Hormonal modulation of ageing skin microstructure and mechanical properties

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Abbreviations

Aebp1- AE Binding Protein 1

AFM - Atomic force microscopy

AGE- Advanced glycation end-products

Ahsg- Alpha 2-HS Glycoprotein

AP-1- Activator protein 1

Aspn- Asporin

Bgn- Biglycan

BMI – Body mass index

Capg- Capping Actin Protein, Gelsolin Like

Cma1- Chymase 1

Col1a1-Collagen Type I Alpha 1 Chain

Col1a2- Collagen Type I Alpha 2 Chain

Col2a1-Collagen Type II Alpha 1 Chain

Col3a1- Collagen Type III Alpha 1 Chain

Col4a2- Collagen Type IV Alpha 2 Chain

Col5a1- Collagen Type V Alpha 1 Chain

Col5a2- Collagen Type V Alpha 2 Chain

Col6a1-Collagen Type VI Alpha 1 Chain

Col6a2- Collagen Type VI Alpha 2 Chain

Col7a1- Collagen Type VII Alpha 1 Chain

Col11a1-Collagen Type XI Alpha 1 Chain

Col14a1- Collagen Type XIV Alpha 1 Chain

cbEGF- calcium binding epidermal growth factor

Cys- Cysteine

DBD- DNA binding domain

Dcn- Decorin

DEJ-Dermo-epidermal junction

DNA –Deoxyribonucleic acid

DPN-2,3-bis(4-hydroxyphenyl)-propionitrile

Dpt- Dermatopontin

DTT- Dithiothretol

EBP- Elastin binding protein

ECL - Enhanced chemiluminescence

ECM- Extracellular matrix

EGF- Epidermal growth factor

EMILIN- Elastin microfibril interface located protein

EMILIN-1- Elastin microfibril interfacer 1

ER – Estrogen Receptor

ERE- Estrogen response element

ERK-Extracellular signal–regulated kinases

Fbn1- Fibrillin 1

Fbn2- Fibrillin 2

Fbln5- Fibulin 5

Fn1- Fibronectin 1

GAG- Glycosaminoglycans

Gsn- Gelsolin

HDF - Human dermal fibroblasts

Hpx- Hemopexin

HRT- Hormone replacement therapy

Hspg2- Heparan Sulfate Proteoglycan 2

IGF-1 - insulin-like growth factor-1

Itih1- Inter-Alpha-Trypsin Inhibitor Heavy Chain 1

kDa- Kilodalton

Lama2- Laminin Subunit Alpha 2

Lama3- Laminin Subunit Alpha 3

Lama4- Laminin Subunit Alpha 4

Lama5- Laminin Subunit Alpha 5

Lamb1- Laminin Subunit Beta 1

Lamc1- Laminin Subunit Gamma 1

LBD-ligand binding domain

Lgals1- Galectin 1

Lgals3- Galectin 3

LOX- Lysyl oxidase

LOXL1 - Lysyl oxidase like

LTBP- Latent transforming growth factor β-binding protein

Lum- Lumican

MAGP-1-3 Microfibril associated glycoprotein-1

MAPK- Mitogen-activated protein kinase

MCP- Macrophage Chemoattractant protein

MIP- Macrophage Inflammatory protein

MMPs - matrix metalloprotienases

Nid1- Nidogen 1

Nid2- Nidogen 2

OCT – Optimum cutting temperature formula

Ogn- Osteoglycin

Ovx – Ovariectomised

PCR- Polymerase chain reaction

PDGF- Platelet-derived growth factor

PPT- 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)

Postn- Periostin

PSR - Picosirius red

RNA - Ribonucleic acid

ROS- Reactive oxygen species

SASP- Senescence-associated secretory phenotype

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SERMS – Selective Estrogen Receptor Modulators

Serpina1d- Serpina1d

Serpina3k- Serpin Family A Member 3

Sfn- Stratifin

Shc - SHC-transforming protein 1

SLRPs - Small leucine rich proteoglycans

SPRRs - Small proline-rich proteins

Src- Proto-oncogene tyrosine-protein kinase

TB - Transforming growth factor beta binding

TGF-β - Transforming growth factor beta

Tgfbi- Transforming Growth Factor Beta Induced

TIMPs - Tissue inhibitor of metalloproteinases

TNFα- Tumor necrosis factor alpha

Trp - Tryptophan

Tyr- Tyrosine

UVR – Ultra violet radiation

VEGF- Vascular endothelial growth factor

Abstract

Ageing tissue is characterised by an alteration in mechanical properties, with tissues becoming increasingly fragile, stiff and less resilient over time. This can lead to a loss of function in tissues which undergo a high degree of stretch and recoil, such as the skin, blood vessels and lungs. These changes are attributed to remodelling of the dermal extra cellular matrix (ECM) proteins which endow tissue with its mechanical properties. Principally the fibrillar collagens provide tensile strength, complemented by the elastic fibres which confer resilience and allow tissues to recoil following deformation. Intriguingly, at the time of menopause, which sees the cessation of the majority of circulating estrogen, skin undergoes accelerated ageing. This strongly suggests a direct link between circulating estrogen and youthful skin. Using an ovariectomised (Ovx) mouse model of human menopause the link between ageing, estrogen deficiency and the dermal ECM proteins was explored. Mechanical testing revealed a significant reduction in the tensile strength, Young's modulus and stress relaxation time of estrogen deprived tissue, indicating postmenopausal skin would be weaker, more lax and less able to withstand sustained force. Corresponding histological analysis highlighted the elastic fibres are dramatically reduced by estrogen deprivation with seemingly little effects on the fibrillar collagen abundance or alignment. Direct comparison between Ovx and age, reveals age affects mechanical properties in a completely opposing way, with aged tissue higher in tensile strength, Young's modulus and stress relaxation time compared to control and Ovx. Proteomic analysis by mass spectrometry confirmed elastic fibres to be highly sensitive to estrogen levels. Additionally small leucine rich proteoglycans (SLRP's) were also significantly reduced by estrogen deficiency, which could affect collagen fibrillogenesis and leave tissue weaker and more susceptible to damage. Despite the opposing effects on mechanical properties, ageing and estrogen deficiency had comparable effects on ECM abundance, suggesting the amount of ECM is not a predictor of mechanical properties; however disparity may be in part due to increased advanced glycation end product crosslinking. Further investigation suggests the mechanism for elastic fibre degradation may be via significant subcutaneous adipose hypertrophy and/or increased gelatinase activity. Both estrogen replacement and stimulation of estrogen receptor α with the agonist 2,3-bis(4-hydroxyphenyl)-propionitrile (PPT) were found to prevent elastic fibre degradation and adipose hypertrophy. Additionally these treatments were also found to induce key elastic fibre proteins in both the mouse model and cultured human dermal fibroblasts. A deeper understanding of estrogen mediated ECM remodelling offers opportunities for targeted pharmacological intervention to slow the effects of menopause and ageing.

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Chapter 1

Introduction

1 Introduction

Age related changes in the mechanical properties of tissues can profoundly affect human morbidity. Dynamic tissues such as those within the cardiovascular, cutaneous and respiratory systems require optimal mechanical function to efficiently fulfil their role within the body. However, ageing can lead to degeneration and over-stiffening of these systems. It is the extracellular matrix (ECM) proteins which are principally involved in endowing tissues with their mechanical functions. The dermis of skin is a readily accessible tissue, which is rich in ECM, and prone to age related changes manifesting as an increase in wrinkling and laxity. A crucial event in skin ageing is the menopause, which sees the cessation of the majority of circulating estrogen in females, and the rapid onset of the signs of ageing.

1.1 Skin structure and function

Broadly speaking the skin can be divided into three main layers (Figure 1.1). The outermost epidermis provides a barrier to external challenges such as pathogens and chemicals, as well as mechanical damage (Winsor, 1944). This layer is highly cellular and avascular and is composed of layers of differentiating keratinocytes. Below this is the vascular dermis, a layer rich in ECM. These two layers are connected at the dermal-epidermal junction, a structure composed of hemidesmosomes and fibrils to anchor the layers together. The deepest layer of the skin termed the hypodermis is mainly composed of adipocytes which function to store fat as both an active energy store and passively for thermoregulation. In addition, it acts as a protective, cushioning layer against mechanical insult.

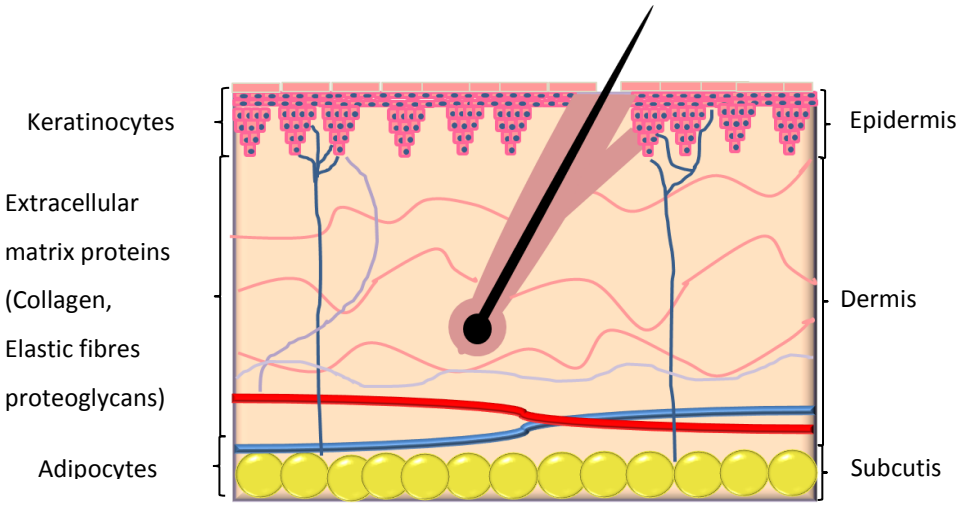

Figure 1.1. Simple overview of skin structure. The 3 main layers of the skin are the outermost epidermis, an avascular keratinocyte-rich layer; the dermis, rich in ECM proteins, blood vessels, nerves and appendages such as hair follicles and sebaceous glands; and the subcutis, made up of adipocytes.

1.1.1 Epidermis

The human epidermis can be further divided into four layers (five in palmar plantar skin); the stratum corneum, the stratum lucidum (a thick, clear layer of dead cells only found in palmar and plantar skin), the stratum granulosum, the stratum spinosum and the stratum basale (Wickett, 2006). Keratinocytes which comprise 95% of the epidermis, originate from a pool of progenitor cells in the basal layer (stratum basale) differentiating as they move up through the layers towards the stratum corneum (Lai-Cheong, 2009). During their progression through this programmed process of terminal differentiation, keratinocytes synthesize major structural components of the epidermal barrier, such as keratin, filaggrin, loricrin and a large family of small proline-rich proteins (SPRRs). This differentiation is regulated by a calcium gradient with keratinocytes becoming increasingly permeable to calcium as they move through the epidermis (Eckert and Rorke, 1989). Ultimately, the cells die, extruding a mixture of lipids and forming an impermeable, highly cross-linked cell envelope: the stratum corneum (Eckert and Rorke, 1989). It is the unique composition of the stratum corneum which confers the majority of skin's barrier function (Winsor, 1944), and is the outermost protection from pathogens, chemical and mechanical damage. In human skin the stratum corneum is comprised of approximately 10 layers of flattened keratinocytes (Lai-Cheong, 2009). The remaining (non-keratinocyte) 5% of epidermal cells consists of melanocytes, Langerhans cells and Merkel cells (Lai-Cheong, 2009). Melanocytes reside in the basal layer where they synthesise melanin in organelles termed melanosomes. These melanosomes are the primary defence against UVR, forming a protective melanin cap over the nuclear DNA (Lai-Cheong, 2009). Langerhans cells are epidermal-specific antigen presenting cells (Holikova et al., 2001) while Merkel cells are thought to be the touch receptors of the skin, found in complex with nerve fibres where it is speculated that they respond to gentle indentation (Maricich et al., 2009).

1.1.2 Dermal epidermal junction

Linking the epidermis to the dermis below is a specialist area of anchoring proteins providing both a physical and signalling link between the two layers. This specialist basement membrane consists mainly, but not exclusively, of laminins, collagen IV and VII, nidogen, perlecan and fibulins 1 and 2 to create a scaffold to the collagen fibrils of the dermis, this is linked to the basal keratinocytes of epidermal region by hemidesmosomes (Burgeson and Christiano, 1997, Timpl, 1996).

