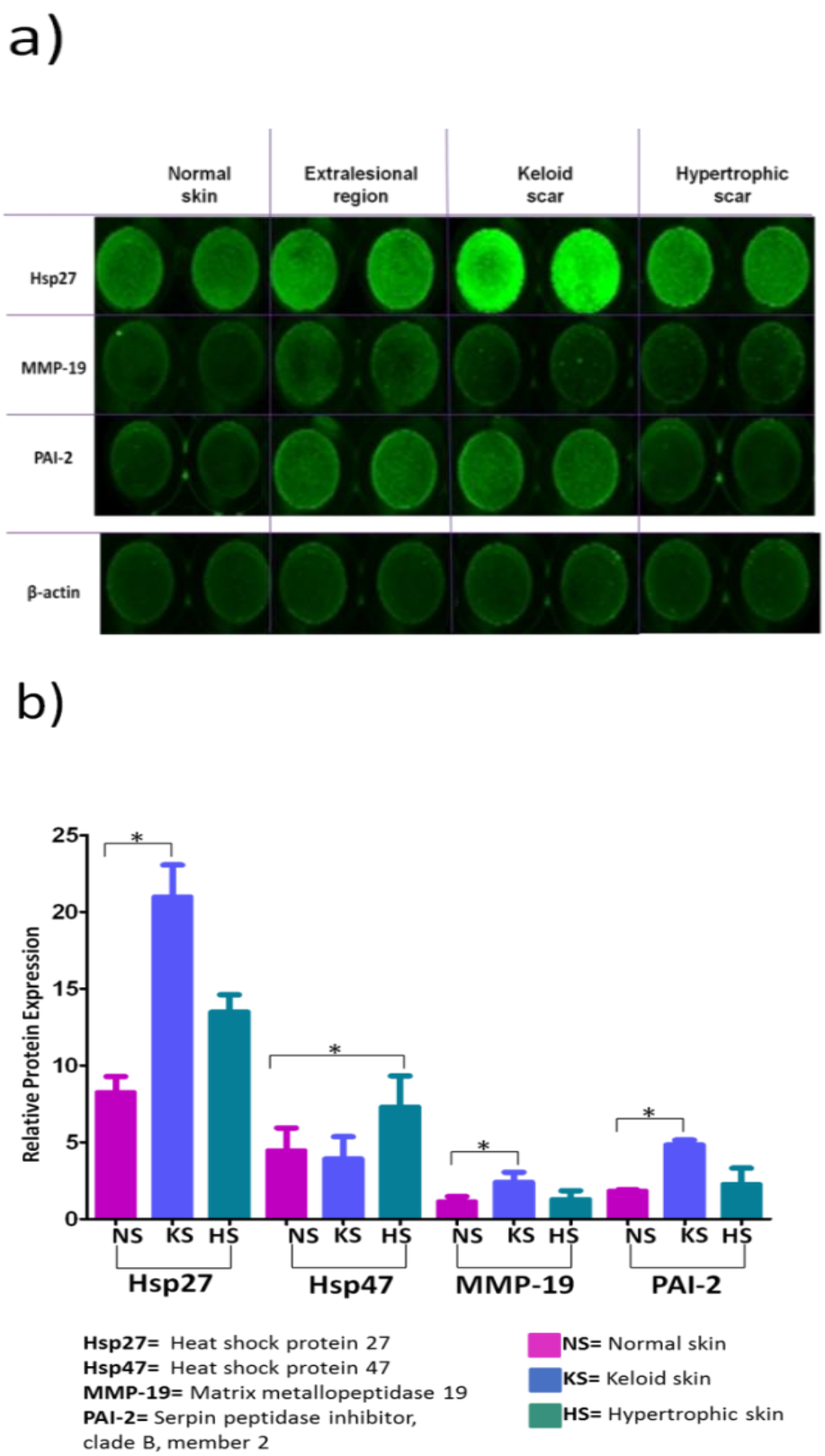


Figure 4.12 Cellular protein levels of tension-related in keloid and hypertrophic scar compared to normal skin evaluated by In-cell western blotting (n=14).

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

Serum-starved normal skin, keloid and hypertrophic fibroblasts (n=14) were seeded in 96-well plates (1 x 10^4 cells/well) and grown up to 24 hr and processed for In Cell Western blotting. After blocking, the ECM proteins were revealed by incubation with their respective antibodies, followed by a second incubation with IRDye 800-labelled secondary antibodies. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (green) from 96-well plates is shown in panel **a)** of Figure 4.13; in panel **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 ± S.E.M. of three independent experiments. *p <0.05 indicates significant up-regulation of the ECM related proteins expression when compared to normal skin fibroblasts (n=14). Whereas, $^{\#}$p <0.05 signifies a significant down-regulation of the ECM proteins expression when compared to their respective control (normal skin fibroblasts) (n=14). Figure 4.13 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 abnormal scar cell types ($p<0.05$). α2β1-integrin and collagen I were strongly expressed in keloid fibroblasts compared to normal cells.

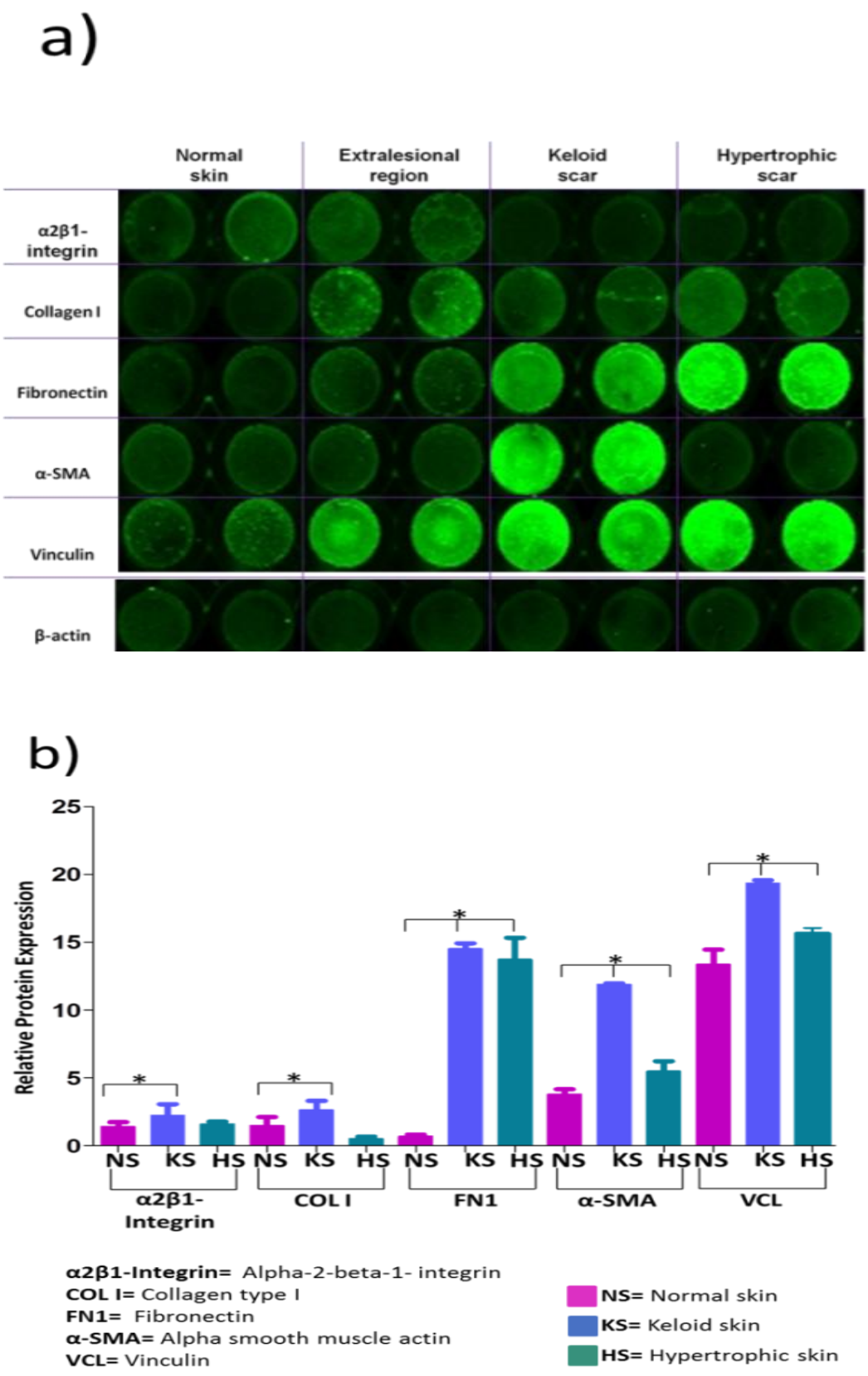


Figure 4.13 Cellular protein levels of ECM-related in keloid and hypertrophic scar compared to normal skin evaluated by In-cell western blotting (n=14).

4.6.4 Characterisation of cytokines protein expression in keloid and hypertrophic fibroblasts

Serum-starved primary fibroblasts were seeded in 96-well plates (1 x 10^4 cells/well) and grown up to 24 hr. The cells were fixed with 4% formaldehyde /phosphate-buffered saline. After blocking, the TGF-β family were revealed by incubation with their respective antibodies, followed by a second incubation with IRDye 800-labelled secondary antibodies. A representative output infrared image of keloid and hypertrophic fibroblasts for neuropeptides expression (green) from 96-well plates is shown in panel **a)** of Figure 4.14; in panel **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 ± S.E.M. of three independent experiments. *p <0.05 indicates significant up regulation of the transcription factor related proteins expression when compared to normal skin fibroblasts (n=14). As can be seen from the results of the in-cell western blotting assay shown in Figure 4.14, TGF-β2 protein levels were significantly higher in both keloid and hypertrophic fibroblasts compared to normal cells. Furthermore, TGF-β1 was up-regulated in keloid cells whereas TGF-β3 expression was lower in hypertrophic fibroblasts.

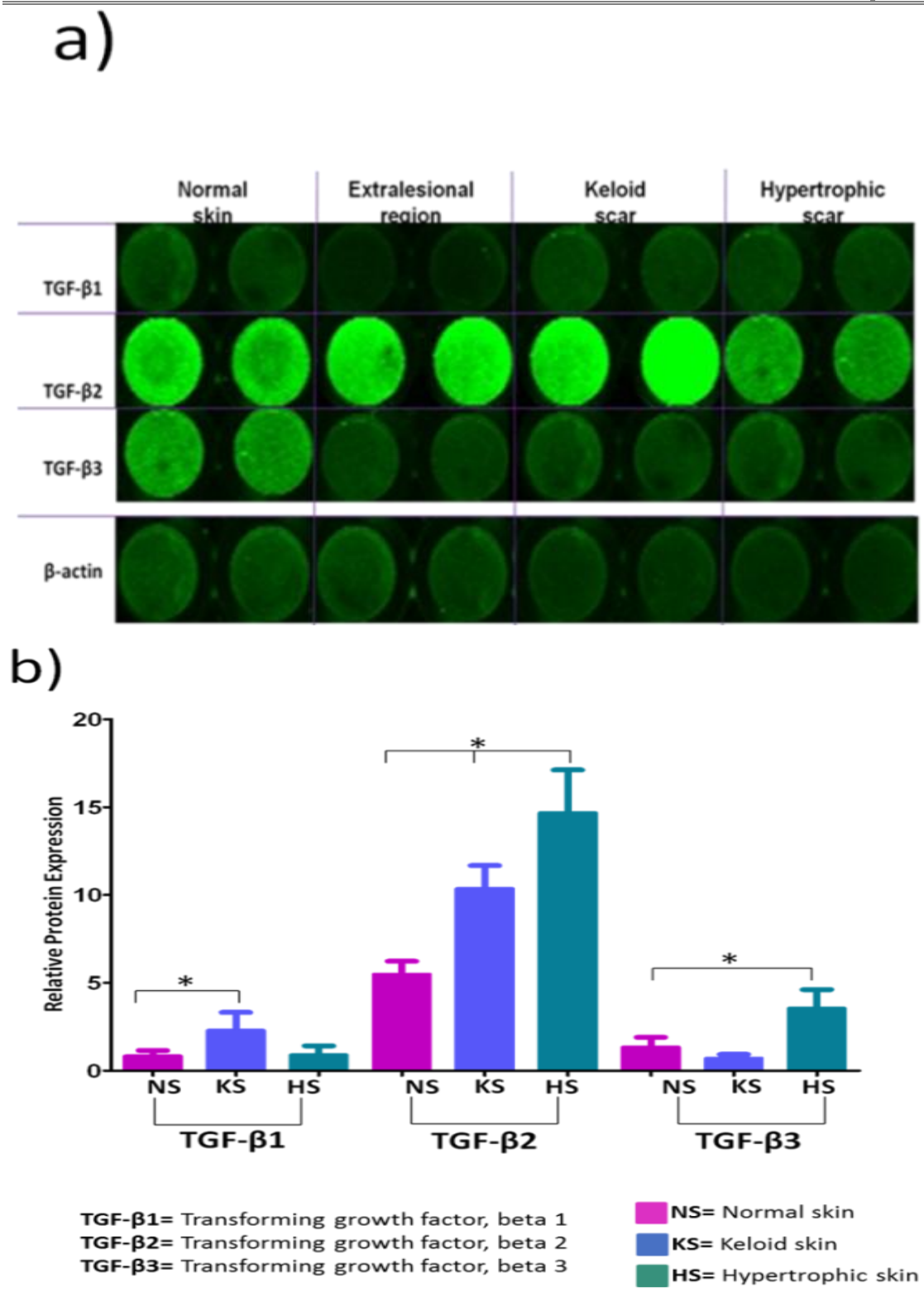


Figure 4.14 Cellular protein levels of cytokines in keloid and hypertrophic scar compared to normal skin evaluated by In-cell western blotting (n=14).

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

Total RNA was extracted from tissue biopsies and fibroblasts from keloid samples as described in the chapter III (sections 3.4.3-3.4.5). The expression of the top 5 up regulated tension related genes was normalised to an internal reference gene (RPL32). The results are expressed as mean ± S.E.M. of triplicates of independent experiments (n = 14). *p <0.05 indicates a significant increased difference between the mRNA expression among the scars collected from different body areas (n=14). Figure 4.15 shows the gene expression for five molecules, α2β1-integrin, Hsp27, PAI-2, MMP-19 and CGRP, which my analysis showed were significantly up-regulated in keloid scar tissue samples and fibroblasts. The results are presented based on the location from which the scar tissue samples and cells were collected. It can be seen upon inspection of Figure 4.15 that the highest expression for the 5 genes shown in both tissue and cells was found to be in the samples taken from the sternum.

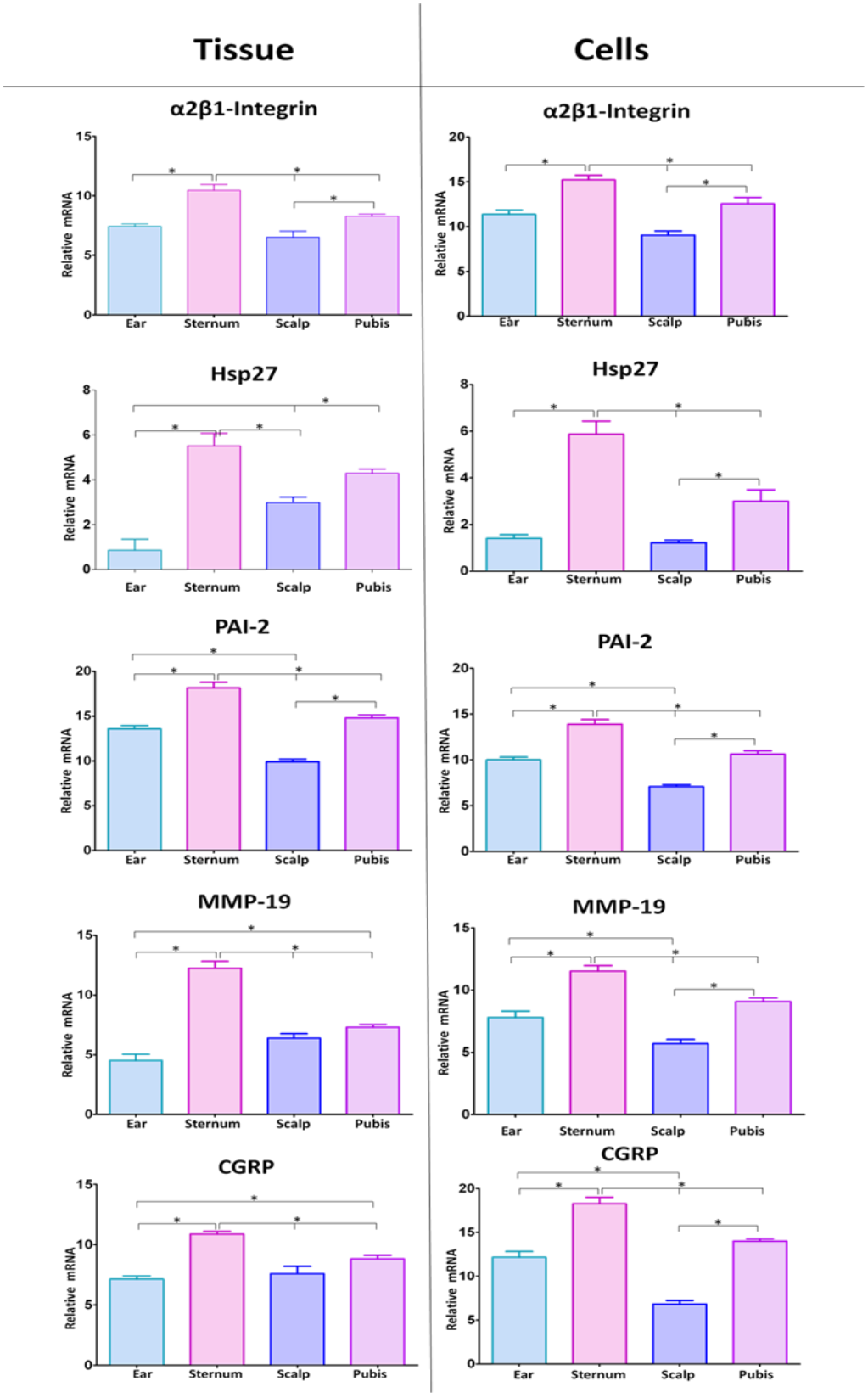


Figure 4.15 Tissue and cellular mRNA expression for the top 5 up-regulated biomarkers in keloid scar compared to normal skin evaluated by qRT-PCR (n=14).

4.8 Discussion

In the present chapter, a list of 21 candidate biomarkers were selected following an extensive literature review in addition to using my own unpublished microarray data. I 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 4.2 provide a list of candidate biomarkers that may be involved in the development and differentiation of HS and KS.

I 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 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 the phenomenon has been identified and discussed by other investigators[5, 17, 131, 161]. Indeed, Shih, *et al*. 2010, demonstrated that transcriptomic data obtained from primary fibroblast cultures does not usually correlate exactly to its respective tissue biopsy sample data. It has also been suggested that culturing conditions exert a profound impact on gene expression levels[162]; and the passage number during culturing could significantly affect gene and protein expression levels in primary fibroblast cultures[22, 163-165]. The five biomarkers found to be consistently up-regulated in KS are strongly associated with mechanical tension[3, 166-168].

In addition, the results obtained at the 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 was highest in the samples taken from the sternum, which, correlates with other studies previously undertaken in relation to anatomical site and mechanical tension[2, 5, 18, 87, 124].

Table 4.2 Molecules strongly express in raised dermal scar (summary)

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

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[93, 169]. 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. Neuropeptides were included in the study, in order to evaluate the role that the constant stimulation of the sensory skin nerve fibres produced by mechanical stress may play in the transmission of signals from the sensory nerves which results in release of specific neuropeptides in the skin[170, 171]. Neuropeptides binding to their respective receptors located on skin cells' surfaces could induce vasodilatation and vessel permeabilisation evoking inflammatory responses[3, 170, 172]. 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[2, 17, 59, 104, 171, 173-176]. I 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[166]. Hsps also regulate the proliferative phase during wound healing and promote new tissue formation. Hsps acts as cellular chaperones that modulate cell death signals such as the FAS-mediated apoptotic pathway, allowing cells to adapt to gradual changes in their environment and to survive in otherwise lethal conditions[174, 177-180]. In addition, MMPs have been implicated in angiogenesis, scar resorption, inflammation, re-epithelialisation and remodelling phases of wound healing[102, 133]. MCPs, transcription factors and cytokines are also linked to the regulation of inflammatory processes and cell recruitment in normal wound healing[176].

Interestingly, a correlation between neuropeptides, HSPs, MMPs and MCPs and skin tension has been proposed as potentially promoting the development and progression of abnormal scars such as KS and HS[17], as they are prone to develop frequently at highly tensioned anatomic locations[25]. Several researchers have suggested that the site of injury has been found to substantially influence scar formation and wound closure [59] with abnormal scar formation tending to occur more frequently in body areas subjected to greater mechanical forces[3, 181]. In this context, the sternum, shoulders and suprapubic region are body zones considered as strong candidates for the development of abnormal scarring[3, 26, 30, 139, 182]. Furthermore, the development of pathologic healing processes has been linked to mechano-signal transduction[87] whereby mechanical stress signals are traduced into biomechanical signals resulting in cellular responses that promote aberrant scar development[85, 102, 134]. Despite this knowledge, target molecules that promote and differentiate abnormal scar types have not been clearly identified to date.

Based on the results presented in the current chapter, it is proposed that the regulation of both aberrant scarring processes are closely related to pathways that regulate mechanisms including proliferation and migration, angiogenesis, ECM degradation, inflammation, communication and cell survival among others[3, 157, 171, 181, 183-185] 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, I 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. However, in order to provide further validation, additional studies are required to fully explore the mechanisms involving these biomarkers in KS and HS pathogenesis.

Chapter V: Tension-related proteins in keloids and their role as ECM regulators

5.1 Introduction

Keloid disease (KD) is an abnormal fibroproliferative lesion that grows in a quasi-neoplastic manner, extends beyond the confines of the original wound margin, does not regress over time, and invades the nearby unaffected skin[3, 90, 93, 137, 186, 187]. The mechanism of KD formation is poorly understood and there is no specific treatment to cure these lesions[90, 93]. Clinical observation has shown that some keloid lesions tend to develop at anatomical sites where increased skin tension is present such as sternum, shoulders, and upper back[93]. It has been suggested that skin tension generated in the remodelling stage of the wound healing process may influence KD behaviour, development and spreading[24, 93, 157, 188]. Thus, there are site-specific morphologies of keloid scarring. For example, butterfly-shaped KS are frequently observed in the sternal area[93]. Several studies have demonstrated that, when fibroblasts are subjected to mechanical forces, this leads to increased focal adhesion complexes and integrin receptors are involved in this process[189, 190]. Moreover, mechanical stimulation has been shown to affect cellular gene expression, protein synthesis and proliferation rates[90, 191]. In addition, fibroblasts under mechanical tension have a higher expression of certain tension-related proteins (e.g., heat shock proteins, integrins, and cytokines) and produce excessive extracellular matrix components[157, 179].

Interestingly, a large number of tension-related proteins in the skin have been shown to be over-expressed during the wound healing process[192] (Table 5.1). Recently, it has been reported that tension-related proteins such as heat shock protein 27 (Hsp27)[193] lead to increased extracellular matrix synthesis and are closely related to progression of the fibrotic process[194-196]. To this end, it was hypothesised that over-expression of tension-related proteins and excessive extracellular matrix deposition may be influenced by mechanical tension produced in the skin during wound healing that may lead to keloid formation.

In the present study, a panel screening of tension-related proteins was performed that showed over-expression of certain tension-related proteins in keloid disease and

convincingly demonstrated that knockdown of these proteins by RNAi technology decreased extracellular matrix synthesis in keloid fibroblasts. The findings from this study offer further insight into understanding keloid disease pathobiology and potential options for future treatment of keloids. It is envisaged that RNA interference could be targeted toward tension-related proteins found in keloid tissue.

5.2 Materials and methods

5.2.1 Patient data and tissue collection

Keloid samples (n=10) and normal skin samples (n=4) from the sternum (Table A5 and A6) were collected and processed as previously described in chapter III section 3.3. A step-by-step flow-chart referring to the experimental design can be found in Figure 5.1.

5.2.2 Primary keloid and normal skin fibroblast culture establishment

Fibroblasts from passage 0 (p0) to passage 3 (p3) were used for all experiments carried out in this study. Fibroblasts primary cultures were grown as previously described in chapter III (section 3.4.2, page 60). Earlier fibroblast passages were employed based on previously published data[160] in order to minimise variability and have optimal consistency in data, only cells up to P3 were utilised in this study.

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

RNA extraction, cDNA synthesis, and quantitative reverse-transcriptase polymerase chain reaction were performed as described in chapter III (Sections 3.4.3-3.4.5). The list of primers used in this study is shown in the appendix section Table 1A. The mRNA expression was normalised with reference to gene ribosomal protein L32 (RPL32).

Table 5.1 Subset of tension-related genes with increased expression in skin

Gene name (Gene symbol)	Biological function	Chromosomal location
α2β1-Integrin (ITGA2)	Cell adhesion, migration, and signalling by providing transmembrane links between the extracellular matrix and the cytoskeleton interactions between fibroblasts and Collagen I [186, 192]	5q11.2
Actin, alpha 1, skeletal muscle (ACTA1, α-SMA)	Myofibriblasts marker. Fibroblasts contraction[137]	1q42.13
Calcitonin-related polypeptide alpha (CALCA, CGRP)	Up regulation of TGF-B controls proliferation, cellular differentiation and vasodilatation[3]	11p15.2
Chemokine (C-C motif) ligand 2 (CCL2; MCP-1)	Influence collagen fibre formation *in-vivo*[186]	17q11.2-q12
Collagen, type I, alpha 1 (COL1A1)	Main structural element of the ECM, forming a relaxed network of cross-linked fibres throughout the dermis to maintain tissue integrity[108]	17q21.33
Cysteine-rich, angiogenic inducer, 61 (Cyr61)	Mediates cell adhesion; induces adhesive signalling; promotes fibroblasts survival[192]	1p22.3
Fibronectin 1 (FN1)	ECM rearrangement[197-199]	2q34
Heat shock protein 27 (Hsp 27, HSPB1)	Protein folding, actin binding proteins[174]	7q11.23
Heat shock protein 60 (HSPD1; Hsp 60)	Protein folding and mycobacterial unfolding, organelle translocation[174]	2q33.1
Heat shock protein 70 (HSPA1A; Hsp 70)	Enhanced resistance to ischemic injury[174]	6p21.3
Heat shock protein 90kDa alpha (cytosolic), class A member 1; Heat shock protein 90 (HSP90AA1; Hsp 90)	Maintenance of proteins such as steroid receptors in an inactive form until appropriate[174]	14q32.33
Hyaluronan and proteoglycan link protein 1 (HAPLN1)	Maintaining the integrity and supporting the functions of the ECM[200]	5q14.3
Hyaluronan and proteoglycan link protein 2 (HAPLN2)	Maintaining the integrity and supporting the functions of the ECM[200]	1q23.1
Matrix metallopeptidase13	Key role in degradation of the collagen matrix[201]	11q22.3

;Collagenase 3 (MMP-13)		
Matrix metallopeptidase 19 (MMP-19)	Extracellular matrix degradation[93]	12q14
Matrix metallopeptidase 3; Stromelysin 1, progelatinase (MMP-3)	Wound Contraction and ECM degradation. Collagen catabolism[93]	11q22.3
Neuropeptide Y (NPY)	Induce keratinocyte proliferation. Neovascularisation. Angiogenesis[192]	7p15.1
Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2; PAI-2)	ECM remodelling. ECM degradation inhibitor. Cell protection against apoptosis[168, 202]	18q21.3
Serpin peptidase inhibitor, clade H; Heat shock protein 47 (Hsp 47, SERPINH1)	Closely related with fibrosis, Collagen specific molecular chaperone[174]	11q13.5
Substance-P Tachykinin, precursor 1 (TAC1, SP)	Keratinocyte and fibroblast proliferation. Cell proliferation[170]	7q21-q22
Tenascin XB (TNXB)	ECM similar to fibronectin that may promote wound healing[203]	6p21.3
Testis derived transcript (3 LIM domains) (TES)	Focal adhesion and cell-cell communication[204]	7q31.2
Transforming growth factor, beta 1 (TGF-β1)	Fibroblast differentiation. ECM synthesis[137]	19q13.2; 19q13.1
Transforming growth factor, beta 2 (TGF-β2)	Increase cell proliferation in keloids[93]. Anti-apoptotic function[205]	1q41
Transforming growth factor, beta 3 (TGF-β3)	Down-regulate scarring and fibrosis *in-vivo*[205]	14q24

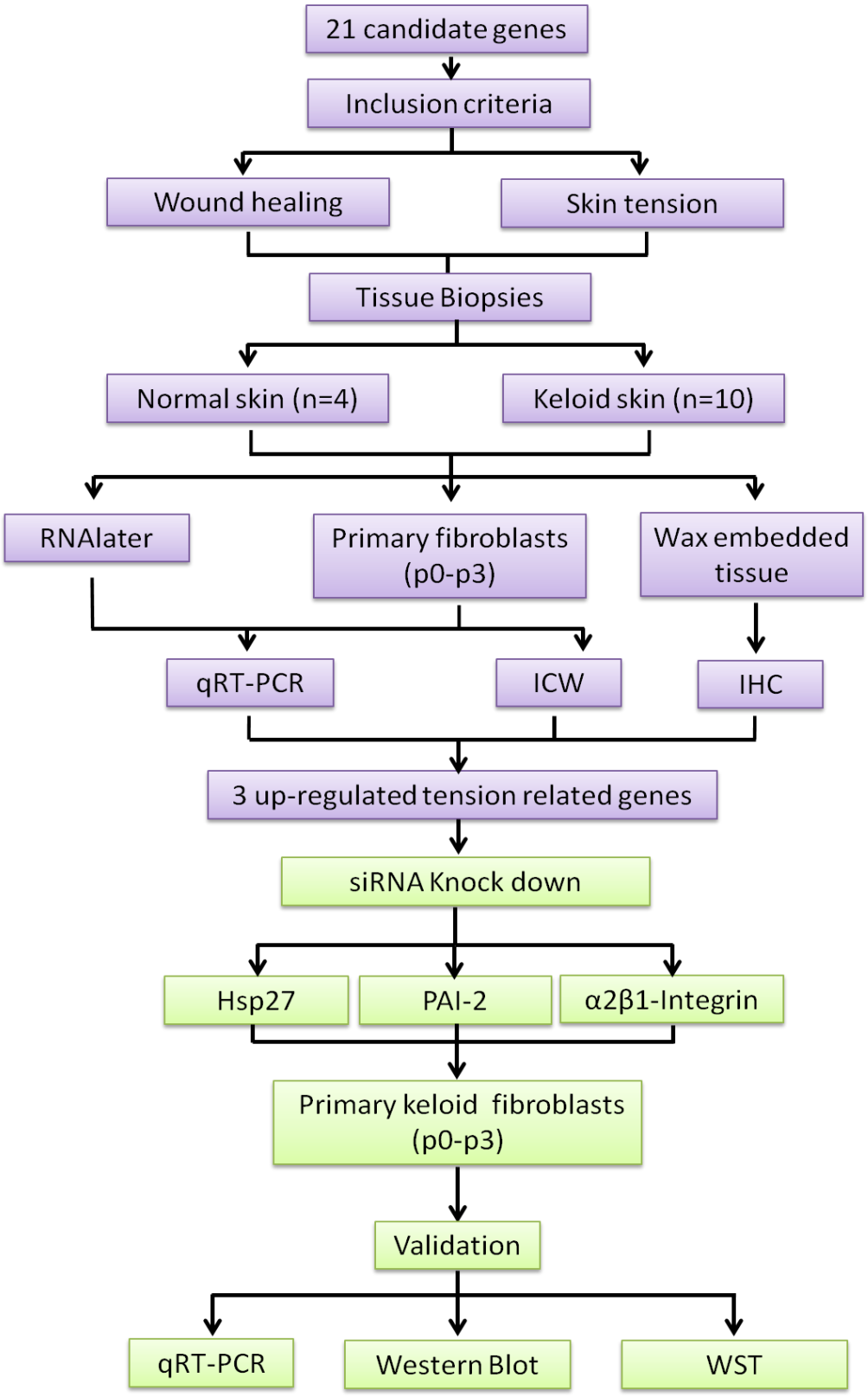


Figure 5.1 Study design to determine potential tension-related biomarkers for KD.
The flowchart presents a summary of the steps taken to perform this study.

5.2.4 Immunohistochemistry (IHC)

Immunohistochemistry was performed as detailed in chapter III (Section 3.4.1). Tissue sections were cut into 5μm slices, fixed onto glass slides, and dried at room temperature overnight. Tissue sections were deparaffinised, rehydrated, and treated with primary antibodies overnight at 4°C followed by secondary antibodies. The list of the primary and secondary antibodies employed in this study is given in Tables 2A and 3A in the appendix.

5.2.5 High throughput In-Cell Western Blotting (ICWB) and quantitation

In-cell Western blotting was carried out using the standard protocol as described previously in chapter III (Section 3.4.6). Actin was used as a loading control; the panels of primary and secondary antibodies used in this study are listed in the appendix Tables 2A and 3A.

5.2.6 siRNA design and target gene knockdown

The Ambion siRNA target online tool (Silencer PreDesigned and Validated siRNAs) was used to select three "positive" siRNA sequences targeting to human anti-Hsp27, α2β1-integrin, PAI-2, and scrambled siRNA (Applied Biosystems, Warrington, United Kingdom). Keloid fibroblasts ($1x10^5$ cells /well) from six different patients were cultured in 24-well plates in triplicate and transfection was carried out in suspension using siRNA complexes prepared with HiPerFect Transfection Reagent according to the manufacturer's protocol (Qiagen, West Sussex, United Kingdom). The cells were then incubated for 48 hr at 37°C. Forty-eight hours after transfection, fibroblasts were collected by scraping. The RNA extraction, cDNA synthesis, and quantitative reverse-transcriptase polymerase chain reaction were performed as described in chapter III. The gene expression levels were normalised with RPL32.

5.2.7 Western Blotting

Forty-eight hours after transfection, keloid fibroblasts were collected using cell scrapers. Cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with phosphatase inhibitors cocktail (Protease Inhibitor Cocktail Set III, EDTA-Free; Calbiochem, Nottinghamshire, United Kingdom). The total amount of protein was quantified with the BCA protein assay kit (Thermo Scientific, Waltham, Mass.). Equal amounts of proteins (100μg) were denatured and resolved on 4 to 12% Bis-Tris gels (Invitrogen, Carlsbad, Calif.) and electrophoresed according to the manufacturer's instructions. The proteins were blotted on polyvinylidene difluoride membranes from polyacrylamide gels using iBlot Dry Blotting System (Life Technologies, Paisley, United Kingdom). Membranes were incubated for 1 hr at room temperature in Odyssey Blocking Buffer (LI-COR Biosciences, Cambridge, United Kingdom). After the blocking step, membranes were incubated overnight at 4°C with the respective primary antibodies followed by secondary antibody incubation for 1 hr at 37°C. The panels of primary and secondary antibodies used in this study are listed in appendix Tables A1 and A2. The polyvinylidene difluoride protein membranes were developed using Amersham ECL prime Western blotting detection kit (GE Healthcare, Buckinghamshire, United Kingdom). The membranes were exposed to Kodak X-OMAT x-ray film (Sigma Aldrich). Densitometry was performed with Image J software (National Institutes of Health, Bethesda, Md.), and the statistical analysis was undertaken using Prism software (GraphPad Software, Inc., San Diego, California.). The optical densities of Hsp27, α2β1-integrin, PAI-2, and collagen I were normalised to α-tubulin.

5.2.8 Cell Viability/Metabolic Activity Detection (Water-Soluble Tetrazolium Salt-1)

At 48 hr after siRNA transfection, cell viability/metabolic activity was tested using water-soluble tetrazolium salt-1 assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

5.2.9 Statistical Analysis

The statistical analysis was performed as described previously in chapter III (Section 3.4.10).

5.3 Results

5.3.1 Differential Expression of Tension-Related Genes in Keloid Fibroblasts Compared with Fibroblasts from Normal Skin

Twenty one skin-tension–related candidate genes (Table 4.2) that have been reported to be significantly up-regulated ($p < 0.01$) during the wound healing process in gene expression studies using cDNA microarray technology [24, 90] were chosen in order that a comprehensive analysis of their association with keloid disease at mRNA and protein levels could be performed. For this purpose, keloid tissue samples from the sternum were compared with normal skin from the same anatomical site by quantitative reverse-transcriptase polymerase chain reaction. At the mRNA level, results showed that of the 21 genes screened, a subset of 14 genes were up-regulated (including α2β1-integrin, Hsp27, Hsp70, PAI-2, and tenascin XB) and seven genes were down-regulated in keloid tissue compared with normal skin tissue samples including Hsp60, matrix metalloproteinase 19, and matrix metalloproteinase 3, among others (see Table 4.2). For further validation, the expression of the 14 up-regulated genes in keloid tissue was investigated in fibroblasts isolated from keloid and normal skin biopsy specimens taken from the same patients (n=10). The expression of the tension-related genes (Hsp27, PAI-2, and α2β1-integrin) was normalised to an internal reference gene (RPL32). The results are expressed as mean ±SEM of triplicates from three independent experiments. Differences between mRNA expression in tissue biopsy specimens from normal skin (NS) versus mRNA expression from keloid scars (KS) and the mRNA expression in fibroblasts from normal skin (NF) versus keloid fibroblasts (KF) were all significant at the $p < 0.05$ level (*). The quantitative reverse-transcriptase polymerase chain reaction results showed significant up-regulation of Hsp27, PAI-2, and α2β1-integrin in samples of both tissue and cells, consistent with the results obtained from the keloid tissue (Figure 5.2).

Table 5.2 Significant up-regulation/down-regulation tension-related genes within the keloid

Up regulated Gene name	Gene symbol	Keloid/normal (Relative mRNA)	p value	Chromosomal location
α2-Integrin	**ITAGA2**	**1.35 ± 0.57**	**0.003**	**5q11.2**
Chemokine (C-C motif) ligand 2	CCL2	0.93 ± 0.25	0.327	17q11.2-q12
Heat shock protein 27	**Hsp27 (HSPB1)**	**3.03 ±0.86**	**0.027**	**7q11.23**
Heat shock protein 47	SERPIN H1	0.34 ± 0.52	0.042	11q13.5
Heat shock protein 70	HSPA1A	1.42 ± 0.24	0.099	6p21.3
Hyaluronan & proteoglycan link protein 1	HAPLN1	1.08 ± 0.42	<0.0001	5q14.3
Hyaluronan & proteoglycan link protein 2	HAPLN2	0.87 ± 0.23	0.560	1q23.1
Matrix metallopeptidase 19	MMP-19	1.02 ± 0.45	0.478	12q14
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	**PAI-2 (SERPIN B2)**	**1.12 ± 0.83**	**0.008**	**18q21.3**
Substance-P Tachykinin, precursor 1	TAC1	1.09 ± 0.23	0.050	7q21-q22
Tenascin XB	TNXB	1.48 ± 0.50	0.110	6p21.3
Transforming growth factor, beta 1	TGF-β1	0.87 ± 0.86	<0.0001	19q13.2; 19q13.1
Transforming growth factor, beta 2	TGF-β2	0.84 ± 0.83	0.009	1q41
Transforming growth factor, beta 3	TGF-β3	0.92 ± 0.50	0.007	14q24
Down regulated Gene name	**Gene symbol**	**Keloid/normal (Relative mRNA)**	**p value**	**Chromosomal location**
Calcitonin-related polypeptide alpha	CALCA	1.83 ± 1.46	<0.0001	11p15.2
Cysteine-rich, angiogenic inducer, 61	Cyr61	2.74 ± 1.03	0.001	1p22.3
Heat shock protein 60	HSPD1	0.97 ± 0.52	0.033	2q33.1
Heat shock protein 90	HSP90A A1	1.93 ± 0.30	0.060	14q32.33
Matrix metallopeptidase 3	MMP-19	1.02 ± 1.46	0.478	12q14
Matrix metallopeptidase 13	MMP-13	0.54 ± 1.09	0.001	11q22.3
Neuropeptide Y	NPY	0.93 ± 1.24	0.654	7p15.1

Genes in dashed boxes are those that showed consistent up-regulation in both tissue and cells.

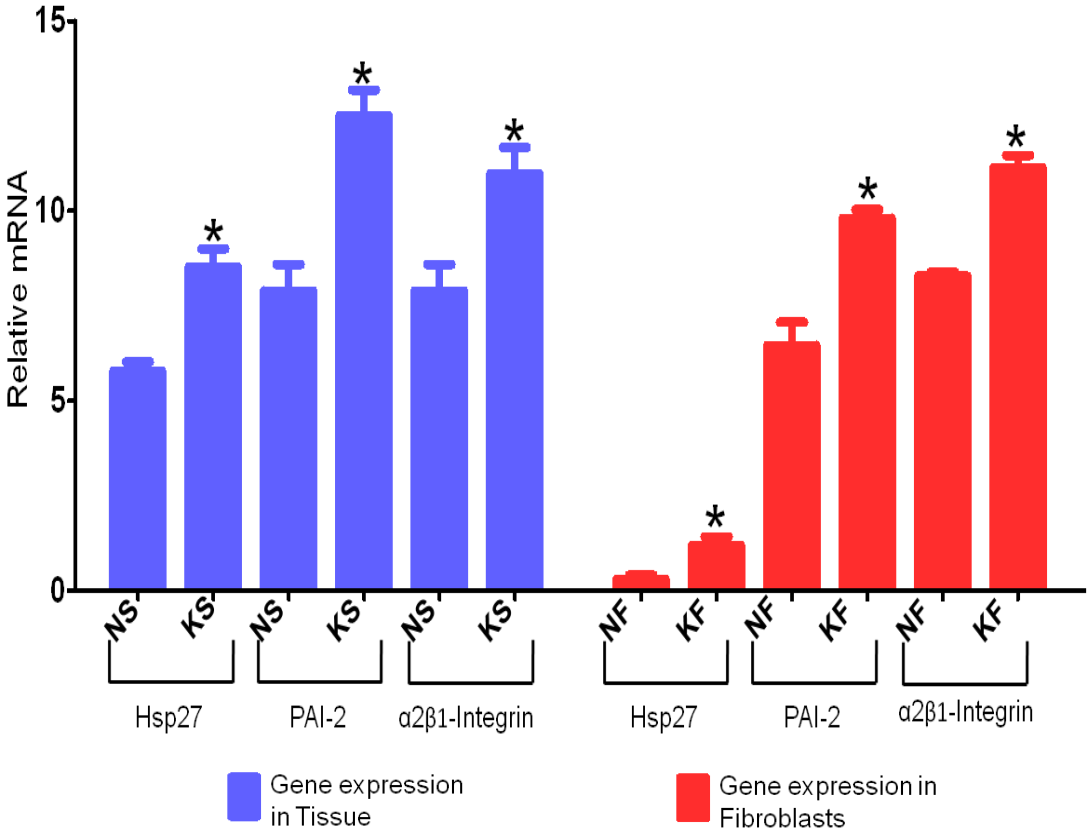


Figure 5.2 Up-regulation of tension-related genes in keloid tissue and fibroblasts (NS n=4; KS n=10).

