Glucocorticoid Metabolism and Function in Ageing Skin

By

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SUMMARY

The continuing demographic shift towards a greater proportion of elderly individuals in the populations of developing nations and associated increased morbidity is fuelling the drive for increased research into healthy lifespan.

Excessive circulating glucocorticoid (GC) levels (e.g. Cushing's Syndrome) often cause hypertension, osteoporosis, central obesity, muscle weakness, skin thinning and poor wound healing - conditions also commonly associated with the ageing process. Studies in our group investigating the enzyme 11-beta hydroxysteroid dehydrogenase type 1 (11β-HSD1), that generates active GC and regulates their local tissue-specific concentrations, have previously demonstrated increased levels in bone cells derived from older compared to younger donors.

This thesis provides exciting new research that reproduces this observation in the organ most noticeably affected by ageing - skin. 11β-HSD oxoreductase activity (i.e. GC-activating) was found to be elevated in skin from older compared to younger donors - with increased enzyme levels in dermal fibroblasts (cells crucial to collagen production and skin remodelling). Activity also was inducible by GC exclusively in cells from older individuals, further increasing dermal cortisol generation potential with advancing age. Evidence is presented for 11β-HSD1-specific regulation of GC target genes in dermal fibroblasts that may be dysregulated during ageing and for novel GC targets in skin that also correlate with enzyme activity. Finally, initial studies characterizing the phenotype of the ageing 11β-HSD1-null mouse reveal exciting morphological alterations in skin structure that are more similar to those of young animals than aged controls.

GC are often used to treat a wide range of common skin disorders in the elderly including xerosis (dry skin), bullous pemphigoid (blistering) and psoriasis. However, elderly patients also have an increased risk of developing treatment side-effects including skin thinning, susceptibility to bruising and tearing and increased infection risk.

In addition to gaining knowledge of a potential contributor to the skin ageing process, the findings presented in this thesis provide support for development of a novel therapeutic target that may minimize side-effects associated with GC therapy in the elderly and may reduce the ageing process in skin and its associated adverse pathologies.

For Dr Sanda Jacomin

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ABBREVIATIONS

11β-HSD(1/2)	11 beta-hydroxysteroid dehydrogenase (type 1/2)
11-DHC	11-dehydrocorticosterone
ACTH	Adrenocorticotrophic hormone
AME	Apparent mineralocorticoid excess
AMP	Antimicrobial peptide
AP	Activating protein
APC	Antigen-presenting cell
BER	Base excision repair
CPD	Cyclobutane pyrimidine dimers
CR	Caloric restriction
CRF	Wellcome Trust Clinical Research Facility
CRH	Corticotrophin-releasing hormone
DBD	DNA-binding domain
DEJ	Dermal-epidermal junction
DHEA(S)	Dehydroepiandrosterone (sulphate)
DSB	Double-stranded break
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FSH	Follicle-stimulating hormone
GAG	Glycosaminoglycan
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GC	Glucocorticoid
GH	Growth hormone
GR	Glucocorticoid receptor
H6PD	Hexose-6-phosphate dehydrogenase
HDF	Primary human dermal fibroblasts
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HRE	Hormone response element
IGF	Insulin-like growth factor
IL	Interleukin
IFN	Interferon
IRS	Inner root sheath
LBD	Ligand-binding domain
LH	Luteinizing hormone
LXR	Liver X receptor
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MR	Mineralocorticoid receptor
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NBF	Neutral buffered formalin
NER	Nucleotide excision repair

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NHEJ	Non-homologous end joining
NKT	Natural killer T
NR	Nuclear receptor
ORS	Outer root sheath
PARP	Poly-(ADP ribose) polymerase
PBS	Phosphate buffered saline
PE	Photo-exposed
PP	Photo-protected
PPAR	Peroxisome proliferator-activated receptor
PPARGC1A	Proliferator-activated receptor gamma coactivator 1-alpha
ROS	Reactive oxygen species
SC	Stem cell
SIRT	Sirtuin
T2D	Type 2 diabetes
TGF	Transforming growth factor
T_h1	Type 1 helper T-cell
T_h2	Type 2 helper T-cell
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
WT	Wild-type

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Skin

1.1.1 Overview

Skin is the largest organ of the human body, weighing on average 5kg and covering a surface area of $2m^2$. It acts as a mechanical barrier, protecting the underlying tissues from the external environment (such as destructive solar radiation), whilst preventing water loss from within through secretion of sebum. It is a protective envelope, producing defensins and cathelicidins (endogenous antibiotics) which provide resistance against bacteria, viruses and fungi, and containing sentinel immune cells which patrol the epidermis and guard against invading pathogens.

Skin aids in the maintenance of temperature homeostasis through vasoconstriction or vasodilation of its extensive vasculature and by producing approximately one litre of sweat each hour during moderate exercise. Pheromones released from apocrine sweat glands play a role in sociosexual communication and attraction.

Subcutaneous fat (around 80% of total body fat in non-obese individuals) acts as a calorific reserve in addition to providing thermal insulation and cushioning against physical trauma. Dermal adipose tissue is also thought to contribute to endocrine regulation of hunger and energy metabolism by releasing the hormone leptin. Skin further contributes to metabolism by synthesizing vitamin D.

Collectively these roles define skin as a vital, highly versatile organ, structurally engineered to meet a diverse range of physiological requirements.

1.2 Skin structure

1.2.1 Key features

The structure of human skin is generally subdivided into three categories; the uppermost, stratified, highly cellular, epidermis (0.05-1mm) which undergoes a continual process of proliferation and differentiation, the underlying dermis (0.5-5mm) composed primarily of connective tissue and the subcutaneous - *panniculus adiposus* - layer which functions as an adipose depot (Burns *et al*., 2010, Figure 1-1). This is separated from the subdermal structures by a thin layer of striated muscle, the vestigial *panniculus carnosus*. The dermal-epidermal junction (DEJ) is normally undulating, with the epidermis projecting into the dermis forming "rete ridges" which provide mechanical support.

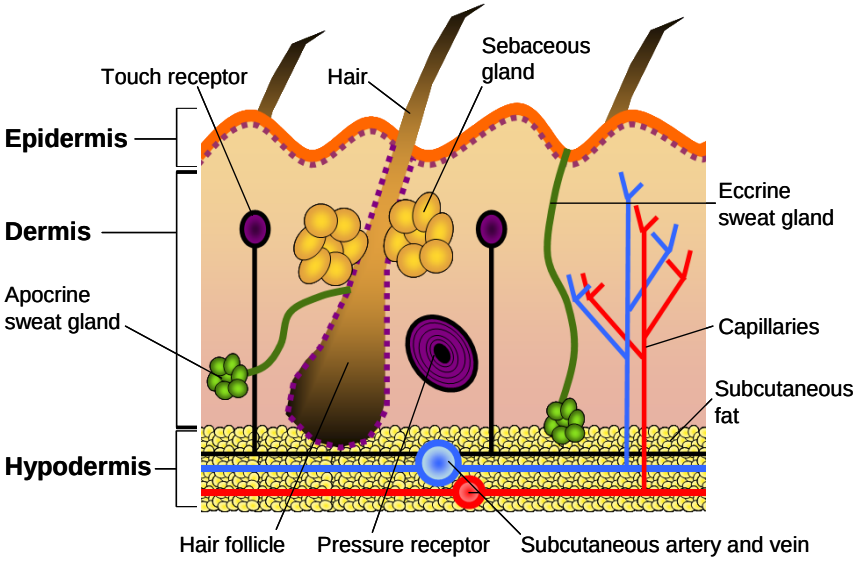


Figure 1-1 The skin and associated appendages

Cells line the basement membrane at the DEJ, anchoring the epidermis to the superficial dermis below through protein and glycoprotein projections, and giving rise to keratinocytes above, which differentiate into the spinous layer, granular layer and stratum corneum before culminating in the process of desquamation. Originating from the Latin term *desquamare*, meaning "to scrape the scales of a fish", desquamation describes the shedding of dead,

anuclear keratinocytes (corneocytes) following approximately 30 days of differentiation. Additionally to keratinocytes, the epidermis contains pigmentory melanocytes, immunological Langerhans' cells and somatosensory Merkel cells.

Hair follicles are dermal appendages present in all but glabrous skin (palms and soles), varying with body site from the large follicles of the scalp to the vellus-producing follicles of the forehead. The epithelium surrounding follicles is continuous with the epidermis and follicles undergo periodic cycling, enveloping a small papilla of dermis during the active phase. Hair follicle motion is regulated by arrector pili smooth muscle bundles which anchor between the epidermis and follicle wall. Sebaceous glands located at the apex of the pilary canal secrete sebum which functions to moisturize the skin and hair and are associated with apocrine glands in some body parts (e.g. axilla). Epidermally-derived eccrine sweat glands are also present in varying degrees of density (100-600/cm^2) throughout the body.

The dermis mainly consists of a densely packed protein and polysaccharide matrix providing the skin with a high affinity for water retention (around 60% of the total weight). The vast proportion of the protein component is collagen (80-85%), conferring a high tensile strength, with elastin and elastin-associated microfibrils making up the remainder. Embedded within this matrix are dermal fibroblasts, which comprise the majority of the dermal cell population, and a variety of surveillance immune cells including mast cells, monocytes and macrophages. The dermis is also highly vascularised and benefits from a rich blood supply.

1.2.2 Epidermal compartment

1.2.2.1 Keratinocyte differentiation

The majority of the epidermis is composed of non-proliferating terminally differentiating keratinocytes of basement membrane basal cell origin. During their transit, keratinocytes form

four well-defined layers (Figure 1-2) characterized by a range of layer-specific microstructures, proteins and signalling molecules; the *stratum basale* (basal layer), *stratum spinosum*, (spinous layer), *stratum granulosum* (granular layer) and *stratum corneum* (cornified layer). Additionally, palmoplantar skin contains a further layer between the granulosum and corneum - the *stratum lucidum*.

The basal layer is the only actively proliferating keratinocyte epidermal layer composed primarily of basement membrane-bound multipotent progenitor stem cells (further discussed in section 1.4) and is generally one-cell thick. The 10-14nm cuboidal cells can be characterized by a K5/K14-keratin filament-containing cytoskeleton and a high prevalence of adherens intercellular junctions. The *stratum basale* is succeeded by the spinous layer - its name attributable to the high density of desmosomes, molecular complexes of cell-to-cell adhesion proteins that link to cytoskeletal K1/K10-keratin filaments anchoring neighbouring keratinocytes to each other. Additionally, transglutaminases produced in the spinous layer catalyse the cross-linking of proteins rendering them insoluble - such as involucrin, a constituent of the cornified envelope of corneocytes (Candi *et al*., 2005). The subsequent granular layer contains intracellular keratohyalin granules, lipid-filled lamellar granules which discharge their contents into the extracellular space conferring corneocyte barrier function though lipid membrane formation (Feingold, 2007) and L granules containing loricrin - a major component of cornified envelope.

The uppermost stratum corneum is formed by keratin filament alignment and disulphide cross-link formation - a process assisted by fillagrin (keratohyalin granule protein) which causes keratin filament aggregation. Increasing intracellular calcium concentrations induce formation of the cornified envelope through desmosomal protein remodelling to form an internal scaffold onto which involucrin, loricrin and other small proline-rich proteins are

incorporated and transglutaminase cross-linked (Figure 1-8). Finally, during desquamation, lamellated intercellular lipids and desmosomal interconnections are degraded and the resulting flattened corneocytes, lacking nuclei and cytoplasmic organelles are removed through friction (Milstone, 2004).

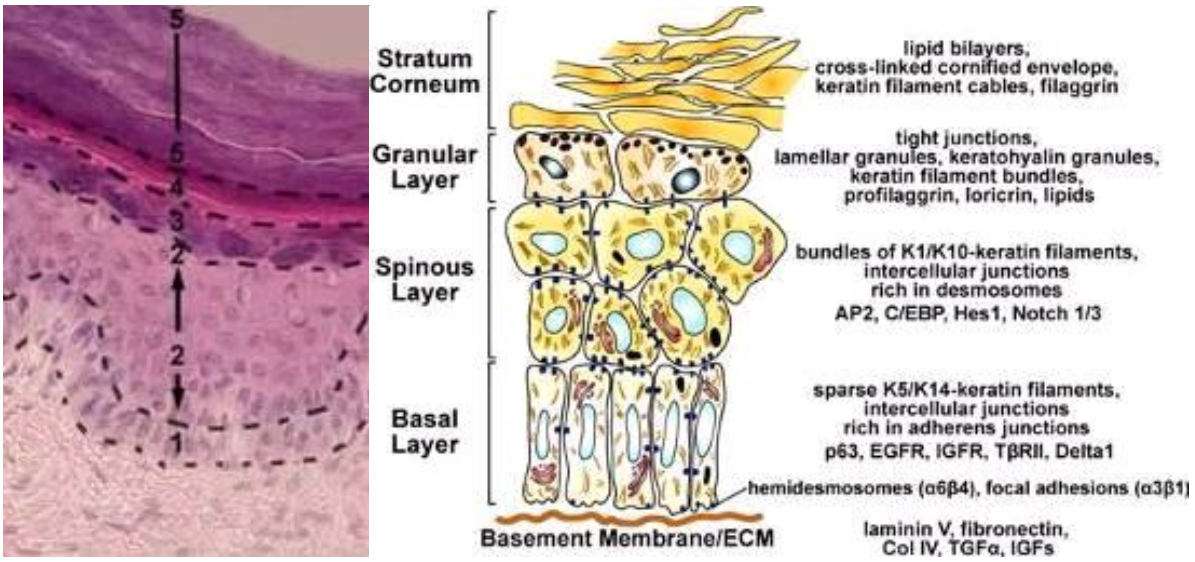


Figure 1-2 The process of epidermal differentiation. Human palmoplantar epidermis depicting basal layer (1), spinous layer (2), granular layer (3), lucidar layer (4) and cornified layer (5) http://histol.narod.ru/atlas-en/skin-en.htm. Illustration annotations describe layer-specific structures, macromolecules, transcription factors and signalling molecules (Fuchs, 2008)

Keratinocyte differentiation is positively regulated by peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR) pathways which stimulate lipid synthesis, lamellar body formation and secretion (Schmuth *et al*., 2008). Concomitantly, these pathways are also anti-inflammatory, suppressing proliferation whilst inducing growth arrest, apoptosis and terminal differentiation thus maintaining normal epidermal turnover in a non-inflamed state.

1.2.2.2 Melanocytes

Epidermal melanocytes produce melanin pigment and transfer it to neighbouring keratinocytes and hair follicles via melanosomes. Two types of melanin are produced,

brown/black eumelanin and yellow/red phaeomelanin, with varying proportion and distribution resulting in a global mosaic of colour diversity.

Melanin functions to absorb ultraviolet (UV) light, protecting DNA, cellular components and subdermal structures from sun-induced damage. UV exposure is associated with an increase in functional melanocyte number, melanosome synthesis and transfer of melanin to neighbouring keratinocytes, resulting in a post-exposure browning of the skin known as delayed tanning (Ortonne, 1990).

Melanin pigment production is regulated, in part, by melanocortin-1 receptor activation which promotes eumelanin formation at the expense of phaeomelanin. Regulation of such receptors has been shown to influence photo-protection in addition to skin colour (Carlson *et al*., 2007).

Changes in melanocyte signalling pathways can induce dramatic phenotypic alterations. For example, downregulation of Notch signalling is associated with precocious hair greying attributable to reduced melanocyte precursor cells (melanoblasts), implicating Notch signalling as an integral regulator of melanocyte regeneration during hair follicle cycling (Schouwey and Beermann, 2008).

1.2.2.3 Langerhans' cells

Dendritic Langerhans' cells play a crucial role in epidermal immune surveillance and are a first line of defence against invading pathogens. Through lengthening and recoiling of dendritic processes between keratinocytes (dendrite surveillance extension and retraction cycling habitude - dSEARCH) they patrol the epidermal environment for foreign antigens (Mohr and Takashima, 2007). Upon contact, Langerhans' cells engulf foreign bodies, process them for antigen presentation and interact with lymphocytes by secretion of cytokines such as

interleukin (IL)-1 to initiate an immune response, as well as upregulating dSEARCH for increased antigen sampling.

Interestingly, Langerhans' cells are more likely to induce an anti-inflammatory type 2 helper T- cell (T_h2)-mediated response rather than the pro-inflammatory type 1 helper T-cell (T_h1)-mediated response typically associated with pathogen recognition. This is further supported by Langerhans' cell-deficient mice which appear to have enhanced contact hypersensitivity. Furthermore, keratinocytes have been implemented as potentiators of both innate and adaptive immune responses through antimicrobial peptide (AMP), cytokine and chemokine production (Nestle *et al*., 2009). Collectively, recent findings are redefining the roles of Langerhans' cells to include induction of tolerance and regulatory properties that counteract the emerging pro-inflammatory actions of keratinocytes (Asahina and Tamaki, 2006).

1.2.2.4 Merkel cells

Merkel cells are located throughout the epidermis, amongst basal keratinocytes particularly in hairy skin and tactile areas of glabrous skin, forming close connections (touch spots) with unmyelinated sensory nerve endings at the base of rete ridges. Interestingly, photo-exposed (PE) skin contains around twice as many Merkel cells compared to photo-protected (PP) skin. Rich in neurosecretory granules, Merkel cells are involved in somatosensory signalling by acting as mechanoreceptors for the sensation of touch, although there is no clear evidence for direct synaptic transmission at Merkel cell-neurite complexes (Lucarz and Brand, 2007).

1.2.3 Pilosebaceous unit

Pilosebaceous units (hair) are subcategorised into four classes: terminal (scalp and beard), apopilosebaceous (axilla and groin), sebaceous (face, back and chest) and vellus (the

remaining majority of skin). The dermal papilla locates to the follicular base and is encompassed by a rich germinative cell matrix which generates the hair fibre innermost medulla, cortex and surrounding cuticle. Lining the cuticle is the inner root sheath (IRS) composed of three layers of cells that undergo keratinisation (IRS cuticle, Huxley layer and Henle layer) and provide support for the hair fibre, terminating at the sebaceous gland. The IRS is separated from the outer root sheath (ORS), which is continuous with the epidermis, by a companion cell layer (Schlake, 2007, Figure 1-3).

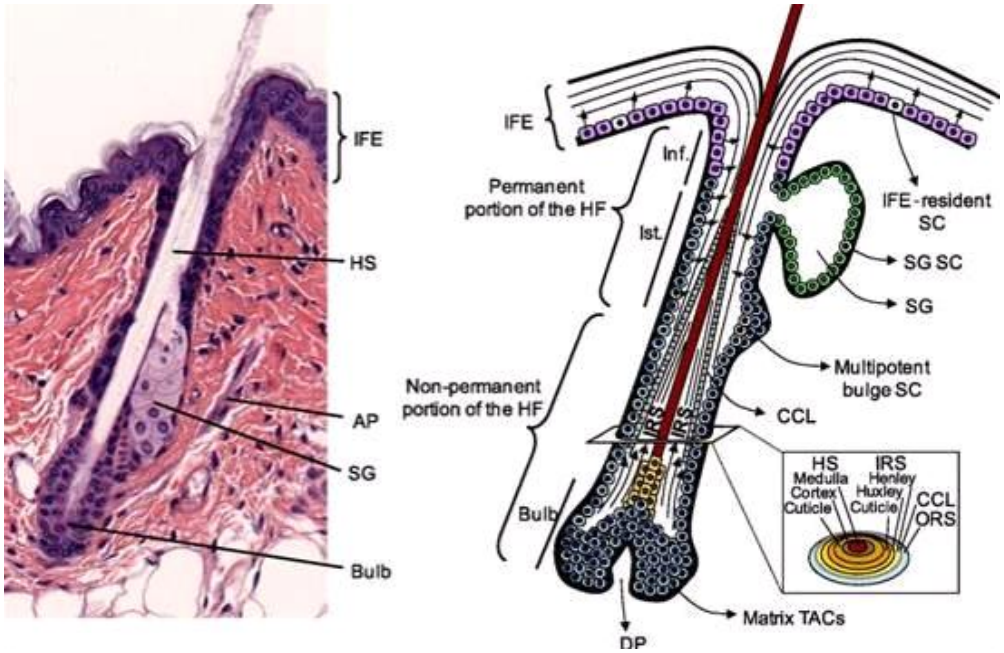


Figure 1-3 Pilosebaceous unit structure. Mouse hair follicle (dorsal skin) stained with haematoxylin/eosin in late catagen/telogen and schematic representation of the hair follicle. AP, arrector pili; CCL, companion cell layer; DP, dermal papilla; HF, hair follicle; HS, hair shaft; IFE, interfollicular epidermis; Inf, infundibulum; Ist, Isthmus; SC, stem cells; SG, sebaceous gland; TAC, transient amplifying cell (Margadant *et al*., 2010)

A dense hair coat is essential in most mammals, assisting in temperature homeostasis and protection from the external environment. Hair requires continuous renewal throughout life, achieved through cycling of the lower section of the pilosebaceous unit through phases of growth (anagen), regression (catagen) and rest (telogen), whilst the infundibulum, isthmus, bulge and sebaceous gland remain stable.

Following hair loss, a new follicle is formed and grows during anagen through the upward proliferation and differentiation of bulb matrix cells which also form the IRS downwards, adding stability to the elongating hair. During catagen, the hair shaft stops growing and both differentiation and proliferation processes are halted. Matrix cells undergo apoptosis accompanied by follicle degeneration and regression, moving the bulb upwards until it reaches the bulge. At this stage of telogen, the follicles are quiescent awaiting anagen re-initiation when the old hair is lost, and new matrix cells are re-formed following interaction between dermal papilla cells and bulge stem cells (Fuchs *et al*., 2006, Figure 1-4).

1.2.4 Eccrine and apocrine glands

There are two types of sweat gland in humans: eccrine and apocrine. The former are responsible for the majority of thermoregulatory sweating and are located over nearly the entire body, with highest density in the soles, palms and forehead. Sweat glands consist of a bulbous coil in the lower dermis and a secretory duct which transverses the epidermis and opens at the skin's surface. Perspiration is insensible, originating from water in the blood and evaporating passively at a rate determinable by the ambient temperature and humidity, or active where intense heat, mental/emotional stimulation, exercise and carbon dioxide levels induce active sweating. The neurological pathways involved in regulating perspiration are not fully understood but thought to involve cholinergic stimulation of sympathetic nerve fibres (Shibasaki *et al*., 2006). This leads to secretion of an isotonic precursor fluid lacking proteins but containing sodium and chloride ions which are generally reabsorbed but during periods of intense perspiration can lead to ion loss as the reabsorption mechanisms become overwhelmed.

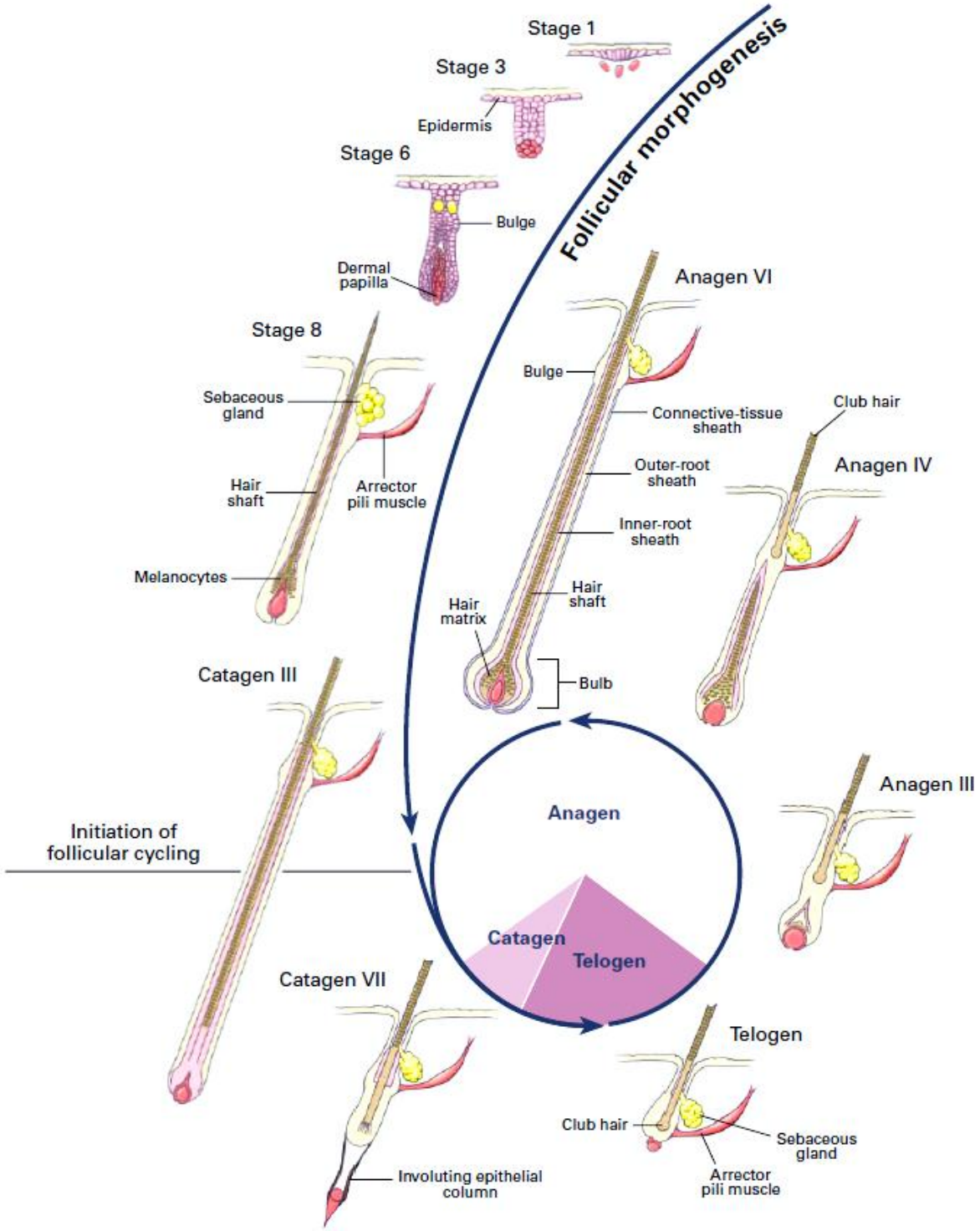


Figure 1-4 The hair cycle. Hair follicles cycle through alternating phases of anagen, catagen, and telogen. Reproduced from Paus and Cotsarelis, 1999

Conversely, apocrine glands are localised exclusively to axillary, genital and mammary skin in association with hair follicles. They have minimal rates of lipid-rich secretions that do not

contribute to temperature homeostasis, and are decantered directly into the hair shaft by excretory ducts which fuse with the epithelium of hair follicles. The role of apocrine glands in humans is unknown.

1.2.5 Dermal compartment

1.2.5.1 Extracellular matrix

The dermal compartment comprises the greatest proportion of skin and, in contrast to the epidermis, is largely acellular, consisting of an extracellular matrix (ECM) composed of a complex network of connective tissues. ECM connective tissue includes collagen fibres providing tensile strength, elastic fibres allowing flexibility and resilience, glycoproteins serving as structural organizers and proteoglycans/glycosaminoglycan (GAG) units which maintain hydration.

The majority of the dermis is composed of several types of collagen with a common triple-helical structure of three varying α-chain (α1-α6) polypeptide monomer subunits with a glycine-X-Y amino acid sequence (Figure 1-5). To date, 29 distinct vertebrate collagen types have been identified (I-XXIX) with 12 of particular relevance to skin. Collagen types I, III and V form large fibrils and together comprise ~95% of dermal collagen (80%, 10% and 5% respectively). These are assembled into an organized structure supported by anchoring collagen type VI microfibrils, network forming collagen type VIII and other fibril-associated collagens with interrupted triple helices such as collagen type XIV. Important membrane-associated collagens include type IV: a component of the basement membrane and dermal vasculature, type VII: the major constituent of anchoring fibrils that interact with other DEJ proteins to form U-shaped loops that ensnare and stabilize larger fibres of the underlying dermis, type XVII: a component of hemidesmosomes that assist in anchoring the epidermal

basal cell layer to the basement membrane and types XIII and XV which are ubiquitously expressed including in epidermis and basement membrane respectively. Most recently, mutations in the gene for a novel putative epidermal collagen type XXIX (*COL29A1*) have been linked to atopic dermatitis (Soderhall *et al*., 2007).

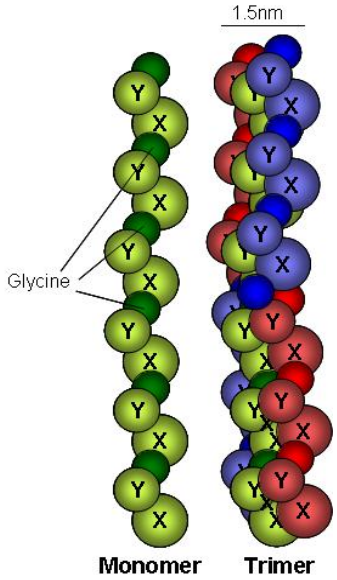


Figure 1-5 Collagen microstructure. Schematic representation of a typical collagen monomer subunit composed of a repeating Glycine-X-Y amino acid sequence and its assembly into a triple-helix

The different collagen types are characterized by considerable complexity and diversity in their structure, their splice variants, the presence of additional, non-helical domains, their assembly and their function (Gelse *et al*., 2003). For example, collagen II is composed of three identical α1 (II)-chains (homotrimer) contributing to the torsional stability and tensile strength of articular cartilage, while the microfibrillar collagen VI is composed of three distinct α1(VI)-, α2(VI)- and α3(VI)-chains (heterotrimer) is also highly disulfide cross-linked and contributes to a network of beaded filaments interwoven with other collagen fibrils.

Additionally to the collagen network, elastin fibres are an integral component of the dermal ECM, although contributing only 2-4% of the total dry weight (Figure 1-6). The elastin system forms a horizontally aligned complex of elastin fibres from which a relatively finer vertical structure of microfibrils (oxytalan fibres) and cross-linked elastin (elaunin fibres)

extend (Midwood and Schwarzbauer, 2002). Other elastin-associated proteins include fibrillins, latent transforming growth factor-β proteins which bind the pro-fibrotic cytokine transforming growth factor (TGF)-β, fibulins (calcium-binding glycoproteins), microfibril-associated glycoproteins/proteins, interface proteins (emilins) and lysyl oxidases which are critical for the cross-linking of elastic fibres.

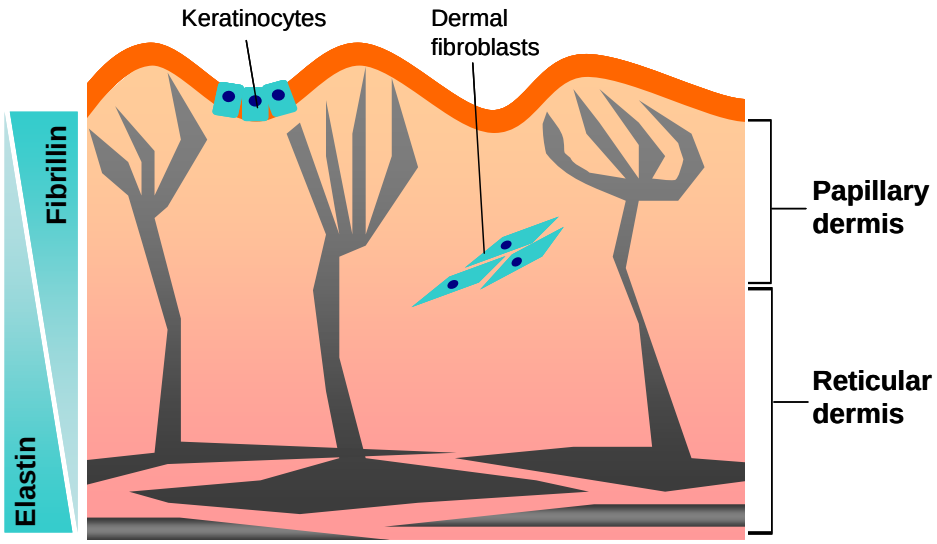


Figure 1-6 Elastin network in human skin. The elastic fibre system in skin is confined to the dermis where composite elastic fibres in the reticular dermis give way to arrays of fibrillin microfibril bundles at the DEJ. The fibrillin microfibrils of the elastic fibre system are synthesised by both keratinocytes and dermal fibroblasts

Proteoglycan macromolecules contribute the remainder of dermal non-cellular structures. Formed by the linkage of unbranched disaccharide GAG to a central protein core, proteoglycan subfamilies are highly variable existing in multiple combinations of core proteins, number of associated GAG units and GAG molecular mass. GAGs are formed by alternating pairs of different monosaccharides (e.g. glucose or galactose) joined by 1-3 or 1-4 serine O-linkage and include heparin, heparan sulphate, hyaluronic acid, keratan sulphate, chondroitin sulphate and dermatan sulphate. Core proteins can be intracellular, membrane bound or extracellular. In the dermis, key proteoglycans include versican (260-370kDa), linked to 10-30 chondroitin/dermatan sulphate GAGs. Versican also contains a hyaluronic

acid binding domain enabling the formation of large aggregates (Figure 1-7). Smaller ECM proteoglycans include type I collagen-associating decorin (36kDa), with a single dermatan sulphate GAG that is also able to bind to tenascin-X, another ECM component with collagen affinity. Therefore, proteoglycan interactions with surrounding structures provide stability and assist the organization of connective tissues architecture.

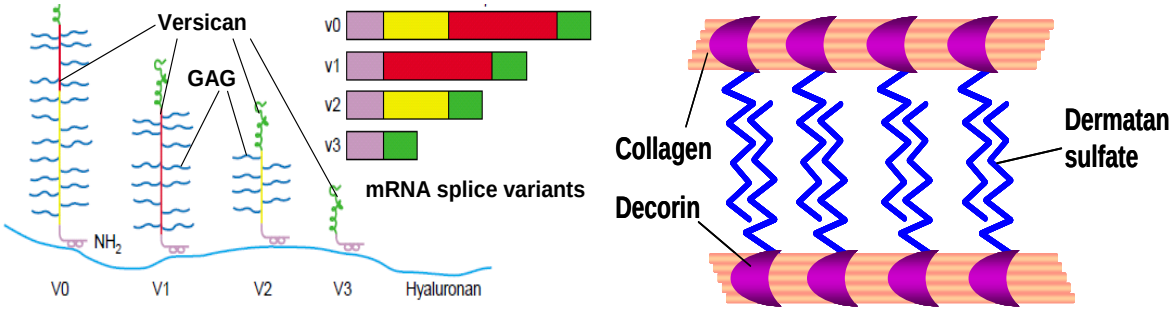


Figure 1-7 Dermal proteoglycans. Versican core protein size is variable due to alternative splicing of the versican mRNA, limiting the number of associated GAG units. All isoforms are capable of binding hyaluronan (Wight, 2002). Decorin is a much smaller proteoglycan that associates with collagen through its protein domain

Proteoglycans have also been found to bind growth factors, cytokines, cell adhesion molecules, growth factor binding proteins and can also function as antiproteases. Additionally, GAGs can also bind directly to other ECM molecules chondroitin and dermatan sulphate, for example, bind to fibronectin and laminin.

Another vital function of proteoglycan/GAG structures is their high affinity for binding water, providing hydration to the skin and facilitating the movement of molecules though the protein-rich matrix.

1.2.5.2 Dermal fibroblasts

Dermal fibroblasts are the principal cells responsible for the production and regulation of ECM macromolecules including collagen, elastin and proteoglycan/GAG with niche-specific product synthesis variations such as greater collagen type III production in papillary (upper) compared to reticular dermis (Sorrel *et al.*, 2007). Fibroblasts express a specific genetic

profile depending on the developmental stage of the donor, anatomical site and signals in the local environment e.g. in wound healing (Chang *et* al., 2002, Rinn *et al*., 2006).

Positional memory is inferred, in part, by the retention of site-specific *HOX* gene expression patterns established during embyogenesis, suggesting fibroblasts have positional memory that enables their function to be tailored exclusively according to environmental requirements. Hence, fibroblasts originating from different skin sites can even be considered as distinct, differentiated cell types. Remarkably, recent findings have demonstrated that fibroblasts can also be induced to become pluripotent stem cells by treatment with certain transcription factors (Takahasi *et al*., 2007).

Fibroblast dysfunction has been implicated in a range of dermal conditions, including connective tissue tumours (keloids) characterized by excessive collagen deposition and a myofibroblast-like cellular phenotype.

1.3 Dermal vasculature, lymphatics and subcutaneous fat

The vasculature of the skin is structured as a subpapillary plexus with capillary/venule loops that reach between rete ridges of the DEJ, originating from vessels in the underlying subcutaneous adipose tissue. Arterioles are distinguishable from venules by the presence of subendothelial elastic tissue. Dermal vasculature provides the skin with nutrients and oxygen, in addition to regulating body temperature through vasodilation (allowing the dissipation of excess heat in a hot climate) or vasoconstriction (reducing dermal blood flow to maintain core temperature in a cold environment).

Lymphatic bulbs terminate in the papillary dermis and form an interconnecting system that drains particulate liquid materials (e.g. proteins) into local lymph nodes. The lymphatic

system is crucial for regular skin function, with abnormalities leading to diseases such as lymphoedemas (Jurisic and Detmar, 2009).

The subcutaneous adipose layer provides mechanical protection, insulation and energy storage. Adipocytes are also involved in endocrine signalling by secreting the appetite-regulating hormone leptin (Farooqi and O'Rahilly, 2008), as well as roles in osteogenesis, angiogenesis and phagocytosis.

1.4 Stem cells

The maintenance of dermal integrity is achieved through distinct resident stem cell (SC) pools occupying the hair follicle bulge, basal epidermal layer and base of sebaceous glands (Braun and Prowse, 2006, Watt and Hogan, 2000). Expression of discrete signalling pathways (Fuchs, 2008) direct the fate of SC towards either self-renewal through proliferation (e.g. c-Myc, integrin β-1, p63, TGF-α, TGF-β (-)) or specialization through differentiation (e.g. Notch, PPAR-α, CCAAT-enhancer-binding protein α/β, activating protein (AP)-2 α/γ).

The exact models for dermal SC propagation are still undergoing clarification. In the case of epidermal SC, evidence exists for both (i) symmetrical proliferation where transient-amplifying cells (derived from a single SC) undergo differentiation following several rounds of lateral division and (ii) asymmetrical proliferation where SC can shift their spindle orientation to perpendicular thus giving rise to either more SC or directly to differentiated spinous layer cells (Lechler and Fuchs, 2005).

In addition to niche-specific dermal SC, skin-derived precursors have been isolated from human skin with multipotent capabilities for differentiation into neurons, glia, and smooth muscle cells (Toma *et al*., 2005). Furthermore, certain dermal fibroblast subsets have been identified with adipogenic, osteogenic, chondrogenic, neurogenic and hepatogenic potential

when cultured with certain inducers (Chen *et al*., 2007), or the ability to be reprogrammed into pluripotent embryonic SC-like precursors (Park *et al*., 2008), increasing the feasibility of donor-specific cell therapies.

1.5 Skin function

Skin provides organisms with a variety of vital functions including physical, chemical and UV radiation barrier function, temperature homeostasis, mechanical protection, immunomodulation, sensory response and sociosexual communication (further detailed below). Severe loss of function has been defined by Irvine as 'skin failure', occurring in several dermatological diseases (Irvine, 1991).

1.5.1 Barrier function

1.5.1.1 Physical

Arguably the most immunologically active organ in the body, the skin's primary role is to form a two-way physical barrier to the exterior environment, preventing both the inward and outward passage of water and electrolytes. This vital function is granted exclusively by differentiated epidermal keratinocytes with the underlying dermis being almost completely permeable. Specifically, the terminally differentiated, cornified stratum corneum keratinocytes (corneocytes) are embedded in a lipid-rich intercellular matrix. The corneocyte envelope develops during keratinocyte differentiation forms an insoluble exoskeleton (Rice and Green, 1977) which is also durable and flexible (Figure 1-8).

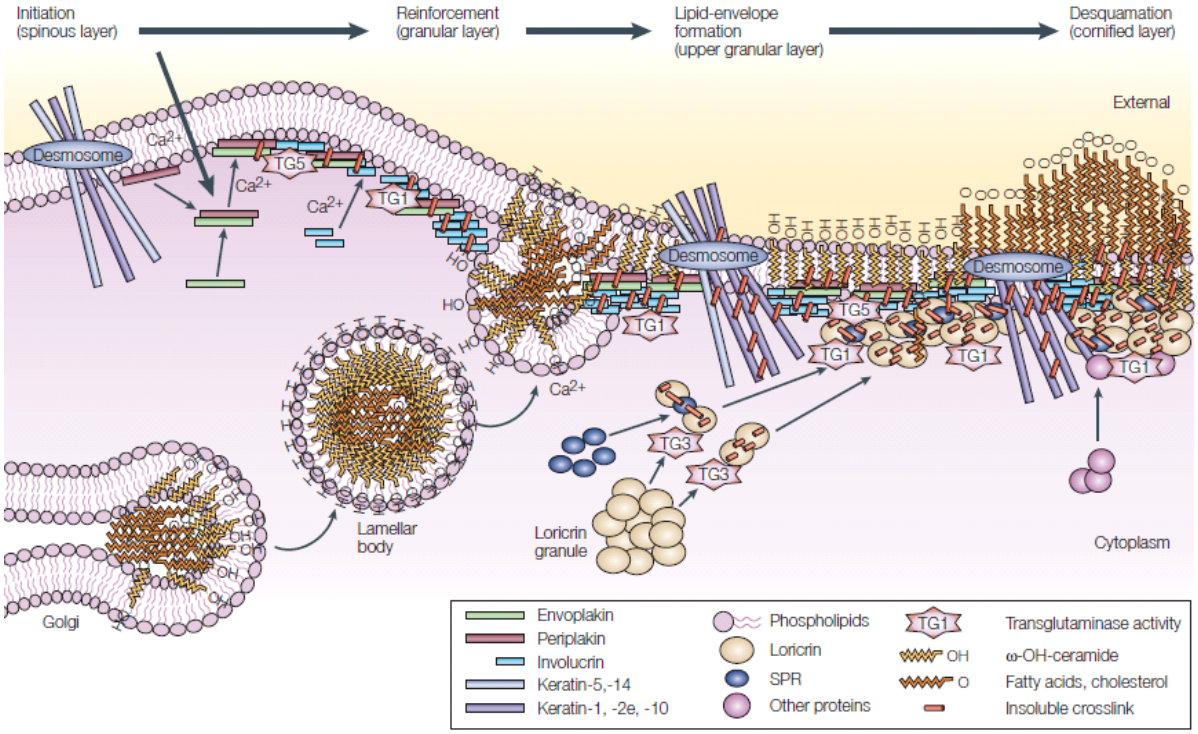


Figure 1-8 Generation of the epidermal cornified cell envelope. Calcium gradient-mediated desmosomal proteins are reorganised to form a scaffold along the plasma membrane used for the external attachment of lipid-rich molecules (cholesterol and ceramide) and internal attachment of loricrin, small proline-rich protein (SPR) cross-linked by transglutaminases (Candi *et al*., 2005)

Additionally, lamellar bodies (membrane coating granules) excrete lipid-rich molecules such as free fatty acids (Schurer and Elias, 1991), cholesterol (Feingold *et al*., 1990) and ceramides (Elias and Brown, 1978) into the extracellular *milieu*, coating the cornified envelope exterior. This highly lipophilic environment is perfectly adapted as an impermeable barrier to polarised molecules, preventing excessive water loss from the body.

1.5.1.2 Chemical

The *stratum corneum* also prevents undesirable invasion by resident skin flora or pathogenic microorganisms. The physical barrier, assisted by continuous desquamation, is also re-enforced by antimicrobial chemical barrier properties. Sebum lipids and glycophospholipids/free fatty acids of the *stratum corneum* are naturally antibacterial and

bacteriostatic respectively, while AMPs coating the skin and associated appendages provides the first line of defence against bacteria, fungi and some viruses (Niyonsaba *et al*., 2006).

Antibiotic α-defensins are expressed in skin by neutrophils with direct antibacterial properties and the ability to stimulate the immune system through increased tumour necrosis factor (TNF)-α/IL-1 expression in bacteria-activated monocytes. Conversely, β-defensins act solely by indirect means, being chemotactic for immature dendritic and memory T-cells, facilitating histamine release and prostaglandin production in mast cells. Mammal-specific cathelicidin also possesses bactericidal abilities following proteolytic activation from its precursor by neutrophil elastase and proteinase. Cathelicidin also functions as a chemoattractant for a host of inflammatory cells.

Cathelicidins and defensins are reported in a variety of dermal environments including sweat gland fluids, duct epithelia and hair follicles (Schittek *et al*., 2001, Fulton *et al*., 1997), providing additional protection in areas where the physical barrier is absent or limited. Furthermore, impaired AMP production has been linked to atopic dermatitis (Ong *et al*., 2002). Importantly, AMP production is upregulated in response to cutaneous injury, both by increased keratinocyte production and from neutrophils that respond during acute inflammation (Gallo *et al*., 2002).

1.5.1.3 UV radiation

The skin also functions as a barrier against damaging solar UV radiation. UVB rays penetrate the superficial epidermis causing acute sunburn which, over the course of chronic lifetime exposure, contributes to photo-ageing and skin cancer while UVA is able to penetrate deeper and is thought to be the primary cause of skin ageing.

Protection against UV is granted by the epidermal melanin barrier and lipid barrier of the *stratum corneum*. A large proportion of UV is absorbed in these structures, reducing the exposure of DNA and minimising the impact on genomic instability.

1.5.2 Temperature homeostasis

The skin is able to detect and respond to extreme changes in ambient temperature in order to maintain a constant core temperature. Warm- and cold-sensitive thermoreceptors are distributed evenly over the skin signalling through the hypothalamus to regulate sweating and shivering. The rich dermal vasculature is also integral to thermoregulation.

However, these processes play only a minor, physiological role in the modulation of core temperature, which is influenced to a much larger degree by behavioural responses such as adding/removing layers of clothing.

1.5.3 Mechanical function

Mechanical protection against blunt physical trauma is granted primarily by the structural properties of the dermis. Elastic fibres allow a reversible initial stretching capacity of 10-50%. Secondary 'viscous extension' stretching occurs if remaining taut for longer periods of time and is irreversible, which causes collagen fibrils to slip relative to each other in contrast to a stationary reorientation toward the load axis during reversible stretching. Moreover, dermal collagens are aligned to form a dense, highly organized structure conferring skin with tensile strength.

Skin can also be reversibly compressed around a pressure point, exerting the force, reducing the pressure on any particular point and leaving a temporary imprint (such as from a pinch) thought to occur as a result of a flow of inter-collagen ground substance flow.

Due to its protein and lipid constituents, the epidermis is also relatively strong, capable of stretching to maintain blister fluid. This contrasts to the relatively weak DEJ with underlying collagen of the papillary dermis particularly susceptible to blistering agents.

1.5.4 Skin as an immune system

Skin plays a crucial role in immunological host defence. Following breach of the physical and chemical barrier, an orchestra of permanently-resident epidermal cells e.g. keratinocytes, Langerhans' cells and dermal cells e.g. natural killer T (NKT)-cells, fibroblasts, dendritic cells, macrophages, mast cells and T-cells are activated to initiate an immune response (Nestle *et al*., 2009, Figure 1-9). Remarkably, the skin contains $2x10^{10}$ T-cells - twice the normal number in circulation. If required, additional transiently-resident cells are recruited from the extensive dermal vasculature.

Traditionally, keratinocytes have been regarded as a passive “brick” contributing primarily a structural role to the integrity of the epidermis. However, research conducted over the past decade has revealed that keratinocyte function also includes the ability to secrete a plethora of inflammatory and immunomodulatory mediators including cytokines, chemokines, peptides and growth factors and associated receptors following injurious stimuli. IL-1 and TNF-α cytokines directly promote endothelial adhesion molecule expression (e.g. E-selectin, intercellular adhesion molecule-1, vascular cell adhesion protein) facilitating leukocyte recruitment from the circulation and indirectly induce Langerhans' cell activation, differentiation and migration by stimulating expression of secondary cytokines e.g. granulocyte-macrophage colony-stimulating factor (GM-CSF). Keratinocyte-derived cytokines are involved in the modulation of T-cells and dendritic cells. Furthermore, secreted chemokines (e.g. monocyte chemotactic protein, cutaneous T-cell-attracting chemokine, eotaxin) regulate lymphocyte, neutrophil, eosinophil, monocyte, macrophage, mast cell and

dendritic cell trafficking. Studies have also implicated keratinocytes in antigen-specific responses through inflammatory stimuli-induced expression of CD80, a molecule typically associated with antigen-presenting cells (APCs), suggesting a further role for keratinocytes in antigen presentation (Wakem *et al*., 2000).

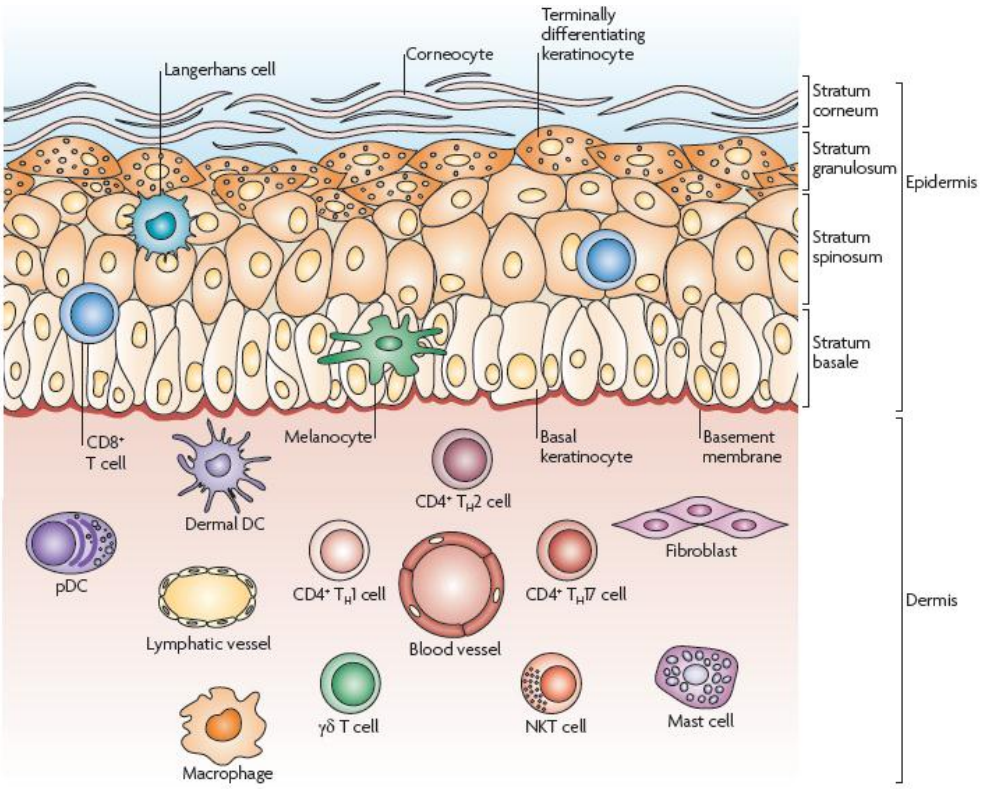


Figure 1-9 Immune cells of the skin. Following stimulation by UV, trauma, irritants or infection, a host of permanently-resident cells become immunologically active including pre-dendritic cells (pDC), fibroblasts, NKT-cells, dendritic cells (DC). Cytokine/chemokine-activated APCs (e.g. DC) then contribute to adaptive immunity by initiating clonal expansion of memory T-cells which also secrete pro-inflammatory mediators further amplifying the immune response (further discussed in text, Nestle et al., 2009)

Dermal-resident NKT-cells are a lymphocytic subset (representing 10% of circulating lymphocytes) capable of inducing apoptosis of tumour and infected cell targets. In contrast to T- and B-cells, NKT-cells are immediate initiators of the innate immune response, lacking the requirement for a proliferative amplification phase. Following target recognition, NKT-cells generate cytokines (e.g. TNF-α, interferon (IFN)-γ, GM-CSF) and chemokines, enhance cytotoxicity and migrate. Virus-induced IFNs (α/β) and IL-12 are also potent activators of NKT-cell cytotoxicity and IFN-γ respectively, highlighting the importance of NKT-cells

during innate defence against viral infections (Biron, 1997). Furthermore, NKT-cells are activated during infection by a host of other cytokines (e.g. TNF-α, IL-15) and chemokines, although the exact mechanisms underlying activation remain elusive (Scott and Trinchieri, 1995). Dysfunction of NKT-cells has been linked to a variety of skin disorders including atopic dermatitis (De Benedetto *et al*, 2009), sarcoidosis (Noor and Knox, 2007) and infection (Smith and Kotwal, 2002).

Similar to keratinocytes, fibroblasts are also capable of playing a dual role by producing structural ECM proteins under physiological conditions and by regulating immunological processes during inflammation and wound healing. Fibroblast-derived GAGs form proteoglycans which can bind cytokines, chemokines and growth factors, increasing their availability to immune modulators whilst collagens, laminins and fibronectin regulate interactions between fibroblasts and inflammatory cells. The functional profile of fibroblasts is dictated by their immediate microenvironment, including structural interactions (e.g. collagen contraction status) and extracellular cytokine, chemokine, protease and growth factor *milieu* (Nagakawa *et al*., 1989, Kovacs, 1991, Xu and Clark, 1996). Furthermore, fibroblasts isolated from hypertrophic scars display an altered response to epidermal growth factor (EGF) and increased collagen production (Garner *et al*., 1993). During wound healing, activated fibroblasts (myofibroblasts) migrate toward the damaged tissue, using ECM proteins as a scaffold, increasing proliferation and production of new ECM components. As the newly synthesized collagens mature and the wound contract, proliferation is gradually inhibited and the myofibroblasts return to a resting state (Grinnell, 1996). In addition to the exclusive role of matrix remodelling during wound repair, dermal fibroblasts secrete pro-inflammatory mediators (e.g. TNF, IL-6) which assist in sustaining the immune response until resolution of the initial trigger.

Immunologically-activated keratinocytes, NKT-cells and fibroblasts secrete a variety of factors that propagate the immune response through the regulation of APCs including macrophages and dendritic cells (differentiated from bone marrow-derived monocytes) which function in both innate immunity whilst processing and presenting foreign- or host-derived antigens to T- and B-cells during acquired immunity. Briefly, macrophages function primarily as professional phagocytic cells, recognizing, engulfing and destroying pathogens through the production of destructive mediators including acidic phagosomes, proteolytic enzymes, matrix metalloproteinases (MMPs) and reactive oxygen species (ROS, Gordon, 2003). Furthermore, microbial stimulation (for example through lipopolysaccharides – LPS), coupled with IFN-γ priming, triggers cytokine release which further stimulate (e.g. IL-1/6, TNF) or inhibit (e.g. IFN-α/β) macrophage function in an autocrine manner, whilst contributing to paracrine regulation of surrounding immune cells, resulting in a highly co-ordinated immune response. Alternatively, macrophages can be activated in a non-microbial manner through antibody and complement receptors, or subsequent to IL-4/13 cytokine stimulation as part of phagocytic humoral immunity. Macrophages are also regulated in an endocrine fashion for example, through GC receptor (GR) signalling, which leads to downregulation of APC function, induction of anti-inflammatory cytokine secretion and production of auto-inhibitory prostaglandins. Macrophage immunosuppression can also be induced by certain pathogens (e.g. human immunodeficiency virus), contributing to sustained infection. Whilst macrophages are initially recruited to sites of inflammation as undifferentiated monocytes, they are poor proliferators, instead remaining quiescent in the skin for months, guarding against future insults.

Dermal dendritic cells are classed according to function, varying in their abilities as APCs, Langerhans' cell precursors, pro-inflammatory mediators and phagocytic cells (Savina and

Amigorena, 2007). Recently, a novel class of pre-dendritic cell producing IFN type 1 has been identified in inflamed skin (Magyarics *et al*., 2008).

Antigen-activated T-cells support the immune response by amplifying T-cell subtype specific cytokine secretion. 'Helper' T-cells secrete either IL-2/IFN-γ (T_h1) assisting cytotoxic and cell-mediated responses, or IL-4/5/9/10/13 (T_h2) aiding antibody-mediated responses. T_h1 and T_h2 T-cells primarily support APCs and B-cells respectively, whilst activated cytotoxic T-cells (IFN-γ-producing T_c1 or IL-4/5-producing T_c2 subtypes) induce apoptosis through mechanisms similar to those implemented by NKT-cells. Selection of appropriate T-cell response (i.e. preferential subset expression) is tailored to meet the specific requirements of the inflammatory trigger (UV, bacterial, viral etc.) by inter-subset cross-regulation such as T_h1 cytokines inhibiting T_h2 cells and vice versa.

The inflammatory responses in skin are further regulated by numerous other cellular components including transiently-resident phagocytic neutrophils, circulatory basophils and eosinophils and permanently-resident granulated mast cells which facilitate innate immunity by increasing vascular permeability, inducing neutrophil recruitment and promoting a T_h2 T-cell phenotype. Further dissection of the multitude of cellular components and associated mediators involved in the complex process of cutaneous inflammation and immunity is beyond the scope of this thesis and will not be further discussed here.

1.6 Ageing

1.6.1 Overview

Ageing can be defined simply as the accumulation of changes in an organism or object over time. However, in humans, ageing refers to a complex multidimensional process of physical, psychological, and social change. It involves a progressive loss of biological function

accompanied by decreasing fertility and increasing mortality with advancing age (Bowen and Atwood, 2004).

Ageing affects every tissue in everybody, although the rate of ageing is highly variable both between organs in the same individual and between the same organs in different individuals based on differing environmental and genetic modifying factors. The most obvious symptoms of ageing manifest as changes in our external physical appearance in tissues such as skin (e.g. wrinkling, dryness, age spots), hair (greying, thinning, balding), fat (e.g. omental distribution, increased storage) and muscle (e.g. decreased strength and mass). Internal changes affect tissues including bone (e.g. decreased mass, increased porosity, osteoporosis), heart (e.g. blood pressure, cardiovascular disease), reproductive organs - particularly females (e.g. menopause) and brain (e.g. decreased reaction time and memory, Alzheimer's disease). These physical changes are attributable to alterations in the homeostatic control of processes such as metabolism and immunity (Figure 1-10) - largely influenced by a modified hormonal regulation repertoire (Bowen and Atwood, 2004).

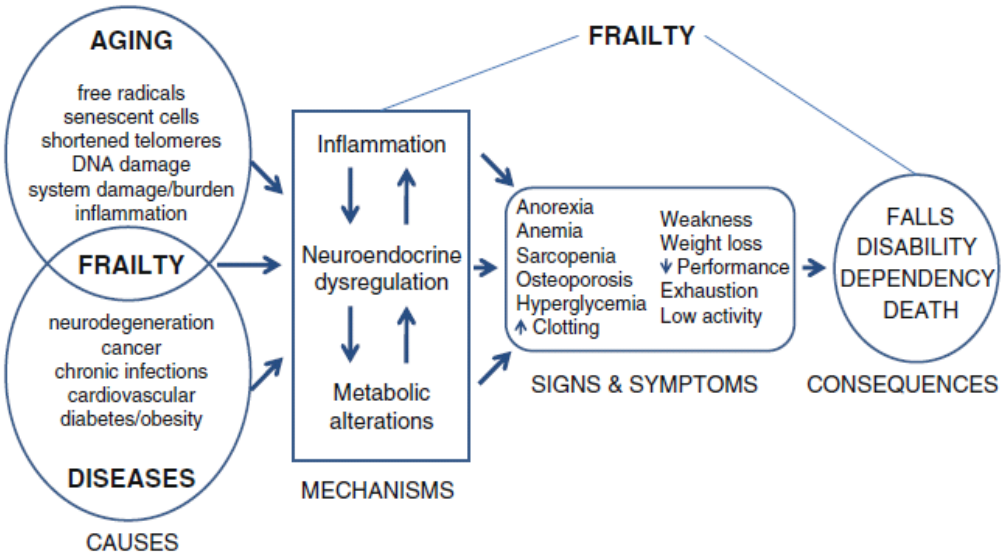


Figure 1-10 Age-related diseases. Schematic representation of the relationship between ageing and associated diseases which result in consequences associated with frailty. Reproduced from Fulop *et al*., 2010

Age-related dysregulation of homeostasis culminates in associated pathologies such as increased susceptibility to infection, neurodegenerative disease, type 2 diabetes (T2D), atherosclerosis and chronic heart failure (Fulop *et al*., 2010). It should be stressed, however, that although all organisms are subjected to ageing, similarly to the variation in physical changes occurring at a tissue level, individuals differ in their tolerance to ageing with regards to exhibiting disease. This variation is based on the ability of individuals to cope with the traditionally non-modifiable internal changes associated with chronological ageing and the superimposing contribution of modifiable external factors termed intrinsic and extrinsic ageing respectively.

With the ever-increasing proportion of elderly individuals within the populations of developed countries resulting from increased life-expectancy, closely followed by the ongoing transition of developing countries, research into the causes of ageing and age-related diseases will be an absolute necessity in the forthcoming years.

1.6.2 Chronological (intrinsic) ageing

Chronological ageing encompasses the ageing of all organs including skin, independently of external influences and its causes are far less clear than those of extrinsic ageing.

1.6.2.1 Oxidative metabolism

A plausible contributor to intrinsic ageing is the generation of ROS during oxidative metabolism (Sohal and Weindruch, 1996), primarily mitochondrial in origin, with effects similar to those of extrinsic ageing such as DNA oxidation resulting in mutation, protein oxidation with loss of function and oxidation of membrane lipids leading to reduced transport and/or membrane signalling. A major consequence of these effects is a reduction in cellular

antioxidant capacity causing further oxidative damage, an exacerbation of the ageing phenotype (Harman, 2001) and ultimately, organismal death.

Molecular oxygen is a primary source of cellular stress but equally indispensable for aerobic life. In addition to the cellular damage inflicted by metabolism- and photochemically-derived ROS, it has been proposed that ROS act as subcellular signals that may regulate a "molecular clock" that determines the pace of ageing (Sohal and Allen, 1990). Hyperoxia (O_2 above 5%) has been shown to cause toxicity in a variety of organisms and decreased proliferation of cells (Davies, 2000) including vascular smooth muscle cells (VSMCs, Absher *et al*., 1994), and unlike hydrogen peroxide, at no point is hyperoxia stimulatory to cell growth. The underlying causes of this toxicity are not fully known but studies have demonstrated an impact on specific points of the cell cycle (Balin *et al*., 1978) and a stimulation of telomere shortening (Von Zglinicki *et al*., 1995) as well as regulation of gene expression and signal transduction pathways (Monteiro and Stern, 1996, Gillespie *et al*., 2010, Hsieh *et al*., 2010, Circu and Aw, 2010).

1.6.2.2 Replicative senescence

The process of cellular replicative senescence is also postulated to contribute to endogenous ageing (Cristofalo *et al*., 2004), and may also be involved in extrinsic ageing. In contrast to cancer cell lines, somatic cells in culture are capable of only a limited number of divisions (Hayflick, 1965), morphologically enlarged and flattened, sensitized to contact inhibition, unresponsive to mitogenic treatment and express beta-galactosidase upon senescence (Dimri *et al*., 1995). Senescent fibroblasts express increased MMPs and decreased tissue inhibitor of MMPs (TIMPs, Millis *et al*., 1992, West *et al*., 1989) alongside decreased expression of ECM components such as elastin, laminin and several forms of collagen (Linskens *et al*., 1995)

shifting their function from matrix-synthesizing to matrix-degrading. The mechanisms underlying replicative senescence are highly complex and involve the dysregulation of multiple systems including signal transduction, cellular stress, apoptosis and telomere shortening. Alterations in lipid-mediated signalling involve increased release of growth-inhibitory prostaglandins and ceramide (Venable *et al*., 1995) whilst stimulatory second messengers such as diglyceride are decreased (Venable *et al*., 1994). This leads to defects in signal transduction including the downregulation of phosphatidylinositol 3-kinase /protein kinase B and Raf/MEK/ERK pathways - essential for normal proliferative response to mitogenic stimulation.

Regarding the former, the protein kinase $p70^{s6k}1$, phosphorylated in its active form, is involved in regulating translation of mRNAs containing polypyrimidine tracks through ribosomal protein S6 phosphorylation. Activation of this kinase is reduced in senescent fibroblasts (Zhang *et al*., 2000) leading to decreased translation of mRNAs such as components of translational machinery (Brown and Schreiber, 1996). Additionally, treatment with phosphatidylinositol 3-kinase inhibitor in early passage fibroblasts induces growth arrest and senescence (Tresini *et al*., 1998).

Activation of the Raf/MEK/ERK pathway is also compromised during senescence with a dramatic reduction in phosphorylated (activated) ERK in the nucleus of late-passage fibroblasts (Park *et al*., 2000, Tresini *et al*., 2001). Concomitantly, there is reduced expression of, and failure to induce upon mitogenic stimulation, the ERK target early response gene c-fos (Seshadri and Campisi, 1990, Irving *et al*., 1992, Riabowol *et al*., 1992) which is essential for cell-cycle progression. The downregulation of ERK signalling may occur through the increased activity of MAP kinase phosphatases (MKP) which dephosphorylate and deactivate

MAP kinases (Torres *at al*., 2003). The signalling pathways affected by replicative senescence are illustrated in Figure 1-11.

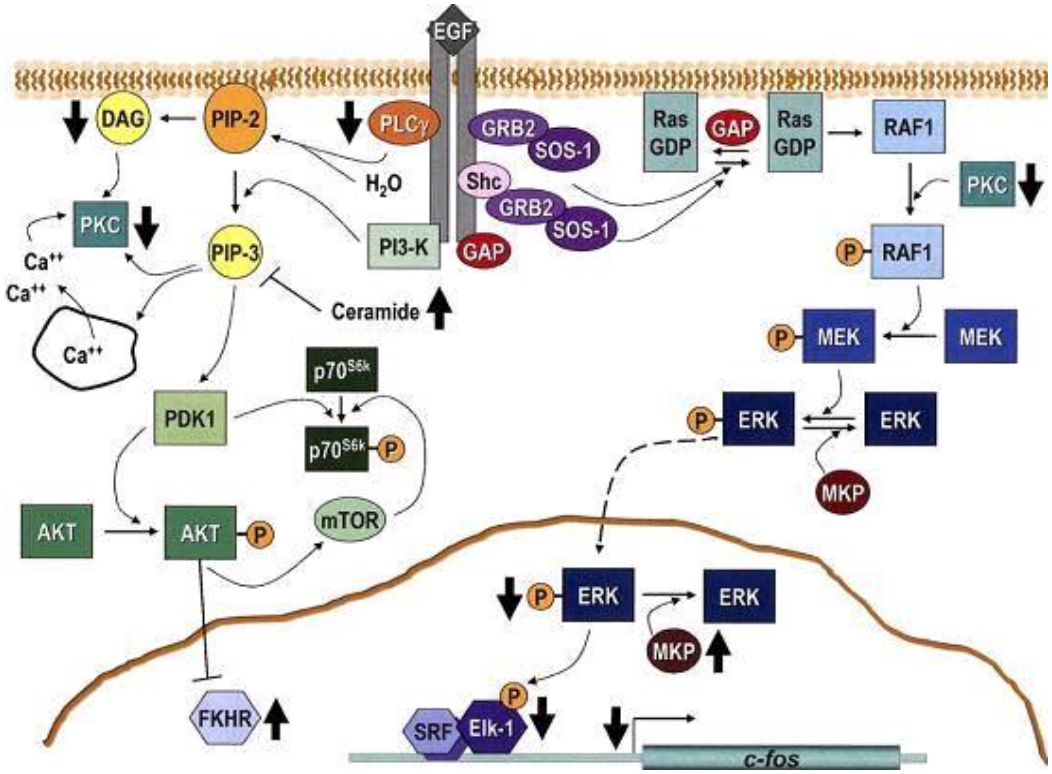


Figure 1-11 Signal transduction pathways activated by receptors with tyrosine kinase activity. The EGF receptor is shown as an example. Events following receptor ligation and autophosphorylation include formation of multi-protein complexes, phospholipid turnover, calcium mobilization and activation of protein kinases, phosphatases and transcription factors. Some of these events are illustrated above with an emphasis on pathways known to be affected by replicative senescence. Bold arrows indicate signalling molecules with altered expression and/or activity in senescent cultures (Cristofalo et al., 2004)

As cellular senescence is believed to contribute to the ageing process, it seems implausible that senescence is associated with a downregulation of c-fos-mediated AP-1 signalling *in vitro* while *in vivo* evidence suggests AP-1 signalling increases with ageing (Fisher *et al*., 1998, Walter and Sierra, 1998, Xiao and Majumdar, 2000, Jung *et al*., 2009). This disparity is explained by the formation of functional Jun/Jun AP-1 homodimers (additionally to Fos/Jun heterodimers) as c-jun expression increases with ageing *in vivo* (Chung *et al.*, 2000) but is not altered during replicative senescence (Irving *et al*., 1992), while c-fos decreases with replicative senescence (described above) but remains unchanged with *in vivo* ageing (Fisher *et*

al., 1998). Furthermore, c-fos, c-jun and AP-1 were found to be fully responsive to mitogenic stimuli in same-passage fibroblasts from centenarians compared to young donors (Grassilli *et al*., 1996), suggesting the mitogenic response deficit postulated to occur with replicative senescence does not translate to *in vivo* ageing. The inconsistent "loss of function *in vitro*" but "gain of function *in vivo*", regarding AP-1 and ageing (the cause of which remains unclear), highlights the need to exercise caution when attempting to interpret data generated using replicative senescence as a model of ageing.

1.6.2.3 Proteolysis

The proteasome is a multicatalytic protease composed of a catalytic core and a regulatory subunit that confers ubiquitin specificity and adenosine triphosphate dependence. It functions primarily to degrade abnormal, misfolded, biologically inactive proteins produced during processes such as oxidative damage.

Several studies report a decrease in proteasomal function with age in epidermis (Bulteau *et al*., 2000), T lymphocyte subsets (Ponnappan, 2002), muscle (Husom *et al*., 2004), retina (Louie *et al*., 2002) and in heart, lung, kidney, liver and spinal cord (Keller *et al*., 2000). This results in an accumulation of ubiquitin-conjugated protein aggregates that impede a variety of cellular processes (Lindner and Demarez, 2009). The mechanisms underlying reduced proteasomal activity with age remain to be fully elucidated but are thought to involve alterations in the quantity/composition of proteasomal subunits, modification causing loss of function (e.g. oxidation) and inhibition by damaged proteins that accumulate with age (Carrard e*t al*., 2002).

The proteasome is not only responsible for the removal of undesirable proteins but also functions to regulate the normal turn-over of proteins involved in cell cycle progression (King

et al., 1996), gene expression (Muratani and Tansey, 2003), apoptosis (Naujokat and Hoffmann, 2002), antigen presentation (Lehner and Cresswell, 1996) and signal transduction (Coulombe *et al*., 2003). This suggests the age-related decrease in proteasomal activity is a plausible contributor to the ageing phenotype.

1.6.2.4 Apoptosis

Apoptosis is a highly conserved process of programmed cell death that prevents the sporadic lysis and release of cellular content into the extra-cellular environment as occurs in necrosis. This prevents release of degenerative enzymes and the onset of inflammation reactions. Specific proteases (caspases) and nucleases are activated by a wide range of stimuli including excessive DNA damage, serum withdrawal and steroid hormone treatment, resulting in the degradation of cellular structures.

While apoptosis is essential for the normal proliferation and amelioration of dysfunctional or damaged (including cancerous) cells, it is also implicated to be defective and a causative agent in ageing (Campisi, 2003). Evidence to support this includes mice with transgenic defects in apoptotic pathways that die prematurely of cancer (Wu and Pandolfi, 2001) and, conversely, aged rats that have reduced hepatocyte apoptosis compared to young rats following induced DNA damage (Suh *et al*., 2002). Equally, no clear consensus exists regarding apoptosis rates during cellular senescence with studies reporting both increased (Wang *et al*., 1995, Seluanov *et al*., 2001) and decreased (DeJesus *et* al., 2002, Zhang *et* al., 2002) resistance to apoptosis. It is therefore likely that age-related alterations in apoptosis are regulated by a variety of tissue and cell-type specific responses to complex signalling pathways.

1.6.2.5 Telomeres

In humans, telomeres are nucleoprotein complexes composed of several kilo-bases of double-stranded sequence repeats (or up to 400 bases of single-stranded repeats at the 3'-end overhang) of the sequence TTAGGG that cap the ends of chromosomes. Forty years ago, Olovnikov proposed the Theory of Marginotomy - which suggests the 5'-end progeny strand of linear chromosomes would shorten upon each round of replication and may contribute to the finite number of replications of cells in culture (Olovnikov, 1973). The process of telomere erosion is controlled by telomerase, a nuclear protein with reverse transcriptase activity which replaces lost TTAGGG units following replication. Telomerase activity is associated with cellular longevity in normal physiology (Klapper *et al*., 1998) alongside immortal cells and cancer (Kim *et al*., 1994).

Although initially hypothesized that promoting telomerase expression would defer cellular senescence and increase organismal longevity, studies have revealed a propensity towards malignancy in cultures with induced telomerase activity, albeit possessing unlimited proliferative capacity (Wang *et al*., 2000). This suggests that replicative senescence, through mechanisms such as telomere shortening, has evolved to prevent cancer. Many ongoing studies are investigating the signalling process between telomere shortening and the induction of senescence with emerging evidence suggesting that "uncapped" telomeres (lacking telomerase activity) are recognized as double stranded DNA breaks (di Fagagna *et al*., 2003).

As telomere length and telomerase regulation is known to have such a profound effect on the longevity of cells in culture, it may appear surprising that studies comparing telomere length in proliferating and non-proliferating human tissues have only demonstrated a modest decrease with age (Aikata *et al*., 2000, Kveiborg *et al*., 1999). Furthermore, Rudolph *et al* reported that telomerase-deficient transgenic mice did not display attenuated survival rates

compared to WT controls until after several generations (Rudolph *et al*., 1999), although this could be due to the telomere length being over four-fold longer than in humans.

In addition to conflicting data regarding cell signalling pathways (e.g. AP-1), the discordance between *in vitro* modulation of telomere length and limited *in vivo* evidence casts further doubt on the occurrence of replicative senescence during ageing, and its relevance as a model to study age-related processes *in situ* (Rubin, 2002). Finally, the fact that the senescent phenotype has not been confirmed in healthy tissues *in vivo* is consistent with this view (Severino *et al*., 2000).

1.6.2.6 Genomic instability

Genomic instability increases with organismal age and contributes to the ageing phenotype through several mechanisms including somatic mutations in extranuclear mitochondrial DNA (mtDNA, Lee *et al*., 2010), decreased cellular DNA repair capacity (Gorbunova *et* al., 2007) and chromosomal abnormalities. The age-related changes associated with these mechanisms are described in more detail below.

1.6.2.7 mtDNA mutations

Mitochondria are particularly susceptible to age-induced genomic instability as mtDNA has a greater mutation rate than nuclear DNA due to a lack of protecting histones, limited repair mechanisms and proximity to the high rate of ROS generation through oxidative phosphorylation. Progressive damage to the human fibroblast mtDNA replication control region in the form of somatic mutations is reported in normal old, but not young, donors (Michikawa *et al*., 1999). Additionally, a correlation between specific mtDNA mutations and donor age has been described for a range of human tissues including liver (Yen *et al*., 1991),

brain (Corraldebrinski *et al*., 1992) and diaphragm (Torii *et al*., 1992) alongside other species such as rhesus monkey muscle (Schwarze *et al*., 1995) and mouse muscle and brain (Chung *et al*., 1994 and Brossas *et al*., 1994 respectively).

Lee *et al*. demonstrated that not only does the mutation detection rate increase with human age (beginning around the mid-thirties), but also varies by tissue, with mutations arising earlier and in greater proportions in highly energy-demanding tissues such as muscle compared to testis (Lee *et al*., 1994), further highlighting the role of oxidative metabolism (and ROS) on the acceleration of ageing. Moreover, the proportion of mutant mtDNA correlates with oxidative modification levels (Lezza *et al*., 1999) in human brain while a common age-related mtDNA deletion can be directly induced with a sub-lethal dose of oxidative stress in human dermal fibroblasts (Dumont *et al*., 2000).

The causal role of mtDNA mutations to the ageing phenotype has been extensively studied in animal models where their induction resulted in a reduced lifespan and signs of premature ageing such as weight loss, reduced subcutaneous fat, alopecia, kyphosis (curvature of the spine), sarcopenia, osteoporosis, anaemia, thymic involution, testicular atrophy associated with depletion spermatogonia, reduced fertility, loss of intestinal crypts, heart enlargement, and hearing loss (Trifunovic *et al*., 2004, Kujoth *et al*., 2005). The mutant mice did not show any increased sensitivity to or signs of oxidative stress compared to wild-type (WT) controls, suggesting that oxidative stress is causative of age-related mtDNA mutations rather than a consequence. Further analysis revealed that cellular proliferation and senescence rates were not accountable for the premature ageing phenotype, but rather the extent of apoptosis which increased more rapidly during ageing of the mutant mice (Kujoth *et al*., 2005). Recently, there is an ongoing debate regarding whether point mutations or deletions are the main contributing factor to the phenotype of these mice (Vermulst *et al*., 2007, Edgar *et al*., 2010). Although

these studies provide strong evidence for a causal role of mtDNA mutations in the ageing process, there is arguable uncertainty around the applicability of transgenic mouse as models of natural ageing and the mechanisms involved remain unclear.

One possibility is that mitochondrial dysfunction may result in an age-related defect in retrograde (mitochondria-to-nucleus) signalling (Finley and Haigis 2009), striking at the heart of cellular systems and causing dysregulation in energy production, oxidative stress and cell survival. Transcriptional pathways implicated in this process include peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A, master transcriptional regulator of mitochondrial function and biogenesis), sirtuin (SIRT)-1 (protein deacetylase regulator of energy metabolism and oxidative capacity), AMPK (protein kinase sensor of cellular energy status) and mTOR (mitochondrial energy production). In addition to evidence suggesting these pathways are integral to correct mitochondrial function, emerging studies are reporting a link to lifespan in model organisms such as yeast, worms and flies and dysregulation in mammalian ageing (Figure 1-12). Currently, alterations in retrograde signalling pathways and increases in mitochondrial ROS production with age (further exacerbating mtDNA, protein and lipid damage, Short *et al.*, 2005) are fundamental areas of research focus for the elucidation of molecular mechanisms involved in ageing.

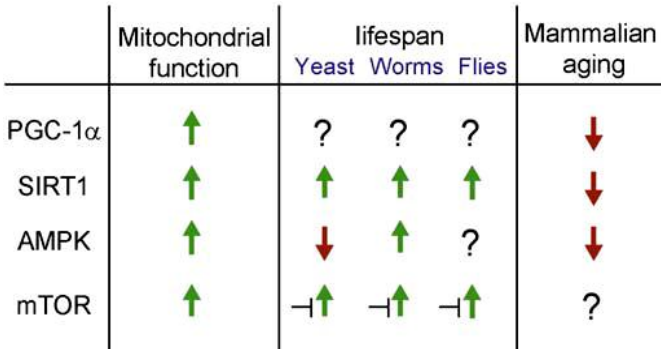


Figure 1-12 The role of major mitochondrial regulators on lifespan and ageing. All four proteins are capable of increasing measures of mitochondrial function, including mitochondrial number, oxidative phosphorylation capacity and fatty acid oxidation. Expression of the SIRT1 ortholog, Sir2, increases lifespan in yeast, worms and flies; AMPK increases lifespan in worms but decreases yeast lifespan, and inhibition of mTOR increases lifespan in all three model organisms. For mammalian ageing, arrows indicate how a given protein's expression or activity changes (reproduced from Finley and Haigis, 2009)

1.6.2.8 Decreased DNA repair mechanisms

As with mtDNA, nuclear DNA damage accumulates with age, occurs at an increased rate in older organisms and characteristically includes genomic rearrangements (Stuart and Glickman, 2000, Vijg and Dolle, 2002). Normally, mispaired bases are corrected by the mismatch repair (MMR) system (Figure 1-13), which would otherwise result in the introduction of point mutations. Defects in MMR have been associated with cellular senescence of T-cell clones *in vitro* (Annett *et al*., 2005) and mutations in MMR genes have been implicated in microsatellite destabilization, the incidence of which increases during human ageing (Karran, 1996, Neri *et al*., 2005).