1.1.3 Dermis

Below the epidermis lies the dermis, the thickest layer of the skin, the upper region is termed the papillary dermis and the deeper area the reticular dermis. This area is relatively acellular and rich in ECM components, principally collagen fibres, elastic fibres, proteoglycans and

glycoproteins. It is this complex network of proteins which endows skin with its mechanical properties, conferring strength and flexibility (Gosline et al., 2002, Muiznieks and Keeley, 2013). The majority of the dermis is made up of collagen, endowing the skin with its characteristic strength and toughness. Collagen I accounts for approximately 90% of dermal collagen, while the remaining 10% is type III (Epstein and Munderloh, 1978). Elastic fibres, the other major component of the dermis, extend from the basement membrane layer to the subcutaneous layer and provide the skin with elastic qualities and the ability to return to a normal configuration after stretching (Wood, 1985, Wysocki, 1999). In addition, the dermis contains two other location-specific types of elastic fibres: superficial oxytalan fibres that extend from the dermal-epidermal junction and elaunin fibres which morphologically resemble elastin, and are comprised of bundles of fibrotubules that are connected to the elastic fibres of the reticular dermis (Cotta-Pereira et al., 1976).

The dermis also contains and extensive network of blood vessels, which along with their important role in thermoregulation support the epidermis above. The major cell type in the dermis is the fibroblast, which is responsible for the secretion of many of the ECM proteins. However it also contains mast cells, macrophages, lymphocytes, melanocytes and skin appendages such as hair follicles, sweat glands and sebaceous glands (Lai-Cheong, 2009).

1.1.4 Hypodermis

The hypodermis, sometimes termed the subcutaneous layer, is the deepest part of the skin and is comprised mainly of adipocytes, which have a large lipid-rich cytoplasm. Between these large round cells is a network of connective tissue containing blood vessels and nerves (Kanitakis, 2002). The hypodermis plays a role in the storage of fat to provide both energy and thermal insulation and additionally acts as a cushioning layer to protect the underlying bones and muscles from mechanical damage. The subcutaneous layer is composed of white adipose, which in mice lies below the panniculus carnosus and in humans forms the lowest layers of adipocytes (Driskell et al., 2014). There is also a second layer of adipose tissue found within the dermis which fluctuates dramatically and synchronously with hair cycling (Chase et al., 1953). As the hair follicle grows the adipocytes surround the growing follicle, increasing in both number and size, via differentiation of pre-adipocytes and hypertrophy as they fill with lipids (Rivera-Gonzalez et al., 2014). Pre-adipocytes are determined from pluripotent stem cells and have a fibroblast like morphology before a complex transcriptional cascade leads to terminal differentiation of the mature adipocyte filled with lipid (Rosen and MacDougald, 2006). This dynamic role for adipocytes in skin, where they fluctuate so dramatically is distinct from other tissues in which they have a much slower turnover (Splading et al., 2008). Alongside their roles in fat storage, to provide energy, the adipocytes are also important for secreting adiopkines,

such as leptin and adiponection. These are important in the regulation of food intake, metabolism and insulin resistance (Lafontan et al., 2012). The regulation of adipose homeostasis can be linked to hormonal changes, particularly thyroid where defective signalling increases adipocyte differentiation from preadipocyte cells (Carmean et al., 2013) Adipocytes are also known to be sensitive to estrogen signalling, which when reduced leads to increased adipose tissue (Heine , 2000, Nilsson 2007) .

Overall the skin is a large and complex tissue, in order to study the mechanisms involved in driving estrogen deficiency induced ageing it is necessary to employ an animal model such as the mouse.

1.1.5 Comparison of murine and human skin

Practically, the mouse offers a valuable tool to study skin, due to its similarities in structure, comparable genetics and the ability to manipulate both surgically and genetically (Bockamp et al., 2002). However it is important to note that there a number of species-specific differences in skin structure between rodents and humans (Figure 1.2). Most notably, rodents are loose skinned with no direct association between the skin and underlying subcutaneous fascia, while humans, non-human primates and pigs are not (reviewed in (Davidson, 1998, Dorsett-Martin, 2004). Other differences include a higher density of appendages, a far reduced epidermal and dermal thickness and a lack of rete ridges (epidermal thickenings that extend downward between dermal papillae) in rodents (Treuting et al., 2012, Gudjonsson et al., 2007). Despite these differences rodents have proved to be an effective model of skin and ageing in humans (Hwang et al., 2011). The mouse has extensively been used in studies of estrogen deprivation, hormone replacement and signalling modulation effects on cutaneous healing (Emmerson et al., 2010, Campbell et al., 2010, Ashcroft and Ashworth, 2003). Dermal collagen distribution is similar in humans and mouse (Junqueira et al., 1983) and although histologically the elastic fibre network of the mouse appears finer than in human dermis the molecular composition and structure appear to be very similar (Parry, 2005) and the mouse model has been extensively used for elastic fibre research (Reviewed in Kielty et al., 2002a).

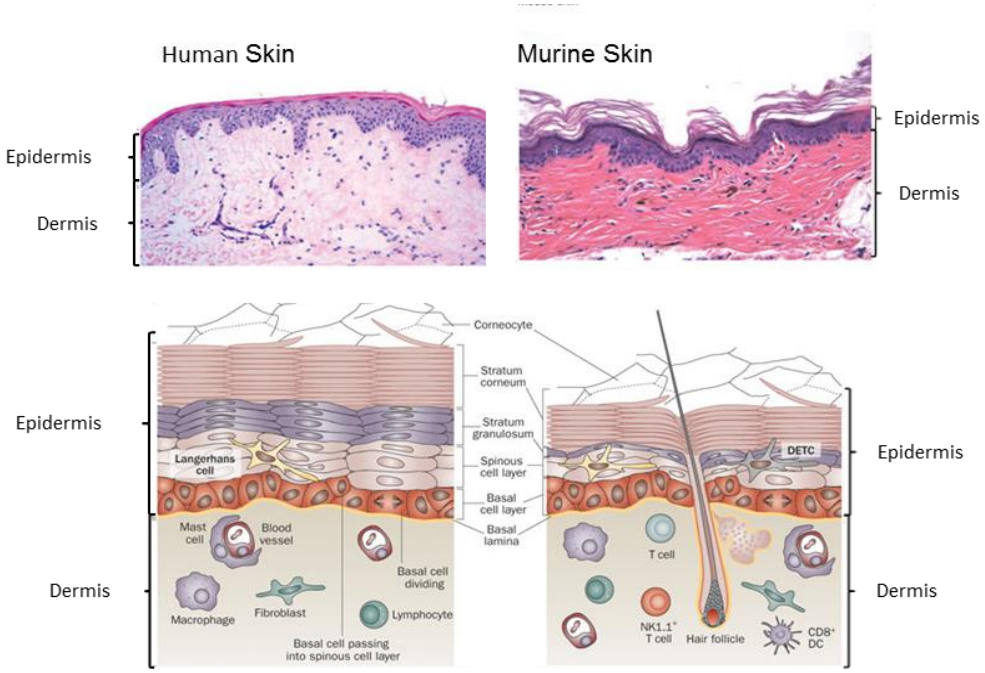

Figure 1.2 . Comparison of human and murine skin structure. Human and mouse skin are very similar in structure, the most notable difference is human skin has a thicker epidermis with rete ridges and far less hair follicles (Adapted from (Wagner et al., 2010))

1.1.5.1 Mouse and human hair

One major difference between mouse and human skin is the abundance of hair and the density of the hair follicles within the skin. These are complex appendages often referred to as miniature organs with roles not only in hair growth but also in immune response and wound repair (Christoph, 2000, Langton, 2008). The hairs go through continuous regular cycling stages of growth termed anagen, regression termed catagen, resting termed teleogen and shedding termed exogen. In the mouse this is a synchronous cycle occurring approximately every 25 days as a wave moving caudal to rostral (Muller-Rover et al., 2001). In the human however the hair cycle is both much longer and asynchronous. The length of the hair represents the length of the anagen phase, which in human scalp can be around 8 years (Chase 1954). The human has different types of hair such as the small fuzzy hairs known as lanugo hair as well as very specific regional hair such as the eyebrows and eyelashes (Hess et al., 1990). Despite these differences the molecular signals which control morphogenesis of hair follicles are evolutionarily conserved from drosophila to mammals (Paus and Cotsarelis 1999) however with the hair follicles contributing to many processes they are an important consideration in skin research.

1.2 Extracellular matrix proteins

The age-related mechanical changes in skin are largely attributed to changes in the ECM proteins (Daly and Odland, 1979). The ECM is principally made up of a mesh of fibrous proteins and proteoglycans and also contains a number of enzymes, cytokines, inhibitors and adhesive glycoproteins (Frantz et al., 2010). It provides a physical scaffold for the cellular constituents of tissues while also playing a role in cell signalling and adhesion. It is the complex assembly of the ECM protein networks, which give tissues their mechanical properties such as stiffness and resilience. The fibrillar collagens make up around 70% of the dermal ECM (Lai-Cheong, 2009) and provide tensile strength which is complimented by the elastic fibres. By contrast, elastic fibres make up only 2% of the dermis (Hwang et al., 2011) but are essential to drive recoil back to resting state following deformation of a tissue (Kielty et al., 2002a). The proteoglycans resist compressive forces (Perrimon and Bernfield, 2001) and are important in the maintenance of hydration (Naylor et al., 2011). Adhesive proteins such as fibronectins and laminins are crucial for mediating cell attachment and tissue organisation, interacting with cells via transmembrane proteins such as the integrins. Cell-matrix interactions play key roles in migration and cell attachment as well as differentiation and gene expression (Gelse et al., 2003). ECM proteins are synthesised by fibroblasts and degraded by matrix metalloprotienases (MMPs), with MMPs being regulated by tissue inhibitor of metalloproteinases (TIMPs).

1.2.1 Collagens

Collagens are a large family of structural extracellular proteins, characterised by their Gly-X-Y peptide repeat (often X is Proline and Y is Hydroxyproline) and a right handed triple helical conformation composed of 3 α-chains (Gordon and Hahn, 2010). The α-chains can be identical forming a homotrimer or different forming a heterotrimer. The triple helical molecules, termed tropocollagen, can then self-assemble into larger aggregates such as fibrils, and the structure is stabilised by hydrogen bonds forming between the high levels of hydroxyproline residues. Collagens are further characterised by this supramolecular arrangement (Table 1.1). Fibrillar collagens make up around 70% of dermal ECM, where they have important roles in providing tensile strength. The most abundant dermal collagen is type I which makes up almost 90% of the collagen content (Gordon and Hahn, 2010). The second most abundant is collagen type III making up almost all the remaining 10% (Gordon and Hahn 2010). Both collagen types I and III assemble into large fibril bundles, type I larger then type III. In human papillary dermis, these bundles are thinner and form a meshwork and as they move down into the reticular dermis they become thicker and less tightly woven forming a 'basket weave' orientation (Lavker et al., 1987, Graham et al., 2010). By electron microscopy collagen fibrils appear to have a banded pattern with a regular periodicity between the bands termed the D-period. This is due to the way in

which they assemble into fibrils and is found to be 67nm in tendon and has been reported to be 65nm in skin (Fang et al., 2012).

However, the skin contains at least 12 other collagen types which perform a range of crucial functions. These include the ubiquitous collagen type VI found throughout the whole dermis, in a number of different assemblies, such as microfibrils, hexagonal networks and broad bands. Although its exact function is unknown it is thought to be important for integrating and bridging ECM components (Birk and P, 2005). In contrast to collagen VI, collagen VII is highly localised to the basement membrane and has a specific role as an anchoring protein forming a bond between the dermis and epidermis (Steplewski et al., 2012).

Table 1.1 Collagen suprastuctures Adapted from (Birk and Bruckner, 2005)

Collagen Suprastructure	Collagen Type
Fibril	I, II, III, V, XI, XXIV, XXVII
Fibril-associated network	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
Network	IV, VI, VII, X
Anchoring	VII
Transmembrane	XIII, XVII, XXII, XXV
Muliplexin	XV, XVIII

A key protein family associated with the correct formation of collagen fibrils and assemblies is small lucine rich proteoglycans.

1.2.2 Small leucine rich proteoglycans (SLRPs)

This family of proteins is characterised by a small protein core and a conserved region of lucine-rich repeats. The SLRPs have crucial roles in the correct organisation of collagen fibres which are found in very different arrangements dependent on their location. The SLRP family is further divided into classes based on their evolution, connecting different collagens together, such as large collagen I fibrils to the smaller collagen structure and number of leucine repeats they contain (Iozzo, 1999) Class I SLRPs are biglycan, decorin and asporin. Decorin may have roles in controlling fibril diameter, although reported findings are conflicting: one report concludes decorin reduces growth and increases diameter (Kuc and Scott, 1997) while another concludes it reduces fibril diameter (Douglas et al., 2006) and VI fibrils (Nareyeck et al., 2004).