Protein expression was also investigated for these three genes at the tissue and cellular levels by immunohistochemistry and in-cell Western blotting, respectively. To this end, five micron tissue sections were stained for each marker, whereas serum-starved primary fibroblasts (n=10) were seeded into 96-well plates (1x 10^4 cells/well) and allowed to grow for 24 hr respectively. All samples were processed as previously described in the materials and methods section of this chapter. The results show significant over-expression of Hsp27, PAI-2, and α2β1-integrin in keloid biopsy samples when compared to normal skin (Figure 5.3), whereas significant over-expression of these three proteins was also observed in keloid fibroblasts compared with fibroblasts from normal skin (Figure 5.4). A representative output infrared image of keloid fibroblasts for tension-related proteins (*green*) from 96-well plates is shown (*above the bar graphs*). Bar graphs represent the quantification of expression of Hsp27, PAI-2, and α2β1-integrin, normalised to the β-actin loading control, from three independent experiments. The

results are expressed as mean ± SEM. * indicates a significant difference ($p < 0.05$) in protein up-regulation when compared with their relative expression in fibroblasts from normal skin. Moreover, expression of Hsp70 and tenascin XB were not significantly up-regulated ($p > 0.05$) at protein level in keloid fibroblasts compared with fibroblasts from normal skin (data not shown).

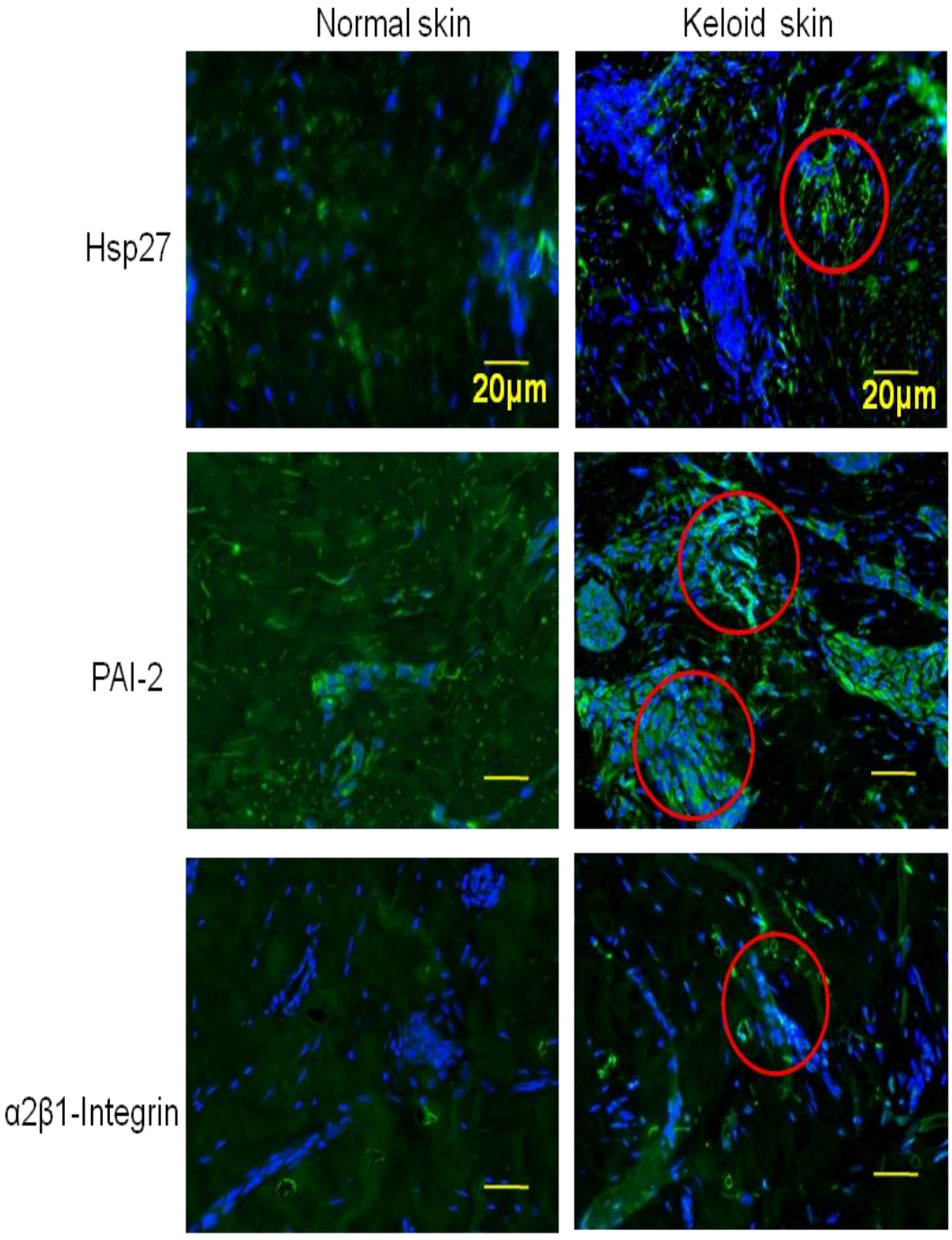


Figure 5.3 Expression pattern of Hsp27, PAI-2, and α2β1-integrin in keloid tissue compared with normal skin (NS n=4; KS n=10).

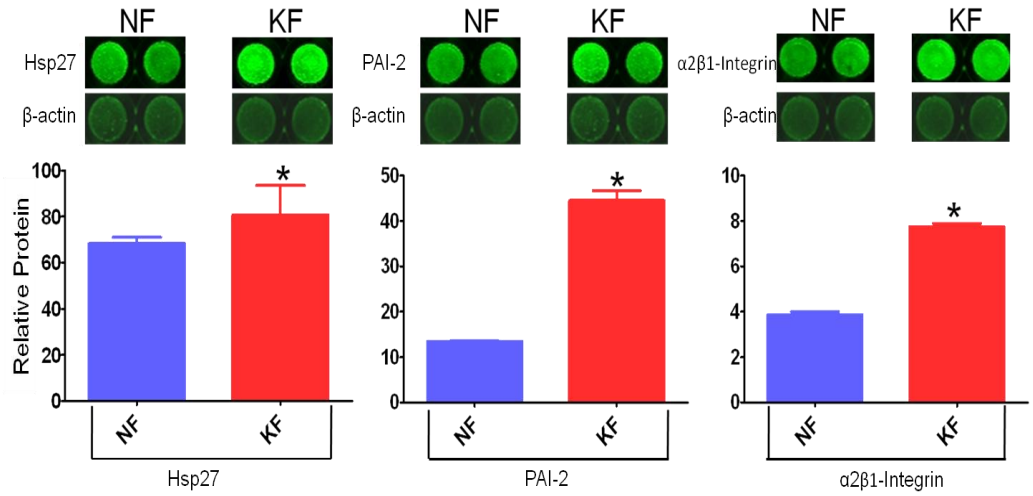


Figure 5.4 Hsp27, PAI-2, and α2β1-integrin protein expression levels in keloid fibroblasts (NS n=4; KS n=10).

5.3.2 Effect of Hsp27 on Extracellular Matrix Expression in Primary Keloid Fibroblasts

To better understand the influence of the Hsp27 expression in keloid fibroblasts on extracellular matrix production, a knockdown of Hsp27 was performed using the small interference RNA (siRNA) technique. Primary keloid fibroblasts (n=6) were transfected with HSp27/siRNA (25 nM and 50 nM). Forty eight hr after transfection, proteins were extracted and subjected to Western blot analysis. Figure 5.5 (*Above*) represents a typical autoradiogram of protein expression. Bar graphs represent densitometry analysis of average protein expression from three experiments, normalised to α-tubulin. Data are expressed as mean ± SEM. * indicates a significant difference (p<0.05) between scrambled siRNA versus the Hsp27/siRNA-transfected group. Transfection of Hsp27/siRNA significantly down-regulated Hsp27 at mRNA and protein levels in keloid fibroblasts compared with the vehicle (scrambled siRNA) group in a dose-dependent manner (p<0.05). To understand the effect of Hsp27 knockdown on extracellular matrix expression, I further assessed collagen I, fibronectin, and α-smooth muscle actin expression levels in keloid fibroblasts. Interestingly, collagen I was significantly down-regulated at the mRNA and protein levels ($p \leq 0.03$) (Figure 5.5), in a dose-dependent manner, in

Hsp27 knockdown in keloid fibroblasts. Similarly, fibronectin and α-smooth muscle actin (Figure 5.6) were significantly reduced in a dose-dependent manner following Hsp27 knockdown in keloid fibroblasts. Therefore, I concluded that inhibition of over-expressed Hsp27 showed significant reduction in extracellular matrix in keloid fibroblasts.

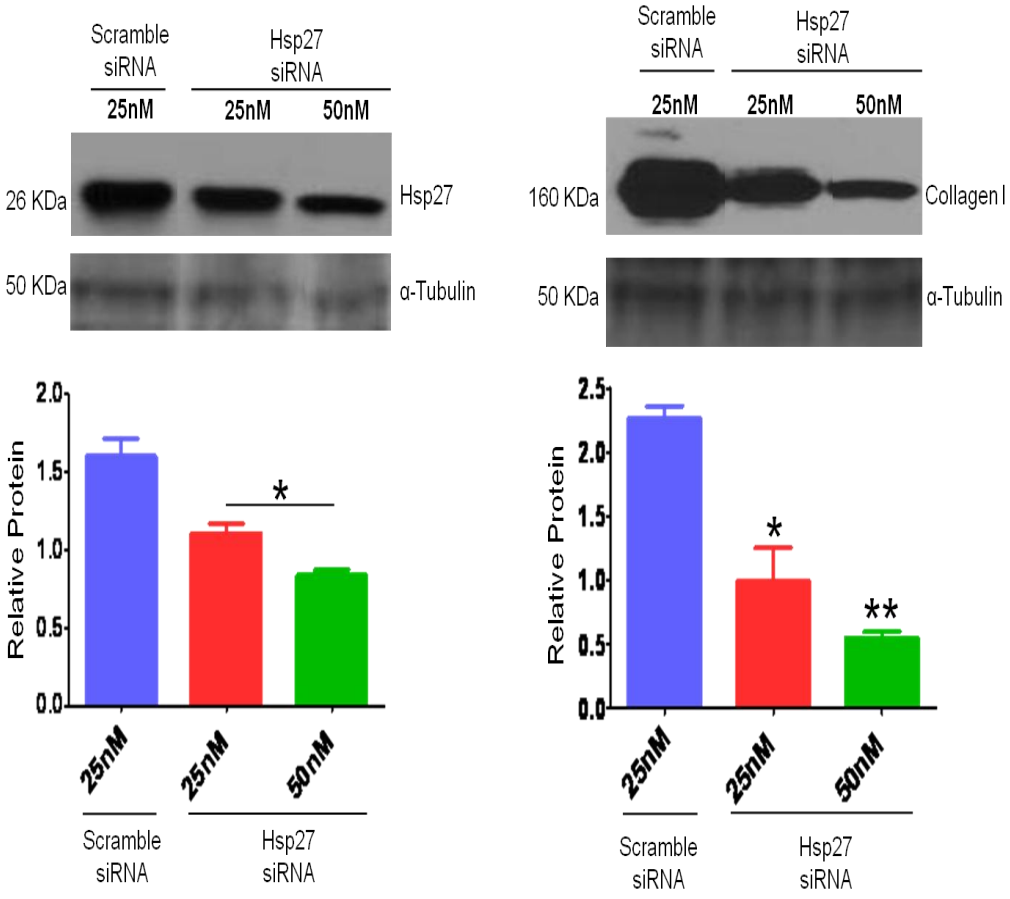


Figure 5.5 Analysis of Hsp27 and collagen I protein expression in keloid fibroblasts following Hsp27/siRNA transfection (n=6).

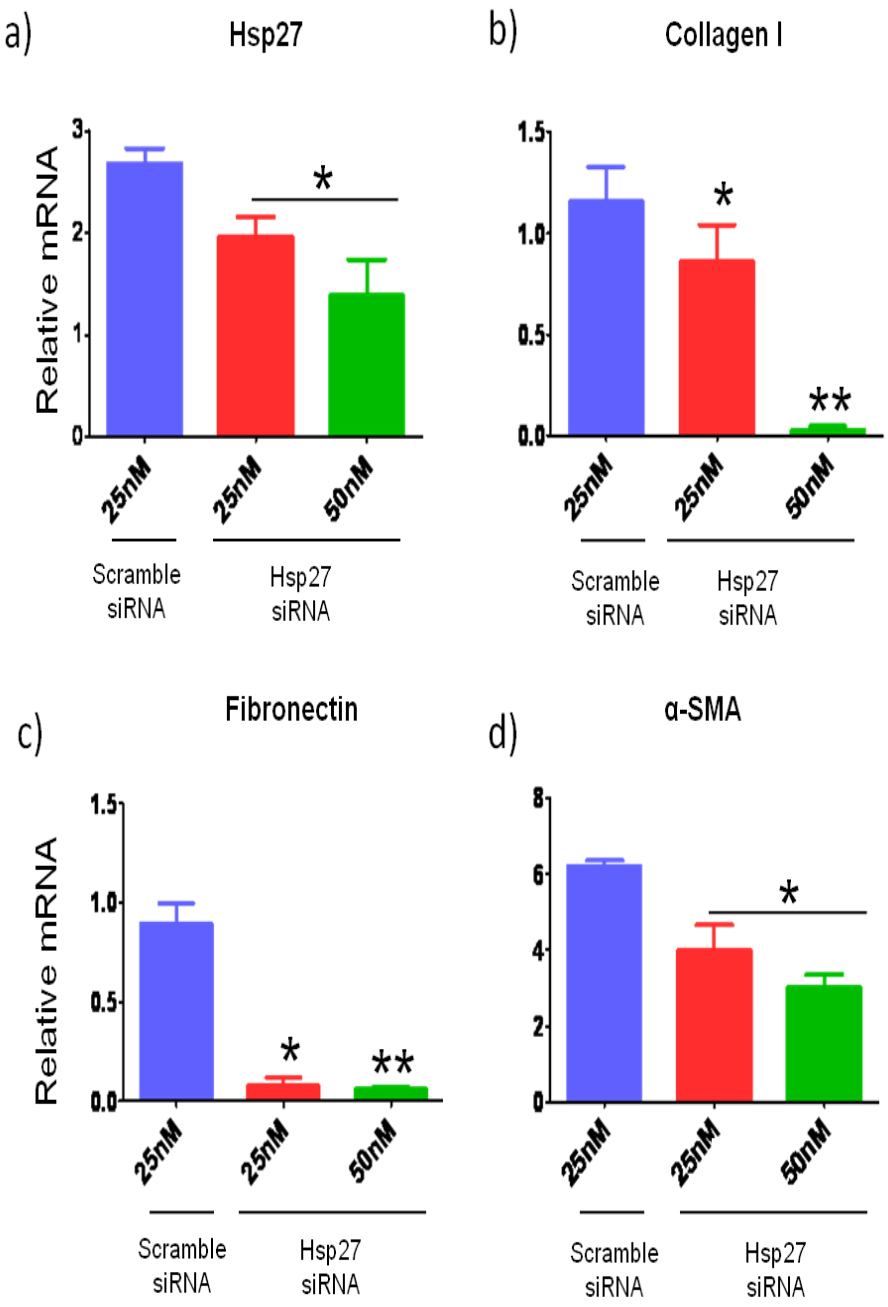


Figure 5.6 Effect of knockdown of Hsp27 on keloid phenotypic markers (n=6).

5.3.3 Alteration of Collagen I Expression in Primary Keloid Fibroblasts Following PAI-2 Knockdown

Proteins or mRNA were extracted from keloid fibroblasts (n=6) 48 hr after PAI-2/siRNA transfection and then subjected to Western blot and qRT-PCR analysis

respectively. Figure 5.7 (*Above*) represents a typical autoradiogram of protein expression. *Bar graphs* represent the average densitometry values of PAI-2 and collagen I protein expression, normalised to α-tubulin, from three independent experiments. Data are expressed as mean ± SEM. * indicates a significant difference ($p < 0.05$) between the scrambled siRNA–transfected versus the PAI-2/siRNA–transfected group. Importantly, because PAI-2 was up-regulated in keloid fibroblasts compared with fibroblasts from normal skin, I evaluated the molecular effect of PAI-2 on extracellular matrix metabolism in keloid fibroblasts. Forty-eight hr after transfection, the PAI-2 expression was significantly (p <0.05) reduced at the mRNA and protein levels in keloid fibroblasts in a dose dependent manner. Down-regulation of PAI-2 significantly reduced collagen I production at the mRNA and protein levels (Figure 5.7). I also assessed the molecular effect of PAI-2 on fibronectin and α-smooth muscle actin at the mRNA level, which showed a significant dose-dependent reduction in fibronectin and α-smooth muscle actin (Figure 5.8). Therefore, my results demonstrated that knockdown of PAI-2 reduced extracellular matrix deposition in keloid fibroblasts.

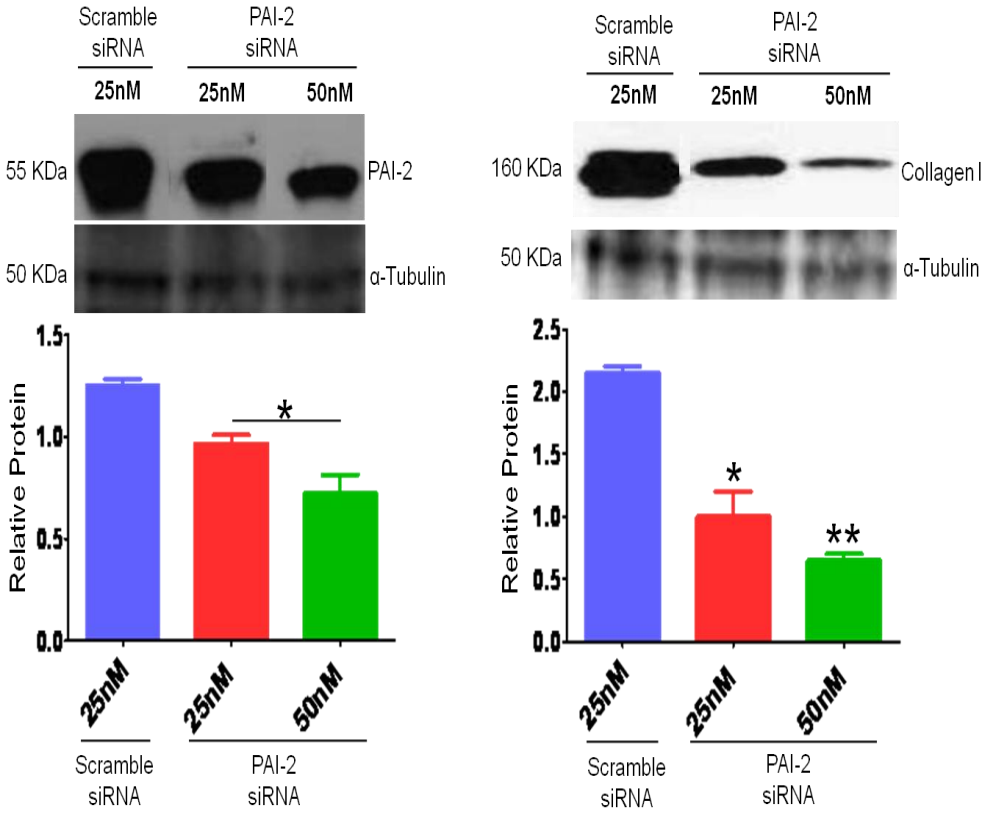


Figure 5.7 Analysis of PAI-2 and collagen I protein levels in keloid fibroblasts following siRNA transfection (n=6).

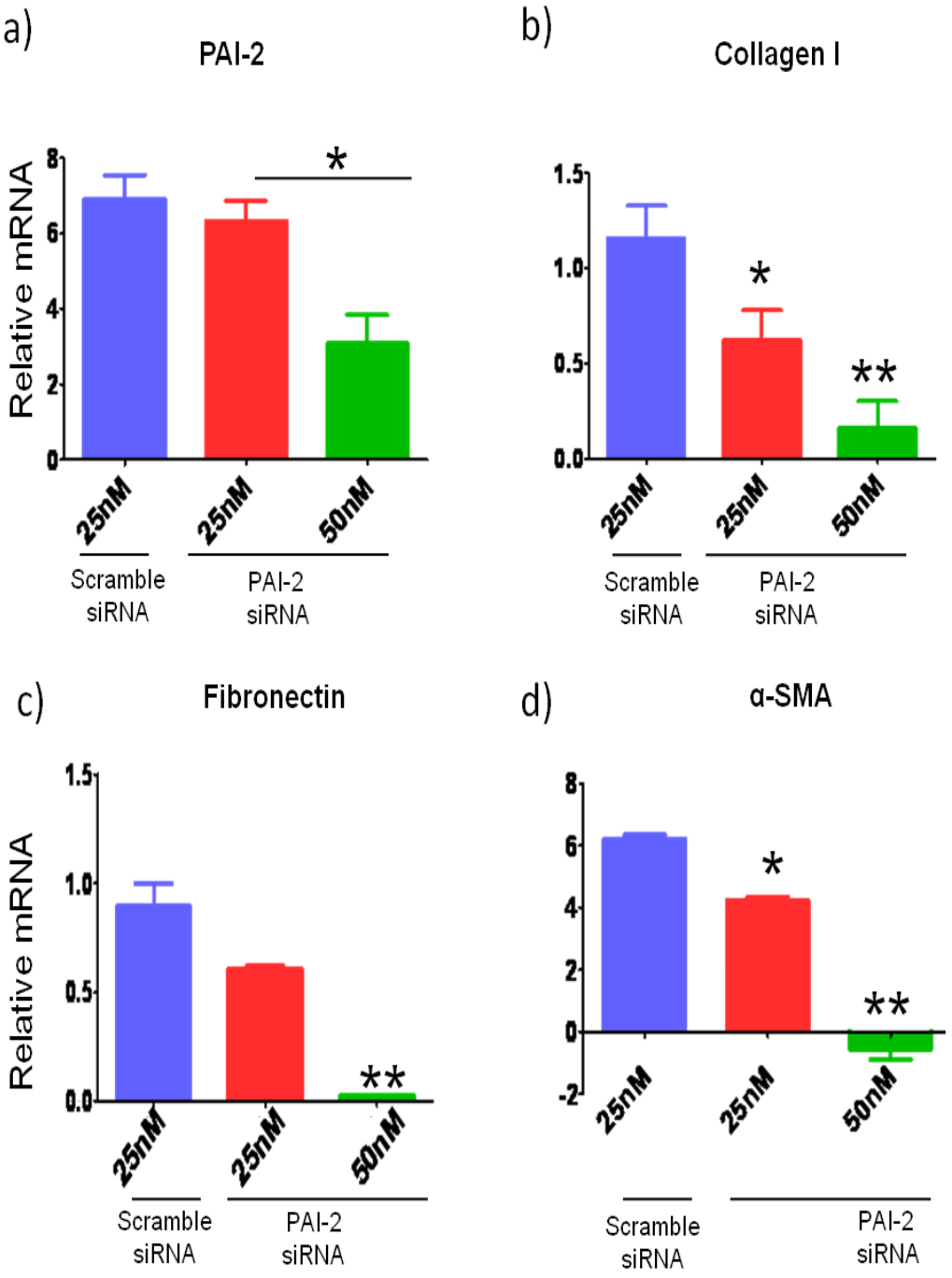


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Figure 5.8 Inhibition of PAI-2 by siRNA down-regulates extracellular matrix in primary keloid fibroblasts (n=6).

5.3.4 Knockdown of α2β1-Integrin Significantly Reduced Extracellular Matrix Deposition, Cell Attachment, and Mobility in Primary Keloid Fibroblasts

α2β1-Integrin is expressed in keloid fibroblasts, which may suggest its potential involvement in extracellular matrix rearrangement and deposition in addition to influencing cell attachment and mobility. Therefore, I evaluated the effect of knocking down α2β1-integrin expression by siRNA transfection. Forty-eight hr after transfection, α2β1-integrin mRNA and protein levels were significantly altered ($p < 0.05$) compared with the vehicle group (scrambled siRNA); importantly, collagen I was also strongly modified at the gene and protein levels (Figure 5.9). To further investigate the influence of α2β1-integrin on extracellular matrix genes, I tested the effect of α2β1-integrin inhibition on the expression of fibronectin and α-smooth muscle actin, showing significant reduction of the mRNA levels ($p < 0.05$) (Figure 5.10). My results demonstrated that the expression of α2β1-integrin influenced cell attachment and mobility in keloid fibroblasts.

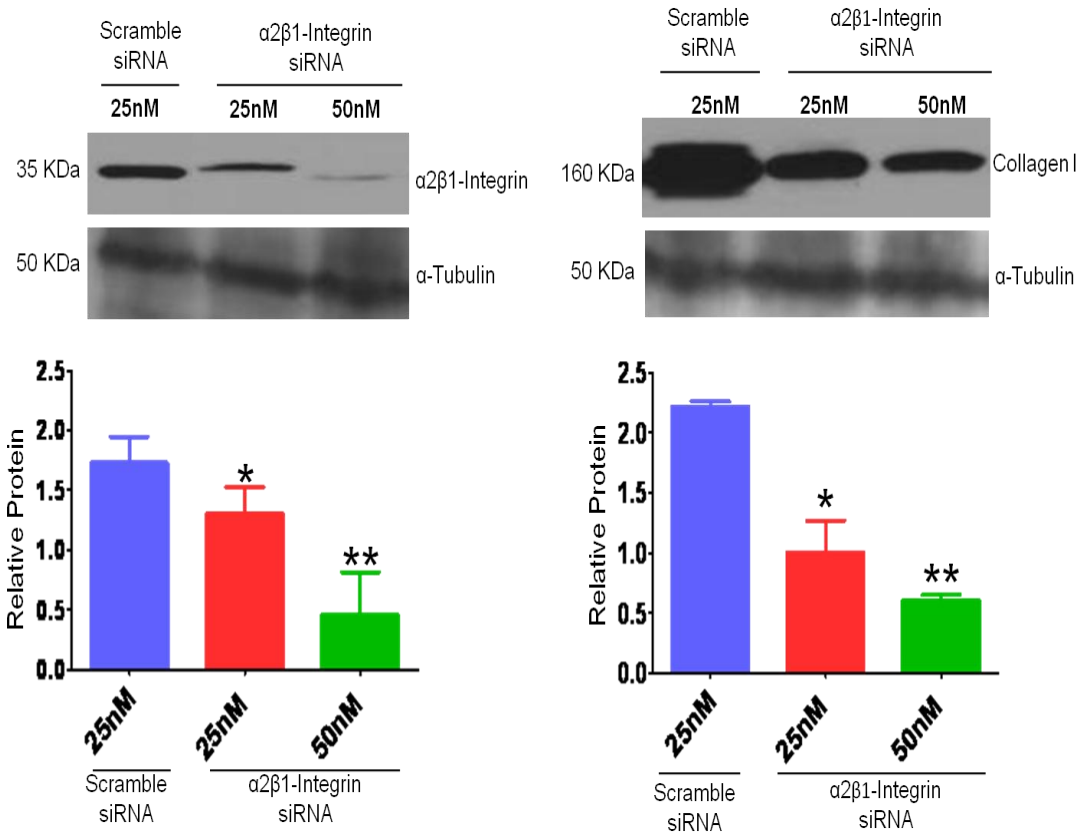


Figure 5.9 Knockdown of α2β1-Integrin and collagen I expression at the protein level in keloid fibroblasts (n=6).

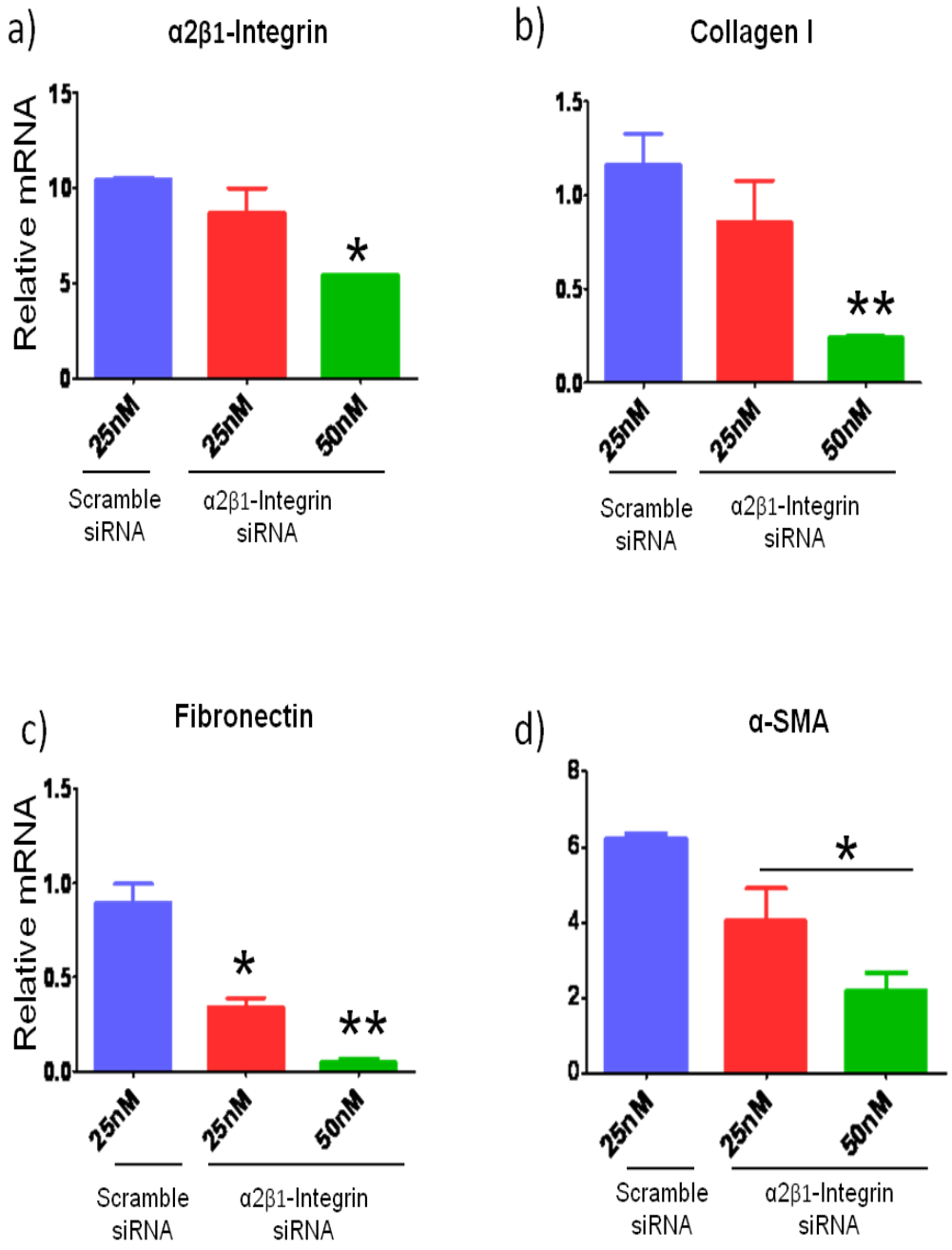


Figure 5.10 Effect of knockdown of α2β1-Integrin on extracellular matrix accumulation in primary keloid fibroblasts (n=6).

5.3.5 Knockdown of Hsp27, PAI-2, and α2β1-Integrin at the mRNA and Protein Levels Does Not Influence Viability/Metabolic Activity of Keloid Fibroblasts

After siRNA transfection, viability/metabolic activity of keloid fibroblasts was determined by using the water-soluble tetrazolium salt-1 assay. Interestingly, when compared with the vehicle group (scrambled siRNA), keloid fibroblasts showed no statistically significant difference in viability/metabolic activity at 48 hr after siRNA transfection in any of the groups. These results suggested that the suppression of Hsp27, PAI-2, or α2β1-integrin at both mRNA and protein levels had no significant influence on the viability and metabolic activity of keloid fibroblasts (Figure 5.11).

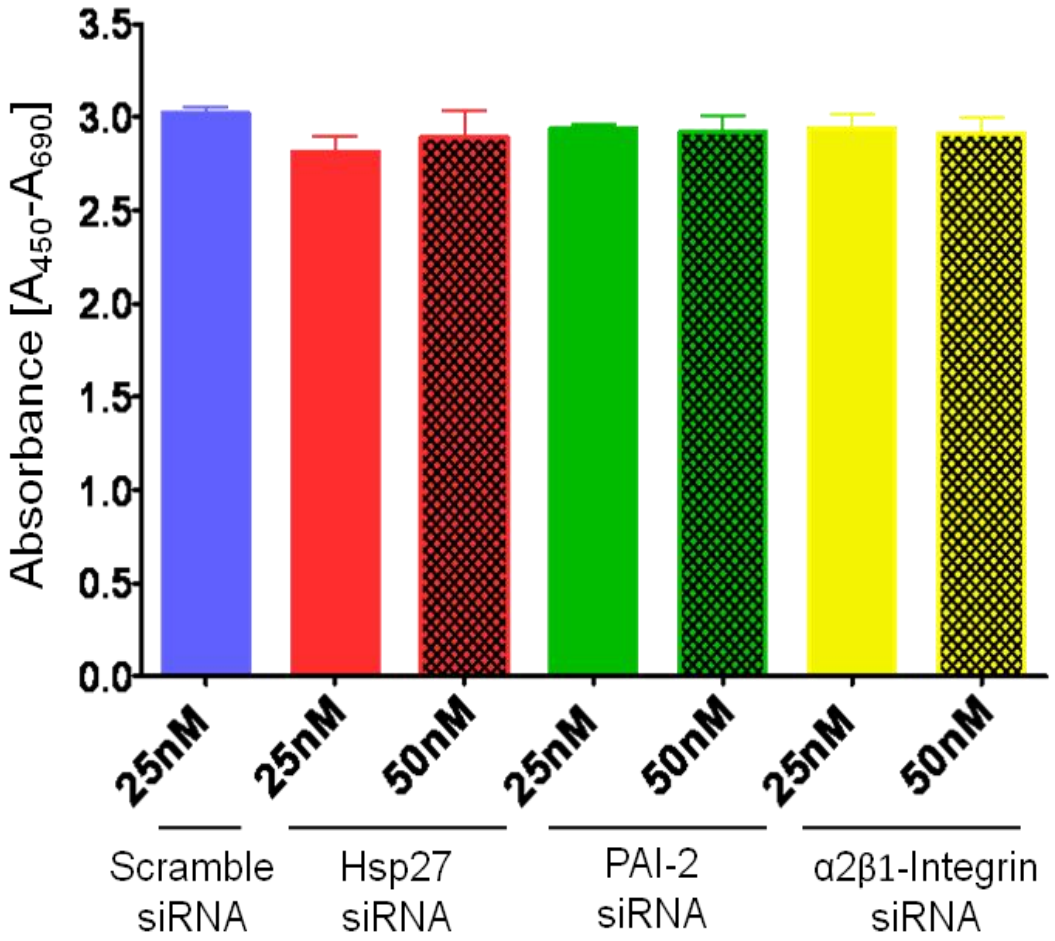


Figure 5.11 Effect on viability/metabolic activity of primary keloid fibroblasts following suppression of Hsp27, α2β1-integrin, and PAI-2 (n=6).

5.4 Discussion

In the present study, there were significant differences in the expression of a number of tension-related genes in keloids at the mRNA and protein level when compared with normal skin. Moreover, the knockdown of the expression of Hsp27, PAI-2, and α2β1-integrin by siRNA attenuated not only the expression of mRNA and protein levels themselves but also extracellular matrix regulation in primary keloid fibroblasts. My findings with Hsp27, PAI-2, and α2β1-integrin concur with previous studies that showed higher expression of these tension-related proteins in keloid fibroblasts[3, 138].

Mechanical tension induces α2β1-integrin activation, which serves as a transmembrane link between the extracellular matrix and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts[133, 206]. Among the proteins implicated in this process, focal adhesion kinase appears to be a key candidate. Wong *et al*. 2011 described the role of focal adhesion kinase in the development of the fibrotic process following mechanical force stimuli of the extracellular-related kinase–monocyte chemoattractant protein-1 inflammatory pathway, which induced fibroblast activation and survival and extracellular matrix production[24, 82, 189]. Actin stabilisation and polymerisation is also mediated by α2β1-integrin[138]. Furthermore, it has been reported that α2β1-integrin regulates collagen degradation by the activation of matrix metalloproteinases. In this study, knocking down the expression of α2β1-integrin demonstrated its effect on the mRNA expression of extracellular matrix components, in addition to protein (Figure 4.9) and mRNA (Figure 4.10) levels of collagen I in keloid fibroblasts.

The mechanotransduction signal also activates Hsp27 by means of p38 mitogen-activated protein kinase[137, 207]. The heat shock protein activation promotes actin stabilisation by unknown mechanisms, focal contact formation, cell contraction, migration, and survival[137, 179, 180, 193, 207]. Collagen degradation is also regulated by PAI-2, which negatively regulates matrix metalloproteinases, leading to extracellular matrix accumulation. PAI-2 is also involved in cell migration mediated by integrins[168]. In this study, I showed that targeting Hsp27 by siRNA down-

regulated extracellular matrix in keloid fibroblasts. Consistent with my results, previous work has shown that down-regulation of the mRNA expression of Hsp27 caused a significant decrease in the expression of collagen I[207]. I also showed that inhibition of PAI-2 significantly reduced the expression of collagen, fibronectin, and α-smooth muscle actin in a dose-dependent manner in keloid fibroblasts. To this end, α2β1-integrin and Hsp27 may be key upstream regulators of extracellular matrix production, whereas PAI-2 regulates extracellular matrix degradation (Figure 5.12)[159].

Wound healing is considered a complicated process that involves multiple phases. Interestingly, it has been suggested that KS may develop following a dysregulation in the inflammatory phase, conversely other authors have suggested that a prolonged proliferative phase tends to result in keloid development. Furthermore, keloids show heterogeneity, as lesions differ in appearance from patient to patient depending on the anatomical site[24]. Thus, I decided to limit my samples to one anatomical location (the sternum). However, future investigation, involving different anatomical locations and samples from different stages of keloid development, will be of value. Another potential limitation of my study was the total number of samples used for keloid (n = 10) and for normal skin (n = 4); a larger number of samples that include different ethnicities would be beneficial. My research represents a preliminary but detailed study focused on the identification of mechanosensory genes related to keloid disease from a specific anatomical location (the sternum) obtained at one time point in mature KS in steady state conditions. To this end, there is insufficient information to provide a clear explanation for when these genes are up-regulated during the healing process. However, based on previous reports, I can speculate that these genes are potentially activated during the earlier stages of wound healing[208]. This unique study may offer a potential therapeutic option based on RNAi, providing the possibility of an endogenous and specific mechanism of gene silencing for keloid disease as described previously for other diseases[9]. The RNAi-based therapy in animal models has also shown benefits in several diseases involving lung, subcutaneous tissue, muscle, eye, and the nervous system[209].

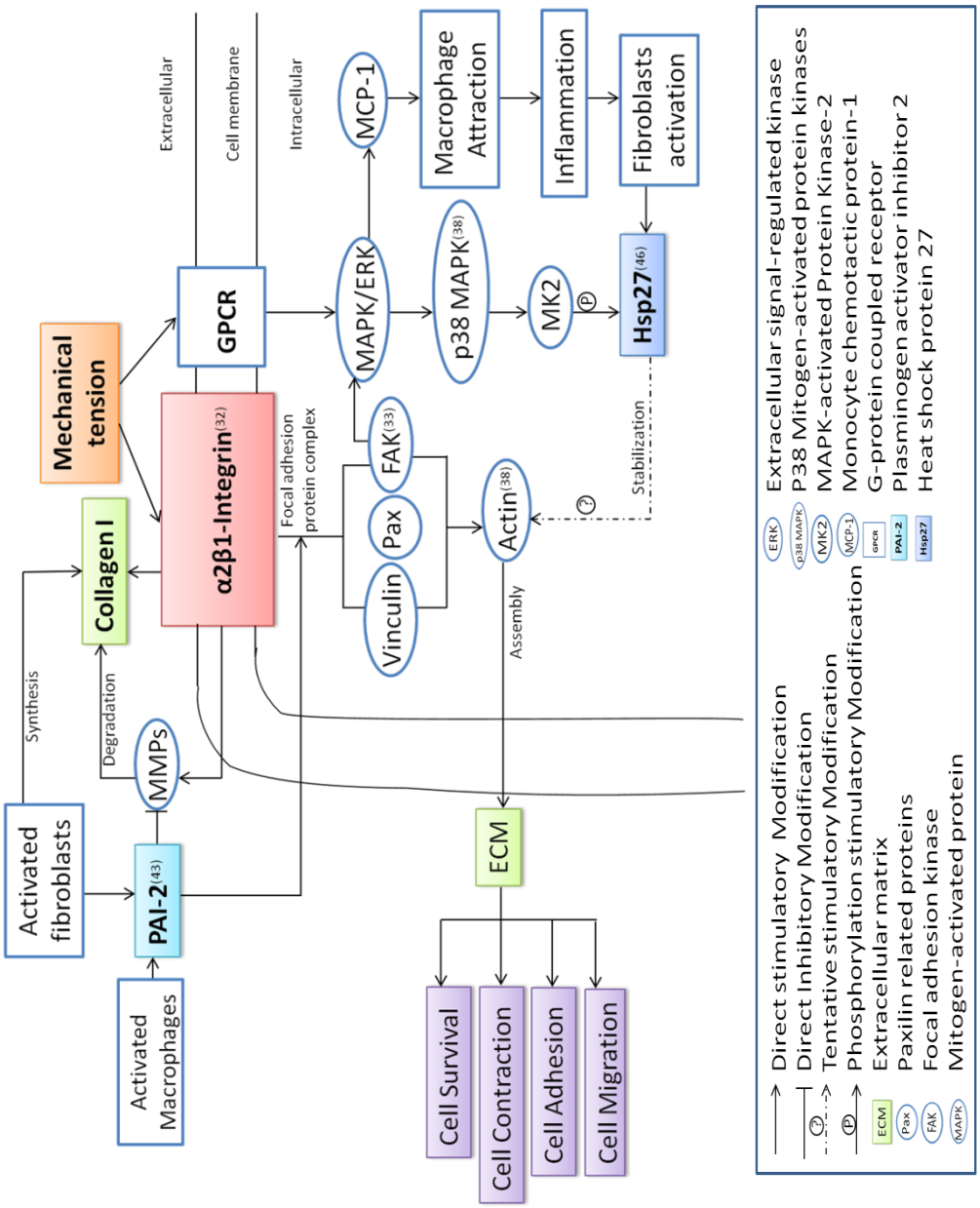


Figure 5.12 Potential molecular links between α2β1-integrin, PAI-2, and Hsp27.
Mechanical tension induces α2β1-integrin activation, which serves as a transmembrane link between the extracellular matrix and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts. The mechanotransduction signal also activates Hsp27 by means of p38 mitogen-activated protein kinase. PAI-2 promotes extracellular matrix accumulation by negative regulation of matrix metalloproteinases. PAI-2 is also linked with the regulation of cell migration mediated by integrins.