Similarly, age-related alterations have been documented for base excision repair (BER) mechanisms (Figure 1-13) which function to remove lesions affecting one DNA strand and utilising the complementary strand to "fill the gap", particularly during repair of oxidised bases produced through ROS damage. Senescent human fibroblasts and leukocytes from older donors contain greater numbers of abasic sites (Atamna *et al*., 2000) - produced following glycosylase cleavage of the damaged base - suggesting a failure in the subsequent repair process; abasic endonuclease excision to leave a single-stranded gap, DNA polymerase-β base re-incorporation and ligation by DNA ligase. Indeed, reduced abundance of DNA polymerase-β in aged rodent tissues (Cabelof *et al*., 2002) and of abasic endonuclease in aged mouse germ cell extracts (Intano *et al*., 2002) has been reported. Furthermore, upon treatment with oxidizing hydrogen peroxide or a methylating agent, the number of abasic sites rose faster in young cells (early passage fibroblasts or young donor-derived leukocytes) suggesting a further defect in the initial mutation detection or excision by DNA glycosylases (Atamna *et al*., 2000).

Moreover, following γ-radiation, tissues from older mice contain higher levels of oxidized guanine than younger animals (Hamilton *et al*., 2001). As a key repair mechanism for oxidative damage, BER was analyzed in tissue extracts from young and old animals by incubating protein extracts with radiolabelled oligonucleotides containing single base lesions. A reduction in cleavage products indicated reduced glycosylase incision activity in the brains of older mice and interestingly, this defect was much greater in mtDNA compared to nuclear DNA (Imam *et al*., 2006). These results have also been corroborated using *in vitro* senescence culture of human fibroblasts (Shen *et al*., 2003). Alongside enzymatic dysfunction, age-related BER insufficiency may be caused by a lack of recognition of or response to DNA damage. For example, in old mice and senescent human fibroblasts, the translocation of glycosylase and abasic endonuclease into the nucleus and mitochondria is impaired (Szczesny *et al*., 2003, 2004). Moreover, impaired enzymatic recognition of single strand breaks by poly-(ADP ribose) polymerase (PARP) to initiate the repair process has been implicated in age-related genomic instability (Burkle *et al*., 2004) and defects in proteasome function (Bakondi *et al*., 2011). Conversely, PARP activation is associated with diabetic neuropathy (Stevens *et al*., 2011) suggesting enzyme levels must be tightly controlled to prevent disease.

Nucleotide excision repair (NER) removes short oligonucleotide sequences containing a damaged base (Figure 1-13), typically bulky lesions caused by carcinogens and UV exposure. NER is further classified into global genome repair or transcription coupled repair where corrections can be made in the transcribed strand of active genes and both systems require intricate multi-protein complexes. Studies exploring links between NER and cellular senescence have shown conflicting results *in vitro*. Following UV-induced cyclobutane pyrimidine dimers (CPD) in human fibroblast and trabecular osteoblast DNA no difference in the rate of NER was observed between early and late passage cells (Christiansen *et al*., 2000),

while another group demonstrated reduced UV-induced CPD removal in senescent fibroblasts (Boyle *et al*., 2005).

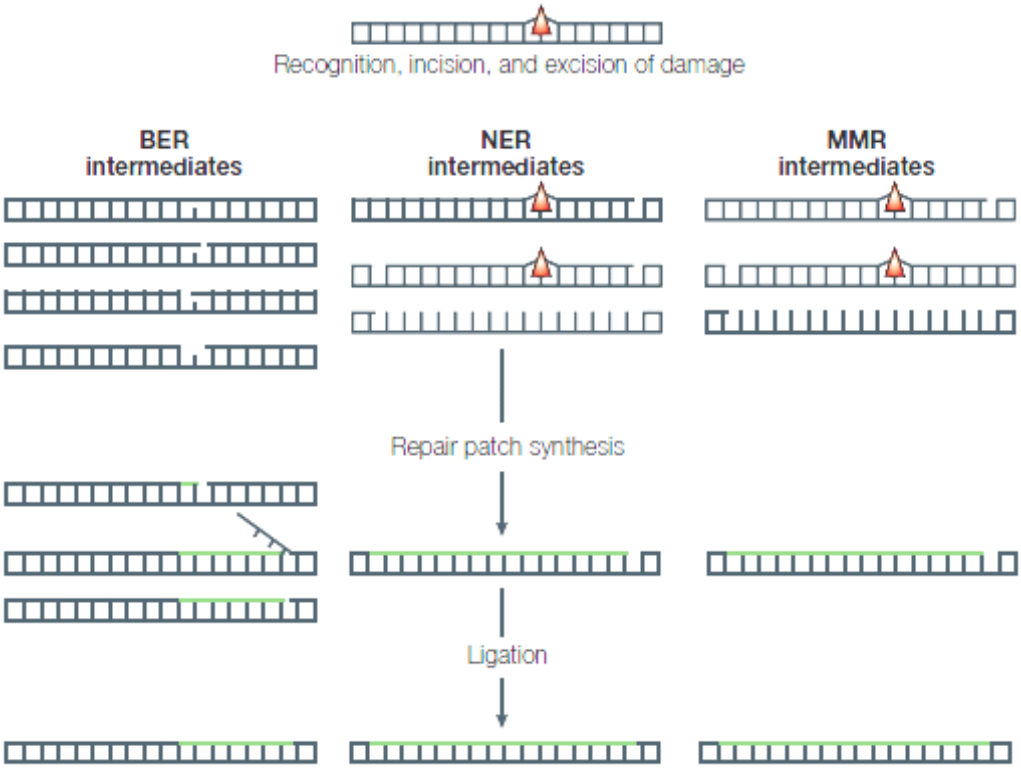


Figure 1-13 Excision repair pathways for DNA damage. The three main excision repair pathways in human cells - BER, NER and MMR - proceed through similar steps to restore the normal DNA sequence. Following recognition of altered DNA bases, incision of DNA is achieved by endonuclease activation. Subsequent displacement or degradation of single-stranded DNA sections containing the damage occurs via enzymes with helicase and exonuclease activity. Repair replication of the resulting DNA gap and strand ligation restores the double-stranded DNA molecule. The many repair enzymes that are involved in each specific step are tightly coupled and may be regulated or inducible by DNA damage response pathways (Cline and Hanawalt, 2003)

Conversely, studies comparing NER according to donor age have consistently demonstrated an age-related decrease in NER efficacy. UV-irradiated plasmids containing a damaged, inactive chloramphenicol acetyltransferase, transfected into human peripheral blood lymphocytes from donors of varying age displayed higher rates of reactivation following successful DNA repair in young donor-derived cells (Wei *et al*., 1993).

A similar study design in human dermal fibroblasts also reported increased introduction of new mutation in plasmids transfected into older donor cells in addition to reduced plasmid reactivation (Moriwaki *et al*., 1995), suggesting that not only is NER more ineffective with

age, but also more inefficient. Interestingly, repair efficiency of telomeric DNA is also reduced in fibroblasts from older human donors (Kruk *et al*., 1995). Furthermore, animal studies report CPD removal by NER is lower in aged rat hepatocytes (Guo *et al*., 1998). CPD and pyrimidine photoproduct removal rates are also decreased in dermal fibroblasts from older human donors (Goukassian *et al*., 2000), with removal also reduced in aged mouse round spermatids in a cell-type specific manner (Xu *et al*., 2005). Increased CPD removal rates have also been documented in younger skin epidermis *in situ* following upper arm UVB radiation (Yamada *et al*., 2006). Moreover, photoproducts in UV-irradiated skin are induced at a higher frequency in older human donors (Xu *et al*., 2000). Recently, studies inducing mutations in NER genes have reported accelerated ageing in mice (Niedernhofer *et al*., 2006).

1.6.2.9 Chromosome abnormalities

Chromosome abnormalities are the most severe form of genomic instability and if unrepaired, threaten the survival of the cell. Double stranded breaks (DSBs) are the most lethal type of DNA lesion and cause chromosomal loss, however, if incorrectly repaired they lead to genomic rearrangements - the incidence of which increases with age (Dolle *et al*., 1997, Vijg and Dolle, 2002, Ramsey *et al*., 1995, Tucker *et al*., 1999).

DSBs are repaired either by homologous recombination, where the sister chromatid serves as a template to complete the faulty locus, or by non-homologous end joining (NHEJ) which fuses two broken ends with little regard for sequence homology. The latter is highly error prone and often leads to deletions or insertions of filler DNA (Sargent *et al*., 1997). Additionally, DSBs situated between two direct repeats can be repaired by single strand annealing resulting in mutagenic deletion of the sequence between the repeats. These three mechanisms of DSB repair are illustrated in Figure 1-14.

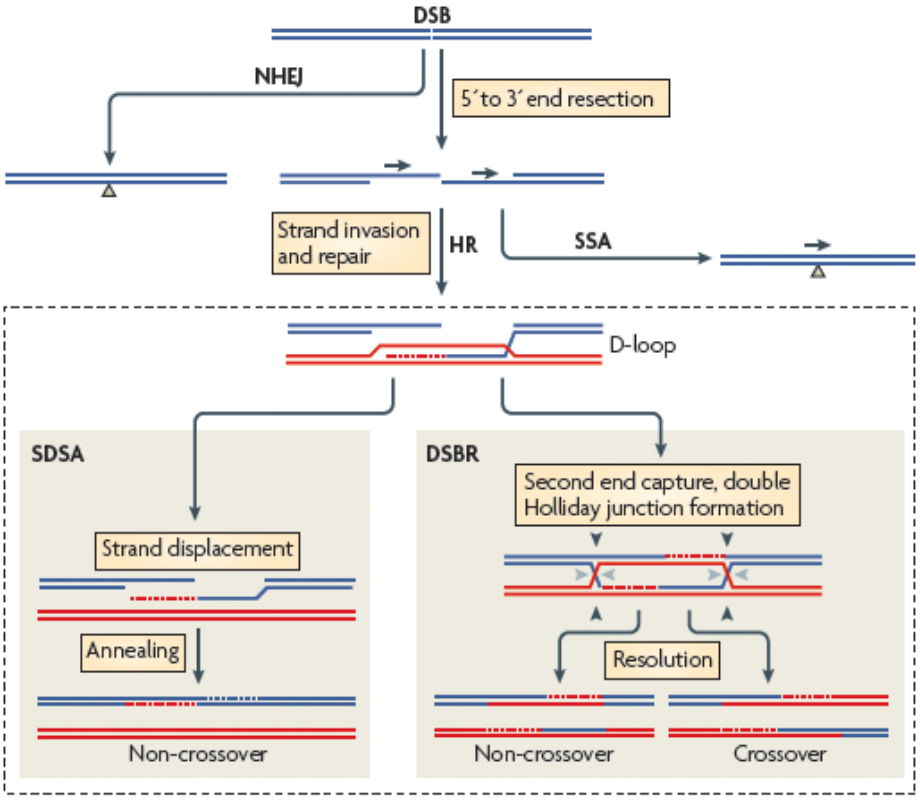


Figure 1-14 Double-strand break repair. Breaks are efficiently repaired in mammalian cells by homologous recombination (HR), NHEJ or single strand annealing (SSA). HR initiates with end resection, producing a 3′ single-stranded end that invades the sister chromatid template to initiate repair. Alternative HR pathways can ensue from the displacement loop (D-loop) intermediate: synthesis-dependent strand annealing (SDSA) and DSB repair (DSBR). In SDSA, the newly synthesized strand displaces to anneal to the other DNA end, resulting in a non-crossover outcome with the template DNA unchanged. In DSBR, the second DNA end is 'captured' by the D-loop forming a double Holliday junction, which can result in a non-crossover (cleavage at black or grey arrowheads) or a crossover (cleavage at black arrowheads on one side and grey arrowheads) outcome. SSA takes place when end resection occurs at sequence repeats (arrowheads) to provide complementary single strands that anneal, giving rise to a product with a single copy of the repeat and a deletion of intervening sequences, often with mutagenic outcomes (Moynahan and Jasin, 2010)

Studies investigating age-related changes in DSB repair are limited. Seluanov *et al*. recently reported that the efficiency of NHEJ is reduced by up to 4.5-fold in pre-senescent and senescent human fibroblasts, with higher precise ligation frequency in younger cells and extended deletions in older cells (Seluanov *et al*., 2004). Reduced NHEJ efficiency is also documented in ageing rat brain neurons (Vyjayanti and Rao, 2006) and interestingly in nuclear cortical extracts from brains of Alzheimer's disease - a highly publicised age-related condition (Shackelford, 2006).

Age-related abnormalities in NHEJ DSB repair may, in part, be due to alterations in the availability and function of accessory proteins involved in the process. For example, levels of the protein Ku which recognizes and binds DSB are reduced in ageing rat testis (Um *et al*., 2003), older donor-derived human lymphocytes (Ju *et al*., 2006) and senescent human fibroblasts (Seluanov *et al*., 2007). Furthermore, an age-related decline in nuclear targeting and DNA binding affinity of Ku has been demonstrated in human peripheral blood mononuclear cells (Frasca *et al*., 1999).

Although there is strong evidence for reduced efficiency of NHEJ DSB repair with age, it may also be the case that this potentially mutagenic pathway is more heavily relied upon than the efficacious homologous recombination repair route. As the latter is restricted to the G2/M phase of the cell cycle when a sister chromatid is available, the major pathway for DSB repair in mammalian G1/G0 (senescent) cells may shift to favor NHEJ (Saleh-Gohari and Helleday, 2004). It remains to be defined whether this occurs in ageing organisms where the presence of pre-senescent and senescent cells remains controversial.

1.6.3 Endocrine Dysfunction

Ageing is associated with a multitude of hormonal changes that constitute a decline in endocrine function involving the responsiveness of tissues as well as reduced hormone secretion from peripheral glands. These changes are highly complex with endocrine functions so intertwined that alterations in one system often affecting others. Changes in gonadal hormone secretion in healthy ageing are among those most well established and comprise the menopause in females and andropause in males.

During the reproductive period, the hormones of the hypothalamic-pituitary-gonadal (HPG) axis are in balance, but the age-related decline in reproductive organ function triggers a decrease in gonadal inhibin secretion in both females (Welt *et al*., 1999) and males, causing

an increase in activin signalling (Baccarelli *et al*., 2001). This induces HPG axis drive with increased gonadotrophin releasing hormone and subsequent gonadotrophin secretion (Baccarelli *et al*., 2001). Additionally, the age-related decrease in gonadal oestrogen and testosterone sex steroid production (Couzinet and Schaiso, 1993, Baccarelli *et al*., 2001) causes a further loss of hypothalamic feedback inhibition and also stimulates gonadotrophin release from the pituitary (Figure 1-15).

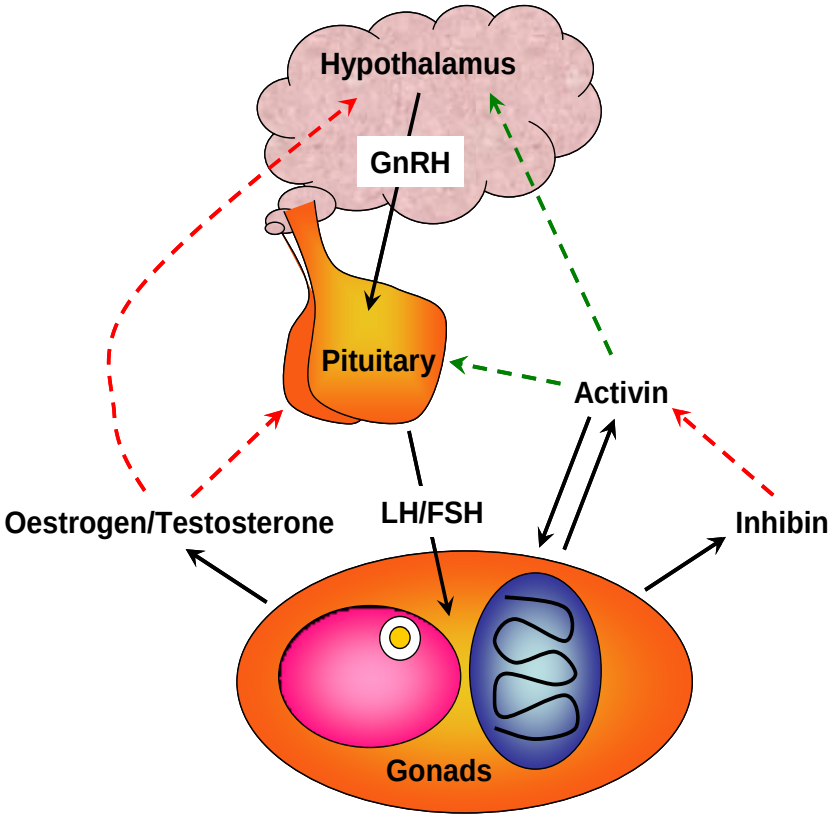


Figure 1-15 The HPG axis. Activins in the periphery stimulate the hypothalamus to secrete gonadotrophin-releasing hormone (GnRH). This stimulates the anterior pituitary to secrete the gonadotrophins LH and FSH which bind to gonadal receptors to stimulate oogenesis/spermatogenesis, sex steroid and inhibin production. Inhibin binds to activin receptors to downregulate the HPG axis. During menopause and andropause, inhibin and sex steroid production decrease as a result of reduced gonadal function, removing the HPG negative feedback control and causing an increase in LH/FSH secretion in a futile attempt to restore reproductive capabilities. Adapted from Bowen and Atwood, 2004

In females, this manifests as a 3- to 4-fold and 4- to 18-fold increase in circulating luteinizing hormone (LH) and follicle-stimulating hormone (FSH) respectively, greatest during the onset of menopause and remaining elevated for the next 4-5 years before declining but never

reaching levels seen during the reproductive years (Chakravarti *et al*., 1976). In males, the loss in reproductive function is more gradual, reflected in a more blunted increase in LH and FSH of 2- and 3-fold respectively (Neaves *et al*., 1984).

The circulating levels of anabolic/lipolytic growth hormone (GH) and its downstream mediator insulin-like growth factor (IGF)-1 also decline during ageing - a process referred to as the somatopause (Corpas *et al*., 1993). Data suggests an age-dependent decrease in endogenous hypothalamic GH releasing hormone output (Russel-Aulet *et al*., 1999); reduced physical activity and increased adiposity contribute to this decline (Vahl *et al*., 1996). As young adults with GH deficiency exhibit physical attributes resembling the ageing phenotype which are improved by long-term recombinant human GH therapy (Carrol and Christ, 1998), it was suggested that the elderly have genuine GH deficiency and would therefore benefit from GH replacement. However, whist improvements in body composition have been reported (e.g. muscle mass), functionality (e.g. muscle strength) was unaffected (Blackman and Harman, 2002). Furthermore, concerns have been raised regarding adverse side effects associated with therapy including arthralgia, carpal tunnel syndrome, oedema, hyperglycaemia and cancer risk (Vance, 2003).

Moreover, dwarf mice deficient in pituitary hormone secretion (including GH) are sterile but have an extended lifespan (Brown-Borg *et al*., 1996) - prompting the hypothesis that decreased GH signalling contributes to increasing longevity. Indeed, transgenic mice overexpressing GH and acromegalic individuals who secrete large amount of GH die prematurely (Wolf *et al*., 1993, Ohalloran and Shalet, 1995), whilst GH receptor-null mice display increased longevity (Coschigano *et al*., 2000) and individuals with Laron Syndrome (primary GH resistance or insensitivity) have a normal life expectancy (Laron, 2004).

These studies suggest that GH signalling is associated with decreased life expectancy and that the reduced secretion of this hormone during ageing may be affected by other factors prolonging lifespan. A recent theory proposes that LH/FSH may be involved in promoting the GH/IGF-1 signalling pathway at the expense of reduced life expectancy in favour of reproduction and growth (Bowen and Atwood, 2004). Thus the increased LH/FSH signalling in post-reproductive years, responding to decreased gonadal function in an attempt to maintain reproductive function, may also lead to increased activation of the IGF-1 signalling pathway causing increased negative feedback to suppress GH secretion and contributing indirectly to the age-associated decline of this hormone. Moreover, GH secretion is also inhibited by activins (Bertherat *et al*., 1995), which are known to drive the age-related increase in LH/FSH thus potentially also contributing directly to a decrease in GH production as the HPG axis becomes hyperactivated. A recent study that may assist in unifying these theories demonstrated that transplanting reproductively viable organs from young into senescent rats significantly extended their lifespan (Cargill *et al*., 2003), providing compelling evidence that increasing germ cell number increases longevity.

In addition to the menopause, andropause and somatopause, levels of dehydroepiandrosterone (DHEA) and its predominantly circulating sulphate-bound form (DHEAS) markedly decrease after the age of 25 (Orentreich *et al*., 1984) - a process referred to as the adrenopause. Histomorphological analysis of adrenal specimens suggests that ageing results in alterations within the adrenal cortex, resulting in a reduction in the size of the *zona reticularis*, this being responsible for the diminished production of DHEA (Parker *et al*., 1997). As well as an abundant circulating adrenal androgen, DHEA(S) is also thought to act directly as a neurosteroid that may have cardioprotective, antidiabetic, anti-obesity and immunoenhancing properties (Yen and Laughlin, 1998), prompting much debate on the anti-ageing properties of

DHEA and its potential as a 'hormone of youth' (Baulieu, 1996). Although correlations between declining DHEA(S) levels and age-associated pathologies such as cardiovascular disease, breast cancer, low bone mineral density, depressed mood, T2D and Alzheimer's disease have been reported, a causal relationship has not been established, casting doubt over the usefulness of DHEA replacement therapy in individuals with only a relative decline in circulating DHEA levels (including ageing), although benefits have been reported in conditions of adrenal insufficiency (Arlt, 2004). Indeed, studies assessing the effect of oral DHEA in otherwise healthy older subjects did not conclusively report any improvements in well-being, cognition or sexuality (Allolio and Arlt, 2002).

Age-associated alterations in hypothalamo-pituitary-thyroid axis function have also been reported, although, as with DHEA, it has been challenging to disseminate these from indirect alterations caused by simultaneous thyroid or nonthyroidal illness, or other physiological or pathophysiological states whose incidence increases with age. Thyroid hormone clearance decreases with age, but thyroid hormone secretion is also reduced, leading to unchanged total and free serum thyroxine (pro-hormone) concentrations. In contrast, serum total and free triiodothyronine concentrations decrease with healthy ageing, although these changes do not occur until after the eighth decade of life. This reduction is believed to be mostly due to reduced peripheral conversion of thyroxine to triiodothyronine, due either to the direct effect of non-thyroidal illness or to ageing itself, although decreased thyroid stimulating hormone secretion has also been reported (Mariotti *et al*., 1993).

Finally, whilst sex steroid, GH, DHEA and triiodothyronine concentrations are reported to decline, there is evidence for increased hypothalamic-pituitary-adrenal (HPA) axis (Figure 1-16) drive with resultant increased circulating cortisol levels (Van-Cauter *et al*., 1996, Seeman *et al*., 1997), although this increase is modest and some studies have failed to detect it

(Waltman *et al*., 1991). There are also complex age-related changes in the HPA axis response to stress, with a prolonged response in older subjects (Bergendahl *et al*., 2000) and diminished sensitivity to GC-mediated negative feed-back through the HPA axis, although this more pronounced in females (Laughlin and Barrett-Connor, 2000).

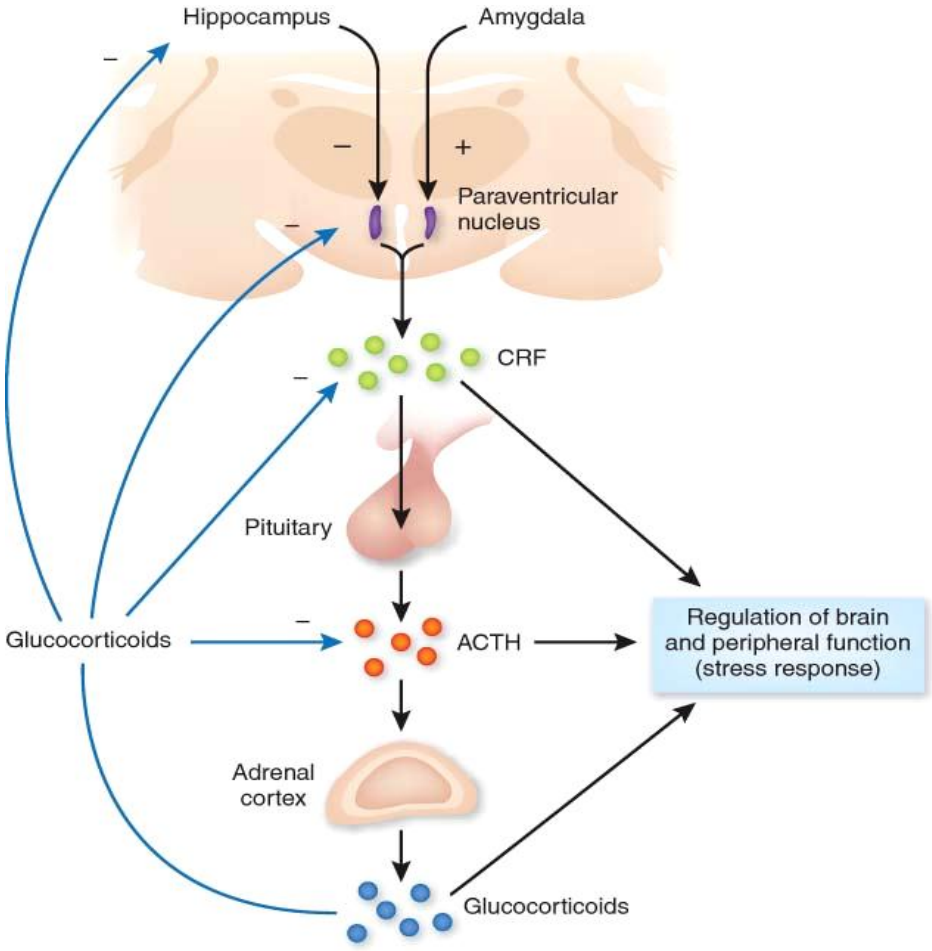


Figure 1-16 The HPA axis. In response to stress, corticotrophin-releasing hormone (CRH) is secreted from the hypothalamic paraventricular nucleus which stimulates the pituitary to release adrenocorticotrophic hormone (ACTH). This in turn stimulates the adrenal secretion of GC (cortisol in humans and corticosterone in rodents) which feed back to the level of the hippocampus, hypothalamus and pituitary to dampen excess activation of the HPA axis. Reproduced from Hyman, 2009

1.6.4 Extrinsic ageing

1.6.4.1 Photo-ageing

Solar UV radiation is the primary contributor to the acceleration of the skin ageing process and also arguably the most aggressive form of extrinsic ageing. Through the photochemical

generation of ROS, a host of cellular components are initiated by increasing pro-inflammatory cytokines e.g. IL-1/6, vascular endothelial growth factor (VEGF) and TNF-α (Rabe *et al*., 2006) which activate protein kinase signal transduction pathways e.g. AP-1 (Angel *et al*., 2001) and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB, Abeyama *et al*., 2000), regulating enzymes which synthesize and degrade structural proteins in the dermis including MMP1, MMP3, and MMP9 (Fisher and Voorhees 1998), reducing the structural integrity and functional properties of the skin.

These detrimental pathways can be activated following only a minimal, sub-erythemal dose of UV, with 5-15min exposure in midday sun on alternate days being sufficient to maintain elevated MMP levels. Furthermore, inflammation attracts neutrophils which burrow destructively through the ECM and produce free-radicals increasing the skin's ROS load, whilst angiogenesis is promoted by increased VEGF levels and contributes to UV-associated telangiectases. In addition, ROS including superoxide anion, peroxide, and singlet oxygen directly modify DNA, proteins and lipids in a detrimental manner.

UV radiation also induces hydrogen peroxide generation in skin by upregulating nicotinamide adenine dinucleotide phosphate (NADP) oxidase, which catalyzes the reduction of molecular oxygen to superoxide anion. This is then converted to hydrogen peroxide which has reduced cellular toxicity (Fisher *et al*., 2002). Hydrogen peroxide acts to induce multiple signalling pathways and leads to premature senescence *in vitro* (Frippiat *et al*., 2000), although low concentrations can stimulate growth (Irani *et al*., 1997). It is a cofactor for peroxidases and also has potential for conversion to the hydroxyl radical and singlet oxygen, the latter of which is able to upregulate collagenase (MMP1) expression in human dermal fibroblasts (Scharffetterkochanek *et al*., 1993).

In addition to the degradation of mature collagen by the upregulation of MMPs, UV radiation also directly impacts on *de novo* collagen synthesis by downregulating procollagen I and III expression in primary human dermal fibroblasts (Fisher *et al*., 2000). This occurs through several mechanisms, for example, (i) by upregulating the transcription factor AP-1 (Pfundt *et al*., 2001) which binds to and sequesters factors required for a procollagen transcription initiation complex (ii) by sequestering a signalling protein activated by TGF-β, a major pro-fibrotic cytokine (Chung *et al.*, 1996) and (iii) by downregulating the TGF-β receptor (Quan *et al*., 2001).

Interestingly, c-jun expression was found to be elevated in PP buttock skin from >80 year old compared to that from 18-28 year olds (Chung *et al.*, 2000), suggesting that upregulation of AP-1 signalling is a mediator common to both photo-ageing and chronological ageing, likely driven by similar mechanisms such as UV- or metabolism-induced generation of ROS respectively. Additionally, AP-1 is known to upregulate MMP1 (collagenase), MMP3 (stromelysin-1) and MMP9 (92-kD gelatinase, Angel *et al*., 2001) and these matrix-degrading enzymes are also elevated in both PE and chronologically aged (Varani *et* al., 2000) skin further supporting a role for AP-1 signalling in skin ageing.

UV radiation further accelerates the ageing process by inducing genomic instability in the form of mtDNA mutation (Berneburg *et al*., 1999) and causes the disruption of many other structural components of the skin besides collagen including the elastin fibre network, anchoring fibrils, proteoglycans and GAGs (Bernstein and Uitto, 1996).

1.6.4.2 Diet

The benefits of a balanced diet to maintaining optimal organismal health and nutrition are well documented (Finch, 2010) and as such play a key role in modifiable extrinsic longevity.

Furthermore, restriction of calorific intake was first described over eight decades ago to increase lifespan in rats (McCay *et al*., 1935) and has since been a focus for research into healthy ageing. Studies have demonstrated increased lifespan through caloric restriction (CR) in a diverse range of species including yeast, worms, flies and rodents (Fontana *et al*., 2010) but in humans, although shown to reduce risk factors for diabetes, cardiovascular disease, and cancer, its influence on lifespan is still not clearly established (Fontana and Klein, 2007).

Much progress has been made in unveiling the molecular mechanisms underlying the contribution of CR to increasing longevity, centred on a shift from a state of growth and proliferation to maintenance and repair (Walford *et al*., 1987). Perhaps unsurprisingly, evidence is emerging for the suppression of common pathways (e.g. IGF-1) that are activated by reproductive hormones at the expense of extending lifespan. Indeed, the decrease in LH/FSH observed during CR (Veldhuis *et al*., 1993, Bergendhal *et al*., 1998) has been postulated to drive longevity in an attempt to increase the chance of reproductive success, acting as an evolutionary mechanism to preserve fertility until environmental conditions (e.g. food supply) become favourable again (Holliday, 1989, Bowen and Atwood, 2004). Additional mechanisms through which CR functions to increase longevity include 1) reduced metabolism and oxidative stress (Brand, 2000) with decreased mitochondrial superoxide radical and hydrogen peroxide generation, slower accumulation of oxidative damage, decreased alkane production and a delayed loss of membrane fluidity (Sohal and Weindruch, 1996), 2) altered body composition with decreased fat mass and increased lipid oxidation to preserve carbohydrate (Heilbronn *et al*., 2006) and a reduction in age-related loss of beneficial brown adipose tissue function (Valle *et al*., 2008) leading to reduced mortality from obesity-related diseases such as diabetes and heart disease (Poirier *et al*., 2006) and 3) reduced systemic inflammation (Spaulding *et al*., 1997), partly through reduced adiposity (Fontana,

2009). Recently, the importance of reduced body temperature as a mediator of these mechanisms has been described (Carrillo and Flouris, 2011).

1.6.4.3 Other environmental influences

Although research into extrinsic factors contributing to ageing (particularly of the skin) have traditionally focused on solar radiation, with the terms 'extrinsic ageing' and 'photo-ageing' used synonymously, there is growing evidence that other environmental factors (e.g. diet) are also involved. Such studies, however, remain challenging as individual influences are difficult to extrapolate from other factors and must contribute a large, independent effect in order to be identified. Furthermore, many environmental influences may require chronic, often lifelong exposure to contribute an effect (i.e. smoking one cigarette or drinking one glass of wine in a lifetime is unlikely to contribute to an alteration in lifespan or healthy ageing), and the very nature of these highly variable lifestyle choices between different individuals makes such assessments difficult to validate, power and quantify.

However, in addition to solar radiation and diet, several other independent extrinsic factors have been reported to contribute to the ageing process. Smoking is strongly associated with a variety of cardiovascular and pulmonary diseases and cancers and is the single largest preventable cause of death and disability in developed countries. In addition to health complications, there is ample evidence for contribution to premature ageing in skin including poor wound healing, wrinkling, squamous cell carcinoma, psoriasis, hair loss and oral cancers (Freiman, 2004). Premature greying of hair is also accelerated by smoking (Stamp, 1995). Mechanisms involved in the manifestation of these changes include the induction of genomic instability by increasing the rate of mtDNA mutations (Lee *et al*., 1999) - a key factor underlying intrinsic ageing. In skin, tobacco smoke has been shown to induce premature skin

ageing by upregulating MMPs through the aryl hydrocarbon receptor pathway - also involved in mediating toxicity of UVB photoproducts (Morita *et al*., 2009). Similarly, airborne particle pollution also correlates with signs of extrinsic ageing such as pigment spots (Vierkotter e*t al*., 2010) which may result through increased oxidative stress and mitochondrial damage (Li *et al*., 2003).

Other extrinsic factors that may be involved in the ageing process include alcohol consumption, possibly through HPA axis activation (Spencer and Hutchison, 1999) and altered social interactions (Nilsson, 1996), although further detail on these and other environmental influences is beyond the scope of this thesis.

1.6.5 Summary

Ageing is a complex multi-factorial process, in its most simplified form, resulting from a systemic imbalance of damage and repair ultimately leading to an accumulation of unrepairable genomic damage. This causes dysregulation of normal gene transcription patterns (Bahar *et al*., 2006), further exacerbating the imbalance and culminating in a cascading decline of age-related physiology. Reduced genomic integrity and dysfunctional systems may trigger a compensatory increase in apoptotic pathways, potentially reducing the SC pool and diminishing the capability for cellular renewal (Gatza *et al*., 2007).

1.7 Skin ageing

1.7.1 Overview

As organisms age, most organs and tissues undergo a chronological process of change resulting in a diminished capacity to function normally. In no other organ is this inevitable progression more obviously apparent than in the skin. Obvious signs include atrophy, laxity,

wrinkling, sagging, dryness, yellowness, various blemishes and sparse, greying hair. The mechanisms underlying skin ageing are highly complex and encompass a multitude of endogenous (e.g. genetic mutation, cellular metabolism, hormonal environment) and exogenous (e.g. UV exposure, ionizing radiation, chemicals, toxins, pollutants) factors. Changes resulting from exogenous ageing occur primarily in PE skin such as the face, neck, décolletage and hands while those resulting from endogenous ageing are typical of PP areas and are often also considered to be representative of internal organ ageing. Importantly, exogenous ageing superimposes on endogenous ageing causing an acceleration of ageing processes that is clearly visible when comparing PE and PP skin.

1.7.2 The phenotype of ageing skin

1.7.2.1 Structural alterations

There are several morphological differences between chronological (intrinsic) and exogenous (extrinsic) skin ageing. Intrinsically aged skin undergoes a gradual thinning of the epidermis - up to 50% by 80 years of age (Lavker, 1979) and a flattening of DEJ rete ridges, although these observations are complicated by morphological epidermal variations depending on anatomical site. Dermal atrophy is moderate compared to PE skin, with decreased cellularity especially dermal fibroblasts (Varani *et al*., 2001). Intrinsically aged skin appears thin, finely wrinkled, smooth, dry, unblemished, sallow, and pale, with some loss of elasticity. Conversely, extrinsically aged epidermis undergoes thickening attributable to processes comparable to chronic wounding and repair. PE skin is characterized by deep wrinkles, laxity, roughness, sallowness, increased fragility, blister formation, pigmentary changes, teleangiectases, impaired wound healing, and benign and malignant growths.

1.7.2.2 Cellular alterations

At a cellular level, dermal fibroblasts from aged skin adopt a stellate phenotype with a highly activated endoplasmic reticulum with increased biosynthetic activity (Ma *et al*., 2001) and there is a decrease in fibroblast number and size. Epidermal keratinocytes become less evenly aligned on the basement membrane with increased irregularities in size, shape, staining properties and a decrease in cellular turnover (Cerimele *et al*., 1990) consistent with a reduction in epidermal repair and wound healing (Ashcroft *et al*., 1995). Age-spots are caused by alterations in melanocyte distribution with increased localized proliferation at the DEJ.

1.7.2.3 Molecular alterations

The key morphological differences between intrinsically and extrinsically aged skin are attributable to a host of alterations at the molecular level. In the epidermis, keratinocytes of the *stratum corneum* undergo irreversible terminal differentiation resulting in an increase of the molecular marker involucrin which is found to be more highly expressed in PE skin *in vivo* compared to PP (Bosset *et al*. 2003). Additionally, basal keratinocytes displayed reduced expression of β1-integrins (transmembrane receptors that assist in anchoring neighbouring keratinocytes to each other and to the basal membrane) in PE than in PP skin (Bosset *et al*., 2003). These data give examples of two epidermal processes; an increased level of keratinocytes terminal differentiation and reduced keratinocyte cell adhesion, which exacerbate the underlying morphological phenotype of intrinsically aged skin and contribute to the phenotype of PE skin.

Structural proteins of the dermis are also affected by extrinsic ageing. Fibrillin is truncated and depleted in the upper dermis of PE skin (Watson *et al*., 2001) as is collagen VII, which forms anchoring fibrils at the DEJ (Contet-Audonneau *et al*., 1999). Additionally, aged PE

skin undergoes solar elastosis, where an increase is elastin promoter activity (Bernstein *et al*., 1996), coupled with decreased fibrillin-1 expression, results in a heavy deposition of dysmorphic elastin fibres in the upper dermis that is not as apparent in PP aged skin (Figure 1-17), although age-induced disintegration and increased abnormal fibres still occur. Furthermore, versican (an elastin-associating chondroitin-sulphate proteoglycan) expression is also increased in areas of solar elastosis. Elastic fibres altered though photo-ageing contain deposits of lysozyme which has been shown to correlate with their basophilic degeneration (Suwabe *et al*., 1999). Over time, the normal collagen composition of the dermal matrix in PE skin is replaced by dysfunctional elastic tissue and contributes to the phenotype of photo-ageing.

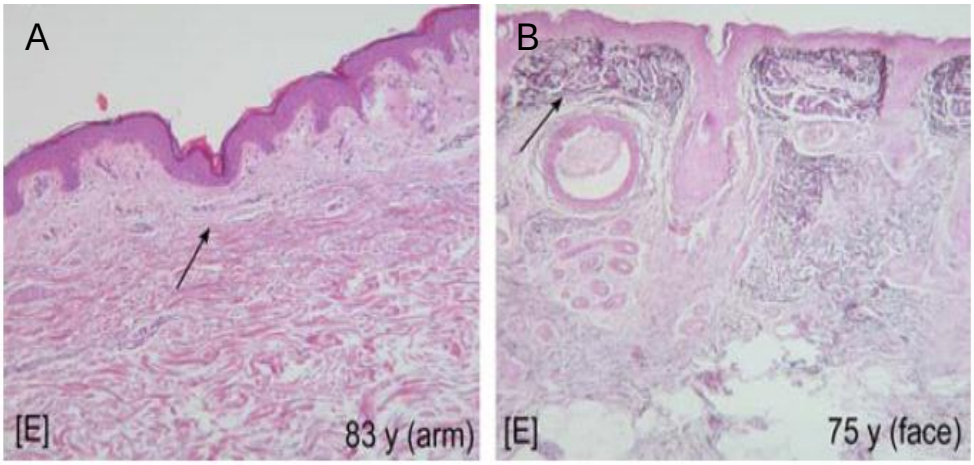


Figure 1-17 PP and PE skin samples of two healthy female subjects. Following staining with elastica [E], PP (inner upper arm) skin from an 83-year old (**A**) displays reduced accumulation of abnormal elastic tissue (arrow) and increased rete ridge formation compared to PE skin from the face of a 75-year old (**B**). Reproduced from (Makrantonaki and Zouboulis, 2007)

Expression of the most abundant structural protein of the ECM, collagen type I, diminishes with age in PE skin (Griffiths *et al*., 1993) leading to a loose, erratic arrangement with poor tensile properties instead of the densely packed, highly organized composition of PP tissue (Bernstein *et al*., 1996). Studies suggests that the reduction in collagen type I is the result of increased degradation rather than reduced production (Varani *et al*., 2001).

Intrinsically aged skin also contains reduced abundance of collagen (although more moderate than in PE skin) and also in elastic fibres, lacking the solar elastosis of PE cutis (Braverman and Fonferko, 1982).

GAGs (e.g. hyaluronic acid) are involved in the maintenance of dermal hydration and are more highly expressed in PE skin (Bernstein *et al*., 1996). The paradox that PE skin is less hydrated while having greater GAG expression may be explained by a change in the distribution of these proteins. Typically located diffusely between collagen bundles in the dermis of PP skin, it is thought that during solar elastosis GAGs become dysfunctionally associated with abnormal elastotic material - a hypothesis supported by the presence of a hyaluronic acid binding domain in versican. Therefore, water content is increased in PE skin - a somewhat surprising concept as lax PE skin is often attributable to a lack of water. In keeping with a lack of solar elastosis, the water content of intrinsically aged skin is similar to non-aged skin (Waller and Maibach, 2006).

1.7.2.4 Dermal appendage alterations

Greying hair bulbs contain fewer melanocytes with abnormal cytoplasmic vacuoles and only lightly melanized melanosomes (Commo *et al*., 2004). The reduced melanin production is linked to a lack of tyrosinase enzyme - involved in the initial stages of melanin pigment synthesis. Hair also becomes sparser with increasing age, particularly scalp hair which undergoes a transformation of terminal to interterminate then vellus hairs with follicle miniaturization resulting in balding. Occurrence of balding is caused by alterations in local androgen levels (androgenic alopecia) and is much more common in males (Otberg *et al*., 2007).

Sebaceous gland sebum production also declines after the seventh decade in males, and earlier in females with the onset of menopause (Zouboulis and Boschnakow, 2001). Eccrine gland numbers are also reduced and produce less sweat.

1.7.2.5 Skin immunity alterations

Age-related changes in skin immune components include reduced numbers of epidermal Langerhans' cells which are also less responsive to migratory stimuli (e.g. TNF-α, Bhushan *et al*., 2002). T- and B-cell mitogenic responses are also impaired, although cell numbers remain constant (Thivolet and Nicolas, 1990).

1.7.3 Skin disease in old age

Skin conditions in the elderly occur as a result of the interplay of several factors associated with the ageing process including changes in its structure and function, cumulative exposure to environmental insults especially UV radiation, indirect effects of ageing or age-related disease in other organs, increased frailty and reduced wound healing. These factors contribute to an increased incidence of skin complaints in older people. For example, over two thirds of over 50-year olds and 83% of over 80-year olds reporting with a skin condition of some kind (Beauregard and Gilchrest, 1987).

Pruritus, or itching, is a common symptom in old age and can be so severe that, if untreated, may rapidly deteriorate a patient’s quality of life. In the majority of cases, a primary cutaneous disease (e.g. xerosis) or systemic disorder (e.g. renal disease) explains this symptom (Reich *et al*., 2011) with the remainder classified as displaying an intractable senile pruritus, accompanied by difficult and often unsatisfactory management regimes.

The texture of skin in the elderly is often described as “dry” and senile xerosis is often a primary cause for pruritus - particularly in winter and especially pronounced on the legs. The surface of the skin adopts a “cracked paving” appearance known as asteatotic eczema, which may be partly due to reduced epidermal water content in the elderly (Potts *et al*., 1984).

Psoriasis onset often peaks in later life (Bonifati *et al*., 1998), typically around the late 50s, and is less well tolerated in older individuals than when presenting earlier in life - when the disease is also more clearly associated with a family history. The mechanisms underlying this increased prevalence are largely unknown and often complicated by associated co-morbidities and other primary pathologies.

Leg ulcers are also a common skin complaint amongst older subjects (Nelzen *et al*., 1991) mostly associated with venous hypertension and complicated by poor wound healing (Tallman *et al*., 1997). Studies have reported an age-related decrease in the migratory response to hypoxia in keratinocytes isolated from older donors, with a concomitant lack of MMP1, MMP9 and TIMP1 induction hypothesized to contribute to reduced re-epithelialisation (Xia *et al*., 2001).

1.8 Glucocorticoids

1.8.1 Overview

Glucocorticoids (GC) form a class of adrenal steroid hormones that bind the ubiquitously expressed GR and regulate a multitude of biological processes. GC are used extensively in pharmacology due to their potent anti-inflammatory and immunosuppressant properties, although their indiscriminate actions affecting almost all tissue types are associated with a broad range of systemic side-effects, diminishing their therapeutic potential for long-term treatment. In humans, the most important GC is cortisol (hydrocortisone); although a range of

synthetic GC (e.g. dexamethasone, prednisolone) with increased potency are commonly used as therapies. The chemical structures of cortisol and dexamethasone are illustrated in Figure 1-18.

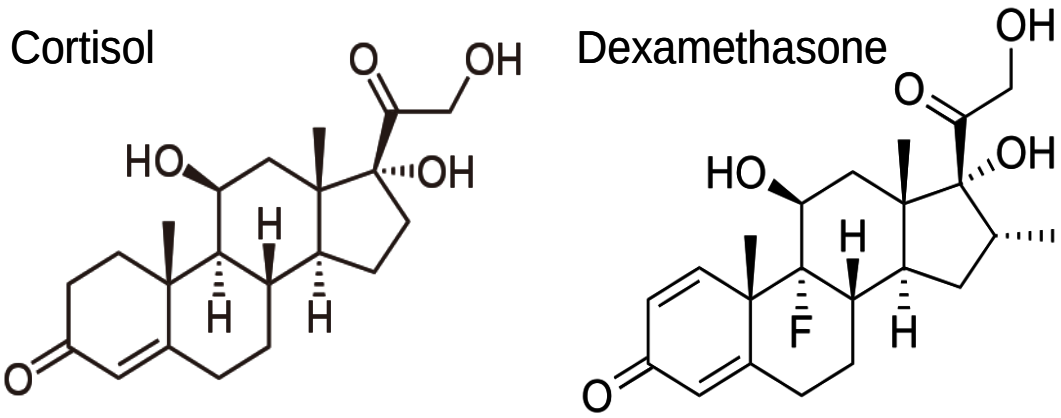


Figure 1-18 The chemical structures of endogenous (cortisol) and synthetic (dexamethasone) GC

1.8.2 GC synthesis

Cortisol is synthesized from the common steroidal precursor cholesterol in the *zona fasiculata* of the adrenal cortex. Through the actions of highly related yet discrete steroidogenic enzymes, cholesterol undergoes minor modifications regarding number, location and type of active side groups or number of carbon atoms to yield several similarly structured moieties with individual functional properties. In addition to cortisol, these include the 21-carbon progestagens (e.g. progesterone) and the mineralocorticoid aldosterone both synthesized in the *zona glomerulosa*, and the sex steroids; 19-carbon androgens and 18-carbon oestrogens produced in the *zona reticularis* (Figure 1-19). Secreted cortisol then enters systemic circulation bound to corticosteroid binding globulin and serum albumin - the majority of cortisol is transported in this manner (>90%) with the concentration of the remaining free cortisol of approximately 1ug/dL - only the latter is functionally active and able to activate the GR.

Cortisol production is regulated by the HPA axis; adrenal secretion is stimulated by pituitary-derived adrenocorticotrophic hormone (ACTH) which is positively regulated by hypothalamic corticotrophin releasing hormone (CRH). GC, in turn, decrease both CRH and ACTH secretion resulting in a classical negative feedback regulatory loop (Makino *et al*., 1995).

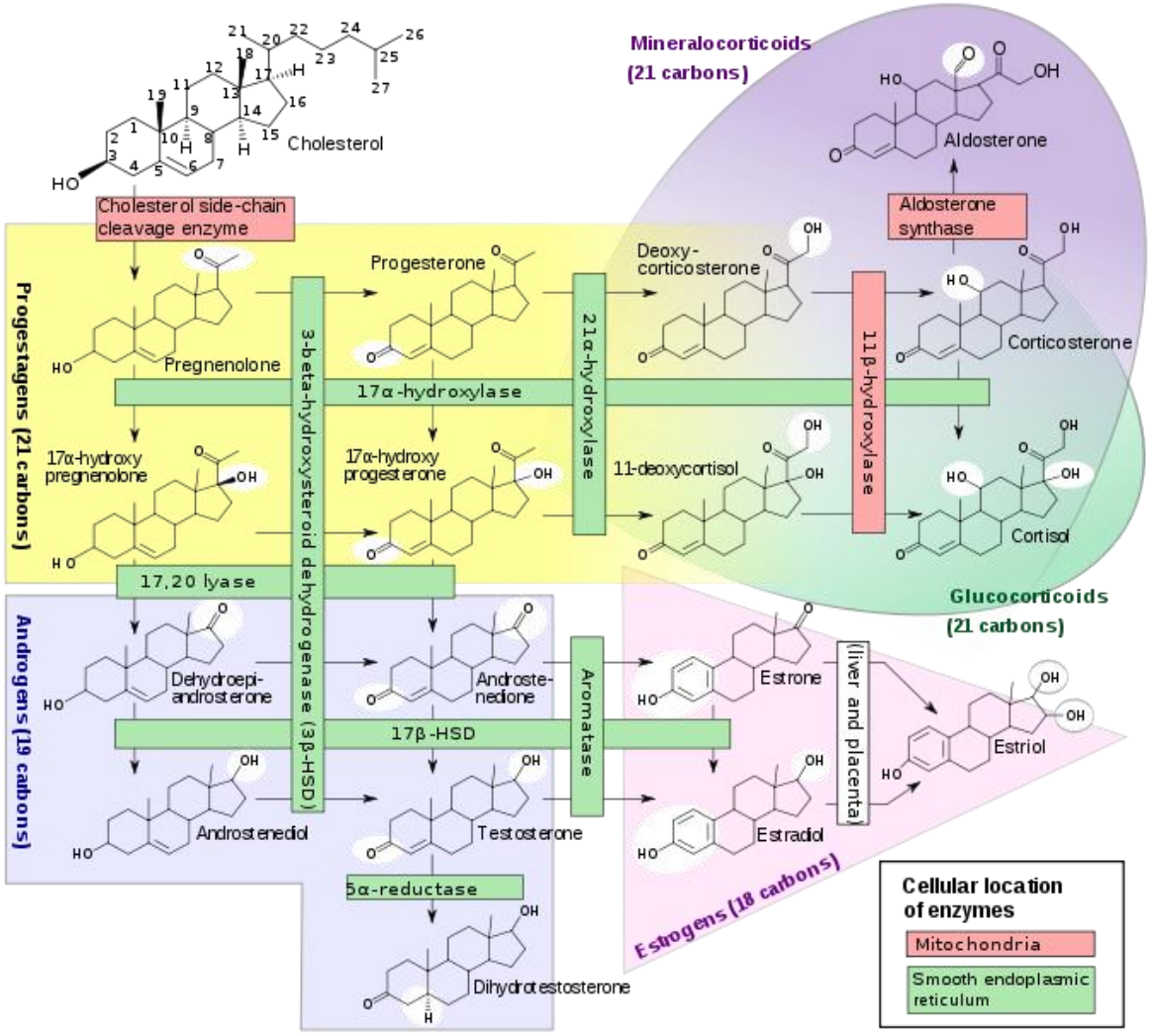


Figure 1-19 Major pathways human steroidogenesis. The common precursor cholesterol is sequentially modified by the enzymes to synthesize the steroid hormones

Adrenal cortisol secretion displays a circadian (diurnal) rhythm reflecting the light-dark cyclical metabolic requirements. This rhythm superimposes on the HPA axis through central nervous system control with various neurotransmitters e.g. noradrenaline and gamma-aminobutyric acid negatively regulating hypothalamic CRH release. Furthermore, melatonin,

secreted at night from the pineal gland, also inhibits CRH production, while CRH functions to inhibit melatonin secretion during the day. Consequently, cortisol levels are low in late evening, continuing to decline into the first hours of sleep, then increasing in the third to fifth hour of sleep, reaching peak levels between 06.00 and 08.00h, after which the concentrations gradually decline during the day excluding transient bursts in response to eating and exercise (Krieger *et al*., 1971).

1.8.3 GR action

The GR belongs to a large superfamily of nuclear receptors (NRs) that mediate a transcriptional response in response to binding of a hormonal or alternative metabolic ligand (Evans, 1988). NRs share several structural elements including an n-terminal regulatory domain with ligand-independent activating function, a double zinc finger DNA-binding domain (DBD), a hinge region involved in nuclear localization and cellular distribution, a ligand-binding domain (LBD) with ligand-dependant activating function and a variable c-terminal domain (Kumar and Thompson, 1999).

NRs are classified according to their activating ligands and mechanism of action. Classical type I steroid receptors include those for progestins, oestrogens, androgens, mineralocorticoids and GC. Following ligand binding in the cytosol, type I receptors dissociate from anchoring heat shock proteins, homodimerise (involving the DBD and LBD) and translocate to the nucleus where they bind directly to DNA hormone response element (HREs) consisting of two inverted repeat half-sites separated by a variable length of DNA (Figure 1-20).

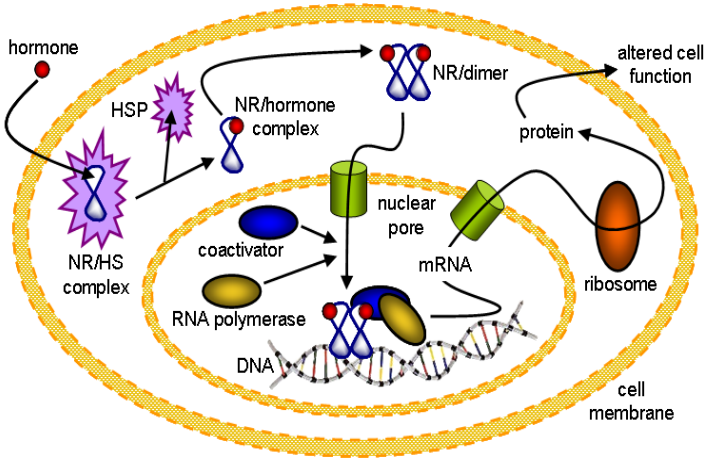


Figure 1-20 Mechanism of class I NR action. Ligand (e.g. cortisol) binds to the LBD of cytoplasmic NRs (e.g. GR) causing dissociation from anchoring heat shock proteins (HSP). NR/hormone complexes then dimerise and translocate to the nucleus where they bind to HREs via the DBD. Association with regulatory proteins results in regulation of target gene expression and altered downstream function

Conversely, type II receptors (e.g. thyroid hormone, all-*trans* and 9-*cis* retinoic acid and vitamin D_3 receptors) are permanently nuclear-resident, switching between corepressor/coactivator proteins in the absence/presence of ligand and bind DNA as heterodimers (typically with retinoid X receptors). Type III receptors consist of recently identified orphan receptors that act similarly to type I receptors by forming homodimers, but differ by binding to direct repeat HREs, while type IV receptors bind to a single half-site HRE (Mangelsdorf *et al*., 1995). Following GC ligand binding (K_d 20-40 nM), the GR regulates target gene expression through two principal mechanisms of action. During transactivation, expression of target genes (e.g. anti-inflammatory lipocortin-1) is upregulated through GR homodimer/ligand-complex binding to HREs following dissociation from cytoplasmic heat shock proteins (Jibard *et al*., 1999) and nuclear translocation.

Alternatively, the ligand-bound GR complex can sequester other cytoplasmic transcription factors (e.g. pro-inflammatory NF-κB, AP-1), preventing their nuclear translocation and indirectly downregulating target genes - a process known as transrepression (Hayashi *et al*., 2004). The ability of the GR to stimulate the expression of some genes in a tissue-specific manner whilst concomitantly repressing others creates a powerful mechanism for generating

diversity of transcriptional control, resulting many pleiotropic effects in development, homeostasis, metabolism and immune function.

1.8.4 Mineralocorticoid receptor action

The mineralocortocoid receptor (MR) has a high and approximately equal affinity (K_d 0.5-2 nM) for the mineralocorticoid ligand, aldosterone, but also for cortisol and their shared precursor corticosterone. However, in contrast to the GR, it exhibits a much more restricted distribution of expression being localized mainly to cells/tissues involved in Na^+/K^+ balance such as the distal renal tubule, sweat glands, parotid glands and colon, but also found in specific brain regions (neurones within the limbic system, entorhinal cortex and, to a lesser extent, the hypothalamus).

As a class I NR, MR ligand binding leads to activation of target genes in a similar manner to the GR (Figure 1-20), regulating sodium transport through the basolateral sodium-potassium adenosine triphosphatase pump and apical sodium channels, culminating in sodium retention, potassium excretion, water retention and raising extracellular fluid volume and hence blood pressure (Sheppard and Funder, 1987).

The shared dual affinity of the MR for both cortisol and aldosterone, the latter being very tightly regulated by the renin-angiotensin system (Foster *et al*., 1979), contributes to the pathological conditions associated with GC excess (where circulating GC concentrations can exceed 300nM) including hypertension and cardiovascular disease, posing a key complication for patients on long-term GC therapy. However, for many years the mechanisms explaining the preference for tissues such as the kidney to be regulated *in vivo* by aldosterone despite their high affinity for GC (further complicated by the higher circulating concentration of GC compared to aldosterone, 1ug/dL compared to 0.006ug/dL respectively) remained frustratingly elusive. Answers to this conundrum were revealed following the discovery of

tissue-specific mechanisms for the delivery of GC to their receptors, allowing the maintenance of discrete physiological functions of GC and mineralocorticoids.

1.8.5 Pre-receptor metabolism of GC

The availability of GC to activate either the GR or MR is controlled at a tissue-specific pre-receptor level by isozymes of the enzyme 11 beta-hydroxysteroid dehydrogenase (11β-HSD), largely independent of circulating GC levels. 11β-HSDs interconvert cortisol and its inactive metabolite cortisone (or corticosterone and 11-dehydrocorticosterone – 11-DHC – in rodents), regulating the availability of GC to the GR in GC target tissues and preventing the inappropriate activation of the MR in aldosterone target tissues. Indeed, 11β-HSD(2) deficiency was first described in 1988 by Stewart *et al*, termed apparent mineralocorticoid excess (AME) and characterised by hypertension, hypokalaemia, suppression of the renin-angiotensin system and impaired inactivation of cortisol to cortisone (Stewart *et al*., 1988).

Since the cloning of two distinct 11β-HSD isoforms, 11β-HSD1 and 11β-HSD2 (Figure 1-21), extensive studies have demonstrated their integral role in the regulation many biological processes (Edwards *et al*., 1996). 11β-HSD2 is a high-affinity, nicotinamide adenine dinucleotide (NAD^+)-dependent, constitutive enzyme that functions exclusively as a dehydrogenase, inactivating cortisol to cortisone. It is this isoform that colocalizes with the MR and protects it from activation by circulating free cortisol (Quinkler and Stewart, 2003) and as such is expressed in mineralocorticoid target tissues such as the kidney, parotid gland, sweat glands, colon and VSMCs. Murine 11β-HSD2 knockout studies revealed high perinatal mortality with severe corticosterone-dependant hypertension and other features of AME in the survivors (Kotelevtsev *et al*., 1999), as do human patients with 11β-HSD2-inactivating mutations (Edwards *et al*., 1996) and animals or humans that have been treated with glycyrrhetinic acid (the active component of liquorice) or its derivative carbenoxolone. 11β-

HSD2 is also expressed in a few non-MR-expressing tissues such as placenta and developing brain, again providing protection from potentially harmful exposure to excessive levels of cortisol/corticosterone (Brown *et al*., 1996).

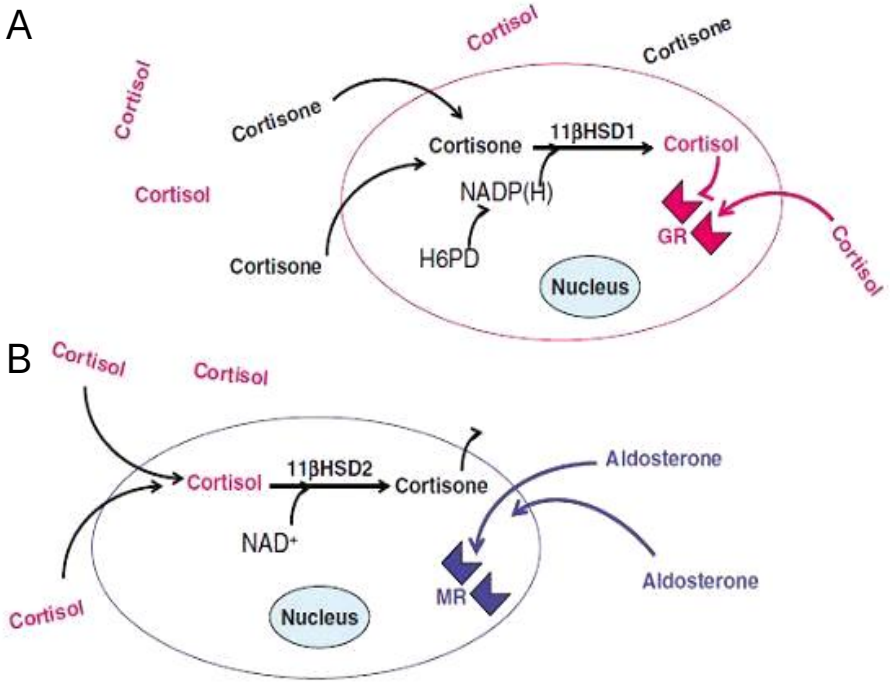


Figure 1-21 Pre-receptor metabolism of GC. The amplification of GC signalling in GR-expressing cells occurs through 11β-HSD1 oxoreductase activity (**A**), whilst prevention of inappropriate MR activation by GC is mediated through 11β-HSD2 dehydrogenase activity which inactivates cortisol (**B**). The reductase activity of 11β-HSD1 is dependent on the local generation of NADPH by hexose-6-phosphate dehydrogenase (H6PD). In rodents, 11β-HSD1 and 11β-HSD2 catalyse the interconversion of corticosterone and 11-DHC respectively

In contrast, 11β-HSD1 is ubiquitously expressed particularly in liver, adipose tissue (especially omental) and brain and is regulated by a wide variety of factors including GC, stress, sex steroids, cytokines and PPAR ligands. In intact cells it functions exclusively as an oxoreductase, activating cortisol from cortisone in an NADPH-cofactor-dependant manner. NADPH is supplied by the enzyme hexose-6-phosphate dehydrogenase (H6PD) which co-localizes with 11β-HSD1 in the endoplasmic reticulum (Draper *et al*., 2003). In homogenized tissues, the cofactor concentration gradient provided by H6PD is disrupted and 11β-HSD1 switches to dehydrogenase activity, with oxoreductase activity restored following exogenous restoration of NADPH concentrations. However, *in vivo* 11β-HSD1 functions as an

oxoreductase, amplifying the local concentration of active GC in steroidal target tissues such as the liver.

Although phenotypic studies in the 11β-HSD1-null mouse revealed higher circulating corticosterone levels (due to impaired HPA axis negative feedback), the animals are resistant to diet- and stress-induced hyperglycaemia seen in WT littermates, supporting the hypothesis that 11β-HSD1 regulates target tissue function independently of circulating GC levels. Furthermore, knockout animals displayed improved circulating cholesterol profiles with raised high-density lipoproteins, reduced low-density lipoproteins and lowered triglycerides, driven by decreased hepatic gluconeogenesis and lipid β-oxidation and potentially also through attenuated GC-dependant functions in visceral adipose tissue (Seckl, 2004). 11β-HSD1-null mice also display reduced weight gain when placed on a high-fat diet which, together with altered fat deposit distributions favouring subcutaneous rather than visceral deposition, is indicative of a mouse model protected against certain aspects of metabolic disease. Further evidence, including 11β-HSD1 inhibitor studies displaying improved insulin secretion rates, has contributed to a consensus that 11β-HSD1 may be involved in the development of insulin resistance, obesity and other aspects of metabolic dysfunction, highlighting its potential use as a therapeutic target in these common endocrine disorders. Recently, 11β-HSD1 blockade has demonstrated further exciting applications to conditions of impaired cognitive function, with elderly subjects displaying improved brain performance in the treated group, further corroborated by genetic studies indicating an association between certain 11β-HSD1 haplotypes and Alzheimer's disease. Interestingly, these studies implicate 11β-HSD1 inhibition as being pharmaceutically relevant in traditionally non-metabolic GC target tissues such as the brain, emphasizing its role as a key regulator in a variety of global processes and the requirement for site-specific therapeutic targeting.

1.8.6 GC function

1.8.6.1 Normal physiology

The ubiquitous nature of GR expression coupled with the ability to modulate the transcription of ~10% of all known genes through both agonism, and antagonism, translates to a regulatory system capable of affecting a wide variety of biological processes. Primarily, GC are involved in maintenance of homeostasis and in the preparation for, responding to and coping with physical and emotional stress (Sapolsky *et al*., 2000), promoting carbohydrate and protein degradation in muscle and exerting complex effects on lipid reorganisation. Stress responses originate in the central nervous system, increasing hypothalamic CRH secretion which stimulates pituitary ACTH release. ACTH stimulates the adrenals to release cortisol within minutes of the onset of stress, abolishing the circadian rhythm of cortisol secretion if the stressor persists.

1.8.6.2 Metabolic functions

One such 'HPA-stressor' is hypoglycaemia (blood glucose <2.2 mmol/l) which triggers ACTH release and cortisol secretion to raise circulating glucose levels and maintain them in a narrow and tightly regulated range. Glucose release and synthesis (gluconeogenesis) is stimulated in the liver through the upregulation of gluconeogenic enzymes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (Rooney *et al*., 1993), providing the organism with additional energy required to cope with the stressor. GC further facilitate this process by increasing permissiveness to other hepatic gluconeogenic hormones (e.g. glucagon and catecholamines) whilst inhibiting peripheral tissue glucose uptake and pancreatic insulin secretion (Delaunay *et al*., 1997). Furthermore, the metabolism of glucose

to fatty acids and neutral fats by adipose tissue is inhibited and gluconeogenic substrate (e.g. fatty acid, glycerol) release into the circulation is stimulated, for example, by the GC-induced upregulation of hormone-sensitive lipase activity.

1.8.6.3 Immune system functions

GC are also key regulators of immune and inflammatory processes and control many aspects of host defence, acting in a stress-protective manner to limit the pathophysiological consequences of tissue injury and inflammation, preventing them from exacerbating to a point where they threaten host survival (Munck and Narayfejestoth, 1992). GC decrease circulating lymphocytes, monocytes and eosinophils but increase neutrophils, platelets and erythrocytes and also reduce leukocyte migration to inflammatory sites. Pro-inflammatory cytokine secretion (e.g. IL-1, TNF-α) is inhibited by GC - largely through NF-κB and AP-1 transrepression - and also drive the HPA axis to limit the inflammatory response (Beutler *et* al., 1986). GC anti-inflammatory and immunosuppressive properties also endow their use as therapeutics in a wide range of conditions.

1.8.6.4 Other functions

Further endogenous actions of GC include raising blood pressure, in part by altering sensitivity to catecholamines and modulating systemic water and electrolyte balance in an ‘aldosterone-like’ manner. Additional tissue-specific functions include negatively regulating bone metabolism, promoting apoptosis and inhibiting proliferation of bone cells and promoting neurodegeneration of the central nervous system by targeting neurone and glial cells. Furthermore, GC affect mood and behaviour, food intake, body temperature, pain perception and neuroendocrine functions.

1.8.6.5 GC excess

The range of GC target tissues is clearly exemplified by conditions of GC excess, due to either hypersecretion of endogenous steroids (Cushing's syndrome/disease, Newell-Price *et al*., 2006) or prolonged administration of exogenous GC therapies (Figure 1-22). In these circumstances, the adverse effects associated with GC function become exaggerated, causing a plethora of harmful pathologies. The adipose-tissue effects of GC result in a redistribution of fat to the abdominal area (central obesity), face (moon-face) and upper back (buffalo-hump). GC-induced proteolysis causes muscle weakness, whilst excessive stimulation of mechanisms increasing circulating glucose lead to hyperglycaemia, insulin resistance and steroid diabetes. Other metabolic effects of GC excess result in hypertension, increased cholesterol, altered serum lipids, salt and water retention.

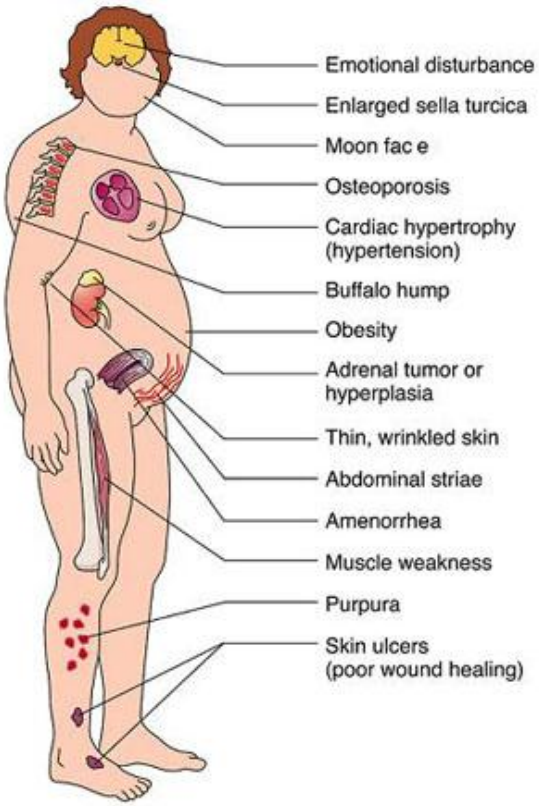


Figure 1-22 Systemic side-effects of GC excess. During, Cushing's syndrome, resulting from an adrenal tumour or hyperplasia, endogenous cortisol levels become elevated, resulting in prolonged and excessive effects in target tissues. These can also occur as a result of exogenous GC administration

Furthermore, the immunologic effects which serve to limit inflammation and assist wound healing are paradoxically reversed during GC excess, with immunodeficiency, poor wound healing, loss of connective tissue, easy bruising, retarded growth and development and osteoporosis. Other endocrine-related changes include menstrual irregularities in females and infertility. Finally, depression and impaired cognitive function can also occur.

Importantly, recent findings indicate that not only substantive, long-term changes, but also subtle variations in the GC *milieu*, if sustained, are capable of inducing potentially hazardous effects and can contribute to the pathogenesis of hypertension, cardiovascular disease, insulin resistance, obesity and T2D (De Kloet *et al*., 1998, Gold *et al*., 2002, Seckl, 2004).

1.9 GC and skin

Pioneering research regarding the potent immunomodulatory benefits of GC was first reported by Hench and co-workers following systemic therapy in patients with rheumatoid arthritis (Hench *et al*., 1949). Following the introduction of topical hydrocortisone in the early 1950s, GC therapy revolutionized the treatment of a wide variety of dermatological disorders. However, alongside the desired therapeutic effects (anti-inflammatory, immune-suppressant, anti-allergic), evidence began to emerge for several adverse side-effects particularly with chronic or inappropriate use (increased infection, muscular myopathy, osteoporosis, HPA insufficiency, metabolic defects, cardiovascular complications, glaucoma). In skin, detrimental thinning was the predominant side-effect, first identified by Epstein and co-workers reporting five patients with atrophic striae in the groins following topical GC treatment (Epstein *et al*., 1963). Subsequently, the adverse effects of GC in skin biology have been extensively documented, including *in vitro* effects on dermal fibroblasts and

keratinocytes, consequences of topical and systemic steroid therapy and dermal phenotype during endogenous GC excess - Cushing's syndrome.

1.9.1 In vitro effects

Primary human fibroblasts treated with the synthetic GC dexamethasone display reduced expression of elastin mRNA (Kahari, 1994). GC treatment (including cortisol and dexamethasone) also induces cellular toxicity primarily in keratinocytes but also in fibroblasts, with varying degrees of potency depending on the GC used (Korting *et al*., 1995). Furthermore, studies conducted using the human keratinocyte cell line HaCat and primary dermal fibroblasts demonstrated adverse outcomes regarding cellular proliferation, chemotaxic migration and fibroblast contraction of collagen lattice gels when treated with synthetic GC (Hein *et al*., 1994, Wach *et al*., 1998), although growth inhibition is not associated with increased foreskin fibroblast apoptosis (Hammer *et al*., 2004). Moreover, dexamethasone treatment protects foreskin fibroblasts against TNF-α-/UV-radiation-/cell-permeable ceramide-induced apoptosis (Hammer *et al*., 2004), whilst foreskin keratinocytes fail to respond to dexamethasone-induced changes in proliferation or apoptosis (including protection). Decreased rates of proliferation are due to anti-mitotic effects in both cell types (Fischer and Maibach, 1971, Hein *et al*., 1994).

GC-induced alterations in ECM components have also been reported. In human skin fibroblasts, hyaluronic acid production is inhibited by dexamethasone (0.5-10nmol/L), an effect reversed by the GR inhibitor RU486 (Smith, 1988). Furthermore, human skin tissue explants treated with hydrocortisone lead to reduced epidermal hyaluronan turnover and content in addition to epidermal thinning and reduced keratinocyte DNA synthesis (Agren *et al*., 1995). More recently, dexamethasone-treated human dermal fibroblasts exhibited a rapid,

near-complete inhibition of hyaluronan synthase 2 (HAS-2) expression with decreased message stability (Zhang *et al*., 2000).

Reductions in fibroblast procollagen type 1 and TIMP1 have also been described following dexamethasone treatment, tilting the balance of collagen metabolism away from accumulation (Slavin *et al*., 1994). The *in vitro* functional consequences of GC treatment are described in more detail in Chapter 5.

1.9.2 Exogenous therapy

1.9.2.1 Topical

The GC-induced dysregulation of genes involved in maintenance of skin homeostasis, integrity and function (e.g. elastin) is believed to contribute to the adverse side-effects associated with topical GC therapy. Of these, skin thinning is the most widely reported, consisting of reductions in both epidermal and dermal thickness as markers of dermal atrophy in rodent (Gniadecki *et al*., 1994, Faergemann *et al*., 2002) and human studies (Lehmann *et* al., 1983, Kolbe *et* al., 2001, Korting *et al*., 2002). Interestingly, GC-induced skin thinning in rats is reversible following prior treatment with the GR inhibitor RU486, whilst the anti-inflammatory effects on ear edema following croton oil treatment were unaffected (Iwasaki *et al*., 1995). This uncoupling of GC anti-inflammatory and atrophogenic functions suggests that the former may be more extensively regulated through non-genomic mechanisms, identifying GR inhibitors as potential therapeutic agents to minimize adverse effects during GC treatment of inflammatory skin conditions (Schacke *et al*., 2004).

Further GC-induced morphological alterations include a flattening of the DEJ (Lehmann *et* al., 1983, Kimura and Doi, 1999), decreased microvasculature, decreased keratinocyte size (Kolbe *et al*., 2001) and rearrangements of the dermal ECM involving ground substance

resorption with subsequent fibrous network collapse and collagen/elastic fiber reorientation (Lehmann *et al*., 1983). Epidermal corneocyte layers are also reduced and contain fewer intercellular lipid lamellae, with reductions in granular layer keratohyalin granules also reported (Sheu *et al*., 1997). Additionally, mast cell numbers become virtually absent (Lehmann *et al*., 1983) - likely contributing to associated reduction in wound healing capabilities.

At a molecular level, topical GC induced a marked decrease in collagen type I and III propeptides and type I mRNA in suction blister fluid and human skin (Oikarinen *et al*., 1998, Nuutinen *et al*., 2003). Tropocollagens I and III were also drastically reduced following subcutaneous dexamethasone injections in rats, with decreases in latent and active collagenase (and corresponding mRNA) and TIMP1 and TIMP2 mRNA also reported (Oishi *et al*., 2002). Recently, hyaluronic acid turnover in human dermis has been evaluated following topical dexamethasone treatment. In support of earlier *in vitro* studies, HAS-2 expression was markedly decreased with a concomitant decrease in dermal hyaluronan content (Gebhardt *et al*., 2010), while hyaluronidase expression and activity was unaffected.

Consequently, topical GC treatment results in skin atrophy characterized by a profound increase in transparency of skin, a cigarette-paper-like consistency with increased fragility, tearing, and bruising (Kimura and Doi, 1999), hypersensitivity to toxicity stimuli (dimethyl sulphoxide and sodium hydroxide), reduced ceramide, cholesterol and free fatty acid content and increased transepidermal water loss (Figure 1-23, Sheu *et* al., 1999, Kolbe *et al*., 2001).

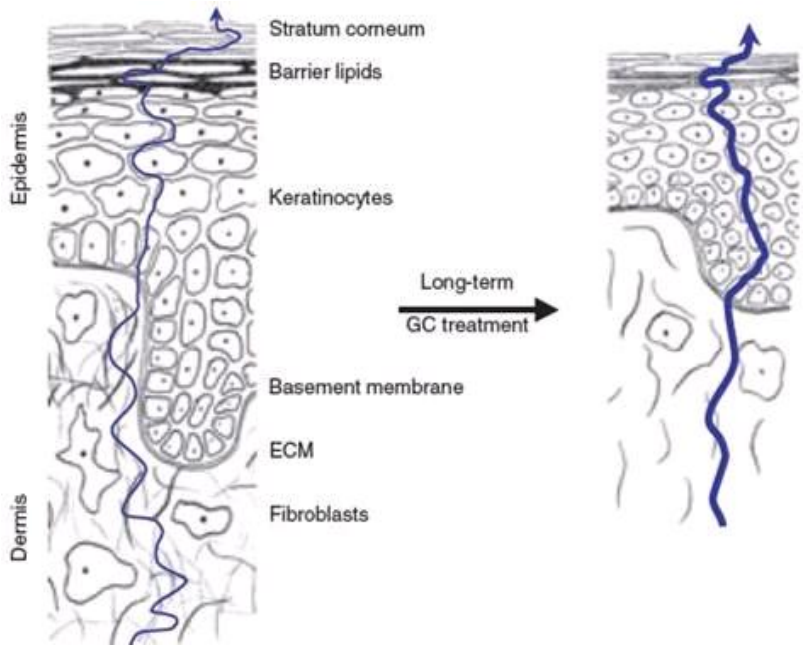


Figure 1-23 Adverse effects of GC therapy. Chronic topical/systemic treatment with GC causes a suppression in fibroblast and keratinocyte proliferation and ECM production. Keratinocyte lipid (free fatty acids, cholesterol, ceramides) production is reduced, resulting in fewer intercellular lipid layers, stratum corneum thinning and increased transepidermal water loss (blue arrow). Reduced dermal integrity and increased water loss ultimately lead to a compromise in barrier function with reduced tensile strength and elasticity (Schoepe *et al*., 2006)

1.9.2.2 Systemic

Systemic GC therapy is associated with a similar range of side-effects in skin as observed with topical treatments. Female patients on chronic oral GC treatment for a variety of dermal pathologies including dermatomyositis, pemphigus vulgaris, pyoderma gangrenosum, and urticarial vasculitis and demonstrated decreased skin thickness in the upper arm, dorsal and ventral forearm regions compared to age-matched controls, whilst male patients also displayed a decrease at the upper arm site (Werth *et al*., 1998). Furthermore, the authors observed a decrease in procollagen-1 mRNA in GC-treated patients. Other studies have also reported decreased collagen type I and III content in human dermis following systemic GC therapy, proposed to be attributable to decreased synthesis, rather than increased degradation (Autio *et al*., 1993, Autio *et al*., 1994).