1.2.3 Elastic fibres

The tensile strength provided by collagen is complemented by the elastic fibre network. These macro-molecular assemblies endow tissue with the ability to passively recoil following deformation (Kielty et al., 2002a). They display a high degree of architectural heterogeneity determined by function; in blood vessels they form concentric sheets of lamellae surrounding

the vessel to confer resilience, in ligaments they form solid rod like structures, whist in elastic cartilage they appear as a fine meshwork (Doliana et al., 1999). In human skin the elastic fibres display a thick, parallel, elastin-rich formation in the reticular dermis which branches into perpendicular fibres towards the papillary dermis (Figure 1.3A-B). These perpendicular fibres, termed oxytalan fibres, branch further into 'candelabra' like structures as they reach the dermo-epidermal junction (DEJ). However in murine skin they do not have the same architecture and instead are found in smaller lengths and more random orientation throughout the dermis (Figure 1.3C). The composition of the elastic fibres changes from elastin rich in the reticular dermis becoming increasingly fibrillin rich through the papillary dermis until they reach the DEJ (Langton et al., 2009).

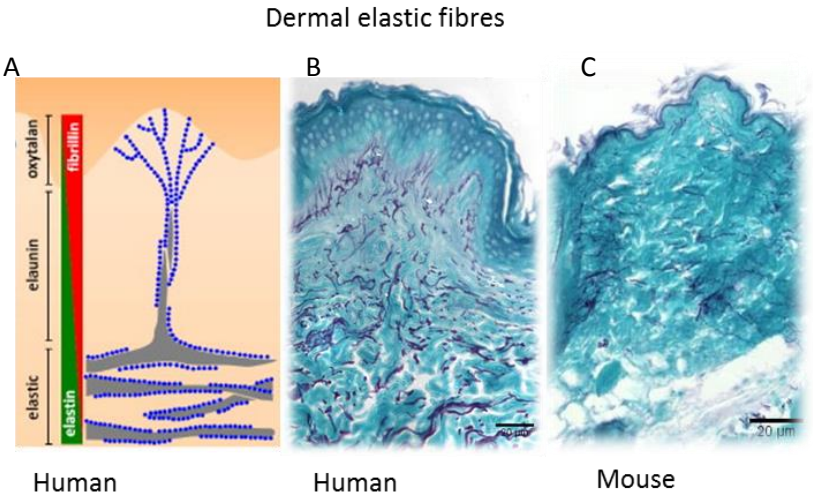

Figure 1.3 . Dermal Elastic fibre system in mouse and human skin. (A) The Human elastic fibre network shows a characteristic arrangement where thick elastic fibres (grey) of the reticular dermis become increasingly fibrillin rich (blue) fibres to form the oxytalan fibres of the papillary dermis. (Adapted from Naylor et al., 2011). Murine skin (C) has a less ordered and abundant elastic fibre system (stained dark purple) then human skin (B).

1.2.4 Composition of elastic fibres

The elastic fibres are composed of a number of molecules, primarily an elastin rich core surrounded by an outer mantel of fibrillin microfibrils, but additionally glycoproteins (MAGP-1), fibulins and EMILIN-1. The elastin core is a highly crosslinked polymer of its monomeric secreted precursor tropoelastin (Sandberg et al., 1971). Crosslinking is catalysed by members of the lysyl oxidase (LOX) family and lysyl oxidase like (LOXL1) proteins to stabilise the fibre (Partridge et al., 1963). The fibrillin microfibrils provide the initial structure for the elastic fibre and act as a scaffold onto which the tropoelastin is deposited and builds (Robb et al., 1999).

1.2.4.1 Elastin

Tropoelastin is a 60-70 kDa protein encoded by a single gene with at least 11 human splice variants identified (Wise and Weiss, 2009), which is suggested to allow fine tuning of the functional properties in different tissues (Kielty, 2006). Tropoelastin is a highly flexible multidomain protein, characterised by repeating hydrophobic and hydrophilic lysine-rich domains. It has approximately 40 lysine residues and around 35 of these become modified by LOX to form crosslinks of the elastin polymer. It is this highly crosslinked structure which gives elastin its stability, insolubility and longevity (Kielty, 2006, Wise and Weiss, 2009).

In the cell, tropoelastin associates with elastin binding protein (EBP) and is secreted to the cell surface. It then dissociates from EBP and aggregates by the process of coacervation. Tropoelastin undergoes crosslinking to form elastin aggregates and it is these polymers which are transferred to the microfibril scaffold. The structure becomes increasingly crosslinked as the elastic fibre builds (Wagenseil and Mecham, 2007). This highly organised process primarily takes place in early development and this process becomes inferior in mature tissues.

1.2.4.2 Fibrillin

The microfibrils which form the scaffold surrounding the elastin core are made up of fibrillin, primarily fibrillin-1. These are large 350kDa glycoproteins (Kielty et al., 2002b) containing 47 epidermal growth factor (EGF)-like domains, 43 of which are calcium binding (cbEGF) domains (Kielty et al., 2005). Binding of calcium is critical to the structural integrity of the fibrillin and gives it a rod like structure (Downing et al., 1996). Fibrillin-1 also contains seven TB/8-cystine motifs and a proline-rich domain, thought to be a flexible region close to the N-terminus. Fibrillin-1 monomers self-polymerise in a head to tail formation through N- and C- terminal interactions to form filaments (Olivieri et al., 2010). This process is preceded by processing of the N- and C- terminus domains by enzymes of the furin and PACE family (Kielty et al., 2002b). The filaments associate laterally such that there are 8 fibrillin-1 filaments in a cross section of a microfibril (Baldock et al., 2001). These assembled microfibrils form parallel bundles to which the tropoelastin aggregates become associated.

Imaging of microfibrils by rotary shadowing electron microscopy and atomic force microscopy shows that isolated microfibrils have a ‘beads on a string’-like appearance with a regular periodicity between the ‘beads’ of 56nm in an untensioned microfibril (Sherratt et al., 2003, Kielty et al., 2002b, Sherratt et al., 2001). Although the average bead to bead distance is found to be 56nm, distances of up to 150nm have been reported after the application of tensile force (Baldock et al., 2001, Keene et al., 1991). This has led to the suggestion that microfibrils

themselves are elastic (Keene et al., 1991). When tissue is extended by 100% the bead to bead distance has been shown to reach 80nm and return to 56nm when untensioned in a revisable unfolding event (Wang et al., 2009, Wess et al., 1998). However extensions over 90nm require the breaking of covalent bonds and therefore cause an irreversible extension (Sherratt et al., 2003). Individual microfibrils have a Young's modulus, a measure of the mechanical properties of a linear elastic fibre, of between 78MPa and 90MPa. This, compared to the Young's modulus of elastin, which is closer to 1MPa, suggests microfibrils to be stiff and have a role in reinforcing the elastic fibre and acting as anchoring proteins (Sherratt et al., 2003).

1.2.4.3 Microfibril associated glycoprotein-1 (MAGP-1)

MAGP-1 was found to be associated with microfibrils in denatured tissue extracts and was therefore identified as a potentially important protein in microfibril structure (Rock et al., 2004). MAGP-1, which binds to the N-terminal of fibrillin-1 molecules and tropoelastin, is important for microfibril organisation (Rock et al., 2004). MAGP-1 is found to be localised close to the microfibril 'beads' binding in a calcium dependent manner, most likely via disulphide bonds (Kielty et al., 2002b, Kielty, 2006). It is the positively charged, C-terminal region of MAGP-1 which is thought to bind to the microfibril and the N- terminal domain has high affinity for TGF-β (Weinbaum et al., 2008, Isogai et al., 2003).

1.2.4.4 Latent transforming growth factor β-binding protein (LTBP)

The LTBP family shares a high level of similarity to the fibrillins; they contain calcium binding epidermal growth factor-like (cbEGF) domains and transforming growth factor β-binding protein like (TB) modules (Sinha et al., 1998, Kielty et al., 2002a). There are 4 members of the LTBP family and LTBP -1, 3 and 4 have been shown to bind TGF-β to form large complexes which are later crosslinked to the ECM (Kielty et al., 2002a). LTBP-2, however, does not bind TGF-β and is found to be associated with microfibrils. LTBP-2 binds via its C –terminal region to the N-terminal region of fibrillin-1. LTBP-2 is a binding partner of fibulin-5 and is believed to have a structural role in elastic fibres (Kielty et al., 2002a).

1.2.4.5 Fibulin-5

Fibulin-5 is a 51 kDa secreted glycoprotein essential for the formation of elastic fibres. It contains 6 cbEGF domains and an integrin binding RGD motif. Fibulin-5 mediates endothelial cell adhesion via binding to integrins αvβ3, αvβ5 and α9β1. Mutations in fibulin-5 cause cutis laxa in humans, highlighting its importance in the elasticity of skin (Kadoya et al., 2005). In young photo-protected skin it is found in the reticular dermis along with the other elastic fibre components and in the papillary dermis it forms candelabra like structures perpendicular to the

epidermis. With age and sun exposure fibulin-5 has been found to be reduced and even disappeared completely from the reticular dermis (Kadoya et al., 2005).

1.2.4.6 Elastin microfibril interface located protein (EMILIN)

EMILIN is a 115kDa glutamic acid- and arginine-rich glycoprotein secreted into the ECM, where it aggregates via disulfide bonds to form a fine network at the interface between the elastin core and the microfibrils (Doliana et al., 1999). In development EMILIN is implicated in the early stages of elastogenesis, given that in the presence of anti-EMILIN antibodies elastin deposition was severely altered (Bressan et al., 1993).

The ECM proteins are one of the major contributors to the mechanical properties of skin providing tensile strength and elastic recoil to allow the tissue to fulfil its roles as a protective barrier.

1.3 Skin Mechanics

Although all layers of the skin contribute to the mechanical properties, with the stratum corneum being the stiffest, it is the dermis which dominates the gross mechanics of the skin (Silver et al., 1992). This is even more pertinent in murine skin where the epidermis is only a few cells thick. However, it is non-homogeneous, anisotropic (directionally dependent characteristics), viscoelastic (displaying both viscous and elastic behaviour) and highly flexible and *in vivo*, subject to both internal and external forces, making calculation of discrete measurements difficult. This has led to the development of a number of different *in vivo* methods being used to measure skin mechanics.

1.3.1 Measuring gross mechanics *in vivo*

A number of different testing methods to measure skin mechanics *in vivo* have been developed. These measure tensile or torsional forces applied parallel to the skin surface, or indentation, impact or elevation perpendicular to the skin surface (Rodrigues, 2001).

Tensile methods include the adherence of two tabs to the skin which are pulled apart by a known force and the distance between them measured. Although this is a relatively simple technique (Rodrigues, 2001). Torsion can be measured by adhesion of a rotatable disk to the skin and a guard ring is used to control the depth of mechanical deformation. The disk is used to apply torque and measures the degree of rotation and the deformation of skin over a period of time (Batisse et al., 2002, Sherratt, 2009).

The most widely used measure of skin elasticity and suppleness is elevation testing via suction. The application of negative pressure to the skin induces deformation, initially elastic

deformation followed by viscoelastic deformation until total deformation is reached. Residual skin elevation can be measured when suction is released and is termed resilient distension. The time the skin takes to recover after removal of suction can be calculated in a ratio with initial deformation to quantify elastic recoil (Hendriks et al., 2003, Sherratt, 2009).

1.3.2 Measuring gross mechanics *ex vivo*

Ex vivo testing allows for more control of the mechanical measurement one is making, as the tissue is no longer subject to the complex forces associated with *in vivo* testing. However they are clearly less physiologically relevant as the skin in removed from its normal environment (Daly and Odland, 1979). One of the most straight forward methods for testing gross mechanics of skin is uniaxial tension testing where a strip of skin is loaded in one direction and yields a stress strain curve as described in the following section. This can be further loaded to failure as a measure of tensile strength (Jacquemoud et al., 2007). Biaxial tension testing has also been used, however this is a far more complex procedure, it does demonstrate that skin mechanical properties differ in different directions (Lanir and Fung, 1974). This anisotropic behaviour was first described by Carl Langer in the 19th century who mapped lines of tension in the skin, now described as Langer lines (Langer, 1978).