A potential mechanism to explain the pathobiology of keloid scar formation under tension may be postulated based on the findings of this preliminary study. After tissue damage occurs, mechanical stress produced during the wound healing process in the injured area promotes cell activation, proliferation, differentiation, and up-regulation of several tension-related proteins, including Hsp27, PAI-2, and α2β1-

integrin. This is followed by an excessive extracellular matrix production and deposition in the keloid-susceptible individual. The increased mechanical tension exhibited in the injured area promotes the establishment of a continuous cycle that increases the activation of these tension-related genes in the wounded area, resulting in the formation of a keloid in the susceptible individual. After tissue damage, mechanical stress placed on the tissue in specific anatomical sites (i.e., sternum) during the wound healing process promotes fibroblast activation. In response to tension, activated fibroblasts synthesise increased levels of stress proteins, certain extracellular matrix components, and α-smooth muscle actin. In this context, stress proteins including Hsp27, PAI-2, and α2β1-integrin may promote cell protection against apoptosis and extracellular matrix component rearrangement and synthesis. Furthermore, mechanical stress may promote differentiation of fibroblasts into myofibroblasts, which also synthesise stress proteins, contributing to extracellular matrix component synthesis and deposition in keloids. High production of extracellular matrix components may promote mechanical stress and support the establishment of a persistent cycle (Figure 5.13).

In conclusion, my study has shown an overexpression of specific tension-related genes and proteins in keloid fibroblasts and tissue. Knockdown of the expression of Hsp27, PAI-2, and α2β1-integrin by siRNA attenuated the expression of not only mRNA and protein levels themselves but also the mRNA and protein levels of key molecules related to extracellular matrix regulation in keloid fibroblasts. This study offers options for a potential therapeutic application of RNAi to modify tension-related proteins for future treatment of keloid disease.

At this point, I can speculate that the most promising molecular candidate in the regulation of KS by mechanical induction may be α2β1-integrin, although further experiments are required to provide a better understanding of the mechano-signalling pathway regulation in keloid scars.

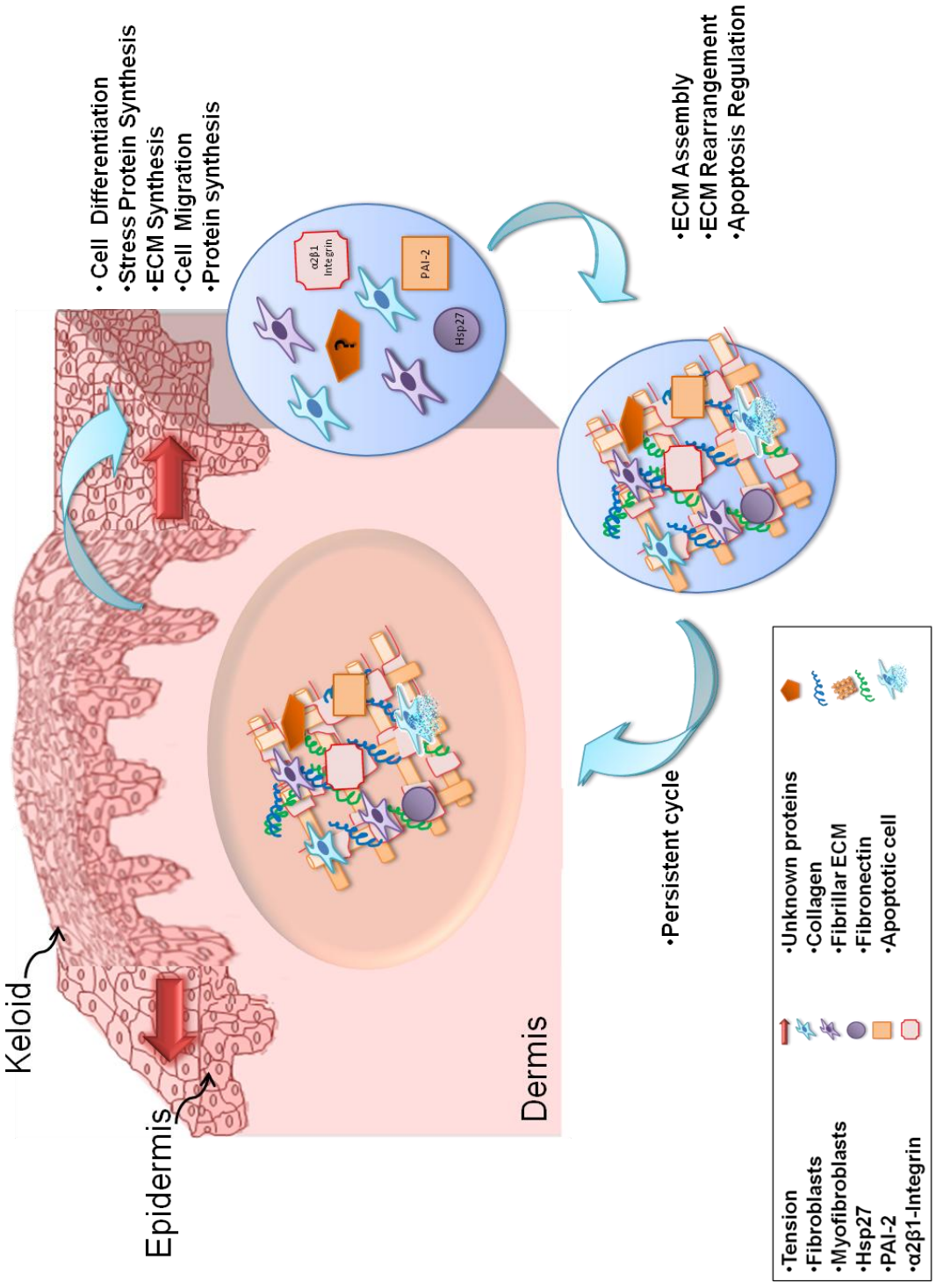


Figure 5.13 Proposed mechanism for the possible role of tension-related proteins in keloid scarring.

My study provides a list of genes that explains the difference between the keloid and hypertrophic scar development processes, as well as options for potential target molecules that could be used to develop new keloid/hypertrophic therapies, although further experiments are required to establish a more comprehensive and complete mechanism to explain the correlation between mechanical skin tension and abnormal scar development and differentiation. In addition, I found tension-related gene expression to be greatest in the sternum, a region known to be subject to high mechanical forces.

The results obtained from this chapter offer valuable information to the design of further experiments that may contribute to provide a more complete explanation to supply a better correlation between the mechanical skin tension and abnormal scar development and differentiation *in-vitro*. These results were employed in the designing and development of a novel *in-vitro* 3D model to further investigate the effect of the skin tension on the progress of KS in the sternum. The details of this model are given in the subsequent chapter, section 6.2.2.

Chapter VI: Skin tension alters keloid fibroblast behaviour

6.1 Introduction

Skin is subject to mechanical forces due to natural body movements[210, 211]. It has been suggested that mechanical forces acting on certain anatomical sites under high tension, such as the sternum may influence the development, both in terms of the behaviour and appearance of keloid scars[1, 17]. Keloid disease (KD) is an abnormal fibroproliferative tumour unique to humans that extends beyond the confines of the original wound margins, does not regress over time and invades the nearby unaffected skin[21, 22]. The mechanism involved in the formation of KS remains unknown and subsequently, its treatment is ill-defined, with poor outcome and high recurrence[4, 212].

Mechanical tension can promote phenotypic alteration in fibroblasts during wound healing[129]. Skin mechano-regulation can induce dysregulation of the expression of several tension-related proteins such as cytokines, growth factors, integrins and extracellular matrix (ECM) components during KD development[90]. A better understanding of the effects of skin mechano-regulation may provide alternatives for prevention of abnormal scar formation in certain anatomical sites prone to high tension.

In the absence of a *reliable* animal model for studying KD; research has focused on the development of new experimental techniques, and models *in-vitro* in order to evaluate the effect of skin tension in repair[213]. To this end, three-dimensional models (3D) have become a useful tool in studying the effects of mechanical tension on skin cells. Collagen type I based 3D-models allow a three-dimensional distribution of force to the cells. Moreover, the evaluation of phenomena such as cell communication, migration, differentiation, adhesion, apoptosis, among others between cells and extracellular matrix (ECM) are evaluated in a closer manner to the *in-vivo* condition. Other advantages of using 3D-models include the ability to semi-quantitatively measure cellular contraction. In the collagen lattice models, a small collagen gel area corresponds to a high cellular contraction effect[133]. Several cell force monitors (CFM) have been used to reproduce tension forces in 3D models. However, these models are rate limiting due to the non-uniform distribution

of the tension over the study model[214]. These CFM are focused on the quantification of the amount of force produced by the cells while contracting the matrix[215, 216] but the amount of force applied to the system (in order to reproduce the normal tension forces in the body and activate the cells populating the gels) is variable. The correlation between the load used and the normal loading conditions in the body is not provided. A device able to reproduce the amount of tension generated *in-vivo* and to provide distribution of the tension all over the model is required. In this study, *in-vivo* and *in-vitro* techniques were combined to develop a 3D-model to mimic *in-vivo* tension in cells and furthermore to compare the effect of mechanical tension on normal and keloid fibroblasts.

6.2 Material and Methods

The study was carried out in three stages: *in-vivo* skin tension force evaluation, determination of optimal tension force application time, and knock down tests. These stages are described in diagrammatic form in Figure 6.1 The results from each stage of the study fed into, and were used in the subsequent stages. The CFM with integrated 3D collagen gel model was used for the second and third tests.

6.2.1 *In-vivo* skin force measurement using photogrammetric analysis

This study was undertaken in order to determine a realistic tension force to be used in the CFM tests. 3D skin deformation measurements were performed on healthy male volunteers (n=4) involving the digitization of a speckle pattern painted on the participant's sternum. All volunteers were invited to participate following informed consent and full ethical approval being obtained prior to the test (detailed ethical approval and informed consent is included in Appendix II section). A series of images of the pattern was obtained and processed using ARAMIS (GOM mbH, Braunschweig, Germany) software. The software tracked the change in position of the discreet area of the pattern that occurred while the volunteers, starting from a standing position, performed, at a self-selected speed, complete maximal breathing cycles (inhalation and expiration) (5 times). Software calculated the 3D deformation occurring in the skin during movement. Two linear sections were

created across the study area for the analysis (Figure 6.2a). The first one (Y-Y') allowed analysis of the skin deformation measured from the top to the bottom of the sternum. The second (X-X'), created along the nipple line, showed the skin deformation approximately parallel to the nipple line. The deforming force in the skin was determined from the measured deformation of the skin and average skin elasticity obtained from published data[217].

6.2.2 Culture force monitor design

The applied tension force *in-vivo* was replicated *in-vitro* using a novel in-house developed cell force monitor. Uniformity in tension field was ensured using an appropriate size and shape of the collagen-cell gel matrix (Figures 6.2b and 6.2c).

The apparatus consisted of a tension application unit, a sensor unit, and a PTFE (polytetrafluoroethylene) base. A Petri-dish containing the rectangular collagen-cell specimen (49 mm×19 mm×0.9 mm) was placed on the PTFE base. At one end of the specimen the tension unit was attached using a small segment of copper wire to one of the two plastic mesh attachment units (19mm×10mm) that had been cast at the two shorter edges of the specimen. The other mesh attachment was connected to the sensor unit. Guiding holes for copper wire were drilled in the Petri-dish wall to ensure alignment of the line-of-action of the tension unit and the sensor unit.

The tension unit consisted of a micrometer screw gauge (accuracy ±0.01 mm) held firmly by a stainless steel stand and holder assembly. By turning the micrometer screw, a tensile force was applied to the attached collagen-cell specimen. The sensor unit consisted of (i) a cantilever copper-beryllium beam (100mm×10mm×0.15mm, Young's modulus E = 290GPa) (Strategic metals LTD, UK) supported in a vertical orientation by a stainless steel rugby-post type framework; (ii) four transducer class strain gauges (gauge factor = 140; resistance ~500 Ω; Micro Measurements, CA) attached at a distance of 20 mm from the top support; (iii) a voltmeter; and (iv) a transducer amplifier.

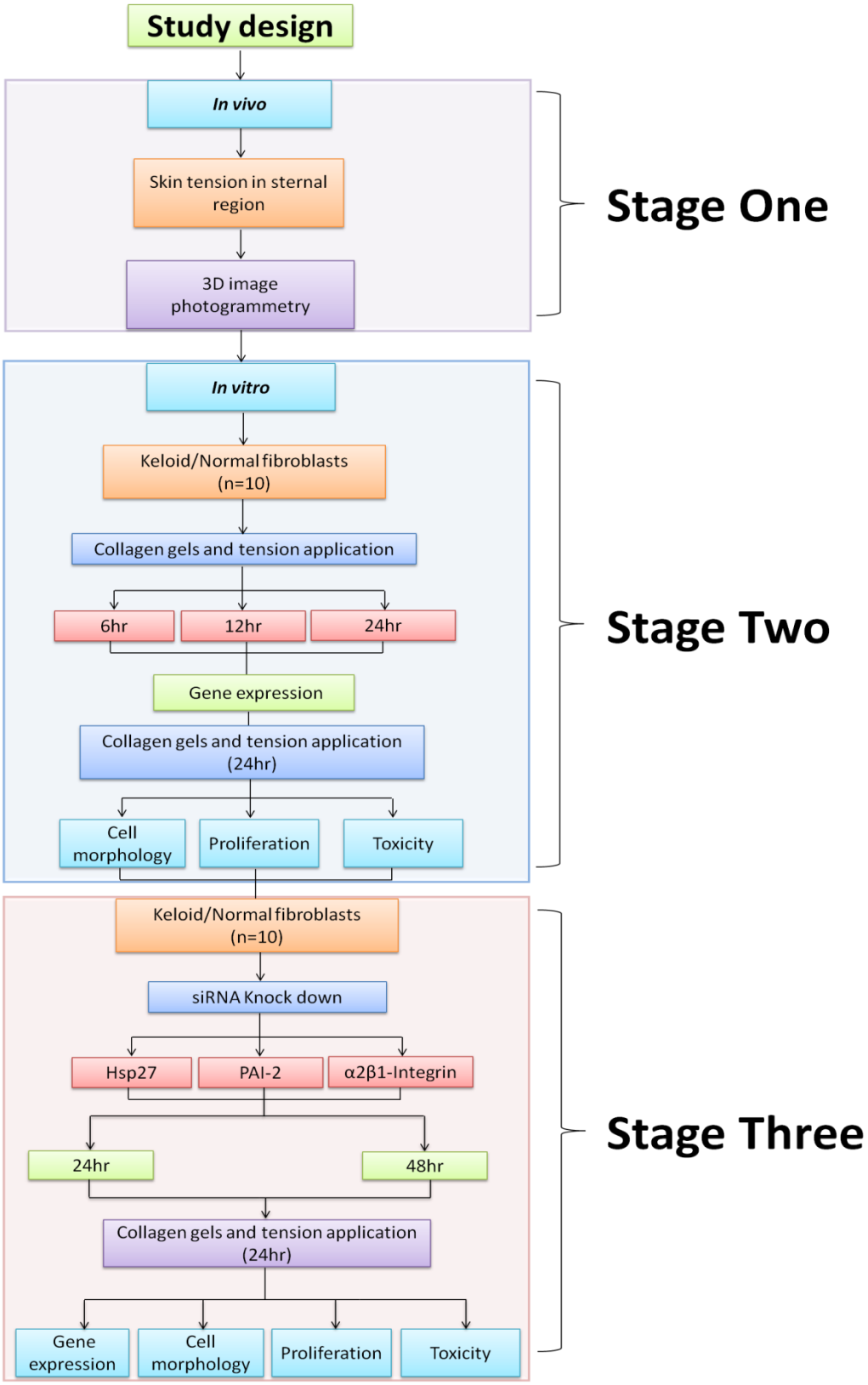


Figure 6.1 Study experimental design.
The flowchart presents a detailed summary of the steps taken to perform this study

The applied tension force in the collagen gel was transferred to the cantilever beam causing deflection and bending of the beam. The bending strain was recorded by the strain gauges, and a digital output was obtained from the voltmeter reading. The transducer amplifier for the strain gauges provided the desired resolution of the reading.

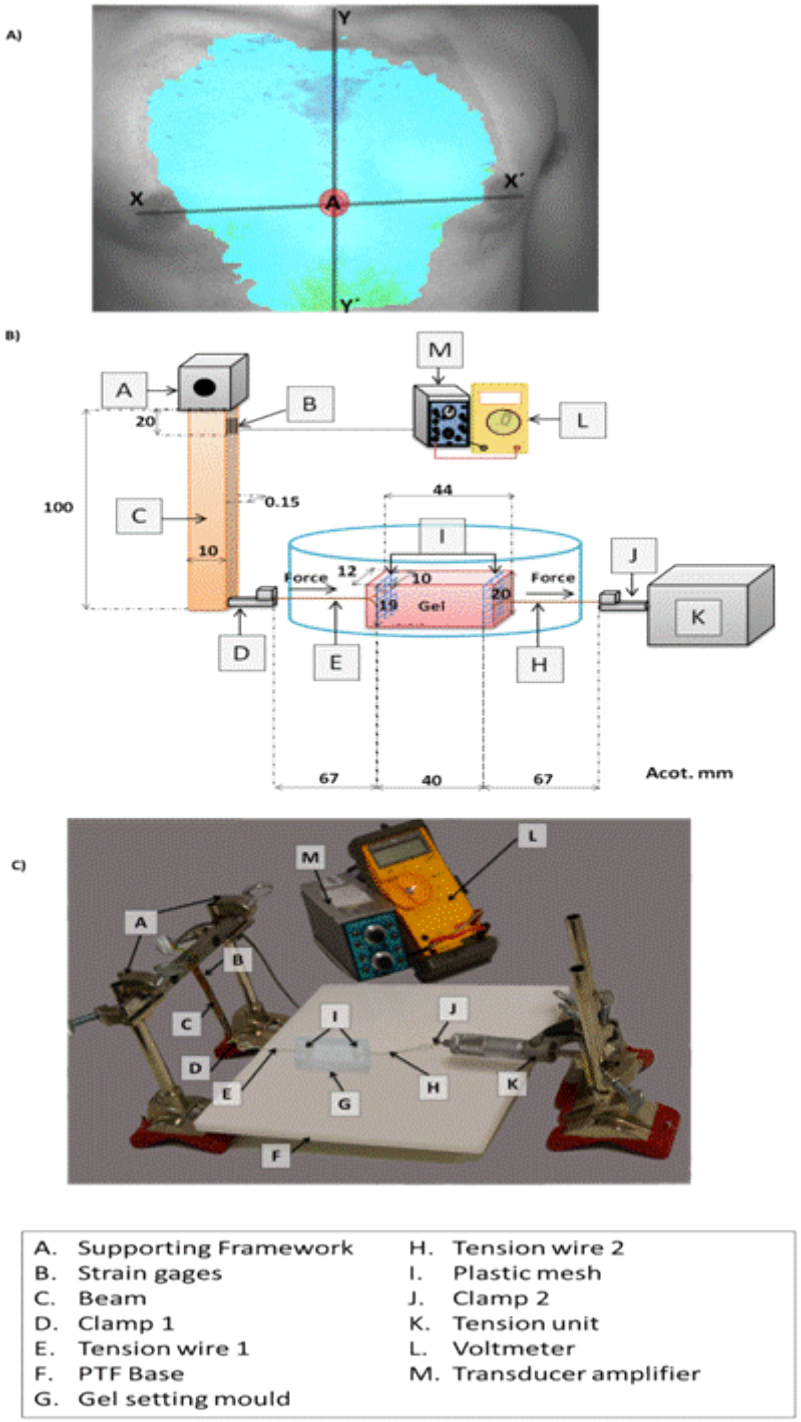
It should be noted here that the set up did not allow for direct measurement of force. To achieve this, the cantilever beam (in horizontal orientation) was calibrated using small weights attached to the free end. A linear relationship between the load and the voltmeter reading (of strain) was obtained for both during loading and unloading of the beam. This calibration line was subsequently used to estimate the amount of tension force applied to the specimen.

The tension force transfer through the gel specimen was ascertained by comparing the applied displacement of the micrometer screw gauge to the displacement of the beam. The beam displacement was calculated by converting the strain reading using simple formulae from structural mechanics. The strain values were recorded by attaching the gauges to a Wheatstone bridge. It was observed that the force dissipation was negligibly small, justifying the use of the PTFE base. In many culture force monitors previously published in the literature; this particular aspect has been completely overlooked[133, 218].

6.2.3 Determination of optimal tension force application time

A preliminary study was undertaken to determine the optimum time to apply the tension force in order to promote the up-regulation of the expression of three target genes Hsp27, α2β1-integrin, PAI-2. The three genes were selected based on the results of a previous study which investigated up-regulation in keloid tissue at gene and protein levels[219]. The preliminary study involved applying the force obtained from the *in-vivo* tests, 35 mN, to the 3D collagen model seeded with either normal or keloid fibroblasts for periods of 6, 12 and 24 hr, and measuring the expression of

the target genes at each time period. Cell morphology, proliferation and toxicity were then evaluated at 24 hr as the gene expression was significantly up-regulated at this time compared to 6 and 12 hr.



A. Supporting Framework	H. Tension wire 2
B. Strain gages	I. Plastic mesh
C. Beam	J. Clamp 2
D. Clamp 1	K. Tension unit
E. Tension wire 1	L. Voltmeter
F. PTF Base	M. Transducer amplifier
G. Gel setting mould	

Figure 6.2 *In-vivo* test and CFM.

A) Image taken from the sternum of a healthy male volunteer during the performance of the *in-vivo*. B) CFM detailed diagram. C) Culture force monitor components.

6.2.4 Patient data and tissue collection

Keloid (n=10) and normal skin samples (n=10) (please see supplementary Table A7) 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, UKL) prior to surgery. All samples were processed as previously described in chapter III (Section 3.3). At the time of surgical excision, the central area of the keloid scar (the middle of the lesion) from the sternum and normal skin from normal scars were collected.

6.2.5 Primary keloid and normal skin fibroblast culture establishment

Fibroblasts from passage 0 (p0) to passage 3 (p3) were used for all experiments carried out in this study as described previously in chapter III (section 3.4.2).

6.2.6 Tension related gene selection criteria

The genes employed in this study (Hsp27, α2β1-integrin, PAI-2) were selected based on my previous findings, chapters IV and V, where convincing up-regulation of tension-related genes was shown in keloid tissue and keloid fibroblasts (KF) after analysing 21 tension-related genes candidates that were selected from three pooled microarray studies[90, 94, 141, 154] for their relationship with wound healing, skin tension and fibrosis.

6.2.7 siRNA design and target gene knock-down

To perform the knock-down of the target genes (Hsp27, α2β1-integrin, PAI-2) normal skin (NF) and keloid fibroblasts were transfected using the methodology previously described in chapter V, section 5.2.6. Transfected cells were collected by trypsinisation after 24 and 48 hr treatment and employed in the preparation of fibroblast populated collagen lattices (FPCL) in further experiments.

6.2.8 3D human fibroblasts populated collagen lattice

To perform the tensional testing, NF and KF were seeded in three dimensional (3D) collagen gels as described previously in chapter III (Section 3.4.9). The polymerized gel was physically detached from the chamber and floated in 10 ml of supplemented DMEM. The cell attachment units were set to the CFM for tension application treatment (24 hr).

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

FPCL were tensioned for 24 hr and employed for RNA extraction. After tension application each fibroblast populated gel was snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Macerated samples were homogenised with trizol (Invitrogen, U.K.). RNA extraction was performed according to the manufacturer's specifications and using RNeasy mini-kit (Qiagen, U.K.). cDNA synthesis, and quantitative reverse-transcriptase polymerase chain reaction were performed as described in chapter III (Sections 3.4.3-3.4.5). The list of primers used in this study is shown in the supplementary Table A8. The mRNA expression was normalised with reference to gene ribosomal protein L32 (RPL32).

6.2.10 Cell morphology and actin rearrangement

FPCG were fixed with 4% formaldehyde for 30 min at 37°C after 24 hr mechanical tension treatment. Cells were permeabilised with 0.1% Triton X-100 and treated for double staining. Primary antibody was incubated overnight at 4°C followed by secondary antibodies (Rhodamine-phalloidin 1 hr and DAPI 15 min). (See Tables A1 and A2 for list of primary and secondary antibodies). Fluorescence images were taken using a high-end wide field microscope (Olympus IX71 microscope).

6.2.11 Cell proliferation assay in 3D models

Proliferation ratios of cells cultured in collagen lattices were measured after 24 and 48 hr siRNA transfection as follows. 40µl of FPCL was added into a 96 well plate

and left to set for 10 min at room temperature. Once set, 100µl of medium was added on top of each well. Control wells were detached from the walls of the wells; whereas the rest of the wells were cultivated under mechanical tension conditions for 24 hr. 10 µl of cell proliferation reagent WST-1 (Roche) was added and incubated for 4 hr at 37°C. Net absorbencies (A450–A690) were calculated.

6.2.12 Cell Viability/Metabolic Activity Detection (Water-Soluble Tetrazolium Salt-1)

Cell viability ratios were measured in FPCL after 24 and 48 hr siRNA transfection using water-soluble tetrazolium salt-1 assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

6.2.13 Statistics

The statistical analysis was performed as described previously in chapter III (Section 3.4.10).

6.3 Results

6.3.1 *In-vivo* skin tension calculation in the sternum using photogrammetry

For the *in-vivo* skin tension tests, a linear axis Y-Y' (Figure 6.3a) was created on the sternum of the volunteers to enable skin deformation measurement from the top to the bottom of the sternum. Axis X-X' was created to estimate skin deformation along the nipple line. The images taken during the tests were analysed to determine the deformation of the skin and tension force within the area defined by the axes and consequently to calculate the tension forces. The point A represents the axes intersection point.

A contour plot of average force over the sternum area calculated from the results of three independent tests performed on each of the four volunteers is shown in Figure 6.3b. As the results were relatively symmetric about the X-X' axes, only the upper half of the sternum is shown in Figure 6.3b. It can be seen from this figure that

larger tension forces (denoted in red) occur in two distinct regions, one running along the nipple line (X-X'), and the other, running vertically, from the top to the bottom of the sternum, along Y-Y'. The tension forces in the nipple line area were higher than those running vertically on the sternum. Comparing Figure 6.3b with Figure 6.3c, a photo of a keloid scar of typical shape found on the sternum, it could be seen that the pattern of distribution of tension force relates closely to the shape of the keloid scar. From the analysis of the skin deformation study, a skin tension force of 35-50mN was selected for use in the subsequent CFM tests. This value was chosen based on the skin tension force values obtained in the tests and upon consideration of the size of the cells and the gel lattice.

6.3.2 Characterisation of the normal skin and keloid fibroblasts response when subjected to mechanical tension in 3D collagen lattice *(In-vitro* I - Stage Two)

The validation of the newly created CFM performance over time was carried out using 3D NF/KF populated collagen lattices (FPCL) (n=10). Following three time point tension applications, the effect on gene expression of Hsp27, PAI-2 and α2β1-Integrin was evaluated by quantitative-PCR (figure 6.4a-6.4c respectively). The level of gene expression of the target genes in NF and KF in the 3D-lattices subjected to tension was time dependent. The application of tension over the evaluated periods, i.e. 6, 12 and 24 hr showed increasing level of gene expression. This trend was seen in the three target genes and was even more evident in KF after 24 hr of tension force application. The expression of the tension-related markers was normalised to RPL32, a housekeeping gene. The results are expressed as mean ± SEM of triplicates from three independent experiments. *$p<0.0001$ indicates significant difference between the mRNA expression. The dashed boxes in Figure 6.4 indicate the group of samples evidently more affected by the treatment.

Hsp27 gene expression levels were significantly increased in KF ($p<0.0001$) compared to NF. Moreover, PAI-2 was up-regulated in both tensioned cell types but was significantly higher in KF ($p<0.0001$) compared to NF. Finally, α2β1-Integrin gene expression levels were also increased significantly in KF ($P<0.05$) compared to NF. Among the three tension-related genes, Hsp27 gene expression level was up-

regulated most compared to others over the three time periods following tension force application in both NF and KF.

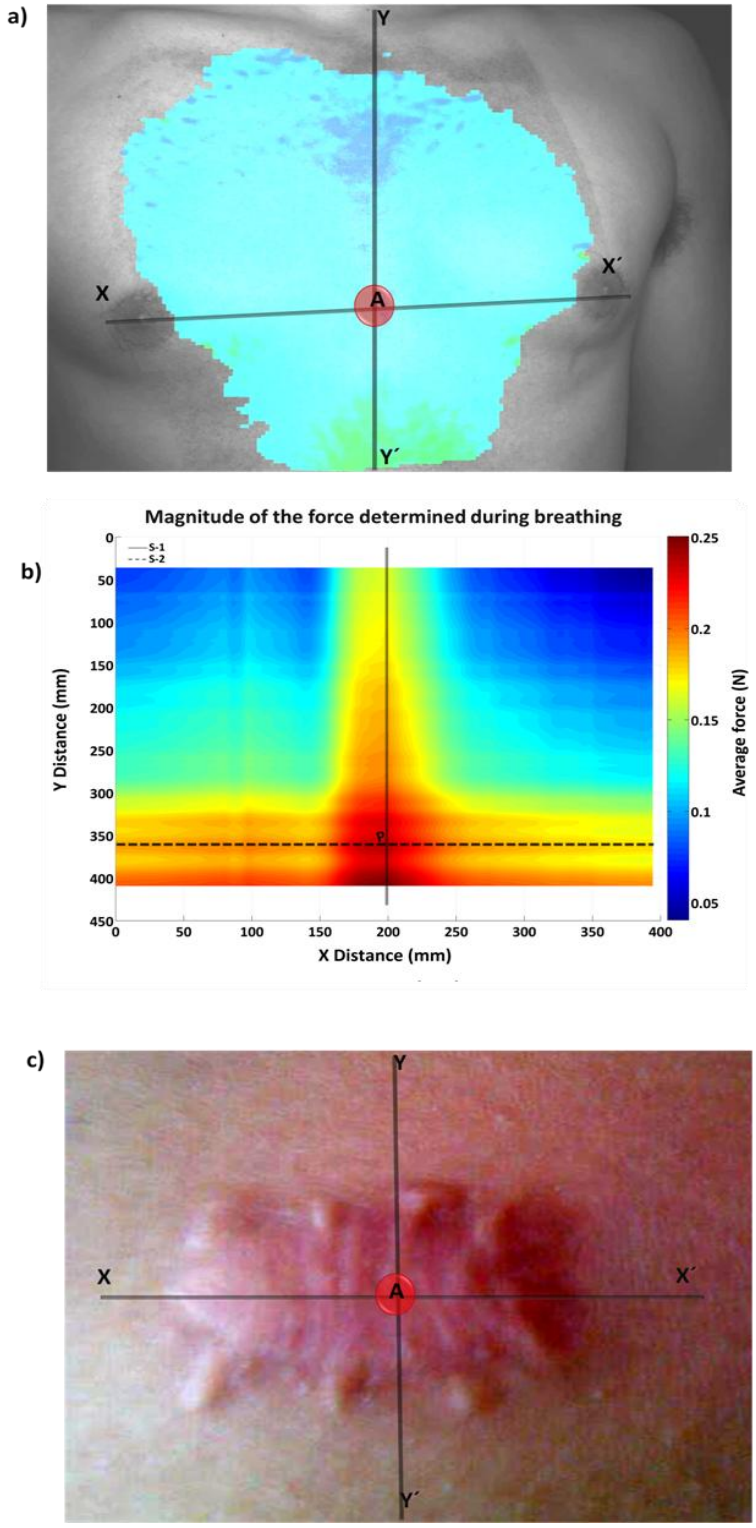


Figure 6.3 *In-vivo* test and butterfly-shaped keloids (Stage I)(n=4).

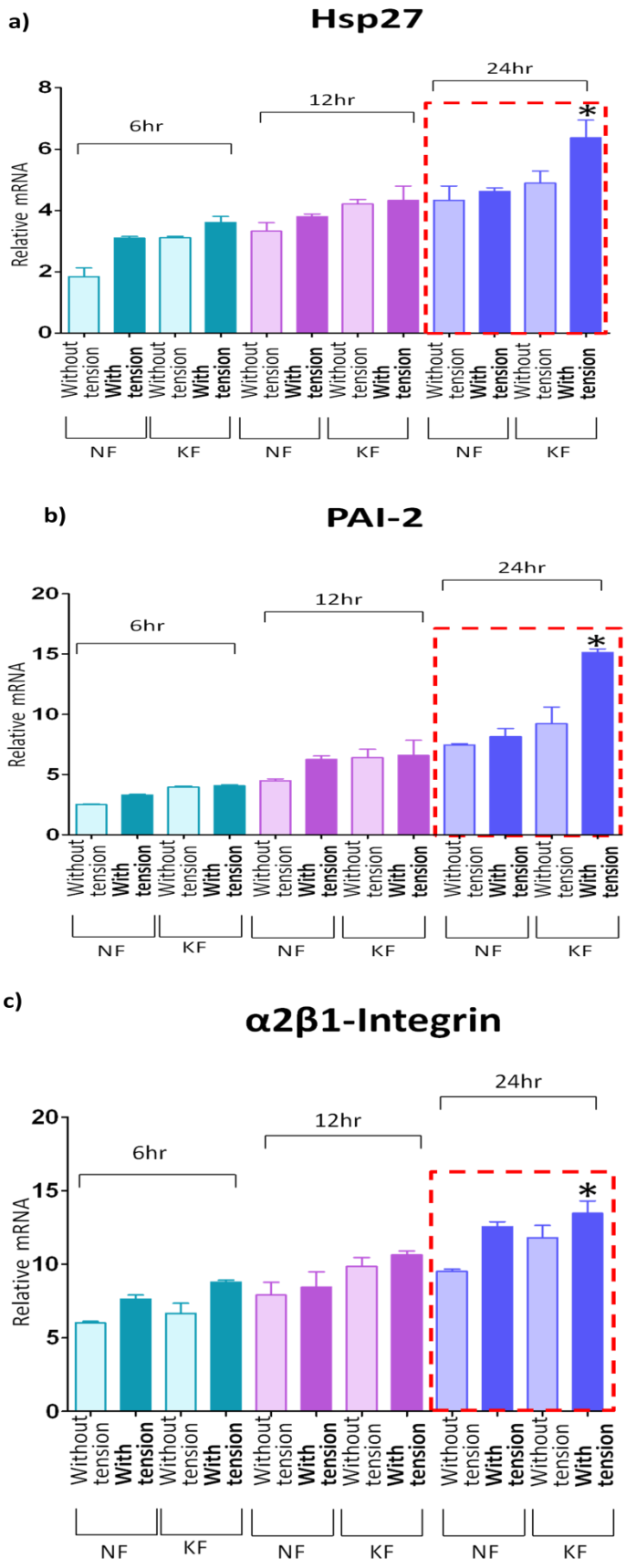


Figure 6.4 Validation of the CFM performance over time using 3D NF/KF populated collagen lattices (FPCL) by qRT-PCR (n=10).

In addition to gene expression, cell morphology was examined following mechanical tension (Figures 6.5a-6.5d). Both cell types showed elongation after being tensioned, however they changed morphology and spread freely when tension was removed. Cells aligned themselves along tension lines following application of force but displayed random orientation when left free to contract (cultured without tension) (Figures 6.5b and 6.5d).

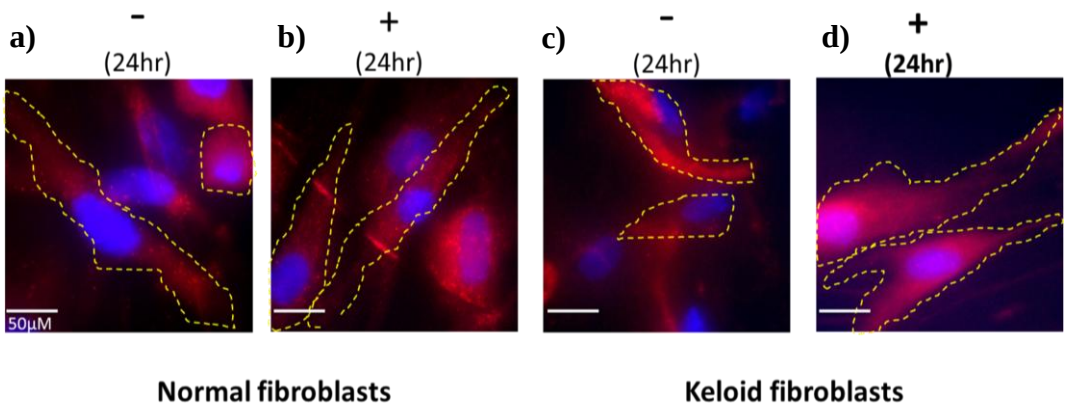


Figure 6.5 Mechanical tension influence cell morphology of NF/KF grown in 3D collagen lattices (n=10).
(Without tension (-), with tension (+))

There was a significant induction of proliferation in both cells types following mechanical tension in the 3D model (n=10). KF proliferation ratios were significantly higher ($p<0.0001$) compared to NF at both time points following tension (24 and 48 hr) (Figure 6.6a). Whereas, no effect in cell viability/metabolic activity was observed in both cell types (Figure 6.6b). The dashed boxes indicate the group of samples evidently more affected by the treatment. Thus, these findings imply skin tension promotes cellular activation, spreading and higher proliferation which is most pronounced in KF. Skin tension also induces the expression of target tension-related genes in both cell types but clearly it is more significant in KF.

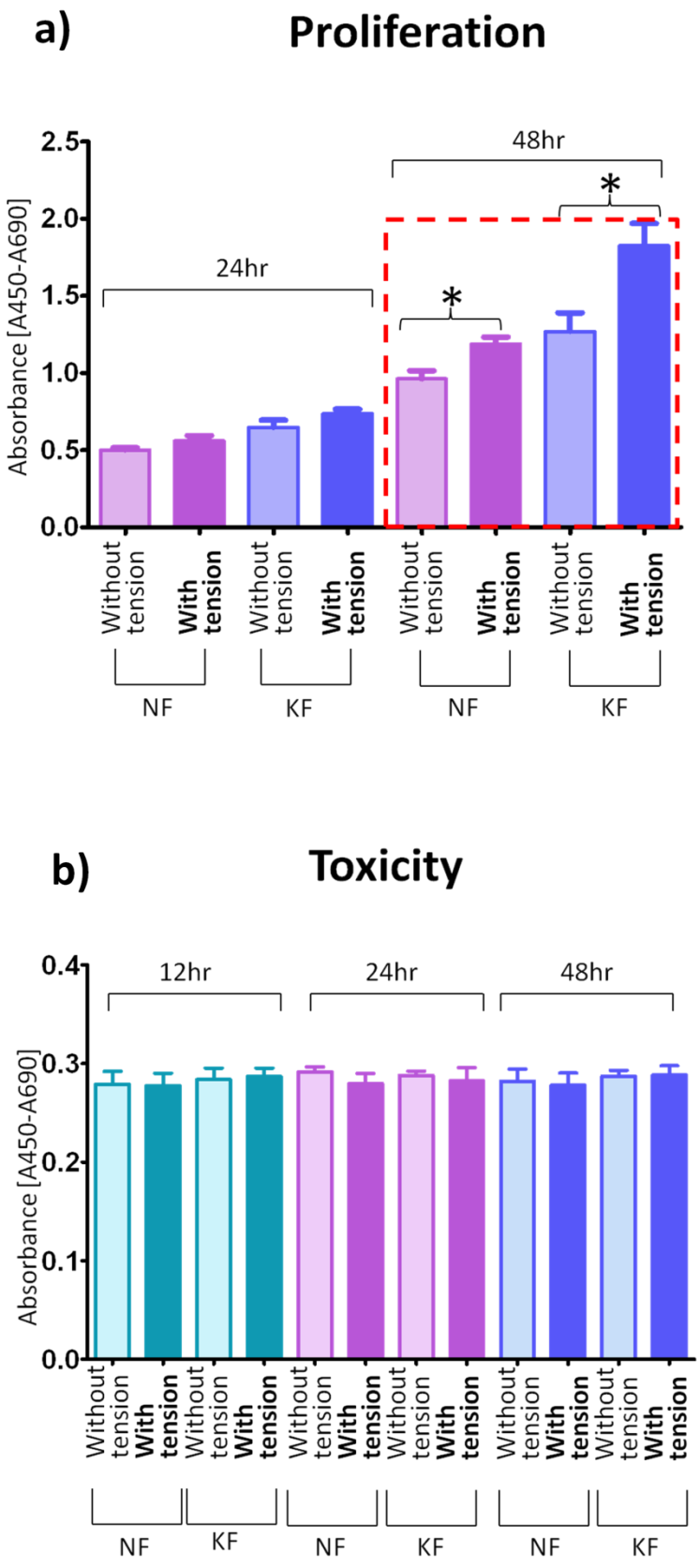


Figure 6.6 Primary fibroblasts viability and metabolic activity characterisation when subjected to mechanical tension in 3D collagen lattice (n=10).