1.9.3 Endogenous glucocortioid excess

GC-induced skin atrophy is also a common pathological feature in conditions of endogenous GC excess (e.g. Cushing's syndrome, Sowers and Lippman, 1985). Murine models of this disorder, with chronic pituitary-adrenal activation driven by continuous CRH production, display skin thinning (in addition to other Cushingoid phenotypes) resulting from CRH-ACTH induced GC hypersecretion (Stenzelpoore *et al*., 1992). In humans, skin thinning is present in >80% of young hypercortisolaemic patients and is a key diagnostic factor in distinguishing Cushing's syndrome from simple obesity (Newell-Price *et al*., 2006).

Interestingly, dermal fibroblasts from patients with Cushing's syndrome display a gain-of function phenotype with increased proliferative capacity, increased collagen gel contraction ability and similar collagen production rates compared to gender- and age-matched normal controls (Pratsinis *et* al., 2001, Zervolea *et al*., 2005, Kletsas *et al*., 2007), suggesting the dermal atrophy associated with this disorder is exclusively due to the hypercortisolaemic *milieu in vivo* and may be reversible following restoration of normal extracellular cortisol concentrations.

1.9.4 Summary

Overall, these adverse effects of GC on skin cell function, following local and systemic therapy or resulting from endogenous GC excess, ultimately culminate in skin that has compromised integrity and barrier function (Sheu *et* al., 1997, Kato *et al*., 2003), which is prone to infection and suffers from reduced rates of wound healing. Clearly, these adverse effects offset the immunological advantages of GC treatment and require further research into developing specialized compounds capable of dissociating the anti-inflammatory and anthropogenic capabilities with increased benefit/risk potential.

1.10 A role for GC in ageing?

Strong evidence now exists for the contribution of GC dysregulation to the ageing process. Whilst acute HPA activation and GC secretion is essential during stress adaptation, excessive exposure to sustained, elevated stress hormone levels has been linked to psychiatric, reproductive, immune, metabolic and cardiovascular disorder predisposition (McEwen, 1998). Basal circulating GC levels are reported to increase significantly with age in ~40% humans, with a similar proportion displaying only moderate increases and the remainder displaying an opposing correlation (Lupien *et al*., 1996). In older people, impaired HPA sensitivity to GC feedback is also documented (Wilkinson *et* al., 2001) in addition to greater serum cortisol levels in the evening and night-time, although circadian rhythmicity is still present to some extent (Van Cauter *et* al., 1996, Ferrari *et al*., 2001). Cortisol levels in cerebrospinal fluid are also raised in older individuals (Guazzo *et al*., 1996). Interestingly, the increase in circulating cortisol is maintained in centenarians, suggesting their longevity more likely results from genetically predetermined improved adaptive mechanisms of coping with the consequences of raised GC levels, rather than from a protective lack of GC hypersecretion with age (Genedani *et al*., 2008). Similarly in rats, a loss in circadian corticosterone secretion patterns has been documented in some (but not other) strains with increasing age, with elevated morning and reduced nocturnal secretion (Honma *et al*., 1996, Audige *et al*., 2002). The causes of this large inter-individual variation are likely to be genetically predetermined (Linkowski *et al*., 1993, Franz *et al*., 2010) as with many other aspects of longevity. Conclusively, age-related hypercortisolism and increased GC resistance has also been documented in other species such as baboons (Sapolsky and Altmann, 1991).

Pathologically, the increase in GC production and altered sensitivity with advancing age is thought to directly contribute to several age-related conditions including osteoporosis -

independently of more traditional causes such as oestrogen decline (Manolagas, 2010, Weinstein *et al*., 2010, Hardy and Cooper 2010), depression and cognitive dysfunction (Sapolskey *et* al., 1986, Sapolsky, 1999, Ferrari and Magri, 2008, Wolkowitz *et al*., 2010), sarcopenia (Dardevet *et al*., 1995, Savary *et al*., 1998) and inflammatory disease (Heffner, 2011).

Additionally, alterations in pre-receptor GC metabolism have recently been reported with age in human bone and mouse hippocampus, with increases in 11β-HSD1 expression that may elevate GC exposure and contribute to age-associated osteoporosis and cognitive impairment (Cooper, 2002, Holmes *et al*., 2010).

Conversely, levels of GR are thought to decrease with age, potentially as a compensatory mechanism for the increased circulatory and pre-receptor-driven local GC concentrations (Sharma and Dutta, 2006). Indeed, polymorphisms in the GR have been described which induce GC resistance and insulin sensitivity, lower cholesterol, reduce immunological markers of mortality (C-reactive protein) and may prolong lifespan (van Rossum *et al*., 2002, 2004).

1.11 Rationale and aims

Clearly, many similarities exist in skin between the consequences of ageing (both chronologically intrinsic and superimposed extrinsic) and those of GC excess (of exogenous topical and systemic, or endogenous overproduction) covering many aspects of skin biology. These include similarities in morphological alterations involving epidermal/dermal organization, cellular composition, proliferative responses and differentiation capabilities, molecular changes in ECM components such as collagens and their regulatory proteins

(MMPs, TIMPs etc.) and downstream consequences on barrier function, homeostatic control, mechanistic integrity and immunological capabilities (Kahan *et al*., 2009).

Although the effects of GC in respect to dermal atrophy have been extensively documented, there is only a limited literature describing the pre-receptor regulation of GC availability in skin. Furthermore, the skin-specific metabolism of cortisol in cells and tissue according to donor age and site has not been investigated, despite evidence supporting a role for GC dysregulation in the ageing process. The ability of pre-receptor metabolism to regulate GC target genes in skin also requires addressing.

Therefore, the primary aims of this thesis are to:

1. Characterize the localization, expression, activity and regulation of 11β-HSDs in murine and human skin.

2. Investigate 11β-HSD1 expression and activity in human dermal fibroblasts and human skin tissue explants from PP (intrinsic ageing) and PE (intrinsic and extrinsic ageing) anatomical locations from young and aged donors and in skin tissue explants from young and aged mice.

3. Examine the pre-receptor regulation of GC target genes through 11β-HSD1 in human dermal fibroblasts and skin tissue explants.

Based on the principal hypothesis that 11β-HSD1 expression and activity increase in skin as a function of age, locally amplifying GC concentrations through pre-receptor reactivation from cortisone, the secondary aim of this thesis is:

4. To characterize the dermal phenotype of the 11β-HSD1-null young and aged mouse compared to WT young and aged animals with regards to markers of ageing.

The secondary hypothesis being that aged 11β-HSD1-null mice will have a dermal phenotype more similar to young WT and knockout animals than aged WT littermates.

CHAPTER 2 MATERIALS AND METHODS

The general reagents and protocols used throughout this thesis are presented here, with specific modifications to individual experiments detailed in their respective chapters. Unless otherwise stated, chemicals were purchased from Sigma Chemicals Co. Ltd. Poole, UK.

2.1 Human cell and tissue preparation

2.1.1 Primary human dermal fibroblasts

Frozen stocks of primary human dermal fibroblasts (HDF) from PP (inner upper arm, n=12) and PE (outer lower arm, n=15) sites from donors of varying age (years ± S.D, 45.9±20.3, n=27) and gender (5 male, 11 female) were obtained with ethical approval and informed consent from Unilever Discover, Bedford, UK.

2.1.1.1 Materials

Phosphate buffered saline (PBS): two tablets of PBS in 400ml distilled water. Autoclave and store at 4ºC.

PBS ethylenediaminetetraacetic acid (EDTA): 400µl 0.5M EDTA into 400ml PBS. Autoclave and store at 4ºC.

Complete media: RPMI-1640 supplemented with 1% (vol/vol) non-essential amino acids, 1% penicillin/streptomycin, 1% sodium pyruvate, 2mmol/l L-glutamine and 10% fetal calf serum (FCS, Labtech International, Sussex, UK).

Trypsin: Trypsin diluted 1/20 into PBS EDTA.

2.1.1.2 Methods

Frozen vials were thawed at 37ºC and the cells were transferred immediately to 5ml PBS (in a 15ml falcon tube), mixed by inversion and centrifuged at 300g for 5min. Following removal of PBS, pellets were resuspended in 10ml pre-warmed complete fibroblast media, seeded in 75cm^2 flasks and incubated at 37ºC in a 5% CO_2 atmosphere. Media was replaced every two days until the cell monolayer reached confluence (Figure 2-1).

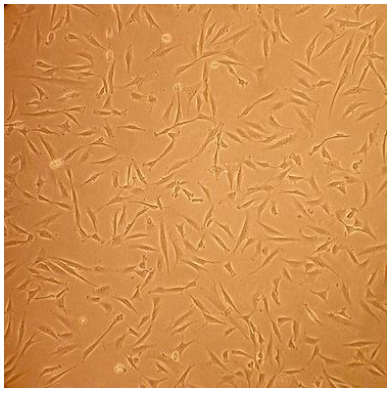


Figure 2-1 HDF in culture

At this point media was discarded, the cells were rinsed briefly in 5ml PBS before replacing with 2ml trypsin and incubating at 37ºC in a 5% CO_2 atmosphere for 5min. Flasks were firmly "tapped" to fully resuspend the cells before neutralizing immediately in 5ml media. Following centrifugation at 300g for 5min and discarding media, pellets were resuspended in 30ml fresh media and passaged into three new 75cm^2 flasks.

Fibroblasts were maintained in this manner up to a maximum of 5 passages. Frozen stocks were regularly replenished by trypsinising cells at 80% confluence, resuspending the pellet in 1ml FCS with 10% dimethyl sulphoxide. All experiments were performed at 80% confluence.

2.1.2 Human skin biopsies

Following application and approval by the Black Country Research Ethics Committee (09/H1202/024), University Hospital Birmingham Research and Development (RRK3890)

and the Wellcome Trust Clinical Research Facility (CRF) Scientific Advisory Committee (study 1010) healthy young (20-30 years) and older (60-75 years) volunteers were recruited from the University of Birmingham and Birmingham "1000 Elders" Group respectively. Subsequently, an ethical amendment was approved to include anyone over 60 years in the older age group. After recruitment, volunteers were provided with a Letter of Invitation and Patient Information Sheet. Interested volunteers were registered with the CRF in advance and signed Consent Forms under supervision by Dr Abd Tahrani before undergoing the 45min procedure. Upon procedure completion, volunteers were provided with a Biopsy Aftercare Sheet and GP Letter. Exclusion criteria were as follows:

- Pregnancy
- Significant past medical history including diabetes, epilepsy and current or past history of active sepsis
- Non-Caucasian
- Pre-existing skin conditions e.g. eczema and psoriasis
- GC therapy in the past 12 months
- Smoking or history of smoking within the past 10 years
- Warfarin medication
- Current participation in another study

40 volunteers completed the process, consisting of 20 young (years ± S.D, 25.7±3.0, 9 female and 11 male) and 20 older (72.2±8.2, 11 female and 9 male) donors.

2.1.2.1 Materials

Surgical trays: For each procedure, surgical trays lined with Supadrape (Westfield Medical Ltd., Radstock, UK) were prepared, containing dressing scissors, pointed forceps, 50 sheets

7.5 x 7.5cm cotton gauze (On Call Medical Supplies, Exmouth, UK) and two pieces of Supadrape prepared with a 5 x 5cm central opening. Items were wrapped in the tray lining, sealed with autoclave tape and trays were wrapped in Supadrape prior to autoclaving.

Biopsy materials: two Stiefel 3mm Biopsy Punch, two termous 19G 1.5in cream needle, two termous 25G 5/8in orange needle (Medisave, Weymouth, UK), two 2ml 2% lidocaine + HCl (National Veterinary Services, Stoke-on-Trent, UK), two 9 x 10cm mepore dressing, two 5 x 5cm inadine dressing, sterile gloves (On Call Medical Supplies, Exmouth, UK), two 5ml syringe and 100% ethanol spray.

2.1.2.2 Methods

The following procedure was conducted by Dr Abd Tahrani (with personal assistance) under sterile conditions. Surgical areas consisted of the midpoint PE side of the lower arm (site 1) and the midpoint PP side of the upper arm (site 2), avoiding scars and hyperpigmented areas.

Site 1 was cleaned with 100% ethanol and covered with Supadrape containing a 5 x 5cm central opening. Lidocaine was loaded into the syringe using a 19G cream needle and injected into the site using a 25G orange needle. Following dispersion of lidocaine, the site was tested for numbness prior to obtaining two 3mm punch biopsies (using cotton gauze to contain bleeding). Samples were placed into 1.5ml eppendorfs containing complete media.

Gauze was applied under pressure until bleeding subsided and the area was dressed with an inadine patch followed by a mepore dressing. The procedure was then repeated on site 2. Samples were analysed immediately or snap-frozen in liquid nitrogen and stored at -80ºC.

2.2 Mouse tissue preparation

2.2.1 Animal housing

All mice were group housed under controlled temperature (21-23ºC) and light 12h light, 12h dark cycle; lights on at 0700h) with *ad libitum* access to standard rodent chow and water. Animal procedures were approved under the British Home Office Animals (Scientific Procedures) Act 1986. 11β-HSD1-null mice were generated as previously described (Semjonous *et al*., 2011) with C57BL/6 WT littermates used as controls. All studies were performed on tissue from male mice.

2.2.1.1 Materials

10% neutral buffered formalin (NBF): 4g sodium monophosphate and 6.5g sodium diphosphate was dissolved in 900ml distilled water before adding 100ml 37% formaldehyde. Stored in the dark at room temperature.

2.2.1.2 Methods

All animal tissue used in this thesis was surplus waste material from experiments designed by other researchers within our group. Skin tissue from mice that had been recently sacrificed by cervical dislocation was obtained from the lower dorsal region following hair removal with clippers (Figure 2-2). Tissue was obtained from WT young (11-20 weeks, n=6) and aged (91-99 weeks, n=5) and 11β-HSD1-null young (11-20 weeks, n=4) and aged (91-99 weeks, n=4) mice. Samples were dissected into 4-10mg pieces and processed immediately, submerged in 1ml 10% NBF and stored in the dark at room temperature or snap-frozen in liquid nitrogen and stored at -80ºC.

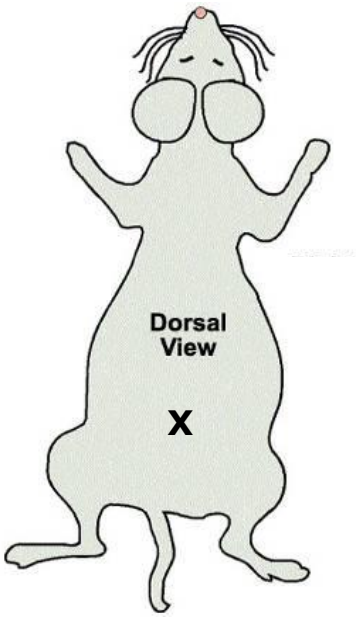


Figure 2-2 Anatomical location of mouse skin tissue explant site. Skin was excised from the lower dorsal region (X) from recently sacrificed animals

2.3 Messenger RNA detection and quantification

2.3.1 Isolation of cellular RNA

Cellular RNA was extracted using the TriReagent system, following the manufacturer's guidelines.

2.3.2 Isolation of skin tissue RNA

Tissue RNA was extracted using an RNeasy Fibrous Tissue kit (Qiagen, Crawley, UK) using a PowerGen 125 homogenizer (Fisher Scientific, Loughborough, UK) according to the manufacturer's protocol.

2.3.3 Generation of cDNA by reverse transcription

Following spectrophotometric quantification and quality control, ~500ng RNA was used in a final volume of 25µl to generate cDNA using the Multiscribe^{TM} Reverse Transcriptase kit according to the manufacturer's guidelines (Applied Biosystems, California, USA). Reaction

mix consisted of 2.5µl Reaction Buffer (10x), 5µl $MgCl_2$ (2.5mM), 5µl dNTPs (1µM), 1.25µl random hexamers, 0.5µl RNase inhibitor, 1.55µl reverse tanscriptase (50U/µl) and made up to 25µl with nuclease-free water. Reactions lacking RNA were used as internal negative controls. Reactions occurred as follows: 24ºC for 10min, 37ºC for 60min, 48ºC for 30min and 95ºC for 5min. cDNA was stored at -20ºC.

2.3.4 Quantification by real time PCR

2.3.4.1 Principle

Quantitative real-time polymerase chain reaction (qPCR) is a method permitting accurate measurement of specific DNA sequences from cell and tissue samples (Bustin, 2000). The TaqMan method employed in this thesis utilises the detection of a fluorescence signal during the amplification of a specific target. A target-specific probe (5'-fluorescently labelled and 3'-quencher labelled) anneals to a DNA sequence between the forward and reverse primers. With both the fluorophore and quencher hybridized to the cDNA in close proximity, no signal is emitted.

As the reaction PCR reaction progresses, the 5'-3' exonuclease activity of *Taq* polymerase degrades the probe, freeing the fluorescent reporter from the quencher and resulting in a detectable signal (Figure 2-3). This signal accumulates with each successive PCR cycle and, during the exponential phase of the cycle, is recorded as the threshold cycle (Ct) value. This value is inversely proportional to the starting DNA target copy number (as a greater starting amount of template will induce a detectable signal in a fewer number of amplification cycles). Moreover, qPCR can utilise multiplex reactions, where a target gene sequence is analysed simultaneously to an internal reference gene (such as ribosomal 18S rRNA) labelled with a different fluorophore. This permits relative quantification of target, resulting in a ΔCt value

(target gene Ct value - reference gene Ct value) which allows for different starting cDNA concentrations by normalising to an internal reference. Comparison of the ΔCt between different samples results in a ΔΔCt value (Figure 2-4) which enables fold-change (*x*) between samples (e.g. treated sample is *x*-fold of untreated sample) to be deducted from the following equation: $x = 2^{-\Delta\Delta Ct} = 2^{-(Ct\ target\ gene - Ct\ control\ gene)_{treated} - (Ct\ target\ gene - Ct\ control\ gene)_{untreated}}$.

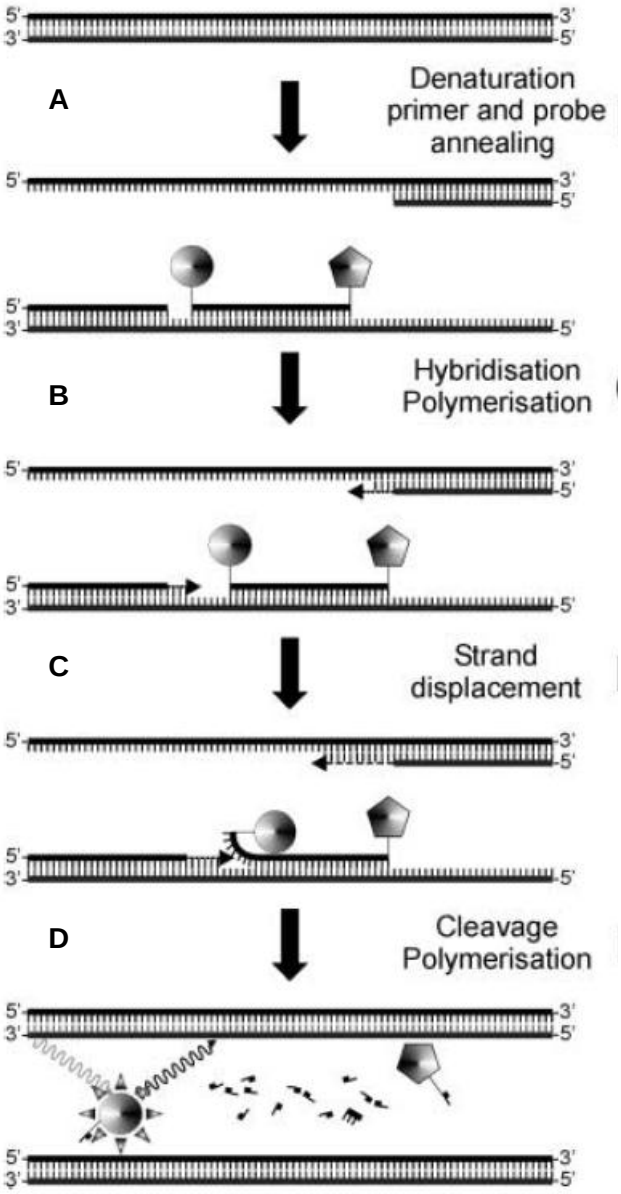


Figure 2-3 The TaqMan qPCR assay. (**A**) During denaturation, the primers and probe anneal. The close proximity of the fluorescent reporter (circle) to the quencher (pentagon) prevents signal emission. (**B**) Polymerisation proceeds at the same temperature as the annealing step. (**C**) The polymerase displaces and hydrolyses the labelled probe. (**D**) The fluorescent dye is released from its proximity to the quencher, and fluorescence is detected. This signal is directly proportional to the number of molecules present at the end of the previous or beginning of the current cycle

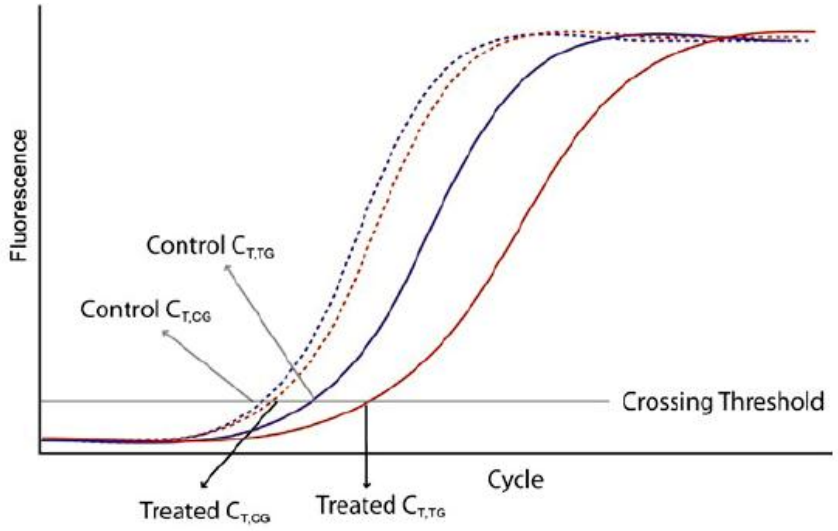


Figure 2-4 Mathematical basis of the $2^{-\Delta\Delta CT}$ method. The $2^{-\Delta\Delta CT}$ method enables relative quantitation (treated sample is X fold of control sample) through measurements of crossing thresholds (CT). As described in the text, the comparative differences between the target gene (TG) of interest and an endogenous control gene (CG) for each sample enable a relative quantitative comparison between the samples. Note that the endogenous control genes (dotted lines) should not vary significantly in CT with treatment. Reproduced from Van Guilder *et al*., 2008

2.3.4.2 Methods

All qPCR reagents were obtained from Applied Biosystems, California, USA. qPCR analyses were performed using an ABI 7500 system (Perkin-Elmer, Cambridge, UK) in 10µl final reaction volumes consisting of 5µl TaqMan Universal PCR mastermix (2x), 0.5µl inventoried FAM-labelled target gene multiplexed with VIC-labelled 18S ribosomal rRNA primers and probe (20x, 25nM final concentration each primer and probe), 3µl nuclease-free water and 1µl cDNA (~25-50ng).

Reactions occurred in 96-well optical plates as follows: 50ºC for 2min, 95ºC for 10min, 44 cycles of 95ºC for 10min followed by 60ºC for 1min. Raw data was obtained as Ct values (the cycle number at which logarithmic PCR plots cross a given threshold line in the exponential reaction phase) and used to determine ΔCt values. Assays were performed in duplicate and the average ΔCt taken for each sample. All data was analysed at this stage prior to transformation through the $2^{-\Delta\Delta CT}$ method.

2.4 11β-HSD isozyme activity assays

2.4.1 Materials

^{3}H-cortisol stock: specific activity 100Ci/mmol, 1mCi/ml (Du Pont, NEN, Netherlands) diluted 1/20 in ethanol for working solution.

^{3}H-corticosterone stock: specific activity 100Ci/mmol, 1mCi/ml (Du Pont, NEN, Netherlands) diluted 1/20 in ethanol for working solution.

^{3}H-cortisone/^{3}H-dehydrocorticosterone stock: generated in-house. 20µl of ^{3}H-cortisol/^{3}H-corticosterone stock was incubated with 250mg of human/mouse placenta (exhibiting high 11β-HSD2 activity) and 500µM NAD^{+} cofactor in 500µl 0.1M potassium phosphate buffer (pH 7.4) in 10ml glass tubes at 37ºC (shaking waterbath) for 3h. Steroids were extracted by adding 5ml dichloromethane, capping and vortexing for 10sec prior to phase separation by 100g centrifugation for 10min. Aqueous phase and protein interface was removed by aspiration and steroids were concentrated under blowing air at 55ºC for 30min. Steroids were resuspended in 100µl dichloromethane and spotted onto silica plates prior to thin layer chromatography (TLC) separation in a 186:14ml chloroform:ethanol mobile phase (90min). Plates were air-dried in a fume hood for 10min and scanned briefly using a Bioscan 200 Imager (LabLogic, Sheffield, UK) to localize the tritiated steroids. The silica surrounding the ^{3}H-cortisone/^{3}H-dehydrocorticosterone was scraped into fresh glass tubes and eluted overnight in 300µl ethanol at 4ºC. Silica was pelleted by centrifugation at 100g for 5min and the ethanol was removed to a clean storage vial. Tritiated steroid concentration was determined by spotting 2µl onto a new silica plate and repeating the TLC procedure to establish experimental working volumes.

2.4.2 Methods

2.4.2.1 HDF studies

HDF 11β-HSD activity was measured at 80% confluence in a 6-well plate format. Assays were performed in duplicate (with post-assay cells from one well contributing to total protein estimation and the other used for RNA extraction) and the average activity calculated.

2ml of fresh complete media containing 200pmol (100nM) substrate (2µl 10^{-4}M cortisone/cortisol in ethanol) enriched with 20,000 cpm tritiated substrate tracer (1.25pmol) was added to each well and incubated at 37ºC in 5% CO_2 for 24h. Media was transferred to a 10ml glass tube containing 5ml dichloromethane and following capping, steroids were extracted into the organic phase by vortexing for 10sec. The phases were separated by centrifugation at 100g for 10min and the aqueous layer (consisting of media and protein interface) was then removed by aspiration. The organic phase was evaporated at 55ºC under blowing air (30min) and dried steroids were subsequently resuspended in 50µl dichloromethane before spotting onto silica plates (2cm from the bottom edge and 1.5cm apart). One drop of non-tritiated standard solution (1mg/ml each of cortisone and cortisol in ethanol) was also added to each spot for visualisation of steroid separation.

Steroids were separated by TLC in a mobile phase of 184:16ml chloroform:ethanol (90min), removed from the TLC tanks to dry in a fume hood (10min) and visualised under UV to confirm steroid separation. Plates were analysed by scanning for 10min/spot using a Bioscan System 200 Imaging Scanner (LabLogic, Sheffield, UK) and percentage conversion was calculated using region counts for individual peaks. Following cellular protein estimation, activity was expressed as amount of substrate converted (pmol) per mg protein per hour.

2.4.2.2 Tissue

Human/mouse skin tissue (4-10mg wet weight weighed prior to onset of assay) 11β-HSD activity was measured in 10ml glass tubes with 500µl of fresh complete media containing 100pmol (200nM) substrate (1µl 10^{-4}M cortisone/cortisol for human samples or corticosterone/11-DHC for mouse samples in ethanol) enriched with 20,000 cpm tritiated substrate tracer (1.25pmol) and incubated at 37ºC in 5% CO_2 for 24h (uncapped). Subsequently, tissue was discarded (or snap-frozen for further analysis where stated) and following addition of 5ml dichloromethane, samples were processed as described above to determine steroid conversion. Activity was expressed as fmol/mg tissue/h.

2.5 Protein estimation

Total soluble protein from cell extracts (required to normalise 11β-HSD enzyme activity levels) was determined using the Bio-Rad RC DC (detergent compatible) protein assay kit (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). This colorimetric assay determines protein concentration following detergent solubilisation, similarly to the well-documented Lowry assay where protein reacts with an alkaline copper tartrate solution followed by the reduction of Folin reagent (Lowry *et al*., 1951). The standard assay is used with samples having protein concentrations between 0.1 and 2.0 mg/ml, measured at 650-750 nm with a standard laboratory microplate reader.

2.5.1 Method

Following removal of media for 11β-HSD substrate conversion determination, cells were rinsed briefly in PBS to remove traces of media. PBS was replaced with 250µl distilled water and freeze-thawed to release intracellular protein. The average absorbance of duplicate

samples of unknown concentration was determined using a BSA standard curve equation (Figure 2-5).

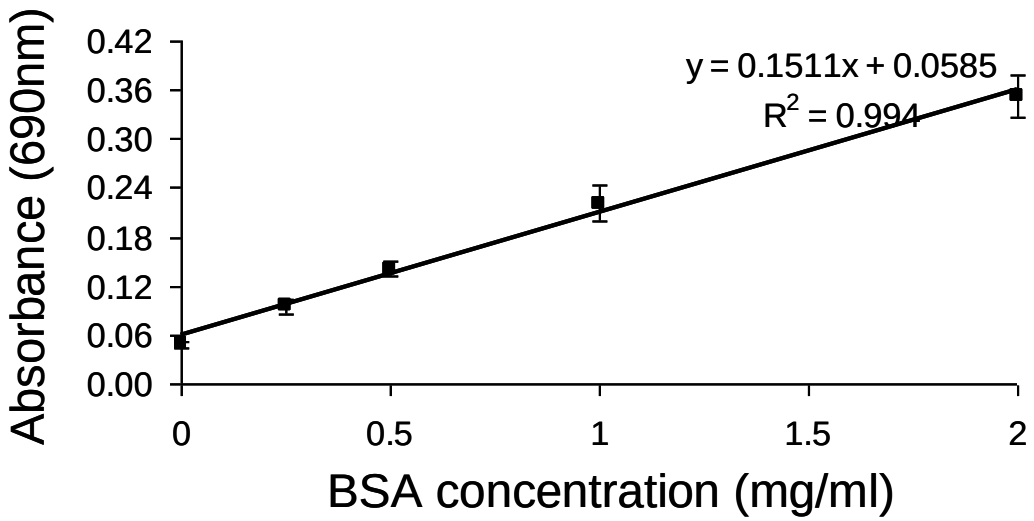


Figure 2-5 Protein standard curve. The absorbance of 0, 0.25, 0.5, 1 and 2mg/ml BSA in distilled water was measured (n=10) and the average absorbance was calculated for each concentration to give the reference standard curve.

5µl of sample (the same volume was used for standard curve) was added to each well (96-well plate) followed by 25µl reagent A (alkaline copper tartrate) and 200µl reagent B (Folin reagent). The plate was incubated at room temperature for 15min before reading at 690nm on a Victor3 1420 multi-label counter (Perkin Elmer, Beaconsfield, UK). Concentrations were read off the standard curve and expressed as mg/ml.

2.5.2 Statistics

Experimental data were analysed using GraphPad Prism software (GraphPad Software Inc., California, USA). A parametric Student's T-test was used to compare data displaying a normal distribution (paired tests were used as appropriate). Non-parametric data was analysed using a Mann-Whitney Rank Sum Test. The null hypothesis was rejected at a significance level of $p<0.05$ (95% confidence).

CHAPTER 3 CHARACTERIZATION OF 11β-HSDs IN SKIN

3.1 Introduction

3.1.1 Skin as a neuro-immuno-endocrine organ

Among the many functions typically attributed to skin, its ability to respond to and regulate local and systemic endocrine processes is often overlooked. This seems somewhat surprising considering the broad spectrum of abnormalities associated with, and in some instances, the primary manifestation of certain endocrine disorders in skin, including Cushing's syndrome, adrenal insufficiency, diabetes, acromegaly, hypopituitarism, hypo/hyperthyroidism, sex hormone excess/deficiency and others (Feingold and Elias, 1987, 1988).

Endocrine regulatory functions of skin include the ability of human epidermal keratinocytes to convert the pro-hormone thyroxine (T_4) to active triiodothyronine (T_3) through deiodination (Kaplan *et al*., 1988) and the photochemical generation of the steroid hormone vitamin D_3 (cholecalciferol) from 7-dehydrocholesterol in the *stratum basale* and *stratum spinosum* (Bouillon *et al*., 1995). The simplicity of these examples is misleading, indeed skin forms a complex bridge between many scientific disciplines as integrated models of analysis such as the neuro-immuno-cutaneous-endocrine (NICE) network (O'Sullivan *et al*., 1998), are beginning to emerge. Associations between nervous system perturbations and skin disease are often seen in clinical practice (Koblenzer, 1996) such as the exacerbation of psoriasis, acne, eczema and urticaria by stress (Gupta *et al*., 1994). Evidence for the mechanisms underlying the connection of abstract stress with a physical clinical (i.e. cutaneous) manifestation centres around shared specific communication molecules common to both the nervous

and immune systems that were identified over two decades ago (Wierdermann, 1987) and since then neuropeptides have gained prominence as a primary signalling mechanism linking the two systems (Reichlin, 1993). In skin, neuropeptides have been identified in both cutaneous nerve fibres and all cells examined including keratinocytes, immunocytes, fibroblasts, Langerhans cells, and endothelial cells (Wallengren *et* al., 1987, Scholzen *et al*., 1998, Di Cornite *et al*., 2007).

3.1.2 Steroidogenesis in skin

In addition to responding to and regulating many elements of neuro-immuno-endocrine function, studies are emerging that demonstrate *de novo* steroid production in skin through the steroidogenic pathway. Keratinocytes are an abundant storage depot for cholesterol - the common steroidal precursor - and are also able to synthesize it (Menon *et al*., 1985), while certain rate-limiting components of the steroidogenesis pathway, such as steroidogenic acute regulatory (StAR) protein, have also been identified in human epidermis by immunofluorescence histochemistry (Slominkski *et al*., 2004). The complete CYP11A1 complex, encoding the mitochondrial enzyme P450scc which converts cholesterol to pregnenolone in three mono-oxygenase reactions, has been identified in human epidermis and keratinocytes (Slominkski *et al*., 2004, Thiboutot *et al*., 2003). Another study reported the presence of CYP11A1, CYP17A1 (17α-hydroxylase) and CYP21A2 (21α-hydroxylase) in skin, but failed to detect CYP11B1 (11β-hydroxylase), the final enzyme in the sequence of cortisol synthesis (Slominksi *et al*., 1996). Others demonstrated the 21α-hydroxylation of progesterone to deoxycorticosterone and associated metabolites in immortalized keratinocytes (Slominski *et al*., 2002).

More recently, Hannen *et al* demonstrated the expression of CYP11A1, CYP17A1, 3β-HSD1, CYP21 and CYP11B1 protein in primary cultures of human keratinocytes (Hannen *et al*., 2011). Furthermore, this study revealed co-migration of tritiated pregnenolone metabolites with progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, corticosterone, cortisone and physiologically relevant amounts of cortisol suggesting the steroidogenesis pathway in keratinocytes is active. In addition to keratinocytes, hair follicles have been reported to express elements of the HPA axis and to synthesise cortisol (Ito *et al*., 2005), sebocytes demonstrate the transformation of 22R-hydroxycholesterol to 17-hydroxypregnenolone (Thiboutot *et al*., 2003), whilst melanocytes have also been shown to produce corticosterone and cortisol (Slominski *et al*., 2005). In dermal fibroblasts, treatment with progesterone was also shown to increase cortisol production (Slominsky *et al*. 2006).

Skin is also known to express enzymes involved in sex steroid steroidogenesis such as the P450 aromatase encoded by CYP19 in dermal fibroblasts (Harada, 1992), hair follicles and sebaceous glands (Sawaya and Penneys, 1997), the 17β-hydroxysteroid dehydrogenases which interconvert androstenedione/testosterone, oestrone/oestradiol and dehydroepiandrosterone/androstenediol in the basement layer of the epidermis (Hikima and Maibach, 2007), sebaceous glands and hair follicles (Labrie *et al*., 2000) and 5α-reductase which converts testosterone into the more potent metabolite dihydrotestosterone (Labrie *et al*., 2000). Only aldosterone synthase, encoded by CYP11B2 and responsible for the conversion of corticosterone to the mineralocorticoid aldosterone has not been detected in skin. Collectively, these studies confirm that skin should indeed be perceived as the largest organ with endocrine function in the human body.

3.1.3 Cortisol metabolism in human skin

Whilst Hannen *et al* were able to demonstrate that skin is capable of *de novo* cortisol synthesis from pregnenolone (Hannen *et* al., 2011), levels produced were low (1.0 ± 0.2 nmol/L over a 24h period) and just within the range required to activate the GR (Dong *et al*., 2006, Charmandari *et al*., 2008). However, cortisol concentrations can be modulated at a cell-specific level by the action of 11β-HSD isozymes which activate (11β-HSD1) and inactivate (11β-HSD2) cortisol from and to cortisone respectively and it is through this mechanism that cells are able to regulate exposure to GC.

Regulation of 11β-HSD activity by a component of human skin was first reported by Hammami and Siiteri in primary whole-cell cultures of foreskin fibroblasts which exhibited predominantly oxoreductase [11β-HSD1] activity with limited dehydrogenase activity (Hammami and Siiteri, 1991).

In contrast, initial studies conducted on isolated human sweat gland ducts and epidermis revealed significant dehydrogenase activity in the former [11β-HSD2], less in the latter and 10-fold lower levels of oxoreductase activity [11β-HSD1] in both structures (Kenouch *et al*., 1994). Here, the co-localization of 11β-HSD dehydrogenase activity with MR protein and mRNA expression was in agreement with its postulated function as a protective mechanism to prevent illicit activation of the MR by cortisol. Subsequent studies confirmed the activity of 11β-HSD2 in human eccrine glands (Bocchi *et al*., 2004).

Following Northern blotting with sheep kidney-isolated 11β-HSD2 cDNA, RNA expression was also detected in ovine skin although relatively weak compared to kidney, placenta and adrenal tissues (Agarwal *et al*., 1995). Similarly, mRNA

expression of cloned 11β-HSD2 was detected in human breast skin, again to a lesser extent compared to kidney, colon and pancreas (Brown *et al*., 1996).

Initial immunohistochemical studies localized 11β-HSD2 to the arterioles (Smith *et al*., 1996), eccrine sweat glands, to a lesser extent to the epidermis and was absent in the sebaceous glands and hair follicles in human skin (Smith *et al*., 1996). Eccrine gland 11β-HSD2 co-localization with MR (luminal cells) and absence of staining in epidermis, sebaceous and apocrine glands (including MR) was confirmed by another study (Hirasawa *et al*., 1997).

In comparison to 11β-HSD2, studies investigating expression, activity and localization of 11β-HSD1 in human skin are relatively sparse. Recently, Hardy *et al* reported 11β-HSD1 mRNA expression in primary dermal fibroblasts cultures from human knee tissue explants (Hardy *et al*., 2006). However, the authors also failed to detect 11β-HSD oxoreductase activity, in contrast to data reported previously for cultured foreskin fibroblasts (Hammami and Siiteri, 1991). Recent immunohistochemical studies localized 11β-HSD1 to the epidermal stratum spinosum, dermis and hair follicles (Hennebert *et al*., 2007), although the activity data in these studies is difficult to interpret as analyses were conducted on tissue homogenates, ameliorating 11β-HSD1 oxoreductase activity (as apparent from the order of magnitude increase in dehydrogenase compared to oxoreductase activity) and preventing its discrimination from 11β-HSD2.

Collectively, these limited studies into 11β-HSD localization, expression and activity were predominantly conducted in human skin cells (dermal fibroblasts) or using *ex vivo* human skin tissue explants with no comparable data available for other species, including mouse.

3.1.4 Skin of mice and men

The laboratory mouse remains the most widely used animal model for biological research based on several advantages including easy housing and maintenance with associated economical benefits, availability of many transgenic models permitting unparalleled opportunities to study disease pathophysiology including many models of human disease (Bedell *et al*., 1997). This is also applicable to many areas of dermatological research such as wound healing (Scheid *et al*., 2000), hair loss (Porter, 2003), pressure ulcers (Wassermann *et al*., 2009) and carcinogenesis (Fraga *et al*., 2004). Furthermore, the severe combined immunodeficient (SCID) mouse model has been used extensively to study a wide range of dermatoimmunological conditions including psoriasis (Raychoudhuri *et al*., 2004, Nickoloff, 2000), chickenpox (Niizuma *et al*., 2003), T-cell migration (Carballido *et al*., 2003), skin graft rejection (Briscoe *et al*., 1999) and allergy (Herz *et al*., 1998).

The relatively short lifespan of mice also endows their use as tool to study many aspects of ageing. In a dermal context, this has been applied to studies of wound healing rates (Chang *et al*, 2007, Agah *et al*., 2004, Ashcroft *et al*., 1997), photo-ageing (Bissett *et al*., 1990, Boyer *et al*., 1992, Bernstein *et al*., 1995, Haratake *et al*., 1997), structure (Jensen *et* al., 2005, Damodarasamy *et al*., 2004, Boyer *et al*., 1991, Ghadially *et al*., 1996), antioxidant capacity (Treiber *et al*., 2011, van Remmen *et al*., 2003) and nutrition (Liang *et al*., 2010, Taylor *et al*., 1995, Reed *et al*., 1996).

Whilst mouse models have been invaluable as tools to develop our understanding of skin biology and disease, there are several key differences between the skin of mice and men that require consideration. Mouse skin is covered in dense hair that follows the same cycling process as human hair: anagen (growth), catagen (regression), and telogen but progresses dorsally from cranial to caudal (Figure 3-1) and is highly

complex (Paus *et al*., 1999, Plikus and Chuong, 2008) while human hair cycling occurs in a mosaic-like manner.

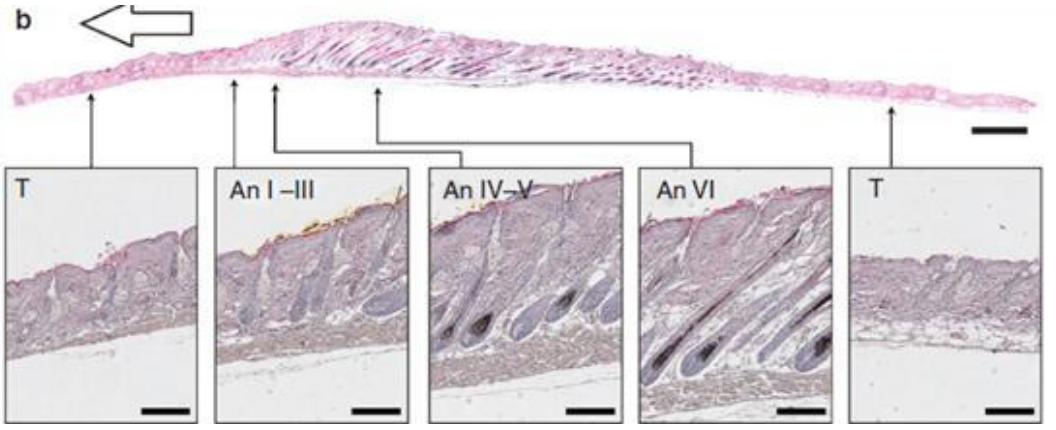


Figure 3-1 Hair cycle wave progression in mice. The region with the most advanced anagen hair follicles (anagen VI in this case) probably started the hair cycle earliest. Adjacent hair follicles to the left are seen in gradient stages from anagen V to I. This represents a spreading wave of hair cycle activation. A boundary of the hair cycle domains forms when a skin region does not respond to the spreading wave and remains in telogen. The blank arrow is used to point to the direction of the spreading waves (Plikus and Chuong, 2008)

Additionally, human hair is either vellus (penetrating to the superficial dermis) or terminal (penetrating to the deep dermis and is pigmented) and can switch between forms in an androgen-sensitive manner whilst mouse hair cannot (Porter, 2003). Another major difference is that mouse skin lacks eccrine sweat glands (potentially relevant to our research due to the high expression of 11β-HSD2 in these structures) and rete ridges/dermal papillae which are both abundant in human skin. Furthermore, mouse skin contains the *panniculus carnosus*, a thin muscle layer underlying the dermis which is absent in human skin and aids wound contraction following injury, while in human skin, wound repair occurs via re-epithelialisation and granulation tissue formation. Gender differences are also more prominent in mouse skin (Azzi *et al*., 2005) and must be controlled for.

Despite these considerations, if correctly accounted for by appropriate study design, mouse models will continue to play a crucial role in progressing our understanding of many human diseases including those that apply to skin.

3.2 Aims

Previous studies have demonstrated that skin is able to respond to, regulate and synthesize key hormones involved in local and systemic neuro- immuno- and endocrine signalling. As GC are known to influence a variety of biological processes in skin and local GC concentrations are tightly regulated by the cell-specific expression and activity of 11β-HSDs, it is not surprising that that the localization of these enzymes in skin tissue and primary cultures of dermal fibroblasts has been attempted. However, whilst there is a substantial volume of data regarding 11β-HSD2 and its co-expression with several MR-rich regions of the human skin - highlighting its role in protecting these aldosterone-sensitive areas from inappropriate GC activation, the data reported for 11β-HSD1 is relatively limited and in some cases (such as the levels of activity in dermal fibroblasts), controversial. **Our study therefore aimed to characterize expression, activity and localization of 11β-HSDs in human skin.**

The advantages of utilizing mice as models of human diseases and to further our understanding of biological processes are well-known and include applications for dermatological conditions and ageing. Our research group has developed several transgenic knockout models for 11β-HSD1 - however the characterization of 11β-HSDs in murine skin in thus far unreported. **Therefore, our study aimed to determine the expression, activity and localization of 11β-HSDs in mouse skin in comparison to human skin.**

While alterations in the expression of 11β-HSDs in skin have the capacity, through altering local GC levels, to modulate a variety of dermatological processes in normal physiology and disease, their regulation has not been investigated with two exceptions. Dexamethasone (100nM) was found to upregulate 11β-HSD oxoreductase

and dehydrogenase activities in cultured human foreskin fibroblasts while a range of other hormones (oestradiol, progesterone, testosterone and triiodothyronine) had little effect on either reaction (Hammami and Siiteri, 1991). Additionally, 11β-HSD oxoreductase activity and 11β-HSD1 mRNA expression was induced in HDF (knee) following treatment with the pro-inflammatory cytokines IL-1 and TNF-α while IL-4 and IFN-γ had no effect (Hardy *et al*., 2006). These limited studies suggest that GC and certain pro-inflammatory cytokines have the potential to escalate local GC activation by upregulating 11β-HSD1 expression and activity. **Our studies will also aim to confirm the regulation of 11β-HSD, H6PD and GR-α expression in HDF.**

3.3 Materials and Methods

3.3.1 Sample preparation and culture

3.3.1.1 Human and mouse skin tissue

Human arm skin biopsies were obtained according to section 2.1.2.

Human knee skin tissue explants were obtained during knee arthoplasty with informed consent and local ethical approval from patients fulfilling the American College of Rheumatology criteria for rheumatoid or osteoarthritis, in collaboration with Dr Rowan Hardy (School of Clinical and Experimental Medicine, University of Birmingham, UK).

Mouse skin tissue explants (WT) were obtained according to section 2.2.

3.3.1.2 Primary human dermal fibroblasts

Cultures of HDF were prepared according to section 2.1.1. In addition, samples were treated as follows:

GC treatment: Upon reaching 80% confluence in a 6-well format, media was replaced with 2ml fresh media containing 100nM cortisol or 100nM dexamethasone (2µl 10^{-4}M per well) in ethanol (final ethanol concentration <0.1%). Treatments were conducted in duplicate and controls were included in each experiment, treated with an equal volume of ethanol alone. Plates were incubated at 37ºC in 5% CO_2 for 48h.

TNF-α treatment: Cells were treated with 10nm/ml TNF-α as for GC treatment.

When determining the effect of GC on 11β-HSD1 activity, treatment was also maintained for total activity assay duration.

3.3.2 Real-time PCR gene expression

RNA extraction, reverse transcription and qPCR were conducted according to section 2.3. The following primer and probe reagents were used (Applied Biosystems, California, USA unless otherwise stated):

Human: 11β-HSD1 (designed and previously optimised in-house) antisense primer 5'-AGG-AAA-GCT-CAT-GGG-AGG-ACT-AG-3', sense primer 5'-ATG-GTG-AAT-ATC-ATC-ATG-AAA-AAG-ATT-C-3' (900nmol/reaction), probe 5'-CAT-GCT-CAT-TCT-CAA-CCA-CAT-CAC-CAA-CA-3' (100-200nm/reaction), 11β-HSD2 (Hs00388669_m1), GR-α (Hs00230813_m1), GR-β (Hs00354508_m1) and H6PD (Hs00188728_m1).

Mouse: 11β-HSD1 (Mm00476182_m1), 11β-HSD2 (Mm0049254_m1), GR-α (Mm00433832_m1) and H6PD (Mm00557617_m1).

Target gene expression assays were used in a multiplex format with 18S rRNA control reagents (Applied Biosystems, California, USA).

3.3.3 11β-HSD activity assays

11β-HSD oxoreductase and dehydrogenase activity assays were conducted for human and mouse skin tissue and HDF according to section 2.4.

3.3.4 Immunohistochemistry

3.3.4.1 Subjects

Paraffin-embedded human arm skin tissue sections were obtained from Unilever Discover, Bedford, UK following local ethical approval and informed consent.

Mouse skin tissue explants stored in 10% NBF according to section 2.2.1.2 were paraffin embedded and sectioned by Dr Charlie Shaikh (Birmingham Midland Eye Centre, City Hospital, Birmingham, UK).

3.3.4.2 Materials

Antigen retrieval: 0.1g calcium chloride in 10ml distilled water (10X stock) stored at 4°C. 1ml calcium chloride stock and 200µl trypsin (2.5%) in 9ml distilled water (final trypsin concentration 0.05%) pH to 7.8 and stored in 1ml aliquots at -20°C.

TBS: 121.2g Tris and 160.4g sodium chloride pH to 7.6 made up to 2l with distilled water (10X stock) and stored at 4°C.

TBSAT: 10ml TBS stock, 3g bovine serum albumin and 50µl Triton X-100 made up to 100ml with distilled water and stored in 5ml aliquots at -20°C.

3.3.4.3 Methods

Paraffin embedded slides were de-waxed by immersion in two 2min xylene washes followed by two 2min 100% ethanol washes and rehydrated in distilled water for 10min taking care not to allow tissue to dry out between transfers.

Tissue was circled with hydrophobic marker, immersed in ~100µl antigen retrieval solution and incubated at 37°C for 20min. Slides were rinsed under running distilled water for 10min followed by inactivation of endogenous peroxidase activity by incubating in 240ml methanol and 4.3ml hydrogen peroxide for 15min before rinsing again under running water.

Sections were blocked in ~100µl TBSAT containing 10% serum from the same host species used to derive secondary antibody (goat serum) for 1h at room temperature. Blocking solution was replaced with 1:20 dilution (in blocking solution, ~30µg/ml) of rabbit anti-human 11β-HSD1 primary polyclonal antibody (Cayman Chemical, Michigan, USA) and incubated at room temperature for 1h. Rabbit isotype-matched (IgG) antibody at the same concentration as the primary was used as a negative control. Slides were washed by gentle agitation in three 1X TBS and 0.05% Tween-20 (TBST) 10min washes.

Sections were incubated with 1:200 dilution (in blocking solution, ~1.25µg/ml) of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary polyclonal antibody (Dako, Glostrup, Denmark) and incubated at room temperature for 30min. Slides were washed in three 10min TBST washes.

Slides were visualised using a 3,3'-diaminobenzidine (DAB) kit according to manufacturer's protocol (Vector Laboratories Inc. California, USA), counterstained with Mayer's haematoxylin (where appropriate) and dehydrated by immersion in two

2min 100% ethanol washes followed by two 2min xylene washes prior to mounting in VectaMount (Vector Laboratories Inc. California, USA) and coverslipping.

11β-HSD2 immunohistochemistry was conducted as for 11β-HSD1, using a sheep anti-human 11β-HSD2 primary polyclonal antibody (The Binding Site, Birmingham, UK) in donkey serum blocking solution, a HRP-conjugated donkey anti-goat secondary (Dako, Glostrup, Denmark) and a sheep isotype control.

3.4 Results

3.4.1 Expression, activity and localization of 11β-HSDs in human skin

Expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD (ΔCt ± S.D) was analyzed in mRNA isolated from full-thickness human arm skin tissue biopsies (Figure 3-2). Expression of 11β-HSD2, GR-α and H6PD mRNA was comparable (14.5±1.6, 15.0±1.6 and 14.1±1.4 respectively, n=21) with 11β-HSD2 expression ~20-fold higher than 11β-HSD1 (14.5±1.6 vs. 19.0±2.0 respectively, $p<0.01$, n=21). Accordingly, 11β-HSD dehydrogenase activity was also higher than oxoreductase activity in *ex vivo* human skin samples (fmol/mg/h, 85.5 vs. 45.3 respectively, $p<0.05$, Figure 3-3) and was further increased following incubation with the 11β-HSD1-specific inhibitor LJ2 to 152.6 fmol/mg/h ($p<0.05$) with a concomitant partial decrease in 11β-HSD1 oxoreductase activity to 14.7 fmol/mg/h ($p<0.05$), demonstrating the close relationship between the two 11β-HSD isozymes in regulating local GC availability. Immunohistochemical analyses in human arm skin sections using a rabbit polyclonal antibody against 11β-HSD1 localized protein expression to epidermal keratinocytes, dermal fibroblasts, microvasculature, hair follicle cells, eccrine sweat glands and sebaceous glands, while only negligible

staining was detected following replacement of the primary antibody with an equimolar isotype control (Figure 3-4).

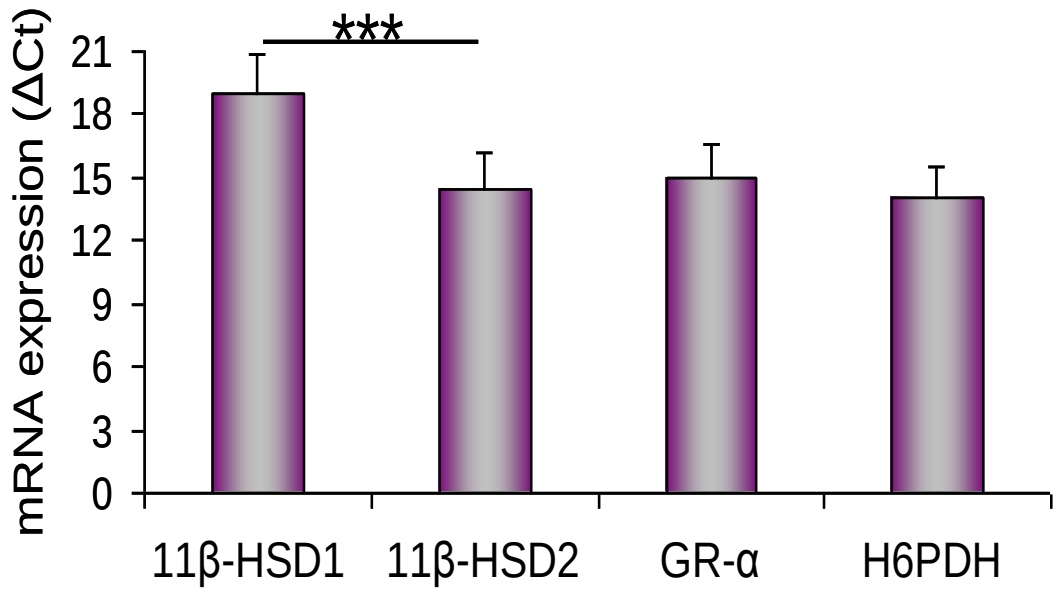


Figure 3-2 Expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD in human arm skin tissue biopsies. Following normalizing for levels of the housekeeping gene 18S rRNA, 11β-HSD2, GR-α and H6PD expression was found to be comparable (ΔCt ± S.D, 14.5±1.6, 15.0±1.6 and 14.1±1.4, n=21) while 11β-HSD2 expression was significantly higher than 11β-HSD1 (14.5±1.6 vs. 19.0±2.0, n=21). Significance *** = $p<0.001$

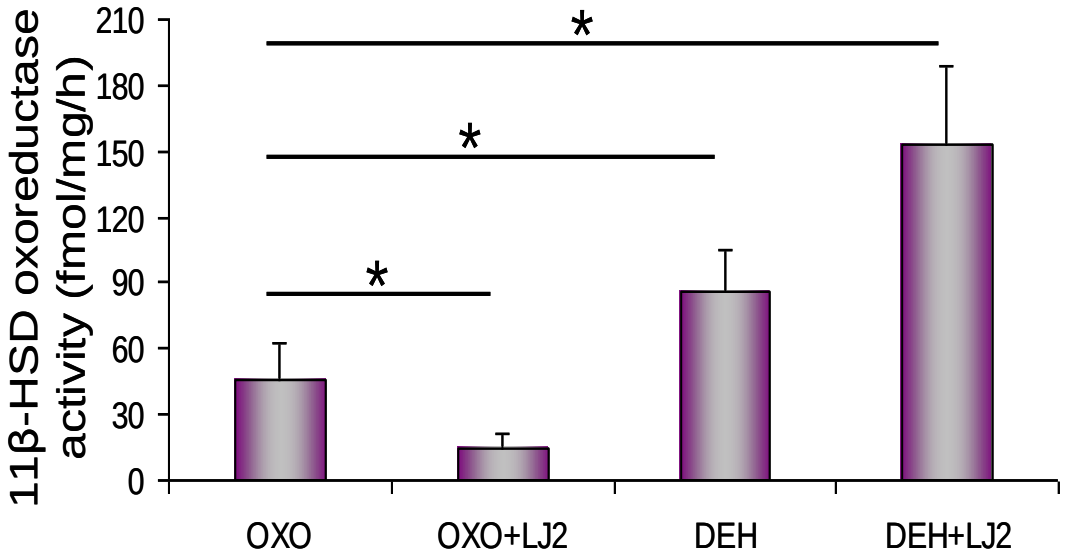


Figure 3-3 11β-HSD activity in human knee skin tissue. In accordance with greater 11β-HSD2 expression, mean 11β-HSD dehydrogenase (DEH) activity (fmol/mg/h) was greater than oxoreductase (OXO) activity in human skin (85.5 vs. 45.3, n=4). Furthermore, co-treatment with an 11β-HSD1-specific inhibitor (LJ2) reduced oxoreductase activity while dehydrogenase activity increased (45.3 vs. 14.7 and 85.5 vs. 152.6 respectively, n=3). Significance * = $p<0.05$

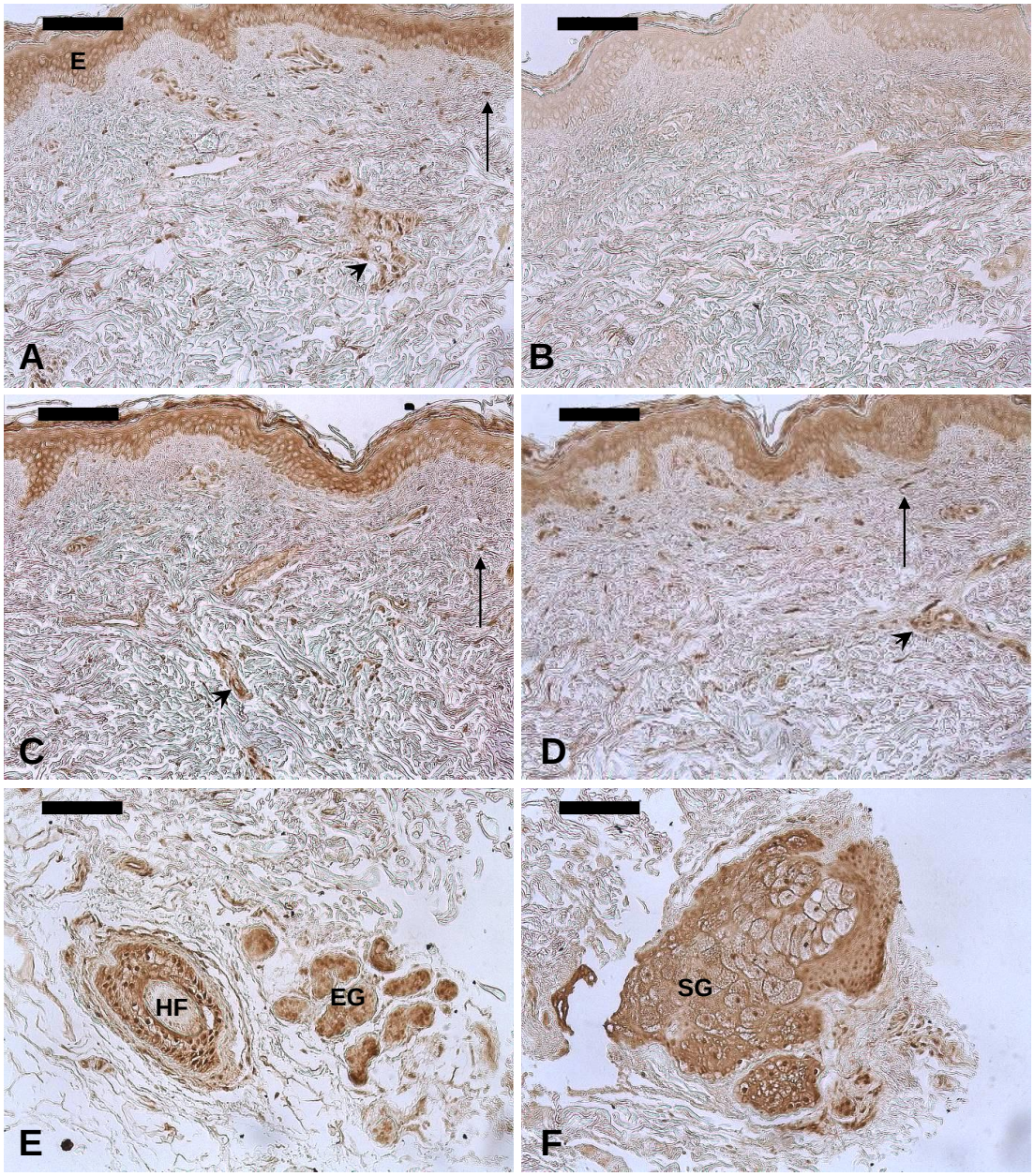


Figure 3-4 11β-HSD1 immunohistochemistry in human skin. Protein expression was detected using an 11β-HSD1 polyclonal antibody in paraffin-embedded sections of human skin (n=3). (**A, C, D**) Positive staining was detected in epidermal keratinocytes (E), dermal fibroblasts (arrows) and microvasculature (arrowheads). (**E**) Staining was also detected in hair follicles (HF), eccrine sweat glands (EG,) and (**F**) in sebaceous glands (SG). (**B**) Negligible staining was observed in equimolar isotype control-treated sections. Scale bar 100μm

Expression of 11β-HSD2 was also detected by immunohistochemistry (sheep polyclonal antibody) in human epidermal keratinocytes and vasculature above isotype control background levels (Figure 3-5).

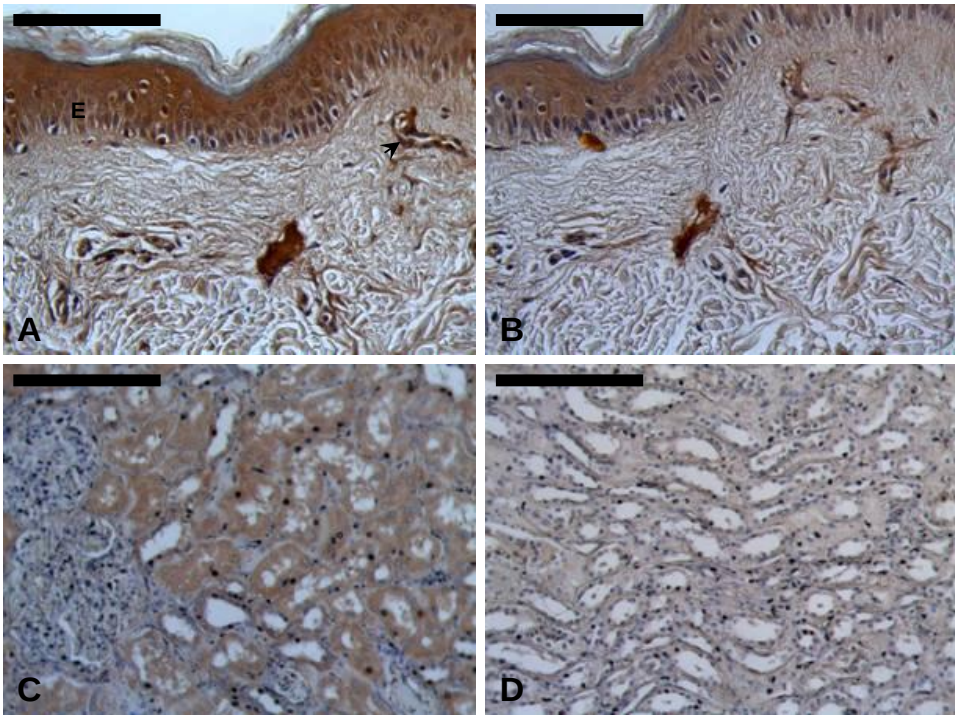


Figure 3-5 11β-HSD2 immunohistochemistry in human skin. Protein expression was detected using an 11β-HSD2 polyclonal antibody in paraffin-embedded sections of human skin (n=3) counterstained with Mayer's haematoxylin. (**A**) Positive staining was detected in epidermal keratinocytes (E) and microvasculature (arrowheads). (**C**) Sections of human kidney were used as a positive control. (**B, D**) Negligible staining was observed in equimolar isotype control-treated sections. Scale bar 100μm

3.4.2 Expression, activity and localization of 11β-HSDs in mouse skin

Murine skin displayed strong mRNA expression (ΔCt ± S.D) of GR-α, which was greater than for H6PD (12.7±1.7 vs. 16.2±1.3, $p<0.001$, n=7, Figure 3-6). 11β-HSD1 mRNA expression was lower compared to H6PD (17.9±1.0 vs. 16.2±1.3, $p<0.01$, n=7, Figure 3-6) but in contrast to human skin, expression of 11β-HSD2 was virtually absent from mouse skin, detectable at low levels in only 2 out of 7 samples (23.9±1.2, Figure 3-6). As expected, the near-absence of 11β-HSD2 mRNA expression in mouse skin translated to almost exclusive 11β-HSD oxoreductase (n=11) compared to dehydrogenase (n=3) activity (fmol/mg/h ± S.D, 340±154.8 vs. 36.7±11.5, $p<0.01$, Figure 3-7). Moreover, treatment with the 11β-HSD1-specific inhibitor LJ2 confirmed a reduction in oxoreductase activity (340±154.8 vs. 60±55.8, $p<0.001$, n=11), whilst only a marginal increase in dehydrogenase activity was recorded (36.7±11.5 vs. 73.3±15.3, $p<0.05$, n=3).

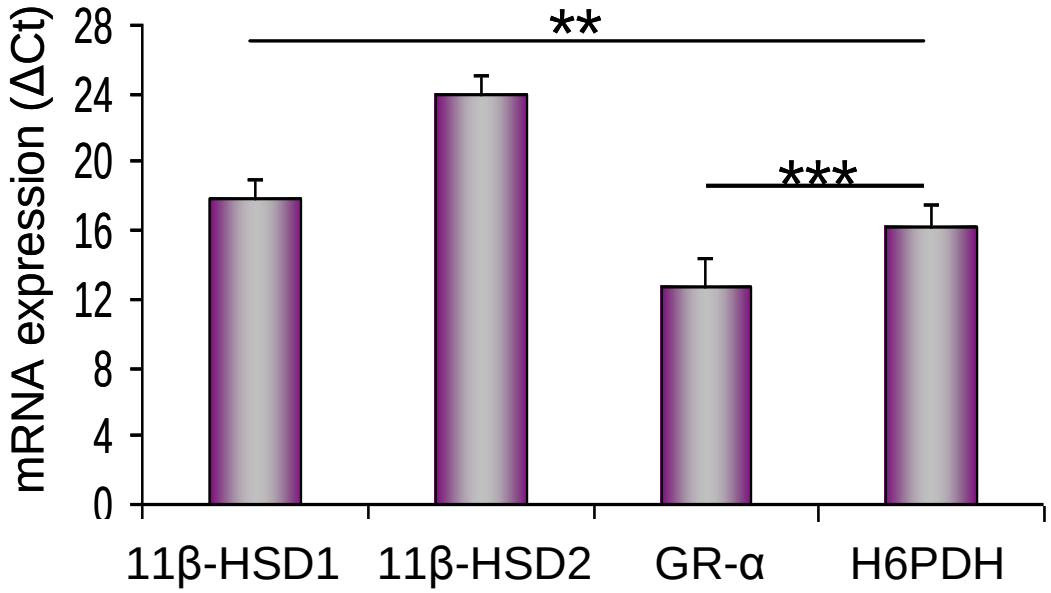


Figure 3-6 Expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD in mouse dorsal skin tissue samples. Following normalizing for levels of the housekeeping gene 18S rRNA, GR-α expression (ΔCt ± S.D) was greater than H6PD (12.7±1.7 vs. 16.2±1.3, n=7) which in turn was greater than 11β-HSD1 (16.2±1.3 vs. 17.9±1.0, n=7). Furthermore, expression of 11β-HSD2 was only weakly detectable in 2/7 samples (23.9±1.2). Significance ** = p<0.01, *** = p<0.001

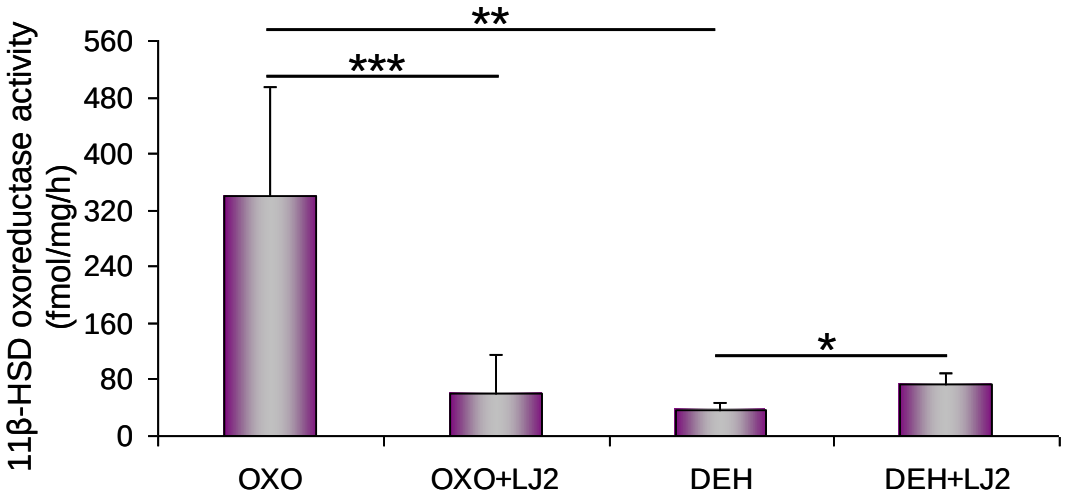


Figure 3-7 11β-HSD activity in mouse dorsal skin tissue. In accordance with lower 11β-HSD2 mRNA expression, mean 11β-HSD oxoreductase (OXO) activity far exceeded dehydrogenase (DEH) activity (fmol/mg/h ± S.D, 340±154.8, n=11 vs. 36.7±11.5, n=3). Furthermore, treatment with an 11β-HSD1-specific inhibitor (LJ2) substantially reduced oxoreductase activity (340±154.8 vs. 60±55.8, n=11), while dehydrogenase activity displayed only a negligible increase (36.7±11.5 vs. 73.3±15.3, n=3). Significance * = p<0.05, ** = p<0.01, *** = p<0.001

Immunohistochemical analyses in mouse dorsal skin sections using a rabbit polyclonal antibody against 11β-HSD1 localized protein expression to epidermal keratinocytes, dermal fibroblasts, hair follicle cells, sebaceous glands,

microvasculature, adipose tissue and *panniculus carnosus* skeletal muscle, while only negligible staining was detected following replacement of the primary antibody with an equimolar isotype control (Figure 3-8).

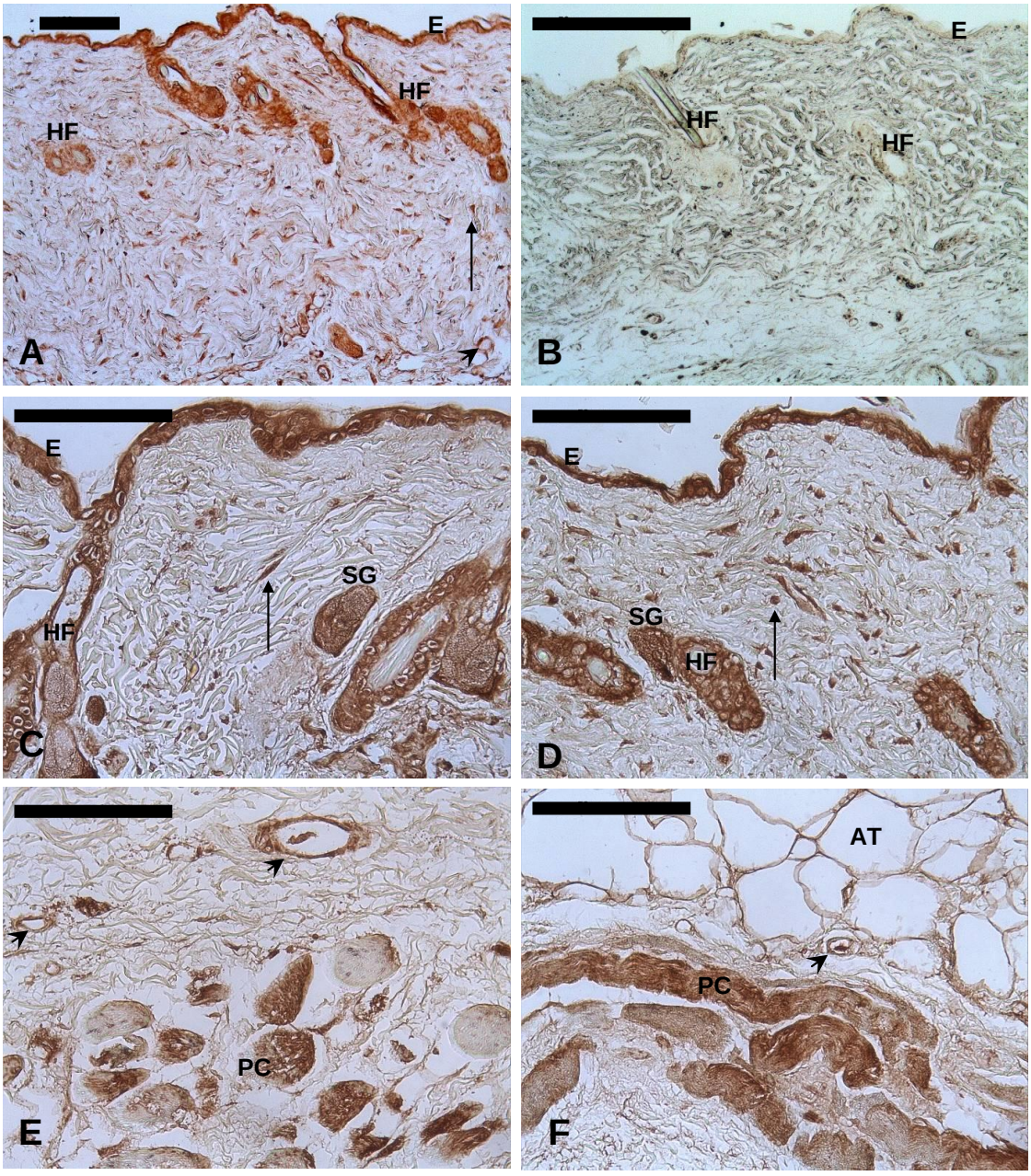


Figure 3-8 11β-HSD1 immunohistochemistry in murine skin. Protein expression was detected using an 11β-HSD1 polyclonal antibody in paraffin-embedded sections of mouse skin (n=8). (**A, C, D, E, F**) Positive staining was detected in epidermal keratinocytes (E), dermal fibroblasts (arrows), hair follicle cells (HF), sebaceous glands (SG), microvasculature (arrowheads), adipose tissue (AT) and *panniculus carnosus* skeletal muscle (PC). (**B**) Negligible staining was observed in equimolar isotype control-treated sections. Scale bar 100μm

11β-HSD2 expression was undetectable by immunohistochemistry in mouse skin (Figure 3-9).

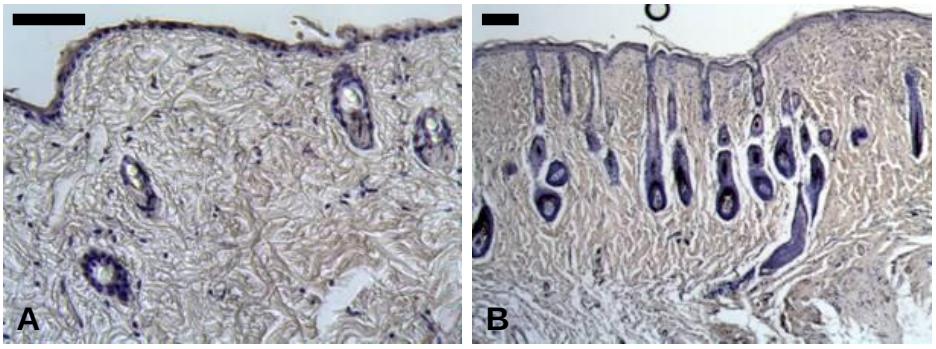


Figure 3-9 11β-HSD2 immunohistochemistry in murine skin. (**A**) Protein expression was undetectable using an 11β-HSD2 polyclonal antibody in paraffin-embedded mouse skin sections (n=3) counterstained with Mayer's haematoxylin. (**B**) Negligible staining was observed in equimolar isotype control-treated sections. Scale bar 100μm

3.4.3 Expression and activity of 11β-HSDs in primary human dermal fibroblasts

Following identification of protein expression by immunohistochemistry in dermal components of mouse and human skin, expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD mRNA was analysed in HDF. Compared to expression in full-thickness human arm skin biopsy mRNA extracts, GR-α levels in HDF (n=18) were higher (ΔCt ± S.D, 9.3±1.2 vs. 15.0±1.6, $p<0.001$) indicative of a potentially highly sensitive GC-responsive cell (Figure 3-10).