1.3.3 Stress strain relationship of skin

When load is applied to skin it yields a typically shaped stress strain curve where stress is load per cross sectional area and strain is % extension (Figure 1.4). This has been described by Gibson *et al* in 3 phases (Gibson et al., 1969). The initial portion corresponds to elastic deformation and during this phase there is realignment of the collagen fibres. The second phase is a viscoelastic curve which has been suggested to represent progressive alignment within the dermis and leads into the linear phase, phase 3, which shows characteristics of aligned collagen fibres and from the linear section an estimate of stiffness can be calculated as the Young's modulus (Stress/Strain).

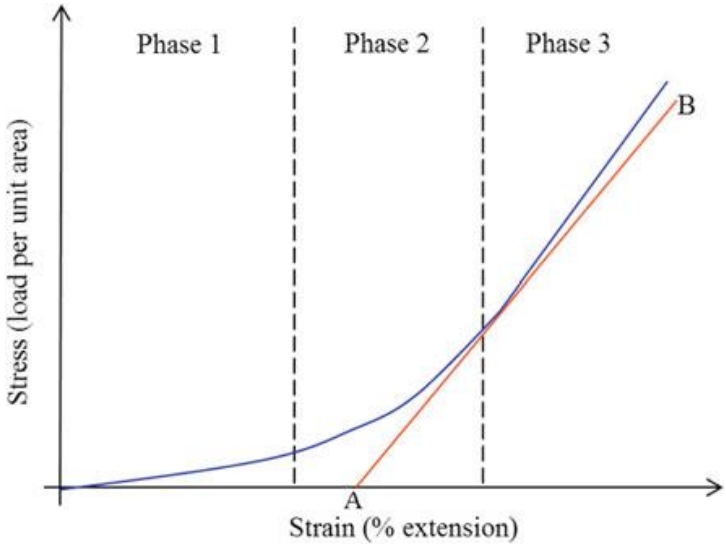

Figure 1.4. A Typical stress stain curve from skin. Phase 1 is referred to as the toe region and corresponds to the initial gross realignment of collagen fibrils and elastic deformation. This is followed by phase 2, where the tissue behaves in a viscoelastic manner as the collagen fibrils continue to align the final phase 3 represents fully aligned collagen under load. The tangent A-B is the Young's modulus and gives an estimation of stiffness (Gibson et al., 1969).

1.3.4 Viscoelastic properties - Stress relaxation

As described by the stress strain curve above skin exhibits viscoelastic behaviour, this means it exhibits both viscous and elastic characteristics, viscous behaviour is associated with a loss of energy whereas elastic behaviour stores energy (Silver et al., 2001). This means that although the tissue will return to its original shape (at least partially) it will take time to do this introducing a time dependent element to the mechanical behaviour. This time dependent element can be measured through stress relaxation which is the time dependent decay of stress (Liu, 2008).

There are clear links between ageing skin and alterations in mechanical properties which have been suggested to be primarily due to alterations in the elastic fibre network (Daly and Odland, 1979), however, since findings vary a great deal between studies it is difficult to draw clear conclusions regarding mechanical remodelling as a key characteristic of ageing skin (Moronkeji and Akhtar, 2015).

1.4 Skin Ageing

The components of the skin, along with all tissues of the body, are subject to chronological ageing due to the passage of time, termed intrinsic ageing. Additionally, skin is also susceptible to a high degree of extrinsic ageing resulting from exposure to the environment, principally ultraviolet radiation (UVR). A combination of intrinsic and extrinsic factors contribute to altered homeostasis in aged skin, which manifest as wrinkling, sagging, fragility, atrophy and increased laxity. The ECM proteins are particularly susceptible to accumulation of damage over time

(Verzijl et al., 2000, Shapiro et al., 1991). Their longevity and extracellular location, allows them to accumulate damage outside of the protective intracellular environment. The half lives of many of the ECM proteins are measured in decades (Sherratt, 2009) and elastin synthesis is greatly downregulated after birth (Zhang et al., 1999) suggesting the elastic fibres are required to last our whole lifetime (Sherratt, 2009). The age-related changes in the mechanical properties of skin, such as stiffening and loss of elastic recoil, are largely attributed to changes in the ECM proteins (Daly and Odland, 1979).

1.4.1 Intrinsic ageing

Clinically, intrinsically aged skin presents with uniform pigmentation, fine wrinkles, loss of elasticity, reduced appendage density and increased fragility (Gilchrest, 1982, Kohl et al., 2011). Histologically it is characterised by a thinning of both the dermis and epidermis along with flattening of the DEJ (Gilchrest, 1982, Jenkins, 2002, Moragas et al., 1993) (Figure 1.5). There is a general loss of ECM, with dermal collagen reported to decrease by 1% per year in adulthood (Fenske and Lober, 1986), and by electron microscopy collagen bundles appear to become less tightly packed and more random in orientation (Lavker et al., 1987). Elastic tissue is gradually lost from the papillary dermis with increasing age (Francis and Robert, 1984). Lavker *et al* (Lavker et al., 1987) distinguishes two phases in this loss, initially a phase of hyperplasia where the number and thickness of elastic fibres increases, followed by a second phase of elastolysis where the thinner fibres of the papillary dermis are lost. Conversely, El-Domyati *et al* (El-Domyati et al., 2002) found that in photoprotected skin the amount of elastic tissue gradually decreased with thinning of the large fibres and shortening of the oxytalan fibres, such that by age 80 few oxytalan fibres could be seen. Reduction in total proteoglycans, which have important roles in binding water, leads to the increasingly dry skin associated with age (Ghersetich et al., 1994, Naylor et al., 2011).

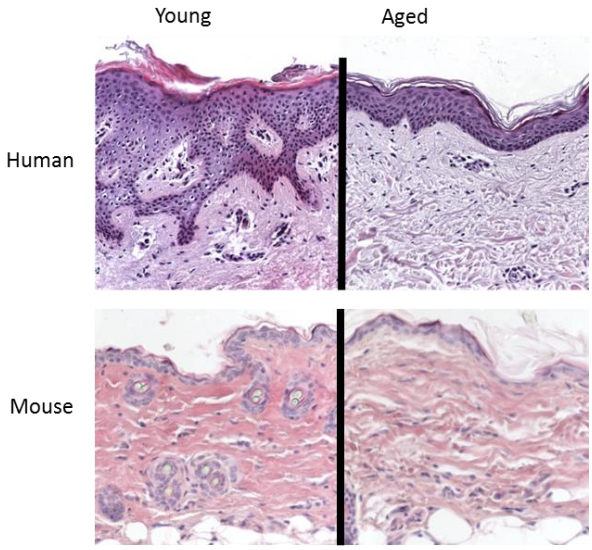

Figure 1.5. Young and aged skin. Photoprotected human skin from young subject has abundant rete ridges. Photoprotected human skin from aged subject shows thinning of the epidermis with a loss of rete ridges and flattening of the DEJ. Mouse skin shows thinning of the epidermis and loss of ECM, though in contrast to human mouse skin does not have rete ridges.

1.4.2 Mechanisms of ageing

Numerous theories have been proposed to drive ageing and these can be broadly split into wear and tear theories, where the underlying premise is that tissue accumulates damage until it essentially wears out, or programmed longevity theories, which propose a genetic preprograming to enter life stages with specific age-related genes (Weinert and Timiras, 2003, Jin, 2010). Some of the proposed theories include the senescence, oxidative stress, endocrine, DNA damage and cross linking theories (Jin, 2010). Although these are described as individual theories, none are totally satisfactory or mutually exclusive, and there is clear involvement of multiple different mechanisms.

1.4.2.1 Senescence

Replicative cellular senescence was first described by Hayflick and Moorhead (1961) as the observation that cells undergo a finite number of cell divisions (Hayflick and Moorhead, 1961). This is now understood to be the result of age associated telomere shortening (Olovnikov, 1996). Teleomeres, which are long tandem repeats at the end of chromosomes, can become shortened by up to 150 base pairs with a single cell division (Harley et al., 1990). When telomeres become critically short cells can no longer divide and enter a senescent state (Hwang et al., 2011). Interestingly murine telomeres are far longer than their human counterparts and mouse cells have far more telomerase activity which actively lengthens telomeres and are therefore far less likely to entre a senescent state. Senescent cells were originally believed to be quiescent, however it is now understood they play an active role in tissue ageing, via an alteration in their expression profile, termed senescence-associated secretory phenotype

(SASP). Senescent cells secrete a number of pro-inflammatory cytokines and proteases which can have deleterious effect on the surrounding tissue (Campisi, 2013).

At the tissue level a slowing of cell division leads to an increasingly ageing cell population: aged fibroblasts in culture are reported to have a 3-fold reduction in population doubling time (Yoon et al., 2004). Further to this aged fibroblasts have a reduced capacity to synthesize ECM proteins and overall are less abundant in aged tissue (Fenske and Lober, 1986).

1.4.2.2 Oxidative stress

Oxidative stress or free radical theory is an accumulation-of-damage theory, where damage occurs to DNA and proteins via the production of reactive oxygen species (ROS). ROS, such as H_2O_2 ,O_2^- ,$^1O_2$, and OH^- , are generated through aerobic metabolism as well as exogenous factors such as UV (Scharffetter-Kochanek et al., 1997). The presence of an unpaired electron makes these highly reactive molecules. The body, therefore, has a number of mechanisms to counteract the ROS produced. These include the antioxidant enzymes catalase and peroxidase and smaller electron donor molecules including NADH, ascorbic acid, tocopherols and carnosine act to scavenge ROS (Kohen, 1999). With increasing age tissue antioxidant levels fall and ROS levels rise (Kohen, 1999, Hu et al., 2000) leading to increased levels and accumulation of ROS-associated tissue damage. ROS can cause damage to DNA, proteins and lipids by different mechanisms, including single strand breaks or crosslinking of nucleic acids, crosslinking or degradation of proteins, addition of carbonyl groups and formation of advanced glycation end-products (AGEs) (Bailey, 2001, Kohen, 1999, Miyata et al., 1997). Additionally ROS can also upregulate MAPK signalling via activation of fibroblast cytokine receptors (Sardy, 2009) leading to AP-1 mediated matrix metalloproteinase (MMP) transcription (Pimienta and Pascual, 2007) as well as directly activating inactive MMPs (Haorah et al., 2007).

The oxidative stress theory has been challenged by Gems and Doonan (Gems and Doonan, 2009) where studies in the lower organisms *C.elegans* and *Drosophila* failed to show an increase or decrease in lifespan with increases in antioxidant or oxidative stress, respectively. However the short natural lifespan and cellular rich nature of these organisms may not provide a good model of the effects of oxidative stress in higher organisms, where some ECM proteins are required to last for decades without the protection of cellular antioxidant mechanisms.

1.4.2.3 Crosslinking

The crosslinking theory was first proposed by Bjorkstein (Bjorksten, 1968) and suggests that it is the accumulation of crosslinks in proteins which causes age-associated damage to tissues. There are a number of crosslinking agents found in the body which could be responsible, including

aldehydes, free radicles, sulphur and quinones, as well as antibodies (Bjorksten, 1968). One of the major ways crosslinking can occur is via glycosylation and AGEs then form crosslinks to one another. With regards to the ECM proteins crosslinking is believed to be a major contributor to age associated tissue stiffening. The initial crosslinks formed by LOX enzymes provide optimal elastic fibre characteristics; however accumulation of additional crosslinks leads to over-stiffened fibres (Bailey, 2001). Additionally, aged tissue is also found to have an increase in LOX/LOXL enzymes which could further contribute to additional crosslinking in elastic fibres and fibrillar collagens (Langton et al., 2012).

1.4.2.4 Endocrine Theory

The endocrine theory of ageing is based on the idea that the ageing is due to changes in hormonal function and the way the body responds to endocrine signals. The theory places the hypothalamic pituitary axis (HPA) as a 'master regulator' to signal the onset and termination of different life stages (Weinert and Timiras, 2003). Signalling via the insulin/IGF-1 pathway is found to have an important role in the regulation of lifespan with a relationship between lowered insulin levels and longevity (Russell et al., 2007), and this could be the driver behind a number of aspects of ageing, via the switching on/off of hormone signalling. Of interest, it has been recently shown that estrogen can also signal through the IGF-1 receptor (IGFR) in the skin, offering one potential mechanism of its involvement in skin ageing (Makrantonaki et al., 2008, Emmerson et al., 2012).