6.3.3 Knock-down of Hsp27, PAI-2 and α2β1-Integrin significantly reduced cell proliferation in mechanical tensioned fibroblasts (*In-vitro* II-Stage III)

The three tension-related genes (Hsp27, PAI-2 and α2β1-Integrin) were knocked-down in order to evaluate their expression in NF and KF, and the effect of these on cellular behaviour when subjected to mechanical tension. Two transfection points were employed in order to optimize the treatment conditions in my 3D-model, transfected cells (n=10) were seeded in collagen gels and subjected to tension for 24 hr. The bar graphs a-c in Figure 6.7 represent the relative mRNA expression of the target genes after 24 and 48 hr siRNA transfection and 24hr tension treatment. Significant reduction in the expression of Hsp27, PAI-2 and α2β1-Integrin at mRNA level, *$p<0.0001$ (*), were shown in both tensioned and un-tensioned systems when compared to their respective controls (scramble transfected fibroblasts). Whereas the plots d-f in Figure 6.7 shows the reduction in the proliferation ratios observed in both cell types after transfection. * indicates a significant difference ($p<0.0001$) among all the samples compared. Moreover, the dashed boxes indicate the group of samples evidently more affected by the treatment. A significant reduction in the expression of the three target genes was observed at the mRNA level after 48 hr transfection and 24 hr of tension application in both cell types. There was down-regulation of the three target genes, which was even more evident in gels subjected to tension and populated with NF. Among the three genes, Hsp27 and α2β1-Integrin gene expression levels were most significantly ($p<0.0001$) down-regulated (Figure 6.5a-6.5c).

Proliferation and toxicity ratios were examined after 48 hr knock-down and 24 hr tension application (Figure 6.5d-6.5f). There was a reduced proliferation rate following knock-down and tension in both NF/KF. Tensioned NF showed lowest ($p<0.0001$) proliferation rates after 48 hr following Hsp27 or α2β1-Integrin knock-down (Figure 6.5D and 6.5F). Moreover, there was no significant effect on cell viability/metabolic activity among the samples (Figure 6.6).

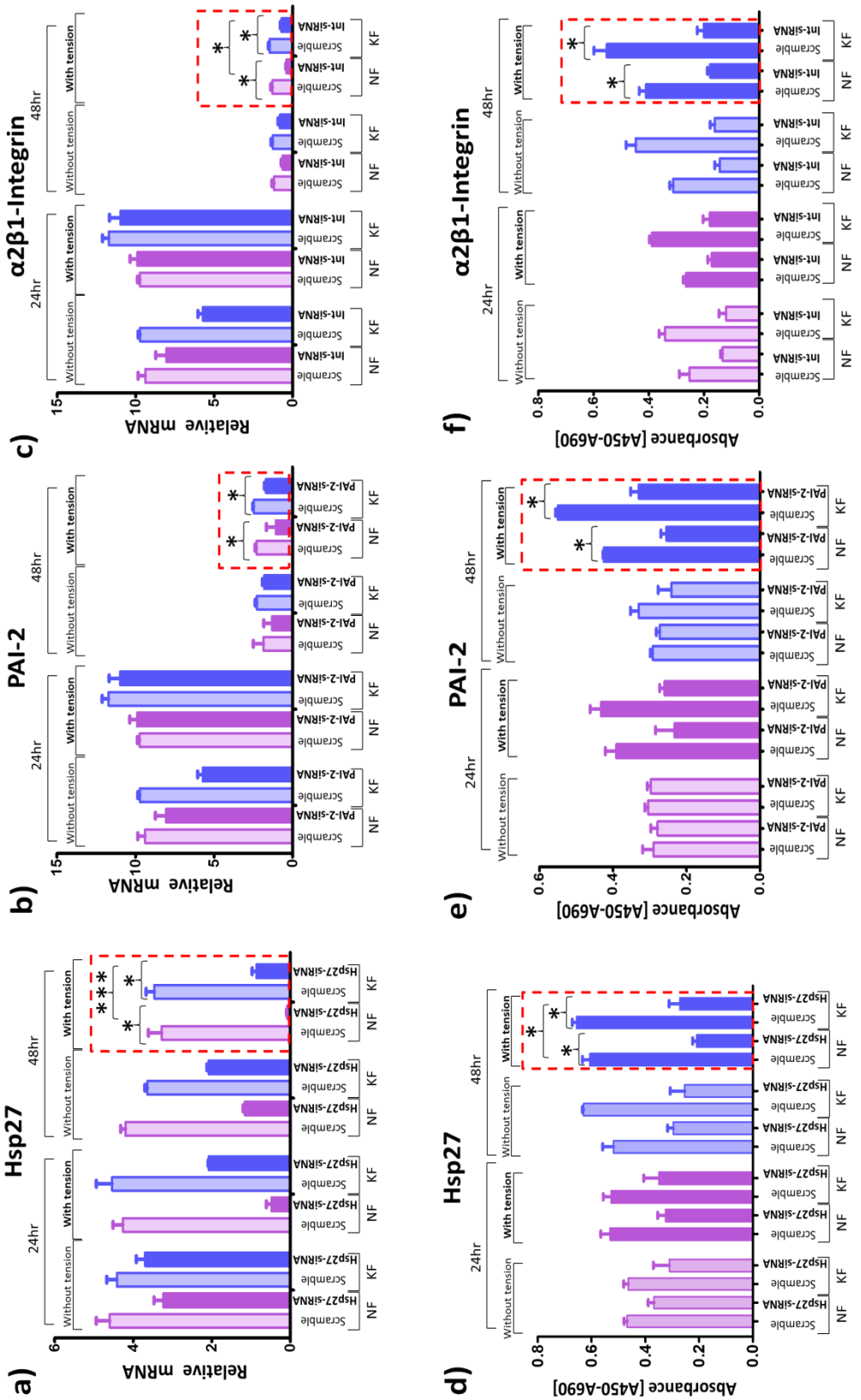


Figure 6.7 Knock-down of Hsp27, PAI-2 and α2β1-integrin reduced cell proliferation on mechanical tensioned fibroblasts (In-vitro II) (n=10).

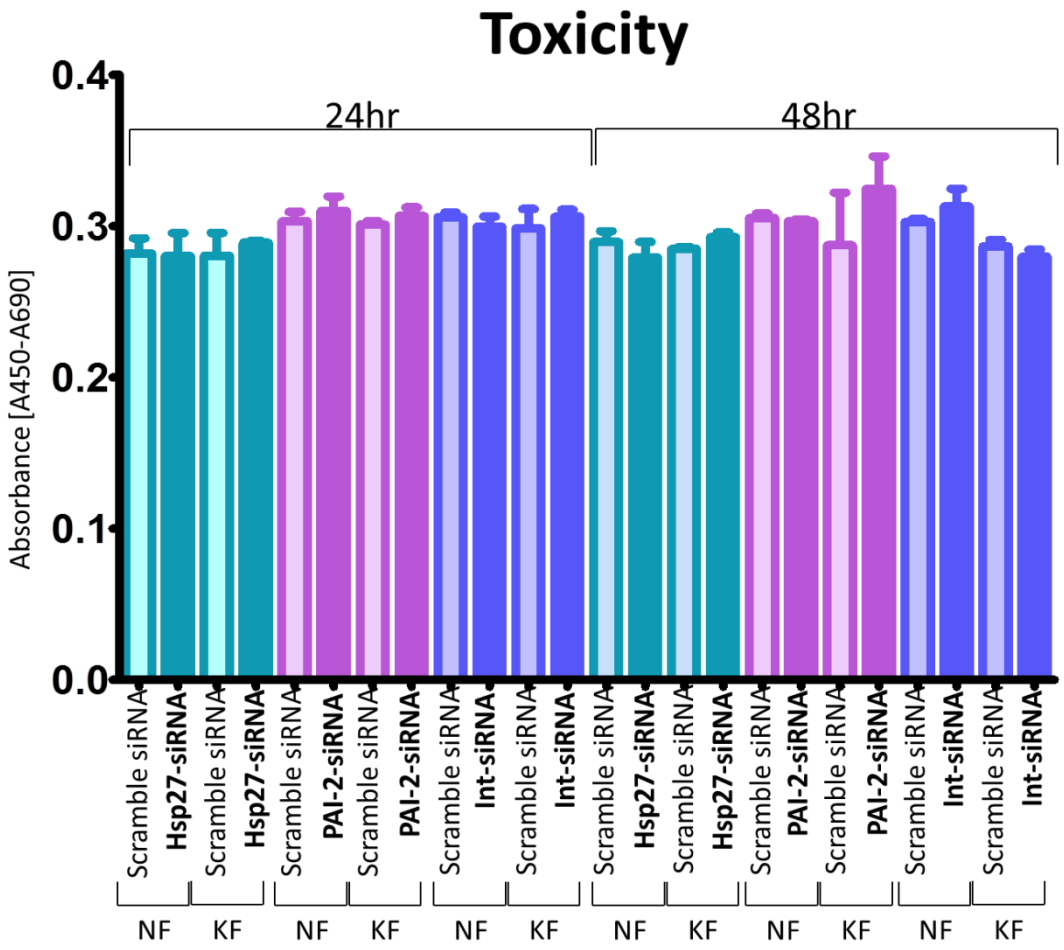


Figure 6.8 Cell viability/metabolic activity detection (n=10).
The bar graph represents the cell viability/metabolic activity following transfection and tension treatment. No statistical effect was detected among the samples.

6.3.4 Effect of down-regulation of tension related genes on mechanical tensioned fibroblast morphology

Fibroblasts morphology was investigated following 48 hr knock-down and 24 hr tension treatment, using phalloidin (red) and DAPI (blue). 3D pictures were taken using a high-end wide-field microscope, Figure 6.9. Interestingly, Hsp27 knock-down produced significant F-actin rearrangement. After transfection, cells were rounded, suggesting that Hsp27 may be closely related with cell spreading under mechanical tension conditions. The same effect was observed in NF but not in KF subjected to tension when α2β1-integrin was down-regulated, suggesting that α2β1-integrin in conjunction with others may be implicated in the mechano-transduction process. No effect on the cell morphology was observed after PAI-2 down-

regulation. No cell alignment along the tension line was observed after knockdown of each gene. Therefore, my results demonstrate that the knock-down of the genes was time and tension dependent; I also conclude that Hsp27 and α2β1-Integrin may be implicated in the regulation of actin rearrangement under tensioned conditions.

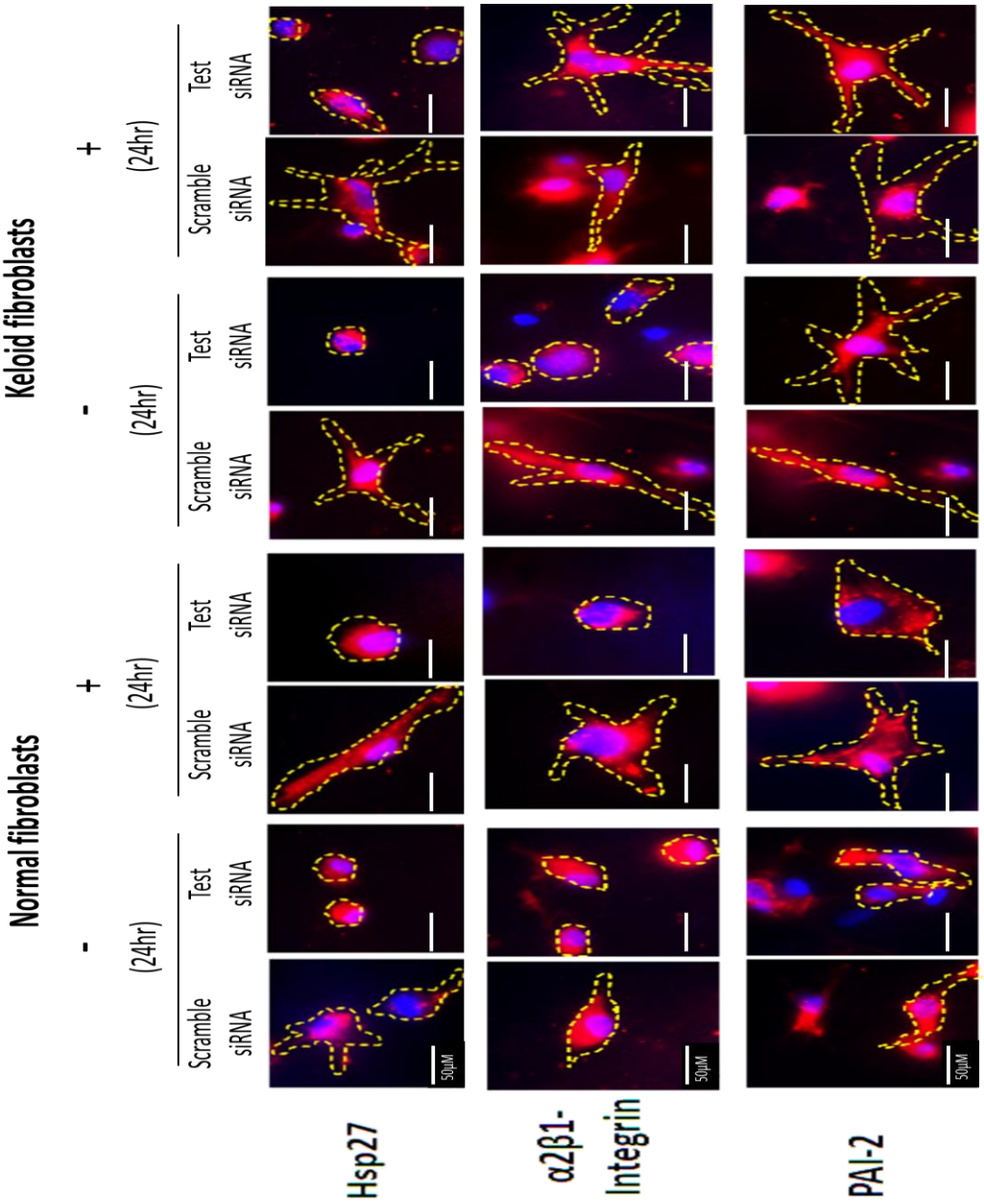


Figure 6.9 Effect of down-regulation of tension related genes on mechanical tensioned fibroblasts morphology (n=10).
(Without tension (-), with tension (+))

6.3.5 Modification of the expression of phenotypic markers after down-regulation of tension-related genes in tensioned 3D models

After 48 hr transfection and 24 hr tension application, I evaluated the effect of knock-down of the expression of my three target genes on the expression of a selection of important extracellular matrix, adhesion and spreading genes. My results showed that the transfection of Hsp27/siRNA and α2β1-integrin/siRNA after 48 hr led to significant ($p<0.001$) down-regulation of the expression of ECM and adhesion-related genes including; fibronectin, collagen I and CD44 and the spreading-related gene neuropilin-1 (Nrp1) in both cell types, however it is clearly more significant in NF left free to contract compared to KF (Figure 6.10 a-h). The same pattern of regulation was observed after PAI-2 knock-down on the expression of fibronectin, collagen I and CD44, but no significant effect on the expression of Nrp1 was observed in both cell types (Figure 6.8I-6.8L). I also evaluated the effect of knocking-down the expression of my target genes on the expression of the myofibroblast marker (α-SMA) in both NF and KF. There was a significant ($p<0.05$) down-regulation of α-SMA gene expression after knock-down of the 3 target genes separately. The down-regulation of α-SMA was more significant in NF compared to KF (Figure 6.10m-6.10o). Thus, Hsp27 and α2β1-integrin appear to be closely related with cell spreading regulation and adhesion under tension (Figure 6.8a-6.8b), whereas PAI-2 does not seem to be related to these phenomena, which support my previous findings regarding the actin cytoskeleton rearrangement (Figure 6.8c).

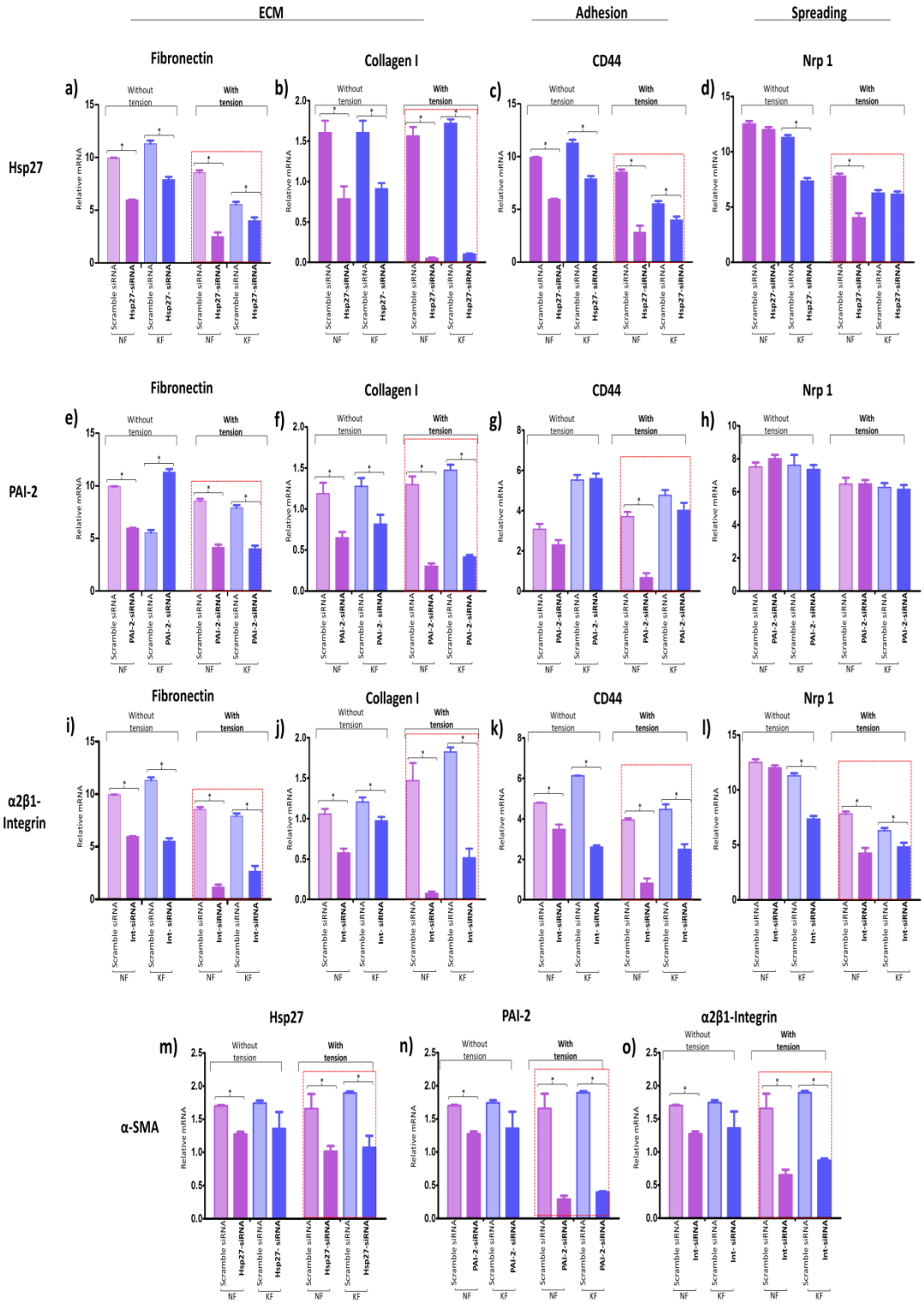


Figure 6.10 Modification of the expression of phenotypic markers after down-regulation of tension-related genes in tensioned 3D-models (n=10).

6.4 Discussion

Photogrammetry and *in-vitro* techniques were employed to create a novel 3D collagen lattice model to mimic tension experienced by NS and KF *in-vivo*. Photogrammetry technique was employed to quantify the amount of force that the skin in the sternum experienced during breathing without disturbing the natural arrangement of the collagen fibres, which frequently happens with methods such as tensile, indentation, torsion and suction testing. I then employed the data obtained *in-vivo* to calculate the size of the collagen lattice needed and to design a novel CFM to reproduce tension distribution throughout the cells populating the collagen gel. Thus, the amount of force applied to the cells in the model mimicked the *in-vivo* tension. I evaluated the newly created CFM by an *in-vitro* 3D-model using normal and keloid fibroblasts seeded in the collagen gels, to investigate the effects of mechanical tension on the expression of three target genes.

The choice of these tension-related genes was based on my previously published study, which showed up-regulation of three tension-related genes, Hsp27, PAI-2 and α2β1-integrin, in both keloid tissue and fibroblast obtained from the sternum[102]. My results demonstrated increased expression of tension-related genes, ECM synthesis, cell spreading and proliferation in cells under tension. Furthermore, I convincingly showed that the knock-down of the expression of these genes in this model influenced not just ECM synthesis but also, adhesion and spreading genes in keloid and normal fibroblasts. These findings provide preliminary evidence for the role of these 3 target genes and the process of mechano-regulation in keloid and normal fibroblasts.

Skin mechanical tension surrounding the connective tissue is considered a crucial regulator of the scarring process. A certain degree of mechanical loading is required to allow a cutaneous wound to contract[220]. Additionally, mechanical tension is thought to be of significance in keloid scar development[85]. Mechanical tension can play an active role during scar formation by affecting the expression of tension-related genes[17, 181]. Keloid morphology and behaviour are affected by its anatomical location and also influenced by the skin tension line distribution (Langer's lines) which is reflected in the development of site-specific morphology seen in KD[221, 2]. Therefore, keloids often (with the exception of the ear lobes) tend to develop in

those body regions constantly subject to skin tension[1, 35, 59, 181, 222]. Butterfly-shaped KS are frequently seen in the sternum, an anatomical body region constantly stretched during breathing[223].

Several molecular and signalling pathways have been shown to be affected by mechano-transduction, including α2β1-integrin where signalling following tension is propagated through integrin-protein complexes allowing the control of the integrin-ligand binding and cell adhesion[85, 128, 133, 218, 224, 225]. Additionally, signals sensed by the cells from the exterior results in cell cytoskeleton reorganisation, phenotypic modification and finally cellular differentiation[128, 224]. I showed here that the down-regulation of α2β1-integrin in FPCL subjected to tension conditions not only altered ECM synthesis but also actin cytoskeleton rearrangement and spreading, factors considered crucial in cell migration and wound healing.

It has also been proposed that mechanical forces activate the FAK-ERK-MCP-1 pathway promoting inflammation in fibrotic processes as well as fibroblast activation[17, 179, 193]. In addition, Hsp27 has been related to cell survival mediated by FAS pathway[98] and several reports suggest the participation of Hsp27 in the actin microfilament stabilisation via MAPK participation[137, 207, 226]. Furthermore, Hsp27 plays an important role in the modulation of endothelial and smooth cell contraction[179]. From the results obtained here, I corroborate the participation of Hsp27 in actin rearrangement under mechanical tension and also note its significant influence on certain ECM molecules - fibronectin and collagen I. Moreover, I suggest a further regulation of the adhesion-related gene CD44 and the spreading-related gene Nrp1 in both NF and KF. The expression of PAI-2 has been suggested to promote cellular protection against apoptosis[98,227]. PAI-2 also plays an active role in the regulation of ECM synthesis and degradation in highly stressed systems[228,138, 158, 159, 229]. Fibroblast differentiation into myofibroblasts is related to the expression of PAI-2 as well[158]. My results showed that collagen I and fibronectin synthesis depend on the expression of PAI-2, whereas no effect was observed on cell spreading, adhesion or actin myofilament arrangement after PAI-2 down-regulation.

I previously showed that α-SMA expression was dependent on the expression of α2β1-Integrin, Hsp27 and PAI-2 in steady state KF[102]. Interestingly, similar results on the expression of α-SMA were observed here following down-regulation of these three genes in my model. Taken together, the results here show that the modulation of the expression of α2β1-Integrin, Hsp27 and PAI-2 may regulate excessive ECM production when subjected to certain tension conditions. Furthermore, I conclude that mechanical tension influences the expression of tension-related proteins and demonstrates induction of ECM synthesis, cell spreading and proliferation in both normal and keloid fibroblasts (Figure 6.9). The following chapter provides an overall discussion of the results achieved during this research project as well as the future perspectives of this research line raised after the data analysis.

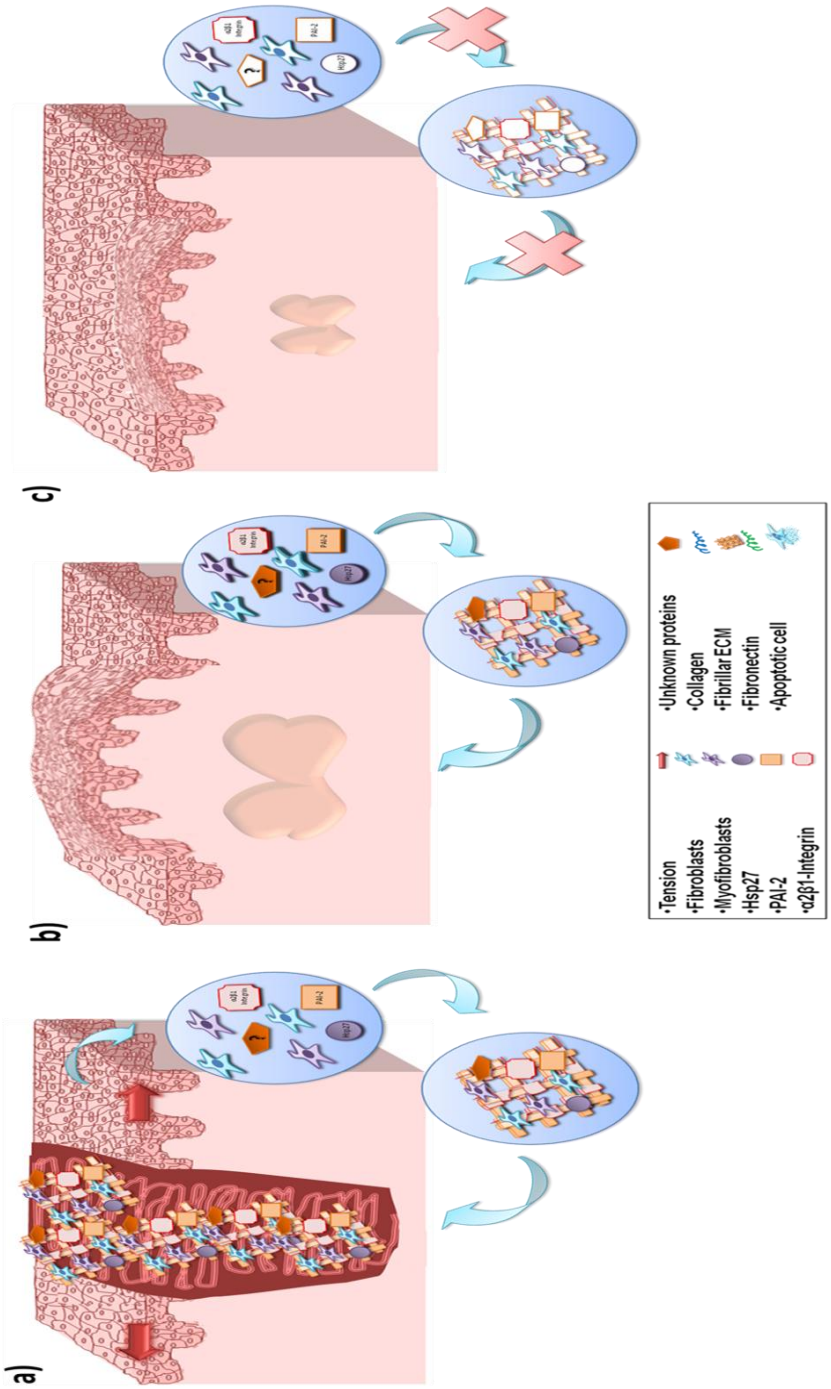


Figure 6.5 Proposed mechanism for the possible role of tension–related genes in keloid scarring.
a) Large amount of ECM components may increase the mechanical tension exhibited in the injured area promoting the establishment of a malignant cycle b) Excessive tension-related genes expression increases tension in the injured area resulting in keloids formation. c) The modulation of the expression of tension-related genes such as Hsp27, PAI-2 and α2β1-Integrin may regulate excessive ECM-production during the keloid formation.

Chapter VII: Discussion

7.1 Discussion and conclusions

To date, the mechanism that triggers the development and growth of KS remains unknown. There is a requirement to elucidate the factors involved in keloid pathogenesis in order to develop an effective treatment that counteracts the disease or perhaps to help in the prevention of such abnormal scar formation. Despite recent speculation, the role that mechanical tension in the skin and connective tissue surrounding a wound plays in the regulation of keloid scar development remains unknown. Mechanical loading is needed to allow wound contraction during normal scarring processes[220, 230,85]. Recent evidence suggests that mechanical tension can be considered as a causative factor that plays an active role during abnormal scarring processes, promoting modification in the expression of several genes often related to inflammation and collagen production during wound healing[181]. Furthermore, the heterogeneity often observed among the KS located along diverse anatomical positions has been correlated to the influence of the skin tension exerted by the distribution of the skin tension lines (Langer's lines) on that particular body region, which is reflected in site-specific morphology[221,2]. Moreover, clinical observation suggests that keloids tend to develop in those body regions constantly subjected to high tensional skin forces due to natural body movements[1, 35, 59, 181, 222]. In this respect, butterfly-like shaped keloids are frequently observed along the sternum, an anatomical region constantly stretched during breathing and subjected to high mechanical tension[231].

The aim of this research was to provide further evidence of the close relationship existing between skin tension and keloid development. To achieve this aim, an integrated approach was taken that combined the screening and characterisation of gene profiles to several tension-related markers with the development of a novel cell force monitor that enabled the application of skin equivalent tensional force to fibroblast-populated 3D-collagen gels.

During the course of this research, the identification of mechano-sensory genes related to keloid disease was investigated. A panel screening of 21 tension-related

markers selected from previously published microarrays and related to wound healing, fibrosis and keloid disease was performed between normal skin biopsies and fibroblasts and abnormal scar (keloid and hypertrophic) tissue and fibroblasts. From the first screening, convincing up-regulation of 5 tension-related genes, Hsp27, PAI-2 and α2β1-integrin, MMP-19 and CPRP in keloid tissue and fibroblasts suggested possible molecular targets involved in the tension regulated keloid formation mechanism. In addition, I found tension-related gene expression to be greatest in the sternum, a region known to be subject to high mechanical forces compared to other anatomical locations such as ear, scalp and pubis (**Chapter IV**). To further validate these findings, the screening of the 21 tension-related markers was assessed in keloid tissue and fibroblasts taken from the sternum. The results demonstrated over expression of 3 (Hsp27, PAI-2 and α2β1-Integrin) of the 21 tension-related markers in keloid tissue and fibroblasts from this location. Following their identification as possible targets, evaluation of the effect of Hsp27, PAI-2 and α2β1-Integrin in terms of regulation of ECM production and deposition was required. In this context, the knock-down of Hsp27, PAI-2 and α2β1-Integrin by RNA interference was found to not only attenuate the expression of mRNA and proteins levels but also certain other ECM molecules, such as Collagen I (**Chapter V**).

It was considered necessary to confirm the obtained results by evaluating the effect of skin tension in a 3D-model, specifically fibroblast-populated collagen lattices. To achieve this aim it was necessary to develop a novel cell force monitor (CFM) capable of mimicking *in-vivo* tension in an *in-vitro* model. The CFM was created based on the measurement of skin tension in the sternum *in-vivo* using photogrammetry. This allowed a more accurate estimation, and subsequent reproduction, of the force that fibroblasts exert in that body region under natural movement conditions. Photogrammetry is a technique based on photographic images employed to determine the study sample geometric properties, this technique provided the opportunity to quantify the amount of force that the skin in the sternum experienced during breathing without disturbing the natural rearrangement of the collagen fibres as frequently happens with methods such as tensile, indentation,

torsion and suction tests, amongst others[46, 62, 127, 232]. From the data obtained *in-vivo*, the size of the collagen lattice to be used and the design of the new CFM, which was able to reproduce a uniform tension distribution throughout the cells populating the collagen gel, were calculated. After the validation of the newly created CFM by an *in-vitro* 3D-model using normal and keloid fibroblasts seeded in the collagen gels, the investigation of the effects of mechanical tension on the expression of the previously identified target genes (Hsp27, PAI-2 and α2β1-Integrin) was performed. It was demonstrated that there was an induction of ECM synthesis, cell spreading and proliferation using FPCL. Furthermore, the results showed that the knock-down of the expression of the tension-related markers Hsp27, PAI-2 and α2β1-integrin on FPCL subjected to tension influenced not just the ECM synthesis but also adhesion and spreading genes in keloid and normal fibroblasts. My results provided strong evidence of the relationship between the 3 target genes and the mechano-regulation in keloid and normal fibroblast (**Chapter VI**).

Several pathways have been related to mechano-transduction, among those, α2β1-integrin has been reported to play a bilateral role conducting the signals inside-out to the cells. Meanwhile, the internal signalling is propagated through integrin-protein complexes allowing the control of the integrin-ligand binding and cell adhesion[224]. The second mechanism of regulation is termed 'outside-in signalling', where the signals sensed by cells from the exterior result in cell cytoskeleton reorganization, phenotypic modification and finally cellular differentiation[224]. The results obtained here show that the down-regulation of α2β1-integrin in FPCL subjected to tension conditions not only affect ECM synthesis but also actin cytoskeleton rearrangement and spreading. These are crucial factors to consider in cell migration and wound healing.

It has also been proposed that mechanical forces activate the FAK-ERK-MCP-1 pathway promoting inflammation on fibrotic processes as well as fibroblast activation[179, 193]. Hsp27 has been related to cell survival mediated by FAS pathway[98] and finally several reports suggest the participation of Hsp27 in actin microfilament

stabilisation via MAPK participation[137, 207, 226]. Furthermore, Hsp27 plays an important role in the modulation of endothelial and smooth cell contraction[179]. The results obtained from this portion of the research corroborated the participation of Hsp27 in the rearrangement of actin under mechanical tension and its role in significantly influencing other ECM molecules, specifically fibronectin and collagen I. Moreover, it was suggested that a further regulation of the adhesion related genes CD44 and Nrp1 was present in both normal and keloid fibroblasts. The regulation observed among the previously mentioned genes was more significantly appreciated in normal fibroblasts.

The expression of PAI-2 has been suggested to promote cell protection against apoptosis[98,227]. PAI-2 also plays an active role in the regulation of ECM synthesis and degradation in highly stressed systems[228,138, 158, 159, 229]. Additionally, fibroblast differentiation into myofibroblasts is related to the expression of PAI-2 [233]. My results showed that collagen I and fibronectin synthesis was dependent on the expression of PAI-2, whereas no effect on the expression of spreading, adhesion or actin myofilament arrangement was observed after PAI-2 down-regulation.

Finally, the results obtained in this research showed that the expression of α-SMA dependents of the expression of the three target proteins, α2β1-Integrin, Hsp27 and PAI-2 in steady state keloid fibroblasts[102]. The same regulation of the expression of α-SMA was observed after the down-regulation of genes. Taking together the results obtained within this thesis, it was convincingly shown that skin tension alters keloid fibroblast behaviour, morphology, mechano-responsive gene expression and extracellular matrix production. In this study it was demonstrated that regulation of the expression of the three tension-related markers, Hsp27, PAI-2 and α2β1-Integrin, in keloid samples by RNAi technology not only attenuated the expression of mRNA and protein levels but also certain other ECM molecules.

Furthermore, it is concluded that mechanical tension influences the expression of tension-related proteins and demonstrates ECM synthesis induction, cell spreading and proliferation in both normal and keloid fibroblasts. The findings from this research offer further insight into understanding KD pathobiology and the potential options for future treatment design. It is envisaged that RNA interference could be targeted towards tension-related genes found in keloid tissue.

7.2 Strength and weakness of this research

The strength of the research presented in this thesis is evident in the combination of *in-vivo* and *in-vitro* techniques resulting in the development of a novel 3D-model able to mimic *in-vivo* tension application. This resulted in *in-vitro* cellular response induction. Moreover, high reproducibility was obtained during the experiments under similar conditions. Other strengths of this study lie in the employment of tissue and primary fibroblast samples obtained from the same patient, and notably, from the same anatomical location. This provided more reliable results in the comparison of mRNA and protein levels between tissue and cells.

A weakness of this research was the use of small sample numbers related to the anatomical locations investigated; this was an inevitable problem due to the availability of samples from that specific anatomical site (sternum) and ethical restrictions in obtaining normal skin from keloid patients. The comparison of samples obtained from KS from different anatomical locations may help to further understand the keloid pathogenesis and heterogeneity.

A further weakness of this study lays in the use of only one, constant force application in order to produce tension in the system. Additional analysis of the effect of different force magnitudes should provide further knowledge of the role that skin tension plays in the keloid development mechanism.

7.3 Future work

The current work has exposed a potential mechano-regulatory mechanism existing in KD and offers further insight into the understanding of keloid pathobiology and identifies potential options for future treatment. However, further questions have been raised regarding the regulation of the mechanism proposed in Figure 7.1 and the existence of a more complex signalling pathway that could be activated in KD. With this in mind, future work could contribute to providing a more complete scenario of the molecular pathways triggered by the effect of skin tension and should provide more reliable candidates to be used as target molecules in the design of drugs and treatments. Moreover, further investigation is required into the effect of the use of force of a higher magnitude to apply tension to the 3D fibroblast populated collagen lattices to fully understand the fibroblast behaviour under tension conditions. In this research, the goal was to mimic the minimum amount of force exerted in the sternum while breathing, in order to create a normal environment for the fibroblasts. Other questions raised from this research refer to the effects that skin tension may have in a more conservative model such as an organotypic culture (Figure 7.2).

The first aim of future work would be to further identify all tension-related signalling pathways relevant to keloid disease and establish the correlation between them by the use of innovative bioinformatics techniques such as the novel developed algorithm "Signalling and Dynamic Regulatory Events Miner (SDREM)"[234]. To further complete this topic it is necessary to identify the key molecule(s) responsible for the regulation of signalling transduction that exists within keloid pathogenesis. This should be followed by the validation of the proposed findings by the use of *in-vitro* techniques such as qRT-PCR and RNA interference, firstly in samples taken from the sternum. Further validation can be conducted in samples obtained from the rest of the diverse anatomical locations where keloids commonly develop.

The second potential aim would be to further understand keloid fibroblast behaviour (differentiation, spreading, migration, ECM production, ECM deposition and phenotypic alterations) when subjected to high tensioned systems such as the

maximum strain (0.25N) performed in the skin of the sternum at the point of maximal inspiration during a normal respiration cycle. To achieve this aim, the use of high tensioned 3D-fibroblasts populated collagen lattices and the combination of microscopy, cytokine arrays, mRNA and protein analysis and RNA interference is envisaged.

Finally, to further complement this research, I propose the development of an organotypic culture model that mimics normal skin characteristics and allows the application of tension. Once achieved, this would allow the characterisation of the effects of tension at a genetic and protein level by the combination of RNA and protein microarrays, qRT-PCR and protein analyses and RNA interference.

In order to provide a better understanding of keloid disease, a more complete statistical analysis of the data should be implemented, such as a multiple regression analysis that allows a better correlation of the multiple variables comprehended within the sample collection. This will provide further evidence of the contribution of variables such as gender, age of the patient, ethnicity, age of the scar and anatomical location during the keloid development and its relationship with skin tension. The sample number for further studies will depend on the homogeneity of the samples, in other words, a greater amount of samples should be included in the study when the higher heterogeneity is observed among them.

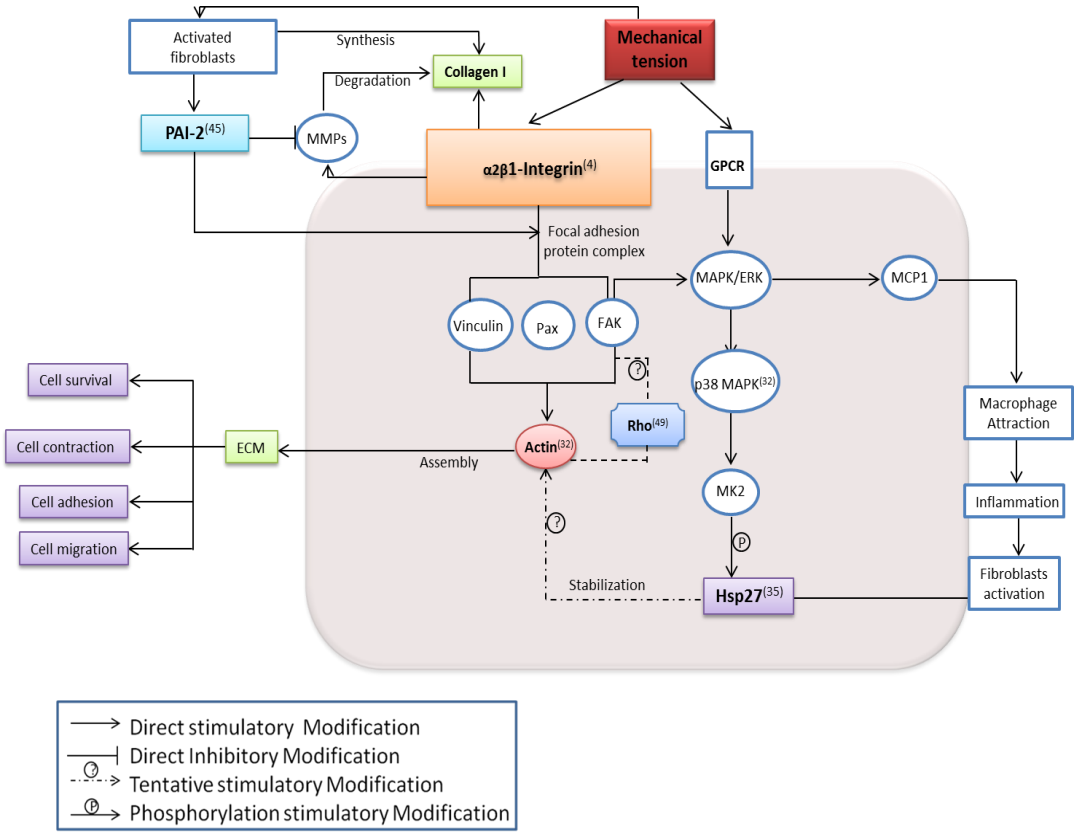


Figure 7.1 Keloid mechano-signalling proposed signalling pathways
Mechanical tension induces α2β1-integrin activation, which serves as a transmembrane link between the extracellular matrix and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts. The mechanotransduction signal also activates Hsp27 by means of p38 mitogen-activated protein kinase. PAI-2 promotes extracellular matrix accumulation by negative regulation of matrix metalloproteinases. PAI-2 is also linked with the regulation of cell migration mediated by integrins.