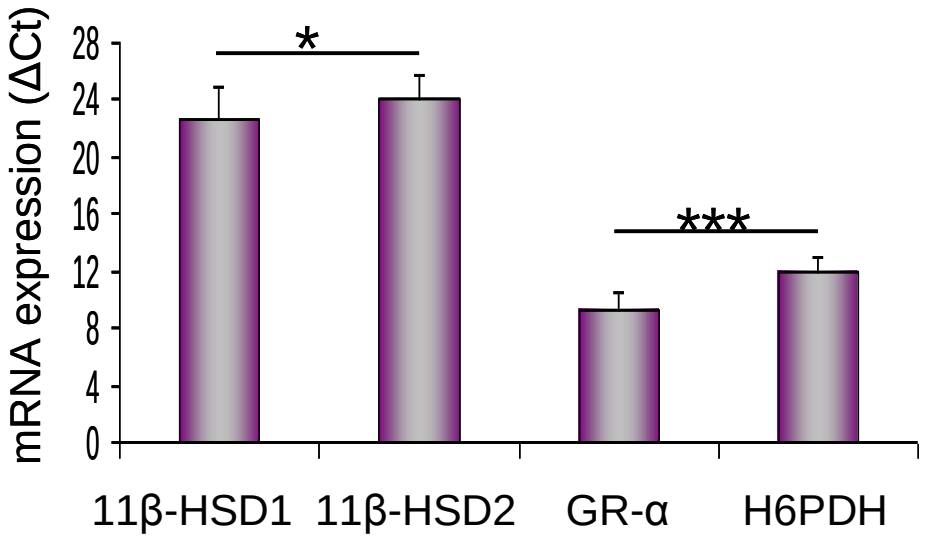


Figure 3-10 Expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD in primary cultures of human dermal fibroblasts. Following normalizing for levels of the housekeeping gene 18S rRNA, GR-α mRNA expression was significantly more abundant than H6PD (ΔCt ± S.D, 9.3±1.2, n=18 vs. 12.0±0.9, n=19) with 11β-HSD1 expression higher than 11β-HSD2 (22.6±2.4, n=27 vs. 24.0±1.7, n=18). Significance * = $p<0.05$, *** = $p<0.001$

Although HDF mRNA levels of 11β-HSD1 (n=27) and 11β-HSD2 (n=18) were both lower than whole skin extracts (22.6±2.4 vs. 18.9±2, $p<0.001$ and 24.0±1.7 vs. 14.5±1.6, $p<0.001$ respectively), the expression of the 11β-HSD1 cofactor NADPH-supplying enzyme H6PD (n=19) was also elevated (12.0±0.9 vs. 14.1±1.4, $p<0.001$, Figure 3-10). This, combined with the high expression of GR-α and greater abundance of 11β-HSD1 compared to 11β-HSD2 mRNA expression (22.6±2.4 vs. 24.0±1.7, $p<0.05$, Figure 3-10) suggests that HDF may be capable of rapidly elevating local GC concentrations through an autocrine positive-feedback mechanism.

Consistent with the relatively low mRNA expression of 11β-HSD1 and 11β-HSD2, 11β-HSD oxoreductase activity was undetectable over 50% of HDF cultures at baseline (12/20), while dehydrogenase activity was undetectable in all HDF cultures tested (data not shown). Activity detectable at baseline in the remaining samples was highly variable (fmol/mg/h ± S.E, 710±263, n=8) and undetectable following co-incubation with the 11β-HSD1-specific inhibitor LJ2 (data not shown). Furthermore, activity was shown to increase in a substrate dose-dependant manner (Figure 3-11).

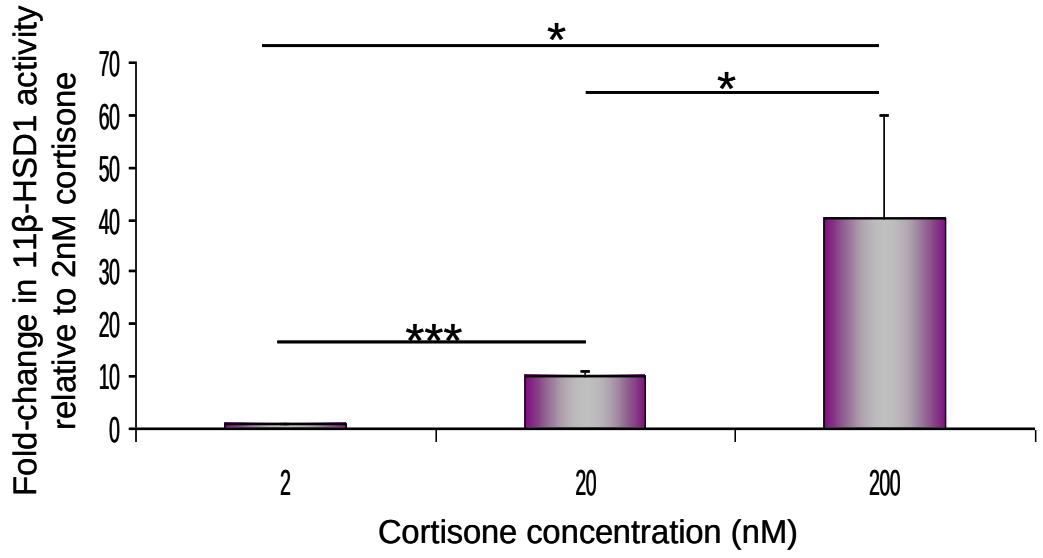


Figure 3-11 11β-HSD1 activity dose response in HDF. 11β-HSD1 activity was shown to increase in a substrate dose-dependant manner relative to the lowest substrate dose (2nM) with an ~10-fold and ~40-fold increase in activity following a 10- and 100-fold increase in substrate concentration respectively (n=3). Significance * = $p<0.05$, *** = $p<0.001$

3.4.4 Regulation of 11β-HSDs in HDF

The ability of HDF to regulate local GC concentrations through modulation of 11β-HSD expression was investigated following treatment with GC and the pro-inflammatory cytokine TNF-α. Expression of 11β-HSD1 mRNA was upregulated by treatment with the synthetic GC dexamethasone (ΔCt ± S.D, 23.2±2.9 vs. 22.0±3.5, $p<0.05$, n=11) and to a comparable degree by the endogenous GC cortisol (22.4±2.5 vs. 20.3±2.5, $p<0.001$, n=22). As the latter is more physiologically relevant, it was used in place of dexamethasone to further demonstrate downregulation of 11β-HSD2 (23.6±1.4 vs. 25.6±1.5, $p<0.001$, n=16) and GR-α (9.3±1.2 vs. 10.8±1.7, $p<0.001$, n=18) while H6PD mRNA expression was not significantly altered (12.0±0.9 vs. 12.4±0.7, p=19).

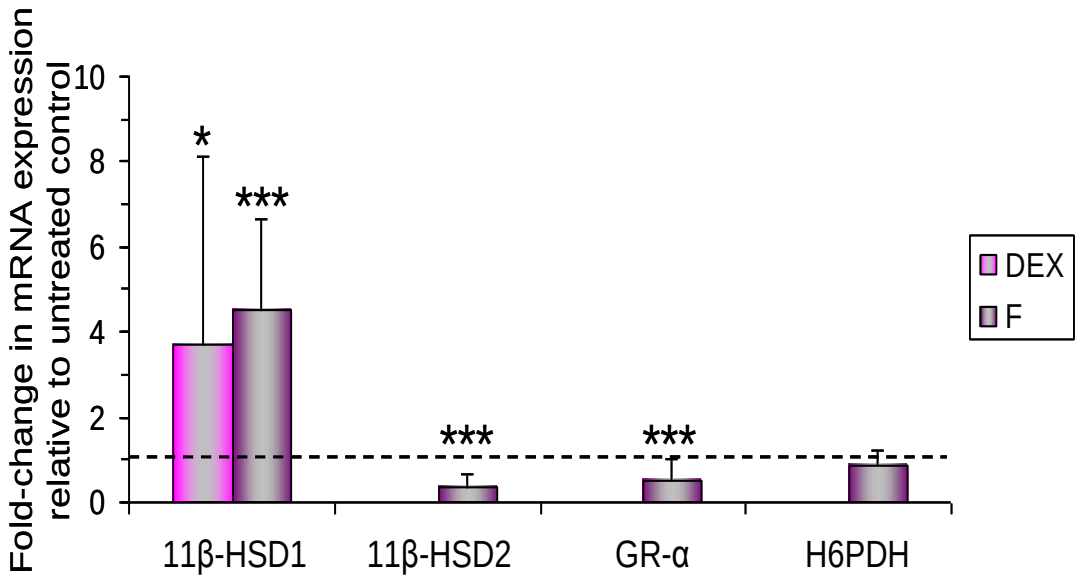


Figure 3-12 Regulation of 11β-HSD1, 11β-HSD2, GR-α and H6PD mRNA expression by GC in HDF. Treatment with 200nM dexamethasone (DEX) or cortisol (F) upregulated 11β-HSD1 expression (ΔCt ± S.D, 23.2±2.9 vs. 22.0±3.5, n=11 and 22.4±2.5 vs. 20.3±2.5, n=22 respectively), while 11β-HSD2 and GR-α were downregulated by cortisol treatment (23.6±1.4 vs. 25.6±1.5, n=16 and 9.3±1.2 vs. 10.8±1.7, n=18 respectively). Expression of H6PD was unaffected by cortisol treatment (12.0±0.9 vs. 12.4±0.7, n=19). Significance * = $p<0.05$, *** = $p<0.001$

Dexamethasone treatment also induced 11β-HSD1 activity (to a variable degree) in several HDF that had undetectable levels at baseline (fmol/mg/h ± S.E, 724±324,

n=5). Additionally, activity was induced to a comparable extent as seen with 11β-HSD1 mRNA expression in HDF where baseline activity was measurable (3.4±2.0 fold increase in activity relative to untreated control, n=5, data not shown). Similarly, treatment with cortisol stimulated a similar induction in 11β-HSD1 activity in several (but not all) HDF cultures with undetectable baseline activity, again with considerable variation between samples (972±370 fmol/mg/h ± S.E, n=8).

Furthermore, treatment with the pro-inflammatory cytokine TNF-α caused a near-significant increase in 11β-HSD oxoreductase activity compared to GC treatment alone (pmol/mg/h ± S.E 2.6±1.0 vs. 1.4±0.7, p=0.07, n=4), and a synergistic increase in combination with GC treatment (Figure 3-13).

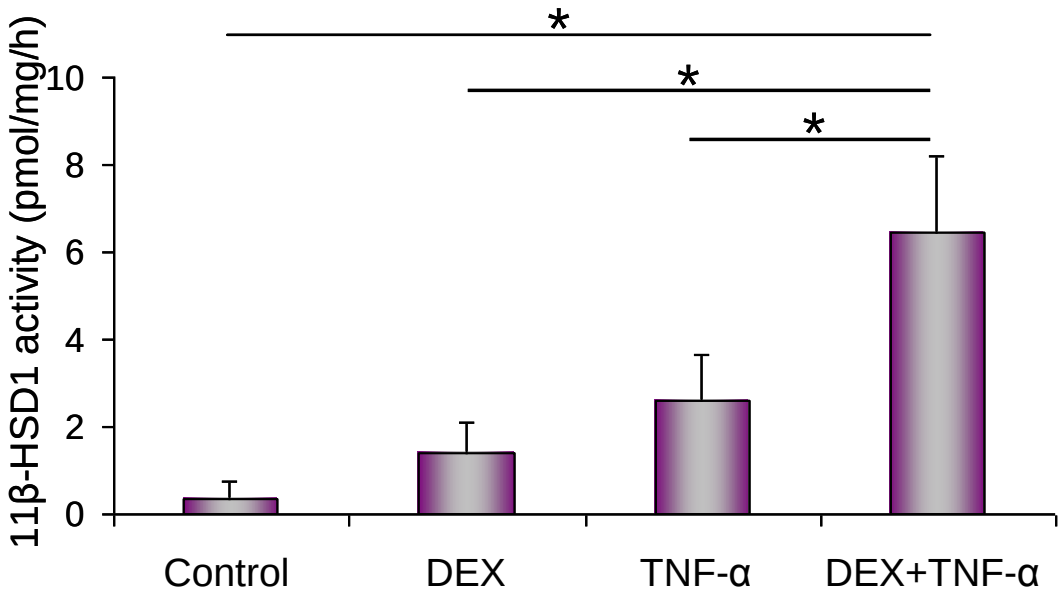


Figure 3-13 11β-HSD1 activity following dexamethasone, TNF-α or combined treatment in HDF. Treatment with the pro-inflammatory cytokine TNF-α upregulated 11β-HSD1 activity to a greater degree than dexamethasone (DEX) treatment alone, although this did not quite reach statistical significance (fmol/mg/h ± S.E 2625±1042 vs. 1405±704, n=4, p=0.07). Combined treatment resulted in a synergistic increase (6468±1723) compared to either DEX or TNF-α treatment alone. Significance * = $p<0.05$

In summary, whilst baseline 11β-HSD dehydrogenase levels were undetectable, oxoreductase activity in HDF was highly variable but inducible by GC/TNF-α treatment/co-treatment (including in samples with undetectable baseline levels).

3.5 Discussion

Our initial immunohistochemical characterization studies describe the localization of GC-metabolizing 11β-HSD protein expression to several discrete components of human and murine skin. 11β-HSD1 protein expression was detected in epidermal keratinocytes, particularly the *stratum spinosum* (located just above the basal *stratum basale* layer) supporting immunohistochemical and enzymatic activity data reported by others (Hennebert *et al*., 2007, Kenouch *et al*., 1994). Subsequently, previously unreported expression was also confirmed in mouse epidermal cells which in contrast to the highly organized human equivalent typically comprise a single keratinocyte layer.

Convincing expression was also detected in the hair follicle ORS cells from both species, in congruence with this layer being continuous with the *stratum spinosum* of the epidermis. Whilst one previous study has also reported 11β-HSD1 protein expression in human skin hair follicles (Hennebert *et al*., 2007), albeit more ambiguously than our data, evidence for expression in the murine counterpart is completely exempt from published literature. Furthermore, expression of 11β-HSD1 protein was observed in the hair follicle auxiliary sebaceous gland structures in both human and murine skin - a finding previously unreported for either species.

In the dermis, 11β-HSD1 protein expression was associated with dermal fibroblasts in both species. Although prior studies have reported mRNA expression in primary cultures of HDF (Hammami and Siiteri, 1991, Hardy *et al*., 2006), our results provide additional supporting evidence for *in situ* protein expression in these cells, whilst being the first group to confirm a similar finding in mouse skin. Additionally, components of human and murine dermal microvasculature such as inner endothelial and outer VSMCs were found to express 11β-HSD1 protein, again previously

unreported for the integumentary circulatory system but extensively characterized in other vasculature (aorta, uterine, chorionic etc.) with known regulatory functions for vascular tone and blood pressure (Brem *et al*., 1995, Alzamora *et al*., 2000, Hammer and Stewart, 2006). Finally, 11β-HSD1 protein expression was also detected in human eccrine sweat glands (absent from mouse skin), consistent with previously reported 11β-HSD oxoreductase activity in these structures (Kenouch *et al*., 1994).

Sub-dermal expression of 11β-HSD1 protein in mouse skin included adipose tissue, known to exhibit 11β-HSD oxoreductase activity (Livingstone *et* al., 2009) and the *panniculus carnosus* skeletal muscle (absent from human skin). Whilst expression in the latter has not been reported previously or in skeletal muscle derived from other anatomical locations in the mouse, expression and activity in human skeletal muscle has been confirmed (Morgan *et al*., 2009). In human samples, subcutaneous adipose expression was undetectable due to the limited depth of the biopsies.

Expression of 11β-HSD2 protein was also detectable in human epidermal keratinocytes, consistent with previous studies demonstrating 11β-HSD dehydrogenase activity and 11β-HSD2 protein expression in this layer (Kenouch *et al*., 1994, Smith *et al*., 1996). Our results also identified 11β-HSD2 protein expression in dermal microvasculature as previously reported (Smith *et al*., 1996).

Although our findings demonstrated protein expression from both 11β-HSD isoforms in human skin, 11β-HSD dehydrogenase activity was ~2-fold greater than 11β-HSD oxoreductase activity, supported by our data indicating 11β-HSD2 mRNA expression levels exceeding those for 11β-HSD1. Kenouch *et al*. also reported greater 11β-HSD dehydrogenase compared to oxoreductase activity in isolated eccrine sweat gland ducts and epidermis (Kenouch *et al*., 1994) while Bocchi *et al*. demonstrated 11β-HSD dehydrogenase levels in sweat gland ducts isolated from 4mm armpit punch

biopsies (Bocchi *et al*., 2004) comparable to those we identified in whole skin biopsies, suggesting that eccrine glands may contribute the majority of 11β-HSD dehydrogenase activity in human skin. Consistent with this, we found almost exclusive 11β-HSD oxoreductase activity and predominantly 11β-HSD1 compared to 11β-HSD2 mRNA expression in mouse skin, known to be largely devoid of eccrine sweat glands except in the foot pads and toes (Johns *et al*., 1995).

Although our results suggest 11β-HSD oxoreductase activity levels in skin are several orders of magnitude lower than those observed in established GC-activating tissues such as omental adipose (Bujalska *et al*., 1997) there are several confounding factors that make a direct comparison challenging. For example, the 11β-HSD dehydrogenase activity in skin contributes to a net 11β-HSD oxoreductase activity that may be a large underestimate of the GC-activating capacity at a cellular level, reflecting the compartmentalized nature of skin compared to the relatively homogenous organization of omental adipose tissue that, additionally, is also devoid of 11β-HSD dehydrogenase activity.

Furthermore, as activity is often expressed as amount of substrate converted (e.g. fmoles) per unit tissue weight (e.g. mg) per unit time (e.g. h), the proportion of tissue mass attributable to cells (and cell type) is also important. In the example stated, omental tissue mass is composed mostly of cells (adipocytes, fibroblasts, macrophages and endothelial cells), while skin consists mostly of structural proteins (collagen, elastin, GAGs etc.) and a substantial amount of water with the cellular component contributing a much smaller fraction to the total tissue mass. Indeed, 11β-HSD1 mRNA expression in omental adipose tissue (Tomlinson *et al*., 2002) is more comparable to our observations in skin. However, at 13-fold greater levels it remains

likely that 11β-HSD oxoreductase activity in adipose cells is still greater than skin cells (although cell type variation is still unaccounted for).

Finally, the large difference in 11β-HSD oxoreductase activity between skin and adipose may be due to its tissue-specific functional role. In simple obesity, accumulation of visceral fat (and increased local GC activation capacity) leads to a protective systemic decrease in 11β-HSD1 activity as indicated by a reduction in the urinary tetrahydrocortisol + 5 alpha-tetrahydrocortisol: tetrahydrocortisone ratio not seen in obese diabetics (Valsamakis *et al*., 2004). In skin, 11β-HSDs are more likely to function at an autocrine or paracrine level, where cells highly sensitive to local GC concentration may only require small changes in GC availability to alter functional properties, such as during an inflammatory response or wound healing.

Our results indicate that GR-α mRNA is expressed in whole skin extracts at a higher but comparable level to that reported for omental adipose tissue (Bujalska *et al*., 2007), whilst we report that expression in HDF cultures is over 300-fold greater. It remains to be elucidated whether this large increase is due to the altered phenotype of HDF in culture or is an indication of GR-α mRNA expression in dermal fibroblasts *in vivo*, with the lower level of expression in whole skin attributable to reduced or absent expression in other resident skin cells. Interestingly, a similar situation exists for H6PD mRNA expression, with levels described by us in whole skin similar to those found in omental adipose tissue (Bujalska *et al*., 2007) but elevated in HDF cultures. These observations suggest that dermal fibroblasts may have the capacity to regulate GC signalling in response to certain, currently unknown external stimuli.

In contrast to GR-α and H6PD, mRNA expression of 11β-HSD1 and 11β-HSD2 was found to be lower in HDF cultures compared to skin tissue, supported by the absence of 11β-HSD dehydrogenase and lower 11β-HSD oxoreductase activity levels in HDF

compared to tissue. This suggests that under culture conditions, dermal fibroblasts exhibit a phenotype tailored towards GC response rather than generation. Following treatment of HDF with GC we found 11β-HSD1 mRNA expression and activity to be upregulated, initiating a positive-feedback auto-regulatory loop that enables HDF to increase local GC concentrations. Concomitantly, the expression of 11β-HSD2 mRNA in HDF is downregulated, preventing the inactivation of newly generated cortisol to its inactive form (cortisone). Whilst GR-α mRNA is also downregulated, expression levels are still much higher than baseline levels in human skin biopsies.

The positive-feedback upregulation of 11β-HSD oxoreductase activity by GC has also been reported for primary cultures of foreskin fibroblasts. Hammami and Siiteri reported a ~3-fold increase in activity following 100nM dexamethasone treatment, a similar induction to that observed in our studies, although untreated cells exhibited much greater baseline activity compared to our observations in arm skin-derived HDF (Hammami and Siiteri, 1991). This discrepancy is likely due to the anatomical origin of the cells used, with genital skin being a site of "immune privilege" - able to tolerate the introduction of antigen without eliciting an inflammatory immune response. High production of anti-inflammatory GC (through elevated 11β-HSD1 activity) is one likely mechanism though which inflammation in specific regions (also including the brain and ocular environment) is minimized, protecting these vital structures from the potentially damaging effects of an inflammatory immune response. Additionally, 11β-HSD1 mRNA expression in HDF derived from knee skin reported by Hardy *et al*. was similar to our findings, with 11β-HSD oxoreductase activity also being undetectable in all donors tested, further supporting this notion (Hardy *et al*., 2006).

Interestingly, we observed mRNA expression of 11β-HSD1 in HDF to be highly variable. Similarly, 11β-HSD oxoreductase conversion (cortisone to cortisol) also

fluctuated between donors from undetectable levels in the majority of samples through various degrees of activity in others. Experimental conditions were unlikely to account for this variation as all cells used were at an early passage (passage 2-5) and maintained under the same culture conditions. Furthermore, this large variation in 11β-HSD1 mRNA expression and activity was maintained following GC-induced upregulation, suggesting some primary cultures are capable of attaining a greater level of GC-activation than others.

In addition to regulation of 11β-HSD1 expression and activity by GC, we investigated regulation by the pro-inflammatory cytokine TNF-α. This was found to act in synergy with GC, further amplifying 11β-HSD oxoreductase activity and hence the GC-activating potential of HDF, with functional implications that may include limiting inflammation and initiating resolution of an inflammatory response (e.g. during sun exposure or wound healing).

In summary, our initial studies characterizing 11β-HSDs in the skin are in support of the view that the integumentary system should be considered an endocrine organ in its own right. We provide evidence to reinforce limited existing immunohistochemical data (Hennebert *et al*., 2007) demonstrating expression of 11β-HSD1 protein in key compartments of human skin (epidermal *stratum spinosum*, hair follicle ORS cells and dermal fibroblasts). Additionally, we describe previously unreported localization to dermal vasculature, eccrine sweat glands and sebaceous glands. We also provide previously unreported quantitative real-time PCR mRNA expression and supporting activity data for both 11β-HSD isozymes in human skin, which are in agreement with limited studies reporting activity in isolated compartments of skin e.g. eccrine sweat gland ducts and epidermis (Kenouch *et al*., 1994, Bocchi *et al*., 2004) and mRNA expression by Northern analysis (Brown *et al*., 1996). We report expression and

activity results for 11β-HSDs in HDF cultures supporting previously published data (Hardy *et al*., 2006) and demonstrating a previously unreported level of variation in 11β-HSD1 mRNA expression and activity between different donors. Furthermore, we present supporting evidence for the ability of HDF to enhance local GC concentrations through positive-feedback upregulation of 11β-HSD1 mRNA expression and activity following GC and TNF-α stimulation, similarly to previous reports (Hammami and Siiteri, 1991, Hardy *et al*., 2006).

Finally, we demonstrate similar findings and provide initial reports of 11β-HSD1 mRNA expression, activity and protein localization in murine skin. Although we highlight major differences compared to human skin (e.g. lack of eccrine sweat glands and concomitant negligible 11β-HSD2 expression and activity), the presence of 11β-HSD1 in similar compartments of human skin (epidermis, dermal fibroblasts, hair follicles, sebaceous glands and microvasculature) opens an avenue for investigating the functional consequences of 11β-HSD1 genetic manipulation and identification of novel roles for GC in skin homeostasis and pathological processes.

CHAPTER 4 AGE / SITE-DEPENDANT EXPRESSION OF 11β-HSDs IN SKIN

4.1 Introduction

4.1.1 11β-HSD dysregulation and disease

Maintenance of homeostasis is crucial for many biological systems especially those under endocrine control. As described in Chapter 1 (Section 4), GC are master regulators of a wide variety of systems and their availability is tightly controlled through several mechanisms including at a pre-receptor level, by 11β-HSD metabolism.

4.1.1.1 Endocrine disorders

The role of pre-receptor metabolism of GC is best exemplified by the monogenic endocrine disorders of apparent cortisone reductase deficiency (ACRD) and AME characterized by functionally inactivating mutations in the 11β-HSD1 co-factor supplying enzyme H6PD (Lavery *et* al., 2008) or 11β-HSD2 respectively (Cooper and Stewart 1998). Recently, dominant negative heterozygous mutations in 11β-HSD1 have also been described, explaining the genetic cause of true cortisone reductase deficiency (CRD) in these patients (Lawson *et al*., 2011).

Extensive animal studies have offered further insights into the consequences of targeted deletion or overexpression of these enzymes. Phenotypically, mice lacking 11β-HSD1 are largely normal, with a compensatory activation of the HPA axis manifesting with adrenal hyperplasia and elevated basal corticosterone levels in some (but not other) strains (Kotelevtsev *et al*., 1997, Harris *et* al., 2001, Carter *et al*.,

2009). Upon closer evaluation it was discovered that this model is resistant to hyperglycaemia upon high-fat feeding or stress challenge with improved glucose tolerance, a more favourable lipoprotein profile and resistance to diet-induced obesity (Kotelevtsev *et al*., 1997, Morton *et al*., 2001, 2004). Interestingly, 11β-HSD1-null mice also display improved age-related cognitive function compared to WT counterparts (Yau *et al*., 2001). Conversely, adipose-specific overexpression of 11β-HSD1 manifests as a full metabolic syndrome in mice with central obesity, glucose intolerance, T2D, dyslipidaemia, hyperphagia and hypertension (Masuzaki *et al*., 2001, 2003). Similarly, 11β-HSD1 overexpression in mouse liver results in modest insulin resistance, fatty liver, dyslipidaemia and hypertension (Paterson *et al*., 2004).

In contrast to the subtle cardio-protective phenotype of 11β-HSD1 knockout animals, H6PD-null mice exhibit a progressive myopathy. Additionally, mice display consistently elevated circulating corticosterone and ACTH levels and raised urinary dehydrocorticosterone metabolites resulting from a switch in 11β-HSD1 activity (oxoreductase to dehydrogenase) presumably due to the absence of NADPH co-factor regeneration within the ER lumen (Lavery *et al*., 2006, 2007, 2008, Bujalska *et al*., 2008).

As expected, the 11β-HSD2-null mouse displays similar characteristics to its human equivalent AME, with hypokalaemia, hypochloraemia, hypotonic polyuria, and marked hypertension, primarily resulting from unregulated activation of MR by corticosterone (Kotelevtsev *et al*., 1999). Alternatively, cardiomyocyte-specific overexpression of 11β-HSD2 in mice results in cardiac hypertrophy, fibrosis and heart failure - a phenotype ameliorated by MR-selective (but not GR) blockade, suggesting that although 11β-HSD2 protects the MR from inappropriate GC activation by inactivating corticosterone/cortisol, a certain degree of GC signaling via the MR is

necessary for normal cardiomyocyte function, although mechanistic details behind this hypothesis require elucidation (Qin *et al*., 2003). Whilst excessive GC signaling is known to contribute to osteoporosis, studies utilizing selective 11β-HSD2 overexpression in rodent osteoblasts revealed detrimental effects on bone volume and architecture in a gender-specific manner, suggesting a threshold GC requirement also exists for optimal bone formation and remodeling, corroborating the cardiomyocyte data (Sher *et al*., 2004).

In addition to the pathological consequences of altered pre-receptor GC metabolism in the monogenic disorders of CRD, ACRD and AME, and insights obtained from genetic manipulation in rodents (that accurately recapitulate many aspects of these human conditions), emerging evidence also implicates GC-metabolizing enzyme dysregulation in the etiology and progression of the metabolic syndrome. For example, 11β-HSD1 is differentially expressed according to adipose depot, with greater activity in omental compared to subcutaneous fat (Bujalska *et al*., 1997), suggesting a plausible mechanism where central adiposity persists in the absence of increased circulatory GC levels and redefines simple obesity as "Cushing's disease of the omentum". 11β-HSD1 expression has also been found to positively correlate with BMI, percentage body fat, and omental adipose tissue area in human females (Michailidou *et* al., 2007) and with BMI in subcutaneous depots (Kannisto *et al*., 2004). Furthermore, overexpression of 11β-HSD2 in visceral adipose tissue confers a degree of resistance to adipose deposition in mice on a high fat diet (Kershaw *et al*., 2005).

11β-HSD1 dysregulation has also been implicated in diabetes and insulin resistance, where a potentially protective, compensatory decrease in cortisol: cortisone urinary metabolite ratio (indicative of decreasing 11β-HSD1 activity) with increasing obesity

in controls was found to be lacking in patients with T2D (Valsamakis *et al*., 2004). Selective 11β-HSD1 inhibitors have demonstrated encouraging results in mouse models of metabolic syndrome, increasing insulin sensitivity whilst decreasing body weight, triglycerides and cholesterol and remarkably in slowing atherosclerosis progression (Alberts *et al*., 2003, Hermanowski-Vosatka *et al*., 2005).

4.1.1.2 Immune dysfunction

The role of GC as regulators of inflammation and immune function is also now known to involve direct modulation by 11β-HSDs (Chapman *et al*., 2009), with postulated roles during the local acute inflammatory response that influence the course and outcome of inflammation resolution (Gilmour *et al*., 2006). Data suggest that during a normal immune response 11β-HSD1 expression and activity is "switched on" following response of immune cells (i.e. differentiation) to pro-inflammatory stimuli such as cytokines (IL-4, IL-13) and LPS (Thieringer *et al*., 2001). Furthermore, 11β-HSD1 expression is associated with monocyte commitment to a T_h1 lineage (Martinez *et al*., 2006). Following monocyte induction (with GM-CSF and IL-4), 11β-HSD1 activity is maintained in differentiated dendritic cells during innate immune system signalling but declines upon adaptive immune response signal activation, demonstrating an intricate and highly sensitive orchestration of local GC levels based on both the differentiation status of immune cells and on microenvironment conditions (Freeman *et al*., 2005).

Conversely, 11β-HSD2 expression is negligible compared to 11β-HSD1 in normal immune cells from both mice (Gilmour *et al*., 2006) and humans (Thieringer *et al*., 2001, Freeman *et al*., 2005) but has been shown to be transiently expressed in peripheral blood mononuclear cells from patients with early rheumatoid arthritis

(Olsen *et al*., 2004) and upregulated in transformed B-cell lines derived from patients with rheumatoid arthritis compared to those derived from their unaffected twins (Haas *et al*., 2006). Additionally, differences in GC activation have been demonstrated in synovial cells from patients with rheumatoid or osteoarthritis (with greater reactivation in the latter, Schmidt *et al*., 2005), while GC activation was shown to positively correlate with inflammation in rheumatoid arthritis (Hardy *et al*., 2008). Moreover, the IL-10-induced upregulation of 11β-HSD1 in normal human macrophages was abolished in those derived from rheumatoid arthritis subjects (Antoniv and Ivashkiv, 2006).

While chronically increased GC activation appears to be a feature of persistent inflammation, studies in the 11β-HSD1-null mouse indicate that impaired GC activation also leads to excessive inflammation and altered resolution following induction of sterile peritonitis (Gilmour *et al*., 2006).

4.1.1.3 Dysregulation during ageing

The inevitable decline of the immune system with ageing is well documented. However, recent studies in murine lymphocytes suggest that this is coupled with an increase in 11β-HSD1 expression and activity (Zhang *et al*., 2005), suggesting a mechanism through which increased GC activation may lead to decreased GC sensitivity and contribute to immune system dysfunction with advancing age.

Similarly, 11β-HSD1 activity was shown to increase as a function of age in human primary osteoblasts (Cooper *et al*., 2002) and was further elevated by GC treatment resulting in a positive-feedback increase in local GC concentrations that may contribute to the decline in bone strength with increasing age and the increased risk of

fracture in elderly patients on systemic GC therapy. 11β-HSD1 expression is also reported to increase with advancing age in mouse bone (Weinstein *et al*., 2010).

More recently, increases in 11β-HSD1 expression have been reported in subcutaneous fat from older (post-menopausal) compared to younger (pre-menopausal) women providing further evidence for increased local GC activation as a general characteristic of the ageing process (Andersson *et al*., 2009). Conversely, Li *et al* describe an increase in omental but not subcutaneous 11β-HSD1 expression with increasing age in children, with no age-associated differences at either site in adults (Li *et al*., 2007).

Holmes *et al* also describe increased 11β-HSD1 expression with age in mouse hippocampus and parietal cortex, correlating with age-related cognitive impairment measured by performance in a water maze. Furthermore, ageing transgenic mice with forebrain-specific 11β-HSD1 overexpression also exhibit a premature, age-associated cognitive decline (Holmes *et al*., 2010). These results may explain the enhanced hippocampal long-term potentiation and spatial learning in aged 11β-HSD1-null mice reported by Yau and colleagues (Yau *et al*., 2007). Subsequently, it was reported that both partial deficiency (11β-HSD1 heterozygote) and temporary inhibition of 11β-HSD1 activity induced cognitive function improvements in ageing mice (Sooy *et al*., 2010).

Studies investigating whether renal 11β-HSD2 activity also increases in an attempt to counteract the increasing 11β-HSD1 levels with age revealed that this is not the case (at least in rats, Audige *et al*., 2002). Moreover, in rat Leydig cells, 11β-HSD2 expression is reported to decrease during ageing, suggesting a loss of protective activity normally provided through blunting the suppressive effects of GC on Leydig cell steroidogenesis (Koeva *et al*., 2009). It would be interesting to investigate

whether this is also coupled with an increase in Leydig cell 11β-HSD1 expression with ageing, resulting in a "double-whammy" effect, further exacerbating local GC concentrations and potentially contributing to the decreasing levels of testosterone observed with advancing age.

4.2 Aims

Previous studies clearly demonstrate that dysregulation of local pre-receptor GC metabolism (through changes in expression of 11β-HSDs) can contribute to the onset and progression of many pathological conditions, ranging from metabolic disease to alterations in immune system function. Over the past decade, evidence has emerged linking local GC production to the ageing process.

Several groups have now reported increases in 11β-HSD1 expression and activity with advancing age in a range of tissues including human subcutaneous fat (Andersson *et al*., 2009), murine lymphocytes (Zhang *et al*., 2005), bone (Weinstein *et al*., 2010) and brain (Holmes *et al*., 2010), since our group initially reported this phenomenon in human osteoblasts (Cooper *et al*., 2002). The lack of compensatory concomitant increase in GC inactivation by 11β-HSD2 in rat kidney (Audige *et al*., 2002), and, conversely, evidence for an increase in 11β-HSD2 expression with age in rat Leydig cells (Koeva *et al*., 2009) suggest that the uninhibited increase in GC reactivation by 11β-HSD1 may be a generalised effect of the ageing process - offering a potential target to combat generic ageing-associated diseases.

Although the adverse effects of increased GC skin exposure are well documented during endogenous Cushing's syndrome or exogenous therapeutic treatment with GC (both systemic and topical), the potential for increased local endogenous production though increased 11β-HSD1 activity in skin ageing has not been investigated.

Therefore, the current study aimed to determine expression and activity of 11β-HSDs in HDF and human full-thickness skin tissue biopsies derived from donors of varying age and in skin tissue explants from young and old mice.

Extrinsic ageing is thought to superimpose on intrinsic (chronological) ageing and in skin is largely attributable to photo-ageing. The mechanisms underlying both forms of ageing are believed to be largely similar (and exacerbated with additional extrinsic insults), however to date no studies have been conducted to examine pre-receptor GC metabolism in the context of extrinsic ageing. **The current study aimed to address this issue by comparing 11β-HSD expression and activity in extrinsically- and intrinsically-aged (PE and PP respectively) HDF and human full-thickness skin tissue biopsies.**

Previously we reported that 11β-HSD1 activity in primary cultures of HDF is largely undetectable due to low levels of 11β-HSD1 mRNA expression, although this expression is readily inducible by GC treatment. **Here, we aimed to determine the GC-mediated positive-feedback in 11β-HSD1 mRNA expression and activity in HDF from donors of varying age.**

Finally, GC signalling is also largely dependant on GR and 11β-HSD1 co-factor (NADPH) availability. We therefore sought to investigate the expression of GR and H6PD in HDF, human skin biopsies and mouse skin explants alongside 11β-HSDs.

4.3 Materials and methods

4.3.1 Sample preparation and culture

Human skin tissue biopsies, mouse skin tissue explants and HDF were prepared according to sections 2.1 and 2.2. HDF were treated with cortisol according to section 3.3.1.2.

4.3.2 11β-HSD activity assays

Human arm skin biopsy 11β-HSD oxoreductase assays were conducted according to section 2.4. However, activity was not normalised for wet tissue weight as we determined this to be an inappropriate correction, particularly when comparing PE to PP sites where we observed increased tissue weight in the former in both young (mg wet tissue ± S.D, 8.3±1.6 vs. 5.7±1.6, $p<0.001$, n=20) and older (7.2±1.4 vs. 5.4±1.6, $p<0.001$, n=20) donors. There was also a significant decrease in PE tissue weight in older compared to young donors (8.3±1.6 vs. 7.2±1.4, $p<0.05$, n=20), but no difference with age in PP samples (5.7±1.6 vs. 5.4±1.6, p=20). Importantly, tissue weight did not positively correlate with 11β-HSD oxoreductase % conversion of cortisone to cortisol within the same site in either age group (Figure 4-1).

Moreover, the surface area of the punch biopsies was standardised to a 3mm diameter, with the changes in weight attributable to increased biopsy depth. As the majority cells in skin are located in the epidermis and upper dermis, with the lower dermis composed mostly of ECM proteins (e.g. collagen), correcting activity for tissue weight would give an inaccurate representation of activity at a cellular level. 11β-HSD oxoreductase activity for these samples was therefore presented as % conversion as a measure of ability of cells within the tissue to activate GC.

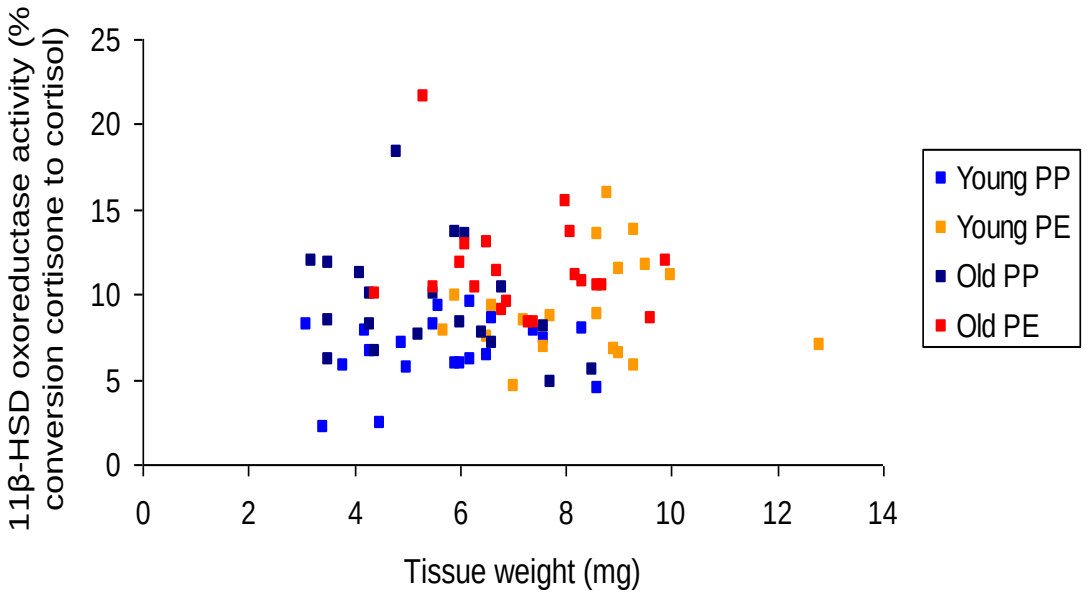


Figure 4-1 11β-HSD oxoreductase activity according to tissue weight in human skin biopsies. No correlation between tissue weight and corresponding 11β-HSD oxoreductase % conversion of cortisone to cortisol was observed for either PE or PP samples in both age groups (n=20 each group)

For human knee skin and mouse skin tissue explants, where the surface area of the tissue taken was not standardised by punch biopsy, and where comparisons between sites were not made, correction for tissue weight was more appropriate.

4.3.3 Real-time PCR gene expression

RNA extraction, reverse transcription and qPCR were conducted according to section 2.3, using the primers and probes described in section 3.3.2.

4.3.4 Quantitative immunohistochemistry

4.3.4.1 Subjects

Frozen human arm skin tissue sections were obtained from Unilever Discover, Bedford, UK following local ethical approval and informed consent.

4.3.4.2 11β-HSD1 monoclonal antibody validation

Quantitative immunohistochemistry studies were conducted using a mouse anti-human 11β-HSD1 monoclonal antibody (Abcam, Cambridge, UK) previously only tested by western blotting against the recombinant full length tagged protein used as immunogen (size 58kDa). As no data was available on the detection of endogenous protein, our validation studies investigated the ability to detect 11β-HSD1 derived from human tissues, compared to the polyclonal antibody used in section 3.3.4.3.

Western blotting: Human liver microsomes were isolated by ultracentrifugation as previously reported (Niculescu and Van Schaftingen, 1998). Microsomal pellets were resuspended in RIPA buffer (50mM Tris pH 7.4, 150mM sodium chloride, 1mM EDTA pH8 and 1% NP-40) stabilised with protease inhibitor cocktail according to manufacturer's guidelines (Roche, Lewes, UK) and ~5µg protein (made up to 15µl with distilled water) was mixed with 15µl 2X loading buffer (1.25ml 0.5M Tris pH6.8, 2ml 10% SDS, 1ml glycerol, 0.5ml β-mercaptoethanol, 0.25ml distilled water and enough bromophenol blue to sufficiently colour the buffer) and heated to 95ºC for 5min. Samples were loaded on graded 4-20% Tris-HCl SDS PAGE ready gels (Bio-Rad, California, USA) and run at 200V for 1h immersed in Tris-Glycine-SDS PAGE Buffer (Geneflow, Fradley, UK) in a Mini-Protean Tetra Cell system according to manufacturer's guidelines (Bio-Rad, California, USA).

Following separation, proteins were transferred to an Immobilon-P PVDF membrane (0.45 µm, 26.5 cm x 3.75 m roll, Millipore Ltd., Watford, UK) at 100V for 1h immersed in Tris-Glycine Electro-blotting Buffer (Geneflow, Fradley, UK) using a Mini Trans-Blot Module according to manufacturer's protocol (Bio-Rad, California, USA). Membranes were incubated in 5ml blocking buffer consisting of 20% milk powder in PBS 0.1% Tween-20 (PBST) under agitation at room temperature for 1h,

rinsed briefly in PBST, and incubated with mouse anti-human 11β-HSD1 monoclonal antibody diluted 1:500 (1-5μg/ml) in PBST or rabbit anti-human 11β-HSD1 polyclonal antibody (Cayman Chemical, Michigan, USA) diluted 1:200 (3μg/ml) in PBST overnight at 5ºC under constant agitation.

Membranes were washed in PBST (three 15min washes) and incubated with secondary antibody (HRP-conjugated goat anti-mouse or anti-rabbit, Dako, Glostrup, Denmark) diluted 1:2000 in PBST, at room temperature for 1h under constant agitation. Following a further three 15min washes, membranes were incubated with Amersham ECL Western Blotting Detection Reagent (1ml per membrane) according to manufacturer's guidelines (GE Healthcare, Little Chalfont, UK), exposed to Kodak MXB photographic film (G.R.I, Rayne, UK) and developed in a dark room.

A single 34kDa band was detected corresponding to monomeric 11β-HSD1 protein on membranes incubated with either polyclonal or monoclonal primary antibody, confirming the ability of the latter to detect endogenous protein.

Immunohistochemistry: *in situ* 11β-HSD1 protein detection in frozen human skin sections was conducted by immunohistochemistry according to section 3.3.4, using the monoclonal primary antibody and an alkaline phosphatase conjugated goat anti-mouse secondary antibody (Dako, Glostrup, Denmark). Fast Red TR/Naphthol AS-MX Tablets were used to visualise the staining according to manufacturer's guidelines. Slides were <u>not</u> dehydrated (as this would remove the alcohol-soluble stain), but air-died for 10min and mounted in aqueous mounting media before coverslipping. Staining was observed as for the polyclonal antibody (section 3.4.1) in epidermal keratinocytes, dermal fibroblasts, microvasculature, hair follicles, eccrine sweat glands and sebaceous glands with negligible staining observed using equimolar isotype control-treated sections (Figure 4-2).

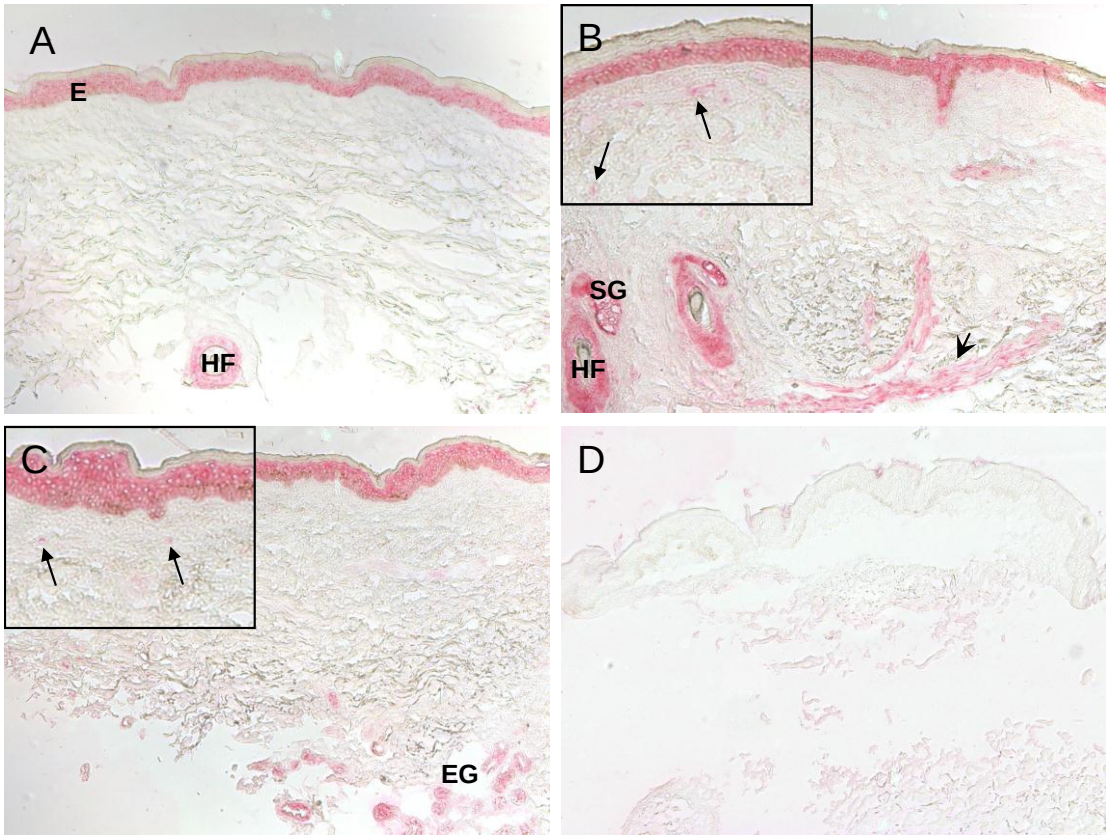


Figure 4-2 11β-HSD1 immunohistochemistry in human skin. Protein expression was detected using an 11β-HSD1 monoclonal antibody in frozen sections of human skin (n=14). (**A, B, C**) Positive staining was detected in epidermal keratinocytes (E), dermal fibroblasts (arrows, inset) and microvasculature (arrowhead). Staining was also detected in hair follicles (HF), eccrine sweat glands (EG,) and sebaceous glands (SG). (**D**) Negligible staining was observed in equimolar isotype control-treated sections. Sections at 10X magnification

4.3.4.3 Quantitation

Stained sections were photographed using a Nuance multispectral imaging system (CRi, part of Caliper Life Sciences Ltd., Runcorn, UK). The system utilizes an optimized, high-throughput tunable filter distinctly matched to the bandwidths of common molecular markers (e.g. Sigmafast fast red).

Nuance image analysis software was used to annotate the DEJ and the *stratum corneum*/*granulosum* interface. The software was programmed to detect the intensity of staining from the DEJ to the *stratum corneum* (5 layers) and from the DEJ to the bottom of the image in layers of fixed width (Figure 4-3). Epidermal staining was defined as the total stain in all 5 layers, while dermal staining was defined as the total

stain in the first 5 layers from the DEJ down into the dermis, in order to avoid inclusion of staining from confounding structures such as microvasculature, glands and hair follicles. Nuance software was also able to determine average epidermal thickness based on the annotations described above.

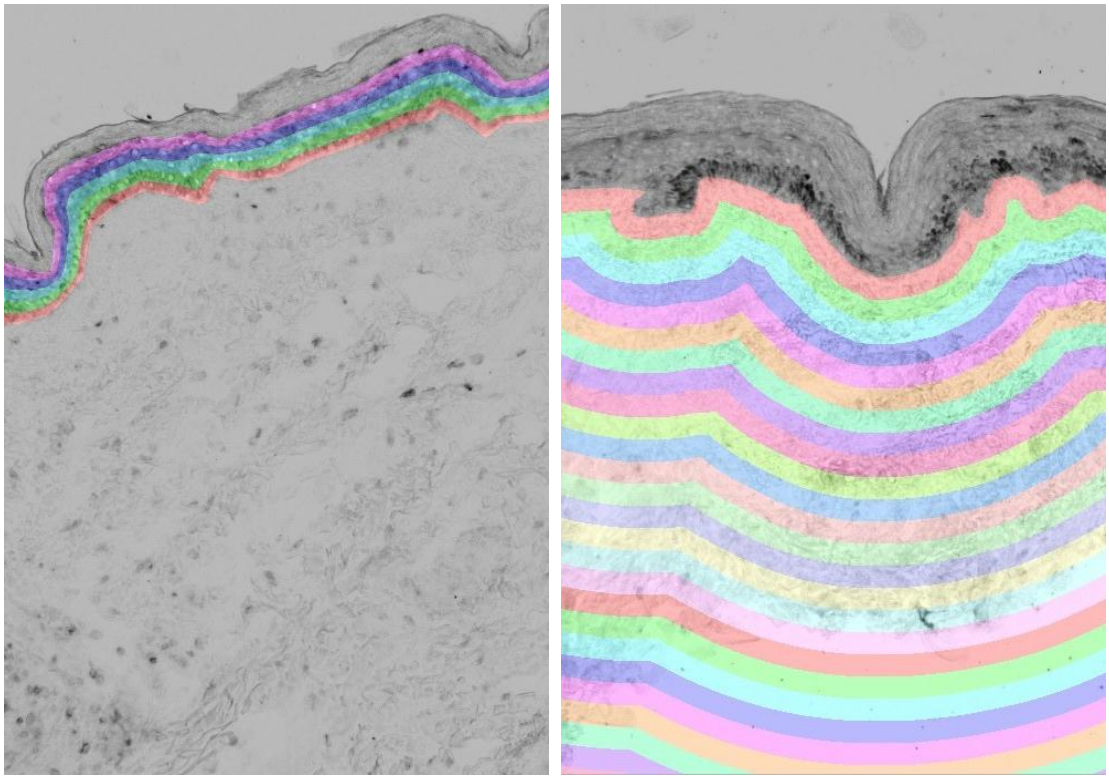


Figure 4-3 Nuance image analysis of immunohistochemically stained slides. Nuance software splits images taken with the spectral camera into user-defined layers, enabling quantification of 11β-HSD1 protein staining on a layer by layer basis (if desired)

4.4 Results

4.4.1 Intrinsic ageing

4.4.1.1 11β-HSD1 expression increases in HDF from older donors

11β-HSD1 mRNA expression displayed a positive correlation with donor age in PP HDF cultures (n=12, r^2=0.59, p<0.01, Figure 4-4). While PE HDF followed a similar trend, this did not reach statistical significance.

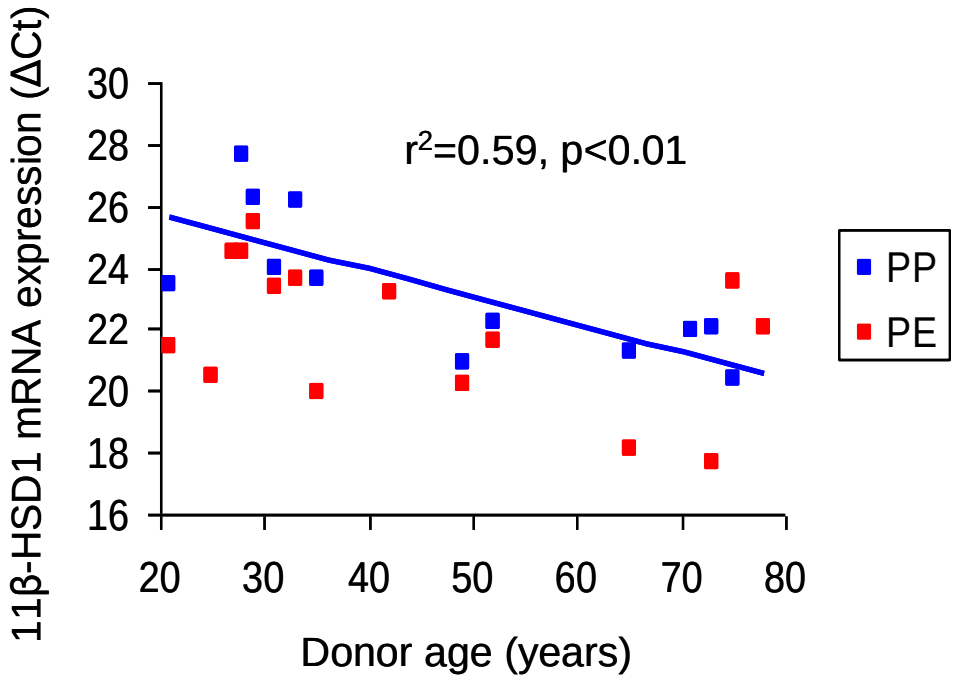


Figure 4-4 Age-related variation of 11β-HSD1 mRNA expression in HDF. Following normalizing for levels of the housekeeping gene 18S rRNA, 11β-HSD1 expression was found to increase with increasing donor age in PP HDF (n=12). Although a similar trend was observed for PE HDF this did not reach statistical significance

Indeed, 11β-HSD1 mRNA expression in PP HDF increased by over 140-fold between the lowest expressing donor (29 years, ΔCt 27.6) and highest expressing donor (75 years, ΔCt 20.4). Interestingly, 11β-HSD2 mRNA expression was also found to increase with donor age in PP HDF (n=10, r^2=0.63, p<0.01, data not shown), although to a lesser degree than 11β-HSD1, with a 26-fold increase between the lowest expressing donor (33 years, ΔCt 26.5) and highest expressing donor (65 years, ΔCt 21.8). Similarly to 11β-HSD1, 11β-HSD2 mRNA expression did not correlate with donor age in PE HDF (n=8, data not shown). Moreover, expression of the 11β-HSD1 cofactor- (NADPH) generating enzyme H6PD and GR-α did not correlate with donor age in either PE (n=9 and n=8 respectively) or PP (n=10) HDF (data not shown).

Following treatment with 100nM cortisol, the age-dependant increase in 11β-HSD1 expression was not only maintained in PP HDF (n=9, r^2=0.6, p<0.05, Figure 4-5), but also induced in PE HDF (n=6, r^2=0.81, p<0.05, Figure 4-5). Additionally, there was a

much greater difference (~560-fold induction) in PP HDF between the lowest expressing donor (28 years, ΔCt 26.6) and highest expressing donor (75 years, ΔCt 17.5). Furthermore, cortisol treatment abolished the correlation between 11β-HSD2 mRNA expression and donor age in PP HDF (n=9) with the lack of correlation between 11β-HSD2 PE HDF (n=8), H6PD and GR-α (PP n=10 and PE n=9) and donor age also remaining unaffected (data not shown).

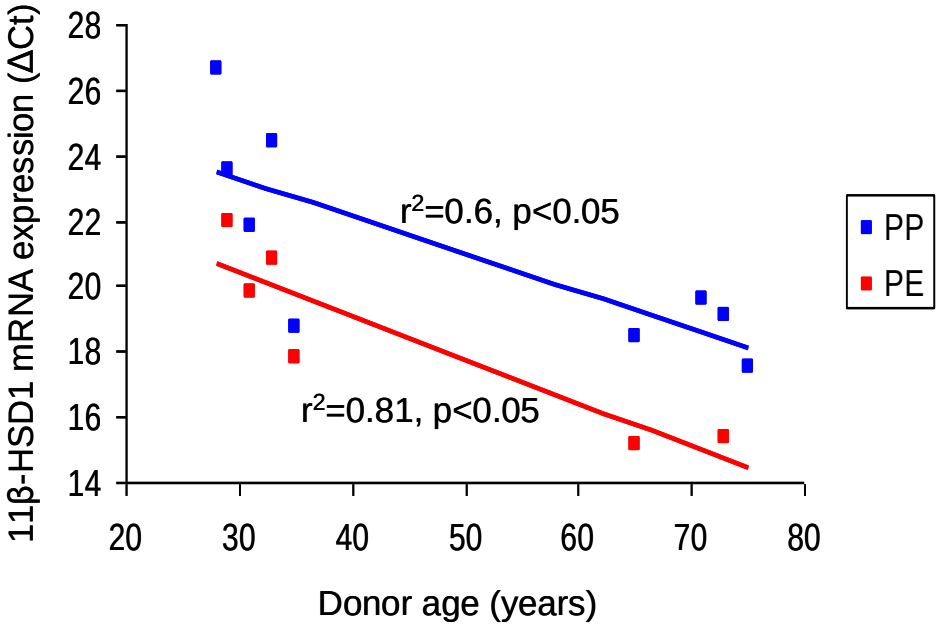


Figure 4-5 Age-related response of 11β-HSD1 mRNA expression in HDF following 100nM cortisol treatment. Following normalizing for levels of the housekeeping gene 18S rRNA, 11β-HSD1 expression was found to increase with increasing donor age in PP (n=9) and PE (n=6) HDF

4.4.1.2 11β-HSD1 activity is induced by GC in HDF from older donors

In order to determine whether the increase in 11β-HSD1 mRNA expression with donor age in HDF resulted in a similar increase in enzyme function, 11β-HSD1 activity assays were performed in the presence and absence of 100nM cortisol stimulation. Whilst 11β-HSD1 activity was undetectable in the majority of unstimulated HDF cultures (Chapter 3), following 100nM cortisol treatment, 11β-HSD1 activity was induced in older donor-derived HDF but remained negligible in

young donor-derived HDF (pmol/mg/h ± S.D, 1.4±1.1 vs. 0.06±0.08 respectively, $p<0.05$, n=5 Figure 4-6, A). This was endorsed by a comparable response in 11β-HSD1 mRNA expression with higher levels in older compared to younger donors (ΔCt ± S.D, 16.6±1.6 vs. 21.4±2.0 respectively, $p<0.01$, n=5, Figure 4-6, B).

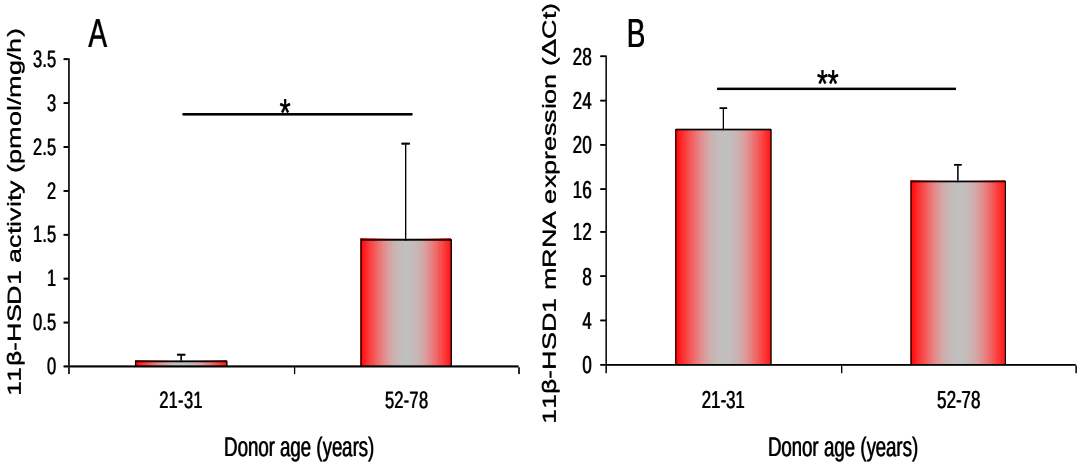


Figure 4-6 Age-related response of 11β-HSD1 activity and mRNA expression in HDF following GC treatment. (**A**) 100nM cortisol treatment induced detectable activity (pmol/mg/h) in HDF (all PE) from older (n=5) but not younger (n=5) donors (1.4±1.1 vs. 0.06±0.08 respectively, $p<0.05$). (**B**) A similar effect was observed in the corresponding levels of 11β-HSD1 mRNA expression (ΔCt ± S.D) following normalizing for 18S rRNA housekeeper expression, with higher levels in older compared to younger donors (16.6±1.6 vs. 21.4±2.0 respectively). Significance * = $p<0.05$, ** = $p<0.01$

4.4.1.3 11β-HSD oxoreductase activity is greater in skin from older donors

Whilst 11β-HSD1 mRNA expression and activity - particularly following positive-feedback stimulation with cortisol - demonstrated a clear positive correlation with donor age in HDF, we sought to determine if this was also the case *in vivo*. 11β-HSD1 activity assays conducted on 3mm punch biopsies from young (20-30 years) and older donors (over 60 years) revealed a similar increase in 11β-HSD oxoreductase activity in older compared to younger donors in both PP (% conversion 100nM cortisone to cortisol ± S.D, 9.5±3.3 vs. 6.7±2.0, $p<0.01$) and PE (11.5±3.0 vs. 9.1±3.0, $p<0.05$) samples (Figure 4-7).

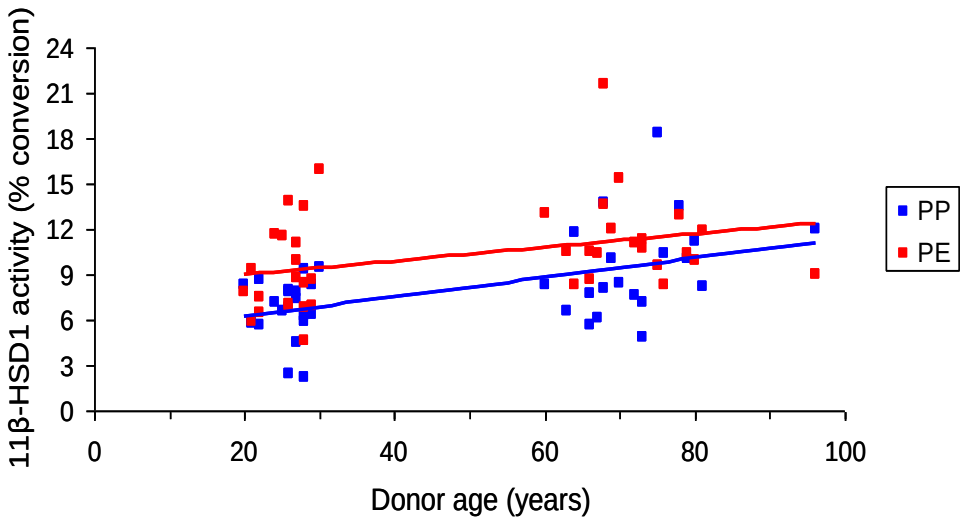


Figure 4-7 Age-related variation of 11β-HSD oxoreductase activity in 3mm human skin punch biopsies. Activity (% conversion 100nM cortisone to cortisol ± S.D) was found to be greater in biopsies obtained from older (n=20) compared to younger (n=20) donors from both PP and PE sites (9.5±3.3 vs. 6.7±2.0, $p<0.01$ and 11.5±3.0 vs. 9.1±3.0, $p<0.05$ respectively)

Although 11β-HSD oxoreductase activity increased with donor age, levels of 11β-HSD1 mRNA expression remained comparable between young and aged donors in skin biopsies derived from both PP (mean age ± S.D, 27.8±1.3, n=4 and 67.5±5.4, n=4, ΔCt ± S.D, 19.5±1.2 vs. 19.6±2.0 respectively) and PE (mean age ± S.D, 25.7±3.4, n=6 and 69.2±6.1, n=5, ΔCt ± S.D, 18.8±2.2 vs. 19.2±2.3 respectively) sites. Furthermore, no significant difference between young and aged donors was observed, from skin biopsies derived from either site, for mRNA expression of 11β-HSD2 (PP, mean age ± S.D, 27.8±1.3, n=4 and 67.5±5.4, n=4, ΔCt ± S.D, 15.7±1.0 vs. 15.0±1.1 respectively, PE, mean age ± S.D, 25.7±3.4, n=6 and 67.3±6.7, n=3, ΔCt ± S.D, 15.0±0.4 vs. 15.2±0.6 respectively), GR-α (PP, mean age ± S.D, 27.8±1.3, n=4 and 67.5±5.4, n=4, ΔCt ± S.D, 16.0±0.6 vs. 14.6±1.1 respectively, PE, mean age ± S.D, 25.9±3.1, n=7 and 67.3±6.7, n=3, ΔCt ± S.D, 15.3±1.5 vs. 16.1±0.3 respectively) or H6PD (PP, mean age ± S.D, 27.8±1.3, n=4 and 67.5±5.4, n=4, ΔCt ± S.D, 14.7±0.8 vs. 14.4±0.7 respectively, PE, mean age ± S.D, 25.7±3.4, n=6 and 67.3±6.7, n=3, ΔCt ± S.D, 14.6±1.0 vs. 15.0±0.2 respectively).

4.4.1.4 11β-HSD1 expression/activity is greater in skin from older mice

As 11β-HSD1 expression and activity was also detected in murine skin, we examined whether the increase in 11β-HSD oxoreductase activity with donor age in humans may also occur in mice. Indeed, we observed a higher level of oxoreductase activity in older (91-99 weeks, n=5) compared to younger (11-20 weeks, n=6) mice (pmol/mg/h ± S.D, 0.44±0.15 vs. 0.26±0.11 respectively, $p<0.05$, Figure 4-8). Furthermore, oxoreductase activity positively correlated with 11β-HSD1 mRNA expression in mouse skin ($r^2=0.6$, $p<0.05$, n=7, Figure 4-8), but not H6PD or GR-α (data not shown).

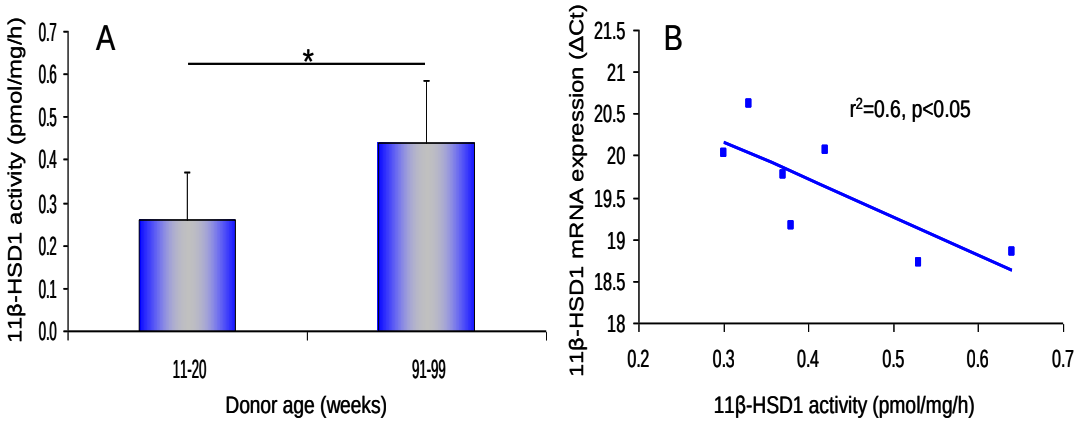


Figure 4-8 Age-related variation in 11β-HSD oxoreductase activity and 11β-HSD1 mRNA expression in full-thickness mouse skin explants. (**A**) Results confirmed increased oxoreductase activity in older (91-99 weeks, n=5) compared to younger (11-20 weeks, n=6) mouse skin (pmol/mg/h ±S.D, 0.44±0.15 vs. 0.26±0.11 respectively, $p<0.05$). (**B**) Activity also positively correlated with 11β-HSD1 mRNA in tissue-matched samples (n=7, $r^2=0.6$, $p<0.05$). Significance * = $p<0.05$

4.4.2 Extrinsic ageing

As 11β-HSD1 expression and oxoreductase activity were shown to increase with donor age in HDF and *ex vivo* skin biopsies (from both PP and PE sources) we next investigated whether this was also the case for donor-matched PE compared to PP samples.

4.4.2.1 11β-HSD1 expression is greater in HDF from PE skin

11β-HSD1 mRNA expression was analysed in HDF obtained from donor-matched PP (inner upper arm) and PE (lower outer arm) skin. Results indicated a 6.5-fold increase in mRNA expression in PE compared to PP samples (ΔCt ± S.D, 21.6±2.7 vs. 23.7±2.3 respectively, $p<0.001$, n=10, Figure 4-9). This was also maintained following 100nM cortisol treatment (19.4±2.5 vs. 22.2±2.5 respectively, $p<0.001$, n=10, data not shown).

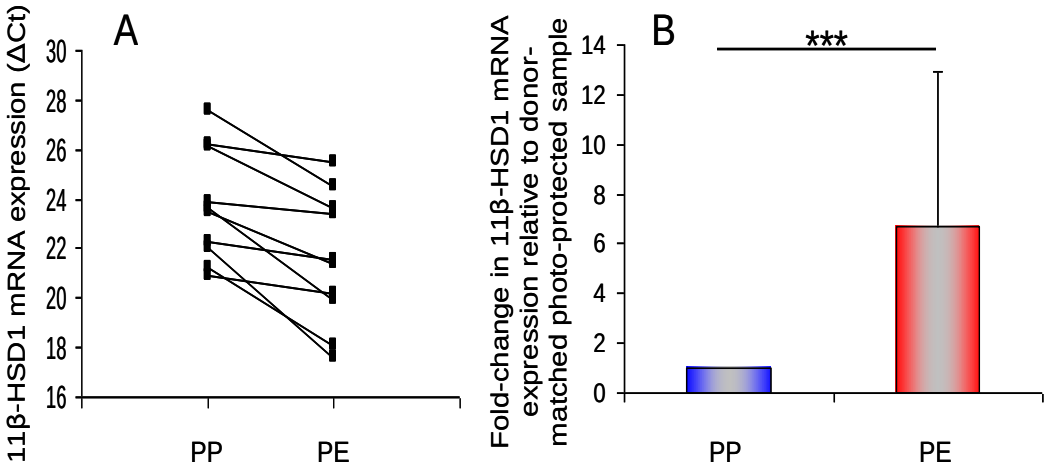


Figure 4-9 Site-related variation of 11β-HSD1 mRNA expression in HDF. (**A**) Following normalizing for levels of the housekeeping gene 18S rRNA, 11β-HSD1 mRNA expression was increased in PE compared to donor-matched PP HDF (ΔCt ±S.D, 21.6±2.7 vs. 23.7±2.3 respectively, $p<0.001$, n=10). (**B**) This corresponded to a 6.5-fold increase in expression in PE samples. Significance *** = $p<0.001$

Conversely, mRNA expression between PP and PE samples was comparable for 11β-HSD2 (ΔCt ±S.D, 24.5±1.6 vs. 24.3±1.7 respectively, n=6), GR-α (ΔCt ±S.D, 9.7±1.1 vs. 9.1±0.9 respectively, n=6) and H6PD (ΔCt ±S.D, 11.9±1.2 vs. 12.1±0.7 respectively, n=7), as was the case following 100nM cortisol treatment (ΔCt ±S.D, 11β-HSD2, 25.6±1.3 vs. 26.0±0.9 respectively, n=5, GR-α, 10.9±1.7 vs. 11.0±1.8 respectively, n=7, H6PD, 12.5±0.8 vs. 12.4±0.7 respectively, n=7, data not shown).

4.4.2.2 11β-HSD1 protein expression is increased in PE human skin

Quantitative analysis of immunohistochemical staining intensity revealed increased 11β-HSD1 protein expression in donor-matched PE compared to PP samples in both the epidermal (0.076±0.042 vs. 0.048±0.034 respectively, $p<0.05$, Figure 4-10 A) and dermal compartments (0.0076±0.004 vs. 0.0043±0.0021 respectively, $p<0.05$, Figure 4-10 B). As expected, endogenous melanin staining (used as a positive control) was also increased in the PE samples (0.023±0.018 vs. 0.0083±0.0043 respectively, $p<0.01$, Figure 4-10 C). Further image analysis demonstrated upregulation of 11β-HSD1 protein expression in the epidermal keratinocytes and induction from negligible levels in dermal fibroblasts from PE samples (Figure 4-11).

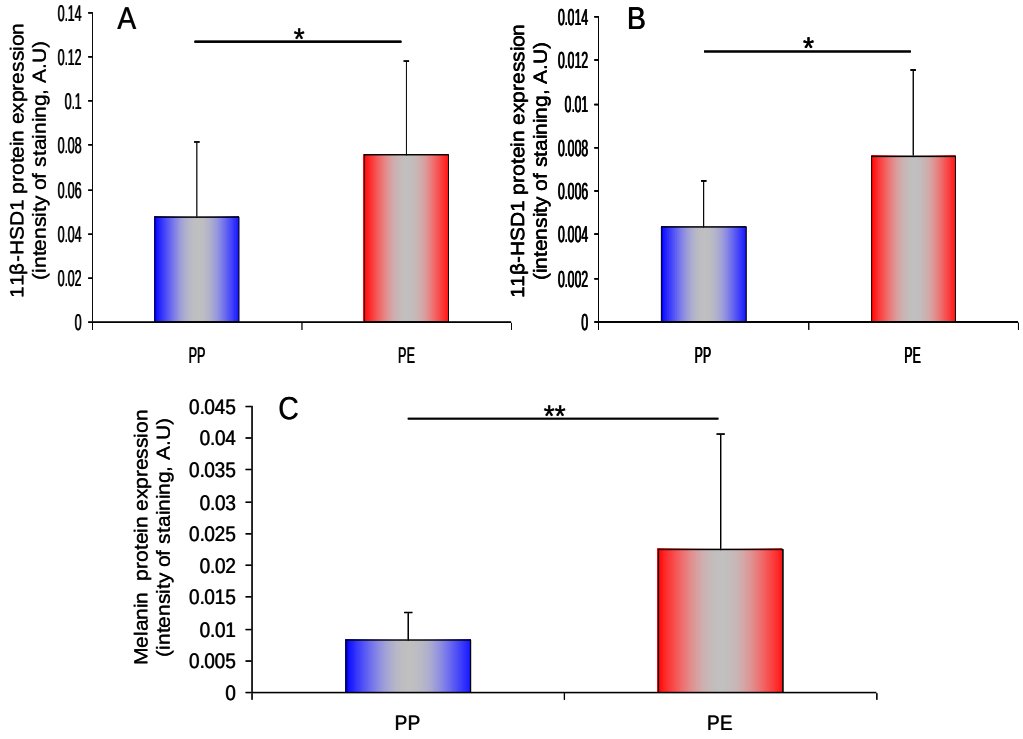


Figure 4-10 Site-related variation of 11β-HSD1 protein expression in human skin sections. (**A**) Epidermal 11β-HSD1 protein expression (as determined by intensity of immunohistochemical staining, A.U±S.D) was greater in donor-matched PE compared to PP skin sections (0.076±0.042 vs. 0.048±0.034 respectively, $p<0.05$, n=14). (**B**) Dermal 11β-HSD1 protein expression was also elevated in PE samples (0.0076±0.004 vs. 0.0043±0.0021 respectively, $p<0.05$, n=14). (**C**) Endogenous epidermal melanin used as a positive control also showed increased staining in PE samples (0.023±0.018 vs. 0.0083±0.0043 respectively, $p<0.001$, n=14). Significance * = $p<0.05$, ** = $p<0.01$

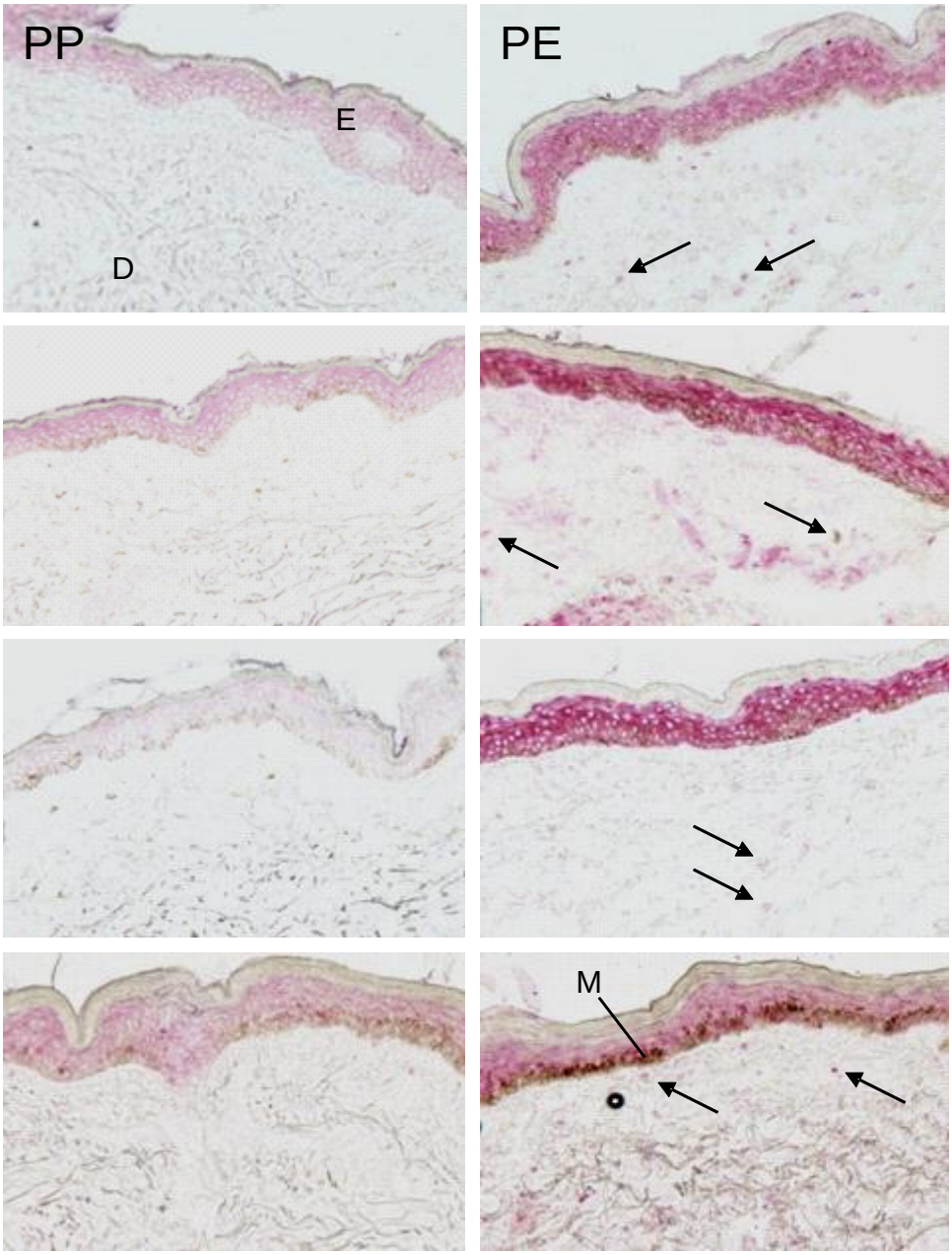


Figure 4-11 Immunohistochemistry of 11β-HSD1 protein expression in PP and PE human skin sections. 11β-HSD1 staining was increased in PE epidermal keratinocytes (E) and in dermal fibroblasts (arrows) compared to donor-matched PP sections (n=14). Melanin staining (M) was also greater in PE sections. D, dermis. 20 X magnification

The average donor age (± S.D) for these samples was 63.6±6.7 years (n=14), with samples from younger donors unfortunately unavailable for comparison of 11β-HSD1 protein expression.