1.4.3 Extrinsic ageing

While all tissues undergo intrinsic ageing, the skin is also uniquely subject to extreme extrinsic ageing, resulting from extensive exposure to environmental factors, predominantly UV exposure. Clinically, extrinsically aged skin presents with deep wrinkles and a leathery appearance, with irregular pigmentation and extensive loss of elasticity (Jenkins, 2002). However, it is changes in the dermal component which has the most profound impact. Fibrillar collagens types I and III are dramatically reduced and become more fragmented leading to reduced tensile strength (El-Domyati et al., 2002, Talwar et al., 1995). There is a loss of fibrillin rich microfibrils at the DEJ, chiefly fibrillin-1 and fibulin 5 (Watson et al., 1999), along with loss of the anchoring protein collagen VII (Tsoureli-Nikita et al., 2006). The loss of fibrillin-1 occurs in minimally photo exposed skin, so can be considered one of the earliest characteristics of photodamage (Langton et al., 2009). In the deeper dermis the response to chronic UV exposure is thought to initially be hyperplastic, where elastic fibres increase in number in response to damage which leads to an accumulation of elastoic disorganised elastic fibres (Watson et al., 1999).

1.4.3.1 Mechanisms of extrinsic ageing

The mechanisms of extrinsic ageing are mainly due to the effects of UVR. UVB is mainly absorbed by the epidermis, and subsequently photoageing is mainly caused by UVA which has a longer wavelength and penetrates deeper into the skin (Biniek et al., 2012). As UVR penetrates the skin the energy is absorbed by molecular regions known as chromophores. The absorption of photon energy leads to an excited state which can lead to a subsequent chemical reaction or change in protein structure directly. Alternatively the absorbed light can excite photosensitisers to a triplet state to form free radical species or energy transfer to O_2 to yield reactive singlet oxygen $^1O_2$ (Thurstan et al., 2012, Poljsak and Dahmane, 2011). Additionally UVR induces activation of cytokines and growth factor receptors (Tsoureli-Nikita et al., 2006) which in turn reactivates protein kinase signalling pathways leading to transcription of MMPs via the AP-1 transcription factor. It was thought that MMP activation was the major cause of UVR induced ECM degradation (Fisher et al., 1996), however the low specificity of MMPs led Sherratt *et al* (Sherratt et al., 2010) to suggest specific loss of the fibrillin 1 and fibulin 5 from the DEJ must be due to a more precise mechanism of UVR-induced degradation. They found by atomic force microscopy (AFM) that exposure of isolated microfibrils to UVB increased 'bead' periodicity and flexibility and also led to fragmentation. The Cystine-Cystine bonds in fibrillin microfibrils play important roles in stabilising the fibrils. However, they are particularly susceptible to UVB damage either by direct absorption or electron capture from other chromophores. It has been proposed this may be a sacrificial role, acting as an endogenous sunscreen to protect other proteins (Hibbert et al., 2015). The other components of the elastic fibres, such as fibulins, LTBPs and the LOX enzymes are also found to be much richer in UVB chromophores than other ECM components such as collagen. The position in the papillary dermis also makes them more susceptible to the effects of the more biological active form of UVR, UVB, which is unable to penetrate to the deeper layers of the skin (Sherratt et al., 2010).

1.4.4 Estrogen and ageing

At the onset of menopause the dramatic decrease in the amount of circulating estrogen correlates with the reported increase in many of the characteristics of skin ageing, such as increased wrinkling, thinning and dryness (Verdier-Sevrain et al., 2006). There is a large body of evidence suggesting that these detrimental effects on skin post-menopause are at least partially reversed by hormone replacement therapy (HRT), which is reported to improve skin thickness, moisture and elasticity (Verdier-Sevrain et al., 2006). This will be explored in more detail in the following section.

1.5 Estrogen

17β-estradiol, discovered in the 1920s (Allen , 1923) is the primary sex hormone in females. The estrogen family actually comprises 3 endogenous hormones, estrone, estradiol and estriol (Figure 1.6) with classical roles in embryonic and post-natal development, regulating sexual characteristics and reproduction (Korach, 1994). The menopause, which occurs at an average age of 51 years in the UK, marks the end of a woman's fertile period with a profound reduction in circulating hormones. Although estrogens have primary functions in reproductive tissues they have effects in many tissues throughout the body (Korach, 1994).

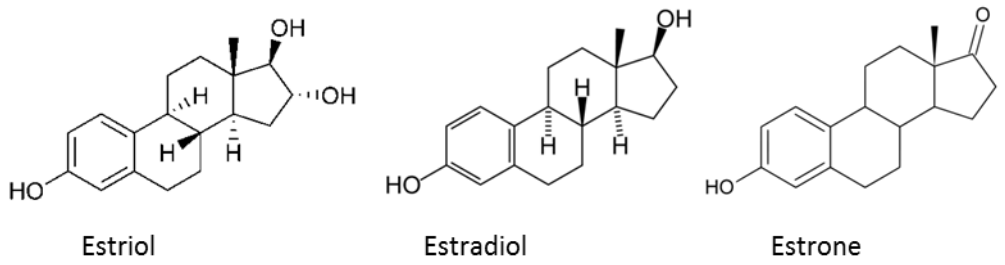

Figure 1.6 Estriol, estradiol and estrone structures. The 3 main endogenous estrogenic hormones are remarkably similar in structure, all containing a phenolic A-ring and hydroxyl D-ring.

Estrogens are predominantly synthesised in the ovaries of pre-menopausal women, with estradiol being the predominant type produced, while post-menopause synthesis shifts to peripheral tissues, with the most abundant estrogen being estrone (Hall and Phillips, 2005) (Figure 1.7). Moreover, alternative signalling (e.g. via IGF-1 crosstalk) may become more important post-menopause. The loss of estrogen following menopause increases the risk of developing a range of age-associated pathologies, eg. osteoporosis and cardiovascular disease (Korach, 1994) but also has profound effects on skin homeostasis and repair (Ashcroft and Ashworth, 2003).

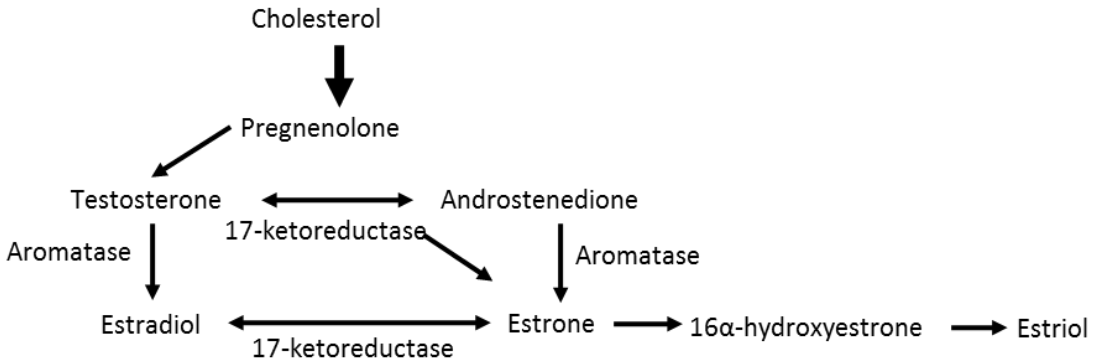

Figure 1.7 The skin contains all the enzymes necessary for the synthesis of estrone, estradiol and estriol from the common precursor cholesterol

The most abundant sex steroid in humans is dehydroepiandrosterone (DHEA) which acts as an intermediate metabolite in the synthesis of many other steroid hormones. Importantly humans (but not rodents) have evolved intracrine mechanisms for the peripheral conversion of DHEA to other steroid hormones including estrogen (Pomari et al., 2016). Following menopause DHEA and DHEA-S (dehydroepiandrosterone-sulphate) provide the major source of steroid hormones in peripheral tissues with DHEA-S circulating at 10,000 times higher concentrations than estradiol in adult females (Labrie et al,. 2000) and the levels in males are almost double that of females. The levels of DHEA fall with age and by age 50 are about 50% that of their peak (Librie et al., 2000) however by 70 years the levels fall dramatically to only 5-20% that of a young adult (Labrie et al., 1995).

1.5.1 Estrogen Signalling

Estrogen signals via two estrogen receptors ERα and ERβ, members of the nuclear hormone receptor family (Figure 1.8). They are distinct proteins, encoded by genes on separate chromosomes. The first receptor to be identified was ERα in 1985 by Walter et al (Walter et al., 1985), however, it was over a decade later that the second receptor ERβ was identified from a prostate cDNA library (Kuiper et al., 1996). The human ERs share a 96% homology in the DNA binding domain (DBD) region highlighting a common function (binding to estrogen response elements [EREs] in target genes to activate transcription). By contrast the receptors share relatively low homology (53%) in their ligand binding domain (LBD), yet bind estrogen with roughly equal affinity (Thornton, 2002). Despite their similar function the two ERs are found to have different distributions in tissues, with distinct and often opposing actions. ERα is thought to be the dominant receptor in reproductive tissues and mammary gland and is also found in the cardiovascular system, bone, skin and brain. ERβ is also found in reproductive tissues but is thought to predominate in peripheral tissues including skin, bone, lung, bladder, heart and kidney (Thornton, 2002). Of direct relevance to my PhD project the human and mouse ERs are functionally homologous, with a very high structural similarity of between 82% and 98% (Enmark and Gustafsson, 1999).

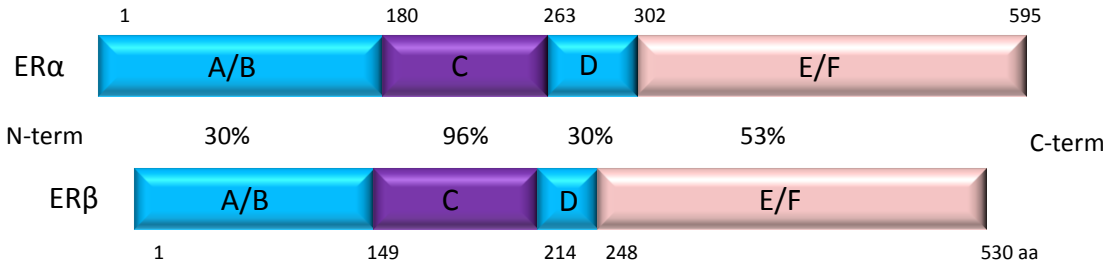

Figure 1.8 Human ER gene structure comparison illustrating high level of similarity in the C-region also termed DNA binding domain (DBD). The E/F region, also known as the Ligand binding domain (LBD) shows much less similarity, explaining how the two receptors can differentially bind the same ligand. Modified (Enmark and Gustafsson, 1999)

Estrogen can signal by a least four different mechanisms, outlined in Figure 1.9. In classical signalling, ligand binding causes intercellular ERs to dimerise and translocate to the nucleus where they act as transcription factors, activating or repressing expression by binding to the estrogen response element (ERE) of target genes, such as c-fos. Secondly, estrogen can induce/repress transcription of genes without an ERE, via alternative transcription factors, such as AP1 or SP1, with examples of such including epidermal growth factor and epidermal growth factor receptor (Hall and Phillips, 2005). Thirdly, ERs can signal in a ligand-independent manner, involving ER phosphorylation mediated by growth factors such as IGF. Indeed, skin ER/IGF crosstalk has recently been shown to be important for wound repair (Emmerson et al., 2012). The final mechanism is poorly characterised but thought to involve membrane ER activation of signalling pathways such as src/shc/ERK (Hall and Phillips, 2005). A further level of complexity is added by the numerous cell-type specific co-activators and co-repressors that can bind to and modulate the ER-DNA complex.