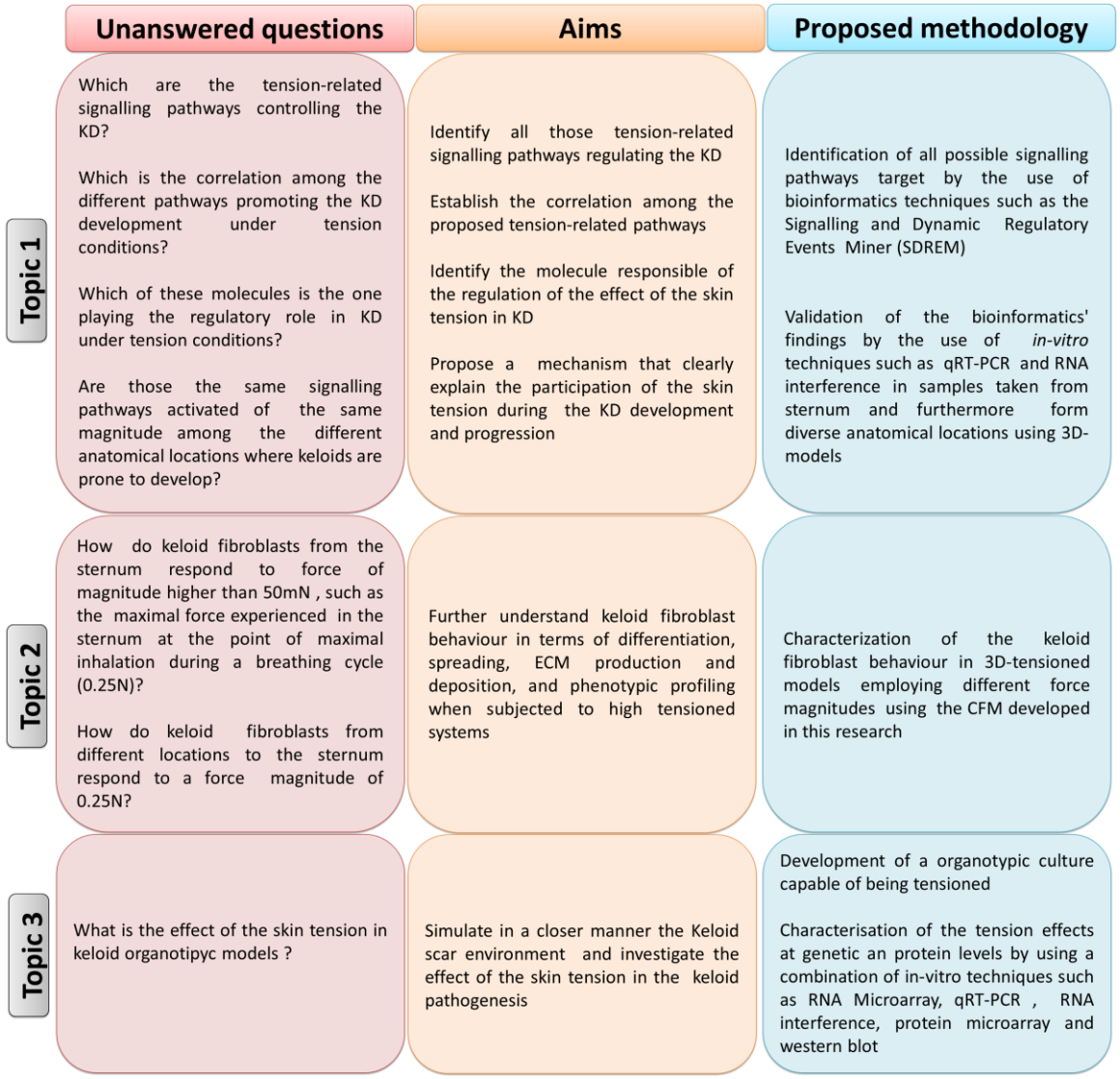


Figure 7.2 Proposed future work
Summary of the questions arisen from the thesis and proposed techniques to achieve the proposed aims

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Appendix I

Table A.1 Primary antibodies

Antibody	Raised Species	Isotype	Clone	Dilution	Product Code	Source
α-Tubulin	Mouse monoclonal	IgG1k	DM1A	1:500	Ab7291	Abcam
β- actin	Rabbit polyclonal	IgG	-	1:200	Ab112053	Abcam
α2β1 Integrin	Mouse monoclonal	IgG1	16B4	1:500	Ab30483	Abcam
Collagen I	Rabbit polyclonal	IgG	-	1:500	Ab59435	Abcam
Hsp 27	Mouse monoclonal	IgG1	-	1:500	mAb 2402	Cell signaling technology
PAI-2	Rabbit polyclonal	IgG	-	1:500	Ab47742	Abcam

Table A.2 Secondary antibodies

Antibody Name	Raised Species	Isotype	Active against	Dilution	Product Code	Company
Donkey-anti mouse IRDye 680CW®	Donkey	IgG	Mouse	1:800	926-32222	Li-Core
Donkey-anti rabbit IRDye 680CW®	Donkey	IgG	Rabbit	1:800	926-32223	Li-Core
Donkey-anti mouse IRDye 800CW®	Donkey	IgG	Mouse	1:800	926-32212	Li-Core
Donkey-anti rabbit IRDye 800CW®	Donkey	IgG	Rabbit	1:800	926-32213	Li-Core
Alexa-Fluor®488 Anti-rabbit (H+L)	Goat	IgG	Rabbit	1:250	SKU# A-11034	Invitrogen
FITC anti-mouse	Goat	IgG	Mouse	1:250	115-097-003	Jackson Immuno-diagnostics

Table A.3 Patient demographic data

Study ID	Gender	Ethnicity	Age (Y)	Site	Age of Scar (Y)
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 A.4 qRT-PCR primers list

Gene/Primer	Gene ID	Sequence 5' to 3'	Primer Position	Amplicon Size (bp)
α-SMA-L	nm_001613.1	ctgttcagccatccttcat	834 - 853	70
α-SMA-R	nm_001613.1	tcatgatgctgttgtaggtggt	882 - 903	70
α2β1 integrin-L	nm_002203.3	gcaggacagaaatcacagttca	1427 - 1448	78
α2β1 integrin-R	nm_002203.3	gcaacaaagtgagtgctttctc	1483 - 1504	78
CGRP-L	nm_001033953.2	cccaccaaggtgtatgcaa	668-686	60
CGRP-R	nm_001033953.2	cccataaggaaacccagagc	708-727	60
Collagen I-L	nm_000088.3	gggattccctggacctaaag	1866 - 1885	63
Collagen I-R	nm_000088.3	ggaacacctcgctctcca	1911 - 1928	63
Cyr61-L	nm_001554.4	ccagtgtacagcagcctgaa	1047 - 1066	110
Cyr61-R	nm_001554.4	ggccggtatttcttcacactc	1136 - 1156	110
Fibronectin-L	nm_212482.1	gccactggagtctttaccaca	3498 - 3518	60
Fibronectin-R	nm_212482.1	cctcggtgttgtaaggtgga	3539 - 3558	60
HAPLN1-L	nm_001884.3	tgctcagcatgggttatgc	1072 - 1090	63
HAPLN1-R	nm_001884.3	agggcgtctctgagtagcag	1115 - 1134	63
HAPLN2-L	nm_021817.2	cgactttatgaccaagagctga	512 - 533	126
HAPLN2-R	nm_021817.2	gccaacaatataagcagctgtg	616 - 637	126
Hsp27-L	nm_001540.3	tccctggatgtcaaccactt	447 - 466	111
Hsp27-R	nm_001540.3	gatgtagccatgctcgtcct	538 - 557	111
Hsp47-L	nm_001235.3	gcgggctaagagtagaatcg	122 - 141	110
Hsp47-R	nm_001235.3	atggccaggaagtggtttg	213 - 231	110
Hsp60-L	nm_199440.1	ggtcttcaggttgtggcagt	997 - 1016	119
Hsp60-R	nm_199440.1	ttcagggtcaatccctcttc	1096 - 1115	119
Hsp70-L	nm_005345.5	cagcagacaccagcagaaaa	1887 - 1906	66
Hsp70-R	nm_005345.5	cttggatccagcttgagagg	1933 - 1952	66
Hsp90-L	nm_005348.3	gggcaacacctctacaagga	661 - 680	76
Hsp90-R	nm_005348.3	cttgggtctgggtttcctc	718 - 736	76
MCP-1-L	nm_002982.3	tcaaactgaagctcgcactc	43 - 62	126
MCP-1-R	nm_002982.3	gtgactggggcattgattg	153 - 171	129
MCP-3-L	nm_006273.2	gcacttctgtgtctgctgct	86 - 105	129
MMP-3-R	nm_002422.3	ctcttgggtatccagctcgt	1169 - 1188	81
MMP-13-L	nm_002427.3	cctggacaagtagttccaaagg	645 - 666	128
MMP-13-R	nm_002427.3	gccggtgtaggtgtagatagga	751 - 772	128
MMP-19-L	nm_002429.2	atgccagacccttgcagtag	974 - 993	76
MMP-19-R	nm_002429.2	ccccctgaaagcataggtc	1030 - 1049	76
PAI-2-L	nm_002575.2	catggagcatctcgtccac	428 - 446	109
PAI-2-R	nm_002575.2	actgcattggctcccactt	518 - 536	109
RPL32-L	nm_000994.3	gaagttcctggtccacaacg	319 - 338	76
RPL32-R	nm_000994.3	gagcgatctcggcacagta	377 – 395	76
SP-L	nm_003182.2	gtttgaaggtgtgggttggt	399-418	63
SP-R	nm_003182.2	accctcttctccctgaatcc	442-461	63
Tenascin-L	nm_019105.6	agggagacttccctgtcctg	103 - 122	119
Tenascin-R	nm_019105.6	catactgggctggcatcatt	202 - 221	119
TGF-β1-L	nm_000660.3	gcagcacgtggagctgta	1362 - 1379	64
TGF-β1-R	nm_000660.3	cagccggttgctgaggta	1408 - 1425	64
TGF-β2-L	nm_003238.1	ccaaagggtacaatgccaac	1194 - 1213	114
TGF-β2-R	nm_003238.1	cagatgcttctggatttatggtatt	1283 - 1307	

TGF-β3-L	nm_003239.2	gggctttggacaccaattac	1755 - 1774	125
TGF-β3-R	nm_003239.2	ggcatagtagcccttaggttca	1858 - 1879	125

Table A.5 Demographic data of keloid tissue samples

Study ID	Gender	Ethnicity	Age (Y)	Site	Age of Scar (Y)	Cause
KD 1	Female	Asian	27	Chest	1	Spot
KD 2	Female	White	18	Chest	15	Chicken pox
KD 3	Male	Black	45	Chest	1	Surgery
KD 4	Female	Black	20	Chest	9	Surgery
KD 5	Male	Asian	74	Chest	11	Surgery
KD 6	Male	Black	42	Chest	3	Industrial accident
KD 7	Female	White	29	Chest	10	Spot
KD 8	Male	Black	36	Chest	5	Surgery
KD 9	Female	Black	35	Chest	9	Spontaneous
KD 10	Female	White	43	Chest	29	Sternal spot

ID, identification; KD, keloid disease.

Table A.6 Demographic data of normal skin tissue samples

Study ID	Gender	Ethnicity	Age (Y)	Site	Sample Collected
NS 1	Female	White	47	Chest	Normal Skin
NS 2	Female	White	39	Chest	Normal Skin
NS 3	Female	White	22	Chest	Normal Skin
NS 4	Male	White	31	Chest	Normal Skin

ID, identification; NS, normal skin.

Table A.7 Demographic data

Study ID	Gender	Ethnicity	Age (Y)	Site	Age of Scar (Y)	Cause
KD 1	Female	Asian	27	Sternum	1	Spot
KD 2	Female	White	18	Sternum	15	Chicken pox
KD 3	Male	Black	45	Sternum	1	Surgery
KD 4	Female	Black	20	Sternum	9	Surgery
KD 5	Male	Asian	74	Sternum	11	Surgery
KD 6	Male	Black	42	Sternum	3	Industrial accident
KD 7	Female	White	29	Sternum	10	Spot
KD 8	Male	Black	36	Sternum	5	Surgery
KD 9	Female	Black	35	Sternum	9	Spontaneous
KD 10	Female	White	43	Sternum	29	Sternal - spot
NS 1	Female	White	47	Sternum	-	Surgery
NS 2	Female	White	39	Sternum	-	Surgery
NS 3	Female	White	22	Sternum	-	Surgery
NS 4	Male	White	31	Sternum	-	Surgery
NS 5	Male	Black	45	Sternum	-	Surgery
NS 6	Male	Black	31	Sternum	-	Surgery
NS 7	Male	Black	25	Sternum	-	Surgery
NS 8	Female	Black	29	Sternum	-	Surgery
NS 9	Male	Asian	33	Sternum	-	Surgery
NS 10	Male	White	42	Sternum	-	Surgery

Table A.8 Primers list

Gene/Primer	Gene ID	Sequence 5' to 3'	Primer Position	Amplicon Size (bp)
α-SMA-L	nm_001613.1	ctgttcagccatccttcat	834 - 853	70
α-SMA-R	nm_001613.1	tcatgatgctgttgtaggtggt	882 - 903	70
α2β1 integrin-L	nm_002203.3	gcaggacagaaatcacagttca	1427 - 1448	78
α2β1 integrin-R	nm_002203.3	gcaacaaagtgagtgctttctc	1483 - 1504	78
CD44-L	nm_001389.1	gacaccatggacaagttttgg	110 - 130	92
CD44-R	nm_001389.1	cggcaggttatattcaaatcg	181 - 201	92
Collagen I-L	nm_000088.3	gggattccctggacctaaag	1866 - 1885	63
Collagen I-R	nm_000088.3	ggaacacctcgctctcca	1911 - 1928	63
Fibronectin-L	nm_212482.1	gccactggagtctttaccaca	3498 - 3518	60
Fibronectin-R	nm_212482.1	cctcggtgttgtaaggtgga	3539 - 3558	60
Nrp1-L	nm_244972.1	ctggaagaggaacaagtgtgg	111 - 131	73
Nrp1-R	nm_244972.1	gtctcaagtcgcctgcatc	165 - 183	73
Hsp27-L	nm_001540.3	tccctggatgtcaaccactt	447 - 466	111
Hsp27-R	nm_001540.3	gatgtagccatgctcgtcct	538 - 557	111
PAI-2-L	nm_002575.2	catggagcatctcgtccac	428 - 446	109

PAI-2-R	nm_002575.2	actgcattggctcccactt	518 - 536	109
RPL32-L	nm_000994.3	gaagttcctggtccacaacg	319 - 338	76
RPL32-R	nm_000994.3	gagcgatctcggcacagta	377 – 395	76

Table A.9 Genes common up-regulated in hypertrophic scars in microarray studies

Gene Symbol	Gene Name	Different Expression in Study
COL5A2	Collagen, type V, alpha 2	Wu 2003, Tsou 2000
COL6A2	Collagen, type VI, alpha 2	Paddock 2003, Tsou 2000
HTRA1	HtrA serine peptidase 1	Wu 2003, Paddock 2003
PTN	Pleiotrophin	Wu 2003, Paddock 2003, Tsou 2000
QPRT	Quinolinate phosphoribosyltransferase	Dasu 2004
PAI-2	Serine (or cysteine) proteinase inhibitor, clade B	Dasu 2004
COL13A1	Collagen, type XIII α-1	Dasu 2004
uPA	Plasminogen activator, urokinase	Dasu 2004
NmU	Neuromedin	Dasu 2004
LGALS3	Lectin, galactose binding protein 3	Dasu 2004
NAGPA	N-acetylglucosamine-I-phosphodiester-α-N-acetylglucosaminidase	Dasu 2004
SECTM1	Secreted and transmembrane I	Dasu 2004
BDKRB2	Bradykinin receptor B2	Dasu 2004
HMOX1	HEME oxygenase (decycling) I	Dasu 2004
IER3	Immediate early response 3	Dasu 2004
FAS	Fatty acid synthase	Dasu 2004
ACAN	Aggrecan	Paddock 2003
COL1A1	Collagen type I alpha 1	Paddock 2003
COMP	Cartilage Oligomeric matrix protein	Paddock 2003
TSP-4	Thrombospondin 4	Paddock 2003
MEGF6	Epidermal growth factor-like-domin, multiple 3	Wu 2003
COL11A1	Collagen , Type XI, alpha 1	Wu 2003
A2AAR	Adenosine A2a receptor	Wu 2003
COL6A2	Collagen, type VI, alpha 2	Wu 2003

Table A.10 Genes common up-regulated in keloid scars in microarray studies

Gene Symbol	Gene Name	Different Expression in Study
ACAN	Aggrecan	Seifert 2008, Naitoh 2005
ANXA1	Annexin A1	Seifert 2008, Hu 2006
C5ORF13	Chromosome 5 open reading frame	Smith 2008, Hu 2006, Naitoh 2005
COL1A1	Collagen, type I, alpha	Seifert 2008, Naitoh 2005, Chen 2003
COL4A1	Collagen, type IV, alpha	Seifert 2008, Satish 2006, Naitoh 2005, Na 2004
COL5A2	Collagen, type V, alpha	Seifert 2008, Chen 2003
COL11A1	Collagen, type XI, alpha	Hu 2006, Naitoh 2005, Chen 2003
DCN	Decorin	Hu 2006, Chen 2003
FAP	Fibroblast activation protein, alpha	Seifert 2008, Naitoh 2005
FN1	Fibronectin	Seifert 2008, Satish 2006, Naitoh 2005, Chen 2003
IGF2	Insulin-like growth factor 2 (somatomedin A)	Smith 2008, Hu 2006,
IGFBP7	Insulin-like growth factor binding protein	Smith 2008, Seifert 2008, Hu 2006
JAG1	Jagged 1 (Alagille syndrome)	Smith 2008, Hu 2006
NGF	Nerve growth factor (beta polypeptide)	Smith 2008, Chen 2003
OGN	Osteoglycin	Smith 2008, Naitoh 2005
P4HA1	Prolyl 4-hydroxylase, alpha polypeptide I	Seifert 2008, Hu 2006
SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	Hu 2006, Na 2004
TGFB1	TGF, beta 1	Seifert 2008, Chen 2003
VCAN	Versican	Seifert 2008, Naitoh 2005, Chen 2003

Table A.11 Genes common up-regulated in keloid and hypertrophic scars in microarray studies

Gene Symbol	Gene Name	Different Expression in Study
A2M	Alpha-2-macroglobulin	Huang 2013, Shih 2010
ACAN	Aggrecan	Huang 2013, Shih 2010
BMP6	Bone morphogenetic protein 6	Huang 2013
C5ORF13	Chromosome 5 open reading frame	Huang 2013, Shih 2010
CALD1	Caldesmon 1	Huang 2013
CALU	Calumenin	Huang 2013
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	Huang 2013
COL10A1	Collagen, type X, alpha 1	Huang 2013
COL1A1	Collagen, type I, alpha 1	Huang 2013, Shih 2010
COL1A2	Collagen, type I, alpha 2	Huang 2013, Shih 2010
COL3A1	Collagen, type III, alpha 1	Huang 2013
COL4A5	Collagen, type IV, alpha 5	Huang 2013
COL5A1	Collagen, type V, alpha 1	Huang 2013, Shih 2010
COL5A2	Collagen, type V, alpha 2	Huang 2013, Shih 2010
COL6A1	Collagen, type VI, alpha 1	
COMP	Cartilage oligomeric matrix protein	Huang 2013
CTGF	Connective tissue growth factor	Huang 2013
DCN	Decorin	Huang 2013
FAM3C	Family with sequence similarity 3, member C	Huang 2013
FN1	Fibronectin 1	Huang 2013, Shih 2010
HIF1A	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	Huang 2013, Shih 2010
HTRA1	Htra serine peptidase 1	Huang 2013
IFI16	Interferon, gamma-inducible protein 16	Huang 2013
IFITM2	Interferon induced transmembrane protein 2 (1-8D)	Huang 2013
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	Huang 2013
INHBA	Inhibin, beta A	Huang 2013
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	Huang 2013
ITGB5	Integrin, beta 5	Huang 2013
LUM	Lumican	Huang 2013
MARCKS	Myristoylated alanine-rich protein kinase C substrate	Huang 2013
MGST3	Microsomal glutathione S-transferase 3	Huang 2013

MMP14	Matrix metallopeptidase 14 (membrane-inserted)	Huang 2013
MMP2	Matrix metallopeptidase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)	Huang 2013
POSTN	Periostin, osteoblast specific factor	Huang 2013, Shih 2010
SEPTIN7	Septin 7	Huang 2013
SFRP2	Secreted frizzled-related protein 2	Huang 2013
SOX11	SRY (sex-determining region Y)-box 11	Huang 2013
THBS4	Thrombospondin 4	Huang 2013
TIMP1	TIMP metallopeptidase inhibitor 1	Huang 2013
TMSB10	Thymosin beta 10	Huang 2013
VCAN	Versican	Huang 2013, Shih 2010
VEGFA	Vascular endothelial growth factor A	Huang 2013
EGFR	Epidermal grwoth factor receptor	Shih 2010
HDGF	Hepatoma-derived growth factor	Shih 2010
SERPINF1	Serpin peptidase inhibitor, clade F	Shih 2010
POSTN	Periostin, osteoblast specific factor	Shih 2010
KRT19	Keratin 19	Shih 2010

Appendix II

UNIVERSITY OF MANCHESTER

COMMITTEE ON THE ETHICS OF RESEARCH ON HUMAN BEINGS

Application form for approval of a research project

This form should be completed by the Chief Investigator(s), after reading the guidance notes.

1. Title of the research

Full title: The effect of skin tension on the formation of keloid scars.

2. Chief Investigator

Title: **Miss**
Forename/Initials: **Edna**
Surname: **Suarez Pozos**
Post: **Postgraduate student**
Qualifications: **Master of Science**
School/Unit: **Bioengineering Group, School of Materials**
E−mail: **Edna.Suarez@postgrad.manchester.ac.uk**
Telephone: 0161 306 2353

3. Details of Project

3.1 Proposed study dates and duration

Start date: **November 2012**

End date: **February 2013**

3.2 Is this a student project?

Yes

If so, what degree is it for? **For PhD requirements**

3.3. What is the principal research question/objective? *(Must be in language comprehensible to a lay person.)*

The aim of the study is to measure *in-vivo* the skin tension in the chest while performing daily movements using three-dimensional (3-D) image analysis.

The aims of the research are to:

1. Accurately determine the skin tension in the chest area while raising the arms.
2. Determine the skin tension in chest during the breathing cycle.

3.4. What is the scientific justification for the research? What is the background? Why is this an area of importance / has any similar research been done? *(Must be in language comprehensible to a lay person.)*

Keloids are benign skin tumours, unique to humans. Keloid disease does not display homogeneous behaviour all across body sections. Clinical observation has shown that some keloid lesions tend to develop in anatomical sites where increased skin tension is observed, such as the chest. The skin tension developed in the chest area promotes site-specific appearance of scars such as the butterfly-shaped keloid scars. The measurement of the skin tension in the chest will be employed to explain the contribution of skin tension to keloid scar development.

3.5. How has the scientific quality of the research been assessed? *(Tick as appropriate)*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi−centre research group
- ☑ Internal review (e.g. involving colleagues, academic supervisor)
- ☐ None external to the investigator
- ☐ Other, e.g. methodological guidelines *(give details below)*

The research proposal was reviewed by my principal supervisor and advisory panel to ensure it was suitable for my course of study and met ethical guidelines.

3.6. Give a full summary of the purpose, design and methodology of the planned research, including a brief explanation of the theoretical framework that informs it. It should be clear exactly what will

happen to the research participant, how many times and in what order. Describe any involvement of research participants, patient groups or communities in the design of the research.

(This section must be completed in language comprehensible to the lay person.)

Cutaneous wound healing is a complicated, multistep process that involves the regulation of several factors in the skin. Generally after suffering dermal injury a scar rises as consequence. There exists a considerable variation in every single scarring process even in the same individual. The anatomical site of the injury is an important factor in scar formation; normally scarring is worse in those body zones subject to more mechanical tension as a result of natural body movements. Areas such as the chest may develop abnormal scarring, such as Keloid scars in some cases. The objective of this study is to measure *in- vivo* the skin tension in the chest while performing daily movements using integrated experimental and computational analysis. All data obtained from this research will be used to validate the force applied to skin cells on previously conducted experiments.

The analysis of skin tension exerted *in-vivo* while performing daily movements will be evaluated by 3-D image analysis, a novel technique that employs photographic images to determine the geometric properties of a study sample.

The study involves digitising an image pattern painted on the participant's chest before, during, and after performing the required movements, in order to record the deformation that occurred in the area of interest. The images will be processed and analysed using ARAMIS (GOM mbH, Braunschweig, Germany) software in order to obtain the value of the linear strain when the skin covering the chest deforms while moving the arms and breathing. The principal process is listed below:

- Camera calibration by capturing a series of images on a calibration panel.

  Subject preparation (anthropometric measurements, cleaning the chest area, and, if necessary, chest shaving, *Shaving will be performed only if the participant presents such amount of hair on the chest that it would interfere with the speckle pattern painting. The participants are free to withdraw at any time if they do not want to follow the procedure). Skin preparation will be performed carefully in order to avoid damage to the participant and hypoallergenic soap and cream will be available to ameliorate any minor skin irritation. A first aid kit will be available to provide the first aid arrangements in the event of someone being cut by the disposable razor blade.*

  In order to perform the random dot pattern painting on the chest only standard clinical agents will be used in contact with participants' skin and hypoallergenic cleaning materials will be on hand to remove

agents in the event of a reaction. Normal aseptic techniques will be adopted. During the speckle pattern painting a pair of goggles will be provided to the participants as well as disposable masks in order to cover the participant's face and reduce the likelihood of harm from splashing.

- Image measurement recording set up.
- Participants will perform the following routine with 2 min rest between sets:
    - Participants will start from a standing position and will perform at self-selected speed a complete maximal breathing cycle (inhalation and expiration) *(5 times with 20s rest between each movement).*
    - Participants will raise their arms starting from a standing position until they achieve 90° shoulder abduction in a frontal plane as show on Figure 1 *(5 times with 20s rest between each movement):*

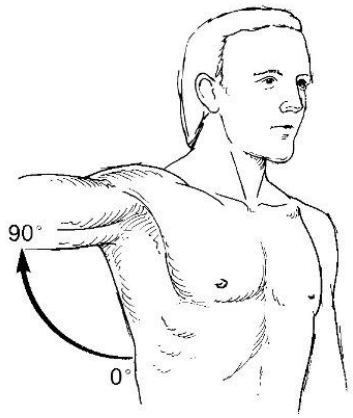


Figure 1:

- Participants will raise their arms starting from a standing position until they achieve 180° shoulder abduction in a frontal plane as show on Figure 2 *(5 times with 20s rest between each movement) :*

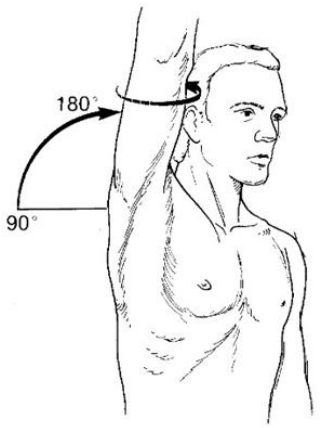


Figure 2:

- Data processing.
- Data results representation.
- Data analysis.

3.6.1. Has the protocol submitted with this application been the subject of review by a statistician in dependent of the research team? *(Select one of the following)*

○ Yes – copy of review enclosed
○
Yes details of review available from the following individual or organisation (give contact details below)
☑ No – justify below

The outcome data for this project will not be analysed using inferential statistics.

3.6.2. If relevant, specify the specific statistical experimental design, and why it was chosen?
N/A

3.6.3. How many participants will be recruited?

4 Healthy male volunteer subjects over the age of 18 will be recruited

3.6.4. How was the number of participants decided upon?

If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.

The aim of this preliminary study is to measure the tension in the skin of the chest while the breathing cycle and arm movements take place. The linear force on skin is described by the equation [1]:

Eq: 1 $$F = K.DSC$$

Where:

- F= Force
- K= Linear stiffness
- DSC= Linear deformation

The K value will be obtained from the literature and is expected to have a coefficient of variation ≤4%, whereas DSC value will be calculated from ARAMIS software tests and is expected to have a coefficient of variation no greater than 1%. So the variability obtained in F will be about 5%. I assume that the 95% confidence interval CI (D) is likely to be ±10% of the mean then in equation [2]:

Eq: 2 $$\frac{\sigma}{D} = \frac{\mathbf{1}}{\mathbf{2}}$$

Where:

- σ= Standard deviation,5%
- D= CI (Confidence Interval), 10%

Using the well-established equation [3] for sample size in descriptive studies [235]:

Eq: 3 $$N = (4\sigma^{\wedge}2\ (Zcrit)^{\wedge}2 \dashv)/D^{\wedge}2$$

Where:

- N= Sample size
- σ = Standard deviation, 5%
- Zcrit = Standard normal deviate for a normal distribution, 1.960
- D = Total width of the expected CI (Confidence Interval), 10%

With 95% confidence the sample size is:

$$N = 4\ (\sigma/_D)^2\ (Zcrit)^2$$

$$N = 4\ \ {}^{1}{}_{2}{}^{2}\ 1.960\ {}^{2} = 4\ \left(1/_4\right)(3.841) = 3.841 \approx 4$$

NOTE: According to the calculation above, a minimum total of 4 participants are necessary.

3.6.5. Describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

The raw data, which consists of the set of 3-D images obtained from the random pattern painted on the chest, will be processed using the ARAMIS platform, in order to calculate the linear strain in the skin from the displacement of data points calculated in the set of 3-D images collected during the test. The resulting data will be used to calculate the skin tension and the mean tension will be used as a representative value of the skin tension over the chest that could be employed to explain the contribution of the skin tension in keloid scar development.

3.7. Where will the research take place?

In the Bioengineering laboratories in the Pariser Building, Sackville Street.

3.8. Names of other staff involved.

Dr Teresa Alonso-Rasgado

Dr Parthasarathi Mandal

Dr Ardeshir Bayat

Dr Alan Walmsley

3.9. What do you consider to be the main ethical issues which may arise with the proposed study and what steps will be taken to address these?

Participants will be asks to elevate their arms from a standing position while a random dot pattern painted on the participant's sternum is recorded electronically. Participants will be required to keep their chest bare in order to allow the dot pattern to be painted and recorded. The procedures will be undertaken in private in the bioengineering laboratory, where privacy will be provided. *Participants may experience some minor discomfort or allergenic reaction to the spray paint, so hypoallergenic spray paint will be used. Also hypoallergenic cleaning agents will be provided to remove any residue from the skin.*

3.9.1. Will any intervention or procedure, which would normally be considered a part of routine care, be withheld from the research participants?

○ Yes ☑ No

If yes, give details and justification

4. Details of Subjects.

4.1. Total Number Total number of subjects will be 4.

4.2 Sex and Age Range There will *be 4 males over 18 years old.*

4.3 Type *Healthy male volunteers* from the general public and MACE postgraduate students.

4.4. What are the principal inclusion criteria? *(Please justify)*

The study will investigate the mechanical tension in the skin over the chest during the execution of daily body movements, such as breathing cycle and arm rising, in the normal population. Consequently, volunteers will be generally healthy with no previous history of skin injury that compromises the study area by modifying the natural skin tension lines.

4.5. What are the principal exclusion criteria? *(Please justify)*

Volunteers with a previous history of skin injury or cicatrisation that compromises the study area by modifying the natural skin tension lines will be excluded from the study.

4.6. Will the participants be from any of the following groups? *(Tick as appropriate)*

- ☐ Children under 16
- ☐ Adults with learning difficulties
- ☐ Adults who are unconscious or very severely ill
- ☐ Adults who have a terminal illness
- ☐ Adults in emergency situations
- ☐ Adults with mental illness (particularly if detained under mental health legislation)
- ☐ Adults with dementia
- ☐ Prisoners
- ☐ Young offenders
- ☐ Adults in Scotland who are unable to consent for themselves
- ☑ Healthy volunteers

- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, medical students.
- ☐ Other vulnerable groups

Justify their inclusion

See above

4.7. Will any research participants be recruited who are involved in existing research or have recently been involved in any research prior to recruitment?

○ Yes ☑ No ○ Not known

If Yes, give details and justify their inclusion. If Not Known, what steps will you take to find out?

4.8 How will potential participants in the study be (i) identified, (ii) approached and (iii) recruited?

Where research participants will be recruited via advertisement, please append a copy to this application

Potential participants will be contacted and invited to take part by the principal researcher Edna Suarez who will meet them at a mutually convenient time and location. A copy of the advertisement provided to the participants is included in appendix C.

At the meeting the nature of the research and the requirements of volunteers will be explained and volunteers will be given an information sheet about the project. Volunteers will be informed of their right to decline to participate, or continue to participate, at any stage, without question. They will also be informed of their right to protection of personal information and the measures taken to ensure their anonymity.

Volunteers will be given as much time as they wish to consider their participation and will be asked to contact the researcher if they decide to participate in the study.

4.9 Will individual research participants receive *reimbursement of expenses* or any other *incentives* or *benefits* for taking part in this research?

○ Yes ☑ No

If yes, indicate how much and on what basis this has been decided

Out of pocket expenses for visits to the laboratory up to a maximum of £10 (ten pounds) per visit will be reimbursed.

5 Details of risks

5.1 Drugs and other substances to be administered

Indicate status, eg full product licence, CTC, CTX. Attach: evidence of status of any unlicensed product; and Martindales Phamacopoeia details for licensed products

DRUG STATUS DOSAGE/FREQUENCY/ROUTE

N/A

5.2 Procedures to be undertaken

Details of any invasive procedures, and any samples or measurements to be taken. Include an questionnaires, psychological tests etc. What is the experience of those administering the procedures?

- **Antropometric data:**
  - Height
  - Weight
  - Length of the sternum
  - Length of arms in horizontal plane with shoulder abducted to 90°

- ***Skin preparation:*** *The skin chest will be shaved (if necessary) and cleaned carefully to ensure paint adhesion and avoid any noise during the image recording. A first aid kit will be available to provide the first aid arrangements in the event of someone being cut by the disposable razor blade. The participants are free to withdraw at any time if they do not want to follow the procedure.*
- ***Dot pattern drawing:*** *every participant will be provide with a disposable mask and a pair of goggles while the dot pattern is painted on the skin using hypoallergenic-spray paint.*

- Performance of the skin tension measurement will be taken by image recording while the required movement are affected.

Researcher Experience:

- MSc E. Suarez: has been working for two years in biomechanics.
- Dr P. Mandal: PhD in structure, dynamics and extreme loading
- Dr A. Bayat : PhD in molecular genetics of scar formation and subsequent work in plastic surgery, skin biology, tissue regeneration and fibrosis
- Dr A. Walmsley: PhD in biomechanics and subsequent work in sport science including extensive biomechanics and athlete assessment.

5.3 *Or* **Activities to be undertaken**

Please list the activities to be undertaken by participants and the likely duration of each

Participants will visit the laboratory once for approximately 2 hr. The participants will complete the informed consent form followed the anthropometic measurements (20 min). Participants will receive one more time the explanation of the procedures to be performed and will be asked to perform several familiarisation trials of the required movements (20 min). A visual pattern will be painted on the participant's sternum section with non-toxic spray paint (10 min). Participants will be video recorded while they complete multiple trials of the breathing cycle and the arm raising movements using an ARAMIS Photogrammetry system (70 min).

5.4 What are the potential adverse effects, risks or hazards for research participants, including potential for pain, discomfort, distress, inconvenience or changes to lifestyle for research participants?

Replace the following text with appropriate explanation of the risks associated with your project.

For participants there are minor risks including an adverse reaction to the spray paint, the shaving cream or alcohol wipes, and some redness or discomfort due to the paint removal.

5.5 Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews/group discussions, or use of screening tests for drugs)?

○ Yes ☑ No

If yes, give details of procedures in place to deal with these issues:

5.6 What is the expected total duration of participation in the study for each participant?

The total time commitment for each participant will not exceed 2 hours.

5.7 What is the potential benefit to research participants?

There is not direct potential benefit for the participants.

5.8 What is the potential for adverse effects, risks or hazards, pain, discomfort, distress, or inconvenience to the researchers themselves? *(If any)*

None.

6. Safeguards

6.1 What precautions have been taken to minimise or mitigate the risks identified above?

Hypoallergenic soap and water will be available to clean the participant's skin in case of minor adverse reactions. Disposables mask and goggles will be provided to protect participant while the dot pattern painting takes place.

6.2 Will informed consent be obtained from the research participants?

☑ Yes ○ No

If Yes, give details of who will take consent and how it will be done. Give details of the experience in taking consent and of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material.

If participants are to be recruited from any of the potentially vulnerable groups listed in Question 4.6, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.

If consent is not to be obtained, please explain why not.

Where relevant the committee must have a copy of the information sheet and consent form.

Written informed consent will be obtained from each participant by Edna Suarez. In addition to the information sheet, the researcher will explain the research procedures to each participant in person and will explain the requirements for informed consent.

The information sheet and consent form are attached, Appendix A and B.

Will a signed record of consent be obtained?

☑ Yes ○ No

If not, please explain why not.

6.4 How long will the participant have to decide whether to take part in the research?

Participants may take as long as they wish. There will be no deadline for inclusion.

6.5 What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs?

Translation and /or interpreters will be considered.

6.6 What arrangements are in place to ensure participants receive any information that becomes available during the course of the research that may be relevant to their continued participation?

Should this become necessary the researcher(s) will contact each participant directly.

6.7 Will the research participants' General Practitioner be informed that they are taking part in the study?

○ Yes ☑ No

If No, explain why not

Participation in the research requires only similar activities to those of daily living and so there is no clinical reason to inform the GP.

6.8 Will permission be sought from the research participants to inform their GP before this is done?

○ Yes ○ No

If No, explain why not

N/A

6.9 What arrangements have been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, participants for (a) *negligent* harm and (b) *non-negligent* harm?

Procedures will be completed to ensure coverage by the University Insurance Policy

7. Data Protection and Confidentiality

7.1 Will the research involve any of the following activities at any stage (including identification of potential research participants)? *(Tick as appropriate)*

☐ Examination of medical records by those outside the NHS, or within the NHS by those who would not normally have access
☑ Electronic transfer by magnetic or optical media, e-mail or computer networks

☐ Sharing of data with other organisations
☐ Export of data outside the European Union
☐ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers
☐ Publication of direct quotations from respondents
☐ Publication of data that might allow identification of individuals
☑ Use of audio/visual recording devices

☑ Storage of personal data on any of the following:

- ☐ Manual files including X-rays
- ☐ NHS computers
- ☐ Home or other personal computers
- ☑ University computers
- ☐ Private company computers
- ☑ Laptop computers

Further details:

7.2 What measures have been put in place to ensure confidentiality of personal data? Give details of whether any encryption or other anonymisation procedures have been used and at what stage?

All personal information and experimental data will be stored on password protected University computers. ONLY THE RESEARCHER WILL HAVE ACCESS TO THE PERSONAL INFORMATION. Participants will be assigned an alphanumeric identifier, which will be used in all analyses.

7.3 Where will the analysis of the data from the study take place and by whom will it be undertaken?

The data will be analysed in the Pariser Building by:

Ms Edna Suarez
Dr Teresa Alonso Rasgado
Dr Parthasarathi Mandal
Dr Ardeshir Bayat
Dr Alan Walmsley

7.4 Who will have control of and act as the custodian for the data generated by the study?

Researcher: Edna Suarez

Head of the Bioengineering group: Dr Teresa Alonso Rasgado

7.5 Who will have access to the data generated by the study?

Ms Edna Suarez
Dr Teresa Alonso Rasgado
Dr Parthasarathi Mandal
Dr Ardeshir Bayat
Dr Alan Walmsley

7.6 For how long will data from the study be stored?

5 Years

Give details of where they will be stored, who will have access and the custodial arrangements for the data:

All collected data will be stored and protected on a password protected University computer. Only the researcher and the Head of the Bioengineering group will have access to the data.

8. Reporting Arrangements

8.1 Please confirm that any adverse event will be reported to the Committee

*A*ny adverse event will be reported to the Committee

8.2. How is it intended the results of the study will be reported and disseminated?

(Tick as appropriate)

- ☑ Peer reviewed scientific journals
- ☑ Internal report
- ☑ Conference presentation
- ☑ Thesis/dissertation
- ☐ Written feedback to research participants
- ☐ Presentation to participants or relevant community groups

☐ Other/none e.g. Cochrane Review, University Library

8.3 How will the results of research be made available to research participants and communities from which they are drawn?

Each participant will receive a summary report of the research findings outlining the study results and their relevance to well-being in the population.

8.4 Has this or a similar application been previously considered by a Research Ethics Committee in the UK, the European Union or the European Economic Area?

○ Yes

☑ No

If Yes give details of each application considered, including:

Name of Research Ethics Committee or regulatory authority:
Decision and date taken:
Research ethics committee reference number:

8.5 What arrangements are in place for monitoring and auditing the conduct of the research?

N/A

Will a data monitoring committee be convened?

○ Yes

☑ No

What are the criteria for electively stopping the trial or other research prematurely?

N/A

9. Funding and Sponsorship

9.1 Has external funding for the research been secured?

○ Yes ☑No

If Yes, give details of funding organisation(s) and amount secured and duration:

Organisation:

UK contact:

Amount (£):

Duration: Months

9.2 Has the external funder of the research agreed to act as sponsor as set out in the Research Governance Framework?

○ Yes ☑ No ○ Not Applicable

9.3 Has the employer of the Chief Investigator agreed to act as sponsor of the research?

☑ Yes ○ No

9.4 Sponsor *(must be completed in all cases where the sponsor is not the University)*

Name of organisation which will act as sponsor for the research:

N/A

10. Conflict of interest

10.1 Will individual *researchers* receive any personal payment over and above normal salary and reimbursement of expenses for undertaking this research?