4.4.2.3 11β-HSD oxoreductase activity is greater in PE human skin

Subsequently, we aimed to determine whether the increase in 11β-HSD1 mRNA expression in donor-matched PE compared to PP HDF may also be the case for human skin 11β-HSD oxoreductase activity *in vivo*. Results demonstrated increased activity in donor-matched PE compared to PP samples from both young (% conversion 100nM cortisone to cortisol ± S.D, 9.1±3.0 vs. 6.7±2.0 respectively, $p<0.001$, n=20) and older (11.5±3.0 vs. 9.5±3.3 respectively, $p<0.05$, n=20) donors (Figure 4-12).

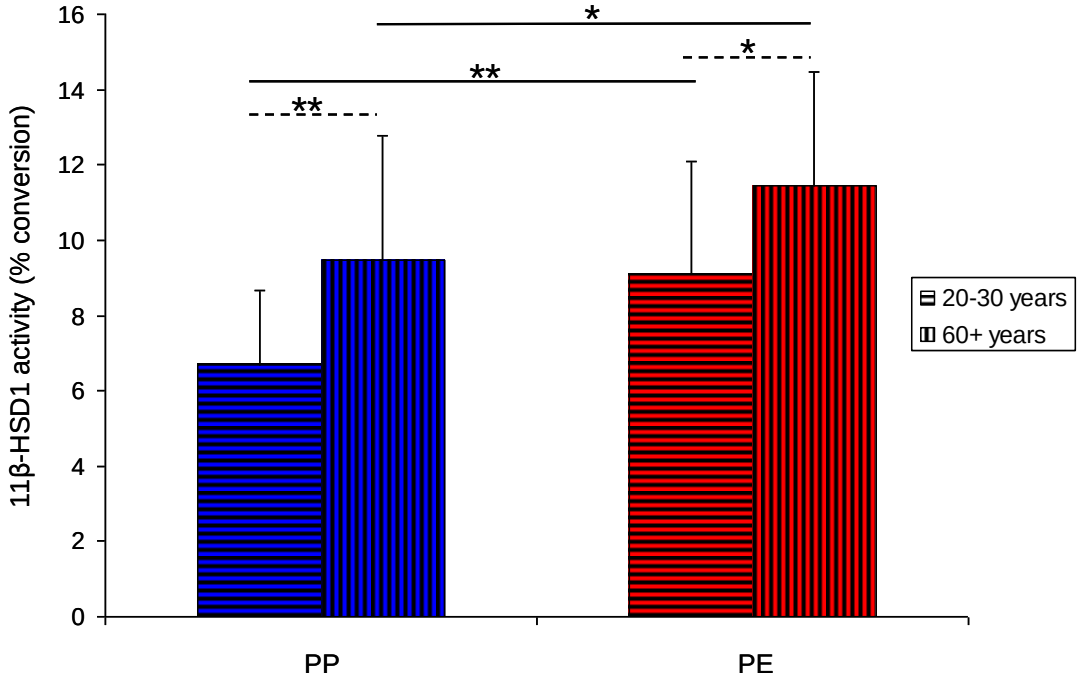


Figure 4-12 Site-related variation in 11β-HSD oxoreductase activity in human 3mm punch biopsies. Conversion of cortisone to cortisol was found to be greater in donor-matched PE compared to PP samples from both young (% conversion 100nM cortisone to cortisol ± S.D, 9.1±3.0 vs. 6.7±2.0 respectively, $p<0.001$, n=20) and older (11.5±3.0 vs. 9.5±3.3 respectively, $p<0.05$, n=20) donors. The significant increase in activity in site-matched older compared to young samples previously described in section 4.4.1.3 is also illustrated for completion (dotted lines). Significance * = $p<0.05$, ** = $p<0.01$

Expression of 11β-HSD1, 11β-HSD2, GR-α and H6PD was also analysed and neither exhibited a site-specific alteration in expression in full-thickness human skin biopsies (data not shown).

4.5 Discussion

Our results provide new evidence for an increase in GC generation in skin during ageing - including increased 11β-HSD1 mRNA expression in HDF, increased 11β-HSD oxoreductase activity and 11β-HSD1 expression following GC treatment in HDF, increased 11β-HSD oxoreductase activity in human full-thickness skin biopsies and increased 11β-HSD oxoreductase activity and 11β-HSD1 expression in mouse full-thickness skin biopsies. These findings are supported by results from other research groups reporting a similar age-associated increase in GC-activating 11β-HSD1 in other tissues.

Zhang *et al* demonstrated a ~2-fold increase in 11β-HSD oxoreductase activity and 11β-HSD1 protein expression in $CD4^+$ T-cells from old (>16 month) compared to young (~3 month) female BALB/c mice (Zhang *et al*., 2005). Although our studies were conducted in male mice, we report a consistent increase in 11β-HSD oxoreductase activity and 11β-HSD1 mRNA expression in full-thickness skin from mice of a comparable age and using a similar 11β-HSD enzymatic activity assay method. An increase in 11β-HSD1 mRNA expression (of a similar magnitude to our observations) in older (>31 month) male and female mouse vertebrae has also been reported (Weinstein *et al*., 2010). Furthermore, 11β-HSD1 mRNA expression has also been shown to increase modestly with age in old (24-27 month) male mouse hippocampus and parietal cortex, although the young control animals were relatively older (6 months) than those used in our studies and could explain much smaller increase in 11β-HSD1 mRNA expression (Holmes *et al*., 2010). GR expression was also found to be unaffected by age in this study, supporting our observations. Overall, these animal studies are in agreement with our findings in mouse skin.

In human studies, Cooper and colleagues reported a positive correlation of 11β-HSD oxoreductase activity (independently of gender) with donor age in primary cultures of trabecular bone osteoblasts (from ~1pmol/mg/h in young to ~7pmol/mg/h in older donors), although 11β-HSD1 mRNA expression only revealed a trend towards an increase with age (Cooper *et al*., 2002). Our studies demonstrate a similar magnitude of increase in 11β-HSD oxoreductase activity in human skin tissue explants although at much lower levels (from ~30fmol/mg/h in young to ~150fmol/mg/h in older donors). This large difference in oxoreductase activity is likely attributable in part to the absence of 11β-HSD2 mRNA expression reported by Cooper *et al* in osteoblasts which is known to be present in human skin (as demonstrated by us and others), inactivating cortisol back to cortisone and resulting in an apparently lower overall activity. More recently, a modest increase in 11β-HSD1 mRNA expression in abdominal subcutaneous adipose tissue from postmenopausal compared to pre-menopausal females has been reported (Andersson *et al*., 2009), although activity was not significantly different between the groups. This may be due to the activity assay being measured solely in the dehydrogenase direction in homogenized tissue, with potential interference from 11β-HSD2 - an issue not addressed elsewhere in the paper. In contrast, Li *et al*., failed to detect a similar age-related increase in adults but reported a weak association between 11β-HSD1 mRNA expression and age in omental fat in children (Li *et al*., 2007), although this was hypothesised to be due to increasing adiposity during early development. These limited humans studies are also in general agreement with our findings in human skin reporting an increase in 11β-HSD1 mRNA expression and 11β-HSD oxoreductase activity as a function of donor age in subcutaneous adipose tissue and primary cultures of osteoblasts.

Although 11β-HSD1 oxoreductase activity was undetectable in the majority of HDF cultures, we observed an age-dependant increase in detectable activity in old but not young donor-derived HDF following GC treatment. This is likely due to the higher baseline levels of 11β-HSD1 mRNA expression in HDF from older donors resulting in significantly greater expression levels following GC-induced upregulation and thus detectable oxoreductase activity. This positive-feedback auto-regulation of local GC metabolism by GC themselves may magnify the exposure of HDF to GC during ageing whilst simultaneously predisposing older individuals to greater GC exposure following treatment with exogenous GC or in states of raised endogenous GC levels (e.g. following physical or emotional stress). This may explain, in part, the increased incidence of skin atrophy in the elderly resulting from topical GC therapy (Oikarinen and Autio, 1991).

The increase in GC-reactivation potential with advancing age due to elevated 11β-HSD1 expression and 11β-HSD oxoreductase activity in the periphery (including skin) may mimic, at a tissue-specific level, the reduced central GC responsiveness (measured by DEX inhibition of LPS-induced cytokine production) reported to occur with ageing following stress (Rohleder *et al*., 2002), leading to an impaired protective response in the elderly against detrimental increases of pro-inflammatory cytokines. Decreased HPA-axis sensitivity to negative feedback cortisol inhibition with ageing (Wilkinson *et al*., 1997) is believed to further exaggerate adrenal cortisol secretion leading to a blunting of the diurnal cortisol section pattern with elevated evening levels (Van Cauter *et al*., 1996). Due to the large surface area of skin it may even be plausible that increased GC reactivation at this site (coupled with a lack of compensatory increase in renal 11β-HSD2 activity with age, Audige *et al*., 2002) could contribute to the elevated systemic cortisol levels with age. Studies have also

demonstrated a link between Alzheimer's disease and decreased GC sensitivity in the skin (Linder *et al*., 1993), while Alzheimer's disease has been associated with increased GC production and altered cortisol metabolism (Rasmuson *et al*., 2001) and 11β-HSD1 expression increases in mouse brain have recently been implicated in declined cognitive function with age (Holmes *et al*., 2010). These studies suggest indirectly that increased GC reactivation with advancing age may indeed lead to decreased GC sensitivity in peripheral tissues such as skin, although 11β-HSD1 expression in the brain and skin of Alzheimer's patients remains to be investigated. Reduced GC sensitivity in skin may in turn contribute to ineffective control of inflammatory mediators, persistent inflammation and reduced wound healing - frequently reported in elderly individuals. Furthermore, the persistent inflammation and uncontrolled levels of inflammatory mediators (e.g., TNF-α, IL-1) resulting from decreased central and peripheral sensitivity to GC with age may also contribute to further exacerbating GC insensitivity though the upregulation of 11β-HSD1 (Cooper *et al*., 2001) potentially resulting in a vicious cycle with increasing GC accumulation (both local and central), decreased beneficial anti-inflammatory function and increased adverse side-effects.

In contrast to the limited but slowly accumulating evidence for an increase in local GC levels with intrinsic ageing through the upregulation of 11β-HSD1, there have been no previous studies examining changes in the levels of this enzyme with respect to extrinsic ageing. Our studies investigating intrinsic ageing in skin offered an opportunity to examine whether 11β-HSD1 may also be involved in potentiating the accelerated ageing associated with the primary causative agent of extrinsic ageing, UV radiation. Intriguingly, our results demonstrate that 11β-HSD1 mRNA expression is indeed consistently higher in donor-matched HDF from PE compared to PP skin,

while levels of 11β-HSD2, GR-α and H6PD remain relatively similar between the two sites. Furthermore, 11β-HSD oxoreductase activity was also higher in donor-matched PE compared to PP skin *ex vivo* strongly supporting the *in vitro* evidence for a causative role of 11β-HSD1-induced GC accumulation in both intrinsic and extrinsic skin ageing. Additionally, our results demonstrate a larger, more statistically significant difference between PE and PP sites in younger volunteers. The more comparable activity between sites in older volunteers is as a result of increased enzyme activity in PP samples with age, supporting the concept of extrinsic ageing superimposing on intrinsic ageing. Although 11β-HSD1 mRNA expression was comparable between donor-matched PE and PP samples, our immunohistochemical studies revealed increased 11β-HSD1 protein expression in the former. This was observed in both epidermal keratinocytes and dermal fibroblasts.

It remains possible that the differences observed between PE and PP 11β-HSD1 mRNA expression and 11β-HSD activity are due to differences resulting from the physical anatomical separation rather than in response to different environmental conditions. Studies have reported differential gene expression patterns in dermal fibroblasts obtained from different body sites. Chang *et al* described notable groups of differentially expressed genes including ECM synthesis, metabolism, and cell signalling pathways that control proliferation, cell migration, and fate determination suggesting that fibroblasts at different locations in the body should be considered distinct differentiated cell types (Chang *et al*., 2002). However, this study has several limitations including a lack of gender- and age-controlled site-matched repeats making the results difficult to interpret in the context of site alone. Experimental replicates (multiples of same-donor samples) were used in lieu of biological replicates and in some cases mRNA expression profiles were more comparable between

different sites from different donors (e.g. abdomen and arm) than site-matched experimental replicates from the same donor suggesting certain anatomical sites may be more similar than the study implies. Unsurprisingly, the greatest expression profile differences existed between foetal- and adult-derived samples, with adult abdomen, arm, back and thigh samples showing relatively similar patterns of expression. Furthermore, dermal fibroblasts were passaged for at least 10 population doublings before mRNA harvest, introducing another potential source of variation into the study. Our studies examining differential 11β-HSD1 mRNA expression in HDF derived from PP and PE sites were designed to limit the impact of potential confounding factors such as these. For example, sites were restricted to the same site (arm) and cultured cells were maintained at a consistently low passage. Although our results in tissue do not indicate that the increased 11β-HSD1 mRNA expression and associated enzymatic activity are a direct consequence of UV exposure, the fact that this difference was especially poignant in young donors supports the notion that this effect is not caused by anatomical demarcation alone. Furthermore, another study investigating positional demarcation in dermal fibroblasts revealed a lack of strong evidence for differential expression related to fibroblast position along the dorsal-ventral axis (Rinn *et al*., 2006) offering additional support with regards to the validity of our observations at these sites. The studies by Rinn *et al* also reported largest differences between foetal- and adult-derived (lung) tissue and relatively similar clustering of expression between HDF derived from adult wrist, forearm, elbow and upper arm in addition to a lack of an expression gradient along the limbs (in spite of lacking same-site inter-individual repeats) further supporting the sites chosen for our studies to examine the effect of UV exposure as being comparatively similar.

Our results support previous studies suggesting a role for increased local GC generation (through 11β-HSD1) during intrinsic ageing, demonstrating for the first time that this is also occurring in mouse and human skin. Intriguingly, our data provides evidence that this may also be the case with extrinsic ageing in humans - offering a novel common mediator through which extrinsic ageing may superimpose on intrinsic ageing. In parallel with the adverse effects associated with 11β-HSD dysfunction in diseases of metabolism and immunity, emerging data, including those presented here, suggest that similar pathways may be involved in the aetiology of ageing and photo-ageing.

In human bone-derived primary osteoblast cultures, increased intracellular 11β-HSD oxoreductase activity with age is postulated to contribute to the increase in fracture risk for idiopathic and GC-induced osteoporosis that occurs with ageing (Cooper *et al*., 2002), in line with the well-known deleterious effects of GC on bone metabolism. In addition to similar age-related changes in murine bone 11β-HSD1 expression, targeted overexpression of 11β-HSD2 in bone cells protected transgenic animals from age-induced osteoblast/osteocyte apoptosis, decrease in bone formation rate and associated microarchitecture erosion, decreased vasculature volume and reduced strength (Weinstein *et al*., 2010), suggesting that realigning the imbalance of GC metabolism that occurs with ageing may lead to dramatic improvements in age-associated decrements in bone function. Indeed, pharmacological inhibition of 11β-HSD1 *in vivo* has been shown to decrease biochemical markers of bone resorption in young individuals (Cooper *et* al., 2000) but this has yet to be investigated in older subjects. Similarly in mouse brain, the increased levels of 11β-HSD1 with age shown to correlate with impaired cognitive performance appear to be causal, as transgenic mice with targeted overexpression of increased intracellular 11β-HSD1 in the

forebrain displayed premature age-associated cognitive decline (Holmes *et al*., 2010), potentially implicating 11β-HSD1 in the age-associated memory impairments also occurring in humans. Our results suggest that the degeneration of skin with advancing age also involves local increases in GC generation through 11β-HSD1, although the 11β-HSD1-specific regulation of GC target genes in skin has yet to be investigated.

Currently, theories to explain these recently emerging data remain speculative and highly complex. One plausible explanation is that of inflamm-ageing, whereby age-associated increases of pro-inflammatory cytokines (Daynes *et al*., 1993, Chen *et al*., 2003, Bruunsgaard and Pedersen, 2003, Viel *et al*., 2001, Casolini *et al*., 2002) may contribute to increased local GC activation by being potent activators of 11β-HSD1 expression and reductase activity (Cooper *et al*., 2001).

Hormonal alterations that occur with ageing are also possible contributors to local GC metabolism dysregulation. One such hormonal alteration is the decrease in adrenal secretion of DHEA and subsequently its sulphated circulating form DHEAS (Sulcova *et al*., 1997). The 7α-hydroxlation of DHEA to the active metabolite 7α-hydroxy-DHEA has been shown to occur in a variety of GC-target tissues including brain (Rose *et al*., 1997), spleen, thymus, intestine, colon, caecum, muscle (Morfin and Courchay, 1994), prostate (Martin *et al*., 2001), joints (Dulos *et al*., 2004) and skin (Morfin and Courchay, 1994, Khalil *et al*., 1993). Furthermore, 11β-HSD1 has recently been shown to interconvert 7α-hydroxy-DHEA and 7β-hydroxy-DHEA via the 7-oxo-DHEA intermediate (Muller *et al*., 2006), raising the possibility that DHEA functions via its metabolites as a tissue-specific anti-GC, limiting the effects of GC particularly following inflammation (Dulos *et al*., 2004). Decreasing circulating levels of DHEA with age could lead to a shift in substrate availability for 11β-HSD1 favouring cortisone over DHEA metabolites and thus contributing to increased local

production of GC during ageing (Muller *et al*., 2006). Additional complexity is introduced when considering the large degree of inter-dependence and cross-talk between systemic processes - one simplified example of such is the potential role that DHEAS plays in protecting against inflamm-ageing during youth through the suppression of cytokines such as IL-6 (Daynes *et al*., 1993). Decreasing levels of DHEA/S with ageing may therefore contribute to elevated local GC concentrations through multiple mechanisms.

The age-related decline in GH and IGF-1 levels may also contribute to increased 11β-HSD1 levels in older individuals. GH, through IGF-1, inhibits 11β-HSD1 activity and enhanced cortisone to cortisol conversion occurs with a reduction in GH levels. This was demonstrated by studying patients with acromegaly (GH excess) who exhibited a relatively low THF+allo-THF/THE ratio (indicative of low systemic 11β-HSD oxoreductase activity) which increased following lowering of GH (and consequently IGF-I) levels (Moore *et al*., 1999). Conversely, studies reported increased global 11β-HSD oxoreductase activity in GH-deficient patients with hypopituitarism (Weaver *et al*., 1994). Interestingly, the dermal phenotype varies greatly between the two disorders. Skin puffiness due to excessive GAG accumulation and oedema, psoriasis (epidermal hyperproliferation), increased vasoconstriction (Ben-Shlomo and Melmed, 2006), hyperelasticity, excessive collagen deposition (Braham *et* al., 2002) and excessive sweating (Lange *et al*., 2001) are common symptoms in patients with acromegaly. In contrast, GH deficiency typically manifests in skin with reduced sweating and epidermal thinning (Lange *et al*., 2001), symptoms also commonly associated with ageing skin.

It is likely that a combination of immune, hormonal and other changes that occur during ageing act in concert to trigger the increase in 11β-HSD1 expression and

oxoreductase activity. Notably, the autocrine upregulation of 11β-HSD1 by GC may also be involved in maintaining and exacerbating local GC concentrations with advancing age. Our results are in support of the view that 11β-HSD1 levels escalate with age independently of tissue type, with skin being the most recent addition to this trend. Although the mechanisms underlying this observation are complex and still poorly understood, 11β-HSD1 dysfunction during ageing is emerging as a potential key mediator of the ageing process, common to the gradual degeneration of multiple tissues and gaining prominence as a possible therapeutic target to combat systemic ageing.

CHAPTER 5 PRE-RECEPTOR REGULATION OF GC TARGET GENES IN SKIN

5.1 Introduction

5.1.1 Overview

The local availability of GC is controlled in a tissue-specific manner by isozymes of the enzyme 11β-HSD. In skin, GC are known to regulate a broad range of target genes including collagen, hyaluronic acid and TIMPs, resulting in detrimental alterations to skin structure and function (section 1.9). However, the molecular mechanisms underlying these alterations have not been examined in detail, with the effects of GC on several elements of skin biology such as collagen biosynthesis and maintenance of homeostasis still awaiting elucidation. Moreover, the pre-receptor regulation of known GC target genes in skin has not been investigated to date. The lack of knowledge in this area combined with our previous findings revealing an increase pre-receptor GC signalling potential in skin during ageing (Chapter 4) prompted the studies presented in this chapter.

5.1.2 Collagen processing and organization in skin

Following translation, individual procollagen-α chains undergo a series of modifications that result in the highly organized structure present in the dermal ECM (Figure 5-1). Firstly, proline and lysine amino acids are hydroxylated by prolyl-4 (P4HA1, P4HA2, P4HA3, and P4HB), prolyl-3 (LEPRE1, LEPREL1 and LEPREL2) and lysyl hydroxylases (PLOD1, PLOD2 and PLOD3) to form the hydroxyproline and hydroxylysine residues characteristic of collagen (Myllyharju, 2008). The collagenous domains of the α-chains then fold into a triple-helical structure, stabilized

by the hydroxyproline residues, and the trimeric molecules are then secreted from the rough endoplasmic reticulum into the extracellular *milieu* assisted by chaperone proteins such as hsp47 (SERPINH1).

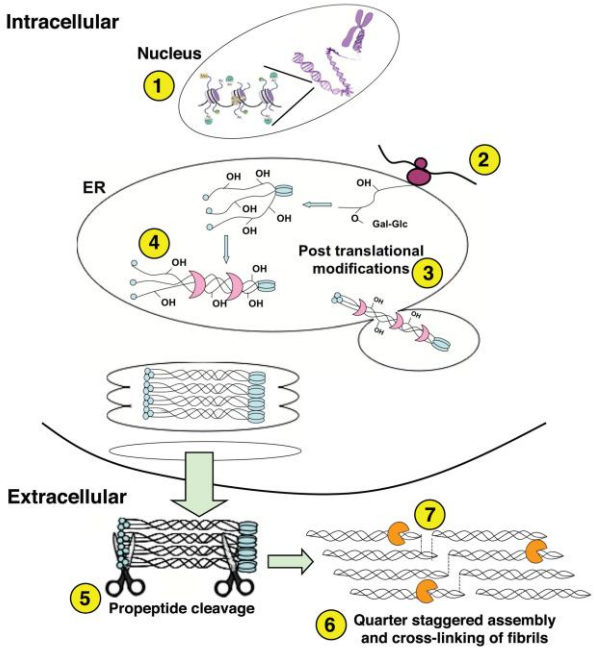


Figure 5-1 Collagen processing pathway. Following transcription of collagen genes (1), expressed mRNA transcripts are translated to procollagen peptides (2) which undergo several post-translational modifications including hydroxylation of proline and lysine residues (3), triple helix formation and association with chaperones such as hsp47 (4) which aid export through the Golgi network and secretion into the extracellular *milieu*. Here, propeptides undergo cleavage of non-collagenous peptide domains through the action of C- and N-terminus endopeptidases (5) with subsequent assembly in a quarter-staggered formation (typical of fibrillar collagens). Tensile properties are attributable to the action of lysyl oxidases which catalyze the formation of lysine and hydroxylysine-derived intramolecular and intermolecular cross-links (6). Degradation of collagen may then occur during tissue remodelling through the action of MMPs (orange crescent, 7). Reproduced from Chen and Raghunath, 2009

Non-collagenous peptide extensions at both ends of the newly secreted collagen molecules are then cleaved by specific proteases (e.g. ADAMTS2, BMP1) and assembled into their tissue-specific supramolecular organization. Although the alignment of collagen molecules in the ECM occurs spontaneously, for example in a quarter-stagger arrangement in the case of fibrillar collagens, the necessary tensile strength is attained through the actions of lysyl oxidases (LOX) and lysyl oxidase-like enzymes (LOXL1, LOXL2, LOXL3 and LOXL4) which catalyze the formation of cross-links at lysine and hydroxylysine residues (Uzawa *et al*., 2003).

5.1.3 Maintenance of skin homeostasis

Over recent years, much progress has been made establishing mechanisms and paracrine interactions between different cell types in the skin to maintain tissue homeostasis and barrier function, best exemplified by examining the process of tissue repair and regeneration following acute injury during wound healing - known to be adversely affected by GC. This highly complex and delicately orchestrated process can be generalized into the early inflammatory response phase, the granulation mid-phase (including epidermal re-epithelialisation and dermal connective tissue generation) and the late tissue remodelling phase (Figure 5-2).

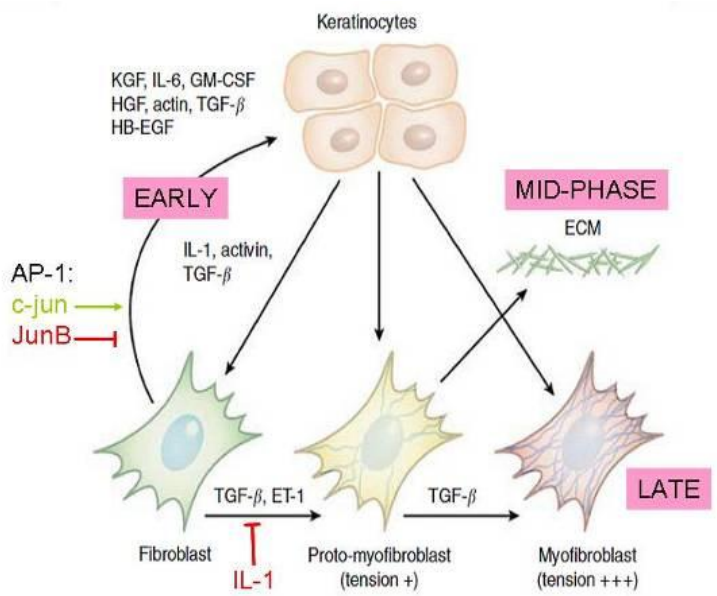


Figure 5-2 Epidermal-mesenchymal signalling in wound healing. Promoted by the initial inflammatory response keratinocyte-derived IL-1 stimulates dermal fibroblasts to secrete several mediators that signal back in a double paracrine manner to regulate keratinocyte phenotype (e.g. stimulating proliferation for re-epithelialisation). Concomitantly, IL-1, through NF-κB, blocks fibroblast differentiation preventing premature ECM remodelling. As inflammatory signalling begins to subside, there is a shift towards increased TGF-β signalling which synergises with mechanical tension build-up to induce fibroblast activation (characterized by increased α-SMA expression typical of myofibroblasts). Thus, epidermal-mesenchymal cross-talk in skin is highly coordinated, enabling efficient remodelling and healing and maintaining effective barrier function. Adapted from Werner *et al.*, 2007.

5.1.3.1 Inflammation as the first response

Studies examining excisional wound healing in mice demonstrated strong induction of IL-1β followed by TNF-α mRNA expression 8 hours post-injury, persisting until mid-

phase (Hubner *et al*., 1996). These pro-inflammatory cytokines are produced by initially by granulocytes (Feiken *et al*., 1995) and maintained thereafter by activated macrophages and keratinocytes (Hubner *et al*., 1996). Surprisingly, Mori *et al*. reported *accelerated* wound healing in TNF-receptor p55 knockout mice displaying enhanced angiogenesis, collagen production and epithelial wound closure with increased TGF-β1, connective tissue growth factor, VEGF-1, VEGF receptor-1 and VEGF-2 expression observed (Mori *et al*., 2002). Moreover, reduced neutrophil and macrophage infiltration was observed, supported by other studies suggesting a beneficial effect on wound healing in mice with reduced leukocyte infiltration (Ashcroft *et al*., 1999) and a negative effect in mice with exaggerated leukocyte infiltration (Ashcroft *et al*., 2000).

Whilst these animal studies suggest a redundant role for TNF-α signalling in wound repair, other early inflammatory mediators play a more active part. Post-injury keratinocyte-derived IL-1 has been shown to stimulate fibroblasts to secrete KGF, IL-6 and GM-CSF (Waelti *et al*., 1992, Maas-Szabowski and Fusenig 1996, Maas-Szabowski *et al*., 1999), a process delicately regulated by the c-jun and JunB AP-1 subunits (Szabowski *et al*., 2000).

5.1.3.2 Regulation of granulation

The importance of fibroblast-derived KGF in promoting epidermal proliferation is highlighted by a dominant-negative mutation in the KGF receptor expressed in basal keratinocytes of transgenic mice. These animals exhibited epidermal thinning, hair follicle abnormalities, dermal fibrosis, and a severe delay in wound re-epithelialisation (Werner *et al*., 1994), whilst subsequent studies involving KGF mutant mice revealed little effect on wound healing (Guo *et al*., 1996) suggesting

other fibroblast-derived factors capable of KGF receptor activation (e.g. FGF10) may be compensating for the lack of KGF.

Similarly, mice deficient in IL-6 display a severely compromised excisional wound healing phenotype, with fewer leukocyte numbers, decreased angiogenesis and reduced collagen accumulation (Lin *et al*., 2003) which was rescued following IL-6 administration (Gallucci *et al*., 2000). Therefore, the epidermal-mesenchymal interaction can be described as a double paracrine cross-talk, with keratinocyte-derived IL-1 stimulating dermal fibroblasts to secrete a host of factors that feedback to keratinocytes and alter their phenotype to promote the wound healing process.

Concomitant with phenotype transition from a resting state to one promoting re-epithelialisation, keratinocytes are stimulated to secrete autocrine-acting growth factors such as TGF-α, an EGFR ligand also associated with the hyperproliferative epidermis recognized as a hallmark of psoriasis (Elder *et al*., 1989). However, its role in wound healing may also be dispensable as mice lacking TGF-α exhibit normal healing with the exception of sites, such as the ear (where supporting granulation tissue in the dermis does not form) suggesting that other growth factors present during the granulation process (e.g. HB-EGF) are able to compensate (Kim *et al*., 2001). Indeed, mice with keratinocyte-targeted inactivation of HB-EGF display delayed epithelial wound closure (Shirakata *et al*., 2005).

In addition to immediate availability from de-granulating platelets, PDGF is synthesised by keratinocytes *de novo* during the granulation phase of tissue repair with reduced PDGF signalling reported in models of reduced wound healing such as diabetic mice (Beer *et al*., 1997).

Whilst several of the growth factors secreted during wound repair are derived primarily from either keratinocytes or dermal fibroblasts, some are expressed in both

cell types following injury. Activins fall into this category, homo- or hetero-dimeric polypeptides that are part of the TGF-β family (Hubner *et al*., 1996). Although *in vitro* studies reported a growth inhibitory effect of activins on keratinocyte proliferation (Seishima *et al*., 1999), wound healing was enhanced following keratinocyte-specific overexpression (Munz *et al*., 1999). This discrepancy may be explained by a requirement for mesenchymal cross-talk, highlighting the challenge faced when attempting to collate *in vitro* and *in vivo* effects. This is further supported by studies comparing the mRNA expression profiles of dermal fibroblasts grown in isolation or in the presence of HaCaTs, with differential expression of a host of growth factor, ECM, protease, and intracellular structural protein transcripts (Shephard *et al*., 2004). Conversely to studies by Munz *et al*., mice overexpressing the soluble activin antagonist follistatin in the epidermis displayed a severe delay in wound healing but decreased resultant scarring (Wankell *et al*., 2001) demonstrating the capacity of growth factors to affect multiple aspects of wound healing.

5.1.3.3 Fibroblast-driven tissue remodelling

Fibroblasts differentiating into myofibroblasts initiate the late-phase of wound healing, with a strong induction in contractile properties as they align in parallel to the mechanical tension building up in the newly formed granulation tissue. During differentiation, fibroblasts acquire expression of α-SMA, driven by exposure to mechanical tension through endothelin-1 (Hinz *et al*., 2001) and TGF-β (Desmouliere *et al*., 1993), but inhibited by IL-1 which is present in the early stages of wound repair (Shephard *et al*., 2004). Indeed, NF-κB activation (following IL-1 and TNF-α stimulation) was found to be a principal inhibitor of TGF-β signalling (Bitzer *et al*., 2000).

5.1.3.4 Summary

These studies demonstrate that molecular mediators of wound healing play specific roles in regulating the sequential phases of repair, through double paracrine mesenchymal-epidermal cross-talk. Increasing our understanding of the highly complex interactions involved in maintaining tissue homeostasis may enable manipulation to improve wound healing. For example, increased keratinocyte proliferation and decreased apoptosis in transgenic animals overexpressing a dominant-negative type II TGF-β receptor mutant in basal keratinocytes improved wound closure rates (Amendt *et al*., 2002).

5.1.4 GC-mediated inhibition of wound healing

Although GC are known to reduce wound healing as observed during topical or systemic therapy, in conditions of endogenous GC excess (Cushing's syndrome) and following physical or emotional stress (DeVries *et al*., 2006), the mechanisms involved in this inhibition are less well defined.

5.1.4.1 GC signalling in wound healing

The anti-inflammatory properties of GC are well documented and occur through several mechanisms (Figure 5-3) including directly blocking the transcription of inflammatory proteins by NF-κB and AP-1 and by inducing the expression of anti-inflammatory proteins such as IκB, annexin-1, and MAPK phosphatase-1 (Rhen and Cidlowski, 2005).

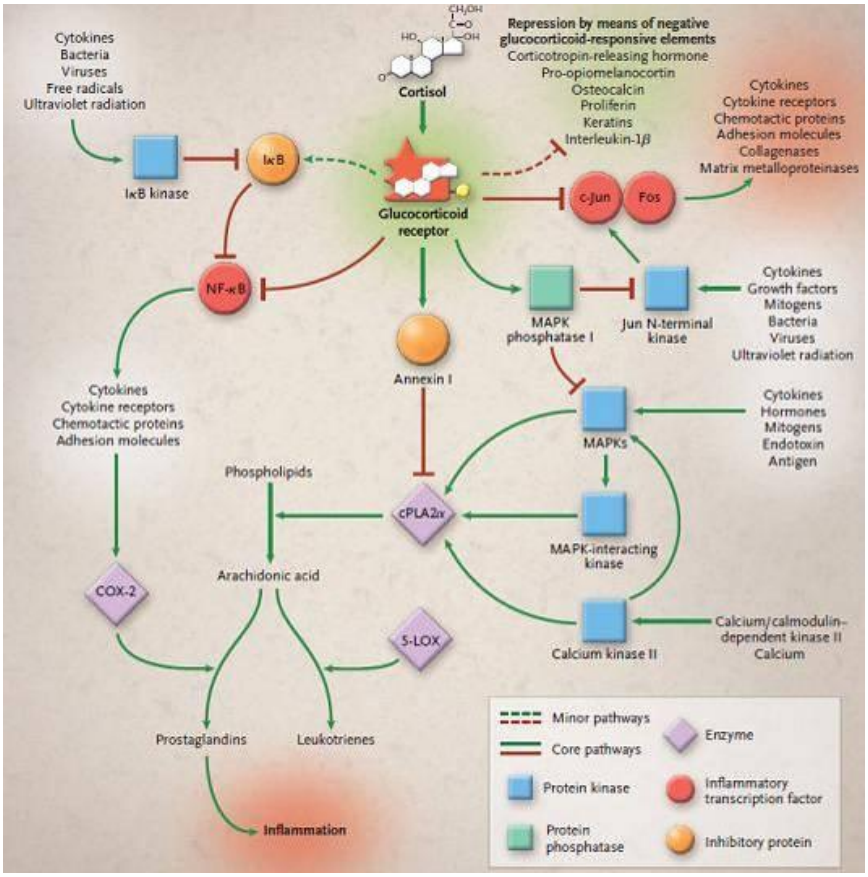


Figure 5-3 Anti-inflammatory GC signalling mechanisms. Activated GR targets pro-inflammatory pathways (e.g. NF-κB) to decrease downstream cytokine expression. Concomitantly, AP-1 signalling through c-Jun and c-Fos transcription factors decreases expression of tissue remodelling mediators (e.g. MMPs). The inability to dissociate GC anti-inflammatory and anti-remodelling properties may contribute to the adverse effects on wound healing when tissues are exposed to excessive GC concentrations (e.g. stress, GC therapy, Cushing's syndrome). Reproduced from Rhen and Cidlowski, 2005

Recently, cortisol synthesis in the epidermis was shown to be increased following tissue injury and after treatment with IL-1. Furthermore inhibition of cortisol synthesis during wound healing increased IL-1 production suggesting it is initially activated as an anti-inflammatory negative feedback mechanism (Stojadinovic *et al*., 2007, Vukelic *et al*., 2011). Interestingly, this study also reported a slight decrease in 11β-HSD2 between 0-24h and a large increase in expression by 48h post-wounding, indicative of a regulatory mechanism to limit active GC availability. Recently, inhibition of wounding-induced *de novo* GC generation by metyrapone (CYP11B1 enzyme inhibitor) was shown to promote wound healing, suggesting the adverse

effects of local GC generation outweigh the anti-inflammatory functions (Vukelic *et al*., 2011).

In collagen gel models of tissue remodelling, GC were found to enhance fibroblast contractility through inhibition of endogenous PGE production (Skold *et al*., 1999) - suggesting, in addition to their anti-inflammatory effects, they may function to regulate several aspects of wound healing, including the promotion of fibroblast contractility in the later stages of wound repair. Conversely, if present early in the wound healing process, GC have been demonstrated to interfere with EGF signalling to inhibit healing (Lee *et al*., 2005) by forming an overriding repressosome that signals keratinocytes to "stop" their activating cycle required for re-epithelialisation and retire to the normal differentiation pathway. Thus the presence of GC in the dermal environment *prior* to wounding or the completion of re-epithelialisation (such as during topical GC therapy or Cushing's syndrome) may impair keratinocyte functions in healing by presenting them with a premature "stop" signal. Further supporting this theory, mice exposed to restraint stress *prior* to wound healing displayed an attenuated immediate IL-1β and KGF response post-wounding, and exaggerated IL-1α and IL-1β levels indicative of persistent inflammation later in the healing process (Mercado *et al*., 2002), suggesting that stress induces alterations in the kinetics of cutaneous proinflammatory cytokine and growth factor gene expression which could impair the quality of healing tissues. Moreover, administration of IL-6 to GC-immunosuppressed mice reversed the impairment in wound healing observed in these animals but, interestingly, delayed healing in vehicle-treated control mice (Gallucci *et al*., 2001) further highlighting the presence of a delicate homeostatic system dependant on the existence of a sensitive equilibrium between pro-inflammatory mediators and anti-inflammatory GC.

5.1.4.2 GC interference with mesenchymal-epidermal signalling

GC have also been shown to interfere with several growth factor signalling pathways during wound healing. GC inhibit KGF signalling (Brauchle *et al*., 1995, Chedid *et al*., 1996, Mercado *et al*., 2002) and induce a keratinocyte proliferation-repressing switch in EGF signalling (Lee *et al*., 2005) during early wound healing. In addition, while all three TGF-β isoforms and their receptors are induced during normal wound healing, GC-treated mice display reduced TGF-β1, TGF-β2 and TGF-β2 receptor and increased TGF-β3 and TGF-β1 receptor mRNA expression (Frank *et al*., 1996). In the epidermis, increased TGF-β3 in GC-treated mice was postulated to contribute to the premature onset of keratinocyte differentiation and inhibition of epithelial cell proliferation resulting in a severe delay in re-epithelialisation seen in these animals. Moreover, exogenous application of TGF-β1 in the treatment of impaired wound healing in rats was sufficient to reverse the GC-induced impediments even when administered 24h in advance (Beck *et al*., 1993). Whilst the adverse effects of GC treatment prior to wounding are clearly exemplified by these studies, a beneficial regulatory role for GC during normal wound healing cannot be excluded. For example, whist reducing the initial inflammatory stages of the repair process, endogenous GC-induced inhibition of TGF-β signalling may enforce the effects of IL-1, acting in synergy to prevent premature tissue remodelling processes. As inflammation subsides, TGF-β signalling increases promoting the later stages of wound repair by suppressing GR-mediated transcriptional activity in a positive-feedback manner and contributing to the neuroprotective, anticatabolic and pro-wound healing properties of the TGF-β family of proteins (Ichijo *et al*., 2005).

Furthermore, IGF-1 signalling has also been recognized as an important component of tissue repair (Sorensen *et al*., 2003, Todorvic *et al*., 2008). Studies have indicated that

the wound microenvironmental IGF-1 system is also adversely affected by GC pre-treatment in rats with reduced IGF-1, IGF-1 receptor and IGF-BP3 mRNA expression rescued by co-treatment with the GR blocker RU486 (Bitar, 2000). Interestingly, this study also reported similar effects in diabetic rats, raising the possibility that GC excess may be a mechanism contributing to non-healing ulcers in sufferers of the condition. Remarkably, RU486 treatment in diabetic rats restored the IGF-1 system to a comparable degree as insulin treatment (Bitar, 2000). Similarly to TGF-β, administration of IGF-1 to GC-treated rats was able to reverse the wound healing impairments observed (Suh *et al*., 1992).

5.1.4.3 Adverse effects on tissue remodelling

Aberrant GC signalling has also been shown to interfere directly with mediators of tissue remodelling. *In vivo* studies revealed that systemic GC treatment resulted in a near-complete inhibition of the elastin-degrading, macrophage-specific, metalloelastase (MMP12) mRNA - normally expressed post re-epithelialisation in deep layers of reconstituted dermis clustering around vascular structures (Madlener *et al*., 1998), whilst levels of stromelysin-1 (MMP3), 92-kDa gelatinase (MMP9), collagenase and TIMP1 were only weakly modulated. Furthermore, stromelysin-2 (MMP10), normally induced by KGF in basal keratinocytes at the wound edge, was found to be excessively upregulated during wound healing in GC-treated mice (Madlener *et al*., 1996). However, this upregulation is likely as a result of GC modulation of MMP10 regulators other than KGF as another study reported decreased KGF expression in GC-treated mice (Brauchle *et al*., 1995). Indeed, increased MMP10 expression may be attributable, in part, to GC-induced IL-6 suppression (Gallucci *et al*., 2001).

The destructive effects of excessive GC signalling in skin is also supported by the phenotype of a transgenic mouse overexpressing GR in the epidermis (and other stratified epithelia) under control of the keratin K5 promoter, manifesting as variable-sized skin lesions ranging from epidermal hypoplasia and underdeveloped dysplastic hair follicles to a complete absence of this tissue (Perez *et al*., 2001).

Collectively, these studies demonstrate that GC are capable of differentially regulating individual MMPs, and their regulators in the dermis, as well as the finely-balanced mesenchymal-epidermal interactions - suggesting that inappropriate GC signalling may cause a dysfunction in the maintenance of homeostasis in skin resulting in wound healing impediments and compromising barrier function.

5.1.5 11β-HSD1 inhibitors and disease

Increased pre-receptor potentiation of GC signalling is now recognized to play a primary role in the progression of several metabolic disorders and as such, 11β-HSD1 inhibitors are gaining support as useful therapeutic tools.

5.1.5.1 Type 2 diabetes

Stimulation of hepatic glucose production is a key function of GC in a manner antagonistic to insulin-mediated peripheral tissue (e.g. adipose and muscle) glucose uptake. Furthermore, GC stimulate lipolysis and increase fatty acid mobilization. Excessive circulating cortisol levels (e.g. Cushing's disease) lead to a host of metabolic complications including visceral obesity, insulin resistance, glucose intolerance, dyslipidaemia, hypertension and increased cardiovascular risk. However, it is widely accepted that GC concentration can also be modulated locally though the action of 11β-HSDs.

Studies investigating tissue-specific generation of cortisol (independently of adrenal secretion) through 11β-HSD1 as a causative agent in T2D have reported an ~2-fold increase in adipose tissue activity derived from obese individuals (Rask *et al*., 2002, Kannisto *et al*., 2004). Together with a wealth of evidence from rodent studies implicating 11β-HSD1 in obesity and metabolic disease (Kotelevtsev *et al*., 1997, Morton *et al*., 2001, Masuzaki *et al*., 2001, Masuzaki *et al*., 2003, Morton *et al*., 2004, Paterson *et al*., 2004, Kershaw *et al*., 2005), support has been rapidly accumulating for clinical trials targeting 11β-HSD1 in these disorders.

Initially trialled in hyperglycaemic mice, 11β-HSD1 inhibitors were shown to reduce fasting blood glucose levels, improve insulin sensitivity and prevent progression of atherosclerosis (Hermanowski-Vosatka *et al*., 2005, Veniant *et al*., 2009). Although trials in man using the liquorice-derived compound carbenoxolone produced modest improvements in insulin sensitivity (Walker *et al*., 1995) and glucose production rates (Andrews *et al*., 2003) the non-specific inhibition of both 11β-HSD isozymes causing potential side-effects such as AME (through decreased 11β-HSD2 activity) called for the development of more effective, specific 11β-HSD1 inhibitors. Subsequent trials in healthy controls demonstrated that 11β-HSD1 inhibitors were safe, well-tolerated and specific, reducing hepatic 11β-HSD1 activity by 37% over 11 days using PF-915275 (Courtney *et al*., 2008).

Recently, a landmark trial has demonstrated increased potency, with complete hepatic and adipose inhibition of 11β-HSD1 by the specific inhibitor INCB13739 leading to improved hyperglycaemia in patients with T2D inadequately controlled by metformin monotherapy, with additional benefits regarding the lipid profile of patients with hyperlipidaemia or hypertriglyceridaemia (Rosenstock *et al*., 2010). Importantly, compensatory HPA axis activation occurred (as indicated by elevated ACTH output)

but generally remained within the reference range with daily rhythmicity, basal cortisol homeostasis, testosterone in men, and free androgen index in women remaining unchanged by INCB13739. A slight elevation in testosterone was observed in females and whist no clinical symptoms of androgen excess were recorded; trials of longer duration will be required to fully assess the potential severity of adverse side-effects in women relating to this measure.

5.1.5.2 11β-HSD1 inhibitors in other disorders

The promising results demonstrated recently for the ability of selective 11β-HSD1 inhibitors to improve clinical measures of T2D in humans has raised the possibility that these compounds could also be applicable to other disorders in which dysregulation of 11β-HSD1 has been reported.

These include age-related cognitive impairment such as Alzheimer's disease where animal studies have demonstrated improved cognition and increased long-term potentiation in aged 11β-HSD1 knockout mice relative to age-matched controls (Yau *et al*., 2007). Additionally, carbenoxolone has also been shown to improve verbal memory in elderly men and T2D (Sandeep *et al*., 2004), reflecting the beneficial effects seen for this non-selective 11β-HSD1 inhibitor for direct outcomes of T2D. Recently, Mohler *et al* published evidence for improved cognitive function in rodents following acute administration of two specific 11β-HSD1 inhibitors. Following treatment, animals exhibited improved memory consolidation and recall in inhibitory avoidance and increased CREB phosphorylation (a transcription factor involved in cognition) in the cingulate cortex in addition to improvements in short-term memory in rat social recognition testing (Mohler *et al*., 2011). Interestingly, these studies were conducted in *young* animals demonstrating that *acute* GC modulation can impact

performance in otherwise healthy subjects, building on previous observations of cognitive enhancement with a selective 11β-HSD1 in *older* rodent subjects after *chronic* lifelong treatment or lasting several days (Sooy *et al*., 2010).

Dysregulation of 11β-HSD1 may also be involved in inflammatory conditions such as rheumatoid arthritis. During an adjuvant-induced inflammatory response in joint synovium tissue of Lewis rats, 11β-HSD1 mRNA expression and 11β-HSD oxoreductase activity is upregulated leading to amplification of local GC concentrations that could reduce inflammation (Ergang *et al*., 2010). This induction appears to occur via pro-inflammatory cytokine stimulation as treatment with TNF-α and IL-1 antagonists reduced 11β-HSD1 upregulation in addition to improving the clinical symptoms (Ergang *et al*., 2010). Furthermore, blockade of GC signalling through carbenoxolone treatment caused an exacerbation of arthritis with an upregulation of pro-inflammatory cytokines (e.g. TNF-α, COX-2), raising the possibility that altered modulation of GC through 11β-HSDs at a tissue-specific level may contribute to persistent non-resolving inflammation (although the non-specificity of carbenoxolone makes interpretation of these results challenging). However, studies investigating the effects of 11β-HSD1-specific modulation in inflammatory disorders are still lacking. In human studies, GC metabolism in synovial explants taken from patients with rheumatoid arthritis and osteoarthritis was highly variable with some individuals exhibiting predominance to activate GC whereas others displayed increased GC inactivation ability (Hardy *et al*., 2008). However, the small study numbers make it difficult to extrapolate an association between GC modulation capacity and inflammation severity. Furthermore, this study suggested increased systemic 11β-HSD1 activation (through urinary metabolite analysis), although the lack of synovial explants from healthy controls cannot exclude the possibility that this

is due to 11β-HSD1 upregulation in other tissues. Although increased local GC activation is a hallmark of resolution of inflammation (Chapman *et al*., 2006), it appears to be ineffective during persistent inflammation. Interestingly, a recent study conducted in murine TNF-α activated primary preadipocytes from ob/ob or diet-induced obese mice and 3T3-L1 preadipocytes demonstrated that 11β-HSD1 overexpression increased iNOS, IL-6 and MCP-1 expression and stimulated NF-κB and MAPK signalling pathways (reversible with carbenoxolone and a selective 11β-HSD1 inhibitor) resulting in potentiation of inflammation - in complete contradiction to its traditional anti-inflammatory properties (Ishii-Yonemoto *et al*., 2010). Whether a similar mechanism occurs in persistent inflammatory disorders remains to be investigated.

5.1.5.3 Potential benefits of 11β-HSD1 inhibitors in skin

Finally, local GC regeneration inhibitors may also have applications in skin disease. Diminished rates of wound healing are a common clinical finding in diabetic subjects, leading to debilitating complications particularly in the lower extremities. In one study, diabetic rats displayed classic symptoms of decreased wound healing ability including reductions in fibroplasia, neovascularisation, inflammatory cell numbers, skin mound tensile strength, PVA sponge hydroxyproline content, and the levels of mRNA transcripts for type I and III collagen. These symptoms were reversed by insulin therapy and, interestingly, by surgical amelioration of GC signalling through adrenalectomy or pharmacological inhibition following treatment with the GR antagonist RU486 (Bitar *et al*., 1999). Interestingly, GC treatment induces cleavage of PARP (demonstrating increased activity during diabetic neuropathy) during GC-mediated apoptotic cell death (Robertson *et al*., 1997) suggesting decreased sensitivity

to GC-mediated regulation of PARP could be involved in the aetiology of diabetic neuropathy although this remains to be investigated.

Furthermore, the inhibitory effect of GC on angiogenesis was reversed with RU486 in subcutaneously implanted polyurethane sponges, healing surgical wounds and in the myocardium of mice (Small *et al*., 2005).

Moreover, 11β-HSD1 knockout mice showed enhanced angiogenesis *in vitro* and *in vivo* within sponges, wounds, and infarcted myocardium (Small *et al*., 2005), suggesting that amelioration of locally produced GC may improve healing of ischaemic or injured tissue through stimulation of angiogenesis.

5.2 Aims

Alterations in availability of GC in the extracellular *milieu* of skin are known to adversely affect several aspects of normal tissue homeostasis such as the inhibition of wound healing. Our previous data suggests that levels of 11β-HSD1 mRNA and 11β-HSD oxoreductase activity increase as a function of donor age in primary cultures of HDF and whole biopsy skin tissue explants, leading to an possible increase in the local availability of active GC. Furthermore, this effect was also shown to be apparent in donor-matched PE compared to PP samples in both young and old subjects, suggesting that increased local GC production may be a common mediator of changes in skin integrity and function in both ageing and photo-ageing. Moreover, wound healing is also known to be reduced in ageing skin, although the underlying mechanisms remain unclear.

Whilst the GC regulation of several target genes in skin has been previously investigated, particularly in response to supraphysiological concentrations of potent, synthetic GC, the potential for this to occur at pre-receptor level with physiological

concentrations of endogenous GC has not. **Our studies therefore aimed to determine whether modulation of 11β-HSD1 activity with the use of specific inhibitors could regulate the expression of GC target genes at a pre-receptor level.**

As we demonstrated levels of 11β-HSD1 activity to increase with chronological and extrinsic ageing in human skin tissue explants, **we aimed to examine possible associations between 11β-HSD oxoreductase activity in these samples and expression of GC target genes** that may partly explain the age-related alterations in skin integrity and function.

Finally, while studies have reported GC-mediated changes in the expression of genes such as collagen, MMPs and TIMPs, the regulation of other important genes involved in collagen biosynthesis and structural organization (see Section 5.1.2) - a feature of skin that is known to be greatly affected during ageing - has not been addressed. Furthermore, the effect of GC on many genes involved in the maintenance and regulation of skin tissue homeostasis (see Section 5.1.3) also remains to be investigated. **Our studies therefore aimed to identify novel GC target genes involved in these processes through which alteration in pre-receptor modulation of local GC availability may affect skin architecture and function during ageing.**

5.3 Materials and Methods

5.3.1 Sample preparation and culture

Human skin tissue biopsies and HDF were prepared according to section 2.1. HDF were treated with cortisol according to section 3.3.1.2. For pre-receptor regulation of target gene expression, HDF and skin tissue biopsies were incubated with 100nM

cortisone for 72h with/without 1μM the 11β-HSD1-specific inhibitor LJ2 (PF-877423, Pfizer, New York, USA).

5.3.2 Real-time PCR gene expression

RNA extraction, reverse transcription and qPCR were conducted according to section 2.3, using the primers and probes described in section 3.3.2 and the following (Applied Biosystems, California, USA):

Human: COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), MMP1 (Hs00899658_m1), TIMP1 (Hs00171558_m1) and IGFBP5 (Hs00181213_m1)

5.3.3 Fluidigm BioMark targeted gene expression array

5.3.3.1 Principle

The BioMark system (Fluidigm, California, USA) uses a cDNA sample and gene expression assay starting volume of 5μl (loaded on opposing sides of the chip), and distributes these in nanoliter volumes across individual reaction chambers on a central Integrated Fluidic Circuit (IFC) such that each sample can be assayed for each gene. Up to 96 samples and standard TaqMan gene expression assays (Applied Biosystems, California, USA) can be loaded, enabling up to 9216 qPCR reactions per run.

Due to the micro-volumes involved, specific target amplification is required to detect target gene expression.

5.3.3.2 Methods

RNA extraction and reverse transcription were conducted according to sections 2.3.1, 2.3.2 and 2.3.3.

Specific target amplification: conducted in 5µl volumes consisting of 2.5µl TaqMan PreAmp Master Mix (2X, Applied Biosystems, California, USA), 1.25µl pooled TaqMan gene expression assay (1µl each assay and 4µl 1X TE buffer, 0.2X final assay concentration) and 1.25µl cDNA (replaced with 1X TE for negative control). Following brief vortexing and centrifugation, reactions occurred as follows: 95ºC for 10min, followed by 14 cycles of 95ºC for 15sec and 60ºC for 4min. Samples were subsequently diluted 1:5 with 20µl 1X TE buffer. Following preamplification, 1µl of each sample was pooled and serially diluted in 1X TE buffer (1:10, 1:100, 1:1000 and 1:10000) for the standard curve.

Sample Mix: Samples (in triplicate) and standards (in duplicate) were prepared for loading onto the chip in 8µl volumes, in a 96-well plate, by combining 4µl TaqMan Universal PCR Master Mix (2X), 0.4µl GE Sample Loading Reagent (20X, Fluidigm, California, USA) and 3.6µl preamplified cDNA/standard. Plates were vortexed, centrifuged and stored at 5ºC until required.

Assay Mix: Gene expression assays were prepared in 8µl volumes (or scaled up for multiple chips) in a 96-well plate by combining 4µl TaqMan Gene Expression Assay (20X) and 4µl Assay Loading Reagent (2X, Fluidigm, California, USA), giving final concentrations of 9µM and 2µM for the primers and probe for each assay respectively. Plate was vortexed, centrifuged and stored at 5ºC until required.

Prior to loading, chips were primed using the IFC controller according to manufacturer's protocol (Fluidigm, California, USA) in order to close the interface valves and prevent premature mixing of samples and assays. Chips were then loaded (within 60min of priming) with 5µl Sample Mix and 5µl Assay Mix (leaving 3µl excess each), taking care not to introduce air bubbles into the inlets and loaded using

the IFC controller. Loaded chips were run on a BioMark qPCR reader according to manufacturer's protocol (Fluidigm, California, USA).

Following standard curve verification, ΔCt values were determined for each sample by normalizing to the geometric mean of 4 housekeeping genes (RPL13, TBP, B2M and PPIA) and the average of triplicates ΔCts calculated.

5.3.3.3 Gene expression assays

The following 96 human gene expression assays were used (Applied Biosystems, California, USA):

Collagen biosynthesis and processing: COL1A1 (Hs00164004_m1), COL3A1 (Hs00943809_m1), PEPD (Hs00944654_m1), LEPRE1 (Hs00223565_m1), PLOD1 (Hs00609368_m1), PLOD2 (Hs00168688_m1), PLOD3 (Hs00153670_m1), LEPREL1 (Hs00216998_m1), LEPREL2 (Hs00204607_m1), P4HA1 (Hs00914594_m1), P4HA2 (Hs00990001_m1), P4HA3 (Hs00420085_m1), P4HB (hs00168586_m1), SERPINH1 (Hs00241844_m1), LOX (Hs00184700_m1), LOXL1 (Hs00935937_m1), LOXL2 (Hs00158757_m1), LOXL3 (Hs01046945_m1), LOXL4 (Hs00260059_m1), ADAMTS2 (Hs00247973_m1), BMP1 (Hs00986789_m1), DCN (Hs00754870_s1), MMP1 (Hs00899658_m1), MMP2 (Hs01548727_m1), MMP3 (Hs00968305_m1), MMP8 (Hs01029057_m1), MMP10 (Hs00233987_m1), MMP12 (Hs00899662_m1), MMP13 (Hs00233992_m1), TIMP1 (Hs00171558_m1), TIMP2 (Hs00234278_m1), TIMP3 (Hs00165949_m1), TIMP4 (Hs00162090_m1)

Structural components: HAS1 (Hs00987418_m1), HAS2 (Hs00193435_m1), HAS3 (Hs00193436_m1), HYAL1 (Hs00201046_m1), HYAL2 (Hs00186841_m1), HYAL3 (Hs00185910_m1), HYAL4 (Hs00202177_m1), HYALP1 (Hs00395402_m1), SPAM1 (Hs00162139_m1), FBN1 (Hs00171191_m1), FBN2 (Hs00266592_m1),

ELN (Hs00355783_m1), HSD17B7 (Hs00996127_m1), VIM (Hs00185584_m1), MYH11 (Hs00224610_m1)

Stress response: NR3C1 (Hs00353740_m1), HSD11B1 (Hs01547870_m1), HSD11B2 (Hs00388669_m1), H6PD (Hs00188728_m1), GSTM1 (Hs01683722_gH), GSTT1 (Hs00184475_m1), HSF1 (Hs00232134_m1), HSPB1 (Hs03044127_g1), SOD1 (Hs00533490_m1), SOD2 (Hs00167309_m1), SOD3 (Hs00162090_m1), HMOX1 (Hs01110250_m1), SIRT1 (Hs01009006_m1), NFE2L2 (Hs00232352_m1)

AP-1 signalling: FOSB (Hs00171851_m1), FOSL1 (Hs04187685_m1), FOSL2 (Hs00232013_m1), DUSP1 (Hs00610256_g1), MAPK8 (Hs00177083_m1), MAPK9 (Hs00177102_m1), MAPK11 (Hs00177101_m1)

Cell cycle control and growth factors: CDKN2A (Hs00923894_m1), TP53 (Hs01034249_m1), FGF7 (Hs00384281_m1), TGFB1 (Hs00998133_m1)

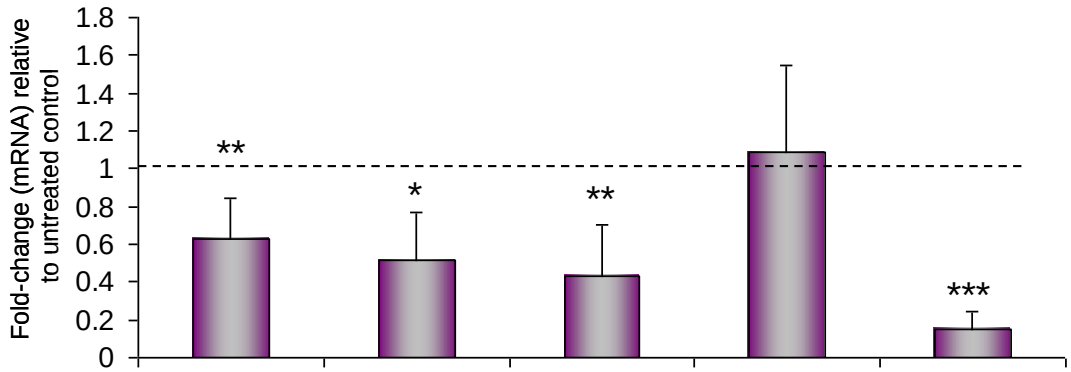
Other signalling: IGF1 (Hs01547656_m1), IGF1R (Hs00609566_m1), IGFBP1 (Hs00426285_m1), IGFBP2 (Hs01040719_m1), IGFBP3 (Hs00181211_m1), IGFBP4 (Hs00181767_m1), IGFBP5 (Hs01052296_m1), IGFBP5 (Hs00181231_m1), IGFBP6 (Hs00942697_m1), IGFBP7 (Hs00266026_m1), PPARA (Hs00231882_m1), PPARG (Hs00234592_m1), PPARGC1A (Hs01016724_m1), AR (Hs00171172_m1), ESR1 (Hs00174860_m1), ESR2 (Hs00230957_m1), PGR (Hs00172183_m1), CXCL1 (Hs00236937_m1)

Housekeepers: RPL13 (Hs00744303_s1), ACTB (Hs99999903_m1), TBP (Hs00427621_m1), B2M (Hs00984230_m1), PPIA (Hs04194521_s1)

5.4 Results

5.4.1 GC regulation of target genes in HDF

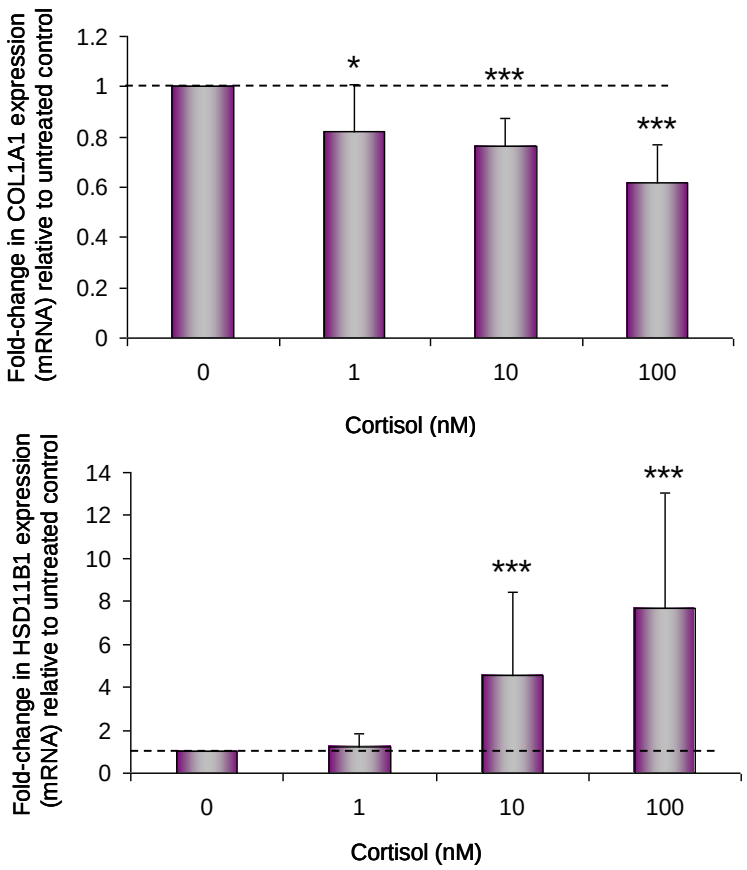
Our earlier studies demonstrated the autoregulatory positive-feedback induction of 11β-HSD1 mRNA expression and 11β-HSD oxoreductase activity in HDF following GC treatment leading to increased generation and potential accumulation of cortisol in the cellular microenvironment (Chapter 3). Subsequently, we aimed to confirm the GC-mediated regulation of several known target genes; COL1A1, COL3A1, MMP1, TIMP1 and IGFBP5, that play key roles in the structure and/or functional properties of skin. Results indicated a decrease in COL1A1, COL3A1, MMP1 and IGFBP5 with no significant effect in TIMP1 mRNA expression (Figure 5-4).



ΔCt	COL1A1	COL3A1	MMP1	TIMP1	IGFBP5
C	8.1	7.7	11.6	8.1	11.3
S.D	0.7	1.0	2.0	1.4	1.3
F	8.9	8.8	13.0	8.2	14.2
S.D	1.1	1.6	2.6	1.4	1.6

Figure 5-4 GC-regulated target gene expression in HDF. Following 100nM cortisol treatment and normalizing for 18S rRNA, mRNA gene expression of collagen 1 (COL1A1, n=8), collagen 3 (COL3A1, n=6), MMP1 (n=6) and insulin-like growth factor binding protein 5 (IGFBP5, n=6) were downregulated whilst TIMP1 (n=6) was unaffected. Significance * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$

Furthermore, COL1A1 mRNA expression was inhibited even at 1nM cortisol concentrations, an order of magnitude lower than those required to induce 11β-HSD1 expression (Figure 5-5).



ΔCt	Control	1nM	10nM	100nM
COL1A1	8.0	8.3	8.4	8.7
S.D	0.9	0.8	0.9	1.1
HSD11B1	24.0	23.8	22.1	21.3
S.D	2.8	2.8	3.2	3.2

Figure 5-5 GC dose-response effect on target gene expression. Following normalizing for 18S rRNA, mRNA gene expression of collagen 1 (COL1A1) and 11β-HSD1 (HSD11B1) was shown to be regulated in a dose-dependant manner in HDF (n=9). Treatment with 10 and 100nM cortisol downregulated COL1A1 expression and upregulated HSD11B1. Furthermore, 1nM cortisol was also able to regulate COL1A1 but not HSD11B1. Significance * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$

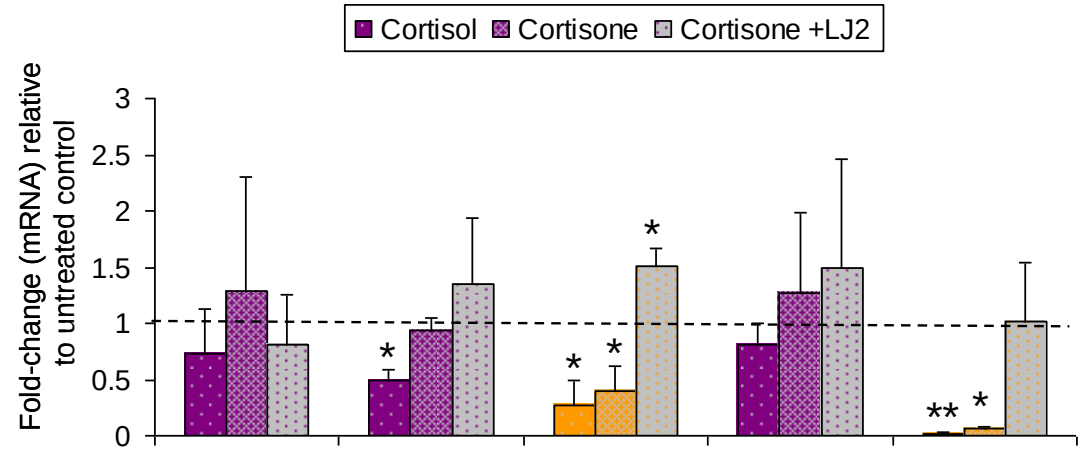
These results confirm the GC-mediated regulation of known GC target genes and indicate that differences in sensitivity to local GC concentrations may exist between different genes and, as suggested by the large variation in response for the same gene, also between different donors.

5.4.2 11β-HSD1-specific regulation of GC target genes in HDF

Next, we examined the possibility that these GC target genes could be regulated by GC at a pre-receptor level. Experiments were conducted in representative HDF from

one donor that displayed detectable 11β-HSD oxoreductase activity levels at baseline (539 ± 331 fmol cortisol/mg cells/h, n=4). Although there was considerable variation in 11β-HSD oxoreductase activity between experimental replicates, the resulting concentrations of cortisol generated following 72h incubation with 100nM cortisone were within the range required to regulate target gene expression (19.4 ± 11.9nM, n=4).

Indeed, cortisone was shown to downregulate MMP1 and IGFBP5 to a similar degree as 100nM cortisol in these cells, and importantly, this regulation was prevented by co-incubation with the 11β-HSD1-specific inhibitor LJ2 (Figure 5-6). However, cortisone failed to replicate the cortisol-induced downregulation of COL3A1, whilst COL1A1 and TIMP1 did not respond to cortisol treatment in these cells (Figure 5-6), again possibly reflecting the variation in response to GC between different donors.



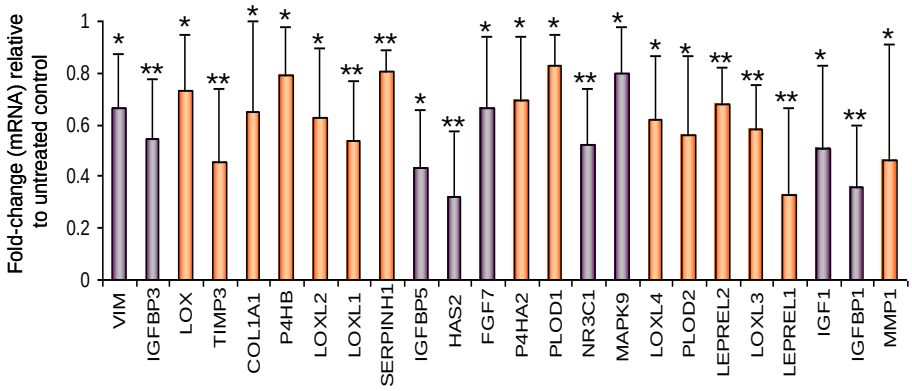
ΔCt	COL1A1	COL3A1	MMP1	TIMP1	IGFBP5
C	7.7	8.2	12.3	9.5	13.8
S.D	0.7	0.3	0.3	0.4	1.1
F	8.3	9.2	14.5	9.9	19.3
S.D	0.6	0.1	1.9	0.6	0.9
E	7.6	8.3	13.8	9.3	18.0
S.D	0.8	0.4	1.2	0.7	1.9
E+LJ2	8.1	7.8	11.9	9.1	13.9
S.D	0.2	0.8	0.4	0.7	0.6

Figure 5-6 11β-HSD1-specific regulation of GC target gene expression. Following 72h incubation with 100nM cortisone and normalizing for 18S rRNA (n=3), MMP1 and IGFBP5 mRNA expression was downregulated to a similar extent to that seen following 100nM cortisol treatment. No regulation was observed following co-incubation of cortisone with the 11β-HSD1-specific inhibitor LJ2. Significance * = $p<0.05$ and ** = $p<0.01$

5.4.3 Novel GC target genes in HDF

As our results demonstrated that previously known GC target genes (e.g. MMP1) are capable of regulation through modulation of 11β-HSD oxoreductase activity, subsequent studies used a targeted gene expression array approach to investigate the GC regulation of other potential GC targets involved in maintenance of dermal integrity and homeostasis in HDF. The selected candidates included proteins involved in collagen maturation (e.g. endopeptidases, decorin), collagen structural organization (e.g. prolidase, prolyl 3- and 4-hydroxylases, lysyl oxidases and hydroxylases), collagen degradation (e.g. MMPs and TIMPs), other structural components (e.g. fibrillin, elastin, hyaluronic acid synthases), stress response components (e.g. superoxide dismutases, heat shock proteins) and dermal signalling molecules (e.g. TGF-β, KGF/FGF7, MAPKs). Furthermore, a set of known GC-regulated genes were included as internal controls (e.g. 11β-HSD1, MMP1, COL1A).

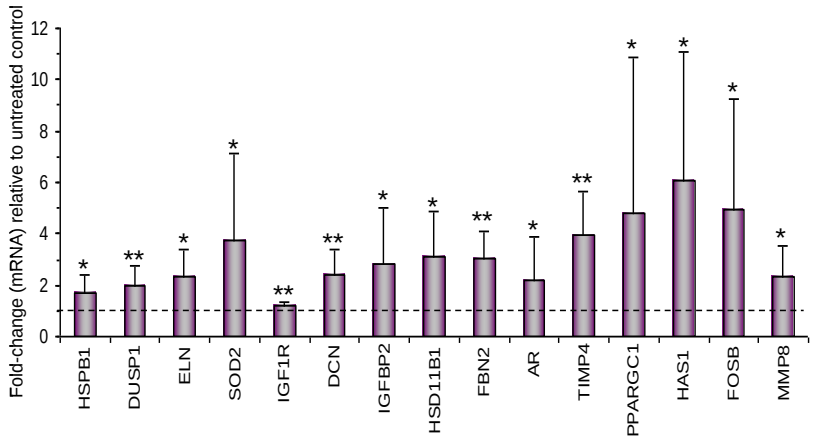
Following treatment with 100nM cortisol, the mRNA expression of 24 genes was downregulated. The majority of these were genes involved in collagen biosynthesis, maturation and organization - including 5/5 lysyl oxidases which catalyze collagen cross-link formation, 4/7 prolyl hydroxylases involved in stabilization of newly formed procollagen triple helices and 2/3 lysyl hydroxylases responsible for the formation of hydroxylysine residues required for effective collagen crosslinking (Figure 5-7). Furthermore, expression of the collagen chaperone hsp47 (SERPINH1) was also downregulated by GC treatment. The internal controls reported a decrease in GR-α (NR3C1), COL1A1, MMP1 and IGFBP5 expression as expected from our earlier studies. In addition, mRNA expression of a further 15 genes was upregulated by GC treatment (Figure 5-8).



ΔCt	VIM	IGFBP3	LOX	TIMP3	COL1A1	P4HB	LOXL2	LOXL1	SERPINH1	IGFBP5	HAS2	FGF7
C	-6.7	-6.5	-5.2	-3.4	-2.8	-2.4	-1.8	-1.5	-1.4	-1.0	-1.0	-0.9
S.D	0.2	0.6	0.3	0.6	0.5	0.3	1.1	0.6	0.6	2.5	0.9	0.7
F	-6.0	-5.6	-4.7	-2.1	-2.0	-2.1	-1.0	-0.6	-1.1	0.4	1.1	-0.2
S.D	0.6	0.4	0.4	0.9	1.0	0.2	0.9	0.8	0.6	2.6	0.9	0.7

P4HA2	PLOD1	NR3C1	MAPK9	LOXL4	PLOD2	LEPREL2	LOXL3	LEPREL1	IGF1	IGFBP1	MMP1
-0.7	-0.3	0.0	1.0	1.3	1.5	2.6	4.5	4.6	7.6	10.2	12.6
0.4	0.3	0.1	0.3	0.5	1.1	0.3	0.9	0.9	2.2	1.1	1.7
-0.1	0.0	1.0	1.4	2.1	2.5	3.1	5.3	6.6	8.8	12.0	14.1
0.3	0.3	0.6	0.3	0.4	0.4	0.4	0.8	0.9	2.0	1.3	0.9

Figure 5-7 Genes downregulated by GC treatment of HDF. Following 100nM cortisol treatment (F) and normalizing for housekeeper gene expression, 24 genes were found to be downregulated in HDF (n=6) relative to untreated controls (C). These included a large proportion of collagen biosynthesis regulators and remodelling enzymes (orange bars). Genes listed in order of increasing baseline expression as detailed in the table. Significance * = p<0.05, ** = p<0.01



ΔCt	HSPB1	DUSP1	ELN	SOD2	IGF1R	DCN	IGFBP2
C	-2.6	-1.6	-0.5	-0.2	-0.1	0.0	1.3
S,D	0.5	0.4	0.9	1.0	0.3	0.7	1.5
F	-3.3	-2.5	-1.5	-1.7	-0.3	-1.2	0.1
S.D	0.5	0.7	0.8	1.0	0.3	0.5	0.9

HSD11B1	FBN2	AR	TIMP4	PPARGC1	HAS1	FOSB	MMP8
5.2	5.3	6.2	8.1	10.2	12.3	13.0	15.5
1.1	0.9	1.0	0.6	1.0	2.1	0.9	1.9
3.9	3.8	5.3	6.3	8.6	10.4	11.1	14.0
0.3	0.6	1.4	0.6	1.6	2.1	1.8	1.7

Figure 5-8 Genes upregulated by GC treatment of HDF. Following 100nM cortisol treatment (F) and normalizing for housekeeper gene expression, 15 genes were found to be upregulated in HDF (n=6) relative to untreated controls (C). Genes listed in order of increasing baseline expression as detailed in the table. Significance * = p<0.05, ** = p<0.01

The function of these genes was more variable, ranging from stress response resistance (e.g. HSPB1, DUSP1, SOD2) to signalling modulators (AR, PPARGC1, FOSB) and collagen remodelling enzymes (DCN, TIMP4, MMP8). Surprisingly, expression of elastin (ELN), fibrillin (FBN2) and the hyaluronic acid synthesis enzyme HAS2 was also upregulated - suggesting that these structural proteins may not be as susceptible to GC-induced atrophy as the collagens. Again, as shown in our previous studies expression of 11β-HSD1 (HSD11B1) was upregulated by GC treatment and served as a positively regulated internal control for the arrays.

5.4.4 11β-HSD1-specific regulation of GC target genes in human skin ex vivo

Our results have shown that 11β-HSD oxoreductase activity increases with ageing and photo-ageing in human skin and that GC are capable of modulating a range of target genes, including at a pre-receptor level in HDF. Subsequently, we investigated the possibility that increased 11β-HSD oxoreductase activity in human skin may have a direct effect on GC target genes through 11β-HSD1 and that this may contribute to the pathological consequences of skin ageing and photo-ageing.

Using the same targeted gene expression array employed for the *in vitro* studies described above, we examined the relationship between 11β-HSD oxoreductase activity and expression of GC target genes in tissue-matched 100nM cortisone-treated human skin biopsies. Our results revealed a correlation between enzyme activity and target gene expression in several genes including the lysyl oxidase LEPREL1 which was found to decrease with increasing enzyme activity ($n=16$, $r^2=0.36$, $p<0.05$, Figure 5-9).