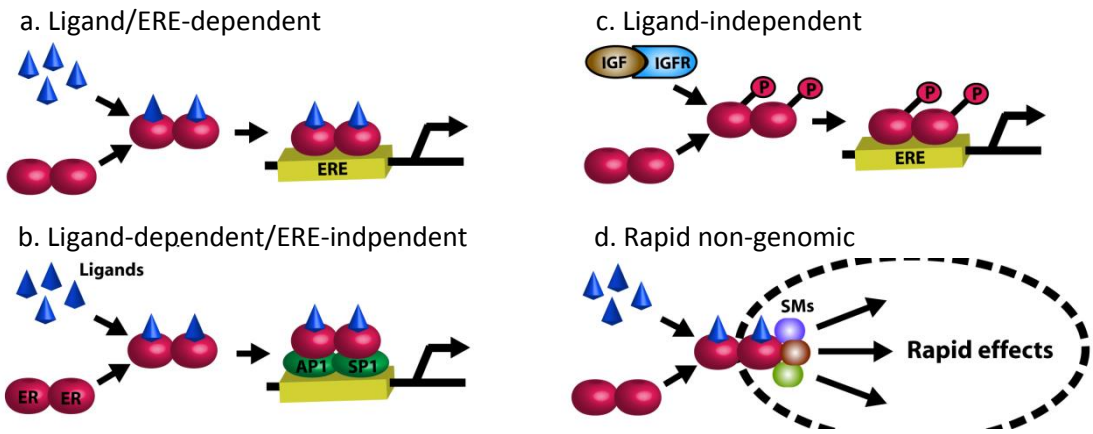

Figure 1.9 Four main mechanisms of estrogen signalling have been described (illustrated): a) classical-ligand dependent; b) ERE-independent involving intermediary transcription factors (eg. AP1); c) classical ligand-independent involving growth factor-mediated receptor phosphorylation and; d) a rapidly transduced membrane receptor pathway that remains poorly characterised. (Heldring et al., 2007)

1.5.2 Estrogen effects on skin

Over the last two decades numerous studies have documented the cutaneous changes that occur post-menopause (see Emmerson and Hardman, 2012 for a recent review), where macroscopic changes include reduced epidermal and dermal thickness and increased wrinkling and sagging. Functionally, post-menopausal skin is less elastic, dry, flaky, bruises easily and is both more susceptible to wounding and less able to heal in a timely manner (Brincat et al., 1985). Initial studies exploring compositional changes in human skin, reported a decline in collagen content, by an average of 1-2% per year post-menopause (Brincat et al., 1987), leading to a reduction in dermal thickness and density which is correlated to post-menopausal years and

not chronological age (Brincat et al., 1985). Skin elasticity measured by suction is reduced by 0.55% per year following menopause (Sumino et al., 2004) and an intriguing early study also showed that elastic fibres are degraded prematurely in a cohort of women who had entered early menopause (at 30-37yrs) (Bolognia et al., 1989). Many of these changes can be prevented, or even reversed by estrogen treatment. Post-menopausal women have been treated with systemic estrogen for decades, in the form of hormone replacement therapy (HRT). Studies have shown that collagen content is significantly increased in the skin of post-menopausal women receiving HRT compared to those who were not (Brincat et al., 1987, Sauerbronn et al., 2000).

HRT has also been linked to increased epidermal thickness post-menopause (Hall and Phillips, 2005, Rauramo and Punnonen, 1969) and mechanistically estrogen increases keratinocyte proliferation (Kanda and Watanabe, 2004) and, at least in vitro, prevents H_2O_2 induced apoptosis (Kanda and Watanabe, 2003). A large study of nearly 4000 women found post-menopausal skin to be significantly dryer, with estrogen use functionally reversing this (Dunn et al., 1997, Verdier-Sevrain et al., 2006). HRT has been shown to significantly increase the water retention in the stratum corneum (Pierard-Franchimont et al., 1995) and epidermal sphingolipids are only altered in the aged skin of females (Denda et al., 1993), and again found to be reversed by HRT (Callens et al., 1996, Sator et al., 2001). The dermis is also in part responsible for skin hydration and in this region there are changes in polysaccharides and reduced sebum levels post-menopause (Pochi et al., 1979), with HRT improving sebum production by 35% compared to without (Callens et al., 1996). Polysaccharides are increased by estrogen treatment (Grosman et al., 1971) and glycoproteins are found to be increased with both the high levels of estrogen associated with pregnancy (Danforth et al., 1974) and HRT. A number of studies have explored skin extensibility in humans as a measure of elasticity. Pierard et al (Pierard et al., 1995b) found HRT to limit menopause-induced increase in forearm skin extensibility (loss of elastic recoi), later quantified by Sumino et al (Sumino et al., 2004) as a 5.2% increase in forearm skin elastic recoil following 12 months of HRT. Henry *et al* (Henry et al., 1997) report similar effects on facial skin extensibility and elasticity following HRT.

Further studies reports effects of local topical estrogen replacement in improving skin thickness (Shah and Maibach, 2001); increasing skin moisture content and barrier function (Pierard et al., 1995b); inducing dermal production of collagen I (Son et al., 2005); increasing the number, thickness and improving the orientation of dermal elastin fibres (Punnonen and Kuurne, 1987) and improving the macroscopic appearance of facial skin (Brincat et al., 1985) with a significant reduction in fine face wrinkles (Creidi et al., 1994). At a cellular level estrogen stimulates keratinocyte proliferation, inhibits apoptosis, dampens skin protease levels and protects against oxidative damage (Brincat, 2000). Collectively, these studies provide strong evidence that

hormonal changes post-menopause play a key role in accelerating matrix changes that are a normally characteristic of ageing, an effect that can be reversed by exogenous estrogen treatment. However, human studies provide little scope for functional exploration of the underlying mechanism, due to the wide variation of human genetics and confounding factors such as health, medication and lifestyle.

1.5.3 Mouse models provide insight into the effects of estrogen on skin

Murine models provide an opportunity to mechanistically address hormonal aspects of skin ageing. Here the surgical menopause model of ovariectomy (Ovx) has been important in rodents as a pre-clinical model of numerous aspects of menopause-associated human pathology, including osteoporosis, neurodegeneration and cardiac dysfunction, and as a model of peri-menopausal symptoms, such as depression and hot flushes. Surprisingly, little skin research has been carried out with this model. Ovx is known to enhance sensitivity of rat skin to UV damage with accelerated photoageing, measured as wrinkling, a loss of elasticity and damage to elastic fibres (Tsukahara et al., 2001). This is supported by a mouse study where effects on skin extensibility and elastic recoil were measured. Here, ovariectomy significantly increased recoil time in UV exposed skin, associated with elevated tissue elastase activity (Tsukahara et al., 2004). Of note the recent work of Fang *et al* (Fang et al., 2012) who have combined cryo-sectioning with AFM, has revealed nanoscale changes in the morphology of dermal collagen fibrils in ovine Ovx samples. The second major advantage of mice is their genetic tractability. Indeed, mice have been generated lacking one or both of the ERs (Cicatiello et al., 1995, Dupont et al., 2000, Korach, 1994, Krege et al., 1998, Lubahn et al., 1993, Shughrue et al., 2002), or aromatase (ArKO) which disrupts estrogen biosynthesis (Fisher et al., 1998), or more complex Cre/LoxP conditional ER nulls (Campbell et al., 2010) and point mutants (Jakacka et al., 2002, Lee et al., 2001, Swope et al., 2002). Phenotypic analysis of these mice had provided, and continues to provide, insight into ER-mediated physiological roles in a range of tissues (summarised in Table 1.2). Of particular relevance ER null mice display a number of skin phenotypes. First to be reported were effects on hair follicles. In 2002 Moverare *et al* (Moverare et al., 2002), reported a role for ERα, but not ERβ, in regulating hair cycle. In a subsequent study Ohnemus *et al* (Ohnemus et al., 2005) show that ERβ does in fact play a role, regulating catagen induction. In the same studies ERα was suggested to be important for estrogen's effects on epidermal thickness. More recently ERs have been shown to play important roles in skin wound repair, where ERβ predominates. Conditional null ERβ (K14-cre;ER$\beta^{L2/L2}$) mice highlight a key role for epidermal ERβ in mediating estrogen's beneficial effects on healing in Ovx mice (Campbell et al., 2010). More recently still inflammatory cell-specific ERα has been shown to be important for eliciting the anti-inflammatory effects of estrogen during wound repair (Campbell et al., 2014).

Table 1.2 Phenotypic changes in ER null mice

	ERαKO		ERβKO	
System	♂	♀	♂	♀
Reproductive (Emmen 2003)(Emmen and Korach, 2003) (Krege 1998)(Krege et al., 1998) (Dupont 2000)(Dupont et al., 2000) (Antal et al.,2008)(Antal et al., 2008)	Infertile	Infertile. Ovarian cysts. No corpora lutea. Hypoplastic uterus	Fertile. $ER\beta_{ST}^{(L2/L2)}$ infertile	Subfertile. Few corpora lutea. Less ovulation. Smal litters.
Cardiovascular (Otsuki 2003)(Otsuki et al., 2003) (Fliegner et al.,2010)(Fliegner et al., 2010)	No histological cardiovascular abnormalities		Systolic and diastolic hypertension. Cardiac fibrosis and heart failure	
Brain (Hill et al., 2009)(Hill and Boon, 2009)	Apoptosis of neurons in the frontal cortex of *ArKO* mice			
Social Interaction (Choleris 2006)(Choleris et al., 2006) (Nomura 2006)(Nomura et al., 2006) (Ogawa 1998)(Ogawa et al., 1998)	-	Impaired social discrimination	Increased aggression	Reduced social discrimination. Anxiety.
Bone (Korach 1994)(Korach, 1994) (Walker 2004)(Walker and Korach, 2004)	Decreased bone density Shorter bones.		No difference compared to WT	Increased bone density
Lung (Morani et al., 2006)(Morani et al., 2006) (Shim et al., 2003)(Shim et al., 2003)	-	-	Alveoli defects and inflammation	

1.5.4 Estrogen receptor agonists and Selective Estrogen Receptor Modulators (SERMs)

Since publication of the Million Women Study drew links between HRT and increased risk of developing certain cancers (Beral, 2003, Beral et al., 2007) there has been drive to develop pharmacological modulators of ERs. These include receptor-specific agonists which are experimentally invaluable, and the more clinically-relevant mixed agonist/antagonists known as SERMs. The experimental ERα agonist PPT and the ERβ agonist DPN have proved important for understanding the roles of ERs in wound healing, where it has been shown that ERβ signalling promotes healing via epidermal cells (Campbell et al., 2010) and ERα drives macrophages toward a pro healing phenotype (Campbell et al., 2014). A different ERβ ligand, WAY-200070, has been shown to dampen inflammation and MMP expression in UV-damaged skin (Chang et al., 2010). The SERMs tamoxifen, raloxifene and the naturally occurring phytoestrogen genistein have also been shown to promote skin healing (Emmerson et al., 2010, Hardman et al., 2008). Additionally, tamoxifen has also been shown to improve scarring in burn patients by reducing collagen synthesis via a reduction in TGF-β signalling. (Gragnani et al., 2010) Genistein has also been reported to improve the gross skin changes in ovariectomised rats (Polito et al., 2012). This

body of evidence clearly illustrates that ERs have important roles in the maintenance of skin structure and function, however the mechanisms remain to be fully elucidated.

1.6 Wound healing

Following injury the skin undergoes a complicated and co-ordinated sequence of events to repair itself, comprising of a number of overlapping phases with interaction between a wide range of cell types, ultimately leading to a healed wound. The first phase, haemostasis, occurs immediately following injury to prevent blood loss. This is followed by the inflammatory phase to prevent infection, then the proliferative phase occurs, within days of injury, to close the wound and the remodelling phase can persist for several years with the ultimate formation of mature scar tissue.

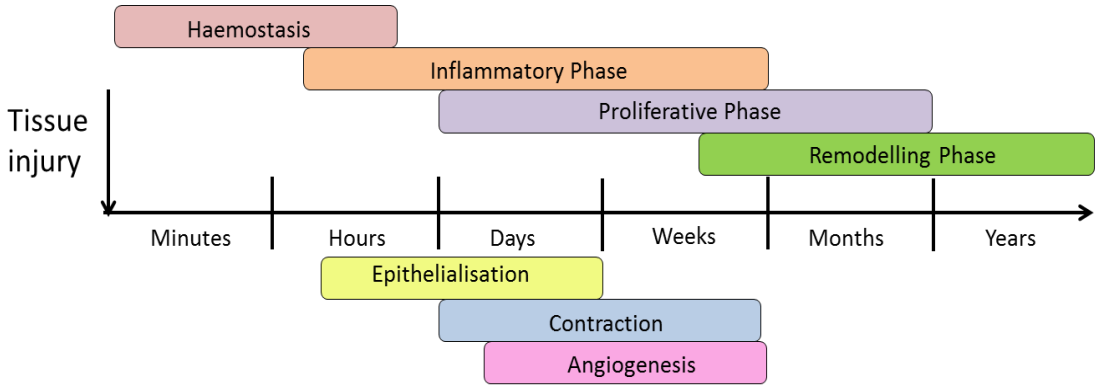

Figure 1.10. An overview of the timeline of the stages of wound healing. Haemostasis begins immediately after an injury and this is followed by infiltration of inflammatory cells in the first 24 hours. Then after a couple of days the proliferative phase begins to restore the damaged tissue. The final remodelling stage then begins and can last years as a more permanent collagen matrix is laid down.