○ Yes ☑ No

If Yes, indicate how much and on what basis this has been decided:

10.2 Will the host organisation or the researcher's department(s) or institution(s) receive any payment of benefits in excess of the costs of undertaking the research?

○ Yes ☑ No

If Yes, give details:

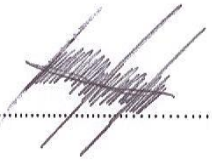
10.3 Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share-holding, personal relationship etc.) in the organisation sponsoring or funding the research that may give rise to a possible conflict of interest?

O Yes ☑ No

If Yes, give details:

11. Signatures of applicant(s)

Edna Suarez



..... 27/Nov/2012.

Signed Date

....

Signed Date

12 Signature by or on behalf of the Head of School

The Committee expects each School to have a pre-screening process for all applications for an ethical opinion on research projects. The purpose of this pre-screening is to ensure that projects are scientifically sound, have been assessed to see if they need ethics approval and, if so, go to the relevant ethics committee. It is **not** to undertake ethical review itself, which must be undertaken by a formal research ethics committee.

The form must therefore be counter-signed by or on behalf of the Head of School to signify that this pre-screening process has been undertaken

I approve the submission of this application

..... 20 October 2012

Signed by or on behalf of the Head of School Date

APPENDIX A

Measurement of skin tension in the chest

Participant Information Sheet

You are invited to take part in a research study as part of a student project for a PhD. The objective of this research is to measure *in-vivo* the skin tension in the chest while performing daily movements using three-dimensional (3-D) images. Before you decide to take part in this research it is important for you to clearly understand the aim of the investigation as well as what it involves. Please take your time to read the following information carefully and feel free to request more information from the researchers if you find something difficult to understand or there are further issues you wish to discuss before you make a decision.

Who will conduct the research?

MSc Edna Suarez Pozos
Dr Parthasarathi Mandal
Dr Ardeshir Bayat

Dr Teresa Alonso Rasgado
Dr Alan Walmsley

Pariser Building, University of Manchester, Sackville street, Manchester M13 9PL.

Title of the Research

The effect of skin tension on the formation of keloid scars

What is the aim of the research?

The aim of this research is to measure the skin tension in the chest while performing daily movements using photographic images from a dot pattern painted on the chest to determinate the properties of the skin.

Why have I been chosen?

You have been chosen because you are healthy *adult male* with no previous history of scarring, skin, muscle or joint problems.

What would I be asked to do if I took part?

You will be asked to visit Bioengineering Laboratories in Pariser Building, Sackville Street, University of Manchester once for approximately 2 hours during which time you will be ask to take part in the following procedures:

1. A full explanation of the measures and preparatory activities that will be undertaken as part of this research will be provided by the researcher.

2. Then you will be asked to take part in some preparatory activities which will include measuring your height, weight, and length of extended arms in horizontal plane with respect the chest. If necessary, the researcher will need to clean and, *if necessary, shave your chest to allow more accurate speckle pattern painting that permits better image collection and more precise data calculations. You are free to withdraw at any time if you do not want to follow the procedure.*

3. The research will locate your clavicle, the manubrium and sternum. Once located, the researcher will paint a random dot pattern on your chest using a spray can in order to delineate the study, as shown in Figure 1:

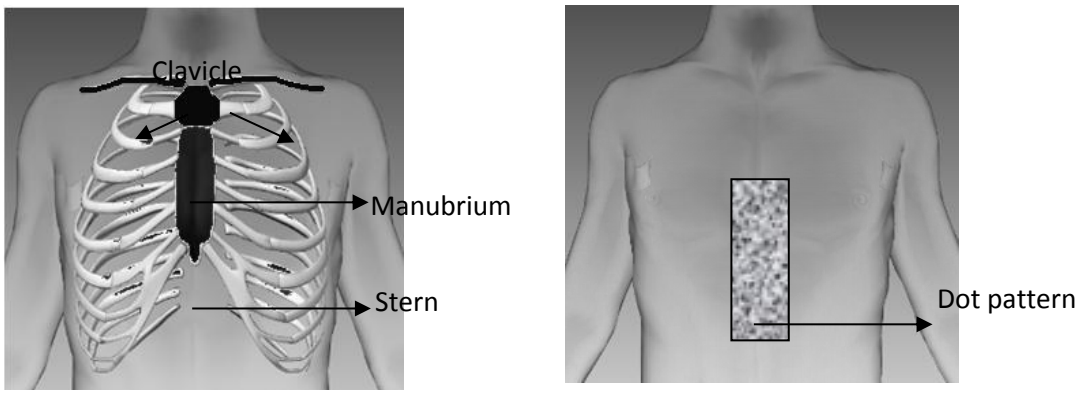


Figure 1

4. Once the preparatory activities are complete the researcher will invite you to *perform natural body movements such as breathing and arm raising as follow:*

- You will start from a standing position and will perform at self-selected speed a complete maximum breathing cycle (inhalation and expiration). *(This movement will be repeated 5 times with 20 seconds rest between repetitions).*
- You will be asked to raise your arms starting from a standing position until you achieve a 90° shoulder abduction as shown in Figure 2 *(This movement will be repeated 5 times with 20 seconds rest between repetitions):*

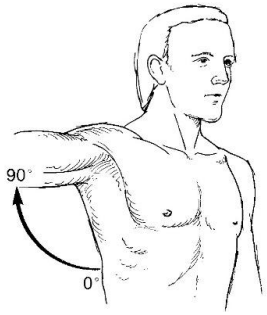


Figure 2

- You will be asked to raise your arms starting from a standing position until you achieve a 180° shoulder abduction as shown in Figure 3 *(This movement will be repeated 5 times with 20 seconds rest between repetitions):*

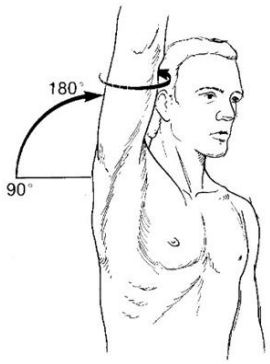


Figure 3

5. All trials described above will be recorded and analysed.
6. Once all activities are complete the pattern will be carefully removed and the data will be analysed.

What happens to the data collected?

The data will be processed by the researcher (Edna Suarez) and will also be shared with the other researchers involved in the investigation named at the end of this information sheet. The results of the research will be submitted as part of a PhD thesis and will be published in a scientific journals and conferences. However, no names will be used in any report or documents, so that no-one will know that you have taken part in the study.

How is confidentiality maintained?

All written personal information will be kept in a locked filing cabinet and electronic data on a protected computer. The information will retained for five years and at the end of this time all information will be destroyed (the data base of personal and research information will be securely deleted from all computers, and any hard copy will be shredded). You will be only identified by a code in any process and analyses.

What happens if I do not want to take part or if I change my mind?

You are not under any obligation to take part in this research. If you decide to be a volunteer of this project you are still free to leave the study at any time you wish without detriment to yourself.

Will I be paid for participating in the research?

You will not receive any payment for being part of the investigation, but if you have any out of pocket expenses, these will be refunded on presentation of receipts. There will be a maximum amount of ten pounds per person.

What is the duration of the test?

This research will take around 2 hr in total.

Where will the research be conducted?

In the Bioengineering Laboratories in the Pariser Building, Sackville Street, University of Manchester

Will the outcomes of the research be published?

The results of the trials will be published in a scientific journal.

Criminal Records Check (if applicable)

N/A

Contact for further information

For more information contact:

Ms Edna Suarez
Postgraduate Student
Floor D, Pariser Building
School of Mechanical Aerospace and Civil Engineering
University of Manchester
Sackville Street
Manchester M13 9PL
Email: Edna.Suarez@postgrad.manchester.ac.uk
Telephone: 01613062353

What if something goes wrong?

The protocol used to carry out this research does not present any risk other than minor discomfort, but if you have any complaint during or after the test, you can contact the person in charge immediately. The University has insurance that covers any harm to participants caused by the research test.

If you wish to make a formal complaint about the conduct of the research you should contact any of the people given at the end of the information sheet.

What if I want to complain?

If you wish to complain you need to contact the researcher in the first instance

Research student: Edna Suarez

If you are still not satisfied then you can contact one of my supervisors listed below

The supervisors of the research are:

Dr Teresa Alonso Rasgado
Dr Parthasarathi Mandal
Dr Ardeshir Bayat

Dr Alan Walmsley

Dr Teresa Alonso-Rasgado
Room C8, Pariser Building
School of Mechanical Aerospace and Civil Engineering
University of Manchester
Sackville Street
Manchester M13 9PL
Ph 0161 306 3857
E-mail: teresa.rasgado@manchester.ac.uk

Or the Head of School:

Prof Andrew Gibson
Room B1, George Begg Building
School of Mechanical Aerospace and Civil Engineering
University of Manchester
Sackville Street
Manchester M13 9PL
Ph 0161 306 9202
E-mail: andrew.gibson@manchester.ac.uk

If the above persons are still not able to address your concerns then you may contact the university research governance office

Dr Andrew Walsh,
Head of Research and Development
Christie Building,
University of Manchester,
Oxford Road,
Manchester, M13 9PL.
Ph 0161 3052068
E-mail: andrew.m.walsh@manchester.ac.uk

Contact for further information:

If you are still interested in taking part of this research, then contact me the researcher named below for further information:

MSc Edna Suarez
Postgraduate student
Room D, Pariser building
School of Mechanical Aerospace and Civil Engineering
University of Manchester
Sackville Street
Manchester M 13 9PL
E-mail: edna.suarez@postgrad.manchester.ac.uk
Telephone: 01613062353

MANCHESTER 1824

The University of Manchester

APPENDIX B

The effect of skin tension on the formation of keloid scars

CONSENT FORM

If you are happy to participate please complete and sign the consent form below

Please Initial Box

1. I confirm that I have read the attached information sheet on the above project and have had the opportunity to consider the information and ask questions and had these answered satisfactorily.	
2. I understand that my participation in the study is voluntary and that I am free to withdraw at any time without giving a reason and without detriment to any treatment/service	

to take part in the above project

Name of participant	Date	Signature

Name of person taking consent	Date	Signature

MANCHESTER 1824

The University of Manchester

APPENDIX C

The effect of skin tension on the formation of keloid scars

I am looking for healthy *male* volunteers to take part in a study investigating the skin tension over the chest while performing daily movements such as breathing and raising the arms.

In order to be eligible you need to:

- *Be a male* aged over 18;
- Be healthy with no previous history of scarring or skin disruption, muscle or joint problems and

You will be required to attend the Bioengineering Laboratories in the Pariser Building, Sackville Street, University of Manchester once for about 2 hours. During that time you will be asked to perform some simple movements of your body while your movements are recorded.

I will reimburse any out of pocket expenses you have to participate in the research on presentation of receipts.

If you agree to participate, please send an email to this address: Edna.Suarez@postgrad.manchester.ac.uk.

I will contact you and explain step by step the simple tests and the procedure that you need to follow. If you still want to take part I will then invite you to attend the laboratory just once for about 2 hours in order to complete the research. Thank you in advance.

Experiments will be conducted at the University of Manchester by Edna Suarez under the supervision of Dr Teresa Alonso, Dr Parthasarathi Mandal, Dr Ardeshir Bayat and Dr Alan Walmsley.

This study has been approved by the Senate Ethics Committee 1 at the University of Manchester

For Further Information Contact details

If you would like further information please contact Edna Suarez:

Phone: 01613062353
E-mail: Edna.Suarez@postgrad.manchester.ac.uk

Appendix III

EXPERIMENTAL

Up-Regulation of Tension-Related Proteins in Keloids: Knockdown of Hsp27, α2β1-Integrin, and PAI-2 Shows Convincing Reduction of Extracellular Matrix Production

Edna Suarez, M.Sc.
Farhatullah Syed, Ph.D.
Teresa Alonso-Rasgado, Ph.D.
Parthasarathi Mandal, Ph.D.
Ardeshir Bayat, M.B., B.S., Ph.D.
Manchester, United Kingdom

Background: Keloid disease is a fibroproliferative disorder, with an ill-defined treatment that is characterized by excessive extracellular matrix deposition. Mechanical tension promotes deposition of extracellular matrix and overexpression of tension-related proteins, which is associated with keloid disease. The aim of this study was to investigate the effect of tension-related proteins on extracellular matrix steady-state synthesis in primary keloid fibroblasts.
Methods: Keloid fibroblasts ($n = 10$) and normal skin ($n = 4$) fibroblast cultures were established from passages 0 to 3. A panel of 21 tension-related genes from microarray data were assessed at mRNA (quantitative reverse-transcriptase polymerase chain reaction) and protein (in-cell Western blotting) levels. Three genes were significantly altered in keloid tissue and fibroblasts, and their functional role was assessed using siRNA knockdown.
Results: Hsp27, α2β1-integrin, and PAI-2 were significantly up-regulated ($p < 0.05$)in keloid tissue and fibroblasts compared with normal skin. Hsp27, α2β1-integrin, and PAI-2 expression was inhibited by RNA interference. Both the mRNA and protein levels of Hsp27, α2β1-integrin, and PAI-2 significantly decreased ($p < 0.05$) in keloid fibroblasts at 48 hours after transfection. After down-regulation of Hsp27, α2β1-integrin, and PAI-2, the expression of intracellular extracellular matrix was significantly reduced ($p < 0.05$). Water-soluble tetrazolium salt-1 assay showed that transfection of Hsp27, α2β1-integrin, and PAI-2 did not influence the viability/metabolic activity of keloid fibroblasts.
Conclusions: This study demonstrates overexpression of key tension-related proteins in keloid tissue and keloid fibroblasts. Knockdown of Hsp27, PAI-2, and α2β1-integrin by RNA interference attenuates the expression of mRNA and protein levels and certain other extracellular matrix molecules. (*Plast. Reconstr. Surg.* 131: 158e, 2013.)

Keloid disease is an abnormal fibroproliferative lesion that grows in a quasineoplastic manner, extends beyond the original wound margin, does not regress over time, and invades the nearby unaffected skin.[1–7] The mechanism of keloid formation is poorly understood, and there is no specific treatment to cure these lesions.[8,9] Clinical observation has shown that some keloid lesions tend to

From Plastic and Reconstructive Surgery Research, School of Translational Medicine, Manchester Institute of Biotechnology, and the Bioengineering Group, School of Mechanical, Aerospace, and Civil Engineering, University of Manchester, and the University Hospital of South Manchester NHS Foundation Trust, Faculty of Medical and Human Sciences, Institute of Inflammation and Repair, University of Manchester, Manchester Academic Health Science Centre.
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The first two authors contributed equally to this work.

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Disclosure: *The authors have no financial interest to declare in relation to the content of this article.*

Supplemental digital content is available for this article. Direct URL citations appear in the text; simply type the URL address into any Web browser to access this content. Clickable links to the material are provided in the HTML text of this article on the *Journal*'s Web site (www.PRSJournal.com).

develop in anatomical sites where increased skin tension is present, such as the chest, shoulders, and upper back.[3,10] It has been suggested that skin tension generated in the remodeling stage of the wound healing process may influence keloid behavior, development, and spreading.[3,11–13] Thus, there are site-specific morphologies of keloid scarring. For example, butterfly-shaped keloid scars are frequently observed in the sternal area.[14] Several studies have demonstrated that, when fibroblasts are subjected to mechanical forces, this leads to increased focal adhesion complexes and integrin receptors in this process.[15,16] Moreover, mechanical stimulation has been shown to affect cellular gene expression, protein synthesis, and proliferation rates.[17,18] In addition, fibroblasts under mechanical tension have a higher expression of certain tension-related proteins (e.g., heat shock proteins, integrins, and cytokines) and produce excessive extracellular matrix components.[13,19]

Interestingly, a large number of tension-related proteins in the skin have been shown to be overexpressed during the wound healing process.[3,20] (**See Table, Supplemental Digital Content 1,** which shows a subset of tension-related genes with increased expression in skin, ***http://links.lww.com/PRS/A642***.) Recently, it has been reported that tension-related proteins such as heat shock protein 27 (Hsp27)[21] lead to increased extracellular matrix synthesis and are closely related to progression of the fibrotic process.[22–24] To this end, it was hypothesized that overexpression of tension-related proteins and excessive extracellular matrix deposition may be influenced by mechanical tension produced in the skin during wound healing that may lead to keloid formation.

In the present study, a panel screening of tension-related proteins was performed that showed overexpression of certain tension-related proteins in keloid disease and convincingly demonstrated that knockdown of these proteins by RNAi technology decreased extracellular matrix synthesis in keloid fibroblasts. The findings from this study offer further insight into understanding keloid disease pathobiology and potential options for future treatment of keloids. It is envisaged that RNA interference could be targeted toward tension-related proteins found in keloid tissue.

PATIENTS AND METHODS

Patient Data and Tissue Collection

Keloid samples ($n = 10$) and normal skin samples ($n = 4$) (Tables 1 and 2) were obtained after acquiring informed consent from all patients (full ethical approval was obtained from the local hospital, university, and regional NHS Ethics Committee) before surgery. All keloid patients were confirmed to have clinical and pathologic evidence of keloid disease as described previously.[2] Briefly, a scar was considered a keloid if the lesion had presented with typical phenotypic behavior with invasion into the boundary of the original lesion, which had been present for more than 1 year, had not regressed over time, and had not responded to conventional treatments, whereas the control group corresponded to normal skin obtained from tissue adjacent to a normal scar (a normal scar was considered to be a flat scar in a patient with no previous history of keloid scarring).[2,25] At the time of surgical excision, the central area of the keloid scar from the chest and normal skin from normal scars were collected. A step-by-step flowchart referring to the experimental design can be found in the supplementary information. (**See Figure, Supplemental Digital Content 2,** which is a summary of steps taken to determine potential tension-related biomarkers for keloid disease, ***http://links.lww.com/PRS/A643***.)

Table 1. Demographic Data of the Keloid Tissue Samples Used in This Study*

Study ID	Sex	Ethnicity	Age (yr)	Age of Scar (yr)	Cause
KD 1	F	Asian	27	1	Spot
KD 2	F	White	18	15	Chickenpox
KD 3	M	Black	45	1	Surgery
KD 4	F	Black	20	9	Surgery
KD 5	M	Asian	74	11	Surgery
KD 6	M	Black	42	3	Industrial accident
KD 7	F	White	29	10	Spot
KD 8	M	Black	36	5	Surgery
KD 9	F	Black	35	9	Spontaneous
KD 10	F	White	43	29	Sternal, spot

ID, identification; KD, keloid disease; F, female; M, male.
*All samples were taken from the chest.

Table 2. Demographic Data of the Normal Skin Tissue Samples Used in This Study*

Study ID	Sex	Age (yr)
NS 1	F	47
NS 2	F	39
NS 3	F	22
NS 4	M	31

ID, identification; NS, normal skin; F, female; M, male.
*All subjects were white; all samples were taken from the chest.

Primary Keloid and Normal Skin Fibroblast Culture Establishment

The tissue was collected in Dulbecco's Modified Eagle Medium (Sigma-Aldrich, St. Louis, Mo.), and fibroblasts were extracted using collagenase type I solution, 0.5 mg/ml (Roche Diagnostics, West Sussex, United Kingdom) as described previously.[2] Fibroblasts from passage 0 to passage 3 were used for all experiments carried out in this study. We decided to use fibroblasts taken from earlier passages because of published data from our laboratory.[2] We previously established that phenotypic alterations were observed in keloid fibroblasts as the number of passages increased, and others have also suggested that cell growth and phenotypic alterations could result from fibroblasts aging through multiple passsages.[26] Thus, to minimize variability and have optimal consistency in data, only cells up to passage 3 were used in this study.

RNA Extraction, cDNA Synthesis, and Quantitative Reverse-Transcriptase Polymerase Chain Reaction

RNA extraction, cDNA synthesis, and quantitative reverse-transcriptase polymerase chain reaction were performed as described previously.[2,27] The list of primers used in this study are shown. (**See Table, Supplemental Digital Content 3,** for a list of primer pairs used in this study for quantitative reverse-transcriptase polymerase chain reaction, ***http://links.lww.com/PRS/A644***.) The mRNA expression was normalized with reference to gene ribosomal protein L32 (RPL32).

Immunohistochemistry

Immunohistochemistry was performed as described previously.[28] Briefly, tissue biopsy specimens were fixed in 4% volume/volume formalin and embedded in wax. Tissue sections were cut into 5-μm slides, fixed onto glass slides, and dried at room temperature overnight. Tissue sections were deparaffinized, rehydrated, and treated with primary antibodies overnight at 4°C followed by secondary antibodies. (**See Table, Supplemental Digital Content 4,** for a list of primary antibodies used in this study, ***http://links.lww.com/PRS/A645***; and **see Table, Supplemental Digital Content 5,** which is a list of secondary antibodies used in this study.)

High-Throughput In-Cell Western Blotting and Quantitation

Eighty to 90 percent confluent primary keloid fibroblasts and fibroblasts from normal skin were starved in 0.2% serum Dulbecco's Modified Eagle Medium for 24 hours, and then trypsinized and counted using fluorescence-activated cell sorting (Accuri C6 Flow Cytometer System; BD, Oxford, United Kingdom), with 1×10^4 cells inoculated into each well of a 96-well plate. The cells were then grown to confluence for 24 hours in Dulbecco's Modified Eagle Medium. After 24 hours, the cells were washed once with phosphate-buffered saline and then fixed using 4% formaldehyde for 20 minutes at room temperature. In-cell Western blotting was carried out using the standard protocol.[27] β-Actin was used as a loading control; the panels of primary and secondary antibodies used in this study are listed in **Tables, Supplemental Digital Content 4** and **Supplemental Digital Content 5,** ***http://links.lww.com/PRS/A645*** and ***http://links.lww.com/PRS/A646***.

siRNA Design and Target Gene Knockdown

The Ambion siRNA target online tool (Silencer PreDesigned and Validated siRNAs) was used to select three "positive" siRNA sequences targeting to human anti-Hsp27, $\alpha 2\beta 1$-integrin, PAI-2, and scrambled siRNA (Applied Biosystems, Warrington, United Kingdom). Keloid fibroblasts (1×10^5 cells/well) from six different patients were cultured in 24-well plates in triplicate and transfection was carried out in suspension using siRNA complexes prepared with HiPerFect Transfection Reagent (Qiagen, West Sussex, United Kingdom) according to the manufacturer's protocol. The cells were then incubated for 48 hours at 37°C. Forty-eight hours after transfection, fibroblasts were collected by scraping. The RNA extraction, cDNA synthesis, and quantitative reverse-transcriptase polymerase chain reaction were performed as described previously.[2] The gene expression levels were normalized with RPL32.

Table 3. Significant Up-Regulation/Down-Regulation Tension-Related Genes within the Keloid

Gene Name	Gene Symbol	Keloid/Normal (relative mRNA)	p	Chromosomal Location
Up-regulated				
α2-integrin	ITAGA2	1.35 ± 0.57	0.003	5q11.2
Chemokine (C-C motif) ligand 2	CCL2	0.93 ± 0.25	0.327	17q11.2-q12
Heat shock protein 27	Hsp27 (HSPB1)	3.03 ± 0.86	0.027	7q11.23
Heat shock protein 47	SERPINH1	0.34 ± 0.52	0.042	11q13.5
Heat shock protein 70	HSPA1A	1.42 ± 0.24	0.099	6p21.3
Hyaluronan and proteoglycan link protein 1	HAPLN1	1.08 ± 0.42	<0.0001	5q14.3
Hyaluronan and proteoglycan link protein 2	HAPLN2	0.87 ± 0.23	0.560	1q23.1
Matrix metallopeptidase 19	MMP-19	1.02 ± 0.45	0.478	12q14
Serpin peptidase inhibitor, clade B (ovalbumin), member 2	PAI-2 (SERPINB2)	1.12 ± 0.83	0.008	18q21.3
Substance-P tachykinin, precursor 1	TAC1	1.09 ± 0.23	0.050	7q21-q22
Tenascin XB	TNXB	1.48 ± 0.50	0.110	6p21.3
Transforming growth factor beta 1	TGF-β1	0.87 ± 0.86	<0.0001	19q13.2; 19q13.1
Transforming growth factor beta 2	TGF-β2	0.84 ± 0.83	0.009	1q41
Transforming growth factor beta 3	TGF-β3	0.92 ± 0.50	0.007	14q24
Down-regulated				
Calcitonin-related polypeptide alpha	CALCA	1.83 ± 1.46	<0.0001	11p15.2
Cysteine-rich, angiogenic inducer, 61	Cyr61	2.74 ± 1.03	0.001	1p22.3
Heat shock protein 60	HSPD1	0.97 ± 0.52	0.033	2q33.1
Heat shock protein 90	HSP90AA1	1.93 ± 0.30	0.060	14q32.33
Matrix metallopeptidase 3	MMP-19	1.02 ± 1.46	0.478	12q14
Matrix metallopeptidase 13	MMP-13	0.54 ± 1.09	0.001	11q22.3
Neuropeptide Y	NPY	0.93 ± 1.24	0.654	7p15.1

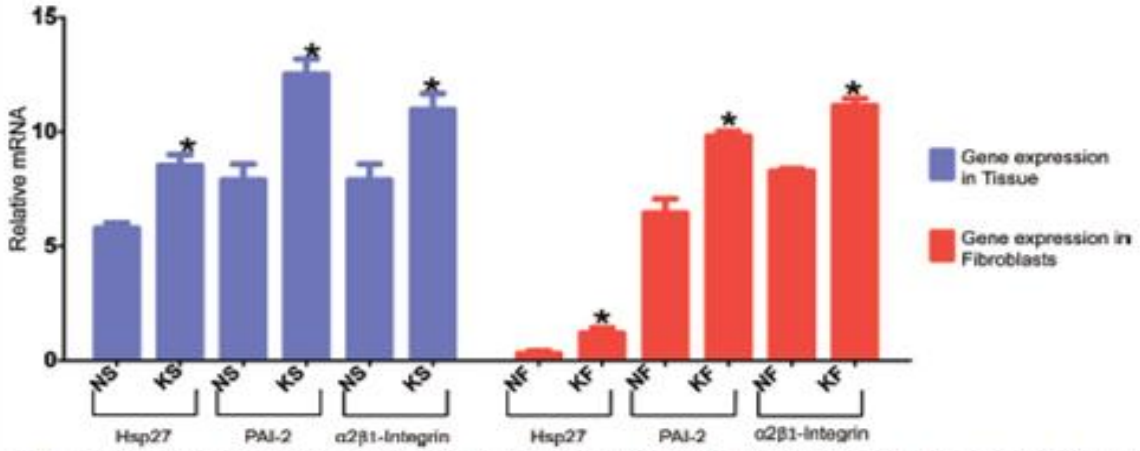

Fig. 1. Up-regulation of tension-related genes in keloid tissue and fibroblasts. Total RNA was extracted from tissue biopsy specimens as described in the Patients and Methods section. The expression of the tension-related genes including Hsp27, PAI-2, and α2β1-integrin were normalized to an internal reference gene (RPL32). The results are expressed as mean ± SEM of triplicates from three independent experiments ($n = 10$). *$p < 0.05$ indicates a significant difference between mRNA expression in tissue biopsy specimens from normal skin (*NS*) versus mRNA expression from keloid skin (*KS*) and the mRNA expression in fibroblasts from normal skin (*NF*) versus keloid fibroblasts (*KF*).

Western Blotting

Forty-eight hours after transfection, keloid fibroblasts were collected using cell scrapers. Cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich) supplemented with phosphatase inhibitors cocktail (Protease Inhibitor Cocktail Set III, EDTA-Free; Calbiochem, Nottinghamshire, United Kingdom). The total amount of protein was quantified with the BCA protein assay kit (Thermo Scientific, Waltham, Mass.). Equal amounts of proteins (100 μg) were denatured and resolved on 4 to 12% Bis-Tris gels (Invitrogen, Carlsbad, Calif.)

and electrophoresed according to the manufacturer's instructions. The proteins were blotted on polyvinylidene difluoride membranes from polyacrylamide gels using iBlot Dry Blotting System (Life Technologies, Paisley, United Kingdom). Membranes were incubated for 1 hour at room temperature in Odyssey Blocking Buffer (LI-COR Biosciences, Cambridge, United Kingdom). After the blocking step, membranes were incubated overnight at 4°C with the respective primary antibodies (**See Table, Supplemental Digital Content 3,** ***http://links.lww.com/PRS/A644***) followed by the secondary antibody incubation for 1 hour at 37°C. The polyvinylidene difluoride protein membranes were developed using Amersham ECL prime Western blotting detection kit (GE Healthcare, Buckinghamshire, United Kingdom). The membranes were exposed to Kodak X-OMAT x-ray film (Sigma-Aldrich). Densitometry was performed with ImageJ software (National Institutes of Health, Bethesda, Md.), and the data were analyzed with Prism software (GraphPad Software, Inc., San Diego, Calif.). The optical densities of Hsp27, α2β1-

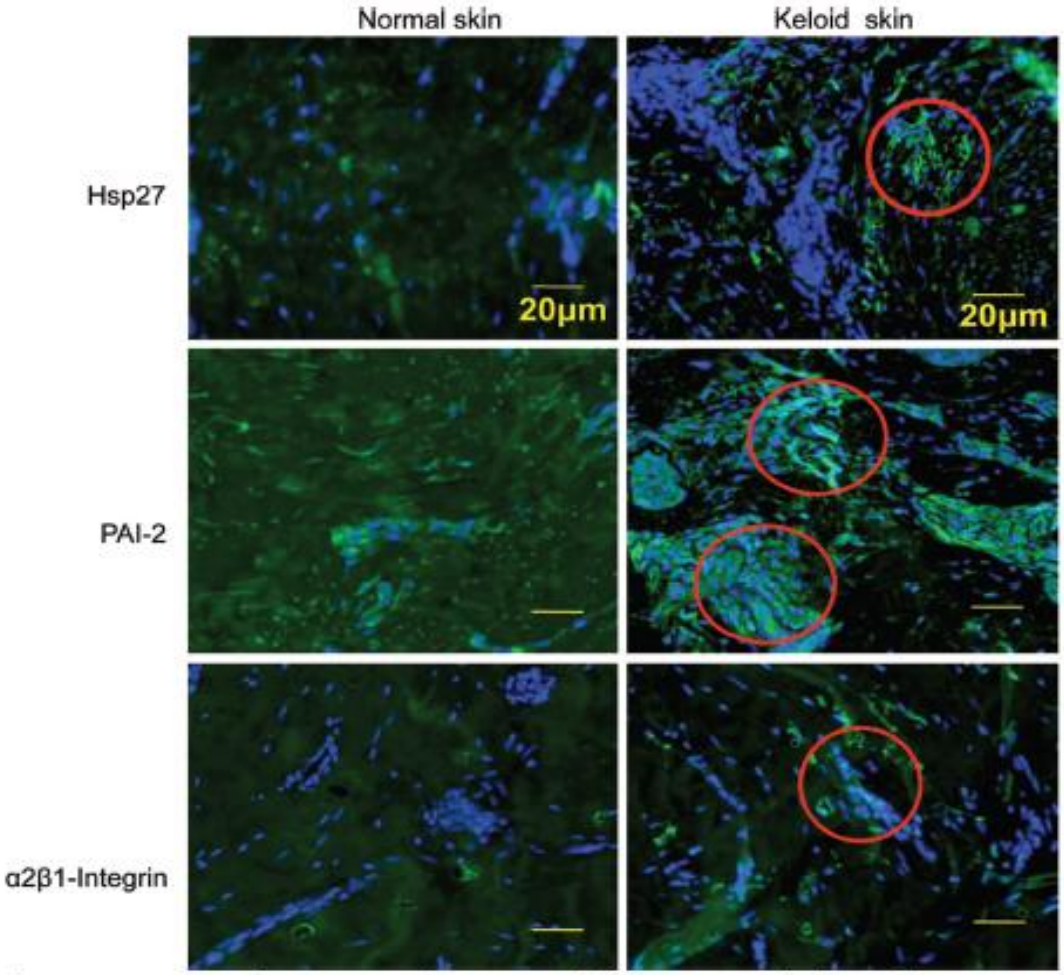

Fig. 2. Expression pattern of Hsp27, PAI-2, and α2β1-Integrin in keloid tissue compared with normal skin. Five-micron tissue sections were stained for each marker. An overexpression of Hsp27, PAI-2, and α2β1-Integrin was observed in keloid reticular dermis when compared with normal skin.

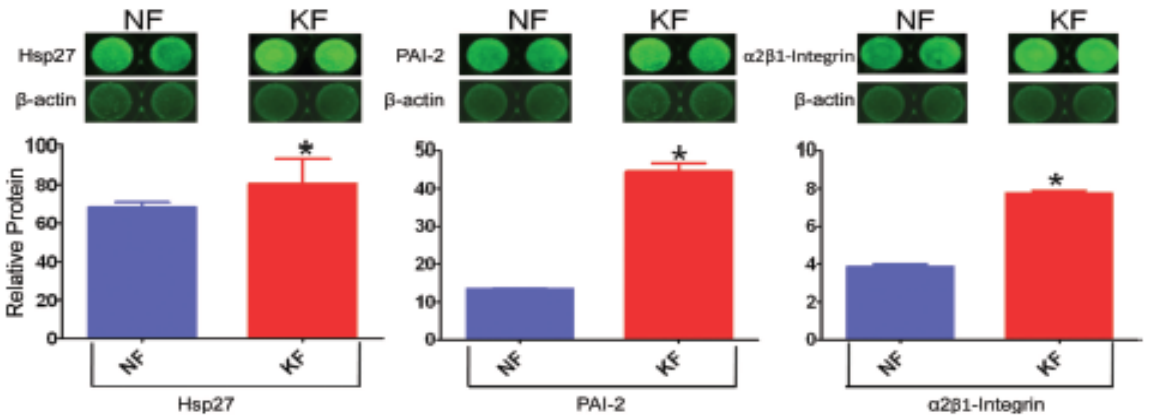

Fig. 3. Hsp27, PAI-2, and α2β1-integrin protein expression levels in keloid fibroblasts. Serum-starved keloid fibroblasts were seeded into 96-well plates (1×10^4 cells/well) and allowed to grow for 24 hours. The cells were fixed with 4% formaldehyde/phosphate-buffered saline. After blocking, Hsp27, PAI-2, and α2β1-integrin were revealed by incubation with their respective antibodies, followed by incubation with IRDye-800 (LI-COR)–labeled secondary antibodies. A representative output infrared image of keloid fibroblasts for tension-related proteins (*green*) from 96-well plates is shown (*above*). Bar graphs represent the quantification of expression of Hsp27, PAI-2, and α2β1-integrin, normalized to the β-actin loading control, from three independent experiments. The results are expressed as mean ± SEM. *$p < 0.05$ indicates a significant difference in protein up-regulation when compared with their relative expression in fibroblasts from normal skin.

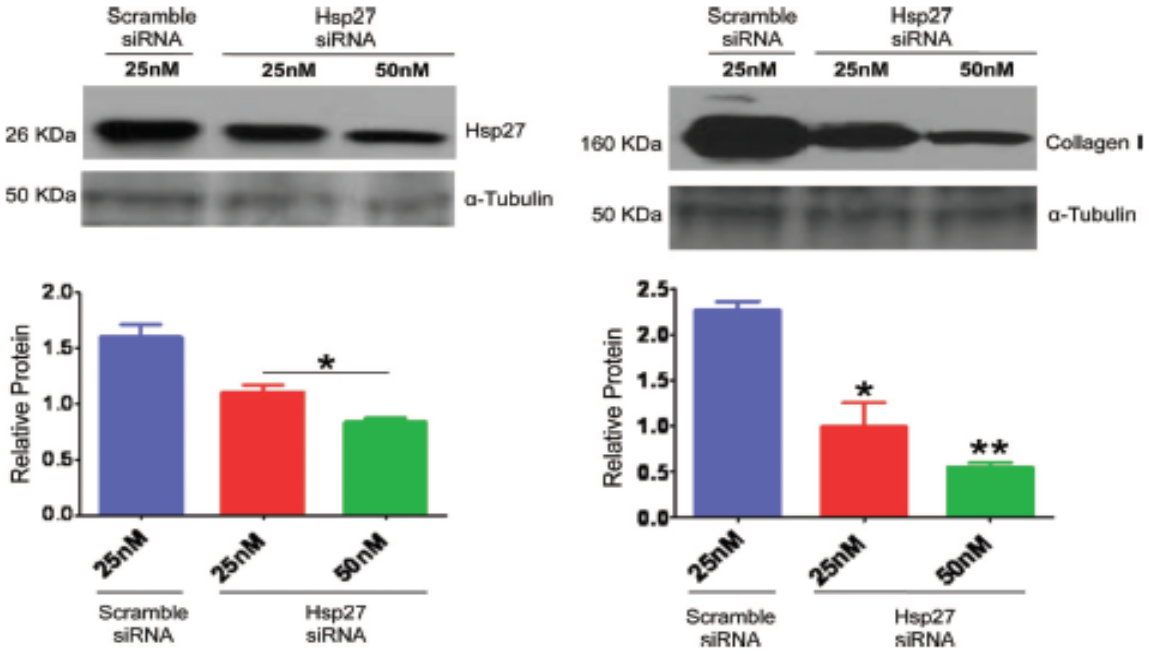

Fig. 4. Analysis of Hsp27 and collagen I protein expression in keloid fibroblasts following Hsp27/siRNA transfection. Keloid fibroblasts ($n = 6$) were transfected with HSp27/siRNA (25 nM and 50 nM). Forty-eight hours after transfection, proteins were extracted and subjected to Western blot analysis. (*Above*) A typical autoradiogram of protein expression. Bar graphs represent densitometry analysis of average protein expression from three experiments, normalized to α-tubulin. Data are expressed as mean ± SEM. *$p < 0.05$ indicates a significant difference between scrambled siRNA versus the Hsp27/siRNA-transfected group.

integrin, PAI-2, and collagen I were normalized to α-tubulin.

Cell Viability/Metabolic Activity Detection (Water-Soluble Tetrazolium Salt-1)

At 48 hours after siRNA transfection, cell viability/metabolic activity was tested using water-soluble tetrazolium salt-1 assay (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as the mean ± SEM of at least three independent experiments. To determine statistical differences, a one-way analysis of variance GraphPad Prism 4 software was used for a Tukey multiple comparison posttest. The

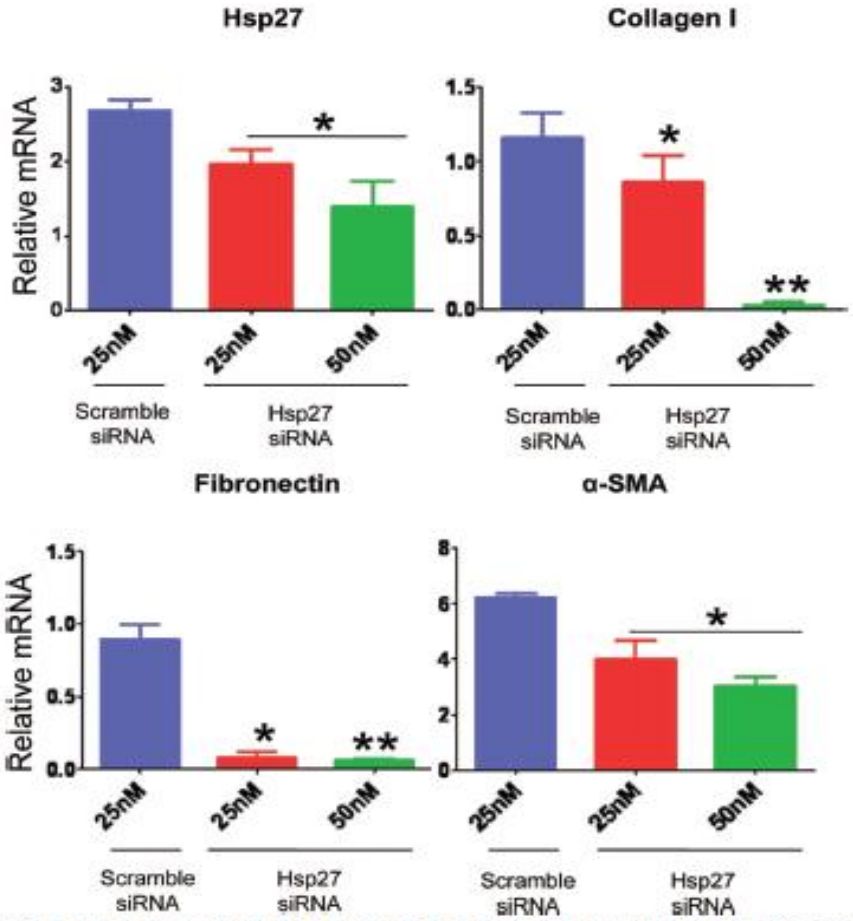

Fig. 5. Effect of knockdown of Hsp27 on keloid phenotypic markers. After 48 hours of Hsp27/siRNA transfection (25 nM and 50 nM), total RNA was extracted from keloid fibroblasts ($n = 6$) (passages 0 to 3). The expression of Hsp27, collagen I, fibronectin, and α-smooth muscle actin mRNA was determined by quantitative reverse-transcriptase polymerase chain reaction. The expression of the above markers was normalized to RPL32, a housekeeping gene. The results are expressed as mean ± SEM of triplicates from three independent experiments. *$p < 0.05$ indicates significant difference between mRNA expression in scrambled siRNA versus mRNA expression in the Hsp27/siRNA group.

difference between the means for all conditions was considered statistically significant at $p < 0.05$.