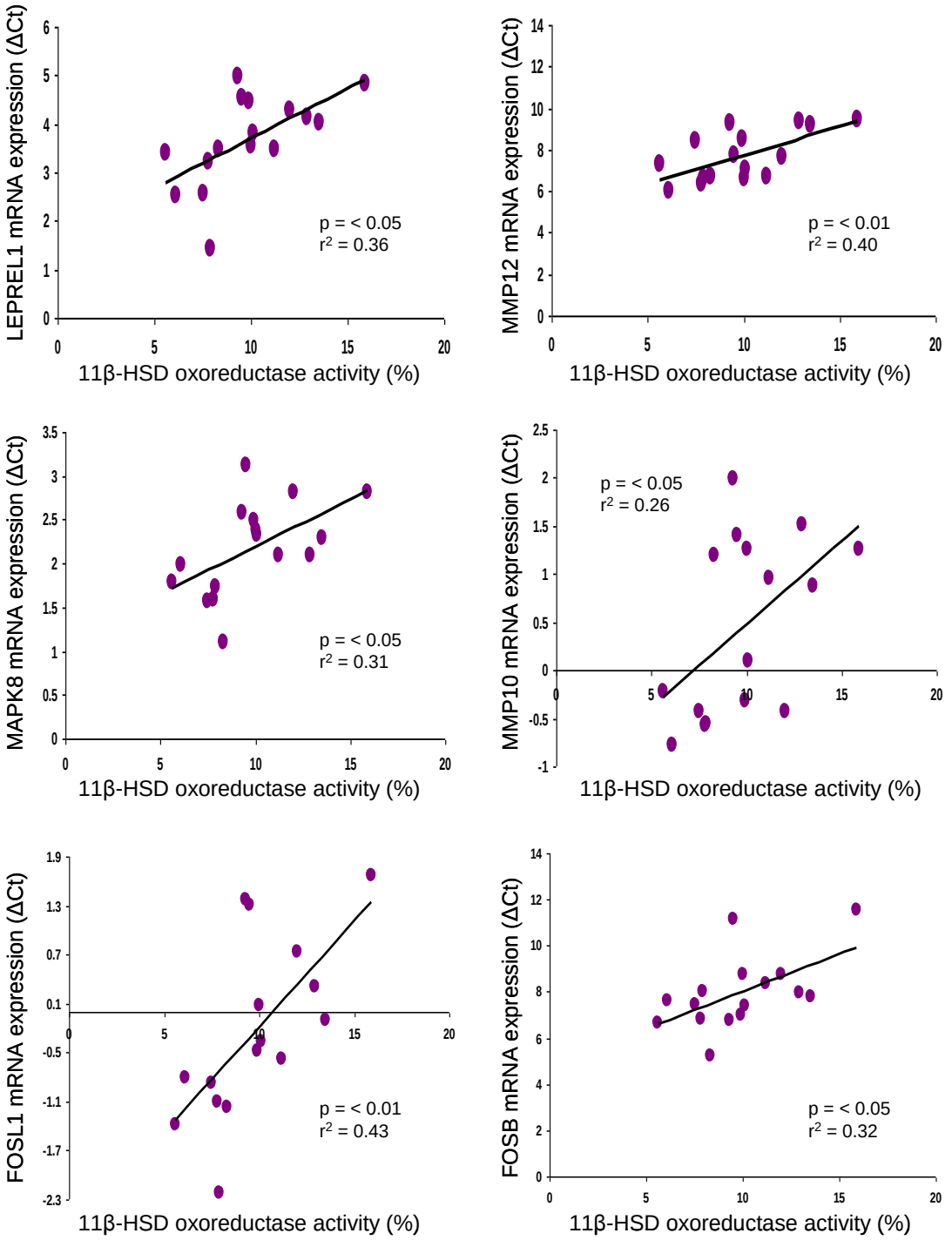


Figure 5-9 11β-HSD oxoreductase activity associates with gene expression in cortisone-treated human skin biopsies. Following 100nM cortisone treatment and normalizing for housekeeper gene expression, mRNA expression of LEPREL1, MMP10 and MMP12 (involved in collagen processing and remodelling) and MAPK8, FOSB and FOSL1 (involve in AP-1 signalling) was found to decrease with increasing 11β-HSD oxoreductase activity in human skin biopsies (n=16)

The expression of collagen remodelling MMP10 and MMP12 (n=16, r^2=0.26, p<0.05 and r^2=0.40, p<0.01 respectively, Figure 5-9), AP-1 complex regulatory proteins MAPK8, FOSB and FOSL1 (n=16, r^2=0.31, p<0.05, r^2=0.32, p<0.05 and r^2=0.43,

$p<0.01$ respectively, Figure 5-9) and stress resistance genes DUSP1, HMOX1, SIRT1 and NFE2L2 ($n=16$, $r^2=0.30$, $p<0.05$, $r^2=0.30$, $p<0.05$, $r^2=0.41$, $p<0.01$ and $r^2=0.48$, $p<0.01$ data not shown) displayed a similar association between increased enzyme activity and decreased mRNA expression. Conversely, mRNA expression of the antioxidant enzyme SOD3, oestrogen receptor ESR1 and the GC inactivating enzyme HSD11B2 ($n=16$, $r^2=0.25$, $p<0.05$, $r^2=0.41$, $p<0.01$ and $r^2=0.34$, $p<0.05$ respectively) was found to increase with increasing 11β-HSD oxoreductase activity (data not shown).

5.5 Discussion

The ability of GC to downregulate collagen mRNA expression is widely accepted as a fundamental component of the resultant detrimental consequences in skin following increased GC exposure of therapeutic origin (Slavin *et al*., 1994, Oikarinen *et al*., 1998, Oishi *et al*., 2002, Nuutinen *et al*., 2003, Jacques *et al*., 2010). Furthermore, the endogenous GC cortisol has also been shown to decrease type 1 pro-collagen expression in HDF following physiological (Oikarinen *et al*., 1983) and supraphysiological (Hamalainen *et al*., 1985) treatment, reaching a maximal 50% reduction in expression attributable to decreased mRNA stability rather than reduced transcription.

Consistent with these studies, we observed a dose-dependant decrease in type-1 procollagen-α1 (COL1A1) mRNA expression, reaching ~40% downregulation following treatment with physiological concentrations of cortisol (100nM) in HDF. Our results also demonstrated a similar reduction in type 3 procollagen-α1 (COL3A1), supported by previous findings reporting a decrease in the propeptide of type-3 collagen following *in vivo* topical or systemic GC therapy (Oikarinen *et al*., 1992,

Autio *et al*., 1994). Interestingly, Oishi *et al*. reported a greater reduction in collagen type-3 compared to type-1 (Oishi *et al*., 2002) and although we observed a similar trend towards a greater reduction in COL3A1 mRNA expression following GC treatment this was not statistically significant.

Likewise, the reduction in the interstitial type-1, -2 and -3 collagenase (MMP1) expression by GC treatment has been previously identified in skin explants (Koob *et al*., 1980, Oishi *et al*., 2002) and dermal fibroblasts (Bauer *et al*., 1985). The mechanism for this repression has been elucidated to be strongly dependant on AP-1 inactivation (Jonat *et al*., 1990, Karin and Chang, 2001), as AP-1 serves as a potent enhancer for collagenase expression. Our data are in support of these findings, as MMP1 mRNA expression was also downregulated by GC treatment in HDF. However, we found TIMP1 expression not to be regulated by GC treatment, in contrast to rodent *in vivo* (Oishi *et al*., 2002) and human dermal fibroblast studies (Slavin *et al*., 1994) which reported a decrease in expression. Alternatively, unpublished observations by Madlener *et al*. suggest that TIMP1 expression is only weakly regulated in dexamethasone-treated mouse models of wound healing (Madlener *et al*., 1998). These conflicting data may be attributable to differences in experimental model employed, GC potency and/or concentration used, but in our *in vitro* system physiological concentrations of endogenous cortisol do not significantly affect TIMP1 mRNA expression in HDF.

Although GC are known to adversely affect collagen expression, the most substantial reductions in expression are often seen following potent topical synthetic GC application. Additionally, Bernstein *et al*. reported that although striking alterations in papillary dermis architecture between donor-matched PE compared to PP skin existed with deformed collagen fibres of varying diameters and increased elastin deposits in

the former (Figure 5-10), collagen gene expression remained essentially unchanged (Bernstein *et al*., 1996). This study also demonstrated that collagen protein expression (type-1 and type-3) is decreased in PE skin, suggesting that post-translational events, rather than changes in transcription, are responsible for the collagen phenotype of PE skin.

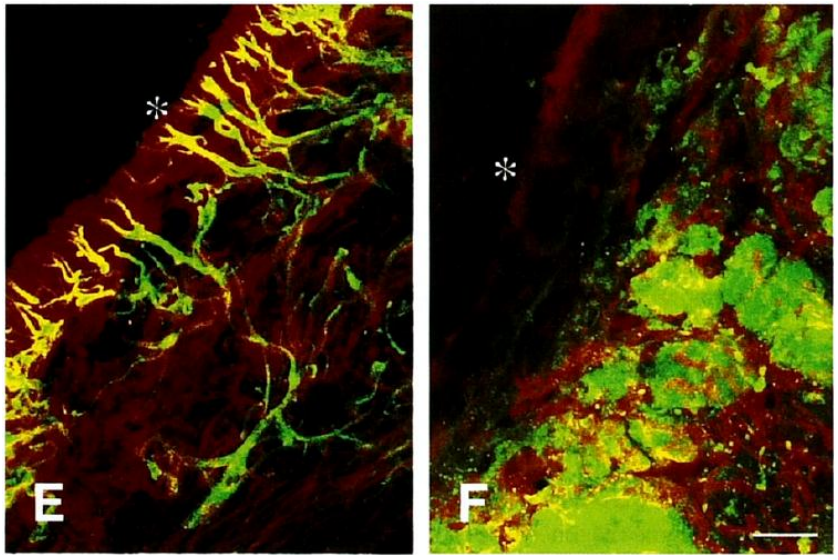


Figure 5-10 Structural differences between PP and PE human skin. Donor-matched PP (buttock, E) and PE (neck, F) skin samples stained for collagen (red) and elastin (green) fibres reveal a highly organized structure in PP skin, with a dense band of collagen below the DEJ (*) absent from PE skin. The latter also contains increased deposits of elastin, although the complete lack of structure is seen. Scale bar 10μM. Reproduced from Bernstein *et al*., 1996.

Our results also demonstrated insulin-like binding protein 5 (IGFBP5) to be regulated by cortisol in HDF, with >80% reduction in mRNA expression following physiological GC treatment, as previously demonstrated for the synthetic GC dexamethasone (Conover *et al*., 1995). Initially associated with conditions of excessive fibrosis such as idiopathic pulmonary fibrosis (Pilewski *et al*., 2005), IGFBP5 has been shown to induce collagen and fibronectin production and promote fibroblast to myofibroblast differentiation in murine skin (Yasuoka *et al*., 2006). Interestingly, increased IGFBP5 expression has been linked to HDF replicative senescence (Yoon *et al*., 2004) and our observations regarding the negative regulation of this gene by GC (combined with the age-related increase in GC activating

capability of skin) could represent a compensatory mechanism to prevent senescence-associated fibrotic disease (Garcia *et al*., 2007). Additionally, IGFBP5-induced senescence in human umbilical endothelial cells was dependant on induction of the tumour suppressor p53 (Kim *et al*., 2007). More recently, increased IGFBP5 expression was reported in keloid-derived HDF, which are also largely resistant to GC (Russell *et al*., 2010).

Our earlier studies demonstrated an increased capacity for HDF from older individuals and from donor-matched PE compared to PP sites to generate the active GC cortisol through increased 11β-HSD1 mRNA expression and activity. Here, we investigated whether this increase is capable of specific modulation of target gene expression, and therefore potentially contributing to an altered GC-induced regulatory profile in old and PE fibroblasts. Indeed, the negative regulation of MMP1 and IGFBP5 by cortisol was reproduced to a comparable degree with cortisone, and prevented by co-incubation of cortisone with the 11β-HSD1-specific inhibitor LJ2. The lack of TIMP1 regulation by cortisol was also consistent with a lack of 11β-HSD1-specific regulation.

However, although cortisol was shown to downregulate COL1A1 and COL3A1 in a range of HDF strains, only COL3A1 was significantly downregulated by cortisol in the strain used in our inhibitor studies - although this is more likely as a result of the low experimental replicates rather than due to a lack of GC responsiveness. Furthermore, neither COL1A1 nor COL3A1 was regulated by cortisone. This may be attributable to a reduced sensitivity of collagen mRNA expression to the levels of active GC generated through 11β-HSD1 which, although predicted to be high enough to elicit a response in a range of fibroblast strains (COL1A1 was significantly downregulated by 1nM cortisol), may not be sufficient in the strain used for our

inhibitor studies. Dose-response experiments for MMP1 and IGFBP5 would aid in clarifying this issue. Nevertheless, our results provide the first evidence for 11β-HSD1-specific regulation of GC target genes in HDF and a possible mechanism through which the age-related increases in GC activation capability of fibroblasts may impact on their ability to maintain effective homeostasis.

As the structural organization of collagen is known to be grossly altered in skin during ageing and photo-ageing, arguably more so than the production of collagen itself, we developed a targeted mRNA expression array to investigate whether any of the modulators of normal collagen biosynthesis and remodelling are regulated by GC in HDF. Our results revealed a striking effect on many elements of the collagen biosynthesis pathway following treatment with physiological concentrations of cortisol (Figure 5-11). In addition to downregulation of previously identified collagen targets (COL1A1, MMP1), cortisol treatment reduced expression of 4 prolyl hydroxylases (LEPREL1, LEPREL2, P4HA2 and P4HB), 2 lysyl hydroxylases (PLOD1 and PLOD2), a collagen chaperone (SERPINH1) and all 5 lysyl oxidases (LOX, LOXL1, LOXL2, LOXL3 and LOXL4).

The regulation of prolyl hydroxylase by GC in skin was previously limited to studies in rats demonstrating a decrease in activity (Cutroneo and Counts, 1975, Benson and Luvalle, 1981). Recently, Vranka *et al*. characterized the phenotype of the prolyl 3-hydroxylase 1 (LEPRE1) null mouse which, in addition to bone and tendon abnormalities displayed differences in skin architecture including a less densely packed dermis with fewer collagen fibrils in the skin of newborn knockout mice and skin thinning in the knockout adult containing clumped areas of collagen fibrils with some gaps and spaces in between collagen fibrils absent in the WT controls.

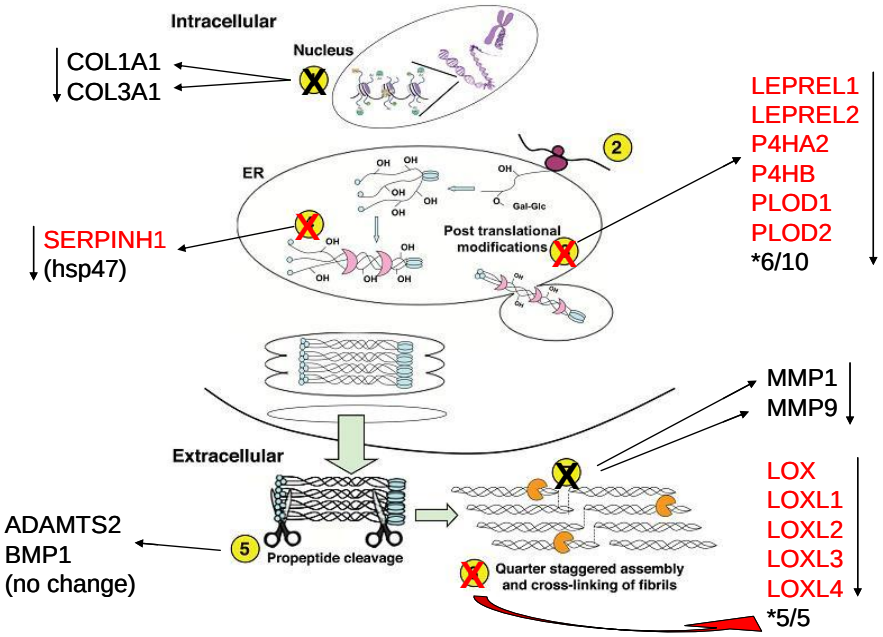


Figure 5-11 GC treatment affects multiple elements of the collagen biosynthesis pathway in HDF. In addition to the widely accepted downregulation of collagen transcription e.g. COL1A1 and COL3A1 and collagen degrading enzymes e.g. MMP1, MMP9 (black crosses), our studies identified novel GC targets in collagen biosynthesis involving a reduction in post-translation hydroxylation enzymes LEPREL1, LEPREL2, P4HA2, P4HB, PLOD1 and PLOD2, the collagen chaperone hsp47 (SERPINH1) and all lysyl oxidase enzymes LOX, LOXL1, LOXL2, LOXL3 and LOXL4 responsible for inter- and intra-fibril cross-link formation (red crosses). Modified from Chen and Raghunath, 2009

These findings are echoed by mutations in LEPRE1 in humans which cause a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta (Cabral *et al*., 2007), with patients also exhibiting overmodification of type I procollagen chains in dermal fibroblast cultures from affected individuals (Willaert *et al*., 2009). Prolyl 4-hydroxylase downregulation by GC in HDF is another novel finding. This enzyme is also critical in the correct folding of newly synthesized collagen propeptides, which are retained in the ER in the case of vitamin C deficiency - as vitamin C is an essential co-factor for the enzyme (Walmsley *et al*., 1999).

Likewise, lysyl hydroxylase regulation by GC in HDF has not previously been reported. However, a deficiency in the activity of this enzyme (PLOD1) is known to cause Ehlers Danlos syndrome type VI characterized by neonatal kyphoscoliosis, generalized joint laxity, skin fragility, and severe muscle hypotonia at birth (Yeowell and Walker, 2000). Alternatively, mutations in PLOD2 cause Bruck syndrome (van

der Slot *et al*., 2003), which particularly affects bone collagen cross-linking (Bank *et al*., 1999), although PLOD2 overexpression has also been linked to skin fibrosis (van der Slot *et al*., 2003). More recently, a mutation in the third lysyl hydroxylase isozyme has been described (Salo *et al*., 2008) which features thin skin and blistering components of disease manifestation.

GC-induced downregulation of SERPINH1 has been reported for primary human lung fibroblasts (Goulet *et al*., 2007) and we provide additional evidence that this is also the case for HDF. Hsp47 monitors the integrity of the triple helix of type I procollagen at the ER/cis-Golgi boundary and, when absent, the rate of transit from the ER to the Golgi is increased and helical structure is compromised as seen in patients deficient in SERPINH1 who present with an additional severe variant of osteogenesis imperfecta, although lacking a skin phenotype (Christiansen *et al*., 2010).

Our results indicated a decrease in all lysyl oxidase isozyme mRNA expression following GC treatment in agreement with previous rodent studies reporting a decrease in enzyme activity after local GC administration (Benson and Luvalle, 1981), although a more recent study failed to record any effect on mRNA expression in human skin following *in vivo* treatment with the potent synthetic GC betamethasone-17-valerate (Oikarinen *et al*., 1998). Decreased lysyl oxidase activity has been identified as a cause of another Ehlers Danlos syndrome variant - type IX and with the clinically distinct Menkes syndrome both involving skin laxity and hyperextensibility (Kiuvaniemi *et al*., 1985).

Collectively, the identification of these regulators of collagen biosynthesis as novel GC targets in HDF raises the possibility that altered local GC availability could contribute to several aspect of altered collagen structural organization in ageing skin.

Our results demonstrate that hyaluronan synthase 2 (HAS2) exhibited the strongest inhibition in expression following GC treatment of HDF. Although previous groups have demonstrated the ability of dexamethasone to inhibit this enzyme in HDF (Zhang *et al*., 2000) and *in vivo* human skin (Gebhardt *et al*., 2010), providing a mechanism through which GC inhibit hyaluronic acid production, our data provide additional evidence that physiological concentrations of endogenous GC have the same effect. Moreover, IGF-1 has been identified as a positive regulator for HAS-2 (Kuroda *et al*., 2001) and our studies demonstrated negative regulation of this growth factor by cortisol, potentially identifying part of the mechanism through which GC inhibit HAS2 expression.

The cytoskeletal microfilament vimentin was also downregulated by cortisol in our *in vitro* studies. Previous reports into the GC-mediated regulation of this highly expressed structural protein are lacking, however a global knockout mouse model failed to detect any morphological differences in the absence of vimentin compared to WT control animals, including skin (Colucciguyon *et al*., 1994) and remarkably even during induction of skin remodelling following wounding. Furthermore, a recent study revealed a stiffening in HDF derived from older donors characterized by a shift from monomeric G-actin to polymerized, filamentous F-actin, but no significant changes in the vimentin content (Schulze *et al*., 2010), suggesting that the modest decrease in vimentin following GC treatment we observed is unlikely to contribute to any phenotypic modification in skin structural properties during ageing. However, further detailed analyses reported significant defects in wound healing (Eckes *et al*., 2000) and endothelial cell stability (Nieminen *et al*., 2006). Vimentin glycation has also been implicated recently in the skin ageing process (Kueper *et al*., 2007), and the role GC play (if any) in this modification also remains to be elucidated.

Although GC-mediated IGF-1 regulation in HDF is previously unreported, a reduction in IGF-1 signalling in senescent fibroblasts *in vitro* and geriatric dermis has been suggested to contribute to an inappropriate UVB response by epidermal keratinocytes that may lead to an increased risk of ageing-related non-melanoma skin cancer (Lewis *et al*., 2010). In addition to IGF-1, we found other element of the IGF-1/IGF-1R signalling axis to be negatively regulated by cortisol in HDF, namely IGFBP-3 and IGFBP-1. These results are in support of previous findings reporting dexamethasone-induced downregulation of IGFBP-3 in human fibroblasts (Conover *et al*., 1995). IGFBP-3 is similar to IGFBP-5 in its pro-fibrotic functions (Pilewski *et al*., 2005) although IGF-independent functions in processes including retinoid X receptor alpha-mediated apoptosis of cancer cells have been reported (Lee and Cohen, 2002).

In addition to the potential interference in IGF signalling, KGF mRNA expression was also decreased by GC treatment - previously unreported in human experimental models. Although this may be involved in normal regulation of tissue remodelling shifting away from the early signalling required for re-epithelialisation, increased local GC *prior* to wounding may be detrimental to initiation of the healing process (Ishimoto and Ishibashi, 2002, Mercado *et al*., 2002). Additionally, we demonstrated downregulation of the AP-1 regulatory kinase MAPK9 (which phosphorylates c-Jun) following GC treatment in HDF, suggesting that this signalling pathway is also affected by GC treatment in skin - although previously not investigated. As c-Jun/AP-1 signalling is implicated in the early stages of wound healing (Angel *et al*., 2001), preceding the anti-inflammatory GC response, GC-induced repression of c-Jun may be involved in the normal progression of tissue repair. As c-Jun/AP-1 signalling is known to positively regulate KGF secretion by mesenchymal cells, the downregulation of c-Jun by cortisol we observed may be responsible, in part, for the

concomitant decrease in KGF. Moreover, AP-1 activation is strongly implicated in MMP activation - potentially explaining the decrease in MMP1 observed in our *in vitro* studies. Interestingly, the GC-induced suppression of c-Jun phosphorylation and concomitant reduction in AP-1 activity was absent in the skin biopsies from corticosteroid-resistant asthmatics (Sousa *et al*., 1999).

Our studies also identified several genes upregulated by cortisol in HDF. In contrast to the strikingly negative regulation of collagen biosynthesis, the mRNA expression of other structural components including elastin, decorin and fibrillin-2 was increased by GC treatment. Human elastin promoter activity activation by GC has been demonstrated in the skin of transgenic mice and in primary dermal fibroblasts isolated from these animals (Ledo *et al*., 1994). However, our results oppose previous reports demonstrating decreased elastin expression by dexamethasone in HDF (Kahari, 1994, Russell *et al*., 1995), a discrepancy potentially due to cells being obtained from different anatomical sources. Fibrillin is known to associate with elastin, and our results demonstrate an increase in fibrillin-2 expression, although GC-mediated fibrillin regulation was previously unknown. Our results support previous findings describing an increase in decorin mRNA expression following dexamethasone treatment (Kahari *et al*., 1995). In contrast to HAS-2, we found HAS-1 mRNA expression to increase following cortisol treatment, although as this isozyme is less active and expressed at much lower levels than HAS-2 (Itano *et al*., 1999, Zhang *et al*., 2000) it is unlikely to be able to compensate for the decrease in HAS-2 expression. Regulators of collagen remodelling MMP8 and TIMP4 were also positively regulated by cortisol treatment in our study. Although traditionally perceived as a neutrophil-specific derived collagenase, MMP8 production by HDF has also been reported (Reuben *et al*., 2001). The levels of expression we detected were

relatively low; however, the previously unreported GC-induced increase in expression may contribute to increased collagen-I and -III degradation. TIMP4 is a potent inhibitor of MMP2 (Bigg *et al*., 2001) - a type IV collagenase associated with primary skin melanoma (Vaisanen *et al*., 1998). Our results reveal a previously unreported ~4-fold increase in TIMP4 mRNA expression following cortisol treatment, suggesting that GC may exhibit anti-tumorigenic properties by limiting MMP2 activity in HDF.

In contrast to the inhibitory effects on c-Jun (via MAPK9 downregulation), the AP-1 component FosB was upregulated by cortisol treatment in HDF. However, whilst an active AP-1 complex can form from Jun homodimers, Fos proteins can only form active transcription AP-1 complexes as heterodimers with Jun proteins and thus the GC-induced increase in FosB is unlikely to result in increased AP-1 signalling although alterations in target gene expression resulting from increased AP-1 heterodimer availability cannot be excluded. Other genes involved in cell signalling upregulated following cortisol treatment included PPARGC1, a transcription factor co-activator protein - including for the GR (Knutti *et al*., 2000) - known to potentiate GC signalling (Jang *et al*., 2007) and the androgen receptor, shown to form heterodimers with the GR, possibly resulting in mutual inhibition of transcriptional activity and thus limiting GC signalling (Chen *et al*., 1997). Alternatively, androgen receptor signalling is thought to suppress wound healing (Lai *et al*., 2009) and may therefore act in synergy with GC signalling. This is supported by observation that cutaneous wounds heal more slowly in elderly males than in elderly females (Ashcroft and Mills, 2002) and that castrated male rats display beneficial wound healing profiles compared to WT controls (Gilliver *et al*., 2007).

Finally, several genes involved in stress resistance were also upregulated by cortisol treatment in HDF. Manganese superoxide dismutase (SOD2) is an important

antioxidant enzyme that attenuates oxidative free radicals in the mitochondria by catalyzing the formation of hydrogen peroxide from superoxide radicals. Although the GC-mediated regulation of SOD2 mRNA expression in HDF has not been investigated to date, recent studies strongly implicate this antioxidant enzyme in the ageing process with fibroblast-specific null mice exhibiting several phenotypic traits associated with premature ageing (Treiber *et al*., 2011). Furthermore, the ability of SOD2 to reduce mitochondrial oxidative stress by de-activating superoxide suggests this enzyme also possesses tumour-suppressing functions (van Remmen *et al*., 2003, Trimmer *et al*., 2011). Intriguingly, recent proteomic studies implicate SOD2 overexpression in fibroblast senescence and skin ageing (Ferchiu *et al*., 2011) - suggesting, as is often the case in biological systems, that regulatory molecules function optimally within a defined range of expression. The stress-induced hsp27 (HSPB1) has been implicated in wound healing (Laplante *et al*., 1998) and more specifically, fibroblast-mediated wound contraction (Hirano *et al*., 2002). Although the regulation by GC in HDF has not previously been examined, our observation that GC treatment induces a modest increase in mRNA expression suggests that GC may also be involved in modulating these processes through HSPB1. We also observed an increase in mRNA expression of the dual specificity phosphatase (DUSP1) which dephosphorylates (and inactivates) the MAP kinases ERK2 and p38 (Abraham and Clark, 2006). Amongst other regulatory functions, ERK2 and p38 are upstream activators of c-Fos and c-Jun transcription respectively, in addition to phosphorylating (and activating) assembled AP-1 complexes, suggesting DUSP-1 is a central GC target involved in decreased AP-1 signalling in HDF (Figure 5-12).

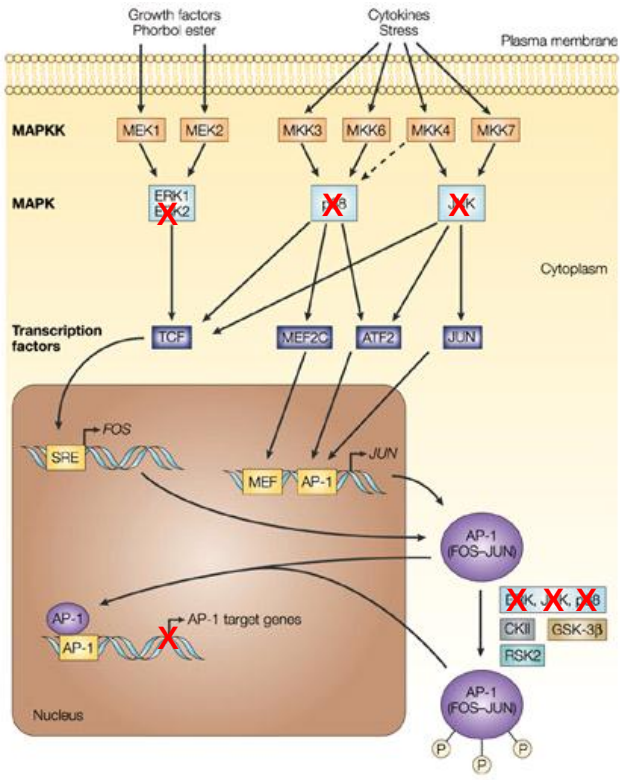


Figure 5-12 GC treatment affects multiple elements of the AP-1 signalling pathway in HDF. Our studies identified novel GC targets in AP-1 signalling involving a reduction in the MAP kinase JNK-2 (MAPK-9) and an decrease in the MAPKs ERK-2 and p38 by upregulating their inactivating phosphatase DUSP-1. These kinases are also involved in phosphorylation (and activation) of AP-1 complexes once formed suggesting their inactivation by GC (red crosses) are likely to decrease the expression of AP-1 target genes. Modified from Eferl and Wagner, 2003

Although GC are known to inhibit MAPK expression (Clark and Lasa, 2003) and DUSP-1 potentiation by GC has been described for a variety of tissues and cell types including HeLa cells (Lasa *et al*., 2002), mast cells (Jeong *et al*., 2003), adipocytes (Bazuine *et al*., 2004), osteoblasts (Engelbrecht *et al*., 2003), breast and lung epithelial cells (Wu *et al*., 2005, Hermoso *et al*., 2004), T-lymphocytes (Li *et al*., 2006), rheumatoid synovial fibroblasts (Toh *et al*., 2004) and myeloid cells (Abraham *et al*., 2006), our results demonstrate that this is also true for HDF. Having identified several new GC target genes in HDF that may interfere not only with fibroblast function, but affect whole skin tissue biology by disrupting mesenchymal-epidermal cross-talk, we investigated whether any of the implicated pathways were also affected in human skin biopsies *ex vivo* as a function of 11β-HSD oxoreductase activity.

Remarkably, we identified several genes that displayed a significant negative correlation with 11β-HSD oxoreductase levels including LEPREL1 - supporting our earlier observations in HDF.

mRNA expression of other genes involved in collagen remodelling found to be downregulated as a function of enzyme activity included the proteoglycan- and fibronectin-degrading stromelysin-2 (MMP10) and the elastase MMP12 which were also downregulated by cortisol treatment in our *in vitro* studies although not quite reaching statistical significance. In contrast to our (previously unreported) observations in human skin and HDF, MMP10 expression was increased by GC treatment in a mouse model of wound healing (Madlener *et al*., 1996, Galluci *et al*., 2001) although high expression in human skin has been associated with certain skin cancers (Kerkela *et al*., 2001, Fernandez-Figueras *et al*., 2007, Boyd *et al*., 2009), further endorsing the potentially anti-tumorigenic properties of GC. Alternatively, MMP10 also plays an important role in wound healing (Krampert *et al*., 2004) identifying stromelysin-2 as another target through which GC impede this vital function of skin. Similarly, MMP12 activity is also important in the wound healing process but, in contrast to MMP10, has also been reported to be downregulated by GC treatment in murine excisional skin wounds (Madlener *et al*., 1996). Additionally, our results provide the first evidence that this may also the case in human skin and dermal fibroblasts. Although traditionally expressed in macrophages, MMP12 has also been implicated in carcinogenesis, with increased expression in transformed keratinocytes in cutaneous squamous cell carcinoma (Kerkela *et al*., 2000).

In support of our *in vitro* studies, elements of the AP-1 signalling pathway are also negatively correlated with 11β-HSD oxoreductase levels in human skin biopsies, specifically, the MAP kinase JNK1 (MAPK8) and the AP-1 heterodimer components

Fra-1 (FOSL1) and FosB which have not previously been described as GC targets in skin. Moreover, these elements were not regulated in HDF, or in the case of FosB, differentially regulated compared to the whole skin correlates - suggesting that GC may exert different effects on differing compartments of skin, or that mesenchymal-epidermal communication must be enabled to permit the regulation of certain GC targets. Indeed, keratinocytes express JNK-1 and -3 but not JNK-2 (Adachi *et al*., 2003), while enhanced FOSL1 expression has recently been linked to psoriasis - a skin disorder primarily caused by keratinocyte hyperproliferation (Sobolev *et al*., 2011).

DUSP-1 expression was also found to be negatively associated with 11β-HSD oxoreductase activity, although this is in contrast with the positive regulation by GC observed in our HDF cultures. In addition, expression of the stress resistance genes for hsp32 (HMOX1), SIRT1 and the transcription activator NFE2L2 were negatively associated with 11β-HSD oxoreductase activity in human skin biopsies. In skin, HMOX1 has been shown to be involved in the adaptive response to UVA-induced oxidative stress in HDF (Vile *et al*., 1994), with our studies suggesting GC may interfere with this response rendering skin enriched in local GC concentrations more vulnerable to the damaging effects of photo-exposure. The histone deacetylase SIRT1 has been shown to inhibit GR binding to GREs in target genes (Amat *et al*., 2007); therefore the 11β-HSD oxoreductase activity-associated decrease in SIRT1 mRNA expression may serve as a mechanism to potentiate GC regulation of target genes. Moreover, in keratinocytes, SIRT1 exerts anti-proliferative effects, antagonising IL-22 induced effects associated with psoriasis, with decreased SIRT1 expression in psoriatic lesions (Sestito *et al*., 2011) - in this respect increased GC availability may promote epidermal fibrosis, consistent with increased risk of psoriasis in the elderly.

Although traditionally anti-inflammatory, the GC-related decrease in SIRT1 expression may promote inflammation as SIRT1 has recently been reported to possess anti-inflammatory properties (Barroso *et al*., 2011). Collectively, evidence for the role of SIRT1 as an anti skin-ageing enzyme (Lee *et al*., 2010) is exciting in the context of a negative association with 11β-HSD oxoreductase activity and an increase in this activity with ageing and photo-ageing in skin as we have demonstrated. Finally, we observed a negative correlation with increasing 11β-HSD oxoreductase activity for the oxidative stress response gene inducer NFE2L2, also recently established to be involved in skin wound healing processes (Braun *et al*., 2002), skin morphogenesis and cancer (Beyer *et al*., 2007).

Collectively, our functional studies demonstrate for the first time (to our knowledge) that GC target genes in skin can be regulated directly through modulation of 11β-HSD1 activity. We demonstrate previously unreported GC target genes including those involved in collagen biosythesis and remodelling, AP-1 signalling pathway and stress resistance, and moreover that these genes are regulated by physiological concentrations of the endogenous GC cortisol in HDF, raising the possibility that these genes too can be modulated in an 11β-HSD1-specific manner. Finally, we provide preliminary evidence of an association between 11β-HSD oxoreductase activity levels in *ex vivo* human skin biopsies and the expression of novel GC target genes, raising the possibility that the age-related increase in GC activating potential we described earlier may be responsible for the modulation of key regulators of skin function and homeostasis - particularly in the response to stress. Results of 11β-HSD1-specific inhibitor studies in *ex vivo* human skin biopsies are eagerly awaited to confirm the age-related 11β-HSD1-specific regulation of novel GC target genes in skin.

CHAPTER 6 11β-HSD1-NULL MOUSE DERMAL PHENOTYPE

6.1 Introduction

6.1.1 The 11β-HSD1 knockout mouse

First described over a decade ago by Kotelevtsev *et al*., the 11β-HSD1-null mouse was found to resist hyperglycaemia provoked by obesity or stress despite compensatory, increased adrenal corticosterone production (Kotelevtsev *et al*., 1997), suggesting that intracellular regeneration of corticosterone in the tissues from inert circulating 11-DHC contributes more to effective intracellular corticosterone concentrations than plasma corticosterone itself. This study raised the notion of 11-DHC and cortisone as pro-hormones, with plasma levels not subjected to wide diurnal fluctuations (Walker *et al*., 1992) or sequestration by corticosteroid binding globulin (Gayrard *et al*., 1996), and which may possess higher tissue specificity because they can act only on tissues expressing 11β-HSD1. Moreover, 11β-HSD1-null mice have a more favourable lipid and lipoprotein profile with improved insulin sensitivity, glucose tolerance and a potentially atheroprotective phenotype compared to their WT counterparts (Morton *et al*., 2001). 11β-HSD1-null mice are also resistant to diet-induced visceral obesity (Morton *et al*., 2004, Wamil *et al*., 2011).

Intriguingly, age-related improvements in cognitive function have also been reported in 11β-HSD1-null mice (Yau *et al*., 2001, 2011), regardless of elevated circulating GC levels, providing initial evidence for reversal of tissue dysfunction in aged animals by limiting local GC reactivation. Recently, supporting studies have provided evidence that 11β-HSD1 mRNA expression is indeed increased in the ageing mouse brain (Holmes *et al*., 2010). Conversely, age-related bone loss occurred at a similar

rate in both 11β-HSD1-null and WT mice (Justesen *et al*., 2004), although a similar increase is bone cells (osteoblasts) from aged humans has been reported (Cooper *et al*., 2002). More recently, increased GC concentration in the brain has been linked to alcohol consumption in rodents (Little *et al*., 2008), proposing a novel mechanism for the alcohol-induced adverse effects on brain functions (such as memory loss). Although direct effects of alcohol intake on 11β-HSD1 levels in the brain have not been investigated, the proposed mechanism is supported by other studies demonstrating increased hepatic 11β-HSD1 expression and activity in patients with alcoholic liver disease (Ahmed *et al*., 2008).

Furthermore, knockout mice do not appear to display any overt adverse effects in the functional capacities of adult tissues examined thus far including vasculature (Hadoke *et al*., 2001), bone (Justesen *et al*., 2004) and skeletal muscle (Semjonous *et al*., 2011). Indeed knockout animals display enhanced angiogenesis in healing surgical wounds and following myocardial infarction (Small *et al*., 2005, McSweeney *et al*., 2009, 2010), offering a previously uncharacterized therapeutic approach to improve healing of ischaemic or injured tissue.

However, lack of 11β-HSD1 enzyme activity appears detrimental in murine foetal tissue development exemplified by defects in lung maturation (Hundertmark *et al*., 2002), may impede the rapid clearance of apoptotic cells during resolution of inflammation in adult mice (Gilmour *et al*., 2006, Chapman *et al*., 2006) and increases susceptibility to endotoxaemia - with increased weight loss and serum pro-inflammatory cytokine (e.g. TNF-α, IL-6) levels - following LPS challenge (Zhang and Daynes, 2007). Although it appears that the majority of phenotypic consequences following global 11β-HSD1 inactivation are positive, a more detailed analysis is

required to examine potentially adverse effects particularly relating to reduced anti-inflammatory responses subsequent to initiation of an immune response.

6.2 Aims

Our studies have demonstrated an increase in the GC activating capacity of human and murine skin from older individuals as a result of increased 11β-HSD1 mRNA expression and oxoreductase activity. Furthermore, in HDF and human skin biopsies *ex vivo* we provide evidence that GC target genes (including several not previously identified in skin) are regulated in an 11β-HSD1-specific manner, supporting our hypothesis that increased expression of 11β-HSD1 in older individuals may play a significant role in the deterioration of skin structure and function.

Although we found several biological pathways to be regulated by GC in our functional studies, genes involved in collagen biosynthesis, processing and remodelling were identified as a cluster that may be particularly susceptible to negative regulation by increases in local GC concentrations. To investigate this further *in vivo,* **we aimed to examine histological differences between young and old mouse skin in WT and 11β-HSD1-null mice, with particular attention to collagen expression, organization and integrity.**

6.3 Materials and methods

6.3.1 Sample preparation and culture

11β-HSD1-null mice were generated in-house as previously reported (Semjonous *et al*., 2011). Mouse skin tissue explants stored in 10% NBF according to section 2.2.1.2 were paraffin embedded and sectioned by Dr Nicola Carpenter (Unilever Discover, Bedford, UK).

6.3.2 Histology

Analysis of young and aged WT and 11β-HSD1-null mouse skin sections (n=4) stained with Mayer's haematoxylin and Eosin was assisted by the late Professor William Parish (Unilever Discover, Bedford, UK). Slides were examined for epidermal and dermal integrity, collagen fibre density and assembly, dermal cell density and overall appearance. Dermal cell count was also determined numerically by counting the nuclei in a 40X magnified field of view (n=4).

6.3.3 Collagen analysis

Slides were stained for collagen using a Masson Trichrome kit according to manufacturer's guidelines. Collagen stain intensity and epidermal thickness was determined using the Nuance system according to section 4.3.4.3.

6.3.4 Real-time PCR gene expression

RNA extraction, reverse transcription and qPCR were conducted according to section 2.3, using the primers and probes described in sections 3.3.2 and the following (Applied Biosystems, California, USA):

Mouse: COL1A1 (Mm00801666_g1) and IGFBP5 (Mm00516037_m1)

6.4 Results

6.4.1 Aged 11β-HSD1-null mouse skin is more comparable to that of young WT mice

Histological analysis between young and aged WT mice revealed striking differences in dermal integrity (Figure 6-1).

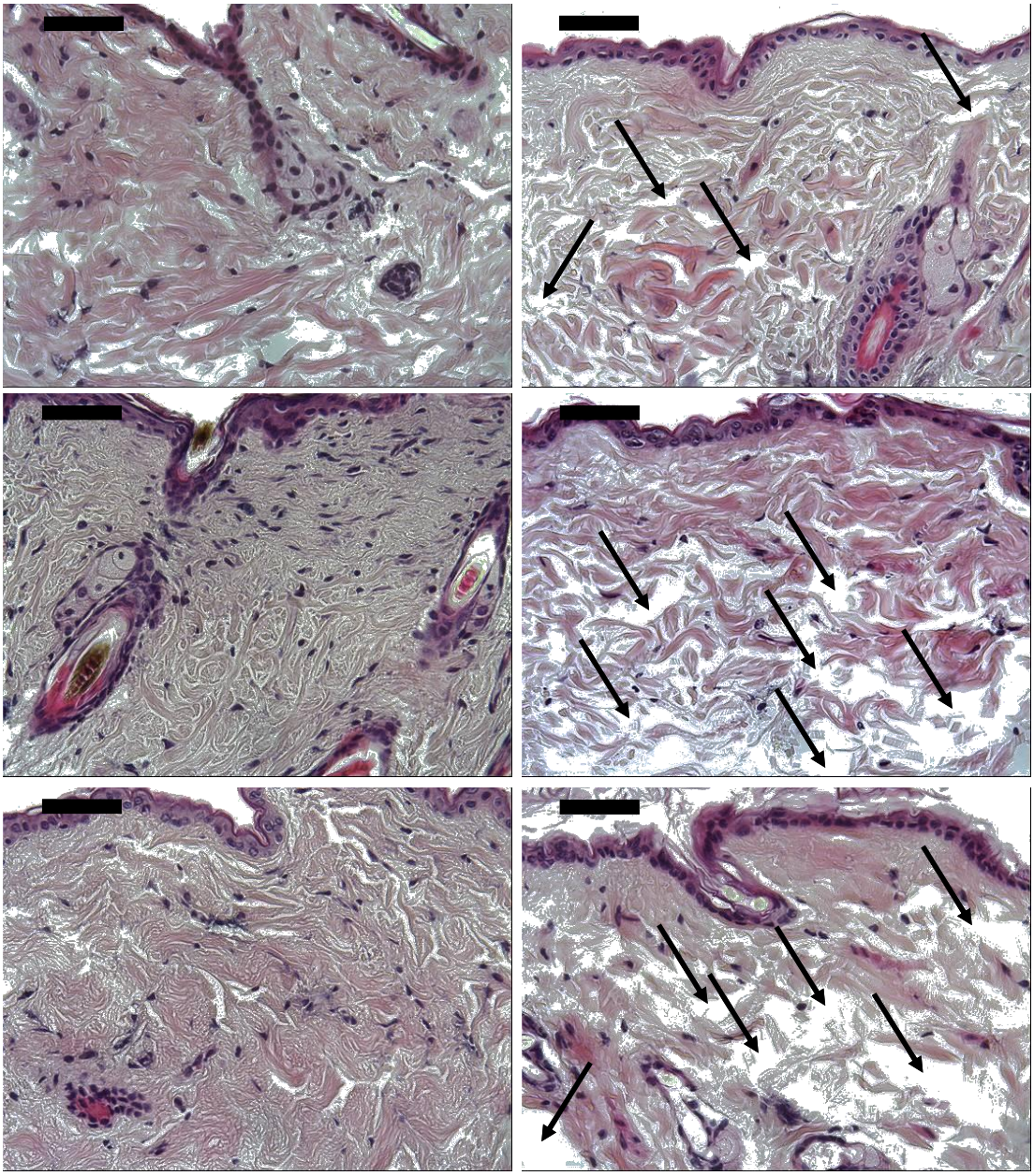


Figure 6-1 Changes in mouse skin histology with age (WT). Comparison of young WT (n=4) and old WT (n=4) skin sections revealed a grossly altered dermal organization of collagen in the aged individuals with a loss of structure and tissue integrity (arrows). Scale bar = 50μm

In the latter, the collagen network appeared "loose" with noticeable atrophy and a "sponge-like" appearance, characterized by large, vacant inter-fibril spaces. In some areas of aged tissue the collagen meshwork appeared "shredded" with little / no structural integrity or organization. The epidermis also appeared thinner and mostly one cell deep in older individuals, although these differences were much more subtle compared to the gross abnormalities in the dermis.

Strikingly, these age-related changes in dermal integrity were indistinguishable between young and aged 11β-HSD1-null mice, which displayed a similar histological skin profile (Figure 6-2), suggesting that the adverse structural modifications present in older skin may indeed be as a consequence of increased local GC generation via 11β-HSD1.

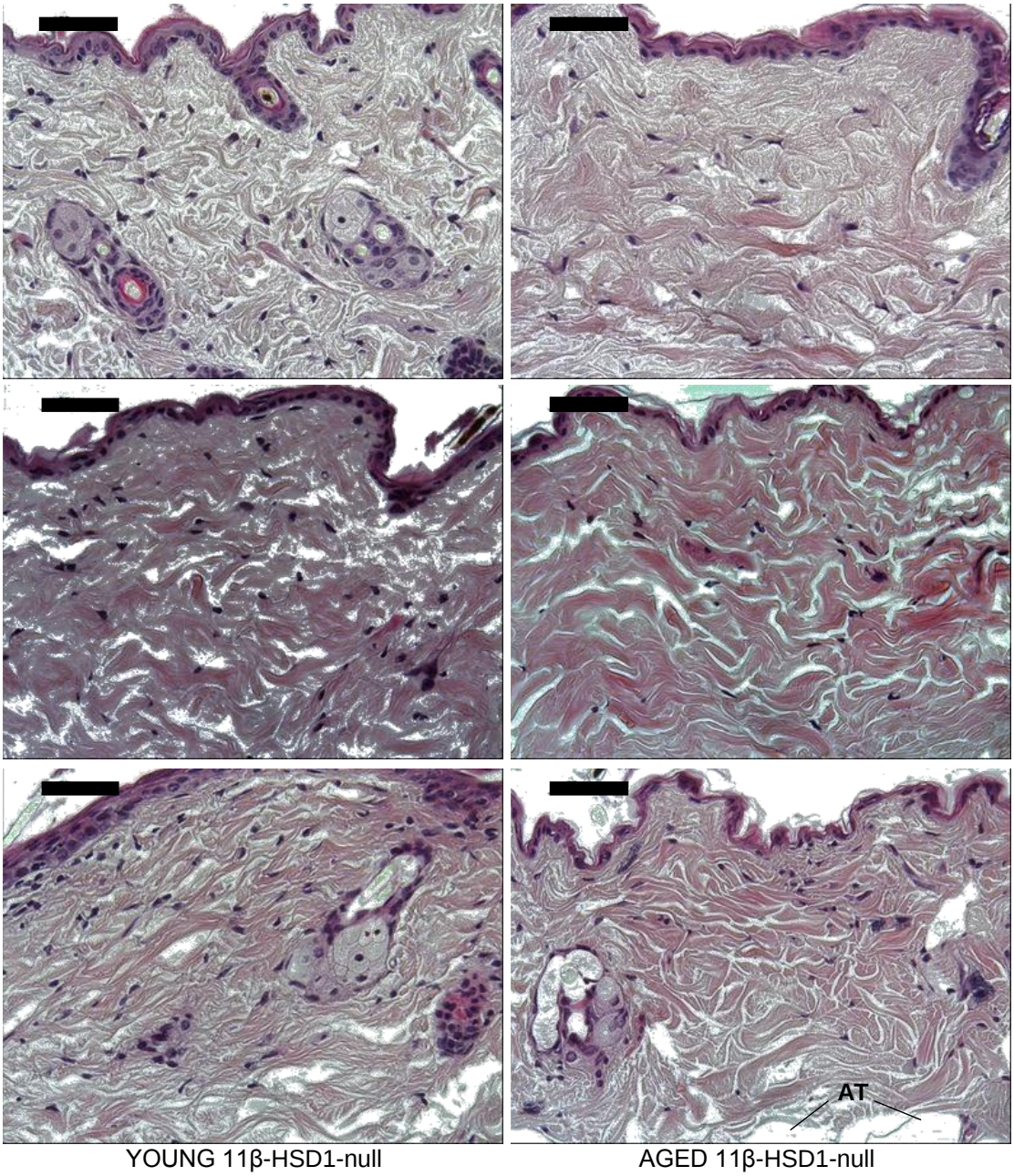


Figure 6-2 Changes in mouse skin histology with age (11β-HSD1-null). In striking contrast to the WT animals, comparison of young 11β-HSD1-null (n=4) and old 11β-HSD1-null (n=4) skin sections revealed similarities in dermal organization of collagen with comparable structure and tissue integrity. AT, adipose tissue. Scale bar = 50μm

Compared to aged WT skin, aged 11β-HSD1-null sections exhibited improved dermal integrity, with collagen fibres adopting a more densely-packed, orderly organization. Independent blinded analysis by Professor William Parish (Unilever Discover, Bedford, UK) identified the aged knockout sections as having better integrity than the WT counterparts on all occasions (n=4). Moreover, an aged knockout section was identified as having the best overall structure, whilst an aged WT section was selected as exhibiting the worst atrophy (Figure 6-3).

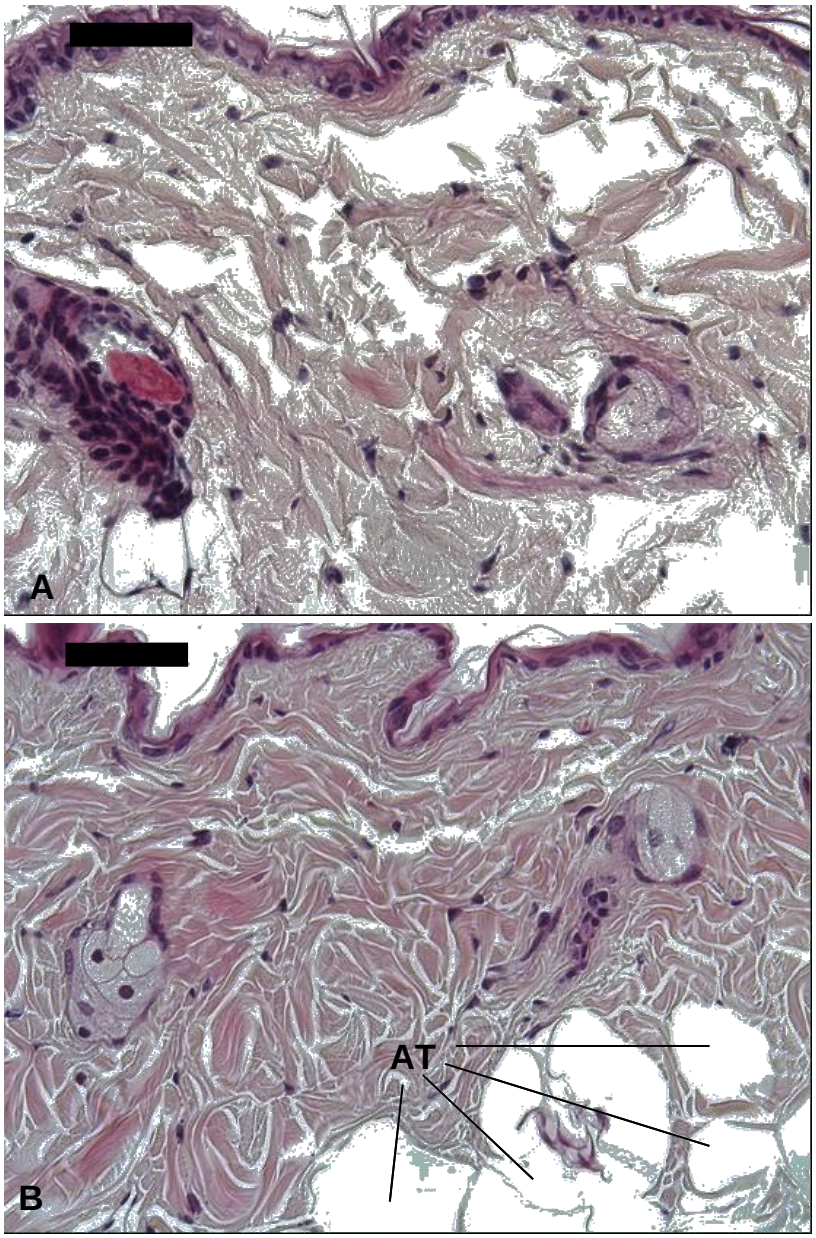


Figure 6-3 Improved skin histology in aged 11β-HSD1-null mice. Striking improvement in dermal structural organization in aged knockout (**B**) compared to aged WT (**A**) mice that revert to a similar integrity as observed in young animals. AT, adipose tissue. Scale bar = 50μm

Histology studies also revealed fewer dermal cells in aged WT (n=4) mice compared to young WT (n=3) and young knockout (n=4) animals (42±12 vs. 71±19 and 89±16, $p<0.05$ and $p<0.01$ respectively). Interestingly, dermal cell numbers were not statistically different between young WT and aged knockout (n=4) mice (Figure 6-4), but the latter remained lower than young knockout animals (59±17 vs. 89±16, $p<0.05$) suggesting that local GC generation decreases dermal cell proliferation independently of age.

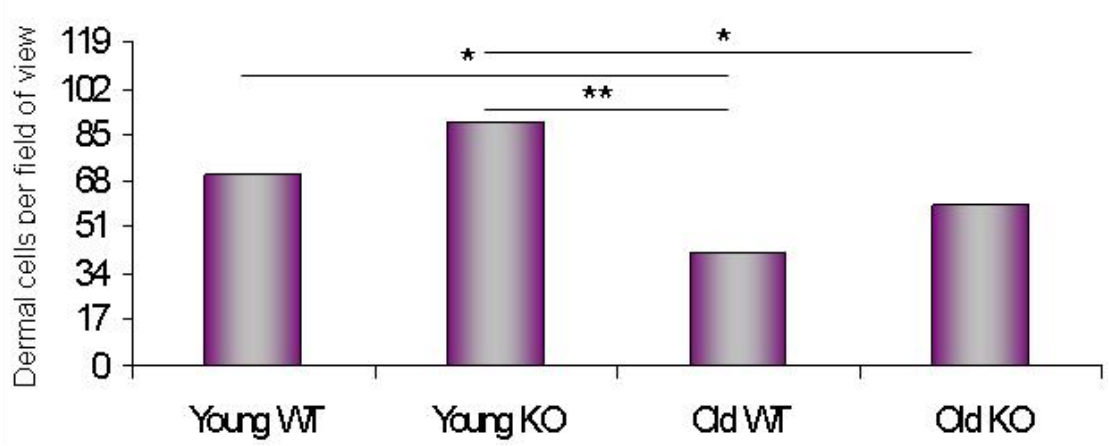


Figure 6-4 Dermal cell count according to mouse age and genotype. Dermal cell numbers in the papillary dermis were reduced in aged WT (n=4) mice compared to young WT (n=3) and knockout (n=4) animals (42±12 vs. 71±19 and 89±16 respectively). However, aged knockout (n=4) mice displayed reduced cell numbers only compared to young knockout animals (59±17 vs. 89±16). Significance * = $p<0.05$, ** = $p<0.01$

6.4.2 Collagen density is improved in 11β-HSD1-null mice

Although our histological analysis provided evidence for altered dermal organization, collagen density was more difficult to interpret from these studies. We therefore repeated the analysis using Masson Trichrome staining to detect changes in collagen content based on the intensity of staining. Using this approach, our results indicated that collagen density is also increased in both young (Figure 6-5) and aged (Figure 6-6) knockout mice compared to WT counterparts.

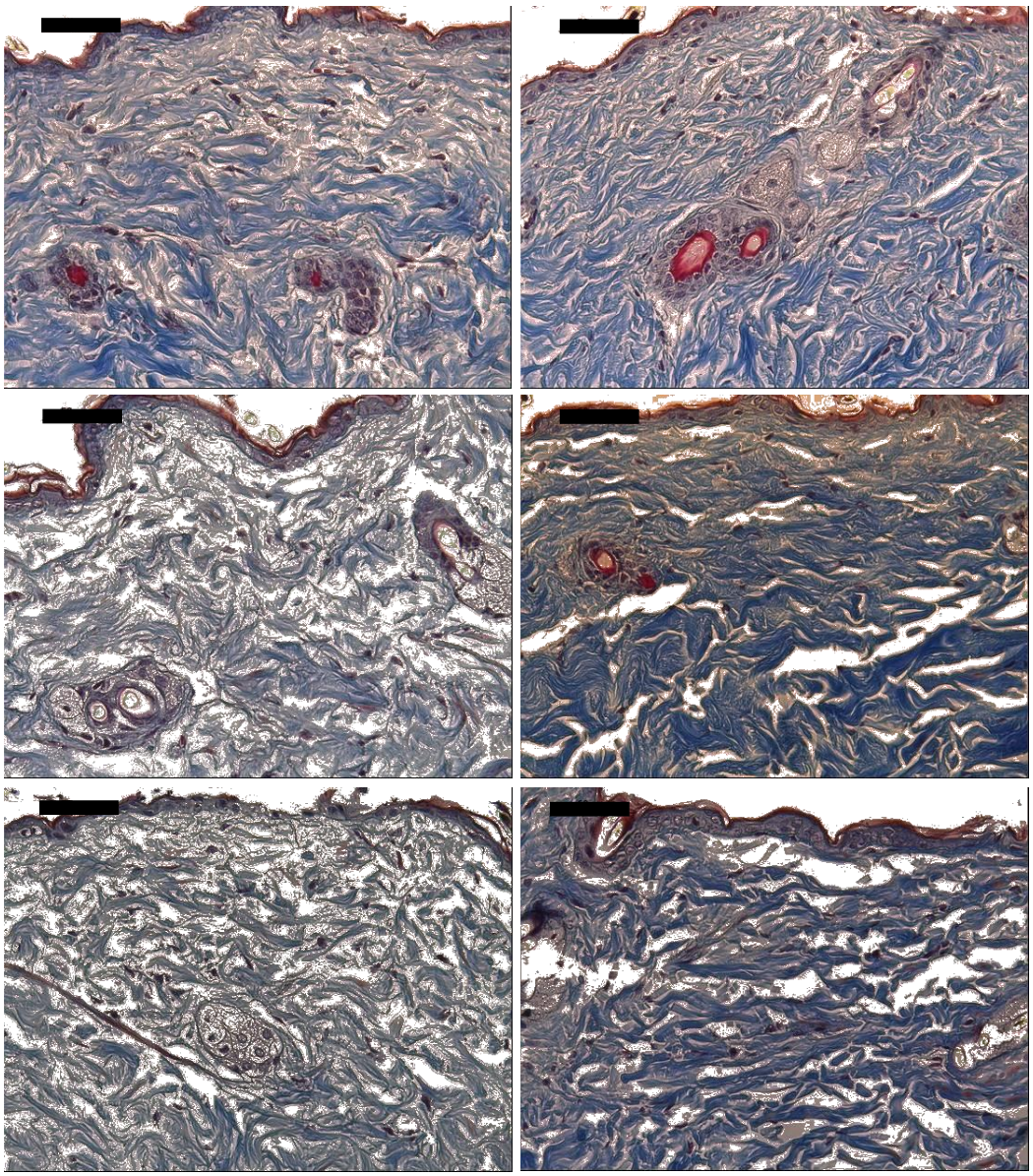


Figure 6-5 Masson trichrome staining in young WT and knockout mice. Collagen staining was more intense in 11β-HSD1-null mice compared to WT counterparts (n=4). Scale bar = 50μm

Semi-quantitative analysis of the staining supported these observations, with increased intensity of collagen staining detected in aged knockout compared to WT mice (A.U, 0.15±0.05 vs. 0.07±0.02, $p<0.05$, n=4, Figure 6-7).

A similar trend towards increased collagen staining intensity was also observed for young knockout compared to young WT mice, although this did not reach statistical significance (0.15±0.05 vs. 0.09±0.02, p=0.07, n=4, Figure 6-7). Surprisingly, collagen staining intensity did not differ between young and aged mice, suggesting

that the balance between collagen synthesis and degradation is not affected by age in mice or by the increased local GC generation in older animals.

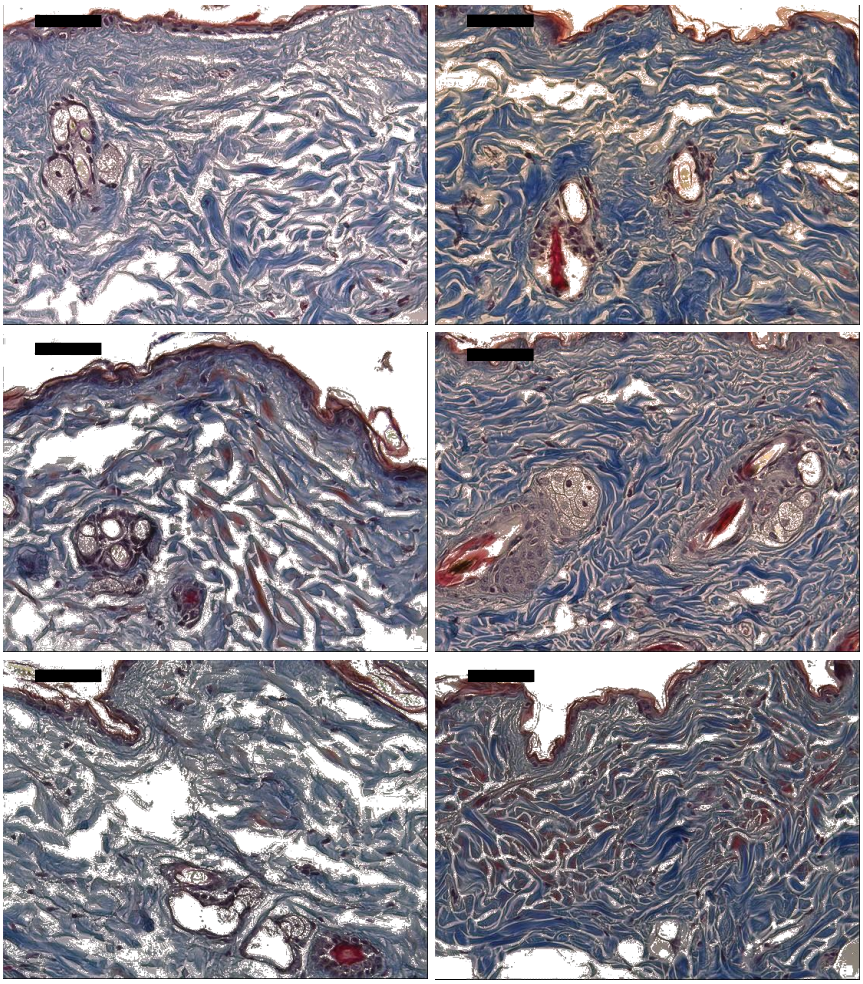


Figure 6-6 Masson trichrome staining in aged WT and knockout mice. Similarly to young mice, collagen staining was more intense in aged 11β-HSD1-null mice (n=4) compared to WT counterparts. Scale bar = 50μm

6.4.3 Epidermal thickness

Epidermal thickness (ET) was also analyzed and found to be similar between young and aged mice both WT (μm, 12.5±1.7 vs. 11.4±3.6, n=4) and knockout (11.7±1.1 vs. 13.8±4.0, n=4) and was also comparable between age-matched WT and knockout animals (data not shown). However, ET was more variable in the older age groups (both genotypes) suggesting an age- and/or genotype-specific effect may be detectable by increasing the power of the study.

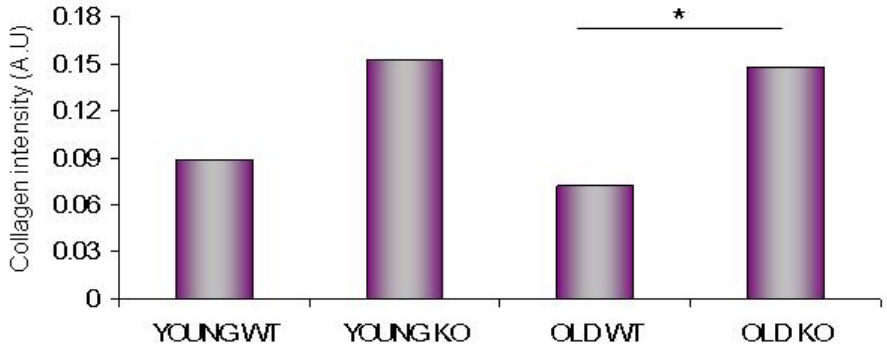


Figure 6-7 Collagen staining intensity according to mouse age and genotype. Collagen intensity was enhanced in aged knockout compared to aged WT mice (A.U, 0.15±0.05 vs. 0.07±0.02 n=4). A similar trend was observed for young knockout compared to young WT mice, although this did not reach statistical significance (0.15±0.05 vs. 0.09±0.02, n=4). Significance * = $p<0.05$

6.4.4 qPCR expression studies

As expected, 11β-HSD1 mRNA expression was negligible in both young (n=3) and aged (n=3) knockout mice (ΔCt±S.D, 18.3±0.4 vs. 22.6±2.1, $p<0.05$ and 17.5±1.3 vs. 23.3±2.2, $p<0.01$ respectively) compared to young (n=3) and aged (n=4) WT controls (Figure 6-8).

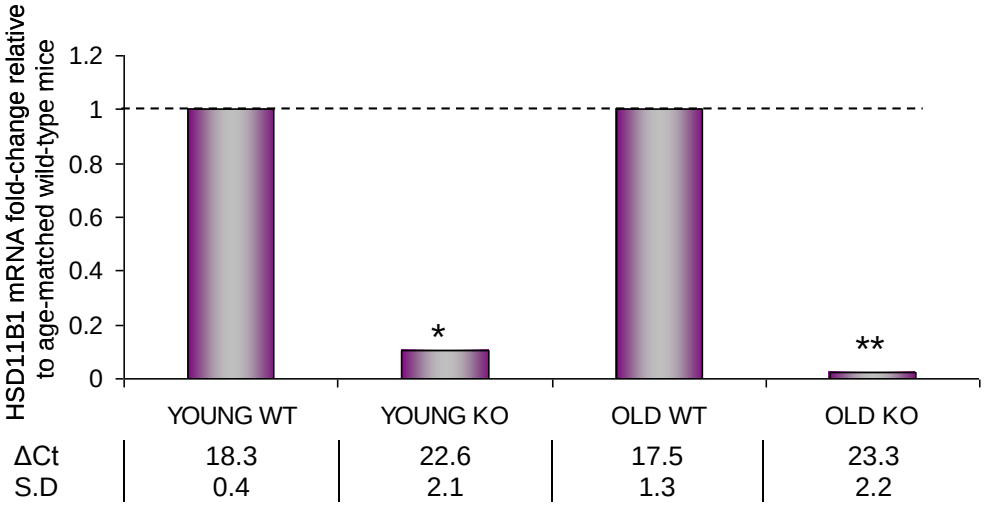


Figure 6-8 11β-HSD1 (HSD11B1) mRNA fold-change relative to age-matched WT control. HSD11B1 mRNA expression was decreased in both young and aged knockout mice (n=3) compared to young (n=3) and aged (n=4) WT controls. Significance * = $p<0.05$ and ** = $p<0.01$

However, no significant changes in GR-α, H6PD, COL1A1 or IGFBP5 mRNA levels between young WT (n=3), young knockout (n=3), aged WT (n=4) and aged knockout (n=4) mouse skin tissue samples were detected (data not shown).

6.5 Discussion

Our preliminary studies investigating the morphological phenotype of 11β-HSD1-null mice revealed a striking improvement in age-related dermal atrophy. The manifestation of intrinsically aged murine skin has been reported, although high costs associated with long-term animal housing has contributed to far fewer studies compared to research into extrinsic ageing. A recent study evaluating changes in the structural properties of type 1 collagen derived from aged mice reported decreased fibril formation rates, diameter and density with increased malleability and contractility (Damodarasamy *et al*., 2010). These reports correlate with *in vivo* findings of increased dermal atrophy in rodent (Liang *et al*., 2010) and human (Varani *et al*., 2006) skin with chronological ageing. Our results are in agreement with these studies, describing a "loosely" packed, disorganized collagen network in aged compared to young WT mice. Remarkably, these morphological features characteristic of aged skin were absent in the 11β-HSD1-null mice, which displayed a dermal phenotype more comparable to that of young counterparts, supporting our hypothesis that increased local GC activating capacity in skin during ageing and photo-ageing may be directly involved in the structural and functional changes implicated in these processes.

Additionally to potential enhancement of tensile properties of skin from aged 11β-HSD1-null mice directly attributable to improvements in dermal integrity, benefits regarding the function of dermal fibroblasts in maintaining effective homoeostasis could also be investigated. Indeed, several studies have demonstrated the ability of the ECM microenvironment (provided by mechanical properties of young relative to aged collagen) to influence dermal fibroblast synthesis of molecules that regulate cell/ECM attachment, repair and turnover (Balas and Davidson, 2001, Varani *et al*., 2006,

Damodarasamy *et al*., 2010). Moreover, a "looser" ECM has been linked to improved tumour growth and angiogenesis in older subjects (Sprenger *et al*., 2008).

Although it is still debatable whether age-related collagen breakdown results from an increase in MMP activity, decreases in MMP inhibition by TIMPs, or changes in associated proteins that modulate collagen stability (Hornebeck, 2003, Fisher *et al*., 2008), there is a general consensus that collagen expression and density decreases in skin (and other tissues) as a function of ageing (Boyer *et al*., 1991, Sayama *et al*., 2010). Moreover, GC are known to have the same effect as previously described. Our hypothesis that the increased 11β-HSD1 expression and activity with age in skin may contribute directly to the observed decrease in dermal collagen content is also supported by our initial *in vivo* studies demonstrating a significant increase in collagen staining in 11β-HSD1-null mice. This was true for aged - and displayed a similar trend for - young mice, suggesting that GC are involved in negative regulation of collagen content independently of age, and that inhibition of local GC activation may increase collagen production in young subjects additionally to combating the age-related collagen decline.

We also observed an age-related decrease in the dermal cell content in WT mice, whist aged knockout animals displayed a comparable number of cells to young WT mice. Decreased dermal fibroblast numbers *in vivo* have been reported for aged skin (Gunin *et al*., 2011), with decreased proliferative potential in cells obtained from aged individuals in culture (Bruce and Deamond, 1991). The same effect also occurs as a result of GC treatment *in vitro* (Wach *et al*., 1998). Our results provide initial evidence supporting a link between decreased cellularity as a direct effect of local GC generation during ageing. Similarly to the results observed for collagen density, it appears that dermal cellularity may also be boosted in young knockout mice.

Although this was not statistically significant directly, young knockout mice exhibited increased cell numbers compared to aged knockouts which themselves did not differ from the young WT animals, suggesting the increase in age-matched young knockout may be resolved by improving study power. As dermal fibroblasts are the primary cell type responsible for collagen synthesis, the increase in collagen density in knockout mice may be as a result of increased fibroblast proliferation. However, analysis of COL1A1 mRNA expression was not significantly altered in these mice suggesting increased collagen synthesis may not be responsible for the increased collagen density observed. Furthermore, mRNA expression of IGFBP5, recently demonstrated to induce type 1 collagen expression in human fibroblasts (Lecca *et al*., 2011), was also unaffected by age or genotype in our mouse model. Alternatively, collagen density may be increased as a result of increased MMP and/or decreased TIMP expression or activity, resulting in inhibition of collagen degradation and we plan to investigate this further in our future studies.

These studies raise the possibility that in addition to improved dermal structural organization, collagen and cellular content, aged 11β-HSD1-null mice may display other advantageous skin characteristics ranging from improved wound healing to cancer resistance, adding to the previously documented metabolic benefits. The implication of these improvements within the context of preventing the development of an ageing skin phenotype warrants highly anticipated further research into the dermal phenotype of 11β-HSD1-null mice and the development of selective 11β-HSD1 inhibitors to combat ageing and age-related disease.

CHAPTER 7 FINAL CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

The similarities between changes that occur structurally and functionally in ageing skin and those observed following GC therapy and in patients with elevated levels of endogenous GC (e.g. Cushing's syndrome) prompted the studies presented in this thesis. We hypothesized that an increase in the expression and activity of the GC-activating enzyme 11β-HSD1 in older skin may be involved in mediating some of the adverse changes that occur as a function of age, with the possibility that reducing 11β-HSD1 activity in aged skin may have beneficial outcomes on skin physiology and could reverse the clinical and biological consequences of skin ageing.

Whilst a limited number of studies have provided some evidence for the presence of 11β-HSDs in human skin, the methods were variable and a detailed assessment was lacking. Studies examining activity were primarily in isolated sweat glands (Kenouch *et al*., 1994, Bocchi *et al*., 2004) and although one study examined activity in whole abdominal skin, the samples were homogenized and under the influence of an artificial co-factor regeneration system (Hennebert *et al*., 2007) making it difficult to establish if the reported results were an accurate reflection of the physiological activity levels. Protein expression was previously reported for 11β-HSD2 in eccrine sweat glands (Smith *et al*., 1996, Hirasawa *et al*., 1997) and vasculature (Smith *et al*., 1996) whist one study reported 11β-HSD1 protein expression in epidermal *stratum spinosum*, with debatable expression also reported for the dermis and hair follicles (Hennebert *et al*., 2007). Moreover, mRNA expression had only been reported for 11β-HSD2 in breast skin using semi-quantitative Northern blot analysis (Brown *et al*.,

1996). Surprisingly, comparative studies in other species including mice had also not been reported for 11β-HSDs in skin.

The data presented in Chapter 3 confirmed the presence of mRNA expression and protein and enzyme activity for both 11β-HSD1 and 11β-HSD2 in whole human skin tissue explants in support of our hypothesis that skin is capable of regulating local active GC availability at a cellular level. Our findings that dehydrogenase (11β-HSD2) activity exceeded oxoreductase (11β-HSD1) activity were consistent with the mRNA expression which was greater for 11β-HSD2 than 11β-HSD1. Immunohistochemical studies detected protein expression in the skin structures previously reported (described above), with previously unreported expression also detected in dermal fibroblasts, sebaceous glands and microvasculature. For the first time, the data presented here describes a comparable tissue distribution for 11β-HSD1 in dorsal mouse skin, supporting our observations in human skin, with protein expression detected in epidermal keratinocytes, dermal fibroblasts, hair follicles, sebaceous glands, microvasculature, subcutaneous adipose tissue (below biopsy depth in human skin) and the *panniculus carnosus* (absent in human skin). Interestingly, in contrast to human skin, 11β-HSD2 activity was absent in mouse skin, consistent with negligible 11β-HSD2 mRNA and protein expression, whilst 11β-HSD1 mRNA expression and activity were readily detectable. This difference may be due, in part, to differences between human and mouse skin architecture with an absence of 11β-HSD2-rich eccrine sweat glands in the latter (Johns *et al*., 1995). Nevertheless, the novel finding that 11β-HSD1 is expressed in mouse skin in a comparable manner to human skin endorsed the ability to investigate the effects of modulating GC availability at a pre-receptor level on skin physiology *in vivo*.

Our observations confirmed that HDF are capable of rapidly amplifying local GC concentrations by increasing 11β-HSD1 activity in response to GC treatment in an autocrine positive-feedback manner further enhanced by the presence of TNF-α. Similar effects have been reported by others (Hammami and Siiteri, 1991, Hardy *et al*., 2006), supporting a pivotal role for HDF in resolution of inflammation following disruption of skin barrier function (e.g. wounding). Indeed, in addition to their anti-inflammatory properties (Stojadinovic *et al*., 2007, Vukelic *et al*., 2011), GC have been postulated to coordinate the sequence of wound healing stages by altering cell function (discussed in Chapter 5), for example, by inducing fibroblast contractility (Skold *et al*., 1999). Therefore, during a resting state, the ability of dermal fibroblasts to activate GC (i.e. through 11β-HSD1) is likely to be low, rapidly increasing in response to pro-inflammatory stimuli, before returning to baseline levels upon cessation of stimulatory signals. Our findings in HDF were largely in support of this notion, with low levels of expression and undetectable oxoreductase activity in unstimulated cells.

However, we observed a large degree of variation in the levels of 11β-HSD1 mRNA expression between fibroblasts from different donors and further analysis revealed this correlated with donor age (Chapter 4). This was also true for cells that had been treated with GC, as activity was readily detectible in cells derived from older donors but remained negligible in those sourced from younger individuals. Subsequently, we investigated whether these *in vitro* findings were representative of the situation *in vivo*, and examined 11β-HSD oxoreductase levels in human skin tissue explants obtained from young and older donors. Indeed, we observed an increased capacity to re-activate GC in skin from older individuals, supporting our *in vitro* data.

Moreover, our studies conducted in ageing mice revealed similar results, suggesting that increased 11β-HSD oxoreductase activity may be a common feature of mammalian skin ageing. Whilst the increased 11β-HSD oxoreductase activity observed in human skin reflects the "net" effect of both isozymes resulting either from an increase in 11β-HSD1, a decrease in 11β-HSD2, or a combination of both, our findings in ageing mouse skin (which are devoid of 11β-HSD2 activity and expression) suggest that increased 11β-HSD1 activity may be the principal cause of our findings in human skin. Nevertheless, the increased potential to generate active GC in older skin may be involved in the development of age-related abnormalities in skin physiology (e.g. reduced structural integrity) and function (e.g. poor wound healing).

This hypothesis is supported by studies reporting a role for increased 11β-HSD1 activity in other tissues such as adipose and liver in the development of pathologies such as metabolic syndrome (Bujalska *et al*., 1997, Masuzaki *et al*., 2001, 2003, Paterson *et al*., 2004). 11β-HSDs are also known to be involved in the complex regulation of inflammation and immunity (Chapman *et al*., 2009, Gilmour *et al*., 2006, Freeman *et al*., 2005), and increased 11β-HSD1 activity in ageing mouse lymphocytes has also been reported (Zhang *et al*., 2005), suggesting a mechanism that may be involved in the age-related decline of the immune-system. Whilst the prevalence of metabolic syndrome is widely accepted to increase with ageing (Eckel *et al*., 2005, Rodriguez *et al*., 2005), an association with elevated 11β-HSD1 activity was has only recently been investigated by Honma *et al*., reporting increased 11β-HSD1 expression and lipid accumulation with advancing age in the senescence-accelerated SAMP10 mouse (Honma *et al*., 2011), with increased expression in human subcutaneous adipose tissue as a function of age also reported (Andersson *et*

al., 2009). Elevated 11β-HSD1 activity has also been reported as a function of donor age in primary human osteoblasts and mouse bone (Cooper *et al*., 2002, Weinstein *et al*., 2010), postulated to contribute to age-related increase in osteoporosis and increased fracture risk. Moreover, ageing mouse brain tissue has also been reported to display increased 11β-HSD1 activity, affecting cognition and contributing to the decline in brain function in older mice (Holmes *et al*., 2010, Sooy *et al*., 2010). Collectively, there is now a growing body of evidence that increased activity of the ubiquitously expressed GC-activating 11β-HSD1 isozyme is a common mediator for the ageing process in a variety of tissues (e.g. adipose, liver, bone, brain, and immune cells) with findings presented in this thesis suggesting this is also the case for skin.