1.6.1 Haemostasis

Haemostasis is the prevention of blood loss via the formation of a blot clot, which occurs almost immediately following injury. Surface activation of Hageman factor (Factor XII) and the release of procoagulant from damaged cells, along with the expression of surface membrane coagulation factors on activated platelets mediate the formation of the fibrin clot. At the end of damaged blood vessels thrombin catalyses conversion of soluble fibrinogen to insoluble fibrin, these fibrin strands form a meshwork between aggregated platelets and trap red blood cells to form the clot. The primary aim of this process is to prevent blood loss and act as protection from invading bacteria, but secondary to this it also it provides a matrix scaffold for invading host

cells, required for successful healing. Once a suitable clot has formed this process must then be stopped. This occurs via release of prostacyclin to inhibit platelet aggregation, antithrombin III which inhibits thrombin and protein C which degrades coagulation factors V and VII. The platelets also release platelet-derived growth factor (PDGF) and Transforming growth factor beta (TGF-β). These regulatory proteins act as chemoattractants and mitogens for fibroblasts, and macrophages (Singer and Clark, 1999).

1.6.2 Inflammatory response

The inflammatory response begins very soon after injury and can last for days or weeks with a co-ordinated influx of immune cells. The first immune cells to arrive at the wound site are leukocytes, which initially leak from the damaged vessels. There is also rapid activation of tissue-resident immune cells such as mast cells (Noli and Miolo, 2001), Langerhans cells (Cumberbatch et al., 2000) and γδ T cells (Jameson et al., 2004). Activation of these resident cells triggers a release of cytokines and chemokines and as such begins the active recruitment of immune cells. Degranulation of mast cells prompts vasopermeability to allow an influx of immune cells to the wound site (Noli and Miolo, 2001).

The first cells recruited to the wound site are neutrophils which arrive within minutes. They kill invading bacteria via phagolysosomes (Lee et al., 2003) as well as bursts of reactive oxygen species (Dovi et al., 2004). They also trap microbes in a mesh of extruded fibres which bind to bacteria (Brinkmann et al., 2004). Neutrophils also increase expression of integrins CD11 and CD18 on their surface, which along with factor X promotes attachment to blood vessels and facilitates migration to the wound site.

Except where there is considerable wound contamination the influx of neutrophils ceases within a few days and macrophages become the dominant immune cell type. Nearly all the neutrophils become trapped in the fibrin clot and are then extruded with the scab and the remaining senescent neutrophils are phagocytosed by macrophages (Newman et al., 1982).

Monocytes/macrophages peak at the wound site at around 24-48 hours post wounding. They leave the blood as monocytes and differentiate into macrophages as they alter gene expression in response to their surroundings (Martinez et al., 2006). They can differentiate into either classically (M1) or alternatively activated (M2) macrophages and these two types have distinct roles; M1 are pro-inflammatory in nature whereas M2 are anti-inflammatory and pro-angiogenic and are thought to have a pro-healing phenotype. Both these types of macrophage are present at the beginning of healing and as healing progresses M2 macrophages predominate (Deonarine et al., 2007).

Monocytes are drawn to the wound site in response to a number of chemoattractants such as Macrophage Chemoattractant protein (MCP) and Macrophage Inflammatory protein (MIP). Once in the wound site their main role is as phagocytes, removing matrix and cell debris (Eming et al., 2007). However aside from their role of cleaning the wound site they are also an important source of cytokines and growth factors to regulate the healing process, including but not limited to IL-1, TNFα, Vascular endothelial growth factor and fibroblast growth factors (Singer and Clark, 1999).

1.6.3 Proliferative phase

The proliferative phase involves re-epithelialisation to restore the epidermis, granulation tissue formation to reform the damaged dermal ECM and angiogenesis to re-establish blood flow to the newly formed tissue.

1.6.4 Re-epithelisation

Within hours of injury the keratinocytes at the wound edge begin to proliferate and migrate to close the wound and restore the epithelial barrier (Singer and Clark, 1999). In order to facilitate movement the cells must first undergo a number of changes to alter their adhesion to both one another and the dermis and basement membrane below. The keratin filaments retract back inside the cell and actin-rich pseudopodia form to allow the cells to crawl (Mitchison and Cramer, 1996). The keratinocytes upregulate expression of enzymes such as MMPs to degrade the matrix and allow the cells to "crawl" under the scab, eventually separating the viable tissue below from the transitory clot (Pilcher et al., 1999).

1.6.5 Restoration of the Dermis

Around 4 days after injury new stroma, termed granulation tissue, forms to restore the dermis and replace the fibrin clot. The main contributing cell type is fibroblasts which are recruited from healthy surrounding tissue and begin to divide, or differentiate from pluripotent resident cells (Fernandes et al., 2004). Fibroblasts also differentiate from the bone marrow fibrocytes and the circulating pericytes (Abe et al., 2001, Rajkumar et al., 2006). The fibroblasts begin to synthesise, deposit and remodel new matrix proteins; initially this is mainly fibronectin and collagen type III.

Fibroblasts close to the wound site form stress fibres to allow some contraction of the connective tissue. However, further to this light contractile ability some of the fibroblasts differentiate into contractile myofibroblasts in response to growth factors such as TGFβ and begin to express α-smooth muscle actin. This leads to a stronger contraction of the wound bed to draw the edges of the wound together (Hinz, 2007).

1.6.6 Angiogenesis

The newly forming tissue requires the support of new blood vessels to sustain it. It is the generation of new vessels which gives granulation tissue its granular appearance. There are a wide variety of signalling molecules which control the complex process of angiogenesis; such as VEGF, angiogenin, angiotropin and TGFβ along with many others. The new blood vessels are formed through the migration of endothelial cells in response to these signalling molecules (Singer and Clark, 1999).

1.6.7 Tissue remodelling

This phase involves the transition from a provisional matrix to the formation of a mature scar and can last for over a year. Following the initial rapid closure of the wound, a scar can appear elevated and pigmented. To restore the skin to as close to its original strength and appearance as possible requires a balance of collagen production, breakdown and remodelling and the regeneration of appendages such as hair follicles and sebaceous glands. In many cases the skin is never fully restored and a visible scar remains.

1.7 Estrogen and wound healing

Post-menopausal skin shows a delay in wound healing ability and an increased incidence of the development of chronic wounds, whilst HRT is able to improve healing (Ashcroft et al., 1997) and protect against the development of chronic wounds (Margolis et al., 2002). Additionally, topical estrogen has been shown to accelerate acute wound healing in both male and female elderly patients (Ashcroft et al., 1999). More recently a microarray study revealed that 78% of genes which were differentially expressed in elderly delayed healing wounds versus acute wounds from young males were estrogen regulated (Hardman and Ashcroft, 2008). Thus, estrogen is clearly clinically and physiologically important for effective wound repair.

Studies using the ovariectomised mouse model and estrogen replacement have shown that estrogen has numerous effects on many aspects of healing (Figure 11) (Emmerson et al., 2009). The Ovx mouse wound shows a delay in re-epithelialisation, increased inflammation and protease activity. Short term estrogen replacement is able to promote healing by increasing keratinocyte migration, dampening inflammation and increasing matrix deposition (Ashcroft et al., 1997, Ashcroft and Ashworth, 2003).

1.7.1 Estrogen and inflammation

In acute healing estrogen dampens the inflammatory response and prevents excessive recruitment of neutrophils to the wound site. Down-regulation of L-selectin by estrogen reduces

the homing signal to the wound site (Ashcroft et al., 1999). Subsequent dampening of wound neutrophil-derived elastase prevents excessive degradation of the matrix proteins. Estrogen also has an important function in the switching of macrophages towards a pro healing (TH2) phenotype, signalling predominantly via ERα (Routley and Ashcroft, 2009, Campbell et al., 2014).

1.7.2 Estrogen and re-epithelialisation

One of the key aspects of age-associated delayed healing is failure to restore the epidermal barrier. Estrogen is able to induce mitosis and promote migration of keratinocytes (Verdier-Sevrain et al., 2004, Campbell et al., 2010). In humans 3 months of HRT is able to reverse post-menopausal associated delayed re-epithelisation and individuals showed similar re-epithelialisation to pre-menopausal women (Ashcroft et al., 1997)

1.7.3 Estrogen and matrix deposition

Post-menopausal wounds also exhibit delayed deposition of collagen and subsequent remodelling, an effect which can be reversed by systemic HRT or topical estrogen treatment (Ashcroft et al., 1997, Ashcroft et al., 1999). In vivo estrogen promotes fibroblast proliferation, migration and increases collagen deposition whilst also dampening MMP activity (Stevenson et al., 2008, Ashcroft et al., 1999)

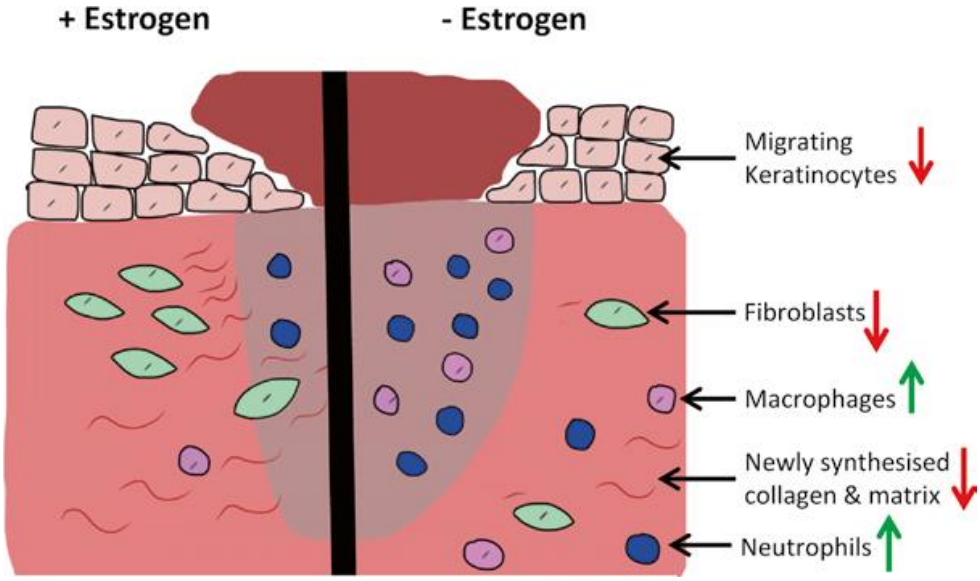

Figure 1.11. Estrogen affects many aspects of wound healing Right estrogen deficiency reduces keratinocyte migration and fibroblast number and increases inflammation leading to reduced matrix and delayed healing (Saville and Hardman, 2015)

1.8 Conclusions, Hypothesis and Aims

As we age the skin undergoes extensive, widespread changes, leading to deterioration in function and appearance. The skin acts as a visible representation, but ageing occurs simultaneously in all tissues of the body. It is known that age leads to alterations in mechanical function of tissues which can have serious health consequences and there is strong evidence to support a clear link between estrogen signalling and skin ageing. However much of the previous work has focussed on alterations in collagen fibrils and there is a lack of detailed understanding as to how estrogen deprivation affects elastic fibres and other matrix proteins.

The hypothesis which drives my project is:-

Estrogen deprivation drives skin ageing and mechanical alterations via loss of ECM homeostasis. Therefore, I hypothesize that estrogen replacement will have a protective effect. I have addressed this via 3 specific aims:

Aim 1 – Determine if

1) Estrogen deprivation induces selective remodelling of the dermal ECM.
2) Which mechanisms could drive ECM remodelling

This will be approached *in vivo* using the ovariectomised mouse model of menopause to understand the effects of estrogen deprivation on the gross mechanical properties of murine skin. Changes will be mapped to alterations in the key ECM proteins particularly changes in the elastic fibre proteins, using both histological and biochemical approaches to ascertain the contribution of catabolic and anabolic processes to alterations in ECM homeostasis with estrogen deficiency.

Aim 2 – Determine if estrogen deprivation models key aspects of skin ageing

This will be answered by comparison of the uniaxial tensile and viscoelastic testing from the ovariectomised model to the chronologically aged mouse. Followed by proteomic comparison (By Mass spectrometry) of the dermal ECM from the aged and ovariectomised mouse to offer a detailed understanding of the effect of these two states on dermal ECM components

Aim 3 - To determine if the effects of estrogen on the elastic fibre network are mediated via ERα or ERβ in both intact and wounded skin.