RESULTS

Differential Expression of Tension-Related Genes in Keloid Fibroblasts Compared with Fibroblasts from Normal Skin

Twenty-one skin-tension–related candidate genes (Table 3) that have been reported to be significantly up-regulated ($p < 0.01$) during the wound healing process in gene expression studies using cDNA microarray technology[17,29–31] were chosen to perform a comprehensive analysis of their association with keloid disease at mRNA and protein levels. For this purpose, keloid tissue samples from the sternum were compared with normal skin from the same anatomical site by quantitative reverse-transcriptase polymerase chain reaction. At the mRNA level, results showed that of 21 genes screened, a subset of 14 genes were up-regulated (including $\alpha2\beta1$-integrin, Hsp27, Hsp70, PAI-2, and tenascin XB) and seven genes were down-regulated in keloid tissue compared with normal skin tissue samples including Hsp60, matrix metalloproteinase 19, and matrix metalloproteinase 3, among others; see Table 3. For further validation, the expression of the 14 up-regulated genes in keloid tissue was investigated in fibroblasts isolated from keloid and normal skin biopsy specimens taken from the same patients. The quantitative reverse-transcriptase polymerase chain reaction results showed significant up-regulation of Hsp27, PAI-2, and $\alpha2\beta1$-integrin in all of the tested samples, consistent with the results obtained from the keloid tissue (Fig. 1). The protein expression was also investigated for these three genes at the tissue and cellular levels by immunohistochemistry and in-cell Western blotting, respectively. A significant ($p < 0.05$) overexpression of Hsp27, PAI-2, and $\alpha2\beta1$-integrin was observed in keloid samples when compared with normal skin (Fig. 2), and significant overexpression of these three proteins—Hsp27, PAI-2, and $\alpha2\beta1$-integrin—was also observed in keloid fibroblasts compared with fibroblasts from normal skin (Fig. 3). Moreover, Hsp70 and tenascin XB were

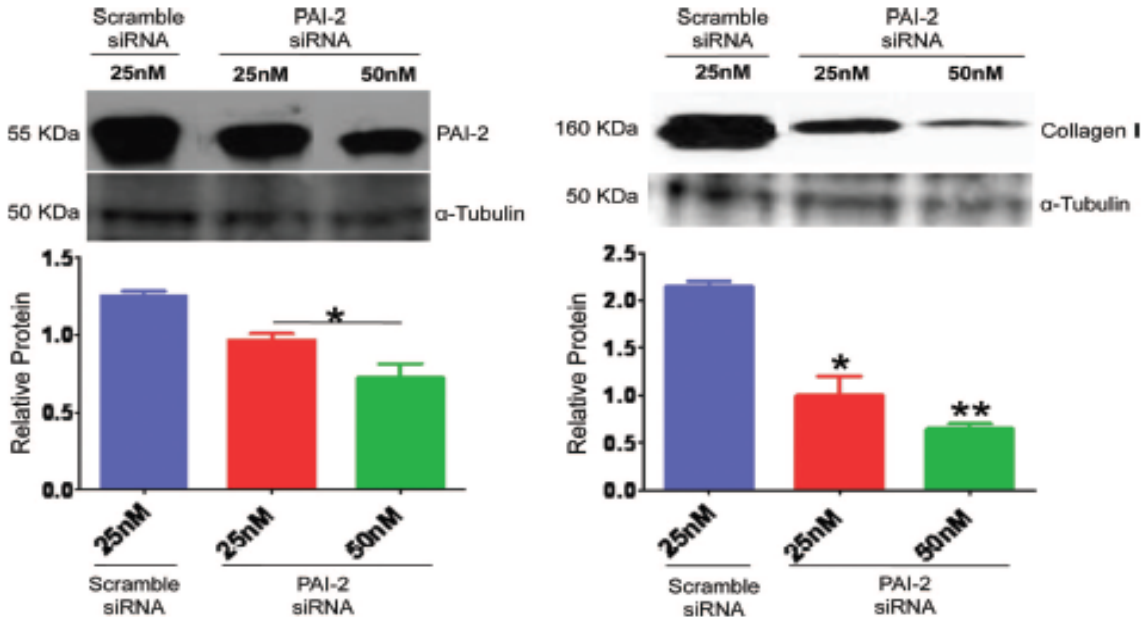

Fig. 6. Analysis of PAI-2 and collagen I protein levels in keloid fibroblasts following siRNA transfection. Proteins were extracted from keloid fibroblasts 48 hours after PAI-2/siRNA transfection and then subjected to Western blot analysis. (*Above*) A typical autoradiogram of protein expression. *Bar graphs* represent the average densitometry values of PAI-2 and collagen I protein expression, normalized to α-tubulin, from three independent experiments. Data are expressed as mean ± SEM. *$p < 0.05$ indicates a significant difference between the scrambled siRNA–transfected versus the PAI-2/siRNA–transfected group.

not statistically significant ($p > 0.05$) at the protein level in keloid fibroblasts compared with fibroblasts from normal skin (data not shown).

To further explore the role of overexpression of these tension-related genes, which are considered to promote excessive extracellular matrix deposition in keloid fibroblasts, we examined the alteration of extracellular matrix components expression following inhibition of Hsp27, PAI-2, and $\alpha 2\beta 1$-integrin expression by using an RNA interference technique.

Effect of Hsp27 on Extracellular Matrix Expression in Primary Keloid Fibroblasts

To achieve a better understanding of the influence of the Hsp27 expression in keloid fibroblasts on extracellular matrix production, a knockdown of Hsp27 was performed using the small interference RNA (siRNA) technique. Transfection of Hsp27/siRNA significantly down-regulated Hsp27 at mRNA and protein levels ($p < 0.05$) compared with the vehicle

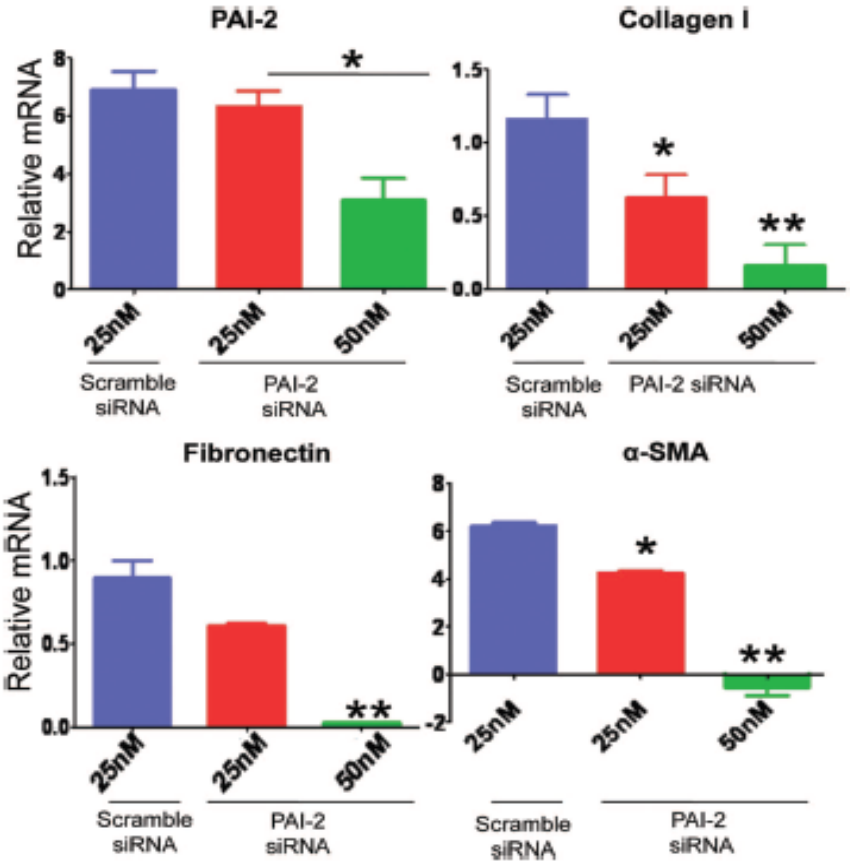

Fig. 7. Inhibition of PAI-2 by siRNA down-regulates extracellular matrix in primary keloid fibroblasts. Forty-eight hours after PAI-2/siRNA transfection, total RNA was extracted from keloid fibroblasts ($n = 6$, passages 0 to 3). The expression of PAI-2, collagen I, fibronectin, and α-smooth muscle actin was examined by quantitative reverse-transcriptase polymerase chain reaction. The expression of extracellular matrix components was normalized to RPL32. The results are expressed as mean ± SEM of triplicates from three independent experiments ($n = 6$). *$p < 0.05$ indicates a significant difference between mRNA expression in scrambled siRNA–transfected versus mRNA expression in the PAI-2/siRNA–transfected group.

(scrambled siRNA) group in a dose-dependent manner in keloid fibroblasts. To understand the effect of Hsp27 knockdown on extracellular matrix expression, we further assessed collagen I, fibronectin, and α-smooth muscle actin expression levels in keloid fibroblasts. Interestingly, collagen I was significantly down-regulated at the mRNA and protein levels ($p < 0.03$) (Fig. 4), in a dose-dependent manner, in Hsp27 knockdown keloid fibroblasts. Similarly, fibronectin and α-smooth muscle actin (Fig. 5) were significantly reduced in a dose-dependent manner following Hsp27 knockdown in keloid fibroblasts. Therefore, we concluded that inhibition of overexpressed Hsp27 showed significant reduction in extracellular matrix in keloid fibroblasts.

Alteration of Collagen I Expression in Primary Keloid Fibroblasts Following PAI-2 Knockdown

Importantly, PAI-2 was up-regulated in keloid fibroblasts compared with fibroblasts from normal skin; thus, we evaluated the molecular effect of PAI-2 on extracellular matrix metabolism in keloid fibroblasts. Forty-eight hours after transfection, the PAI-2 expression was significantly ($p < 0.05$) reduced at the mRNA and protein levels in keloid fibroblasts in a dose-dependent manner. Down-regulation of PAI-2 significantly reduced collagen I production at the mRNA and protein levels (Fig. 6). We also assessed the molecular effect of PAI-2 on fibronectin and α-smooth muscle actin at the mRNA level, which showed a significant dose-dependent reduction in fibronectin and α-smooth muscle actin (Fig. 7). Therefore, our results demonstrated that knockdown of PAI-2 reduced extracellular matrix deposition in keloid fibroblasts.

Knockdown of α2β1-Integrin Significantly Reduced Extracellular Matrix Deposition, Cell Attachment, and Mobility in Primary Keloid Fibroblasts

α2β1-Integrin is expressed in keloid fibroblasts, which may suggest its potential involvement in extracellular matrix rearrangement and depo-

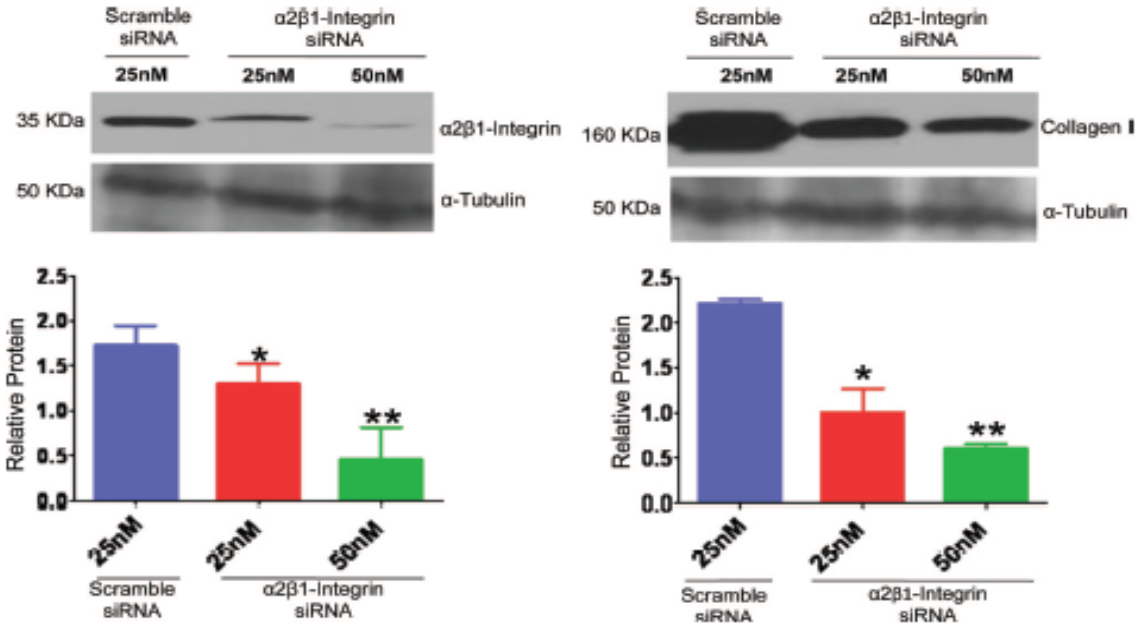

Fig. 8. Knockdown of α2β1-Integrin and collagen I expression at the protein level in keloid fibroblasts. Forty-eight hours after α2β1-Integrin/siRNA transfection, proteins were extracted from keloid fibroblasts ($n = 6$) and subjected to Western blotting. (*Above*) A typical autoradiogram of protein expression. *Bar graphs* show the average densitometry values for α2β1-Integrin and collagen I protein levels after being normalized to loading control α-tubulin. Data are expressed as mean ± SEM from three independent experiments. *$p < 0.05$ for the comparison between keloid fibroblasts scramble transfected versus keloid fibroblasts siRNA/PAI-2 transfected at two different concentrations.

sition in addition to influencing cell attachment and mobility.[1] Therefore, we evaluated the effect of knocking down $\alpha 2\beta 1$-integrin expression by siRNA transfection. Forty-eight hours after transfection, $\alpha 2\beta 1$-integrin mRNA and protein levels were significantly altered ($p < 0.05$) compared with the vehicle group (scrambled siRNA); importantly, collagen I was also strongly modified at the gene and protein levels (Fig. 8). To further investigate the influence of $\alpha 2\beta 1$-integrin on extracellular matrix genes, we tested the effect of $\alpha 2\beta 1$-integrin inhibition on the expression of fibronectin and α-smooth muscle actin, showing significant reduction of the mRNA levels ($p < 0.05$) (Fig. 9). Our results demonstrated that the expression of $\alpha 2\beta 1$-integrin influenced cell attachment and mobility in keloid fibroblasts.

Knockdown of Hsp27, PAI-2, and $\alpha 2\beta 1$-Integrin at the mRNA and Protein Levels Does Not Influence Viability/Metabolic Activity of Keloid Fibroblasts

After siRNA transfection, viability/metabolic activity of keloid fibroblasts was determined by using

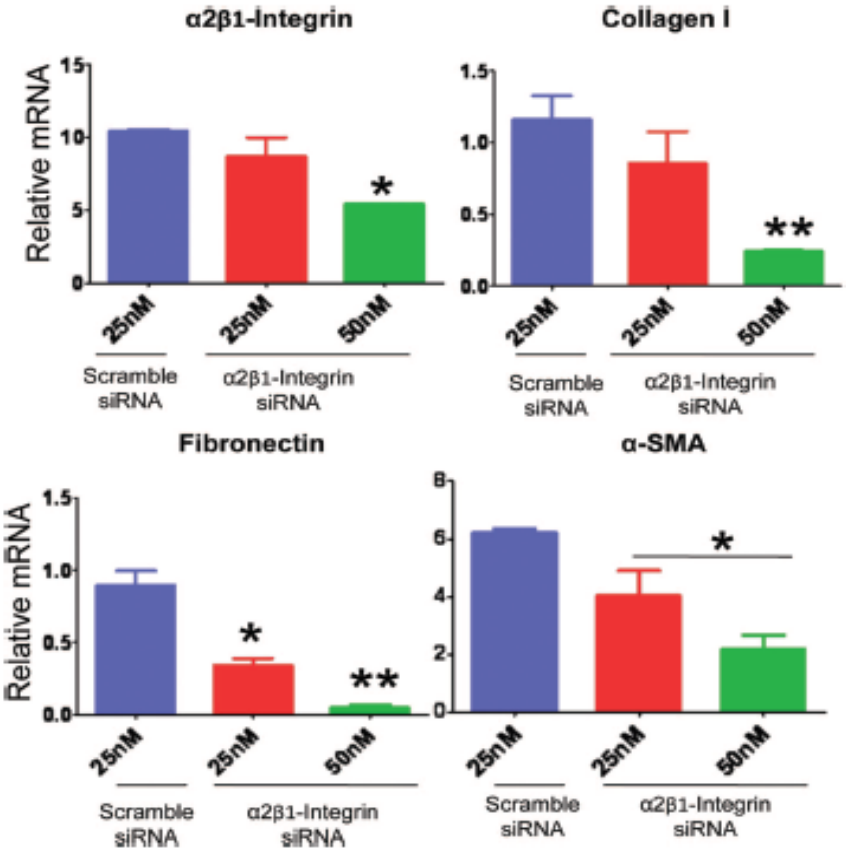

Fig. 9. Effect of knockdown of $\alpha 2\beta 1$-Integrin on extracellular matrix accumulation in primary keloid fibroblasts ($n = 6$) from passages 0 to 3 transfected with two different concentrations, 25 nM and 50 nM, respectively. Forty-eight hours after transfection, total RNA was extracted and examined by quantitative reverse-transcriptase polymerase chain reaction. The expression of extracellular matrix components was normalized to RPL32. The results were expressed as mean ± SEM of triplicates from two independent experiments. *A value of $p < 0.05$ indicates a significant difference between mRNA expression in scramble siRNA–transfected versus mRNA expression in the $\alpha 2\beta 1$-Integrin/siRNA–transfected group.

the water-soluble tetrazolium salt-1 assay. Interestingly, when compared with the vehicle group (scrambled siRNA), keloid fibroblasts showed no statistically significant difference in viability/metabolic activity at 48 hours after siRNA transfection in any of the groups. These results suggested that the suppression of Hsp27, PAI-2, or α2β1-integrin at both mRNA and protein levels had no significant influence on the viability and metabolic activity of keloid fibroblasts (Fig. 10).

DISCUSSION

In the present study, a number of tension-related genes were significantly expressed in keloids at the mRNA and protein levels when compared with normal skin. Moreover, the knockdown of the expression of Hsp27, PAI-2, and α2β1-integrin by siRNA attenuated not only the expression of mRNA and protein levels themselves but also extracellular matrix regulation in primary keloid fibroblasts. Our findings with Hsp27, PAI-2, and α2β1-integrin concur with previous studies that showed higher expression of these tension-related proteins in keloid fibroblasts.[1,7]

Mechanical tension induces α2β1-integrin activation, which serves as a transmembrane link between the extracellular matrix and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts.[1,6] Among the proteins implicated in this process, the focal adhesion kinase appears to be a key candidate. Wong et al. in 2011 described the role of focal adhesion kinase in the development of the fibrotic process following mechanical force stimuli of the extracellular-related kinase–monocyte chemoattractant protein-1 inflammatory pathway, which induced fibroblast activation and survival and extracellular matrix production.[15,32–34] The actin stabilization and polymerization is also mediated by α2β1-integrin.[1] Furthermore, it had been reported that α2β1-integrin regulates collagen degradation by the activation of matrix metalloproteinases.[35–37] In this study, after knocking down the expression of α2β1-integrin, we demonstrated its effect on the mRNA expression of extracellular matrix components, in addition to protein (Fig. 8) and mRNA (Fig. 9) levels of collagen I in keloid fibroblasts.

The mechanotransduction signal also activates Hsp27 by means of p38 mitogen-activated protein kinase.[38,39] The heat shock protein activation promotes actin stabilization by unknown mechanisms and focal contact formation, cell contraction, migration, and survival.[19,21,38–41] The collagen degradation is also regulated by PAI-2, which negatively regulates matrix metalloproteinases, leading to extracellular matrix accumulation. PAI-2 is also involved in cell migration mediated by integrins.[42,43] In the present study, we

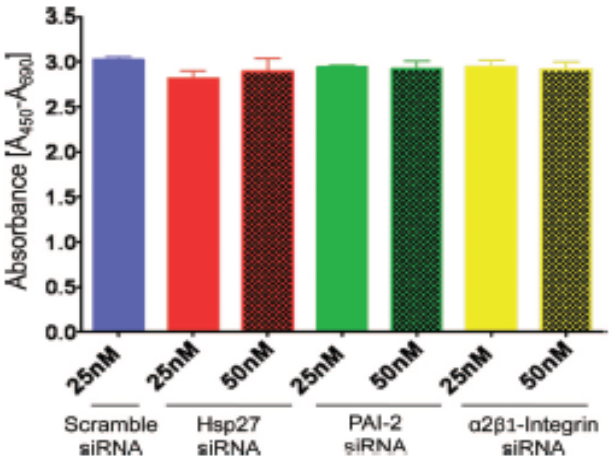

Fig. 10. Effect on viability/metabolic activity of primary keloid fibroblasts following suppression of Hsp27, α2β1-integrin, and PAI-2. *Bar graphs* show the viability/metabolic activity of keloid fibroblasts, 48 hours after transfection with Hsp27, α2β1-integrin, and PAI-2/siRNAs, analyzed by water-soluble tetrazolium salt-1 assay. No statistical difference was detected.

show that targeting Hsp27 by siRNA down-regulated extracellular matrix in keloid fibroblasts. Consistent with our results, previous work has been shown that down-regulation of the mRNA expression of Hsp27 caused a significant decrease in the expression of collagen I.[39] We also showed that inhibition of PAI-2 significantly reduced the expression of collagen, fibronectin, and α-smooth muscle actin in a dose-dependent manner in keloid fibroblasts. To this end, α2β1-integrin and Hsp27 may be key upstream regulators of the extracellular matrix production, whereas PAI-2 regulates extracellular matrix degradation (Fig. 11).[32,44]

Wound healing is considered a complicated process that involves multiple phases. Interestingly, it has been suggested that keloid scars may develop following a dysregulation in the inflammatory phase, whereas other authors have suggested a prolonged proliferative phase.[45] Furthermore, keloids show heterogeneity, as lesions differ in appearance from patient to patient depending on the anatomical site.[14] Thus, we decided to limit our samples to one anatomical location (i.e., the sternum). However, future investigation, involving different anatomical locations and samples from different stages of keloid development, will be of value. Another potential limitation of our study was the total number of samples used for keloid ($n = 10$) and for normal skin ($n = 4$); however, a larger number of samples that include

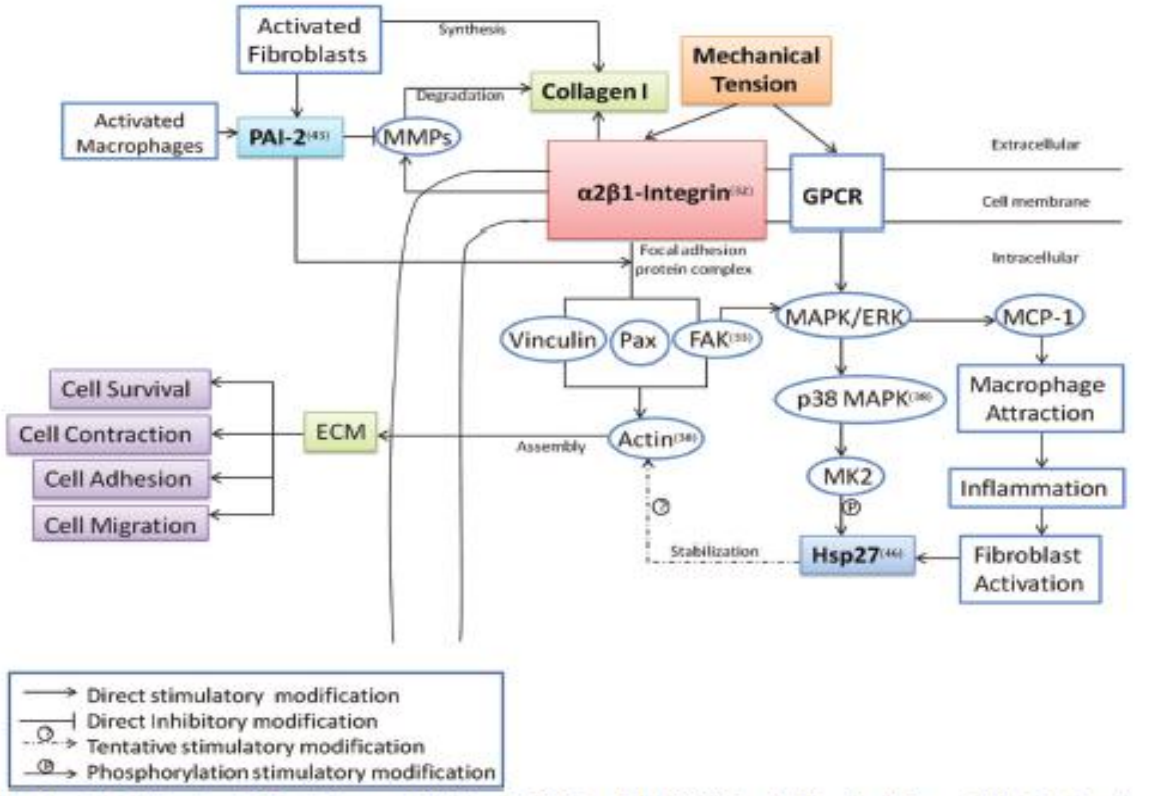

Fig. 11. Potential molecular links between α2β1-integrin, PAI-2, and Hsp27. Mechanical tension induces α2β1-integrin activation, which serves as a transmembrane link between the extracellular matrix and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts. The mechanotransduction signal also activates Hsp27 by means of p38 mitogen-activated protein kinase. PAI-2 promotes extracellular matrix accumulation by negative regulation of matrix metalloproteinases. PAI-2 is also linked with the regulation of cell migration mediated by integrins. *ECM*, extracellular matrix; *Pax*, paxillin-related proteins; *FAK*, focal adhesion kinase; *MAPK*, mitogen-activated protein kinase; *ERK*, extracellular signal-regulated kinase; *p38 MAPK*, p38 mitogen-activated protein kinases; *MK2*, MPAK-activated protein kinase 2; *MCP-1*, monocyte chemotactic protein 1; *GPCR*, G-protein coupled receptor.

different ethnicities can be more beneficial. Our research represents a preliminary but detailed study focused on the identification of mechanosensory genes related to keloid disease from a specific anatomical location (the sternum) obtained at one time point in mature keloid scars in steady-state conditions. To this end, there is insufficient information to provide a clearer explanation for when these genes are up-regulated during the healing process. However, based on previous reports, we can speculate that these genes are potentially activated during the earlier stages of wound healing.[16–18] This unique study may offer a potential therapeutic option based on RNAi, providing the possibility of an endogenous and specific mechanism of gene silencing for keloid disease as described previously for other diseases.[19] The RNAi-based therapy in animal models has also shown benefits in several diseases involving lung, subcutaneous tissue, muscle, eye, and the nervous system.[50]

A potential mechanism to explain the pathobiology of keloid scar formation under tension may be postulated based on the findings of this preliminary study. After tissue damage occurs, mechanical stress produced during the wound healing process in the injured area promotes cell activation, proliferation, differentiation, and up-regulation of several tension-related proteins, including Hsp27, PAI-2, and $\alpha 2\beta 1$-integrin. This is followed by an excessive extracellular matrix production and deposition in the keloid-susceptible individual. The increased mechanical tension exhibited in the injured area promotes the estab-

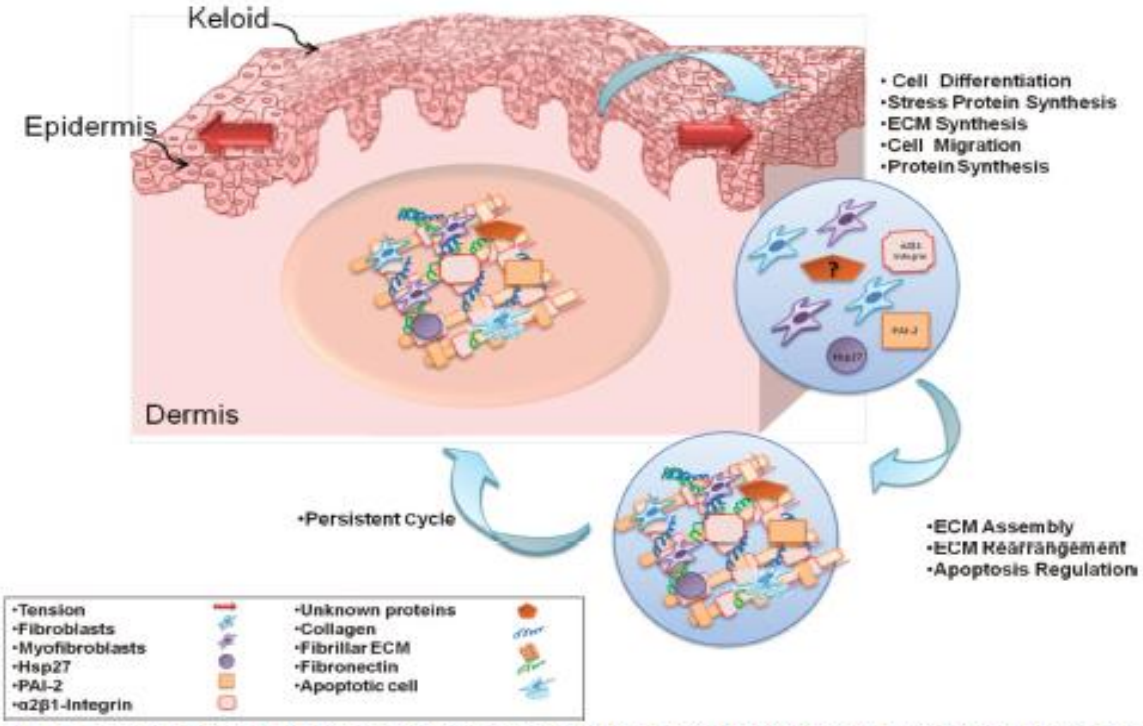

Fig. 12. Proposed mechanism for the possible role of tension-related proteins in keloid scarring. After tissue damage, mechanical stress placed on the tissue in specific anatomical sites (i.e., sternum) during the wound healing process promotes fibroblast activation. In response to tension, activated fibroblasts synthesize increased levels of stress proteins, certain extracellular matrix components, and α-smooth muscle actin. In this context, stress proteins including Hsp27, PAI-2, and $\alpha 2\beta 1$-integrin may promote cell protection against apoptosis and extracellular matrix component rearrangement and synthesis. Furthermore, mechanical stress may promote differentiation of fibroblasts into myofibroblasts, which also synthesize stress proteins, contributing to extracellular matrix component synthesis and deposition in keloids. High production of extracellular matrix components may promote mechanical stress and support the establishment of a persistent cycle.

lishment of a continuous cycle that increases the activation of these tension-related genes in the wounded area, resulting in the formation of a keloid in the susceptible individual (Fig. 12).

CONCLUSIONS

Our study has shown an overexpression of specific tension-related genes and proteins in keloid fibroblasts and tissue. Knockdown of the expression of Hsp27, PAI-2, and $\alpha 2\beta 1$-integrin by siRNA attenuated the expression of not only mRNA and proteins levels themselves but also the mRNA and protein levels of key molecules related to extracellular matrix regulation in keloid fibroblasts. This study offers options for a potential therapeutic application of RNAi to modify tension-related proteins for future treatment of keloid disease. At this point, we can speculate that the most promising molecular candidate in the regulation of keloid scars by mechanical induction may be $\alpha 2\beta 1$-integrin, although further experiments are certainly required to provide a better understanding of the mechanosignaling pathway regulation in keloid scars.

Ardeshir Bayat, M.B., B.S., Ph.D.
Plastic and Reconstructive Surgery Research
School of Translational Medicine
Manchester Institute of Biotechnology
University of Manchester
131 Princess Street
Manchester M1 7DN, United Kingdom
ardeshir.bayat@manchester.ac.uk

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Supplementary Table S1.

Gene name (Gene symbol)	Biological function	Chromosomal location
α2β1-Integrin (ITGA2)	Cell adhesion, migration, and signalling by providing transmembrane links between the extracellular matrix and the cytoskeleton Interactions between fibroblasts and Collagen I[138, 236]	5q11.2
Actin, alpha 1, skeletal muscle (ACTA1, α-SMA)	Myofibriblasts marker. Fibroblasts contraction[237]	1q42.13
Calcitonin-related polypeptide alpha (CALCA, CGRP)	Up regulation of TGF-B (Controls proliferation, cellular differentiation) and Vasodilator[181]	11p15.2
Chemokine (C-C motif) ligand 2 (CCL2; MCP-1)	Influence collagen fibre formation *in-vivo*[186]	17q11.2-q12
Collagen, type I, alpha 1 (COL1A1)	Main structural element of the ECM, forming a relaxed network of cross-linked fibres throughout the dermis to maintain tissue integrity[238]	17q21.33
Cysteine-rich, angiogenic inducer, 61 (Cyr61)	Mediates cell adhesion, Induces adhesive signalling; Promotes fibroblasts survival[239]	1p22.3
Fibronectin 1 (FN1)	ECM rearrangement[197-199]	2q34
Heat shock protein 27 (Hsp 27, HSPB1)	Protein folding, actin binding proteins[174]	7q11.23
Heat shock protein 60 (HSPD1; Hsp 60)	Protein folding and mycobacterial unfolding, organelle translocation[174]	2q33.1
Heat shock protein 70 (HSPA1A; Hsp 70)	Enhanced resistance to ischemic injury[174]	6p21.3
Heat shock protein 90kDa alpha (cytosolic), class A member 1; Heat shock protein 90 (HSP90AA1; Hsp 90)	Maintenance of proteins such as steroid receptors in an inactive form until appropriate[174]	14q32.33
Hyaluronan and proteoglycan link protein 1 (HAPLN1)	Maintaining the integrity and support the functions of the ECM[240]	5q14.3
Hyaluronan and proteoglycan link protein 2 (HAPLN2)	Maintaining the integrity and support the functions of the ECM[240]	1q23.1

Matrix metallopeptidase13 ;Collagenase 3 (MMP-13)	Key role in degradation of the collagen matrix[241]	11q22.3
Matrix metallopeptidase 19 (MMP-19)	Extracellular matrix degradation[9]	12q14
Matrix metallopeptidase 3; Stromelysin 1, progelatinase (MMP-3)	Wound Contraction and ECM degradation. Collagen catabolism[165]	11q22.3
Neuropeptide Y (NPY)	Induce keratinocytes proliferation. Neovascularisation. Angiogenesis[242]	7p15.1
Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2; PAI-2)	ECM remodelling. ECM degradation inhibitor. Cell protection against apoptosis[98, 168]	18q21.3
Serpin peptidase inhibitor, clade H; Heat shock protein 47 (Hsp 47, SERPINH1)	Closely related with fibrosis, Collagen specific molecular chaperone[174]	11q13.5
Substance-P Tachykinin, precursor 1 (TAC1, SP)	Keratinocytes and fibroblasts proliferation. Cell proliferation[170]	7q21-q22
Tenascin XB (TNXB)	ECM similar to fibronectin that may promote wound healing[203]	6p21.3
Testis derived transcript (3 LIM domains) (TES)	Focal adhesion and cell-cell communication[204]	7q31.2
Transforming growth factor, beta 1 (TGF-β1)	Fibroblast differentiation. ECM synthesis[237]	19q13.2; 19q13.1
Transforming growth factor, beta 2 (TGF-β2)	Increase cell proliferation in keloids[165]. Anti-apoptotic function[243]	1q41
Transforming growth factor, beta 3 (TGF-β3)	Down regulate scarring and fibrosis *in-vivo*[243]	14q24

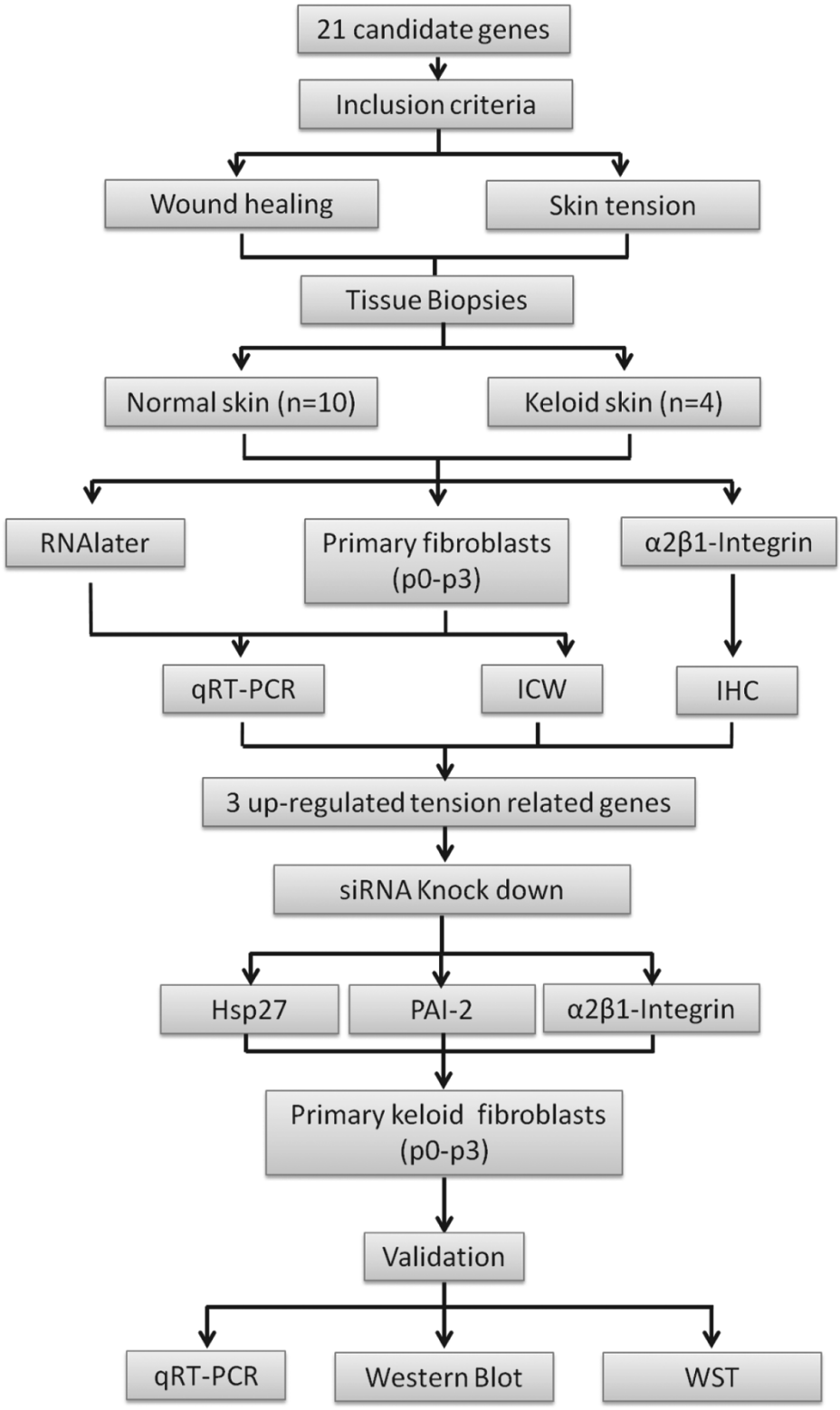
Supplementary Table S2.

Gene/Primer	Gene ID	Sequence 5' to 3'	Primer Position	Amplicon Size (bp)
α-SMA-L	nm_001613.1	ctgttcagccatccttcat	834 - 853	70
α-SMA-R	nm_001613.1	tcatgatgctgttgtaggtggt	882 - 903	70
α2β1 integrin-L	nm_002203.3	gcaggacagaaatcacagttca	1427 - 1448	78
α2β1 integrin-R	nm_002203.3	gcaacaaagtgagtgctttctc	1483 - 1504	78
CGRP-L	nm_001033953.2	cccaccaaggtgtatgcaa	668-686	60
CGRP-R	nm_001033953.2	cccataaggaaacccagagc	708-727	60
Collagen I-L	nm_000088.3	gggattccctggacctaaag	1866 - 1885	63
Collagen I-R	nm_000088.3	ggaacacctcgctctcca	1911 - 1928	63
Cyr61-L	nm_001554.4	ccagtgtacagcagcctgaa	1047 - 1066	110
Cyr61-R	nm_001554.4	ggccggtatttcttcacactc	1136 - 1156	110
Fibronectin-L	nm_212482.1	gccactggagtctttaccaca	3498 - 3518	60
Fibronectin-R	nm_212482.1	cctcggtgttgtaaggtgga	3539 - 3558	60
HAPLN1-L	nm_001884.3	tgctcagcatgggttatgc	1072 - 1090	63
HAPLN1-R	nm_001884.3	agggcgtctctgagtagcag	1115 - 1134	63
HAPLN2-L	nm_021817.2	cgactttatgaccaagagctga	512 - 533	126
HAPLN2-R	nm_021817.2	gccaacaatataagcagctgtg	616 - 637	126
Hsp27-L	nm_001540.3	tccctggatgtcaaccactt	447 - 466	111
Hsp27-R	nm_001540.3	gatgtagccatgctcgtcct	538 - 557	111
Hsp47-L	nm_001235.3	gcgggctaagagtagaatcg	122 - 141	110
Hsp47-R	nm_001235.3	atggccaggaagtggtttg	213 - 231	110
Hsp60-L	nm_199440.1	ggtcttcaggttgtggcagt	997 - 1016	119
Hsp60-R	nm_199440.1	ttcagggtcaatccctcttc	1096 - 1115	119
Hsp70-L	nm_005345.5	cagcagacaccagcagaaaa	1887 - 1906	66
Hsp70-R	nm_005345.5	cttggatccagcttgagagg	1933 - 1952	66
Hsp90-L	nm_005348.3	gggcaacacctctacaagga	661 - 680	76

Hsp90-R	nm_005348.3	cttgggtctgggtttcctc	718 - 736	76
MCP-1-L	nm_002982.3	tcaaactgaagctcgcactc	43 - 62	126
MCP-1-R	nm_002982.3	gtgactggggcattgattg	153 - 171	129
MCP-3-L	nm_006273.2	gcacttctgtgtctgctgct	86 - 105	129
MMP-3-R	nm_002422.3	ctcttgggtatccagctcgt	1169 - 1188	81
MMP-13-L	nm_002427.3	cctggacaagtagttccaaagg	645 - 666	128
MMP-13-R	nm_002427.3	gccggtgtaggtgtagatagga	751 - 772	128
MMP-19-L	nm_002429.2	atgccagacccttgcagtag	974 - 993	76
MMP-19-R	nm_002429.2	ccccccttgaaagcataggtc	1030 - 1049	76
PAI-2-L	nm_002575.2	catggagcatctcgtccac	428 - 446	109
PAI-2-R	nm_002575.2	actgcattggctcccactt	518 - 536	109
RPL32-L	nm_000994.3	gaagttcctggtccacaacg	319 - 338	76
RPL32-R	nm_000994.3	gagcgatctcggcacagta	377 – 395	76
SP-L	nm_003182.2	gtttgaaggtgtgggttggt	399-418	63
SP-R	nm_003182.2	accctcttctccctgaatcc	442-461	63
Tenascin-L	nm_019105.6	agggagacttccctgtcctg	103 - 122	119
Tenascin-R	nm_019105.6	catactgggctggcatcatt	202 - 221	119
TGF-β1-L	nm_000660.3	gcagcacgtggagctgta	1362 - 1379	64
TGF-β1-R	nm_000660.3	cagccggttgctgaggta	1408 - 1425	64
TGF-β2-L	nm_003238.1	ccaaagggtacaatgccaac	1194 - 1213	114
TGF-β2-R	nm_003238.1	cagatgcttctggatttatggtatt	1283 - 1307	
TGF-β3-L	nm_003239.2	gggctttggacaccaattac	1755 - 1774	125
TGF-β3-R	nm_003239.2	ggcatagtagcccttaggttca	1858 - 1879	125

Supplementary Table S3.