The anatomical distribution of skin also enabled us to examine the effect of extrinsic ageing (photo-ageing) on the expression of 11β-HSD1 with the benefit of complete exclusion of inter-individual confounding factors. Indeed, our result revealed increased 11β-HSD1 mRNA expression in HDF derived from PE (lower outer arm) compared to donor-matched PP (inner upper arm) skin independently of donor age, again corroborated by our *ex vivo* findings of increased "net" 11β-HSD oxoreductase activity in PE compared to donor-matched PP human skin biopsies from both young and older individuals. These results provide the fist evidence that extrinsic ageing in skin, which superimposes on (and accelerates) the chronological ageing process, may also be mediated, in part, through increased/prolonged GC exposure as a consequence of increased 11β-HSD1 activity (Chapter 4). Interestingly, 11β-HSD1 expression was upregulated in human pharyngeal mucosa by another factor contributing to extrinsic ageing: tobacco smoke, and moreover, was decreased in tumour tissue compared to unaffected mucosa (Gronau *et al*., 2002). This study provides intriguing evidence that 11β-HSD1 may be upregulated in response to stress as a protective mechanism

against malignancy, offering a plausible reason for the increased activity reported for ageing tissues and our observations for intrinsically and extrinsically aged skin. However, the lack of reports for increased tumour incidence in any of the aged 11β-HSD1-null mouse studies conducted to date suggests that this is not the case; although the possibility that anti-tumorigenic pathways upregulated during ageing may also be driving 11β-HSD1 expression cannot be excluded.

In order to determine the potential functional consequences of increased 11β-HSD oxoreductase activity during ageing, we evaluated the previously unreported pre-receptor regulation of GC target genes in skin (Chapter 5). We demonstrated that the cortisol-regulated genes MMP1 and IGFBP-5 can also be regulated by cortisone through 11β-HSD1 in HDF - and this was preventable by co-incubation with the 11β-HSD1-specific inhibitor LJ2. These results reveal a pivotal role for 11β-HSD1 in skin and suggest that in the presence of substrate, changes in activity levels of this enzyme are capable of modulating target gene expression. Therefore, the increased activity levels with advancing donor age in skin reported in this thesis are likely to have a functional effect on local tissue architecture and function.

Our functional studies in HDF also revealed novel GC target genes though which pre-receptor regulation may exert its effects. These included the negative regulation of several key enzymes in the collagen biosynthesis pathway (e.g. prolyl and lysyl hydroxylases and lysyl oxidases). Defects in collagen structural organization have been reported in ageing skin (Bernstein *et al.*, 1996) and it is of interest to speculate that this may be attributable, in part, to increased local GC signalling resulting from increased pre-receptor activation of cortisol in elderly individuals. Similarly, the impairment in wound healing reported for aged skin (Ashcroft *et al.*, 1995) may be due to previously unreported GC-mediated impairments in AP-1 signalling presented

in this thesis, which may also be exacerbated in older skin as a result of elevated local 11β-HSD1 activity. Moreover, in *ex vivo* human skin tissue biopsies, the expression of several genes involved in the collagen biosynthesis and AP-1 signalling pathways correlated with sample-matched 11β-HSD oxoreductase activity when treated with cortisone. Although these correlations may be coincidental rather than as a direct result of elevated 11β-HSD1 activity, inhibitor studies now underway should determine whether the increased pre-receptor GC metabolism in ageing skin may indeed alter the regulation of a wide range of genes involved in maintenance of dermal homeostasis and function.

11β-HSD1 activity and expression also increased in murine skin as a function of age, and our functional studies in human HDF and tissue explants suggested an adverse impact of increased local GC activation potential on skin biology. Therefore, the final part of this thesis investigated whether global inactivation in the ageing mouse would induce benefits regarding skin structure. Preliminary studies revealed a remarkable reversal in age-induced dermal atrophy, loss of collagen and depletion of dermal cell numbers in the 11β-HSD1-null mouse compared to WT counterparts. These data are in support of our functional studies which identified the collagen biosynthesis pathway as a primary target of GC regulation in human HDF. Most importantly, these results provide unequivocal evidence for a causative role of increased 11β-HSD1 activity in the skin ageing process and suggest that prevention or blockade of this increase with the use of specific 11β-HSD1 inhibitors may minimise the adverse clinical outcomes whilst promoting improved structural and functional properties of ageing skin.

7.2 Future in vitro studies

The *in vitro* studies presented here were focused largely on primary cultures of HDF, due to their ease of maintenance and pivotal role in regulating ECM structure and integrity in the dermal layer of skin. However, our initial characterization studies examining the localization of 11β-HSDs in skin confirmed that these enzymes are also expressed in epidermal keratinocytes. Studies investigating potential age-related changes in keratinocyte 11β-HSD levels will assist in determining the cellular origin of the observed increase in 11β-HSD oxoreductase activity in human skin biopsies. Further immunohistochemistry analyses in skin sections from young and aged donors would also be beneficial in clarifying this issue. Moreover, as the biological functions of the discrete cellular components of skin are largely determined by a highly coordinated inter-cellular cross-talk (e.g. the complex epidermal-mesenchymal feedback regulation involved in wound healing), studies utilizing recently developed full-thickness *in vitro* skin equivalents may provide a more physiologically-relevant experimental setting. For example, this system could be used to examine the effects of modulating dermal fibroblast levels of 11β-HSD1 activity, either by using primary cells derived from aged or young donors or by artificially inducing overexpression or inhibition of activity in young and aged donor-derived fibroblasts respectively to study the effects on dermal structural architecture, dermal fibroblast-epidermal keratinocyte interactions and regulation of wound healing. These studies would provide a more detailed examination of the effects of modulating 11β-HSD1 activity in a more physiologically-relevant human cell based system to complement the 11β-HSD1-null mouse studies in the absence of the ability to modulate 11β-HSD1 levels in humans *in vivo*.

Although the cause underlying the increase in 11β-HSD1 activity in older skin is currently unknown, our *in vitro* findings have provided a starting point for further research into this area. One possible underlying mechanism could be that the altered extracellular *milieu* in aged skin resulting from changes in hormone levels such as IGF-1 which decreases with advancing age and negatively regulates 11β-HSD1 expression (discussed in Chapter 4 and Section 1.6.3) or TNF-α which increase with ageing (Rabe *et al*., 2006) and has been demonstrated in this thesis (Chapter 3) and by others (Cooper *et al*., 2001) to positively regulate 11β-HSD1. However, our studies in HDF demonstrate increased 11β-HSD1 expression and activity in cells derived from older donors which persists in the *absence* of the native *in vivo* extracellular environment suggesting that other mechanisms are responsible for the chronic upregulation of 11β-HSD1 in cells derived from older donors. Moreover, dermal fibroblasts obtained from Cushing's syndrome patients display a comparable or even gain-of-function phenotype in relation to gender and age-matched normal control cells (Pratsinis *et* al., 2001, Zervolea *et al*., 2005, Kletsas *et al*., 2007) suggesting that the dermal atrophy in these patients is as a consequence of the hypercortisolaemic cellular environment *in vivo* and is reversible upon restoration of normal conditions.

An alternative theory may be that 11β-HSD1 expression increases as a consequence of increased cellular stress during replicative senescence postulated to contribute to the ageing process (Cristofalo *et al*., 2004). Evaluating the expression profile of 11β-HSD1 during serial passageing and correlating this with markers of cellular senescence (e.g. beta-galactosidase) in culture and in skin tissue sections obtained from young and older donors may aid in elucidating the underlying mechanisms. Furthermore, this system could be manipulated by treatments which accelerate cellular senescence such as PI3-kinase inhibitors (Tresini *et al*., 1998) or hydrogen

peroxide (Frippiat *et al*., 2000) with the hypothesis that these treatments would also induce 11β-HSD1 expression.

Similarly, this mechanism could explain the increased 11β-HSD1 expression in PE skin derived HDF compared to donor-matched PP cells, as the former may exhibit premature replicative senescence resulting from accelerated extrinsic ageing *in vivo*. Whilst replicative senescence may explain the *in vitro* increase in 11β-HSD1 expression, it is likely that an altered extracellular environment is also involved in the upregulation of 11β-HSD oxoreductase activity reported in human skin *ex vivo*. Therefore, studies investigating the regulation of 11β-HSD1 by factors involved in intrinsic (e.g. GH/IGF-1) and extrinsic (e.g. UV radiation) ageing may also be of value.

Finally, the *in vitro* functional studies identifying novel GC target genes in HDF could be extended to determine whether they are also regulated at a pre-receptor level (similarly to MMP1 and IGFBP-5). Moreover, these studies could also include investigation into GC and pre-receptor mediated changes in expression at the protein level (e.g. Western blot and ELISA analysis) and evaluating GC-induced changes in enzyme activity where possible (e.g. MMP zymography assays). As GC-mediated changes in the expression of several genes involved in phosphorylation-dependant signal transduction were also identified, studies into the post-translational phosphorylation status of these enzymes may also be of interest.

7.3 Future in vivo studies

Certainly, further studies regarding the phenotype of the 11β-HSD1-null mouse are likely to be one of the most promising avenues of research to pursue based on the exciting preliminary data presented in this thesis. Although initial mRNA expression

data for GR-α, H6PD, COL1A1 or IGFBP5 were found not to differ by age or genotype, subsequent data obtained from the *ex vivo* human biopsy studies examining correlations in gene expression with 11β-HSD oxoreductase activity also failed to detect any associations for these genes, suggesting their expression may be relatively stable during skin ageing. Future mRNA and protein expression studies in the 11β-HSD1-null mouse should therefore focus on genes found to correlate with 11β-HSD oxoreductase activity (e.g. MMP10, MMP12 etc.) and/or genes modulated by the 11β-HSD1-specific inhibitor LJ2 identified from ongoing ex vivo human biopsy studies.

Currently, a conditional skin-specific 11β-HSD1-knockout mouse model is lacking. Although careful consideration will be required regarding the specific cellular target for this model (e.g. dermal fibroblast, epidermal keratinocyte or double knockout), the generation of these mice may aid in elucidating whether the beneficial dermal phenotype described in the aged global 11β-HSD1-null mouse is due to prevention of local or systemic GC activation capacity. Moreover, inducible conditional 11β-HSD1-knockout models could be used ameliorate GC activation capacity at different stages of life to determine whether the adverse dermal outcomes associated with increased 11β-HSD1 activity in older mice is reversible by knocking out enzyme activity in aged animals or just preventable by knocking out enzyme activity prior to the onset of the age-related increase. Alternatively, these hypotheses could be investigated by the systemic or topical administration of an 11β-HSD1-specific inhibitor, having the additional benefit of enabling dose-response studies to be conducted.

Such future studies also require assessing the ability of skin from mice lacking 11β-HSD1 (global-, conditional- or inhibitor-mediated) to respond when challenged, for example, during wounding, irritation (e.g. chemical, UV) and pathogenic insult to determine whether removal of GC activating ability in skin has an adverse effect on

the normal functions of skin. These investigations could indicate that whilst the age-related increase in local GC activating capacity may be involved in reduced healing rates in elderly animals, complete ablation of 11β-HSD1 may be equally detrimental, and only a partial inhibition of activity in older animals to match levels in younger mice may be required for optimal results regarding skin physiology and function.

Finally, if the murine *in vivo* studies prove successful, clinical trials with topical inhibitors could be conducted in humans which may lead to a reduction or reversal of the skin ageing process with improvements in the integrity, functional properties and appearance of this vital organ.

REFERENCE LIST

Abeyama, K., Eng, W., Jester, J. V. et al. (2000) A role for NF-kappa B-dependent gene transactivation in sunburn. Journal of Clinical Investigation, 105 (12): 1751-1759.

Abraham, S. M. and Clark, A. R. (2006) Dual-specificity phosphatase 1: a critical regulator of innate immune responses. Biochemical Society Transactions, 34: 1018-1023.

Abraham, S. M., Lawrence, T., Kleiman, A. et al. (2006) Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. Journal of Experimental Medicine, 203 (8): 1883-1889.

Absher, M., Makrides, W., Shapiro, P. et al. (1994) Hyperoxia Inhibits Proliferation of Cultured Rat Tracheal Smooth-Muscle Cells. American Journal of Physiology, 267 (2): L101-L105.

Adachi, M., Gazel, A., Pintucci, G. et al. (2003) Specificity in stress response: Epidermal keratinocytes exhibit specialized UV-responsive signal transduction pathways. Dna and Cell Biology, 22 (10): 665-677.

Agah, A., Kyriakides, T. R., Letrondo, N. et al. (2004) Thrombospondin 2 levels are increased in aged mice: consequences for cutaneous wound healing and angiogenesis. Matrix Biology, 22 (7): 539-547.

Agarwal, A. K., Rogerson, F. M., Mune, T. et al. (1995) Analysis of the human gene encoding the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. Journal of Steroid Biochemistry and Molecular Biology, 55 (5-6): 473-479.

Agren, U. M., Tammi, M. and Tammi, R. (1995) Hydrocortisone Regulation of Hyaluronan Metabolism in Human Skin Organ-Culture. Journal of Cellular Physiology, 164 (2): 240-248.

Ahmed, A., Saksena, S., Sherlock, M. et al. (2008) Induction of hepatic 11 beta-hydroxysteroid dehydrogenase type 1 in patients with alcoholic liver disease. Clinical Endocrinology, 68 (6): 898-903.

Aikata, H., Takaishi, H., Kawakami, Y. et al. (2000) Telomere reduction in human liver tissues with age and chronic inflammation. Experimental Cell Research, 256 (2): 578-582.

Alberts, P., Nilsson, C., Selen, G. et al. (2003) Selective inhibition of 11 beta-hydroxysteroid dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. Endocrinology, 144 (11): 4755-4762.

Allolio, B. and Arlt, W. (2002) DHEA treatment: myth or reality? Trends in Endocrinology and Metabolism, 13 (7): 288-294.

Alzamora, R., Michea, L. and Marusic, E. T. (2000) Role of 11 beta-hydroxysteroid dehydrogenase in nongenomic aldosterone effects in human arteries. Hypertension, 35 (5): 1099-1104.

Amat, R., Solanes, G., Giralt, M. et al. (2007) SIRT1 is involved in glucocorticoid-mediated control of uncoupling protein-3 gene transcription. Journal of Biological Chemistry, 282 (47): 34066-34076.

Amendt, C., Mann, A., Schirmacher, P. et al. (2002) Resistance of keratinocytes to TGF beta-mediated growth restriction and apoptosis induction accelerates re-epithelialization in skin wounds. Journal of Cell Science, 115 (10): 2189-2198.

Andersson, T., Simonyte, K., Andrew, R. et al. (2009) Tissue-Specific Increases in 11 beta-Hydroxysteroid Dehydrogenase Type 1 in Normal Weight Postmenopausal Women. Plos One, 4 (12)

Andrews, R. C., Rooyackers, O. and Walker, B. R. (2003) Effects of the 11 beta-hydroxysteroid dehydrogrenase inhibitor carbenoxolone on insulin sensitivity in men with type 2 diabetes. Journal of Clinical Endocrinology & Metabolism, 88 (1): 285-291.

Angel, P., Szabowski, A. and Schorpp-Kistner, M. (2001) Function and regulation of AP-1 subunits in skin physiology and pathology. Oncogene, 20 (19): 2413-2423.

Annett, K., Duggan, O., Freeburn, R. et al. (2005) An investigation of DNA mismatch repair capacity under normal culture conditions and under conditions of supra-physiological challenge in human CD4+T cell clones from donors of different ages. Experimental Gerontology, 40 (12): 976-981.

Antoniv, T. T. and Ivashkiv, L. B. (2006) Dysregulation of interleukin-10-dependent gene expression in rheumatoid arthritis synovial macrophages. Arthritis and Rheumatism, 54 (9): 2711-2721.

Arlt, W. (2004) Dehydroepiandrosterone replacement therapy. Seminars in Reproductive Medicine, 22 (4): 379-388.

Asahina, A. and Tamaki, K. (2006) Role of Langerhans cells in cutaneous protective immunity: Is the reappraisal necessary? Journal of Dermatological Science, 44 (1): 1-9.

Ashcroft, G. S., Horan, M. A. and Ferguson, M. W. J. (1995) The Effects of Aging on Cutaneous Wound-Healing in Mammals. Journal of Anatomy, 187: 1-26.

Ashcroft, G. S., Horan, M. A. and Ferguson, M. W. J. (1997) Aging is associated with reduced deposition of specific extracellular matrix components, upregulation of angiogenesis, and an altered inflammatory response in a murine incisional wound healing model. Journal of Investigative Dermatology, 108 (4): 430-437.

Ashcroft, G. S., Yang, X., Glick, A. B. et al. (1999) Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nature Cell Biology, 1 (5): 260-266.

Ashcroft, G. S., Lei, K. J., Jin, W. W. et al. (2000) Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. Nature Medicine, 6 (10): 1147-1153.

Ashcroft, G. S. and Mills, S. J. (2002) Androgen receptor-mediated inhibition of cutaneous wound healing. Journal of Clinical Investigation, 110 (5): 615-624.

Atamna, H., Cheung, I. and Ames, B. N. (2000) A method for detecting abasic sites in living cells: Age-dependent changes in base excision repair. Proceedings of the National Academy of Sciences of the United States of America, 97 (2): 686-691.

Audige, A., Dick, B., Frey, B. M. et al. (2002) Glucocorticoids and II beta-hydroxysteroid dehydrogenase type 2 gene expression in the aging kidney. European Journal of Clinical Investigation, 32 (6): 411-420.

Autio, P., Risteli, J., Kiistala, U. et al. (1993) Serum Markers of Collagen-Synthesis and Degradation in Skin Diseases - Altered Levels in Diseases with Systemic Manifestation and During Systemic Glucocorticoid Treatment. Archives of Dermatological Research, 285 (6): 322-327.

Autio, P., Oikarinen, A., Melkko, J. et al. (1994) Systemic Glucocorticoids Decrease the Synthesis of Type-I and Type-Iii Collagen in Human Skin In-Vivo, Whereas Isotretinoin Treatment Has Little Effect. British Journal of Dermatology, 131 (5): 660-663.

Azzi, L., El Alfy, M., Martel, C. et al. (2005) Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone. Journal of Investigative Dermatology, 124 (1): 22-27.

Baccarelli, A., Morpurgo, P. S., Corsi, A. et al. (2001) Activin A serum levels and aging of the pituitary-gonadal axis: a cross-sectional study in middle-aged and elderly healthy subjects. Experimental Gerontology, 36 (8): 1403-1412.

Bahar, R., Hartmann, C. H., Rodriguez, K. A. et al. (2006) Increased cell-to-cell variation in gene expression in ageing mouse heart. Nature, 441 (7096): 1011-1014.

Bakondi, E., Catagol, B., Bak, I. Et al. (2011) Age-related loss of stress-induced nuclear proteasome activation is due to low PARP-1 activity. Free Radical Biology & Medicine, 50 (1): 86-92.

Balin, A. K., Goodman, D. B. P., Rasmussen, H. et al. (1978) Oxygen-Sensitive Stages of Cell-Cycle of Human Diploid Cells. Journal of Cell Biology, 78 (2): 390-400.

Ballas, C. B. and Davidson, J. M. (2001) Delayed wound healing in aged rats is associated with increased collagen gel remodeling and contraction by skin fibroblasts, not with differences in apoptotic or myofibroblast cell populations. Wound Repair and Regeneration, 9 (3): 223-237.

Bank, R. A., Robins, S. P., Wijmenga, C. et al. (1999) Defective collagen crosslinking in bone, but not in ligament or cartilage, in Bruck syndrome: Indications for a bone-specific telopeptide lysyl hydroxylase on chromosome 17. Proceedings of the National Academy of Sciences of the United States of America, 96 (3): 1054-1058.

Barroso, E., Eyre, E., Palomer, X. et al. (2011) The peroxisome proliferator-activated receptor beta/delta (PPAR beta/delta) agonist GW501516 prevents TNF-alpha-induced NF-kappa B activation in human HaCaT cells by reducing p65 acetylation through AMPK and SIRT1. Biochemical Pharmacology, 81 (4): 534-543.

Bauer, E. A., Kronberger, A., Valle, K. J. et al. (1985) Glucocorticoid Modulation of Collagenase Expression in Human-Skin Fibroblast-Cultures - Evidence for Pre-Translational Inhibition. Biochimica et Biophysica Acta, 825 (2): 227-235.

Baulieu, E. E. (1996) Dehydroepiandrosterone (DHEA): A fountain of youth? Journal of Clinical Endocrinology & Metabolism, 81 (9): 3147-3151.

Bazuine, M., Carlotti, F., Tafrechi, R. S. J. et al. (2004) Mitogen-activated protein kinase (MAPK) phosphatase-1 and -4 attenuate p38 MAPK during dexamethasone-induced insulin resistance in 3T3-L1 Adipocytes. Molecular Endocrinology, 18 (7): 1697-1707.

Beauregard, S. and Gilchrest, B. A. (1987) A Survey of Skin Problems and Skin Care Regimens in the Elderly. Archives of Dermatology, 123 (12): 1638-1643.

Beck, L. S., Deguzman, L., Lee, W. P. et al. (1993) One Systemic Administration of Transforming Growth-Factor-Beta-1 Reverses Age-Impaired Or Glucocorticoid-Impaired Wound-Healing. Journal of Clinical Investigation, 92 (6): 2841-2849.

Bedell, M. A., Jenkins, N. A. and Copeland, N. G. (1997) Mouse models of human disease .1. Techniques and resources for genetic analysis in mice. Genes & Development, 11 (1): 1-10.

Bedell, M. A., Largaespada, D. A., Jenkins, N. A. et al. (1997) Mouse models of human disease .2. Recent progress and future directions. Genes & Development, 11 (1): 11-43.

Beer, H. D., Longaker, M. T. and Werner, S. (1997) Reduced expression of PDGF and PDGF receptors during impaired wound healing. Journal of Investigative Dermatology, 109 (2): 132-138.

Ben Shlomo, A. and Melmed, S. (2006) Skin manifestations in acromegaly. Clinics in Dermatology, 24 (4): 256-259.

Benson, S. C. and Luvalle, P. A. (1981) Inhibition of Lysyl Oxidase and Prolyl Hydroxylase-Activity in Glucocorticoid Treated Rats. Biochemical and Biophysical Research Communications, 99 (2): 557-562.

Bergendahl, M., Aloi, J. A., Iranmanesh, A. et al. (1998) Fasting suppresses pulsatile luteinizing hormone (LH) secretion and enhances orderliness of LH release in young but not older men. Journal of Clinical Endocrinology & Metabolism, 83 (6): 1967-1975.

Bergendahl, M., Iranmanesh, A., Mulligan, T. et al. (2000) Impact of age on cortisol secretory dynamics basally and as driven by nutrient-withdrawal stress. Journal of Clinical Endocrinology & Metabolism, 85 (6): 2203-+.

Berneburg, M., Grether-Beck, S., Kurten, V. et al. (1999) Singlet oxygen mediates the UVA-induced generation of the photoaging-associated mitochondrial common deletion. Journal of Biological Chemistry, 274 (22): 15345-15349.

Bernstein, E. F., Fisher, L. W., Li, K. H. et al. (1995) Differential Expression of the Versican and Decorin Genes in Photoaged and Sun-Protected Skin - Comparison by Immunohistochemical and Northern Analyses. Laboratory Investigation, 72 (6): 662-669.

Bernstein, E. F., Brown, D. B., Urbach, F. et al. (1995) Ultraviolet-Radiation Activates the Human Elastin Promoter in Transgenic Mice - A Novel In-Vivo and In-Vitro Model of Cutaneous Photoaging. Journal of Investigative Dermatology, 105 (2): 269-273.

Bernstein, E. F. and Uitto, J. (1996) The effect of photodamage on dermal extracellular matrix. Clinics in Dermatology, 14 (2): 143-151.

Bernstein, E. F., Chen, Y. Q., Kopp, J. B. et al. (1996) Long-term sun exposure alters the collagen of the papillary dermis - Comparison of sun-protected and photoaged skin by Northern analysis, immunohistochemical staining, and confocal laser scanning microscopy. Journal of the American Academy of Dermatology, 34 (2): 209-218.

Bernstein, E. F., Underhill, C. B., Hahn, P. J. et al. (1996) Chronic sun exposure alters both the content and distribution of dermal glycosaminoglycans. British Journal of Dermatology, 135 (2): 255-262.

Bernstein, E. F., Chen, Y. Q., Kopp, J. B. et al. (1996) Long-term sun exposure alters the collagen of the papillary dermis - Comparison of sun-protected and photoaged skin by Northern analysis, immunohistochemical staining, and confocal laser scanning microscopy. Journal of the American Academy of Dermatology, 34 (2): 209-218.

Bertherat, J., Bluetpajot, M. T. and Epelbaum, J. (1995) Neuroendocrine Regulation of Growth-Hormone. European Journal of Endocrinology, 132 (1): 12-24.

Beutler, B., Krochin, N., Milsark, I. W. et al. (1986) Control of Cachectin (Tumor-Necrosis-Factor) Synthesis - Mechanisms of Endotoxin Resistance. Science, 232 (4753): 977-980.

Beyer, T. A., Keller, U. A. D., Braun, S. et al. (2007) Roles and mechanisms of action of the Nrf2 transcription factor in skin morphogenesis, wound repair and skin cancer. Cell Death and Differentiation, 14 (7): 1250-1254.

Bhushan, M., Cumberbatch, M., Dearman, R. J. et al. (2002) Tumour necrosis factor-alpha-induced migration of human Langerhans cells: the influence of ageing. British Journal of Dermatology, 146 (1): 32-40.

Bigg, H. F., Morrison, C. J., Butler, G. S. et al. (2001) Tissue inhibitor of metalloproteinases-4 inhibits but does not support the activation of gelatinase A via efficient inhibition of membrane type I-matrix metalloproteinase. Cancer Research, 61 (9): 3610-3618.

Biron, C. A. (1997) Activation and function of natural killer cell responses during viral infections. Current Opinion in Immunology, 9 (1): 24-34.

Bissett, D. L., Chatterjee, R. and Hannon, D. P. (1990) Photoprotective Effect of Topical Antiinflammatory Agents Against Ultraviolet Radiation-Induced Chronic Skin Damage in the Hairless Mouse. Photodermatology Photoimmunology & Photomedicine, 7 (4): 153-158.

Bitar, M. S., Farook, T., Wahid, S. et al. (1999) Glucocorticoid-dependent impairment of wound healing in experimental diabetes: Amelioration by adrenalectomy and RU 486. Journal of Surgical Research, 82 (2): 234-243.

Bitar, M. S. (2000) Insulin and glucocorticoid-dependent suppression of the IGF-I system in diabetic wounds. Surgery, 127 (6): 687-695.

Bitzer, M., von Gersdorff, G., Liang, D. et al. (2000) A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappa B/RelA. Genes & Development, 14 (2): 187-197.

Blackman, M. B., Sorkin, J. D., Munzer, T. et al. (2002) Growth hormone and sex steroid administration in healthy aged women and men - A randomized controlled trial. Jama-Journal of the American Medical Association, 288 (18): 2282-2292.

Bocchi, B., Kenouch, S., Lamarre-Cliche, M. et al. (2004) Impaired 11-beta hydroxysteroid dehydrogenase type 2 activity in sweat gland ducts in human essential hypertension. Hypertension, 43 (4): 803-808.

Bonifati, C., Carducci, M., Mussi, A. et al. (1998) Recognition and treatment of psoriasis - Special considerations in elderly patients. Drugs & Aging, 12 (3): 177-190.

Bosset, S., Bonnet-Duquennoy, M., Barre, P. et al. (2003) Decreased expression of keratinocyte beta 1 integrins in chronically sun-exposed skin in vivo. British Journal of Dermatology, 148 (4): 770-778.

Bouillon, R., Okamura, W. H. and Norman, A. W. (1995) Structure-Function-Relationships in the Vitamin-D Endocrine System. Endocrine Reviews, 16 (2): 200-257.

Bowen, R. L. and Atwood, C. S. (2004) Living and dying for sex - A theory of aging based on the modulation of cell cycle signaling by reproductive hormones. Gerontology, 50 (5): 265-290.

Boyd, S., Virolainen, S., Parssinen, J. et al. (2009) MMP-10 (Stromelysin-2) and MMP-21 in human and murine squamous cell cancer. Experimental Dermatology, 18 (12): 1044-1052.

Boyer, B., Kern, P., Fourtanier, A. et al. (1991) Age-Dependent Variations of the Biosyntheses of Fibronectin and Fibrous Collagens in Mouse Skin. Experimental Gerontology, 26 (4): 375-383.

Boyer, B., Fourtanier, A., Kern, P. et al. (1992) Uva-Induced and Uvb-Induced Changes in Collagen and Fibronectin Biosynthesis in the Skin of Hairless Mice. Journal of Photochemistry and Photobiology B-Biology, 14 (3): 247-259.

Boyle, J., Kill, I. R. and Parris, C. N. (2005) Heterogeneity of dimer excision in young and senescent human dermal fibroblasts. Aging Cell, 4 (5): 247-255.

Braham, C., Betea, D., Pierard-Franchimont, C. et al. (2002) Skin tensile properties in patients treated for acromegaly. Dermatology, 204 (4): 325-329.

Brand, M. D. (2000) Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Experimental Gerontology, 35 (6-7): 811-820.

Brauchle, M., Rassler, R. and Werner, S. (1995) Suppression of Keratinocyte Growth-Factor Expression by Glucocorticoids In-Vitro and During Wound-Healing. Journal of Investigative Dermatology, 105 (4): 579-584.

Braun, K. M. and Prowse, D. M. (2006) Distinct epidermal stem cell compartments are maintained by independent niche microenvironments. Stem Cell Reviews, 2 (3): 221-231.

Braun, S., Hanselmann, C., Gassmann, M. G. et al. (2002) Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. Molecular and Cellular Biology, 22 (15): 5492-5505.

Braverman, I. M. and Fonferko, E. (1982) Studies in Cutaneous Aging .1. the Elastic Fiber Network. Journal of Investigative Dermatology, 78 (5): 434-443.

Brem, A. S., Bina, R. B., King, T. et al. (1995) Bidirectional Activity of 11-Beta-Hydroxysteroid Dehydrogenase in Vascular Smooth-Muscle Cells. Steroids, 60 (5): 406-410.

Briscoe, D. M., Dharnidharka, V. R., Isaacs, C. et al. (1999) The allogeneic response to cultured human skin equivalent in the hu-PBL-SCID mouse model of skin rejection. Transplantation, 67 (12): 1590-1599.

Brossas, J. Y., Barreau, E., Courtois, Y. et al. (1994) Multiple Deletions in Mitochondrial-Dna Are Present in Senescent Mouse-Brain. Biochemical and Biophysical Research Communications, 202 (2): 654-659.

Brown, E. J. and Schreiber, S. L. (1996) A signaling pathway to translational control. Cell, 86 (4): 517-520.

Brown, R. W., Diaz, R., Robson, A. C. et al. (1996) The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology, 137 (2): 794-797.

Brown, R. W., Chapman, K. E., Kotelevtsev, Y. et al. (1996) Cloning and production of antisera to human placental 11 beta-hydroxysteroid dehydrogenase type 2. Biochemical Journal, 313: 1007-1017.

BrownBorg, H. M., Borg, K. E., Meliska, C. J. et al. (1996) Dwarf mice and the ageing process. Nature, 384 (6604): 33-33.

Bruce, S. A. and Deamond, S. F. (1991) Longitudinal-Study of Invivo Wound Repair and Invitro Cellular Senescence of Dermal Fibroblasts. Experimental Gerontology, 26 (1): 17-27.

Bruunsgaard, H. and Pedersen, B. K. (2003) Age-related inflammatory cytokines and disease. Immunology and Allergy Clinics of North America, 23 (1): 15-+.

Bujalska, I. J., Kumar, S. and Stewart, P. M. (1997) Does central obesity reflect "Cushing's disease of the omentum"? Lancet, 349 (9060): 1210-1213.

Bujalska, I. J., Durrani, O. M., Abbott, J. et al. (2007) Characterisation of 11 beta-hydroxysteroid dehydrogenase 1 in human orbital adipose tissue: a comparison with subcutaneous and omental fat. Journal of Endocrinology, 192 (2): 279-288.

Bujalska, I. J., Hewitt, K. N., Hauton, D. et al. (2008) Lack of hexose-6-phosphate dehydrogenase impairs lipid mobilization from mouse adipose tissue. Endocrinology, 149 (5): 2584-2591.

Bulteau, A. L., Petropoulos, I. and Friguet, B. (2000) Age-related alterations of proteasome structure and function in aging epidermis. Experimental Gerontology, 35 (6-7): 767-777.

Burkle, A., Beneke, S. And Muiras, M. L. (2004) Poly(ADP-ribosyl)ation and aging. Experimental Gerontology, 39 (11-12): 1599-1601.

Bustin, S. A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. Journal of Molecular Endocrinology, 25 (2): 169-193.

Cabelof, D. C., Raffoul, J. J., Yanamadala, S. et al. (2002) Attenuation of DNA polymerase beta-dependent base excision repair and increased DMS-induced mutagenicity in aged mice. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 500 (1-2): 135-145.

Cabral, W. A., Chang, W., Barnes, A. M. et al. (2007) Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. Nature Genetics, 39 (3): 359-365.

Campisi, J. (2003) Cellular senescence and apoptosis: how cellular responses might influence aging phenotypes. Experimental Gerontology, 38 (1-2): 5-11.

Candi, E., Schmidt, R. and Melino, G. (2005) The cornified envelope: A model of cell death in the skin. Nature Reviews Molecular Cell Biology, 6 (4): 328-340.

Carballido, J. M., Biedermann, T., Schwarzler, C. et al. (2003) The SCID-hu Skin mouse as a model to investigate selective chemokine mediated homing of human T-lymphocytes to the skin in vivo. Journal of Immunological Methods, 273 (1-2): 125-135.

Cargill, S. L., Carey, J. R., Muller, H. G. et al. (2003) Age of ovary determines remaining life expectancy in old ovariectomized mice. Aging Cell, 2 (3): 185-190.

Carlson, J. A., Linette, G. P., Aplin, A. et al. (2007) Melanocyte receptors: Clinical implications and therapeutic relevance. Dermatologic Clinics, 25 (4): 541-+.

Carrard, G., Bulteau, A. L., Petropoulos, I. et al. (2002) Impairment of proteasome structure and function in aging. International Journal of Biochemistry & Cell Biology, 34 (11): 1461-1474.

Carrillo, A. E. and Flouris, A. D. (2011) Caloric restriction and longevity: Effects of reduced body temperature. Ageing Research Reviews, 10 (1): 153-162.

Carroll, P. V. and Christ, E. R. (1998) Growth hormone deficiency in adulthood and the effects of growth hormone replacement: A review. Journal of Clinical Endocrinology & Metabolism, 83 (2): 382-395.

Carter, R. N., Paterson, J. M., Tworowska, U. et al. (2009) Hypothalamic-Pituitary-Adrenal Axis Abnormalities in Response to Deletion of 11 beta-HSD1 is Strain-Dependent. Journal of Neuroendocrinology, 21 (11): 879-887.

Casolini, P., Catalani, A., Zuena, A. R. et al. (2002) Inhibition of COX-2 reduces the age-dependent increase of hippocampal inflammatory markers, corticosterone secretion, and behavioral impairments in the rat. Journal of Neuroscience Research, 68 (3): 337-343.

Cerimele, D., Celleno, L. and Serri, F. (1990) Physiological-Changes in Aging Skin. British Journal of Dermatology, 122: 13-20.

Chakravarti, S., Collins, W. P., Forecast, J. D. et al. (1976) Hormonal Profiles After Menopause. British Medical Journal, 2 (6039): 784-787.

Chang, E. I., Loh, S. A., Ceradini, D. J. et al. (2007) Age decreases endothelial progenitor cell recruitment through decreases in hypoxia-inducible factor 1 alpha stabilization during ischemia. Circulation, 116 (24): 2818-2829.

Chang, H. Y., Chi, J. T., Dudoit, S. et al. (2002) Diversity, topographic differentiation, and positional memory in human fibroblasts. Proceedings of the National Academy of Sciences of the United States of America, 99 (20): 12877-12882.

Chapman, K. E., Coutinho, A., Gray, M. et al. (2006) Local amplification of Glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 and its role in the inflammatory response. Neuroendocrine and Immune Crosstalk, 1088: 265-273.

Chapman, K. E., Coutinho, A. E., Gray, M. et al. (2009) The role and regulation of 11 beta-hydroxysteroid dehydrogenase type 1 in the inflammatory response. Molecular and Cellular Endocrinology, 301 (1-2): 123-131.

Charmandari, E., Ichijo, T., Jubiz, W. et al. (2008) A Novel Point Mutation in the Amino Terminal Domain of the Human Glucocorticoid Receptor (hGR) Gene Enhancing hGR-Mediated Gene Expression. Journal of Clinical Endocrinology & Metabolism, 93 (12): 4963-4968.

Chedid, M., Hoyle, J. R., Csaky, K. G. et al. (1996) Glucocorticoids inhibit keratinocyte growth factor production in primary dermal fibroblasts. Endocrinology, 137 (6): 2232-2237.

Chen, F. G., Zhang, W. J., Bi, D. et al. (2007) Clonal analysis of nestin(-) vimentin(+) multipotent fibroblasts isolated from human dermis. Journal of Cell Science, 120 (16): 2875-2883.

Chen, J., Mo, R., Lescure, P. A. et al. (2003) Aging is associated with increased T-cell chemokine expression in C57B1/6 mice. Journals of Gerontology Series A-Biological Sciences and Medical Sciences, 58 (11): 975-983.

Chen, S. Y., Wang, J., Yu, G. Q. et al. (1997) Androgen and glucocorticoid receptor heterodimer formation - A possible mechanism for mutual inhibition of transcriptional activity. Journal of Biological Chemistry, 272 (22): 14087-14092.

Christiansen, H. E., Schwarze, U., Pyott, S. M. et al. (2010) Homozygosity for a Missense Mutation in SERPINH1, which Encodes the Collagen Chaperone Protein HSP47, Results in Severe Recessive Osteogenesis Imperfecta. American Journal of Human Genetics, 86 (3): 389-398.

Christiansen, M., Stevnsner, T., Bohr, V. A. et al. (2000) Gene-specific DNA repair of pyrimidine dimers does not decline during cellular aging in vitro. Experimental Cell Research, 256 (1): 308-314.

Chung, J. H., Kang, S., Varani, J. et al. (2000) Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin in vivo. Journal of Investigative Dermatology, 115 (2): 177-182.

Chung, K. Y., Agarwal, A., Uitto, J. et al. (1996) An AP-1 binding sequence is essential for regulation of the human alpha 2(I) collagen (COL1A2) promoter activity by transforming growth factor-beta. Journal of Biological Chemistry, 271 (6): 3272-3278.

Chung, S. S., Weindruch, R., Schwarze, S. R. et al. (1994) Multiple Age-Associated Mitochondrial-Dna Deletions in Skeletal-Muscle of Mice. Aging-Clinical and Experimental Research, 6 (3): 193-200.

Circu, M. L. and Aw, T. Y. (2010) Reactive oxygen species, cellular redox systems, and apoptosis. Free Radical Biology and Medicine, 48 (6): 749-762.

Clark, A. R. and Lasa, M. (2003) Crosstalk between glucocorticoids and mitogen-activated protein kinase signalling pathways. Current Opinion in Pharmacology, 3 (4): 404-411.

Cline, S. D. and Hanawalt, P. C. (2003) Who's on first in the cellular response to DNA damage? Nature Reviews Molecular Cell Biology, 4 (5): 361-372.

Colucciguyon, E., Portier, M. M., Dunia, I. et al. (1994) Mice Lacking Vimentin Develop and Reproduce Without An Obvious Phenotype. Cell, 79 (4): 679-694.

Commo, S., Gaillard, O. and Bernard, B. A. (2004) Human hair greying is linked to a specific depletion of hair follicle melanocytes affecting both the bulb and the outer root sheath. British Journal of Dermatology, 150 (3): 435-443.

Conover, C. A., Clarkson, J. T. and Bale, L. K. (1995) Effect of Glucocorticoid on Insulin-Like Growth-Factor (Igf) Regulation of Igf-Binding Protein Expression in Fibroblasts. Endocrinology, 136 (4): 1403-1410.

Contet-Audonneau, J. L., Jeanmarie, C. and Pauly, G. (1999) A histological study of human wrinkle structures: comparison between sun-exposed areas of the face, with or without wrinkles, and sun-protected areas. British Journal of Dermatology, 140 (6): 1038-1047.

Cooper, M. and Stewart, P. M. (1998) The syndrome of apparent mineralocorticoid excess. Qjm-An International Journal of Medicine, 91 (7): 453-455.

Cooper, M. S., Walker, E. A., Bland, R. et al. (2000) Expression and functional consequences of 11-beta-hydroxysteroid dehydrogenase activity in human bone. Bone, 27 (3): 375-381.

Cooper, M. S., Bujalska, I., Rabbitt, E. et al. (2001) Modulation of 11 beta-hydroxysteroid dehydrogenase isozymes by proinflammatory cytokines in osteoblasts: An autocrine switch from glucocorticoid inactivation to activation. Journal of Bone and Mineral Research, 16 (6): 1037-1044.

Cooper, M. S., Rabbitt, E. H., Goddard, P. E. et al. (2002) Osteoblastic 11 beta-hydroxysteroid dehydrogenase type 1 activity increases with age and glucocorticoid exposure. Journal of Bone and Mineral Research, 17 (6): 979-986.

Corpas, E., Harman, S. M. and Blackman, M. R. (1993) Human Growth-Hormone and Human Aging. Endocrine Reviews, 14 (1): 20-39.

Corraldebrinski, M., Horton, T., Lott, M. T. et al. (1992) Mitochondrial-Dna Deletions in Human Brain - Regional Variability and Increase with Advanced Age. Nature Genetics, 2 (4): 324-329.

Coschigano, K. T., Clemmons, D., Bellush, L. L. et al. (2000) Assessment of growth parameters and life span of GHR/BP gene-disrupted mice. Endocrinology, 141 (7): 2608-2613.

Coulombe, P., Rodier, G., Pelletier, S. et al. (2003) Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. Molecular and Cellular Biology, 23 (13): 4542-4558.

Courtney, R., Stewart, P. M., Toh, M. et al. (2008) Modulation of 11 beta-hydroxysteroid dehydrogenase (11 beta HSD) activity biomarkers and pharmacokinetics of PF-00915275, a selective 11 beta HSD1 inhibitor. Journal of Clinical Endocrinology & Metabolism, 93 (2): 550-556.

Couzinet, B. and Schaison, G. (1993) The Control of Gonadotropin-Secretion by Ovarian-Steroids. Human Reproduction, 8: 97-101.

Cristofalo, V. J., Lorenzini, A., Allen, R. G. et al. (2004) Replicative senescence: a critical review. Mechanisms of Ageing and Development, 125 (10-11): 827-848.

Cutroneo, K. R. and Counts, D. F. (1975) Anti-Inflammatory Steroids and Collagen-Metabolism - Glucocorticoid-Mediated Alterations of Prolyl Hydroxylase-Activity and Collagen-Synthesis. Molecular Pharmacology, 11 (5): 632-639.

Damodarasamy, M., Vernon, R. B., Karres, N. et al. (2010) Collagen Extracts Derived From Young and Aged Mice Demonstrate Different Structural Properties and Cellular Effects in Three-Dimensional Gels. Journals of Gerontology Series A-Biological Sciences and Medical Sciences, 65 (3): 209-218.

Dardevet, D., Sornet, C., Taillandier, D. et al. (1995) Sensitivity and Protein-Turnover Response to Glucocorticoids Are Different in Skeletal-Muscle from Adult and Old Rats - Lack of Regulation of the Ubiquitin-Proteasome Proteolytic Pathway in Aging. Journal of Clinical Investigation, 96 (5): 2113-2119.

Davies, K. J. A. (2000) Oxidative stress, antioxidant defenses, and damage removal, repair, and replacement systems. Iubmb Life, 50 (4-5): 279-289.

Daynes, R. A., Araneo, B. A., Ershler, W. B. et al. (1993) Altered Regulation of Il-6 Production with Normal Aging - Possible Linkage to the Age-Associated Decline in Dehydroepiandrosterone and Its Sulfated Derivative. Journal of Immunology, 150 (12): 5219-5230.

De Benedetto, A., Agnihothri, R., Mcgirt, L. Y. et al. (2009) Atopic Dermatitis: A Disease Caused by Innate Immune Defects? Journal of Investigative Dermatology, 129 (1): 14-30.

de Kloet, E. R., Vreugdenhil, E., Oitzl, M. S. et al. (1998) Brain corticosteroid receptor balance in health and disease. Endocrine Reviews, 19 (3): 269-301.

DeJesus, V., Rios, I., Davis, C. et al. (2002) Induction of apoptosis in human replicative senescent fibroblasts. Experimental Cell Research, 274 (1): 92-99.

Delaunay, F., Khan, A., Cintra, A. et al. (1997) Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids. Journal of Clinical Investigation, 100 (8): 2094-2098.

Desmouliere, A., Geinoz, A., Gabbiani, F. et al. (1993) Transforming Growth-Factor-Beta-1 Induces Alpha-Smooth Muscle Actin Expression in Granulation-Tissue Myofibroblasts and in Quiescent and Growing Cultured Fibroblasts. Journal of Cell Biology, 122 (1): 103-111.

DeVries, A. C., Craft, T. K. S., Glasper, E. R. et al. (2007) 2006 Curt P. Richter Award winner - Social influences on stress responses and health. Psychoneuroendocrinology, 32 (6): 587-603.

Di Cornite, G., Sabbadini, M. G., Corti, A. et al. (2007) Conversation galante: How the immune and the neuroendocrine systems talk to each other. Autoimmunity Reviews, 7 (1): 23-29.

di Fagagna, F. D., Reaper, P. M., Clay-Farrace, L. et al. (2003) A DNA damage checkpoint response in telomere-initiated senescence. Nature, 426 (6963): 194-198.

Dimri, G. P., Lee, X. H., Basile, G. et al. (1995) A Biomarker That Identifies Senescent Human-Cells in Culture and in Aging Skin In-Vivo. Proceedings of the National Academy of Sciences of the United States of America, 92 (20): 9363-9367.

Dolle, M. E. T., Giese, H., Hopkins, C. L. et al. (1997) Rapid accumulation of genome rearrangements in liver but not in brain of old mice. Nature Genetics, 17 (4): 431-434.

Dong, D. D., Jewell, C. M., Bienstock, R. J. et al. (2006) Functional analysis of the LXXLL motifs of the human glucocorticoid receptor: Association with altered ligand affinity. Journal of Steroid Biochemistry and Molecular Biology, 101 (2-3): 106-117.

Draper, N., Walker, E. A., Bujalska, I. J. et al. (2003) Mutations in the genes encoding 11 beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase interact to cause cortisone reductase deficiency. Nature Genetics, 34 (4): 434-439.

Dulos, J., Verbraak, E., Bagchus, W. M. et al. (2004) Severity of murine collagen-induced arthritis correlates with increased CYP7B activity - Enhancement of dehydroepiandrosterone metabolism by interleukin-1 beta. Arthritis and Rheumatism, 50 (10): 3346-3353.

Dumont, P., Burton, M., Chen, Q. M. et al. (2000) Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. Free Radical Biology and Medicine, 28 (3): 361-373.

Eckel, R. H., Grundy, S. M. and Zimmet, P. Z. (2005) The metabolic syndrome. Lancet, 365 (9468): 1415-1428.

Eckes, B., Colucci-Guyon, E., Smola, H. et al. (2000) Impaired wound healing in embryonic and adult mice lacking vimentin. Journal of Cell Science, 113 (13): 2455-2462.

Edelstein, S. B. and Breakefield, X. O. (1986) Monoamine Oxidase-A and Oxidase-B Are Differentially Regulated by Glucocorticoids and Aging in Human-Skin Fibroblasts. Cellular and Molecular Neurobiology, 6 (2): 121-150.

Edgar, D., Larsson, N. G. and Trifunovic, A. (2010) Point Mutations Are Causing Progeroid Phenotypes in the mtDNA Mutator Mouse. Cell Metabolism, 11 (1): 1-1.

Edwards, C. R. W., Benediktsson, R., Lindsay, R. S. et al. (1996) 11 beta-hydroxysteroid dehydrogenases: Key enzymes in determining tissue-specific glucocorticoid effects. Steroids, 61 (4): 263-269.

Eferl, R. and Wagner, E. F. (2003) AP-1: A double-edged sword in tumorigenesis. Nature Reviews Cancer, 3 (11): 859-868.

Elder, J. T., Fisher, G. J., Lindquist, P. B. et al. (1989) Overexpression of Transforming Growth Factor-Alpha in Psoriatic Epidermis. Science, 243 (4892): 811-814.

Elias, P. M. and Brown, B. E. (1978) Mammalian Cutaneous Permeability Barrier - Defective Barrier Function in Essential Fatty-Acid Deficiency Correlates with Abnormal Inter-Cellular Lipid Deposition. Laboratory Investigation, 39 (6): 574-583.

Engelbrecht, Y., de Wet, H., Horsch, K. et al. (2003) Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. Endocrinology, 144 (2): 412-422.

Epstein, N. N., Epstein, W. L. and Epstein, J. H. (1963) Atrophic Striae in Patients with Inguinal Intertrigo - Pathogenesis. Archives of Dermatology, 87 (4): 450-&.

Ergang, P., Leden, P., Vagnerova, K. et al. (2010) Local metabolism of glucocorticoids and its role in rat adjuvant arthritis. Molecular and Cellular Endocrinology, 323 (2): 155-160.

Evans, R. M. (1988) The Steroid and Thyroid-Hormone Receptor Superfamily. Science, 240 (4854): 889-895.

Faergemann, J., Sarnhult, T., Hedner, E. et al. (2002) Dose-response effects of tri-iodothyroacetic acid (Triac) and other thyroid hormone analogues on glucocorticoid-induced skin atrophy in the haired mouse. Acta Dermato-Venereologica, 82 (3): 179-183.

Farooqi, I. S. and O'Rahilly, S. (2008) Mutations in ligands and receptors of the leptin-melanocortin pathway that lead to obesity. Nature Clinical Practice Endocrinology & Metabolism, 4 (10): 569-577.

Feiken, E., Romer, J., Eriksen, J. et al. (1995) Neutrophils Express Tumor-Necrosis-Factor-Alpha During Mouse Skin Wound-Healing. Journal of Investigative Dermatology, 105 (1): 120-123.

Feingold, K. R. and Elias, P. M. (1987) Endocrine-Skin Interactions - Cutaneous Manifestations of Pituitary Disease, Thyroid-Disease, Calcium Disorders, and Diabetes. Journal of the American Academy of Dermatology, 17 (6): 921-940.

Feingold, K. R. and Elias, P. M. (1988) Endocrine-Skin Interactions. Journal of the American Academy of Dermatology, 19 (1): 1-20.

Feingold, K. R., Man, M. Q., Menon, G. K. et al. (1990) Cholesterol-Synthesis Is Required for Cutaneous Barrier Function in Mice. Journal of Clinical Investigation, 86 (5): 1738-1745.

Feingold, K. R. (2007) The role of epidermal lipids in cutaneous permeability barrier homeostasis. Journal of Lipid Research, 48 (12): 2531-2546.

Ferchiu, F., Sindrilaru, A., Sante, L. et al. (2011) A proteomic approach identifies SOD2 overexpression to be responsible for imbalanced redox signalling in senescent fibroblasts and skin ageing. Experimental Dermatology, 20 (2): 203-204.

Fernandez-Figueras, M. T., Puig, L., Musulen, E. et al. (2007) Expression profiles associated with aggressive behavior in Merkel cell carcinoma. Modern Pathology, 20 (1): 90-101.

Ferrari, E., Cravello, L., Muzzoni, B. et al. (2001) Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates. European Journal of Endocrinology, 144 (4): 319-329.

Ferrari, E. and Magri, F. (2008) Role of neuroendocrine pathways in cognitive decline during aging. Ageing Research Reviews, 7 (3): 225-233.

Finch, C. E. (2010) Evolution of the human lifespan and diseases of aging: Roles of infection, inflammation, and nutrition. Proceedings of the National Academy of Sciences of the United States of America, 107: 1718-1724.

Finley, L. W. S. and Haigis, M. C. (2009) The coordination of nuclear and mitochondrial communication during aging and calorie restriction. Ageing Research Reviews, 8 (3): 173-188.

Fisher, G. J., Talwar, H. S., Lin, J. Y. et al. (1998) Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin in vivo. Journal of Clinical Investigation, 101 (6): 1432-1440.

Fisher, G. J., Datta, S., Wang, Z. Q. et al. (2000) c-Jun-dependent inhibition of cutaneous procollagen transcription following ultraviolet irradiation is reversed by all-trans retinoic acid. Journal of Clinical Investigation, 106 (5): 663-670.

Fisher, G. J., Kang, S. W., Varani, J. et al. (2002) Mechanisms of photoaging and chronological skin aging. Archives of Dermatology, 138 (11): 1462-1470.

Fisher, G. J., Varani, J. and Voorhees, J. J. (2008) Looking older - Fibroblast collapse and therapeutic implications. Archives of Dermatology, 144 (5): 666-672.

Fisher, L. B. and Maibach, H. I. (1971) Effect of Corticosteroids on Human Epidermal Mitotic Activity. Archives of Dermatology, 103 (1): 39-&.

Fontana, L. and Klein, S. (2007) Aging, adiposity, and calorie restriction. Jama-Journal of the American Medical Association, 297 (9): 986-994.

Fontana, L. (2009) Neuroendocrine factors in the regulation of inflammation: Excessive adiposity and calorie restriction. Experimental Gerontology, 44 (1-2): 41-45.

Fontana, L., Partridge, L. and Longo, V. D. (2010) Extending Healthy Life Span-From Yeast to Humans. Science, 328 (5976): 321-326.

Foster, R., Lobo, M. V. and Marusic, E. T. (1979) Studies of Relationship Between Angiotensin-Ii and Potassium-Ions on Aldosterone Release. American Journal of Physiology, 237 (4): E363-E366.

Fraga, M. F., Herranz, M., Espada, J. et al. (2004) A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Research, 64 (16): 5527-5534.

Frank, S., Madlener, M. and Werner, S. (1996) Transforming growth factors beta 1, beta 2, and beta 3 and their receptors are differentially regulated during normal and impaired wound healing. Journal of Biological Chemistry, 271 (17): 10188-10193.

Franz, C. E., York, T. P., Eaves, L. J. et al. (2010) Genetic and Environmental Influences on Cortisol Regulation Across Days and Contexts in Middle-Aged Men. Behavior Genetics, 40 (4): 467-479.

Frasca, D., Barattini, P., Tirindelli, D. et al. (1999) Effect of age on DNA binding of the ku protein in irradiated human peripheral blood mononuclear cells (PBMC). Experimental Gerontology, 34 (5): 645-658.

Freeman, L., Hewison, M., Hughes, S. V. et al. (2005) Expression of 11 beta-hydroxysteroid dehydrogenase type 1 permits regulation of glucocorticoid bioavailability by human dendritic cells. Blood, 106 (6): 2042-2049.

Freiman, A., Bird, G., Metelitsa, A. I. et al. (2004) Cutaneous effects of smoking. Journal of Cutaneous Medicine and Surgery, 8 (6): 415-423.

Frippiat, C., Chen, Q. M., Remacle, J. et al. (2000) Cell cycle regulation in H2O2-induced premature senescence of human diploid fibroblasts and regulatory control exerted by the papilloma virus E6 and E7 proteins. Experimental Gerontology, 35 (6-7): 733-745.

Fuchs, E., Merrill, B. J., Jamora, C. et al. (2001) At the roots of a never-ending cycle. Developmental Cell, 1 (1): 13-25.

Fuchs, E. (2008) Skin stem cells: rising to the surface. Journal of Cell Biology, 180 (2): 273-284.

Fulop, T., Larbi, A., Witkowski, J. M. et al. (2010) Aging, frailty and age-related diseases. Biogerontology, 11 (5): 547-563.

Fulton, C., Anderson, G. M., Zasloff, M. et al. (1997) Expression of natural peptide antibiotics in human skin. Lancet, 350 (9093): 1750-1751.

Gallo, R. L., Murakami, M., Ohtake, T. et al. (2002) Biology and clinical relevance of naturally occurring antimicrobial peptides. Journal of Allergy and Clinical Immunology, 110 (6): 823-831.

Gallucci, R. M., Simeonova, P. P., Matheson, J. M. et al. (2000) Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. Faseb Journal, 14 (15): 2525-2531.

Gallucci, R. M., Sugawara, T., Yucesoy, B. et al. (2001) Interleukin-6 treatment augments cutaneous wound healing in immunosuppressed mice. Journal of Interferon and Cytokine Research, 21 (8): 603-609.

Garcia, C. K., Wright, W. E. and Shay, J. W. (2007) Human diseases of telomerase dysfunction: Insights into tissue aging. Nucleic Acids Research, 35 (22): 7406-7416.

Garner, W. L., Karmiol, S., Rodriguez, J. L. et al. (1993) Phenotypic Differences in Cytokine Responsiveness of Hypertrophic Scar Versus Normal Dermal Fibroblasts. Journal of Investigative Dermatology, 101 (6): 875-879.

Gatza, C., Moore, L., Dumble, M. et al. (2007) Tumor suppressor dosage regulates stem cell dynamics during aging. Cell Cycle, 6 (1): 52-55.

Gayrard, V., Alvinerie, M. and Toutain, P. L. (1996) Interspecies variations of corticosteroid-binding globulin parameters. Domestic Animal Endocrinology, 13 (1): 35-45.

Gebhardt, C., Averbeck, M., Diedenhofen, N. et al. (2010) Dermal Hyaluronan Is Rapidly Reduced by Topical Treatment with Glucocorticoids. Journal of Investigative Dermatology, 130 (1): 141-149.

Gelse, K., Poschl, E. and Aigner, T. (2003) Collagens - structure, function, and biosynthesis. Advanced Drug Delivery Reviews, 55 (12): 1531-1546.

Genedani, S., Filaferro, M., Carone, C. et al. (2008) Influence of f-MLP, ACTH(1-24) and CRH on in vitro Chemotaxis of Monocytes from Centenarians. Neuroimmunomodulation, 15 (4-6): 285-289.

Ghadially, R., Brown, B. E., Hanley, K. et al. (1996) Decreased epidermal lipid synthesis accounts for altered barrier function in aged mice. Journal of Investigative Dermatology, 106 (5): 1064-1069.

Gillespie, M. N., Pastukh, V. M. and Ruchko, M. V. (2010) Controlled DNA "damage" and repair in hypoxic signaling. Respiratory Physiology & Neurobiology, 174 (3): 244-251.

Gilliver, S. C., Ruckshanthi, J. P. D., Atkinson, S. J. et al. (2007) Androgens influence expression of matrix proteins and proteolytic factors during cutaneous wound healing. Laboratory Investigation, 87 (9): 871-881.

Gilmour, J. S., Coutinho, A. E., Cailhier, J. F. et al. (2006) Local amplification of Glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes. Journal of Immunology, 176 (12): 7605-7611.

Gniadecki, R., Gniadecka, M. and Serup, J. (1994) Inhibition of Glucocorticoid-Induced Epidermal and Dermal Atrophy with Kh-1060 - A Potent 20-Epi Analog of 1,25-Dihydroxyvitamin D-3. British Journal of Pharmacology, 113 (2): 439-444.

Gold, P. W., Drevets, W. C. and Charney, D. S. (2002) New insights into the role of cortisol and the glucocorticoid receptor in severe depression. Biological Psychiatry, 52 (5): 381-385.

Gorbunova, V., Seluanov, A., Mao, Z. et al. (2007) Changes in DNA repair during aging. Nucleic Acids Research, 35 (22): 7466-7474.

Gordon, S. (2003) Alternative activation of macrophages. Nature Reviews Immunology, 3 (1): 23-35.

Goukassian, D., Gad, F., Yaar, M. et al. (2000) Mechanisms and implications of the age-associated decrease in DNA repair capacity. Faseb Journal, 14 (10): 1325-1334.

Goulet, S., Bihl, M. P., Gambazzi, F. et al. (2007) Opposite effect of corticosteroids and long-acting beta 2-agonits on serum- and TGF-beta(1)-induced extracellular matrix deposition by primary human lung fibroblasts. Journal of Cellular Physiology, 210 (1): 167-176.

Grassilli, E., Bellesia, E., Salomoni, P. et al. (1996) C-Fos/C-Jun expression and AP-1 activation in skin fibroblasts from centenarians. Biochemical and Biophysical Research Communications, 226 (2): 517-523.

Griffiths, C. E. M., Russman, A. N., Majmudar, G. et al. (1993) Restoration of Collagen Formation in Photodamaged Human Skin by Tretinoin (Retinoic Acid). New England Journal of Medicine, 329 (8): 530-535.

Grinnell, F. (1994) Fibroblasts, Myofibroblasts, and Wound Contraction. Journal of Cell Biology, 124 (4): 401-404.

Gronau, S., Greger, D. K., Jerg, M. et al. (2002) 11 beta-Hydroxysteroid dehydrogenase 1 expression in squamous cell carcinomas of the head and neck. Clinical Otolaryngology, 27 (6): 453-457.

Guazzo, E. P., Kirkpatrick, P. J., Goodyer, I. M. et al. (1996) Cortisol, dehydroepiandrosterone (DHEA), and DHEA sulfate in the cerebrospinal fluid of man: Relation to blood levels and the effects of age. Journal of Clinical Endocrinology & Metabolism, 81 (11): 3951-3960.

Gunin, A. G., Kornilova, N. K., Vasilieva, O. V. et al. (2011) Age-Related Changes in Proliferation, the Numbers of Mast Cells, Eosinophils, and cd45-Positive Cells in Human Dermis. Journals of Gerontology Series A-Biological Sciences and Medical Sciences, 66 (4): 385-392.

Guo, L. F., Degenstein, L. and Fuchs, E. (1996) Keratinocyte growth factor is required for hair development but not for wound healing. Genes & Development, 10 (2): 165-175.

Guo, Z. M., Heydari, A. and Richardson, A. (1998) Nucleotide excision repair of actively transcribed versus nontranscribed DNA in rat hepatocytes: Effect of age and dietary restriction. Experimental Cell Research, 245 (1): 228-238.

Gupta, M. A., Gupta, A. K., Schork, N. J. et al. (1994) Depression Modulates Pruritus Perception - A Study of Pruritus in Psoriasis, Atopic-Dermatitis, and Chronic Idiopathic Urticaria. Psychosomatic Medicine, 56 (1): 36-40.

Haas, C. S., Creighton, C. J., Pi, X. J. et al. (2006) Identification of genes modulated in rheumatoid arthritis using complementary DNA microarray analysis of lymphoblastoid B cell lines from disease-discordant monozygotic twins. Arthritis and Rheumatism, 54 (7): 2047-2060.

Hadoke, P. W. F., Christy, C., Kotelevtsev, Y. V. et al. (2001) Endothelial cell dysfunction in mice after transgenic knockout of type 2, but not type 1, 11 beta-hydromysteroid dehydrogenase. Circulation, 104 (23): 2832-2837.

Hamalainen, L., Oikarinen, J. and Kivirikko, K. I. (1985) Synthesis and Degradation of Type-I Procollagen Messenger-Rnas in Cultured Human-Skin Fibroblasts and the Effect of Cortisol. Journal of Biological Chemistry, 260 (2): 720-725.

Hamilton, M. L., Van Remmen, H., Drake, J. A. et al. (2001) Does oxidative damage to DNA increase with age? Proceedings of the National Academy of Sciences of the United States of America, 98 (18): 10469-10474.

Hammami, M. M. and Siiteri, P. K. (1991) Regulation of 11-Beta-Hydroxysteroid Dehydrogenase-Activity in Human Skin Fibroblasts - Enzymatic Modulation of Glucocorticoid Action. Journal of Clinical Endocrinology & Metabolism, 73 (2): 326-334.

Hammer, F. and Stewart, P. M. (2006) Cortisol metabolism in hypertension. Best Practice & Research Clinical Endocrinology & Metabolism, 20 (3): 337-353.

Hammer, S., Sauer, B., Spika, I. et al. (2004) Glucocorticoids mediate differential anti-apoptotic effects in human fibroblasts and keratinocytes via sphingosine-1-phosphate formation. Journal of Cellular Biochemistry, 91 (4): 840-851.

Hannen, R. F., Michael, A. E., Jaulim, A. et al. (2011) Steroid synthesis by primary human keratinocytes; implications for skin disease. Biochemical and Biophysical Research Communications, 404 (1): 62-67.

Harada, N. (1992) A Unique Aromatase (P-450Arom) Messenger-Rna Formed by Alternative Use of Tissue-Specific Exons-1 in Human Skin Fibroblasts. Biochemical and Biophysical Research Communications, 189 (2): 1001-1007.

Haratake, A., Uchida, Y., Mimura, K. et al. (1997) Intrinsically aged epidermis displays diminished UVB-induced alterations in barrier function associated with decreased proliferation. Journal of Investigative Dermatology, 108 (3): 319-323.

Hardy, R., Rabbitt, E. H., Filer, A. et al. (2008) Local and systemic glucocorticoid metabolism in inflammatory arthritis. Annals of the Rheumatic Diseases, 67 (9): 1204-1210.

Hardy, R. and Cooper, M. S. (2010) Adrenal gland and bone. Archives of Biochemistry and Biophysics, 503 (1): 137-145.

Hardy, R. S., Filer, A., Cooper, M. S. et al. (2006) Differential expression, function and response to inflammatory stimuli of 11 beta-hydroxysteroid dehydrogenase type 1 in human fibroblasts: a mechanism for tissue-specific regulation of inflammation. Arthritis Research & Therapy, 8 (4)

Harman, D. (2001) Aging: Overview. Healthy Aging for Functional Longevity, 928: 1-21.

Harris, H. J., Kotelevtsev, Y., Mullins, J. J. et al. (2001) Intracellular regeneration of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase (11 beta-HSD)-1 plays a key role in regulation of the hypothalamic-pituitary-adrenal axis: Analysis of 11 beta-HSD-1-deficient mice. Endocrinology, 142 (1): 114-120.

Hayashi, R., Wada, H., Ito, K. et al. (2004) Effects of glucocorticoids on gene transcription. European Journal of Pharmacology, 500 (1-3): 51-62.

Hayflick, L. (1965) Limited in Vitro Lifetime of Human Diploid Cell Strains. Experimental Cell Research, 37 (3): 614-&.

Heffner, K. L. (2011) Neuroendocrine Effects of Stress on Immunity in the Elderly: Implications for Inflammatory Disease. Immunology and Allergy Clinics of North America, 31 (1): 95-+.

Heilbronn, L. K., de Jonge, L., Frisard, M. I. et al. (2006) Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals - A randomized controlled trial. Jama-Journal of the American Medical Association, 295 (13): 1539-1548.

Hein, R., Korting, H. C. and Mehring, T. (1994) Differential Effect of Medium Potent Nonhalogenated Double-Ester-Type and Conventional Glucocorticoids on Proliferation and Chemotaxis of Fibroblasts In-Vitro. Skin Pharmacology, 7 (5): 300-306.

Hench, P. S., Kendall, E. C., Slocumb, C. H. et al. (1949) The Effect of A Hormone of the Adrenal Cortex (17-Hydroxy-11-Dehydrocorticosterone - Compound-E) and of Pituitary Adrenocorticotropic Hormone on Rheumatoid Arthritis - Preliminary Report. Proceedings of the Staff Meetings of the Mayo Clinic, 24 (8): 181-197.

Hennebert, O., Chalbot, S., Alran, S. et al. (2007) Dehydroepiandrosterone 7 alpha-hydroxylation in human tissues: Possible interference with type 1 11 beta-hydroxysteroid dehydrogenase-mediated processes. Journal of Steroid Biochemistry and Molecular Biology, 104 (3-5): 326-333.

Hermanowski-Vosatka, A., Balkovec, J. M., Cheng, K. et al. (2005) 11 beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. Journal of Experimental Medicine, 202 (4): 517-527.

Hermoso, M. A., Matsuguchi, T., Smoak, K. et al. (2004) Glucocorticoids and tumor necrosis factor alpha cooperatively regulate Toll-like receptor 2 gene expression. Molecular and Cellular Biology, 24 (11): 4743-4756.

Herz, U., Schnoy, N., Borelli, S. et al. (1998) A Human-SCID mouse model for allergic immune responses: Bacterial superantigen enhances skin inflammation and suppresses IgE production. Journal of Investigative Dermatology, 110 (3): 224-231.

Hikima, T. and Maibach, H. I. (2007) Gender differences of enzymatic activity and distribution of 17 beta-hydroxysteroid dehydrogenase in human skin in vitro. Skin Pharmacology and Physiology, 20 (4): 168-174.

Hinz, B., Mastrangelo, D., Iselin, C. E. et al. (2001) Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. American Journal of Pathology, 159 (3): 1009-1020.

Hirano, S., Rees, R. S. and Gilmont, R. R. (2002) MAP kinase pathways involving hsp27 regulate fibroblast-mediated wound contraction. Journal of Surgical Research, 102 (2): 77-84.

Hirasawa, G., Sasano, H., Takahashi, K. I. et al. (1997) Colocalization of 11 beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. Journal of Clinical Endocrinology & Metabolism, 82 (11): 3859-3863.

Holliday, R. (1989) Food, Reproduction and Longevity - Is the Extended Lifespan of Calorie-Restricted Animals An Evolutionary Adaptation. Bioessays, 10 (4): 125-127.

Holmes, M. C., Carter, R. N., Noble, J. et al. (2010) 11 beta-Hydroxysteroid Dehydrogenase Type 1 Expression Is Increased in the Aged Mouse Hippocampus and Parietal Cortex and Causes Memory Impairments. Journal of Neuroscience, 30 (20): 6916-6920.

Honma, S., Katsuno, Y., Abe, H. et al. (1996) Aging affects development and persistence of feeding-associated circadian rhythm in rat plasma corticosterone. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 40 (6): R1514-R1520.

Honma, T., Yanaka, M., Tsuduki, T. et al. (2011) Increased Lipid Accumulation in Liver and White Adipose Tissue in Aging in the SAMP10 Mouse. Journal of Nutritional Science and Vitaminology, 57 (2): 123-129.

Hornebeck, W. (2003) Down-regulation of tissue inhibitor of matrix metalloprotease-1 (TIMP-1) in aged human skin contributes to matrix degradation and impaired cell growth and survival. Pathologie Biologie, 51 (10): 569-573.

Hsieh, H. L., Wang, H. H., Wu, W. B. et al. (2010) Transforming growth factor-beta 1 induces matrix metalloproteinase-9 and cell migration in astrocytes: roles of ROS-dependent ERK- and JNK-NF-kappa B pathways. Journal of Neuroinflammation, 7

Hubner, G., Brauchle, M., Smola, H. et al. (1996) Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. Cytokine, 8 (7): 548-556.

Hubner, G., Hu, Q. J., Smola, H. et al. (1996) Strong induction of activin expression after injury suggests an important role of activin in wound repair. Developmental Biology, 173 (2): 490-498.

Hundertmark, S., Dill, A., Ebert, A. et al. (2002) Foetal lung maturation in 11 beta-hydroxysteroid dehydrogenase type 1 knockout mice. Hormone and Metabolic Research, 34 (10): 545-549.

Husom, A. D., Peters, E. A., Kolling, E. A. et al. (2004) Altered proteasome function and subunit composition in aged muscle. Archives of Biochemistry and Biophysics, 421 (1): 67-76.

Hyman, S. E. (2009) How adversity gets under the skin. Nature Neuroscience, 12 (3): 241-243.

Ichijo, T., Voutetakis, A., Cotrim, A. P. et al. (2005) The Smad6-histone deacetylase 3 complex silences the transcriptional activity of the glucocorticoid receptor - Potential clinical implications. Journal of Biological Chemistry, 280 (51): 42067-42077.

Imam, S. Z., Karahalil, B., Hogue, B. A. et al. (2006) Mitochondrial and nuclear DNA-repair capacity of various brain regions in mouse is altered in an age-dependent manner. Neurobiology of Aging, 27 (8): 1129-1136.

Intano, G. W., McMahan, C. A., McCarrey, J. R. et al. (2002) Base excision repair is limited by different proteins in male germ cell nuclear extracts prepared from young and old mice. Molecular and Cellular Biology, 22 (7): 2410-2418.

Irani, K., Xia, Y., Zweier, J. L. et al. (1997) Mitogenic signaling mediated by oxidants in ras-transformed fibroblasts. Science, 275 (5306): 1649-1652.

Irvine, C. (1991) Skin Failure - A Real Entity - Discussion Paper. Journal of the Royal Society of Medicine, 84 (7): 412-413.

Irving, J., Feng, J. L., Wistrom, C. et al. (1992) An Altered Repertoire of Fos Jun (Ap-1) at the Onset of Replicative Senescence. Experimental Cell Research, 202 (1): 161-166.

Ishii-Yonemoto, T., Masuzaki, H., Yasue, S. et al. (2010) Glucocorticoid reamplification within cells intensifies NF-kappa B and MAPK signaling and reinforces inflammation in activated preadipocytes. American Journal of Physiology-Endocrinology and Metabolism, 298 (5): E930-E940.

Ishimoto, S. I. and Ishibashi, T. (2002) Induction of growth factor expression is reduced during healing of tympanic membrane perforations in glucocorticoid-treated rats. Annals of Otology Rhinology and Laryngology, 111 (10): 947-953.

Itano, N., Sawai, T., Yoshida, M. et al. (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. Journal of Biological Chemistry, 274 (35): 25085-25092.

Ito, N., Ito, T., Kromminga, A. et al. (2005) Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal (HPA) axis and synthesize cortisol. Faseb Journal, 19 (8): 1332-+.

Iwasaki, K., Mishima, E., Miura, M. et al. (1995) Effect of Ru-486 on the Atrophogenic and Antiinflammatory Effects of Glucocorticoids in Skin. Journal of Dermatological Science, 10 (2): 151-158.

Jacques, E., Semlali, A., Boulet, L. P. et al. (2010) AP-1 overexpression impairs corticosteroid inhibition of collagen production by fibroblasts isolated from asthmatic subjects. American Journal of Physiology-Lung Cellular and Molecular Physiology, 299 (2): L281-L287.

Jang, W. G., Kim, E. J., Park, K. G. et al. (2007) Glucocorticoid receptor mediated repression of human insulin gene expression is regulated by PGC-1 alpha. Biochemical and Biophysical Research Communications, 352 (3): 716-721.

Jensen, J. M., Forl, M., Winoto-Morbach, S. et al. (2005) Acid and neutral sphingomyelinase, ceramide synthase, and acid ceramidase activities in cutaneous aging. Experimental Dermatology, 14 (8): 609-618.

Jeong, H. J., Na, H. J., Hong, S. H. et al. (2003) Inhibition of the stem cell factor-induced migration of mast cells by dexamethasone. Endocrinology, 144 (9): 4080-4086.

Jibard, N., Meng, X., Leclerc, P. et al. (1999) Delimitation of two regions in the 90-kDa heat shock protein (Hsp90) able to interact with the glucocorticosteroid receptor (GR). Experimental Cell Research, 247 (2): 461-474.

Johns, H., Morris, W. J. and Joiner, M. C. (1995) Radiation Response of Murine Eccrine Sweat Glands. Radiotherapy and Oncology, 36 (1): 56-64.

Jonat, C., Rahmsdorf, H. J., Park, K. K. et al. (1990) Antitumor Promotion and Antiinflammation - Down-Modulation of Ap-1 (Fos Jun) Activity by Glucocorticoid Hormone. Cell, 62 (6): 1189-1204.

Ju, Y. J., Lee, K. H., Park, J. E. et al. (2006) Decreased expression of DNA repair proteins Ku70 and Mre11 is associated with aging and may contribute to the cellular senescence. Experimental and Molecular Medicine, 38 (6): 686-693.

Jung, K. J., Lee, E. K., Kim, J. Y. et al. (2009) Effect of short term calorie restriction on pro-inflammatory NF-kB and AP-1 in aged rat kidney. Inflammation Research, 58 (3): 143-150.

Jurisic, G. and Detmar, M. (2009) Lymphatic endothelium in health and disease. Cell and Tissue Research, 335 (1): 97-108.

Justesen, J., Mosekilde, L., Holmes, M. et al. (2004) Mice deficient in 11 beta-hydroxysteroid dehydrogenase type 1 lack bone marrow adipocytes, but maintain normal bone formation. Endocrinology, 145 (4): 1916-1925.

Kahan, V., Andersen, M. L., Tomimori, J. et al. (2009) Stress, immunity and skin collagen integrity: Evidence from animal models and clinical conditions. Brain Behavior and Immunity, 23 (8): 1089-1095.

Kahari, V. M. (1994) Dexamethasone Suppresses Elastin Gene-Expression in Human Skin Fibroblasts in Culture. Biochemical and Biophysical Research Communications, 201 (3): 1189-1196.

Kahari, V. M., Hakkinen, L., Westermarck, J. et al. (1995) Differential Regulation of Decorin and Biglycan Gene-Expression by Dexamethasone and Retinoic Acid in Cultured Human Skin Fibroblasts. Journal of Investigative Dermatology, 104 (4): 503-508.

Kannisto, K., Pietilainen, K. H., Ehrenborg, E. et al. (2004) Overexpression of 11 beta-hydroxysteroid dehydrogenase-1 in adipose tissue is associated with acquired obesity and features of insulin resistance:

Studies in young adult monozygotic twins. Journal of Clinical Endocrinology & Metabolism, 89 (9): 4414-4421.

Kaplan, M. M., Pan, C. Y., Gordon, P. R. et al. (1988) Human Epidermal-Keratinocytes in Culture Convert Thyroxine to 3,5,3'-Triiodothyronine by Type-Ii Iodothyronine Deiodination - A Novel Endocrine Function of the Skin. Journal of Clinical Endocrinology & Metabolism, 66 (4): 815-822.

Karin, M. and Chang, L. (2001) AP-1-glucocorticoid receptor crosstalk taken to a higher level. Journal of Endocrinology, 169 (3): 447-451.

Karran, P. (1996) Microsatellite instability and DNA mismatch repair in human cancer. Seminars in Cancer Biology, 7 (1): 15-24.

Kato, J. S., Fluhr, J. W., Man, M. Q. et al. (2003) Short-term glucocorticoid treatment compromises both permeability barrier homeostasis and stratum corneum integrity: Inhibition of epidermal lipid synthesis accounts for functional abnormalities. Journal of Investigative Dermatology, 120 (3): 456-464.

Keller, J. N., Hanni, K. B. and Markesbery, W. R. (2000) Possible involvement of proteasome inhibition in aging: implications for oxidative stress. Mechanisms of Ageing and Development, 113 (1): 61-70.

Kenouch, S., Lombes, M., Delahaye, F. et al. (1994) Human Skin As Target for Aldosterone - Coexpression of Mineralocorticoid Receptors and 11-Beta-Hydroxysteroid Dehydrogenase. Journal of Clinical Endocrinology & Metabolism, 79 (5): 1334-1341.

Kerkela, E., Ala-aho, R., Jeskanen, L. et al. (2000) Expression of human macrophage metalloelastase (MMP-12) by tumor cells in skin cancer. Journal of Investigative Dermatology, 114 (6): 1113-1119.

Kerkela, E., Ala-aho, R., Jeskanen, L. et al. (2001) Differential patterns of stromelysin-2 (MMP-10) and MT1-MMP (MMP-14) expression in epithelial skin cancers. British Journal of Cancer, 84 (5): 659-669.

Kershaw, E. E., Morton, N. M., Dhillon, H. et al. (2005) Adipocyte-specific glucocorticoid inactivation protects against diet-induced obesity. Diabetes, 54 (4): 1023-1031.

Khalil, M. W., Strutt, B., Vachon, D. et al. (1993) Metabolism of Dehydroepiandrosterone by Cultured Human Adipose Stromal Cells - Identification of 7-Alpha-Hydroxydehydroepiandrosterone As A Major Metabolite Using High-Performance Liquid-Chromatography and Mass-Spectrometry. Journal of Steroid Biochemistry and Molecular Biology, 46 (5): 585-595.

Kim, I., Mogford, J. E., Chao, J. D. et al. (2001) Wound epithelialization deficits in the transforming growth factor-alpha knockout mouse. Wound Repair and Regeneration, 9 (5): 386-390.

Kim, K. S., Seu, Y. B., Baek, S. H. et al. (2007) Induction of cellular senescence by insulin-like growth factor binding protein-5 through a p53-dependent mechanism. Molecular Biology of the Cell, 18 (11): 4543-4552.

Kim, N. W., Piatyszek, M. A., Prowse, K. R. et al. (1994) Specific Association of Human Telomerase Activity with Immortal Cells and Cancer. Science, 266 (5193): 2011-2015.

Kimura, T. and Doi, K. (1999) Dorsal skin reactions of hairless dogs to topical treatment with corticosteroids. Toxicologic Pathology, 27 (5): 528-535.

King, R. W., Deshaies, R. J., Peters, J. M. et al. (1996) How proteolysis drives the cell cycle. Science, 274 (5293): 1652-1659.

Klapper, W., Kuhne, K., Singh, K. K. et al. (1998) Longevity of lobsters is linked to ubiquitous telomerase expression. Febs Letters, 439 (1-2): 143-146.

Kletsas, D., Pratsinis, H., Gioni, V. et al. (2007) Prior chronic in vivo glucocorticoid excess leads to an anabolic phenotype and an extension of cellular life span of skin fibroblasts in vitro. Biogerontology: Mechanisms and Interventions, 1100: 449-454.

Knutti, D., Kaul, A. and Kralli, A. (2000) A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. Molecular and Cellular Biology, 20 (7): 2411-2422.

Koblenzer, C. S. (1996) Psychologic aspects of aging and the skin. Clinics in Dermatology, 14 (2): 171-177.

Koeva, Y., Bakalska, M., Atanassova, N. et al. (2009) Age-related changes in the expression of 11 beta-hydroxysteroid dehydrogenase type 2 in rat Leydig cells. Folia Histochemica et Cytobiologica, 47 (2): 281-287.

Kolbe, L., Kligman, A. M., Schreiner, V. et al. (2001) Corticosteroid-induced atrophy and barrier impairment measured by non-invasive methods in human skin. Skin Research and Technology, 7 (2): 73-77.

Koob, T. J., Jeffrey, J. J., Eisen, A. Z. et al. (1980) Hormonal Interactions in Mammalian Collagenase Regulation - Comparative Studies in Human-Skin and Rat Uterus. Biochimica et Biophysica Acta, 629 (1): 13-23.

Korting, H. C., Hulsebus, E., Kerscher, M. et al. (1995) Discrimination of the Toxic Potential of Chemically Differing Topical Glucocorticoids Using A Neutral Red Release Assay with Human Keratinocytes and Fibroblasts. British Journal of Dermatology, 133 (1): 54-59.

Korting, H. C., Unholzer, A., Schafer-Korting, M. et al. (2002) Different skin thinning potential of equipotent medium-strength glucocorticoids. Skin Pharmacology and Applied Skin Physiology, 15 (2): 85-91.

Kotelevtsev, Y., Holmes, M. C., Burchell, A. et al. (1997) 11 beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proceedings of the National Academy of Sciences of the United States of America, 94 (26): 14924-14929.

Kotelevtsev, Y., Brown, R. W., Fleming, S. et al. (1999) Hypertension in mice lacking 11 beta-hydroxysteroid dehydrogenase type 2. Journal of Clinical Investigation, 103 (5): 683-689.

Kovacs, E. J. (1991) Fibrogenic Cytokines - the Role of Immune Mediators in the Development of Scar Tissue. Immunology Today, 12 (1): 17-23.

Krampert, M., Bloch, W., Sasaki, T. et al. (2004) Activities of the matrix metalloproteinase stromelysin-2 (MMP-10) in matrix degradation and keratinocyte organization in wounded skin. Molecular Biology of the Cell, 15 (12): 5242-5254.

Krieger, D. T., Allen, W., Rizzo, F. et al. (1971) Characterization of Normal Temporal Pattern of Plasma Corticosteroid Levels. Journal of Clinical Endocrinology & Metabolism, 32 (2): 266-+.

KueperO, T., Grune, T., PrahlO, S. et al. (2007) Vimentin is the specific target in skin glycation - Structural prerequisites, functional consequences, and role in skin aging. Journal of Biological Chemistry, 282 (32): 23427-23436.

Kuivaniemi, H., Peltonen, L. and Kivirikko, K. I. (1985) Type-Ix Ehlers-Danlos Syndrome and Menkes Syndrome - the Decrease in Lysyl Oxidase Activity Is Associated with A Corresponding Deficiency in the Enzyme Protein. American Journal of Human Genetics, 37 (4): 798-808.

Kujoth, G. C., Hiona, A. and Pugh, T. D. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science, 309 (5733): 481-484.

Kumar, R. and Thompson, E. B. (1999) The structure of the nuclear hormone receptors. Steroids, 64 (5): 310-319.

Kuroda, K., Utani, A., Hamasaki, Y. et al. (2001) Up-regulation of putative hyaluronan synthase mRNA by basic fibroblast growth factor and insulin-like growth factor-1 in human skin fibroblasts. Journal of Dermatological Science, 26 (2): 156-160.

Kveiborg, M., Kassem, M., Langdahl, B. et al. (1999) Telomere shortening during aging of human osteoblasts in vitro and leukocytes in vivo: lack of excessive telomere loss in osteoporotic patients. Mechanisms of Ageing and Development, 106 (3): 261-271.

Labrie, F., Luu-The, V., Labrie, C. et al. (2000) Intracrinology and the skin. Hormone Research, 54 (5-6): 218-229.

Lai, J. J., Lai, K. P., Chuang, K. H. et al. (2009) Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression. Journal of Clinical Investigation, 119 (12): 3739-3751.

Lange, M., Thulesen, J., Feldt-Rasmussen, U. et al. (2001) Skin morphological changes in growth hormone deficiency and acromegaly. European Journal of Endocrinology, 145 (2): 147-153.

Laplante, A. F., Moulin, V., Auger, F. A. et al. (1998) Expression of heat shock proteins in mouse skin during wound healing. Journal of Histochemistry & Cytochemistry, 46 (11): 1291-1301.

Laron, Z. (2004) Extensive personal experience - Laron syndrome (primary growth hormone resistance or insensitivity): The personal experience 1958-2003. Journal of Clinical Endocrinology & Metabolism, 89 (3): 1031-1044.

Lasa, M., Abraham, S. M., Boucheron, C. et al. (2002) Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. Molecular and Cellular Biology, 22 (22): 7802-7811.

Laughlin, G. A. and Barrett-Connor, E. (2000) Sexual dimorphism in the influence of advanced aging on adrenal hormone levels: The Rancho Bernardo Study. Journal of Clinical Endocrinology & Metabolism, 85 (10): 3561-3568.

Lavery, G. G., Walker, E. A., Draper, N. et al. (2006) Hexose-6-phosphate dehydrogenase knock-out mice lack 11 beta-hydroxysteroid dehydrogenase type 1-mediated glucocorticoid generation. Journal of Biological Chemistry, 281 (10): 6546-6551.

Lavery, G. G., Hauton, D., Hewitt, K. N. et al. (2007) Hypoglycemia with enhanced hepatic glycogen synthesis in recombinant mice lacking hexose-6Phosphate dehydrogenase. Endocrinology, 148 (12): 6100-6106.

Lavery, G. G., Walker, E. A., Tiganescu, A. et al. (2008) Steroid biomarkers and genetic studies reveal inactivating mutations in hexose-6-phosphate dehydrogenase in patients with cortisone reductase deficiency. Journal of Clinical Endocrinology & Metabolism, 93 (10): 3827-3832.

Lavery, G. G., Walker, E. A., Turan, N. et al. (2008) Deletion of hexose-6-phosphate dehydrogenase activates the unfolded protein response pathway and induces skeletal myopathy. Journal of Biological Chemistry, 283 (13): 8453-8461.

Lavker, R. M. (1979) Structural Alterations in Exposed and Unexposed Aged Skin. Journal of Investigative Dermatology, 73 (1): 59-66.

Lavrovsky, Y., Chatterjee, B., Clark, R. A. et al. (2000) Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. Experimental Gerontology, 35 (5): 521-532.

Lawson, A. J., Walker, E. A., Lavery, G. G. et al. (2011) Cortisone-reductase deficiency associated with heterozygous mutations in 11 beta-hydroxysteroid dehydrogenase type 1. Proceedings of the National Academy of Sciences of the United States of America, 108 (10): 4111-4116.

Lecca, M. R., Maag, C., Berger, E. G. et al. (2011) Fibrotic response in fibroblasts from congenital disorders of glycosylation. Journal of Cellular and Molecular Medicine, 15 (8): 1788-1796.

Lechler, T. and Fuchs, E. (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature, 437 (7056): 275-280.

Ledo, I., Wu, M., Katchman, S. et al. (1994) Glucocorticosteroids Up-Regulate Human Elastin Gene Promoter Activity in Transgenic Mice. Journal of Investigative Dermatology, 103 (5): 632-636.

Lee, B., Vouthounis, C., Stojadinovic, O. et al. (2005) From an enhanceosome to a repressosome: Molecular antagonism between glucocorticoids and EGF leads to inhibition of wound healing. Journal of Molecular Biology, 345 (5): 1083-1097.

Lee, H. C., Pang, C. Y., Hsu, H. S. et al. (1994) Differential Accumulations of 4,977 Bp Deletion in Mitochondrial-Dna of Various Tissues in Human Aging. Biochimica et Biophysica Acta-Molecular Basis of Disease, 1226 (1): 37-43.

Lee, H. C., Lim, M. L. R., Lu, C. Y. et al. (1999) Concurrent increase of oxidative DNA damage and lipid peroxidation together with mitochondrial DNA mutation in human lung tissues during aging - Smoking enhances oxidative stress on the aged tissues. Archives of Biochemistry and Biophysics, 362 (2): 309-316.

Lee, H. C., Chang, C. M. and Chi, C. W. (2010) Somatic mutations of mitochondrial DNA in aging and cancer progression. Ageing Research Reviews, 9: S47-S58.

Lee, J. S., Park, K. Y., Min, H. G. et al. (2010) Negative regulation of stress-induced matrix metalloproteinase-9 by Sirt1 in skin tissue. Experimental Dermatology, 19 (12): 1060-1066.

Lee, K. W. and Cohen, P. (2002) Nuclear effects: unexpected intracellular actions of insulin-like growth factor binding protein-3. Journal of Endocrinology, 175 (1): 33-40.

Lehmann, P., Zheng, P., Lavker, R. M. et al. (1983) Corticosteroid Atrophy in Human-Skin - A Study by Light, Scanning, and Transmission Electron-Microscopy. Journal of Investigative Dermatology, 81 (2): 169-176.

Lehner, P. J. and Cresswell, P. (1996) Processing and delivery of peptides presented by MHC class I molecules. Current Opinion in Immunology, 8 (1): 59-67.

Lewis, D. A., Travers, J. B., Somani, A. K. et al. (2010) The IGF-1/IGF-1R signaling axis in the skin: a new role for the dermis in aging-associated skin cancer. Oncogene, 29 (10): 1475-1485.

Lezza, A. M. S., Mecocci, P., Cormio, A. et al. (1999) Mitochondrial DNA 4977 bp deletion and OH(8)dG levels correlate in the brain of aged subjects but not Alzheimer's disease patients. Faseb Journal, 13 (9): 1083-1088.

Li, L. B., Leung, D. Y. M., Hall, C. F. et al. (2006) Divergent expression and function of glucocorticoid receptor beta in human monocytes and T cells. Journal of Leukocyte Biology, 79 (4): 818-827.

Li, N., Sioutas, C., Cho, A. et al. (2003) Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environmental Health Perspectives, 111 (4): 455-460.

Li, X., Lindquist, S., Chen, R. et al. (2007) Depot-specific messenger RNA expression of 11 beta-hydroxysteroid dehydrogenase type 1 and leptin in adipose tissue of children and adults. International Journal of Obesity, 31 (5): 820-828.

Liang, J. A., Pei, X. R., Zhang, Z. F. et al. (2010) The Protective Effects of Long-Term Oral Administration of Marine Collagen Hydrolysate from Chum Salmon on Collagen Matrix Homeostasis in the Chronological Aged Skin of Sprague-Dawley Male Rats. Journal of Food Science, 75 (8): H230-H238.

Lin, Z. Q., Kondo, T., Ishida, Y. et al. (2003) Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. Journal of Leukocyte Biology, 73 (6): 713-721.

Linder, J., Nolgard, P., Nasman, B. et al. (1993) Decreased Peripheral Glucocorticoid Sensitivity in Alzheimers-Disease. Gerontology, 39 (4): 200-206.

Lindner, A. B. and Demarez, A. (2009) Protein aggregation as a paradigm of aging. Biochimica et Biophysica Acta-General Subjects, 1790 (10): 980-996.

Linkowski, P., Vanonderbergen, A., Kerkhofs, M. et al. (1993) Twin Study of the 24-H Cortisol Profile - Evidence for Genetic-Control of the Human Circadian Clock. American Journal of Physiology, 264 (2): E173-E181.

Linskens, M. H. K., Feng, J. L., Andrews, W. H. et al. (1995) Cataloging Altered Gene-Expression in Young and Senescent Cells Using Enhanced Differential Display. Nucleic Acids Research, 23 (16): 3244-3251.

Little, H. J., Croft, A. P., O'Callaghan, M. J. et al. (2008) Selective Increases in Regional Brain Glucocorticoid: A Novel Effect of Chronic Alcohol. Neuroscience, 156 (4): 1017-1027.

Livingstone, D. E. W., Grassick, S. L., Currie, G. L. et al. (2009) Dysregulation of glucocorticoid metabolism in murine obesity: comparable effects of leptin resistance and deficiency. Journal of Endocrinology, 201 (2): 211-218.

Louie, J. L., Kapphahn, R. J. and Ferrington, D. A. (2002) Proteasome function and protein oxidation in the aged retina. Experimental Eye Research, 75 (3): 271-284.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. et al. (1951) Protein Measurement with the Folin Phenol Reagent. Journal of Biological Chemistry, 193 (1): 265-275.

Lucarz, A. and Brand, G. (2007) Current considerations about Merkel cells. European Journal of Cell Biology, 86 (5): 243-251.

Lupien, S., Lecours, A. R., Schwartz, G. et al. (1996) Longitudinal study of basal cortisol levels in healthy elderly subjects: Evidence for subgroups. Neurobiology of Aging, 17 (1): 95-105.

Ma, W., Wlaschek, M., Tantcheva-Poor, I. et al. (2001) Chronological ageing and photoageing of the fibroblasts and the dermal connective tissue. Clinical and Experimental Dermatology, 26 (7): 592-599.

Maas-Szabowski, N., Shimotoyodome, A. and Fusenig, N. E. (1999) Keratinocyte growth regulation in fibroblast cocultures via a double paracrine mechanism. Journal of Cell Science, 112 (12): 1843-1853.

MaasSzabowski, N. and Fusenig, N. E. (1996) Interleukin-1-induced growth factor expression in postmitotic and resting fibroblasts. Journal of Investigative Dermatology, 107 (6): 849-855.

Madlener, M., Mauch, C., Conca, W. et al. (1996) Regulation of the expression of stromelysin-2 by growth factors in keratinocytes: Implications for normal and impaired wound healing. Biochemical Journal, 320: 659-664.

Madlener, M., Parks, W. C. and Werner, S. (1998) Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. Experimental Cell Research, 242 (1): 201-210.

Magyarics, Z., Csillag, A., Pazmandi, K. et al. (2008) Identification of plasmacytoid pre-dendritic cells by one-color flow cytometry for phenotype screening. Cytometry Part A, 73A (3): 254-258.

Makino, S., Schulkin, J., Smith, M. A. et al. (1995) Regulation of Corticotropin-Releasing Hormone-Receptor Messenger-Ribonucleic-Acid in the Rat-Brain and Pituitary by Glucocorticoids and Stress. Endocrinology, 136 (10): 4517-4525.

Makrantonaki, E. and Zouboulis, C. C. (2007) Molecular mechanisms of skin aging - State of the art. Molecular Mechanisms and Models of Aging, 1119: 40-50.

Mangelsdorf, D. J., Thummel, C., Beato, M. et al. (1995) The Nuclear Receptor Superfamily - the 2Nd Decade. Cell, 83 (6): 835-839.

Manolagas, S. C. (2010) From Estrogen-Centric to Aging and Oxidative Stress: A Revised Perspective of the Pathogenesis of Osteoporosis. Endocrine Reviews, 31 (3): 266-300.

Margadant, C., Charafeddine, R. A. and Sonnenberg, A. (2010) Unique and redundant functions of integrins in the epidermis. Faseb Journal, 24 (11): 4133-4152.

Mariotti, S., Barbesino, G., Caturegli, P. et al. (1993) Complex Alteration of Thyroid-Function in Healthy Centenarians. Journal of Clinical Endocrinology & Metabolism, 77 (5): 1130-1134.

Martin, C., Bean, R., Rose, K. et al. (2001) Cyp7b1 catalyses the 7 alpha-hydroxylation of dehydroepiandrosterone and 25-hydroxycholesterol in rat prostate. Biochemical Journal, 355: 509-515.

Martinez, F. O., Gordon, S., Locati, M. et al. (2006) Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. Journal of Immunology, 177 (10): 7303-7311.

Masuzaki, H., Paterson, J., Shinyama, H. et al. (2001) A transgenic model of visceral obesity and the metabolic syndrome. Science, 294 (5549): 2166-2170.

Masuzaki, H., Yamamoto, H., Kenyon, C. J. et al. (2003) Transgenic amplification of glucocorticoid action in adipose tissue causes high blood pressure in mice. Journal of Clinical Investigation, 112 (1): 83-90.

Mccay, C. M., Crowell, M. F. and Maynard, L. A. (1935) The effect of retarded growth upon the length of life span and upon the ultimate body size. Journal of Nutrition, 10 (1): 63-79.

Mcewen, B. S. (1998) Protective and damaging effects of stress mediators. New England Journal of Medicine, 338 (3): 171-179.

McSweeney, S. J., Kozak, A. M., Khaled, H. et al. (2009) Sustained Enhancement of Cardiac Function Follows Increased Recruitment of Pro-angiogenic Macrophages to Healing Infarcts of 11 Beta Hydroxysteroid Dehydrogenase Type 1 Deficient Mice. Circulation, 120 (18): S792-S792.

McSweeney, S. J., Hadoke, P. W. F., Kozak, A. M. et al. (2010) Improved heart function follows enhanced inflammatory cell recruitment and angiogenesis in 11 beta HSD1-deficient mice post-MI. Cardiovascular Research, 88 (1): 159-167.

Menon, G. K., Feingold, K. R., Moser, A. H. et al. (1985) Denovo Sterologenesis in the Skin .2. Regulation by Cutaneous Barrier Requirements. Journal of Lipid Research, 26 (4): 418-427.

Mercado, A. M., Padgett, D. A., Sheridan, J. F. et al. (2002) Altered kinetics of IL-1 alpha, IL-1 beta, and KGF-1 gene expression in early wounds of restrained mice. Brain Behavior and Immunity, 16 (2): 150-162.

Michailidou, Z., Jensen, M. D., Dumesic, D. A. et al. (2007) Omentall 11 beta-hydroxysteroid dehydrogenase 1 correlates with fat cell size independently of obesity. Obesity, 15 (5): 1155-1163.

Michikawa, Y., Mazzucchelli, F., Bresolin, N. et al. (1999) Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science, 286 (5440): 774-779.

Midwood, K. S. and Schwarzbauer, J. E. (2002) Elastic fibers: Building bridges between cells and their matrix. Current Biology, 12 (8): R279-R281.

Milewich, L., Shaw, C. B. and Sontheimer, R. D. (1988) Steroid-Metabolism by Epidermal-Keratinocytes. Annals of the New York Academy of Sciences, 548: 66-89.

Millis, A. J. T., Hoyle, M., Mccue, H. M. et al. (1992) Differential Expression of Metalloproteinase and Tissue Inhibitor of Metalloproteinase Genes in Aged Human Fibroblasts. Experimental Cell Research, 201 (2): 373-379.

Milstone, L. M. (2004) Epidermal desquamation. Journal of Dermatological Science, 36 (3): 131-140.

Mohler, E. M., Browman, K. E., Roderwald, V. A. et al. (2011) Acute Inhibition of 11 beta-Hydroxysteroid Dehydrogenase Type-1 Improves Memory in Rodent Models of Cognition. Journal of Neuroscience, 31 (14): 5406-5413.

Mohr and Takashima (2007) In the skINsight feature titled "Epidermal langerhans cell movement in situ: A model for understanding immunologic function in the skin". Archives of Dermatology, 143 (11): 1438-1438.

Monteiro, H. P. and Stern, A. (1996) Redox modulation of tyrosine phosphorylation-dependent signal transduction pathways. Free Radical Biology and Medicine, 21 (3): 323-333.

Moore, J. S., Monson, J. P., Kaltsas, G. et al. (1999) Modulation of 11 beta-hydroxysteroid dehydrogenase isozymes by growth hormone and insulin-like growth factor: In vivo and in vitro studies. Journal of Clinical Endocrinology & Metabolism, 84 (11): 4172-4177.

Morfin, R. and Courchay, G. (1994) Pregnenolone and Dehydroepiandrosterone As Precursors of Native 7-Hydroxylated Metabolites Which Increase the Immune-Response in Mice. Journal of Steroid Biochemistry and Molecular Biology, 50 (1-2): 91-100.

Morgan, S. A., Sherlock, M., Gathercole, L. L. et al. (2009) 11 beta-Hydroxysteroid Dehydrogenase Type 1 Regulates Glucocorticoid-Induced Insulin Resistance in Skeletal Muscle. Diabetes, 58 (11): 2506-2515.

Mori, R., Kondo, T., Ohshima, T. et al. (2002) Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration. Faseb Journal, 16 (9): 963-974.

Morita, A., Torii, K., Maeda, A. et al. (2009) Molecular Basis of Tobacco Smoke-Induced Premature Skin Aging. Journal of Investigative Dermatology Symposium Proceedings, 14 (1): 53-55.

Moriwaki, S. I., Ray, S., Tarone, R. E. et al. (1996) The effect of donor age on the processing of UV-damaged DNA by cultured human cells: Reduced DNA repair capacity and increased DNA mutability. Mutation Research-Dna Repair, 364 (2): 117-123.

Morton, N. M., Holmes, M. C., Fievet, C. et al. (2001) Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11 beta-hydroxysteroid dehydrogenase type 1 null mice. Journal of Biological Chemistry, 276 (44): 41293-41300.

Morton, N. M., Paterson, J. M., Masuzaki, H. et al. (2004) Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice. Diabetes, 53 (4): 931-938.

Moynahan, M. E. and Jasin, M. (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nature Reviews Molecular Cell Biology, 11 (3): 196-207.

Muller, C., Pompon, D., Urban, P. et al. (2006) Inter-conversion of 7 alpha- and 7 beta-hydroxy-dehydroepiandrosterone by the human 11 beta-hydroxysteroid dehydrogenase type 1. Journal of Steroid Biochemistry and Molecular Biology, 99 (4-5): 215-222.

Muller, C., Hennebert, O. and Morfin, R. (2006) The native anti-glucocorticoid paradigm. Journal of Steroid Biochemistry and Molecular Biology, 100 (1-3): 95-105.

Munck, A. and Narayfejestoth, A. (1992) The Ups and Downs of Glucocorticoid Physiology Permissive and Suppressive Effects Revisited. Molecular and Cellular Endocrinology, 90 (1): C1-C4.

Munz, B., Smola, H., Engelhardt, F. et al. (1999) Overexpression of activin A in the skin of transgenic mice reveals new activities of activin in epidermal morphogenesis, dermal fibrosis and wound repair. Embo Journal, 18 (19): 5205-5215.

Muratani, M. and Tansey, W. R. (2003) How the ubiquitin-proteasome system controls transcription. Nature Reviews Molecular Cell Biology, 4 (3): 192-201.

Myllyharju, J. (2008) Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. Annals of Medicine, 40 (6): 402-417.

Nakagawa, S., Pawelek, P. and Grinnell, F. (1989) Extracellular-Matrix Organization Modulates Fibroblast Growth and Growth-Factor Responsiveness. Experimental Cell Research, 182 (2): 572-582.

Naujokat, C. and Hoffmann, S. (2002) Role and function of the 26S proteasome in proliferation and apoptosis. Laboratory Investigation, 82 (8): 965-980.

Neaves, W. B., Johnson, L., Porter, J. C. et al. (1984) Leydig-Cell Numbers, Daily Sperm Production, and Serum Gonadotropin-Levels in Aging Men. Journal of Clinical Endocrinology & Metabolism, 59 (4): 756-763.

Nelzen, O., Bergqvist, D., Lindhagen, A. et al. (1991) Chronic Leg Ulcers - An Underestimated Problem in Primary Health-Care Among Elderly Patients. Journal of Epidemiology and Community Health, 45 (3): 184-187.

Neri, S., Gardini, A., Facchini, A. et al. (2005) Mismatch repair system and aging: Microsatellite instability in peripheral blood cells from differently aged participants. Journals of Gerontology Series A-Biological Sciences and Medical Sciences, 60 (3): 285-292.

Nestle, F. O., Di Meglio, P., Qin, J. Z. et al. (2009) Skin immune sentinels in health and disease. Nature Reviews Immunology, 9 (10): 679-691.

Newell-Price, J., Bertagna, X., Grossman, A. B. et al. (2006) Cushing's syndrome. Lancet, 367 (9522): 1605-1617.

Nickoloff, B. J. (2000) Characterization of lymphocyte-dependent angiogenesis using a SCID mouse: Human skin model of psoriasis. Journal of Investigative Dermatology Symposium Proceedings, 5 (1): 67-73.

Niculescu, L. and Van Schaftingen, E. (1998) Mannitol 1-phosphate mediates an inhibitory effect of mannitol on the activity and the translocation of glucokinase in isolated rat hepatocytes. Diabetologia, 41 (8): 947-954.

Niedernhofer, L. J., Garinis, G. A., Raams, A. et al. (2006) A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature, 444 (7122): 1038-1043.

Nieminen, M., Henttinen, T., Merinen, M. et al. (2006) Vimentin function in lymphocyte adhesion and transcellular migration. Nature Cell Biology, 8 (2): 156-U41.

Niizuma, T., Zerboni, L., Sommer, M. H. et al. (2003) Construction of varicella-zoster virus recombinants from parent Oka cosmids and demonstration that ORF65 protein is dispensable for infection of human skin and T cells in the SCID-hu mouse model. Journal of Virology, 77 (10): 6062-6065.

Nilsson, P. M. (1996) Premature ageing: The link between psychosocial risk factors and disease. Medical Hypotheses, 47 (1): 39-42.

Niyonsaba, F., Nagaoka, I. and Ogawa, H. (2006) Human defensins and cathelicidins in the skin: Beyond direct antimicrobial properties. Critical Reviews in Immunology, 26 (6): 545-575.

Noor, A. and Knox, K. S. (2007) Immunopathogenesis of sarcoidosis. Clinics in Dermatology, 25 (3): 250-258.

Nuutinen, P., Riekki, R., Parikka, M. et al. (2003) Modulation of collagen synthesis and mRNA by continuous and intermittent use of topical hydrocortisone in human skin. British Journal of Dermatology, 148 (1): 39-45.

O'Sullivan, R. L., Lipper, G. and Lerner, E. A. (1998) The neuro-immuno-cutaneous-endocrine network: Relationship of mind and skin. Archives of Dermatology, 134 (11): 1431-1435.

Ohalloran, D. J. and Shalet, S. M. (1995) Acromegaly - Unraveling A Complex Disease. Growth Regulation, 5 (3): 119-124.

Oikarinen, A. and Autio, P. (1991) New Aspects of the Mechanism of Corticosteroid-Induced Dermal Atrophy. Clinical and Experimental Dermatology, 16 (6): 416-419.

Oikarinen, A., Autio, P., Kiistala, U. et al. (1992) A New Method to Measure Type-I and Type-Iii Collagen-Synthesis in Human Skin Invivo - Demonstration of Decreased Collagen-Synthesis After Topical Glucocorticoid Treatment. Journal of Investigative Dermatology, 98 (2): 220-225.

Oikarinen, A., Haapasaari, K. M., Sutinen, M. et al. (1998) The molecular basis of glucocorticoid-induced skin atrophy: topical glucocorticoid apparently decreases both collagen synthesis and the corresponding collagen mRNA level in human skin in vivo. British Journal of Dermatology, 139 (6): 1106-1110.

Oikarinen, J., Pihlajaniemi, T., Hamalainen, L. et al. (1983) Cortisol Decreases the Cellular Concentration of Translatable Procollagen Messenger-Rna Species in Cultured Human-Skin Fibroblasts. Biochimica et Biophysica Acta, 741 (3): 297-302.

Oishi, Y., Fu, Z. W., Ohnuki, Y. et al. (2002) Molecular basis of the alteration in skin collagen metabolism in response to in vivo dexamethasone treatment: effects on the synthesis of collagen type I and III, collagenase, and tissue inhibitors of metalloproteinases. British Journal of Dermatology, 147 (5): 859-868.

OLOVNIKO.AM (1973) Theory of Marginotomy - Incomplete Copying of Template Margin in Enzymic-Synthesis of Polynucleotides and Biological Significance of Phenomenon. Journal of Theoretical Biology, 41 (1): 181-190.

Olsen, N. J., Sokka, T., Seehorn, C. L. et al. (2004) A gene expression signature for recent onset rheumatoid arthritis in peripheral blood mononuclear cells. Annals of the Rheumatic Diseases, 63 (11): 1387-1392.

Ong, P. Y., Ohtake, T., Brandt, C. et al. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. New England Journal of Medicine, 347 (15): 1151-1160.

Orentreich, N., Brind, J. L., Rizer, R. L. et al. (1984) Age-Changes and Sex-Differences in Serum Dehydroepiandrosterone Sulfate Concentrations Throughout Adulthood. Journal of Clinical Endocrinology & Metabolism, 59 (3): 551-555.

Ortonne, J. P. (1990) The Effects of Ultraviolet Exposure on Skin Melanin Pigmentation. Journal of International Medical Research, 18: C8-C17.

Otberg, N., Finner, A. M. and Shapiro, J. (2007) Androgenetic alopecia. Endocrinology and Metabolism Clinics of North America, 36 (2): 379-+.

Park, I. H., Zhao, R., West, J. A. et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature, 451 (7175): 141-1U1.

Park, W. Y., Park, J. S., Cho, K. A. et al. (2000) Up-regulation of caveolin attenuates epidermal growth factor signaling in senescent cells. Journal of Biological Chemistry, 275 (27): 20847-20852.

Parker, C. R., Mixon, R. L., Brissie, R. M. et al. (1997) Aging alters zonation in the adrenal cortex of men. Journal of Clinical Endocrinology & Metabolism, 82 (11): 3898-3901.

Paterson, J. M., Morton, N. M., Fievet, C. et al. (2004) Metabolic syndrome without obesity: Hepatic overexpression of 11 beta-hydroxysteroid dehydrogenase type 1 in transgenic mice. Proceedings of the National Academy of Sciences of the United States of America, 101 (18): 7088-7093.

Paus, R. and Cotsarelis, G. (1999) The biology of hair follicles. New England Journal of Medicine, 341 (7): 491-497.

Paus, R., Muller-Rover, S., van der Veen, C. et al. (1999) A comprehensive guide for the recognition and classification of distinct stages of hair follicle morphogenesis. Journal of Investigative Dermatology, 113 (4): 523-532.

Perez, P., Page, A., Bravo, A. et al. (2001) Altered skin development and impaired proliferative and inflammatory responses in transgenic mice overexpressing the glucocorticoid receptor. Faseb Journal, 15 (9): 2030-+.

Pfundt, R., Vlijmen-Willems, I., Bergers, M. et al. (2001) In situ demonstration of phosphorylated c-jun and p38 MAP kinase in epidermal keratinocytes following ultraviolet B irradiation of human skin. Journal of Pathology, 193 (2): 248-255.

Pilewski, J. M., Liu, L. X., Henry, A. C. et al. (2005) Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. American Journal of Pathology, 166 (2): 399-407.

Plikus, M. V. and Chuong, C. M. (2008) Complex hair cycle domain patterns and regenerative hair waves in living rodents. Journal of Investigative Dermatology, 128 (5): 1071-1080.

Poirier, P., Giles, T. D., Bray, G. A. et al. (2006) Obesity and cardiovascular disease: Pathophysiology, evaluation, and effect of weight loss - An update of the 1997 American Heart Association Scientific Statement on obesity and heart disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Circulation, 113 (6): 898-918.

Ponnappan, U. (2002) Ubiquitin-proteasome pathway is compromised in CD45RO(+) and CD45RA(+) T lymphocyte subsets during aging. Experimental Gerontology, 37 (2-3): 359-367.

Porter, R. M. (2003) Mouse models for human hair loss disorders. Journal of Anatomy, 202 (1): 125-131.

Potts, R. O., Buras, E. M. and Chrisman, D. A. (1984) Changes with Age in the Moisture-Content of Human-Skin. Journal of Investigative Dermatology, 82 (1): 97-100.

Pratsinis, H., Tsagarakis, S., Zervolea, I. et al. (2002) Chronic in vivo exposure to glucocorticoids prolongs cellular lifespan: the case of Cushing's syndrome-patients' fibroblasts. Experimental Gerontology, 37 (10-11): 1237-1245.

Qin, W. N., Rudolph, A. E., Bond, B. R. et al. (2003) Transgenic model of aldosterone-driven cardiac hypertrophy and heart failure. Circulation Research, 93 (1): 69-76.

Quan, T. H., He, T. Y., Voorhees, J. J. et al. (2001) Ultraviolet irradiation blocks cellular responses to transforming growth factor-beta by down-regulating its Type-II receptor and inducing Smad7. Journal of Biological Chemistry, 276 (28): 26349-26356.

Quinkler, M. and Stewart, P. M. (2003) Hypertension and the cortisol-cortisone shuttle. Journal of Clinical Endocrinology & Metabolism, 88 (6): 2384-2392.

Rabe, J. H., Mamelak, A. J., McElgunn, P. J. S. et al. (2006) Photoaging: Mechanisms and repair. Journal of the American Academy of Dermatology, 55 (1): 1-19.

Ramsey, M. J., Moore, D. H., Briner, J. F. et al. (1995) The Effects of Age and Life-Style Factors on the Accumulation of Cytogenetic Damage As Measured by Chromosome Painting. Mutation Research-Dnaging Genetic Instability and Aging, 338 (1-6): 95-106.

Rask, E., Walker, B. R., Soderberg, S. et al. (2002) Tissue-specific changes in peripheral cortisol metabolism in obese women: Increased adipose 11 beta-hydroxysteroid dehydrogenase type 1 activity. Journal of Clinical Endocrinology & Metabolism, 87 (7): 3330-3336.

Rasmuson, S., Andrew, R., Nasman, B. et al. (2001) Increased glucocorticoid production and altered cortisol metabolism in women with mild to moderate Alzheimer's disease. Biological Psychiatry, 49 (6): 547-552.

Raychaudhuri, S. R., Sanyal, M., Weltman, H. et al. (2004) K252a, a high-affinity nerve growth factor receptor blocker, improves psoriasis: An in vivo study using the severe combined Immunodeficient mouse-human skin model. Journal of Investigative Dermatology, 122 (3): 812-819.

Reed, M. J., Penn, P. E., Li, Y. et al. (1996) Enhanced cell proliferation and biosynthesis mediate improved wound repair in refed, caloric-restricted mice. Mechanisms of Ageing and Development, 89 (1): 21-43.

Reich, A., Stander, S. and Szepietowski, J. C. (2011) Pruritus in the elderly. Clinics in Dermatology, 29 (1): 15-23.

Reichlin, S. (1993) Neuroendocrine-Immune Interactions. New England Journal of Medicine, 329 (17): 1246-1253.

Reuben, P. M., Wenger, L., Cruz, M. et al. (2001) Induction of matrix metalloproteinase-8 in human fibroblasts by basic calcium phosphate and calcium pyrophosphate dihydrate crystals: Effect of phosphocitrate. Connective Tissue Research, 42 (1): 1-+.

Rhen, T. and Cidlowski, J. A. (2005) Antiinflammatory action of glucocorticoids - New mechanisms for old drugs. New England Journal of Medicine, 353 (16): 1711-1723.

Riabowol, K., Schiff, J. and Gilman, M. Z. (1992) Transcription Factor Ap-1 Activity Is Required for Initiation of Dna-Synthesis and Is Lost During Cellular Aging. Proceedings of the National Academy of Sciences of the United States of America, 89 (1): 157-161.

Rice, R. H. and Green, H. (1977) Cornified Envelope of Terminally Differentiated Human Epidermal Keratinocytes Consists of Cross-Linked Protein. Cell, 11 (2): 417-422.

Rinn, J. L., Bondre, C., Gladstone, H. B. et al. (2006) Anatomic demarcation by positional variation in fibroblast gene expression programs. Plos Genetics, 2 (7): 1084-1096.

Robertson, N. M., Zangrilli, J., Fernandes-Alnemri, T. et al. (1997) Baculovirus P35 inhibits the glucocorticoid-mediated pathway of cell death. Cancer Research, 57 (1): 43-47.

Rodriguez, A., Muller, D. C., Engelhardt, M. et al. (2005) Contribution of impaired glucose tolerance in subjects with the metabolic syndrome: Baltimore Longitudinal Study of Aging. Metabolism-Clinical and Experimental, 54 (4): 542-547.

Rohleder, N., Kudielka, B. M., Hellhammer, D. H. et al. (2002) Age and sex steroid-related changes in glucocorticoid sensitivity of pro-inflammatory cytokine production after psychosocial stress. Journal of Neuroimmunology, 126 (1-2): 69-77.

Rooney, D. P., Neely, R. D. G., Cullen, C. et al. (1993) The Effect of Cortisol on Glucose/Glucose-6-Phosphate Cycle Activity and Insulin Action. Journal of Clinical Endocrinology & Metabolism, 77 (5): 1180-1183.

Rose, K. A., Stapleton, G., Dott, K. et al. (1997) Cyp7b, a novel brain cytochrome P450, catalyzes the synthesis of neurosteroids 7 alpha-hydroxy dehydroepiandrosterone and 7 alpha-hydroxy pregnenolone. Proceedings of the National Academy of Sciences of the United States of America, 94 (10): 4925-4930.

Rosenstock, J., Banarer, S., Fonseca, V. A. et al. (2010) The 11-beta-Hydroxysteroid Dehydrogenase Type 1 Inhibitor INCB13739 Improves Hyperglycemia in Patients With Type 2 Diabetes Inadequately Controlled by Metformin Monotherapy. Diabetes Care, 33 (7): 1516-1522.

Rubin, H. (2002) The disparity between human cell senescence in vitro and lifelong replication in vivo. Nature Biotechnology, 20 (7): 675-681.

Rudolph, K. L., Chang, S., Lee, H. W. et al. (1999) Longevity, stress response, and cancer in aging telomerase-deficient mice. Cell, 96 (5): 701-712.

Russell-Aulet, M., Jaffe, C. A., Demott-Friberg, R. et al. (1999) In vivo semiquantification of hypothalamic growth hormone-releasing hormone (GHRH) output in humans: Evidence for relative GHRH deficiency in aging. Journal of Clinical Endocrinology & Metabolism, 84 (10): 3490-3497.

Russell, S. B., Trupin, J. S., Kennedy, R. Z. et al. (1995) Glucocorticoid Regulation of Elastin Synthesis in Human Fibroblasts - Down-Regulation in Fibroblasts from Normal Dermis But Not from Keloids. Journal of Investigative Dermatology, 104 (2): 241-245.

Russell, S. B., Russell, J. D., Trupin, K. M. et al. (2010) Epigenetically Altered Wound Healing in Keloid Fibroblasts. Journal of Investigative Dermatology, 130 (10): 2489-2496.

Saleh-Gohari, N. and Helleday, T. (2004) Conservative homologous recombination preferentially repairs DNA double-strand breaks in the S phase of the cell cycle in human cells. Nucleic Acids Research, 32 (12): 3683-3688.

Salo, A. M., Cox, H., Farndon, P. et al. (2008) A Connective Tissue Disorder Caused by Mutations of the Lysyl Hydroxylase 3 Gene. American Journal of Human Genetics, 83 (4): 495-503.

Sandeep, T. C., Yau, J. L. W., MacLullich, A. M. J. et al. (2004) 11 beta-Hydroxysteroid dehydrogenase inhibition improves cognitive function in healthy elderly men and type 2 diabetics. Proceedings of the National Academy of Sciences of the United States of America, 101 (17): 6734-6739.

Sapolsky, R. M., Krey, L. C. and Mcewen, B. S. (1986) The Neuroendocrinology of Stress and Aging - the Glucocorticoid Cascade Hypothesis. Endocrine Reviews, 7 (3): 284-301.

Sapolsky, R. M. and Altmann, J. (1991) Incidence of Hypercortisolism and Dexamethasone Resistance Increases with Age Among Wild Baboons. Biological Psychiatry, 30 (10): 1008-1016.

Sapolsky, R. M. (1999) Glucocorticoids, stress, and their adverse neurological effects: relevance to aging. Experimental Gerontology, 34 (6): 721-732.

Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews, 21 (1): 55-89.

Sargent, R. G., Brenneman, M. A. and Wilson, J. H. (1997) Repair of site-specific double-strand breaks in a mammalian chromosome by homologous and illegitimate recombination. Molecular and Cellular Biology, 17 (1): 267-277.

Savary, I., Debras, E., Dardevet, D. et al. (1998) Effect of glucocorticoid excess on skeletal muscle and heart protein synthesis in adult and old rats. British Journal of Nutrition, 79 (3): 297-304.

Savina, A. and Amigorena, S. (2007) Phagocytosis and antigen presentation in dendritic cells. Immunological Reviews, 219: 143-156.

Sawaya, M. E. and Penneys, N. S. (1992) Immunohistochemical Distribution of Aromatase and 3B-Hydroxysteroid Dehydrogenase in Human Hair Follicle and Sebaceous Gland. Journal of Cutaneous Pathology, 19 (4): 309-314.

Sayama, A., Soushin, T., Okada, T. et al. (2010) Morphological and Biochemical Changes During Aging and Photoaging of the Skin of C57BL/6J Mice. Journal of Toxicologic Pathology, 23 (3): 133-139.

Schacke, H., Schottelius, A., Docke, W. D. et al. (2004) Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. Proceedings of the National Academy of Sciences of the United States of America, 101 (1): 227-232.

Scharffetterkochanek, K., Wlaschek, M., Briviba, K. et al. (1993) Singlet Oxygen Induces Collagenase Expression in Human Skin Fibroblasts. Febs Letters, 331 (3): 304-306.

Scheid, A., Meuli, M., Gassmann, M. et al. (2000) Genetically modified mouse models in studies on cutaneous wound healing. Experimental Physiology, 85 (6): 687-704.

Schittek, B., Hipfel, R., Sauer, B. et al. (2001) Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nature Immunology, 2 (12): 1133-1137.

Schlake, T. (2007) Determination of hair structure and shape. Seminars in Cell & Developmental Biology, 18 (2): 267-273.

Schmidt, M., Weidler, C., Naumann, H. et al. (2005) Reduced capacity for the reactivation of glucocorticoids in rheumatoid arthritis synovial cells - Possible role of the sympathetic nervous system? Arthritis and Rheumatism, 52 (6): 1711-1720.

Schmuth, M., Jiang, Y. J., Dubrac, S. et al. (2008) Peroxisome proliferator-activated receptors and liver X receptors in epidermal biology. Journal of Lipid Research, 49 (3): 499-509.

Schoepe, S., Schacke, H., May, E. et al. (2006) Glucocorticoid therapy-induced skin atrophy. Experimental Dermatology, 15 (6): 406-420.

Scholzen, T., Armstrong, C. A., Bunnett, N. W. et al. (1998) Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. Experimental Dermatology, 7 (2-3): 81-96.

Schulze, C., Wetzel, F., Kueper, T. et al. (2010) Stiffening of Human Skin Fibroblasts with Age. Biophysical Journal, 99 (8): 2434-2442.

Schurer, N. Y. and Elias, P. M. (1991) The Biochemistry and Function of Stratum-Corneum Lipids. Advances in Lipid Research, 24: 27-56.

Schwarze, S. R., Lee, C. M., Chung, S. S. et al. (1995) High-Levels of Mitochondrial-Dna Deletions in Skeletal-Muscle of Old Rhesus-Monkeys. Mechanisms of Ageing and Development, 83 (2): 91-101.

Scott, P. and Trinchieri, G. (1995) The Role of Natural-Killer-Cells in Host-Parasite Interactions. Current Opinion in Immunology, 7 (1): 34-40.

Seckl, J. R. (2004) Prenatal glucocorticoids and long-term programming. European Journal of Endocrinology, 151: U49-U62.

Seckl, J. R. (2004) 11 beta-hydroxysteroid dehydrogenases: changing glucocorticoid action. Current Opinion in Pharmacology, 4 (6): 597-602.

Seeman, T. E., Mcewen, B. S., Singer, B. H. et al. (1997) Increase in urinary cortisol excretion and memory declines: MacArthur studies of successful aging. Journal of Clinical Endocrinology & Metabolism, 82 (8): 2458-2465.

Seishima, M., Nojiri, M., Esaki, C. et al. (1999) Activin A induces terminal differentiation of cultured human keratinocytes. Journal of Investigative Dermatology, 112 (4): 432-436.

Seluanov, A., Gorbunova, V., Falcovitz, A. et al. (2001) Change of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stabilize p53. Molecular and Cellular Biology, 21 (5): 1552-1564.

Seluanov, A., Mittelman, D., Pereira-Smith, O. M. et al. (2004) DNA end joining becomes less efficient and more error-prone during cellular senescence. Proceedings of the National Academy of Sciences of the United States of America, 101 (20): 7624-7629.

Seluanov, A., Danek, J., Hause, N. et al. (2007) Changes in the level and distribution of Ku proteins during cellular senescence. Dna Repair, 6 (12): 1740-1748.

Semjonous, N. M., Sherlock, M., Jeyasuria, P. et al. (2011) Hexose-6-Phosphate Dehydrogenase Contributes to Skeletal Muscle Homeostasis Independent of 11 beta-Hydroxysteroid Dehydrogenase Type 1. Endocrinology, 152 (1): 93-102.

Seshadri, T. and Campisi, J. (1990) Repression of C-Fos Transcription and An Altered Genetic Program in Senescent Human-Fibroblasts. Science, 247 (4939): 205-209.

Sestito, R., Madonna, S., Scarponi, C. et al. (2011) STAT3-dependent effects of IL-22 in human keratinocytes are counterregulated by sirtuin 1 through a direct inhibition of STAT3 acetylation. Faseb Journal, 25 (3): 916-927.

Severino, J., Allen, R. G., Balin, S. et al. (2000) Is beta-galactosidase staining a marker of senescence in vitro and in vivo? Experimental Cell Research, 257 (1): 162-171.

Shackelford, D. A. (2006) DNA end joining activity is reduced in Alzheimer's disease. Neurobiology of Aging, 27 (4): 596-605.

Sharma, R. and Dutta, D. (2006) Age-Dependent Decrease in Renal Glucocorticoid Receptor Function Is Reversed by Dietary Restriction in Mice. Understanding and Modulating Aging, 1067: 129-141.

Shen, G. P., Galick, H., Inoue, M. et al. (2003) Decline of nuclear and mitochondrial oxidative base excision repair activity in late passage human diploid fibroblasts. Dna Repair, 2 (6): 673-693.

Shephard, P., Martin, G., Smola-Hess, S. et al. (2004) Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. American Journal of Pathology, 164 (6): 2055-2066.

Sheppard, K. and Funder, J. W. (1987) Type-I Receptors in Parotid, Colon, and Pituitary Are Aldosterone Selective Invivo. American Journal of Physiology, 253 (4): E467-E471.

Sher, L. B., Woitge, H. W., Adams, D. J. et al. (2004) Transgenic expression of 11 beta-hydroxysteroid dehydrogenase type 2 in osteoblasts reveals an anabolic role for endogenous glucocorticoids in bone. Endocrinology, 145 (2): 922-929.

Sheu, H. M., Lee, J. Y. Y., Chai, C. Y. et al. (1997) Depletion of stratum corneum intercellular lipid lamellae and barrier function abnormalities after long-term topical corticosteroids. British Journal of Dermatology, 136 (6): 884-890.

Shibasaki, M., Wilson, T. E. and Crandall, C. G. (2006) Neural control and mechanisms of eccrine sweating during heat stress and exercise. Journal of Applied Physiology, 100 (5): 1692-1701.

Shirakata, Y., Kimura, R., Nanba, D. et al. (2005) Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. Journal of Cell Science, 118 (11): 2363-2370.

Short, K. R., Bigelow, M. L., Kahl, J. et al. (2005) Decline in skeletal muscle mitochondrial function with aging in humans. Proceedings of the National Academy of Sciences of the United States of America, 102 (15): 5618-5623.

Skold, C. M., Liu, X. D., Zhu, Y. K. et al. (1999) Glucocorticoids augment fibroblast-mediated contraction of collagen gels by inhibition of endogenous PGE production. Proceedings of the Association of American Physicians, 111 (3): 249-258.

Slavin, J., Unemori, E., Hunt, T. K. et al. (1994) Transforming Growth-Factor-Beta (Tgf-Beta) and Dexamethasone Have Direct Opposing Effects on Collagen-Metabolism in Low Passage Human Dermal Fibroblasts In-Vitro. Growth Factors, 11 (3): 205-213.

Slominski, A., Ermak, G. and Mihm, M. (1996) ACTH receptor, CYP11A1, CYP17 and CYP21A2 genes are expressed in skin. Journal of Clinical Endocrinology & Metabolism, 81 (7): 2746-2749.

Slominski, A., Wortsman, J., Foecking, M. F. et al. (2002) Gas chromatography/mass spectrometry characterization of corticosteroid metabolism in human immortalized keratinocytes. Journal of Investigative Dermatology, 118 (2): 310-315.

Slominski, A., Zjawiony, J., Wortsman, J. et al. (2004) A novel pathway for sequential transformation of 7-dehydrocholesterol and expression of the P450scc system in mammalian skin. European Journal of Biochemistry, 271 (21): 4178-4188.

Slominski, A., Zbytek, B., Szczesniewski, A. et al. (2005) CRH stimulation of corticosteroids production in melanocytes is mediated by ACTH. American Journal of Physiology-Endocrinology and Metabolism, 288 (4): E701-E706.

Slominski, A., Zbytek, B., Szczesniewski, A. et al. (2006) Cultured human dermal fibroblasts do produce cortisol. Journal of Investigative Dermatology, 126 (5): 1177-1178.

Small, G. R., Hadoke, P. W. F., Sharif, I. et al. (2005) Preventing local regeneration of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 enhances angiogenesis. Proceedings of the National Academy of Sciences of the United States of America, 102 (34): 12165-12170.

Smith, R. E., Little, P. J., Maguire, J. A. et al. (1996) Vascular localization of the 11 beta-hydroxysteroid dehydrogenase type II enzyme. Clinical and Experimental Pharmacology and Physiology, 23 (6-7): 549-551.

Smith, R. E., Maguire, J. A., SteinOakley, A. N. et al. (1996) Localization of 11 beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. Journal of Clinical Endocrinology & Metabolism, 81 (9): 3244-3248.

Smith, S. A. and Kotwal, G. J. (2002) Immune response to poxvirus infections in various animals. Critical Reviews in Microbiology, 28 (3): 149-185.

Smith, T. J. (1988) Glucocorticoid Regulation of Glycosaminoglycan Synthesis in Cultured Human-Skin Fibroblasts - Evidence for A Receptor-Mediated Mechanism Involving Effects on Specific Denovo Protein-Synthesis. Metabolism-Clinical and Experimental, 37 (2): 179-184.

Sobolev, V. V., Zolotorenko, A. D., Soboleva, A. G. et al. (2011) Effects of Expression of Transcriptional Factor AP-1 FOSL1 Gene on Psoriatic Process. Bulletin of Experimental Biology and Medicine, 150 (5): 632-634.

Soderhall, C., Marenholz, I., Kerscher, T. et al. (2007) Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. Plos Biology, 5 (9): 1952-1961.

Sohal, R. S. and Allen, R. G. (1990) Oxidative Stress As A Causal Factor in Differentiation and Aging - A Unifying Hypothesis. Experimental Gerontology, 25 (6): 499-522.

Sohal, R. S. and Weindruch, R. (1996) Oxidative stress, caloric restriction, and aging. Science, 273 (5271): 59-63.

Sooy, K., Webster, S. P., Noble, J. et al. (2010) Partial Deficiency or Short-Term Inhibition of 11 beta-Hydroxysteroid Dehydrogenase Type 1 Improves Cognitive Function in Aging Mice. Journal of Neuroscience, 30 (41): 13867-13872.

Sorensen, O. E., Cowland, J. B., Theilgaard-Monch, K. et al. (2003) Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. Journal of Immunology, 170 (11): 5583-5589.

Sorrell, J. M., Baber, M. A. and Caplan, A. I. (2007) Clonal characterization of fibroblasts in the superficial layer of the adult human dermis. Cell and Tissue Research, 327 (3): 499-510.

Sousa, A. R., Lane, S. J., Soh, C. et al. (1999) In vivo resistance to corticosteroids in bronchial asthma is associated with enhanced phosphorylation of JUN N-terminal kinase and failure of prednisolone to inhibit JUN N-terminal kinase phosphorylation. Journal of Allergy and Clinical Immunology, 104 (3): 565-574.

Sowers, J. R. and Lippman, H. R. (1985) Cushings-Syndrome Due to Ectopic Acth Production - Cutaneous Manifestations. Cutis, 36 (4): 351-&.

Spaulding, C. C., Walford, R. L. and Effros, R. B. (1997) Calorie restriction inhibits the age-related dysregulation of the cytokines TNF-alpha and IL-6 in C3B10RF1 mice. Mechanisms of Ageing and Development, 93 (1-3): 87-94.

Spencer, R. L. and Hutchison, K. E. (1999) Alcohol, aging, and the stress response. Alcohol Research & Health, 23 (4): 272-+.

Sprenger, C. C., Plymate, S. R. and Reed, M. J. (2008) Extracellular influences on tumour angiogenesis in the aged host. British Journal of Cancer, 98 (2): 250-255.

Stamp, T. (1995) Grey Hairs, False Teeth, and Bad Bones. Lancet, 345 (8954): 876-876.

Stenzelpoore, M. P., Cameron, V. A., Vaughan, J. et al. (1992) Development of Cushings-Syndrome in Corticotropin-Releasing Factor Transgenic Mice. Endocrinology, 130 (6): 3378-3386.

Stevens, M. J., Li, F., Drel, V. R. et al. (2007) Nicotinamide reverses neurological and neurovascular deficits in streptozotocin diabetic rats. The Journal of Pharmacology and Experimental Therapeutics, 320 (1): 458-464.

Stewart, P. M., Corrie, J. E. T., Shackleton, C. H. L. et al. (1988) Syndrome of Apparent Mineralocorticoid Excess - A Defect in the Cortisol Cortisone Shuttle. Journal of Clinical Investigation, 82 (1): 340-349.

Stojadinovic, O., Lee, B., Vouthounis, C. et al. (2007) Novel genomic effects of glucocorticoids in epidermal keratinocytes - Inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation. Journal of Biological Chemistry, 282 (6): 4021-4034.

Stuart, G. R. and Glickman, B. W. (2000) Through a glass, darkly: Reflections of mutation from lacI transgenic mice. Genetics, 155 (3): 1359-1367.

Suh, D. Y., Hunt, T. K. and Spencer, E. M. (1992) Insulin-Like Growth Factor-I Reverses the Impairment of Wound-Healing Induced by Corticosteroids in Rats. Endocrinology, 131 (5): 2399-2403.

Suh, Y., Lee, K. A., Kim, W. H. et al. (2002) Aging alters the apoptotic response to genotoxic stress. Nature Medicine, 8 (1): 3-4.

Sulcova, J., Hill, M., Hampl, R. et al. (1997) Age and sex related differences in serum levels of unconjugated dehydroepiandrosterone and its sulphate in normal subjects. Journal of Endocrinology, 154 (1): 57-62.

Suwabe, H., Serizawa, A., Kajiwara, H. et al. (1999) Degenerative processes of elastic fibers in sun-protected and sun-exposed skin: Immunoelectron microscopic observation of elastin, fibrillin-1, amyloid P component, lysozyme and alpha(1)-antitrypsin. Pathology International, 49 (5): 391-402.

Szabowski, A., Maas-Szabowski, N., Andrecht, S. et al. (2000) c-jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell, 103 (5): 745-755.

Szczesny, B., Bhakat, K. K., Mitra, S. et al. (2004) Age-dependent modulation of DNA repair enzymes by covalent modification and subcellular distribution. Mechanisms of Ageing and Development, 125 (10-11): 755-765.

Takahashi, K., Okita, K., Nakagawa, M. et al. (2007) Induction of pluripotent stem cells from fibroblast cultures. Nature Protocols, 2 (12): 3081-3089.

Tallman, P., Muscare, E., Carson, P. et al. (1997) Initial rate of healing predicts complete healing of venous ulcers. Archives of Dermatology, 133 (10): 1231-1234.

Taylor, A., Lipman, R. D., Jahngenhodge, J. et al. (1995) Dietary Calorie Restriction in the Emery Mouse - Effects on Life-Span, Eye Lens Cataract Prevalence and Progression, Levels of Ascorbate, Glutathione, Glucose, and Glycohemoglobin, Tail Collagen Breaktime, Dna and Rna Oxidation, Skin Integrity, Fecundity, and Cancer. Mechanisms of Ageing and Development, 79 (1): 33-57.

Thiboutot, D., Jabara, S., McAllister, J. M. et al. (2003) Human skin is a steroidogenic tissue: Steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1). Journal of Investigative Dermatology, 120 (6): 905-914.

Thieringer, R., Le Grand, C. B., Carbin, L. et al. (2001) 11 beta-hydroxysteroid dehydrogenase type 1 is induced in human monocytes upon differentiation to macrophages. Journal of Immunology, 167 (1): 30-35.

Thivolet, J. and Nicolas, J. F. (1990) Skin Aging and Immune Competence. British Journal of Dermatology, 122: 77-81.

Todorovic, V., Pesko, P., Micev, M. et al. (2008) Insulin-like growth factor-I in wound healing of rat skin. Regulatory Peptides, 150 (1-3): 7-13.

Toh, M. L., Yang, Y., Leech, M. et al. (2004) Expression of mitogen-activated protein kinase phosphatase 1, a negative regulator of the mitogen-activated protein kinases, in rheumatoid arthritis - Up-regulation by interleukin-1 beta and glucocorticoids. Arthritis and Rheumatism, 50 (10): 3118-3128.

Toma, J. G., McKenzie, I. A., Bagli, D. et al. (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells, 23 (6): 727-737.

Tomlinson, J. W., Sinha, B., Bujalska, I. et al. (2002) Expression of 11 beta-hydroxysteroid dehydrogenase type 1 in adipose tissue is not increased in human obesity. Journal of Clinical Endocrinology & Metabolism, 87 (12): 5630-5635.

Torii, K., Sugiyama, S., Tanaka, M. et al. (1992) Aging-Associated Deletions of Human Diaphragmatic Mitochondrial-Dna. American Journal of Respiratory Cell and Molecular Biology, 6 (5): 543-549.

Torres, C., Francis, M. K., Lorenzini, A. et al. (2003) Metabolic stabilization of MAP kinase phosphatase-2 in senescence of human fibroblasts. Experimental Cell Research, 290 (2): 195-206.

Treiber, N., Maity, P., Singh, K. et al. (2011) Accelerated aging phenotype in mice with conditional deficiency for mitochondrial superoxide dismutase in the connective tissue. Aging Cell, 10 (2): 239-254.

Tresini, M., Mawal-Dewan, M., Cristofalo, V. J. et al. (1998) A phosphatidylinositol 3-kinase inhibitor induces a senescent-like growth arrest in human diploid fibroblasts. Cancer Research, 58 (1): 1-4.

Tresini, M., Lorenzini, A., Frisoni, L. et al. (2001) Lack of Elk-1 phosphorylation and dysregulation of the extracellular regulated kinase signaling pathway in senescent human fibroblast. Experimental Cell Research, 269 (2): 287-300.

Trifunovic, A., Wredenberg, A., Falkenberg, M. et al. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature, 429 (6990): 417-423.

Trimmer, C., Sotgia, F., Whitaker-Menezes, D. et al. (2011) Caveolin-1 and mitochondrial SOD2 (MnSOD) function as tumor suppressors in the stromal microenvironment A new genetically tractable model for human cancer associated fibroblasts. Cancer Biology & Therapy, 11 (4): 383-394.

Tucker, J. D., Spruill, M. D., Ramsey, M. J. et al. (1999) Frequency of spontaneous chromosome aberrations in mice: effects of age. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 425 (1): 135-141.

Um, J. H., Kim, S. J., Kim, D. W. et al. (2003) Tissue-specific changes of DNA repair protein Ku and mtHSP70 in aging rats and their retardation by caloric restriction. Mechanisms of Ageing and Development, 124 (8-9): 967-975.

Uzawa, K., Yeowell, H. N., Yamamoto, K. et al. (2003) Lysine hydroxylation of collagen in a fibroblast cell culture system. Biochemical and Biophysical Research Communications, 305 (3): 484-487.

Vahl, N., Jorgensen, J. O. L., Jurik, A. G. et al. (1996) Abdominal adiposity and physical fitness are major determinants of the age associated decline in stimulated GH secretion in healthy adults. Journal of Clinical Endocrinology & Metabolism, 81 (6): 2209-2215.

Vaisanen, A., Kallioinen, M., Taskinen, P. J. et al. (1998) Prognostic value of MMP-2 immunoreactive protein (72 kD type IV collagenase) in primary skin melanoma. Journal of Pathology, 186 (1): 51-58.

Valle, A., Guevara, R., Garcia-Palmer, F. J. et al. (2008) Caloric restriction retards the age-related decline in mitochondrial function of brown adipose tissue. Rejuvenation Research, 11 (3): 597-604.

Valsamakis, G., Anwar, A., Tomlinson, J. W. et al. (2004) 11 beta-hydroxysteroid dehydrogenase type 1 activity in lean and obese males with type 2 diabetes mellitus. Journal of Clinical Endocrinology & Metabolism, 89 (9): 4755-4761.

van der Slot, A. J., Zuurmond, A. M., Bardoel, A. F. J. et al. (2003) Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. Journal of Biological Chemistry, 278 (42): 40967-40972.

Van Remmen, H., Ikeno, Y., Hamilton, M. et al. (2003) Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiological Genomics, 16 (1): 29-37.

van Rossum, E. F. C., Koper, J. W., Huizenga, N. A. T. M. et al. (2002) A polyntorphism in the glucocorticoid receptor gene, which decreases sensitivity to Glucocorticoids in vivo, is associated with low insulin and cholesterol levels. Diabetes, 51 (10): 3128-3134.

van Rossum, E. F. C., Feelders, R. A., van den Beld, A. W. et al. (2004) Association of the ER22/23EK polymorphism in the glucocorticoid receptor gene with survival and C-reactive protein levels in elderly men. American Journal of Medicine, 117 (3): 158-162.

Vancauter, E., Leproult, R. and Kupfer, D. J. (1996) Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. Journal of Clinical Endocrinology & Metabolism, 81 (7): 2468-2473.

Vance, M. L. (2003) Retrospective - Can growth hormone prevent aging? New England Journal of Medicine, 348 (9): 779-780.

VanGuilder, H. D., Vrana, K. E. and Freeman, W. M. (2008) Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques, 44 (5): 619-626.

Varani, J., Warner, R. L., Gharaee-Kermani, M. et al. (2000) Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. Journal of Investigative Dermatology, 114 (3): 480-486.

Varani, J., Spearman, D., Perone, P. et al. (2001) Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen in vitro. American Journal of Pathology, 158 (3): 931-942.

Varani, J., Dame, M. K., Rittie, L. et al. (2006) Decreased collagen production in chronologically aged skin - Roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. American Journal of Pathology, 168 (6): 1861-1868.

Veldhuis, J. D., Iranmanesh, A., Evans, W. S. et al. (1993) Amplitude Suppression of the Pulsatile Mode of Immunoradiometric Luteinizing-Hormone Release in Fasting-Induced Hypoandrogenemia in Normal Men. Journal of Clinical Endocrinology & Metabolism, 76 (3): 587-593.

Venable, M. E., Blobe, G. C. and Obeid, L. M. (1994) Identification of A Defect in the Phospholipase-D Diacylglycerol Pathway in Cellular Senescence. Journal of Biological Chemistry, 269 (42): 26040-26044.

Venable, M. E., Lee, J. Y., Smyth, M. J. et al. (1995) Role of ceramide in cellular senescence. Journal of Biological Chemistry, 270 (51): 30701-30708.

Veniant, M. M., Hale, C., Komorowski, R. et al. (2009) Time of the day for 11 beta-HSD1 inhibition plays a role in improving glucose homeostasis in DIO mice. Diabetes Obesity & Metabolism, 11 (2): 109-117.

Vermulst, M., Bielas, J. H., Kujoth, G. C. et al. (2007) Mitochondrial point mutations do not limit the natural lifespan of mice. Nature Genetics, 39 (4): 540-543.

Viel, J. J., McManus, D. Q., Smith, S. S. et al. (2001) Age- and concentration-dependent neuroprotection and toxicity by TNF in cortical neurons from beta-amyloid. Journal of Neuroscience Research, 64 (5): 454-465.

Vierkotter, A., Schikowski, T., Ranft, U. et al. (2010) Airborne Particle Exposure and Extrinsic Skin Aging. Journal of Investigative Dermatology, 130 (12): 2719-2726.

Vijg, J. and Dolle, M. E. T. (2002) Large genome rearrangements as a primary cause of aging. Mechanisms of Ageing and Development, 123 (8): 907-915.

Vile, G. F., Basumodak, S., Waltner, C. et al. (1994) Heme Oxygenase-1 Mediates An Adaptive Response to Oxidative Stress in Human Skin Fibroblasts. Proceedings of the National Academy of Sciences of the United States of America, 91 (7): 2607-2610.

Vonzglinicki, T., Saretzki, G., Docke, W. et al. (1995) Mild Hyperoxia Shortens Telomeres and Inhibits Proliferation of Fibroblasts - A Model for Senescence. Experimental Cell Research, 220 (1): 186-193.

Vranka, J. A., Pokidysheva, E., Hayashi, L. et al. (2010) Prolyl 3-Hydroxylase 1 Null Mice Display Abnormalities in Fibrillar Collagen-rich Tissues Such as Tendons, Skin, and Bones. Journal of Biological Chemistry, 285 (22): 17253-17262.

Vukelic, S., Stojadinovic, O., Pastar, I. et al. (2011) Cortisol Synthesis in Epidermis Is Induced by IL-1 and Tissue Injury. Journal of Biological Chemistry, 286 (12): 10265-10275.

Vyjayanti, V. N. and Rao, K. S. (2006) DNA double strand break repair in brain: Reduced NHEJ activity in aging rat neurons. Neuroscience Letters, 393 (1): 18-22.

Wach, F., Bosserhoff, A., Kurzidym, U. et al. (1998) Effects of mometasone furoate on human keratinocytes and fibroblasts in vitro. Skin Pharmacology and Applied Skin Physiology, 11 (1): 43-51.

Waelti, E. R., Inaebnit, S. P., Rast, H. P. et al. (1992) Coculture of Human Keratinocytes on Postmitotic Human Dermal Fibroblast Feeder Cells - Production of Large Amounts of Interleukin-6. Journal of Investigative Dermatology, 98 (5): 805-808.

Wakem, P., Burns, R. P., Ramirez, F. et al. (2000) Allergens and irritants transcriptionally upregulate CD80 gene expression in human keratinocytes. Journal of Investigative Dermatology, 114 (6): 1085-1092.

Walford, R. L., Harris, S. B. and Weindruch, R. (1987) Dietary Restriction and Aging - Historical Phases, Mechanisms and Current Directions. Journal of Nutrition, 117 (10): 1650-1654.

Walker, B. R., Campbell, J. C., Fraser, R. et al. (1992) Mineralocorticoid Excess and Inhibition of 11-Beta-Hydroxysteroid Dehydrogenase in Patients with Ectopic Acth Syndrome. Clinical Endocrinology, 37 (6): 483-492.

Walker, B. R., Connacher, A. A., Lindsay, R. M. et al. (1995) Carbenoxolone Increases Hepatic Insulin Sensitivity in Man - A Novel Role for 11-Oxosteroid Reductase in Enhancing Glucocorticoid Receptor Activation. Journal of Clinical Endocrinology & Metabolism, 80 (11): 3155-3159.

Wallengren, J., Ekman, R. and Sundler, F. (1987) Occurrence and Distribution of Neuropeptides in the Human-Skin - An Immunocytochemical and Immunochemical Study on Normal Skin and Blister Fluid from Inflamed Skin. Acta Dermato-Venereologica, 67 (3): 185-192.

Waller, J. M. and Maibach, H. I. (2006) Age and skin structure and function, a quantitative approach (II): protein, glycosaminoglycan, water, and lipid content and structure. Skin Research and Technology, 12 (3): 145-154.

Walmsley, A. R., Batten, M. R., Lad, U. et al. (1999) Intracellular retention of procollagen within the endoplasmic reticulum is mediated by prolyl 4-hydroxylase. Journal of Biological Chemistry, 274 (21): 14884-14892.

Walter, R. and Sierra, F. (1998) Changes in hepatic DNA binding proteins as a function of age in rats. Journals of Gerontology Series A-Biological Sciences and Medical Sciences, 53 (2): B102-B110.

Waltman, C., Blackman, M. R., Chrousos, G. P. et al. (1991) Spontaneous and Glucocorticoid-Inhibited Adrenocorticotropic Hormone and Cortisol Secretion Are Similar in Healthy-Young and Old Men. Journal of Clinical Endocrinology & Metabolism, 73 (3): 495-502.

Wamil, M. W., Battle, J. H., Turban, S. et al. (2011) Novel Fat Depot-Specific Mechanisms Underlie Resistance to Visceral Obesity and Inflammation in 11 beta-Hydroxysteroid Dehydrogenase Type 1-Deficient Mice. Diabetes, 60 (4): 1158-1167.

Wang, E. (1995) Senescent Human Fibroblasts Resist Programmed Cell-Death, and Failure to Suppress Bcl2 Is Involved. Cancer Research, 55 (11): 2284-2292.

Wang, J., Hannon, G. J. and Beach, D. H. (2000) Cell biology - Risky immortalization by telomerase. Nature, 405 (6788): 755-756.

Wankell, M., Munz, B., Hubner, G. et al. (2001) Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. Embo Journal, 20 (19): 5361-5372.

Wassermann, E., van Griensven, M., Gstaltner, K. et al. (2009) A chronic pressure ulcer model in the nude mouse. Wound Repair and Regeneration, 17 (4): 480-484.

Watson, R. E. B., Craven, N. M., Kang, S. W. et al. (2001) A short-term screening protocol, using fibrillin-1 as a reporter molecule, for photoaging repair agents. Journal of Investigative Dermatology, 116 (5): 672-678.

Watt, F. M. and Hogan, B. L. M. (2000) Out of Eden: Stem cells and their niches. Science, 287 (5457): 1427-1430.

Weaver, J. U., Thaventhiran, L., Noonan, K. et al. (1994) The Effect of Growth-Hormone Replacement on Cortisol Metabolism and Glucocorticoid Sensitivity in Hypopituitary Adults. Clinical Endocrinology, 41 (5): 639-648.

Wei, Q. Y., Matanoski, G. M., Farmer, E. R. et al. (1993) Dna-Repair and Aging in Basal-Cell Carcinoma - A Molecular Epidemiology Study. Proceedings of the National Academy of Sciences of the United States of America, 90 (4): 1614-1618.

Weinstein, R. S., Wan, C., Liu, Q. L. et al. (2010) Endogenous glucocorticoids decrease skeletal angiogenesis, vascularity, hydration, and strength in aged mice. Aging Cell, 9 (2): 147-161.

Welt, C. K., McNicholl, D. J., Taylor, A. E. et al. (1999) Female reproductive aging is marked by decreased secretion of dimeric inhibin. Journal of Clinical Endocrinology & Metabolism, 84 (1): 105-111.

Werner, S., Smola, H., Liao, X. et al. (1994) The Function of Kgf in Morphogenesis of Epithelium and Reepithelialization of Wounds. Science, 266 (5186): 819-822.

Werner, S., Krieg, T. and Smola, H. (2007) Keratinocyte-fibroblast interactions in wound healing. Journal of Investigative Dermatology, 127 (5): 998-1008.

Werth, V. P., Kligman, A. M., Shi, X. M. et al. (1998) Lack of correlation of skin thickness with bone density in patients receiving chronic glucocorticoid. Archives of Dermatological Research, 290 (7): 388-393.

West, M. D., Pereirasmith, O. M. and Smith, J. R. (1989) Replicative Senescence of Human-Skin Fibroblasts Correlates with A Loss of Regulation and Overexpression of Collagenase Activity. Experimental Cell Research, 184 (1): 138-147.

White, P. C. (2009) Neonatal screening for congenital adrenal hyperplasia. Nature Reviews Endocrinology, 5 (9): 490-498.

Wiedermann, C. J. (1987) Shared Recognition Molecules in the Brain and Lymphoid-Tissues - the Polypeptide Mediator Network of Psychoneuroimmunology. Immunology Letters, 16 (3-4): 371-378.

Wight, T. N. (2002) Versican: a versatile extracellular matrix proteoglycan in cell biology. Current Opinion in Cell Biology, 14 (5): 617-623.

Wilkinson, C. W., Peskind, E. R. and Raskind, M. A. (1997) Decreased hypothalamic-pituitary-adrenal axis sensitivity to cortisol feedback inhibition in human aging. Neuroendocrinology, 65 (1): 79-90.

Wilkinson, C. W., Petrie, E. C., Murray, S. R. et al. (2001) Human glucocorticoid feedback inhibition is reduced in older individuals: Evening study. Journal of Clinical Endocrinology & Metabolism, 86 (2): 545-550.

Willaert, A., Malfait, F., Symoens, S. et al. (2009) Recessive osteogenesis imperfecta caused by LEPRE1 mutations: clinical documentation and identification of the splice form responsible for prolyl 3-hydroxylation. Journal of Medical Genetics, 46 (4): 233-241.

Wolf, E., Kahnt, E., Ehrlein, J. et al. (1993) Effects of Long-Term Elevated Serum Levels of Growth-Hormone on Life Expectancy of Mice - Lessons from Transgenic Animal-Models. Mechanisms of Ageing and Development, 68 (1-3): 71-87.

Wolkowitz, O. M., Epel, E. S., Reus, V. I. et al. (2010) Depression Gets Old Fast: do Stress and Depression Accelerate Cell Aging? Depression and Anxiety, 27 (4): 327-338.

Wu, W., Pew, T., Zou, M. et al. (2005) Glucocorticoid receptor-induced MAPK phosphatase-1 (MPK-1) expression inhibits paclitaxel-associated MAPK activation and contributes to breast cancer cell survival. Journal of Biological Chemistry, 280 (6): 4117-4124.

Wu, X. M. and Pandolfi, P. P. (2001) Mouse models for multistep tumorigenesis. Trends in Cell Biology, 11 (11): S2-S9.

Xia, Y. P., Zhao, Y. A., Tyrone, J. W. et al. (2001) Differential activation of migration by hypoxia in keratinocytes isolated from donors of increasing age: Implication for chronic wounds in the elderly. Journal of Investigative Dermatology, 116 (1): 50-56.

Xiao, Z. Q. and Majumdar, A. P. N. (2000) Induction of transcriptional activity of AP-1 and NF-kappa B in the gastric mucosa during aging. American Journal of Physiology-Gastrointestinal and Liver Physiology, 278 (6): G855-G865.

Xu, G. G., Snellman, E., Bykov, V. J. et al. (2000) Effect of age on the formation and repair of UV photoproducts in human skin in situ. Mutation Research-Dna Repair, 459 (3): 195-202.

Xu, G. G., Spivak, G., Mitchell, D. L. et al. (2005) Nucleotide excision repair activity varies among murine spermatogenic cell types. Biology of Reproduction, 73 (1): 123-130.

Xu, J. H. and Clark, R. A. F. (1996) Extracellular matrix alters PDGF regulation of fibroblast integrins. Journal of Cell Biology, 132 (1-2): 239-249.

Yamada, M., Udono, M. U., Hori, M. et al. (2006) Aged human skin removes UVB-induced pyrimidine dimers from the epidermis more slowly than younger adult skin in vivo. Archives of Dermatological Research, 297 (7): 294-302.

Yasuoka, H., Jukic, D. M., Zhou, Z. H. et al. (2006) Insulin-like growth factor binding protein 5 induces skin fibrosis - A novel murine model for dermal fibrosis. Arthritis and Rheumatism, 54 (9): 3001-3010.

Yau, J. L. W., Noble, J., Kenyon, C. J. et al. (2001) Lack of tissue glucocorticoid reactivation in 11 beta-hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. Proceedings of the National Academy of Sciences of the United States of America, 98 (8): 4716-4721.

Yau, J. L. W., Mcnair, K. M., Noble, J. et al. (2007) Enhanced hippocampal long-term potentiation and spatial learning in aged 11 beta-hydroxysteroid dehydrogenase type 1 knock-out mice. Journal of Neuroscience, 27 (39): 10487-10496.

Yau, J. L. W., Noble, J. and Seckl, J. R. (2011) 11 beta-Hydroxysteroid Dehydrogenase Type 1 Deficiency Prevents Memory Deficits with Aging by Switching from Glucocorticoid Receptor to Mineralocorticoid Receptor-Mediated Cognitive Control. Journal of Neuroscience, 31 (11): 4188-4193.

Yen, S. S. C. and Laughlin, G. A. (1998) Aging and the adrenal cortex. Experimental Gerontology, 33 (7-8): 897-910.

Yen, T. C., Su, J. H., King, K. L. et al. (1991) Aging-Associated 5 Kb Deletion in Human Liver Mitochondrial-Dna. Biochemical and Biophysical Research Communications, 178 (1): 124-131.

Yeowell, H. N. and Walker, L. C. (2000) Mutations in the lysyl hydroxylase 1 gene that result in enzyme deficiency and the clinical phenotype of Ehlers-Danlos syndrome type VI. Molecular Genetics and Metabolism, 71 (1-2): 212-224.

Yoon, I. K., Kim, H. K., Kim, Y. K. et al. (2004) Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology. Experimental Gerontology, 39 (9): 1369-1378.

Zervolea, I., Pratsinis, H., Tsagarakis, S. et al. (2005) The impact of chronic in vivo glucocorticoid excess on the functional characteristics of human skin fibroblasts obtained from patients with endogenous Cushing's syndrome. European Journal of Endocrinology, 152 (6): 895-902.

Zhang, H., Hoff, H., Marinucci, T. et al. (2000) Mitogen-independent phosphorylation of S6K1 and decreased ribosomal S6 phosphorylation in senescent human fibroblasts. Experimental Cell Research, 259 (1): 284-292.

Zhang, J. L., Patel, J. M. and Block, E. R. (2002) Enhanced apoptosis in prolonged cultures of senescent porcine pulmonary artery endothelial cells. Mechanisms of Ageing and Development, 123 (6): 613-625.

Zhang, T. Y., Ding, X. H. and Daynes, R. A. (2005) The expression of 11 beta-hydroxysteroid dehydrogenase type I by lymphocytes provides a novel means for intracrine regulation of glucocorticoid activities. Journal of Immunology, 174 (2): 879-889.

Zhang, T. Y. and Daynes, R. A. (2007) Macrophages from 11 beta-hydroxysteroid dehydrogenase type 1-deficient mice exhibit an increased sensitivity to lipopolysaccharide stimulation due to TGF-beta-Mediated up-regulation of SHIP1 expression. Journal of Immunology, 179 (9): 6325-6335.

Zhang, W., Watson, C. E., Liu, C. et al. (2000) Glucocorticoids induce a near-total suppression of hyaluronan synthase mRNA in dermal fibroblasts and in osteoblasts: a molecular mechanism contributing to organ atrophy. Biochemical Journal, 349: 91-97.

Zouboulis, C. C. and Boschnakow, A. (2001) Chronological ageing and photoageing of the human sebaceous gland. Clinical and Experimental Dermatology, 26 (7): 600-607.

BIBLIOGRAPHY

Publications related to this thesis:

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