Antibody	Raised Species	Isotype	Clone	Dilution	Product Code	Source
α-Tubulin	Mouse monoclonal	IgG1k	DM1A	1:500	Ab7291	Abcam
β- actin	Rabbit polyclonal	IgG	-	1:200	Ab112053	Abcam
α2β1 Integrin	Mouse monoclonal	IgG1	16B4	1:500	Ab30483	Abcam
Collagen I	Rabbit polyclonal	IgG	-	1:500	Ab59435	Abcam
Hsp 27	Mouse monoclonal	IgG1	-	1:500	mAb 2402	Cell signaling technology
PAI-2	Rabbit polyclonal	IgG	-	1:500	Ab47742	Abcam


21 candidate genes
Inclusion criteria
Wound healing
Skin tension
Tissue Biopsies
Normal skin (n=10)
Keloid skin (n=4)
RNAlater
Primary fibroblasts (p0-p3)
α2β1-Integrin
qRT-PCR
ICW
IHC
3 up-regulated tension related genes
siRNA Knock down
Hsp27
PAI-2
α2β1-Integrin
Primary keloid fibroblasts (p0-p3)
Validation
qRT-PCR
Western Blot
WST

Appendix IV

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Wound Repair and Regeneration

YOUNG INVESTIGATOR AWARD

Skin equivalent tensional force alters keloid fibroblast behavior and phenotype

Edna Suarez, MSc[1,2,*]; Farhatullah Syed, PhD[1]; Teresa A. Rasgado, PhD[2]; Alan Walmsley, PhD[2]; Parthasarathi Mandal, PhD[3]; Ardeshir Bayat, MBBS, PhD[1,2,4]

1. Plastic & Reconstructive Surgery Research, Manchester Institute of Biotechnology,
2. Bioengineering Group, School of Materials,
3. Bioengineering Group, School of Mechanical, Aerospace and Civil Engineering, and
4. University Hospital of South Manchester NHS Foundation Trust, Faculty of Medical and Human Sciences, Institute of Inflammation and Repair, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK

Reprint requests:
Dr. Ardeshir Bayat, Manchester Institute of Biotechnology, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK.
Tel: +44 (0)161 306 5177;
Fax: ••;
Email: Ardeshir.Bayat@manchester.ac.uk

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ABSTRACT

Skin tension may influence keloid scar behavior, development, and spreading, e.g., butterfly-shaped keloid disease in the sternum. Here, we developed a three-dimensional (3D) in vitro model to mimic in vivo tension and evaluate keloid fibroblast (KF) behavior and extracellular matrix synthesis under tension. In vivo skin tension measured in volunteers ($n = 4$) using 3D-image photogrammetry enabled prediction of actual force (35 mN). A novel cell force monitor applied tension in a fibroblast-populated 3D collagen lattice replicating the in vivo force. The effect of tension on keloid ($n = 10$) and normal skin ($n = 10$) fibroblasts (NF) at set time points (6, 12, and 24 hours) was measured in *Hsp27*, *PAI-2*, and *α2β1* integrin, tension-related genes demonstrating significant ($p < 0.05$) time-dependent regulation of these genes in NF vs. KF with and without tension. KF showed higher ($p < 0.05$) proliferation post-tension. Knockdown of all three genes in 24 and 48 hours with and without tension showed significant down-regulation in NF vs. KF. Additionally, we show significant ($p < 0.05$) modification of the expression of extracellular matrix-related genes post-tension following down-regulation of *Hsp27*, *PAI-2*, or *α2β1* integrin. Finally, we demonstrate significant alteration in NF compared with KF morphology following knockdown. In conclusion, this study shows induction of tension-related genes expression following mechano-regulation in KFs, with potential relevance to its development and therapy.

Skin is subject to mechanical forces due to natural body movements.[1,2] It has been suggested that mechanical forces acting on certain anatomical sites under high tension, such as the sternum, may influence the development, both in terms of the behavior and appearance, of keloid scars.[3,4] Keloid disease (KD) is a benign yet an abnormal fibroproliferative lesion that is unique to humans and extends beyond the confines of the original wound margins, does not regress over time, and invades the surrounding unaffected skin.[5,6] The mechanism involved in the formation of keloid scars remains unknown and subsequently, its treatment remains ill-defined, with poor outcome and high recurrence.[7,8]

3D	Three-dimensional	MCP-1	Monocyte chemotactic protein-1
α2β1 integrin	alpha-2-beta-1 integrin	MMP	Matrix metalloproteinasis
α-SMA	alpha-smooth muscle actin	mRNA	Messenger RNA
CFM	Cell force monitor	NF	Normal skin fibroblast
ECM	Extracellular matrix	Nrp1	Neuropilin-1
ERK	Extracellular regulated kinase	p3	Passage 3
F	Force	PAI-2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2
FAK	Focal adhesion kinase		
FPCL	Fibroblast-populated collagen lattice	qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Hsp27	Heat shock protein 27	RPL32	Ribosomal protein L32
KD	Keloid disease	S-1	Sternum sagittal plane 1
KF	Keloid fibroblasts	S-2	Sternum sagittal plane 2
MAPK	Mitogen-activated protein kinase	siRNA	Small interfering RNA

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Mechanical tension can promote phenotypic alteration in fibroblasts during wound healing.[9] Skin mechano-regulation can induce dysregulation of the expression of several tension-related proteins such as cytokines, growth factors, integrins, and extracellular matrix (ECM) components during KD development.[10] A better understanding of the effects of skin mechano-regulation may provide alternatives for prevention of abnormal scar formation in certain anatomical sites prone to high tension.

In the absence of a reliable animal model for studying KD, research has focused on development of new experimental techniques and in vitro models in order to evaluate the effect of skin tension in repair.[11] To this end, three-dimensional (3D) models have become a useful tool in studying effects of mechanical tension on skin cells. Collagen type I-based 3D-models allow a 3D distribution of force to the cells. Moreover, the evaluation of phenomena such as cell communication, migration, differentiation, adhesion, apoptosis, among others between cells and ECM are evaluated in a closer manner to the in vivo condition. Several cell force monitors (CFMs) have been used to reproduce tension forces in 3D models. However, there is nonuniform distribution of tension in these study models.[12,13] The CFMs are focused on the quantification of the amount of force produced by the cells while contracting the matrix,[14,15] although the amount of force applied to the system (in order to reproduce the normal tension forces in the body and activate the cells populating the gels) is variable. The correlation between the load used and the normal loading conditions in the body is not provided. Therefore, a device able to reproduce the amount of tension generated in vivo and to provide distribution of the tension all over the model is required.

The genes employed in this study (*Hsp27*, *α2β1* integrin, *PAI-2*) were selected from our previous findings,[16] where convincing up-regulation of tension-related genes was shown in keloid fibroblasts (KFs) and tissue after analyzing 21 tension-related candidate genes. These were selected from three pooled gene expression microarray studies[16] for their relationship with skin tension and healing.

In this study, in vivo and in vitro techniques were combined to develop a 3D model to mimic in vivo tension on cells and furthermore to compare the effect of mechanical tension on normal skin fibroblast (NF) and KFs.

MATERIALS AND METHODS

In vivo skin force measurement using photogrammetric analysis

The study was performed in healthy male volunteers ($n = 4$), aged between 25 and 30 years old. Full ethical approval was obtained from the University of Manchester Research Ethics Committee (ref 12331), prior to start of the study. Speckle pattern created using black paint on the participant's sternum was captured during five cycles of maximal inspiration and expiration. The images were taken using two Phantom high-speed cameras (model v7, Vision Research, Wayne, NJ), angled no more than 25° with respect to each other. The surface movement occurring in the skin was analyzed using ARAMIS (GOM mbH, Braunschweig, Germany) software.

Figure 1A shows a representative image acquired and processed using ARAMIS software, which determines surface strain by tracking the movement of surface elements in 3D. The surface strain on the skin during the performance of normal breathing was measured in four participants. Surface strain along two cross-sections was determined from the point-to-point displacement during the breathing cycle. The first section corresponded to skin deformation between the top and bottom of the sternum in a sagittal plane (S-1). The second section indicated deformation along the line joining the nipples in a transverse plane (S-2). The intersection of these two sections was denoted as point "P".

The minimum strain observed at the intersection of these two sections was used to calculate the tensile stress in the skin

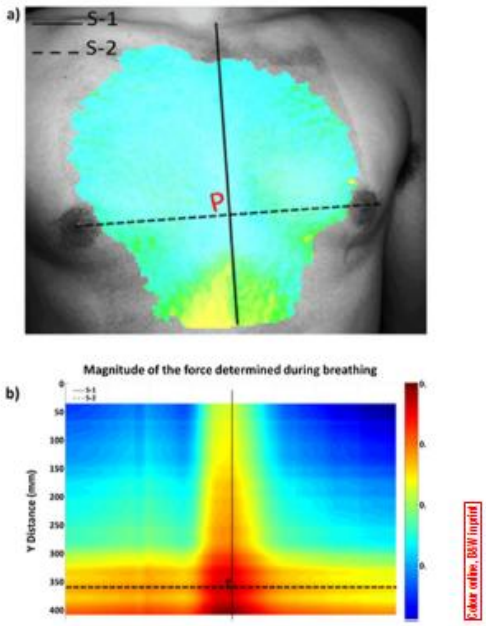

Figure 1. In vivo test and novel cell force monitor (CFM) setup: (A) Image taken from the sternum of a healthy male volunteer during the performance of the in vivo test. Two cross-sections were created, the first one is from the top to the bottom of the sternal area to measure skin deformation in the midline in the mid-axial plane, the second line measures skin deformation along the nipple line in the transverse plane. (B) Image shows surface plot of the average force calculated within the area described by the axes. The pattern of color shows the forces calculated during the test. The largest tension forces are denoted in red, whereas the lowest values appear in blue, as defined by the color bar. (C–D) CFM-detailed diagram and components.

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at point P using a value for the elastic modulus of skin from the literature (3.25 KPa)[17,18] and the square of the average skin thickness (0.2 mm).[7] The average skin thickness was considered constant in order to reduce the number of factors that might affect our calculations (the volunteers had similar build in the chest area, had similar weight, and were of the same ethnic background). The apparent skin tension was estimated from the calculated stress and the calculated cross-sectional area (0.04 mm^2) using $F = \varepsilon EA$. Using the values indicated, a minimum skin tension of 39 mN was measured, which was the basis for the force applied to the collagen gel. The values obtained were interpolated and a contour graph was created as shown in Figure 1B; the positions of the sections S-1 and S2 are also shown on this figure.

Culture force monitor design

The in vivo tension force was replicated in vitro using a novel, in-house-developed CFM. Uniformity in the tension field was ensured using an appropriate size and shape of collagen-cell gel matrix (please see supplementary information Figure S2). Please see Supplementary Materials and Methods 1 for detailed description of this apparatus.

Patient data and tissue collection

Keloid ($n = 10$) and normal skin samples ($n = 10$) (please see supplementary Table S1) were obtained following informed consent from all patients (full ethical approval was obtained from the local hospital, university, and regional National Health Service Ethics Committee in England, UK) prior to surgery. All keloid patients were confirmed to have clinical and pathological evidence of KD as described previously.[6] Briefly, a scar was considered as keloid if the lesion showed typical phenotypic behavior with invasion into the boundary of the original lesion, which had been present for more than 1 year, had not regressed over time, and had not responded to conventional treatment.[19] The control group consisted of normal skin obtained from tissue adjacent to normal scars (a normal scar was considered to be a flat scar in a patient with no previous history of KD).[6,20] At the time of surgical excision, keloid scar and normal skin from the sternum were collected. A flowchart illustrating the experimental design and the step-by-step process can be found in the supplementary information Figure S1.

Primary KF and NF culture establishment

The tissue was collected in Dulbecco's modified Eagle medium (Sigma-Aldrich, ••, UK) and fibroblasts were extracted using collagenase type I solution 0.5 mg/mL (Roche Diagnostics, ••, UK). Fibroblasts from passage 0 to passage 3 (p3) were used for all experiments carried out in this study as described previously by Suarez et al.[16] Briefly, we decided to employ fibroblasts taken from earlier passages because of published data from our lab.[6] We previously established that phenotypic alterations were observed in KF as the number of passages increased and others have also suggested that cell growth and phenotypic alterations could result from fibroblasts aging through multiple passages.[19] Thus, in order to minimize variability and have optimal consistency in data, only cells up to p3 were utilized in this study.

Tension-related gene selection criteria

The genes employed in this study (*Hsp27*, *α2β1* integrin, *PAI-2*) were selected from our previous findings,[16] where convincing up-regulation of tension-related genes was shown in KFs and tissue after analyzing 21 tension-related candidate genes. These were selected from three pooled gene expression microarray studies[16] for their relationship with skin tension and healing.

siRNA design and target gene knockdown

In order to perform small interfering RNA (siRNA) knockdown of the target genes (i.e., *Hsp27*, *α2β1* integrin, *PAI-2*), NF and KF were transiently transfected as described previously (Suarez et al.[16]). siRNA-transfected cells were collected by trypsinization after 24- and 48-hour knockdown and employed in the preparation of fibroblast-populated collagen lattices (FPCLs) in further experiments.

3D human FPCL

To perform the tensional testing, NF and KF were seeded in 3D collagen gels as described previously.[13,21] All those collagen gels that were free to contract were labeled as "without tension" samples (please see Supplementary Materials and Methods 2).

RNA extraction, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction

After application of tension to FPCL for 24 hours, each fibroblast-populated gel was snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Macerated samples were homogenized with TRIzol (Invitrogen, ••, UK). RNA extraction was performed according to the manufacturer's instructions using RNeasy mini-kit (Qiagen, ••, UK). cDNA synthesis and quantitative reverse-transcriptase polymerase chain reaction (PCR) were performed as described previously.[6] The list of primers used in this study is shown in the supplementary Table S2. The messenger RNA (mRNA) expression was normalized with reference to gene ribosomal protein L32 (RPL32).

Cell morphology and actin rearrangement

At 24-hour post-mechanical tension, FPCL was fixed in 4% formaldehyde for 30 minutes at 37 °C. Cells in FPCL were permeabilized with 0.1% Triton X-100 and subjected to double staining. First, FPCL were incubated over night at 4 °C using rhodamine phalloidin (Invitrogen) to stain filamentous actin and then counterstained with DAPI (Invitrogen) (15 minutes, room temperature). Images of 50 µm were taken with a immunofluorescence microscopy using a DeltaVision deconvolution system softWoRx v3.4.5 (Applied Precision, ••, ••), which consists of an Olympus IX-70 inverted microscope with an epi-fluorescence attachment. Images were analyzed using softWoRx v3.4.5 and Image-J software (National Institutes of Health, ••, ••). Images were taken from representative sections of the FPCL as previously described by Verhoekx et al.,[21] with the aim of

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making them comparable among the samples. The cell-spreading area was not formally quantified per cell, but we used cell spreading as a morphological readout for strain response as seen in other publications. However, it remains uncertain whether part of the increased spreading area is simply due to the straining of the cells.[23]

In vitro cell proliferation and viability assays using 3D models

Proliferation ratios of cells cultured in FPCL were measured after 24- and 48-hour siRNA transfection as follows. Forty microliter of FPCL was added into a 96-well plate and left to set for 10 minutes at room temperature. Once set, 100 µL of medium was added on top of each well. Control wells were detached from the walls of the wells, whereas the rest of the wells were cultivated under mechanical tension conditions for 24 hours. Ten microliter of cell proliferation reagent WST-1 (Roche) was added and incubated for 4 hours at 37 °C. Cell viability ratios were measured in FPCL after 24- and 48-hour siRNA transfection using water-soluble tetrazolium salt-1 assay (Roche) according to the manufacturer's instructions. Net absorbencies (A450–A690) were calculated using a spectrophotometer (Molecular Devices, ••, ••).

Statistics

All data were expressed as the mean ± standard error of the mean of at least three independent experiments. To determine statistical differences, a one-way analysis of variance and nonparametric analysis were performed with Bonferroni multiple comparison posttests. The difference between the means for all conditions was considered statistically significant at $p < 0.05$, for which GraphPad Prism5 software (••, ••, ••, ••) was used.

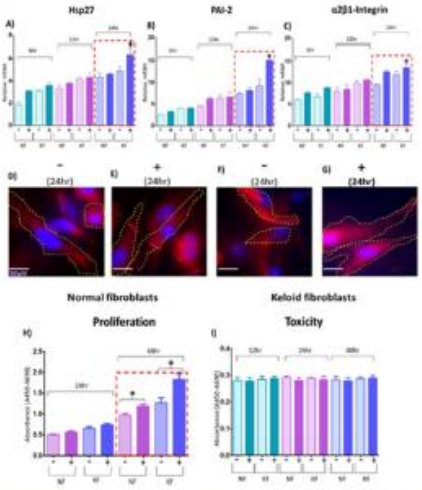
RESULTS

In vivo skin tension calculation in the sternum using photogrammetry

A contour plot of the force produced in vivo in the skin while performing normal breathing is shown in Figure 1B. The average of the force values from three independent tests of each of the four volunteers was plotted. The distribution of the force values was observed higher at the lower end of the sternum. The average of the force calculated values (35 mN) was employed on the design of the novel CFM.

Characterization of NF and KF response when subjected to mechanical tension in 3D collagen lattice (in vitro I)

The validation of the newly created CFM performance over time was carried out using 3D cell-populated collagen lattices (FPCL). Following three-time point tension application, the effect on gene expression of *Hsp27*, *PAI-2*, and *α2β1* integrin was evaluated by quantitative PCR (Figure 2A–C, respectively). The level of gene expression of the target genes in NF



Colour online; B&W in print

Figure 2. Characterization of normal skin fibroblast (NF) or keloid fibroblast (KF) behavior when subjected to mechanical tension in three-dimensional (3D) collagen lattice (in vitro I): Optimal tension force application time evaluation (6, 12, and 24 hours) by mRNA expression modification of (A) *Hsp27*, (B) *PAI-2*, and (C) *α2β1* integrin in NF/KF ($n = 10$) seeded in fibroblast-populated collagen lattice (FPCL). The expression of the tension-related markers was normalized to *RPL32*, a housekeeping gene. The results are expressed as mean ± standard error of the mean (SEM) of triplicates from three independent experiments (*$p < 0.0001$). (D–G) After 24 hours of tensional treatment, the collagen gels were removed from the cell force monitor (CFM), washed with PBS once, fixed in 4% formaldehyde (30 minutes at 37 °C), and treated for rhodamine phalloidin (red) and DAPI (blue) staining. The images show actin cytoskeleton rearrangement/cell spreading after 24-hour tension application. (H) FPCL-populated with NF or KF cells were seeded in 96-well plates and grown for 24 hours and 48 hours under these tension conditions. Proliferation ratios in both cell types under tension at 24-hour and 48-hour tension application were measured using WST-1 reagent following manufacturer's instructions (*$p < 0.0001$). The dashed box indicates the group of samples with more evident effect to the treatment. (I) Cell viability/metabolic activity at the end of 12-, 24-, and 48-hour tension treatment; the net absorbencies (A450–A690) of the water-soluble tetrazolium salt-1 were measured following manufacturer's instructions. (–) without tension/free to contract, (+) with tension. PBS, ••.

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and KF in the 3D lattices subjected to tension was time dependent. The application of tension over the evaluated periods, i.e., 6, 12, and 24 hours, showed increasing level of gene expression. This trend was seen in the three target genes, which was even more evident in KF after 24 hours of tension force application.

Hsp27 gene expression levels were significantly increased in KF ($p < 0.0001$) compared with NF. Moreover, *PAI-2* was up-regulated in both tensioned cell types but was significantly higher in KF ($p < 0.0001$) compared with NF. Finally, *α2β1* integrin gene expression levels were also increased significantly in KF ($p < 0.05$) compared with NF. Among the three tension-related genes, *Hsp27* gene expression level was up-regulated most compared with others over the three time periods following tension force application in both NF and KF.

In addition to gene expression; cell morphology was examined following mechanical tension (Figure 2D–G). Both cell types showed elongation after being tensioned; however, they changed morphology and spread freely when tension was removed. Cells aligned themselves along tension lines following application of force but displayed random orientation when left free to contract (cultured without tension).

There was a significant induction of proliferation in both cells types following mechanical tension in the 3D model. KF proliferation ratios were significantly higher ($p < 0.0001$) compared with NF at both time points following tension (24 and 48 hours) (Figure 2H), whereas no effect in cell viability/metabolic activity was observed in both cell types (Figure 2I). Thus, these findings imply skin tension promotes cellular activation, spreading, and higher proliferation, which are most pronounced in KF. Skin tension also induces the expression of target tension-related genes in both cell types but clearly is more significant in KF.

Knockdown of *Hsp27*, *PAI-2*, and *α2β1* integrin significantly reduced cell proliferation in mechanically tensioned fibroblasts (in vitro II)

The three tension-related genes (*Hsp27*, *PAI-2*, and *α2β1* integrin) were knocked down in order to evaluate their expression in NF and KF and the effect of these on cellular behavior when subjected to mechanical tension. Two transfection points were employed in order to optimize the treatment conditions in our 3D model; transfected cells were seeded in collagen gels and subjected to tension for 24 hours. Significant reduction on the expression of the three target genes was observed at mRNA level after 48 hours of transfection and 24 hours of tension application in both cell types. There was down-regulation of the three target genes that was even more evident in gels subjected to tension and populated with NF. Among the three genes, *Hsp27* and *α2β1* integrin gene expression levels were most significantly ($p < 0.0001$) down-regulated (Figure 3A–C).

Proliferation and toxicity ratios were examined after 48-hour knockdown and 24-hour tension application (Figure 3D and F). There was a reduced proliferation rate following knockdown and tension in both NF and KF. Tensioned NF showed least ($p < 0.0001$) proliferation rates after 48 hours following *Hsp27* or *α2β1* integrin knockdown. Moreover, there was no significant effect on cell viability/metabolic activity among the samples (Figure S3).

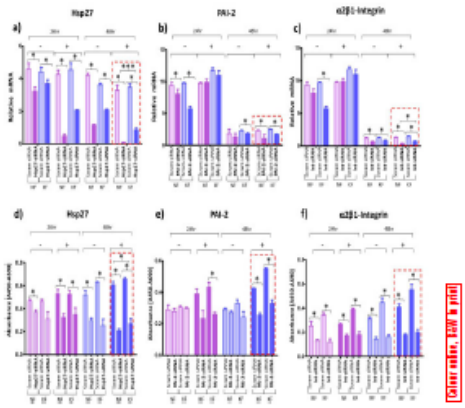

Figure 3. Knockdown of *Hsp27*, *PAI-2*, and *α2β1* integrin reduced cell proliferation on mechanical tensioned fibroblasts (in vitro II): The bar graphs A–C represent the relative mRNA expression of the target genes after 24- and 48-hour small interfering RNA (siRNA) transfection and 24-hour tension treatment using normal skin fibroblast (NF) or keloid fibroblast (KF) ($n = 10$). Significant reduction on the expression of *Hsp27*, *PAI-2*, and *α2β1* integrin at mRNA level, $^*p < 0.0001$, were shown in both tensioned and nontensioned systems when compared with their respective controls (scramble transfected fibroblasts), whereas plots D–F show the reduction on the proliferation ratios observed in both cell types after transfection. * indicates a significant difference ($p < 0.0001$) among all the samples compared. The dashed boxes indicate the group of samples seen to show the most significant effect when subjected to the treatment. (–) without tension/free to contract, (+) with tension.

Effect of down-regulation of tension-related genes on mechanically tensioned fibroblast morphology

Fibroblasts morphology was investigated following 48-hour knockdown and 24-hour tension treatment. Interestingly, *Hsp27* knockdown produced noticeable actin rearrangement. After transfection, cells were rounded, suggesting that *Hsp27* may be closely related with cell spreading under mechanical tension conditions (Figure 4). The same effect was observed in NF, but not in KF, subjected to tension when *α2β1* integrin was down-regulated, suggesting that *α2β1* integrin in conjunction with others may be implicated in the mechano-transduction process (Figure 4). No effect on the cell morphology was observed after *PAI-2* down-regulation. No cell alignment was observed along the tension line after knockdown of each gene (Figure 4). Therefore, our results show that the knockdown of the genes was time and tension dependent; we also conclude that *Hsp27* and *α2β1* integrin may be implicated in the regulation of act in rearrangement under tensioned conditions.

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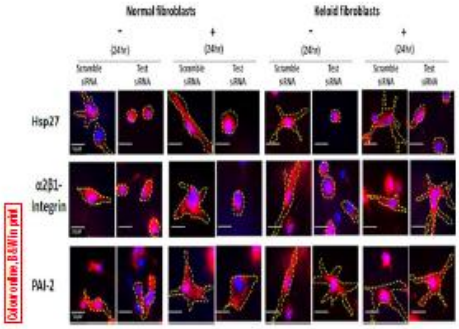

Figure 4. Effect of down-regulation of tension-related genes on mechanical tensioned fibroblast morphology: This figure shows the effect on normal skin fibroblast (NF) or keloid fibroblast (KF) ($n = 10$) morphology after 48-hour knockdown and 24-hour tension treatment by immune staining using phalloidin (red) and DAPI (blue). Three-dimensional (3D) pictures were taken using a high-end wide field microscope. (–) without tension/free to contract, (+) with tension.

Modification of the expression of phenotypic markers after down-regulation of tension-related genes in tensioned 3D models

After 48-hour transfection and 24-hour tension application, we evaluated the effect of knockdown of the expression of our three target genes on the expression of a selection of important ECM, adhesion, and spreading genes. Our results showed that the transfection of *Hsp27*/siRNA and *α2β1* integrin/siRNA after 48 hours led to significant ($p < 0.001$) down-regulation of the expression of ECM-related genes including fibronectin, collagen I, and *CD44* (Figures 5A–C and 6A) and the spreading-related gene neuropilin-1 (*Nrp1*) in both cell types, but is clearly more significant in NF left free to contract compared with KF. Same pattern of regulation was observed after *PAI-2* knockdown on the expression of fibronectin, collagen I, and *CD44*, but no significant effect on the expression of *Nrp1* was observed in both cell types (Figures 5D–F and 6B). We also evaluated the effect of knocking down the expression of our target genes on the expression of the myofibroblast marker (alpha-smooth muscle actin [α-SMA]) in both NF and KF. There was a significant ($p < 0.05$) down-regulation of α-SMA gene expression after knockdown of the three target genes separately. The down-regulation of α-SMA was more significant in NF compared with KF (Figures 5G–I and 6C). Thus, *Hsp27* and *α2β1* integrin appear to be closely related with cell-spreading regulation and adhesion under tension (Figure 7A and C), whereas *PAI-2* does not seem to be related to these phenomena, which likely support our previous findings regarding the actin cytoskeleton rearrangement (Figure 7B).

DISCUSSION

Photogrammetry and in vitro techniques were employed to create a novel 3D collagen lattice model to mimic tension experienced by NS and KF in vivo. Photogrammetry technique was employed to quantify the amount of force that the skin in the sternum experienced during breathing without disturbing the natural arrangement of the collagen fibers, which frequently happens with methods such as tensile, indentation, torsion, and suction testing. We then employed the data obtained in vivo to calculate the size of the collagen lattice needed and to design a novel CFM to reproduce tension distribution throughout the cells populating the collagen gel. Thus, the amount of force applied to the cells in the model mimicked the in vivo tension. We evaluated the newly created CFM by an in vitro 3D model using NF and KF seeded in the collagen gels to investigate the effects of mechanical tension on the expression of three target genes.

The choice of these tension-related genes was based on our previously published study, which showed up-regulation of three tension-related genes, *Hsp27*, *PAI-2*, and *α2β1* integrin,

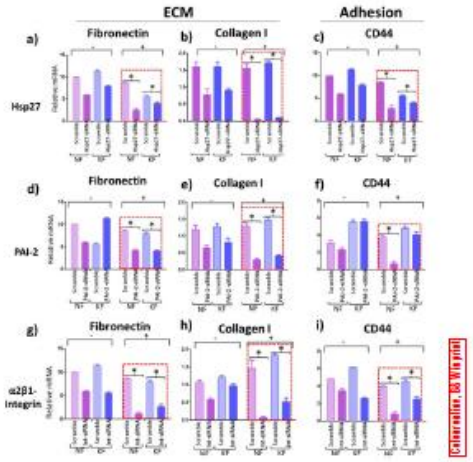

Figure 5. Modification of the expression of phenotypic markers after down-regulation of tension-related genes in tensioned three-dimensional (3D) models: (A–I) After 48-hour *Hsp27*/siRNA, *PAI-2*/siRNA, and *α2β1* integrin/siRNA transfection (50 nM), total RNA was extracted from tensioned normal/keloid-populated collagen gels ($n = 10$) and the expression of fibronectin, collagen I, and *CD44* was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression of the phenotypic markers was normalized to *RPL32*, a housekeeping gene. * indicates significant difference ($p < 0.0001$). Results are expressed as mean ± standard error of the mean (SEM) of triplicates from three independent experiments. Dashed boxes indicate the group of samples seen to show the most significant effect to the treatment. (–) without tension/free to contract, (+) with tension. ECM, extracellular matrix; KF, keloid fibroblast; NF, normal skin fibroblast; siRNA, small interfering RNA.

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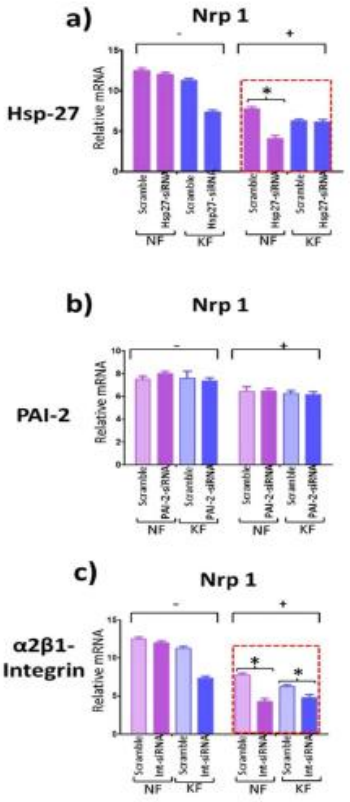

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Figure 6. Modification of the expression of phenotypic markers after down-regulation of the spreading-related gene *Nrp1* in tensioned three-dimensional (3D) models: (A–C) After 48-hour *Hsp27*/siRNA, *PAI-2*/siRNA, and *α2β1* integrin/siRNA transfection (50 nM), total RNA was extracted from tensioned normal/keloid-populated collagen gels ($n = 10$) and the expression of *Nrp1* was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The expression of the phenotypic markers was normalized to *RPL32*, a housekeeping gene. * indicates significant difference ($p < 0.0001$). Results are expressed as mean ± standard error of the mean (SEM) of triplicates from three independent experiments. Dashed boxes indicate the group of samples seen to show the most significant effect to the treatment. (–) without tension/free to contract, (+) with tension. KF, keloid fibroblast; NF, normal skin fibroblast; siRNA, small interfering RNA.

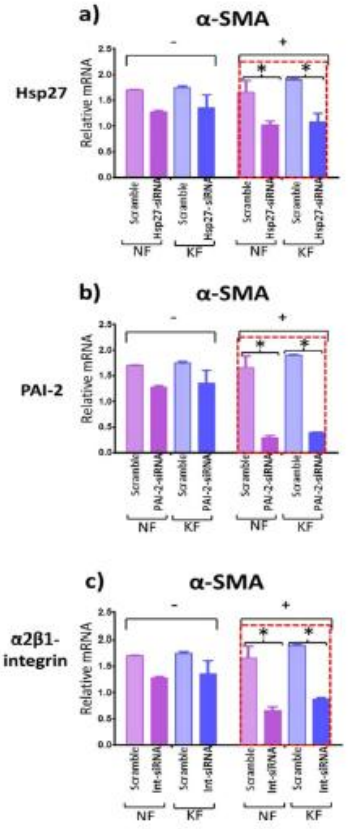

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Figure 7. Modification of the expression of phenotypic markers after down-regulation of the myofibroblast marker alpha-smooth muscle actin (α-SMA) in tensioned three-dimensional (3D) models: (A–C) Bar graphs represent the expression of the myofibroblast marker α-SMA after 48-hour target gene knockdown and 24-hour tension application. * indicates significant difference ($p < 0.05$). Dashed boxes indicate the group of samples seen to show the most significant effect to the treatment. (–) without tension/free to contract, (+) with tension. KF, keloid fibroblast; NF, normal skin fibroblast; siRNA, small interfering RNA.

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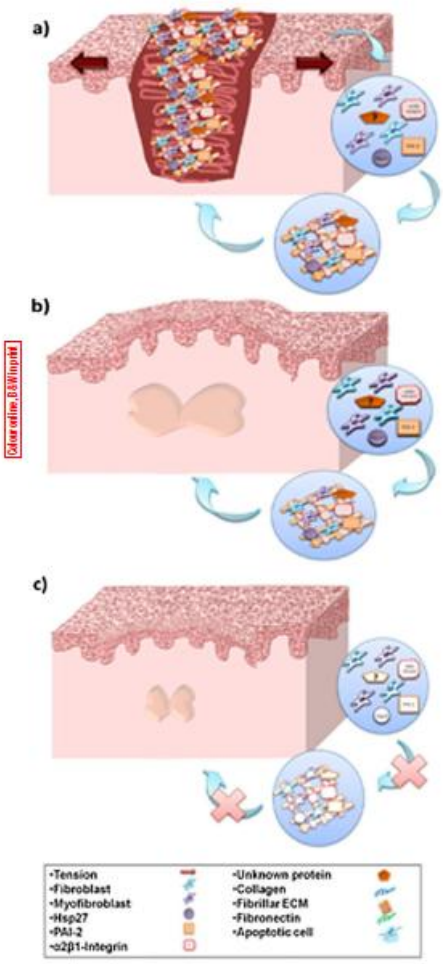

Figure 8. Proposed mechanism for the possible role of tension-related genes in keloid scarring: (A) Continued increase in mechanical tension in the injured area may promote the induction of tension-related genes and subsequently to production of increasing amount of extracellular matrix (ECM) components by susceptible cells such as keloid fibroblasts. (B) Continued mechanical tension may lead to further up-regulation of tension-related genes and excessive deposition of matrix resulting in keloid formation. (C) The modulation (such as inhibition or down-regulation) of the expression of tension-related genes such as *Hsp27*, *PAI-2*, and *α2β1* integrin may regulate excessive ECM production during keloid formation.

in both keloid tissue and fibroblast.[16] Our results here show increased expression of tension-related genes, ECM synthesis, cell spreading, and proliferation in cells under tension. Furthermore, we also showed that knockdown of these genes in this model influenced not just ECM synthesis but also adhesion and spreading-related genes as well as the myofibroblast marker α-SMA in NF and KF. These findings provide preliminary evidence for the role of these three target genes and the process of mechano-regulation in NF and KF.

Skin mechanical tension surrounding the connective tissue is considered a crucial regulator of the scarring process. Certain degree of mechanical loading is required to allow a cutaneous wound to contract.[22] Additionally, mechanical tension is thought to be of significance in keloid scar development.[23] Mechanical tension can play an active role during scar formation by affecting the expression of tension-related genes.[4] Keloid morphology and behavior are affected by its anatomical location and also influenced by the skin tension lines distribution (Langer's lines), which is reflected in development of site-specific morphology seen in KD.[24,25] Therefore, keloids often (exception in the ear lobes) tend to develop in those body regions constantly subject to skin mechanical tension.[3,26,27] Butterfly-shaped keloid scars are frequently seen in the sternum, an anatomical body region constantly stretched during daily breathing.[28]

Several molecular and signaling pathways have been shown to be affected by mechano-transduction, including *α2β1* integrin where signaling following tension is propagated through integrin-protein complexes allowing the control of the integrin-ligand binding and cell adhesion.[12,21,23] Hirano et al. reported that the formation of protein complexes regulated by *α2β1* integrin in the fibroblast membrane is closely involved with the actin filament stabilization during mechano-regulated processes such as wound contraction in rat models.[29] Additionally, signals sensed by the cells from the exterior result in cytoskeleton reorganization, phenotypic modification, and finally cellular differentiation.[21,30] We showed here that the down-regulation of *α2β1* integrin in FPCL subjected to tension conditions not only altered ECM synthesis but also actin-cytoskeleton rearrangement and spreading, factors considered crucial in cell migration and wound healing. The expression of α-SMA was also reduced under tension conditions following *α2β1* integrin knockdown.

It has also been proposed that mechanical forces activate the intracellular pathway FAK-ERK-MCP-1, promoting inflammation in fibrotic processes as well as fibroblast activation.[4,31,32] In addition, the molecular chaperone Hsp27 (heat shock protein 27) has been reported to regulate apoptosis under stress conditions. It also has been shown that Hsp27 post-phosphorylation by mitogen-activated protein kinase (MAPK) pathway can lead to modulation of actin filament.[31,33] Hsp27 has been related to cell survival mediated by FAS pathway,[34] and several reports suggest the participation

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of Hsp27 in the actin microfilament stabilization via MAPK participation.[29,35] Furthermore, Hsp27 plays an important role in the modulation of endothelial and smooth cell contraction.[31] From the results obtained here, we corroborate the participation of Hsp27 in actin rearrangement under mechanical tension and also note its significant influence on certain ECM molecules, fibronectin, and collagen I. Moreover, we suggest a further regulation of the adhesion-related gene *CD44* and the spreading-related gene *Nrp1* in both NF and KF. The expression of *PAI-2* has been suggested to promote cellular protection against apoptosis.[34] *PAI-2* also plays an active role in the regulation of ECM synthesis and degradation in highly stressed systems.[36,37] Fibroblast differentiation into myofibroblasts is related to the expression of *PAI-2* too.[37,38] Our results showed that collagen I and fibronectin synthesis depend on the expression of *PAI-2*, whereas no effect was observed on the cell spreading, adhesion, nor actin myofilament arrangement after *PAI-2* down-regulation (Figure 8A–C).

We previously showed that α-SMA expression was dependent on the expression of *Hsp27*, *α2β1* integrin, and *PAI-2* in steady state KF.[16] Interestingly, similar results on the expression of α-SMA were observed here following down-regulation of these three genes in our model. Taken together, the results here show that the modulation of the expression of *Hsp27*, *α2β1* integrin, and *PAI-2* may regulate excessive ECM production when subjected to certain tension conditions. It is postulated that out of the three target genes that we investigated, *α2β1* integrin may contribute more significantly than *PAI-2* or *Hsp27* in keloid development based on *α2β1* integrin interaction at the intra- and extracellular levels. In addition, we envisage that evaluation of the relationship between *α2β1* integrin and the Rho-GTPase may provide further clues to the role of mechanical tension in keloid development. The interaction of *α2β1* integrin–Rho-GTPase may allow stabilization and contraction of the newly formed ECM during the wound healing process. The activation of the Rho-GTPase signaling pathway may also have a direct impact in the regulation of the tension forces exerted during the healing process[39,40] (Figure 9). Furthermore, we conclude that mechanical tension influences the expression of tension-related proteins and shows induction of ECM synthesis, cell spreading, and proliferation in both NF and KF.

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Conflicts of Interest: The authors have no potential conflict of interests to declare in relation to the content of this article.

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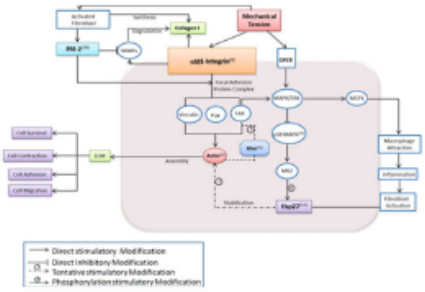

Figure 9. Possible interaction between *α2β1* integrin, *PAI-2*, and *Hsp27* in keloid fibroblast mechano-regulation. Mechanical tension induces *α2β1* integrin activation, which serves as a transmembrane link between the extracellular matrix (ECM) and the cell cytoskeleton, allowing the interaction between collagen and fibroblasts by formation of focal adhesion contacts. Among the proteins implicated in this process, the focal adhesion kinase (FAK) appears to be a key candidate in the development of the fibrotic process following mechanical force stimuli of the ERK-MCP-1 inflammatory pathway, which induced fibroblast activation and survival as well as ECM production.[4] The mechano-transduction signal also activates *Hsp27* via p38 MAPK.[29] The heat shock protein activation promotes actin stabilization by unknown mechanisms, as well as focal contact formation, cell contraction, migration, and survival. The actin stabilization and polymerization is also mediated by *α2β1* integrin.[26] Furthermore, it had been reported that *α2β1* integrin regulates collagen degradation by the activation of metalloproteinases (MMPs). The collagen degradation is also regulated by *PAI-2*, which negatively regulates MMPs leading in the ECM accumulation. *PAI-2* is involved in cell migration mediated by integrins.[39] ERK, extracellular regulated kinase; GPCR, ••; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Materials and Methods 1. ••
Supplementary Materials and Methods 2. ••

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Figure S1. Study design: The flowchart presents a detailed summary of the steps taken to perform this study.

Figure S2. Culture force monitor. (A) This is a drawing to illustrate the components of the design of our novel cell force monitor assembly including dimensions of the device components. (B) This is a photograph of the cell force monitor device.

Figure S3. Viability/metabolic activity detection at 24 or 48 hours post-tension in transiently knockdown *Hsp27*, *PAI-2*, and $\alpha 2\beta 1$ integrin primary fibroblasts. The bar graph represents cell viability/metabolic activity following small interfering RNA (siRNA) transfection and tension treatment using tensioned normal and keloid fibroblasts ($n = 10$). No statistical effect was detected among the samples. (–) without tension/free to contract, (+) with tension. *Hsp27*, NF/KF transfected with siHSP27; integrin, NF/KF transfected with $\alpha 2\beta 1$ integrin; KF, keloid fibroblast; NF, normal skin fibroblast; *PAI-2*, NF/KF transfected with *PAI-2*; sHsp27, NF/KF transfected with scramble *Hsp27*; sintegrin, NF/KF transfected with scramble $\alpha 2\beta 1$ integrin; sPAI-2, NF/KF transfected with scramble *PAI-2*.

Table S1. Demographic data of keloid and normal skin samples employed in the study.

Table S2. List of primer pairs used in this study for quantitative reverse transcription polymerase chain reaction (qRT-PCR).