By utilising selective estrogen receptor agonists to modulate signalling *in vivo* in the Ovx mouse model we will assess the effect of signalling via ERα or ERβ on the elastic fibres in whole skin and

the re-deposition of elastic fibres into the healing wound. Additionally we will analysis the effects of the agonist's *in vitro* using human dermal fibroblast cell culture.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Animal Experiments

All animal studies were carried out in accordance with UK home office regulations under approved licences. Mice were of the C57/BL6 genetic background

2.1.1 Ovariectomy

Mice undergoing bilateral ovariectomy were initially anesthetised via inhalation of isoflurane, nitrous oxide and pure oxygen. The lower ventral area was shaved and cleaned using an alcohol wipe (Seton healthcare, Liverpool, UK). A small horizontal incision was made through the skin, followed by a second incision, at 90^{o} to the first, through the body wall. The uterus was identified by the fat pads and then the horns of the uterus were followed to identify the ovaries. The ovaries were removed using sharp sterile scissors and the body wall sutured with absorbable polyglycolic acid suture (Dexon II Johnson & Johnson). The skin incision was then closed using silk braided non-absorbable sutures (Ethicon Mersilk, Johnson & Johnson). An interperitoneal injection of 1mg/kg buprenorphine was given as an analgesic.

2.1.2 Incisional wounding

Mice undergoing incisional wounding were initially anesthetised via inhalation of isoflurane, nitrous oxide and pure oxygen. The dorsal area was shaved and cleaned using an alcohol wipe (Seton healthcare, Liverpool, UK). Two full- thickness wounds were made 2cm from the base of the skull and 0.5cm either side of the midline (Figure 2.1). Incisions were made through the skin and panniculus carnosus muscle using a sterile scalpel blade. An interperitoneal injection of 1mg/kg buprenorphine was given as an analgesic. The mice were then singularly housed in minimal bedding to prevent interference of the wounds and the wounds allowed to heal by secondary intension (Emmerson et al., 2009).

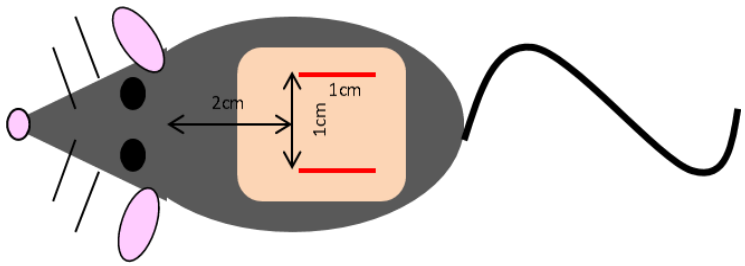

Figure 2.1 Position of dorsal incisional wounds. Two 1cm incisional wounds are made 2cm from the base of the skull and 0.5 cm either side of the midline through the dorsal skin and carnosus muscle.

2.1.3 Tissue harvest

On the day of sacrifice mice were euthanised via rising concentration of CO_2. Wounds were photographed using FujiFilm FinePix S5700 camera (Fuji, Tokoyo, Japan). The wounds were then excised and bisected then either formalin fixed (see appendix) for paraffin wax sectioning, embedded in optimum cryo medium (Klinipath) cooled in liquid nitrogen and stored at -80^{o}C for cryotomy or 'snap frozen' by placing into cryo tubes and into liquid nitrogen prior to storage at -80^{o}C. Pieces of unwounded, shaved, dorsal skin were also collected and in the same manner. The ventral skin was shaved and collected in a full length strip and stored on ice for immediate mechanical testing. The uterus was removed to be weighed to ensure the ovariectomy had been effective. A shrunken uterus indicates systemic estrogen deprivation (Garcia-Perez et al., 2006).

2.1.4 Tissue processing

Following formalin fixation for 24 hours the tissue was transferred to 70% ethanol 30% dH_2O. The tissue was processed on a STP120 (Microm) tissue processor following the program described in Table 2.1

Table 2.1 Tissue processing protocol

Step	Solution	Time (Min)	Temperature(^{o}C)
1	90% IMS	45	Room Temp
2	95% IMS	45	Room Temp
3	100% IMS	45	Room Temp
4	100% IMS	45	Room Temp
5	100% IMS	45	Room Temp
6	Xylene	30	Room Temp
7	Xylene	30	Room Temp
8	Xylene	30	Room Temp
9	Wax	60	60
10	Wax	60	60

IMS and Xylene supplied by Genta Medical, New York

Tissue was embedded into warm paraffin wax (RA Lamb) on a microm embedding station. The tissue was orientated with the cut edge of the downward in the block and cooled until solidified on a cold plate.

2.2 Histological analysis

2.2.1 Tissue sectioning

Slides were prepared from wax embedded fixed tissue. Glass microscope slides were pretreated with Vectabond reagent (Vector Laboratories, UK) (see appendix). Tissue was sectioned to 6µm thickness on a RM2235 microtome (Leica, UK) and floated onto slides in a 50^{o}C flotation bath (Medite TFB45). Slides were dried overnight in a drying oven at 37^{o}C.

2.2.2 Dewaxing and rehydration

Prior to staining slides were submerged in xylene for 20 minutes to remove the wax then rehydrated through decreasing IMS alcohol concentrations 1 minute in each (2x100%, 90%, 70%, 50%) then placed into water before the appropriate staining was carried out.

2.2.3 Immunoperoxidase staining

Following dewaxing and rehydration the slides were heated in a microwave in citrate buffer (see appendix) for 2.5 minutes and allowed to cool for 15 minutes. Slides were then placed in phosphate buffered saline (PBS) for 2 minutes. A hydrophobic barrier was drawn around each section (ImmEdge Pen, Vector Laboratories) and the slides placed in a humidity chamber. Slides were incubated in 0.3% H_2O_2 (Sigma, UK) for 30 minutes to quench endogenous peroxidase activity. Slides were washed by submerging in PBS for 15 minutes. Sections were incubated for 20 minutes in the appropriate blocking serum, based on the host species of the secondary antibody (ABC Kit, Vector Laboratries) to prevent unspecific binding. Excess blocking serum was removed and the appropriate primary antibody was applied over the section and incubated overnight. A PBS control section was also prepared. Table 2.2 shows primary antibody concentrations. Slides were washed by submerging in PBS for 15 minutes then incubated for 30 minutes with the appropriate peroxidase anti IgG secondary antibody (ABC Kit, Vector Laboratories). Slides were washed in PBS for 15 minutes then incubated with ABC reagent for 30 minutes (ABC Kit, Vector Laboratories). Slides washed in PBS for 15 minutes. A drop of Nova Red reagent was pipetted over each section until a colour change was seen. Slides rinsed in water for 3 minutes. Slides were counter stained in Gills Haematoxylin (Vector Laboratories) for 30 seconds then rinsed in running water for 3 minutes. Slides were dehydrated by submerging in increasing alcohol concentrations (50%, 70%, 90%, 100%, 100%) for 1 minute each. Slides were then submerged in xylene for 20 minutes clearing. Slides were mounted with Pertex mounting medium (CellPath, Newton, UK) and glass coverslips.

Table 2.2 Antibodies for immunohistochmical analysis

Antibody	Working dilution	Manufacturer	Host species
Fbn-1	1:200	Santa Cruz (sc-7540)	Goat
Fbn-2	1:100	Genetex(GTX-100261)	Mouse
ER α	1:50	Santa Cruz (Sc-542)	Rabbit
ER β	1:50	Santa Cruz (Sc-8974)	Rabbit
AGE	1:100	ABCam (Ab23722)	Rabbit

2.2.4 Gamori's aldehyde fuchsin stain for elastic fibres

Following dewaxing and rehydration slides were incubated in Lugols Iodine solution for 30 minutes then rinsed in water for 3 minutes. Slides were incubated in 5% sodium thiosulpahte (Sigma, UK) for 5 minutes then rinsed in running water for 10 minutes. Slides were submerged in 90% IMS for 30 seconds then transferred into aldehyde fuchsin (see appendix) for 10 minutes. Slides were rinsed in water and then differentiated in 90% IMS. Slides were dehydrated by submerging in increasing alcohol concentrations (50%, 70%, 90%, 100%, 100%) for 1 minute each. Slides were then submerged in xylene for 20 minutes clearing. Slides were mounted with Pertex mounting medium (CellPath, Newton, UK) and glass coverslips.

2.2.5 Picro-Sirius red staining for organised fibrillar collagen

Slides dewaxed and rehydrated as described above then incubated in picro-sirius red stain for 1 hour. Slides were washed twice in 0.5% acetic acid for 10 minutes each. Slides were dehydrated by submerging in increasing alcohol concentrations (50%, 70%, 90%, 100%, 100%) for 1 minute each. Slides were then submerged in xylene for 20 minutes clearing. Slides were mounted with Pertex mounting medium (CellPath, Newton, UK) and glass coverslips (McConnell et al., 2016).

2.2.6 Massons trichrome staining for collagen

Slides dewaxed and rehydrated as described above then placed in picric acid solution for 1 hour. Slides were rinsed in running water until the water for 2 minutes then dipped in 90% IMS. Slides were stained in Weigert hematoxylin (see appendix) for 10 minutes then rinsed in running water for 10 minutes. Slides were then stained in Biebrich scarlet-acid fuchsin (see appendix) for 2 minutes then rinsed in distilled water. Slides then stained in phosphomolybdic/phospotungstic acid solution (See appendix) for 15 minutes. Then placed in aniline blue solution (See appendix) for 5 minutes and rinsed in distilled water. Slides were submerged in 1% acetic acid for 5 minutes then dehydrated by placing into 100% ethanol (Fisher scientific, UK) and cleared by submerging in xylene for 20 minutes. Slides were mounted with Pertex mounting medium (CellPath, Newton, UK) and glass coverslips.

2.3 Cell Culture

2.3.1 Culture of human dermal fibroblasts

Human dermal fibroblasts were a gift from Epistem (Manchester, UK) initially obtained, under informed consent, from the normal abdominal skin of a 38yr old female donor undergoing surgery.

Cells were cultured in phenol red free DMEM media (Sigma) supplemented with 10% charcoal stripped fetal bovine serum, 4mM L-glutamine (Sigma) and 1% penicillin-streptomycin (10,000 U/ml 10mg/ml stock) (Sigma) and used between passage 6 and passage 10. Both phenol red and unstripped serum have estrogenic activity (Welshons et al 1988, Berthios et al 1986). Cells were incubated at 37^{o}C with 5% CO_2.

2.3.2 Cell culture for Mass spectrometry

Primary dermal fibroblast were plated into 6 well plates at 50 % confluence and allowed to adhere overnight. Fresh media was added on alternate days and half of the samples were supplemented with estrogen into the media at a final concentration of 10^{-7}M. Cell matrix was extracted after 8 days culture using 2M NaCL 25mm ammonium bicarbonate 25mM dithiothretol.

2.4 Quantitative Real-time PCR

2.4.1 RNA Extraction

RNA was extracted from snap frozen tissue by homogenising in Trizol reagent (Life technologies, Invitrogen, UK) or from cultured cells by vortexing in Trizol reagent. A small piece of snap frozen tissue was added to 1 ml of Trizol and homogenised 3x 10 seconds using an ultra Turrax IKA-T1 homogeniser. For cells they were vortexed for 30 seconds in 500ml Trizol. 200µl of chloroform per 1ml of Trizol was added and shaken vigorously and incubated for 2 minutes. Samples were centrifuged at 12,500xg for 15 minutes at 4^{o}C. The colourless upper phase was transferred to an RNAse free tube and an equal volume of 70% ethanol was added mixed by gently inverting. The solution was then purified using a PureLink RNA minikit (Invitrogen, UK) following the manufactures instructions. The solution was added to a spin column and centrifuged 12,000 rpm for 15 seconds and the flow through discarded. The column was washed with 700µl wash buffer 1 and centrifuged 12,000 rpm for 15 seconds. The column was placed into a new tube and washed 2x with 500µl wash buffer II (with ethanol added) by centrifugation at 12,000 rpm for 15 seconds. The flow through was discarded and the column membrane dried by centrifuging at

12,000 rpm for 1 minute. The RNA was eluted into an RNAse free tube by incubating with 30µl RNase free water for 1 minute, then centrifuging for 2 minutes at 12,000 rpm.

2.4.2 RNA quantification and cDNA synthesis

RNA yields were assessed using a Nanodrop 1000 spectrophotometer then cDNA was transcribed from 1µg of RNA (Promega RT kit and AMV-revese transcripate (Roche)) RNA was diluted to 1µg in 10µl of nuclease free water then incubated for 10 minutes at 70^{o}C. Following this samples were incubated on ice for 2 minutes then the reaction mix added containing 4µl RT buffer, 2µl $MgCl_2$, 1µl random hexamer, 0.5µl Rnase inhibitor and 0.5µl reverse transcriptase. Samples then placed in the thermal cycler (TC-412 TECHNE) and incubated for 10 minutes at 25^{o}C, 30 minutes at 55^{o}C then 5 minutes at 85^{o}C.

2.4.3 Quantitative Real-Time PCR analysis (qPCR)

qPCR was performed on an opticon qPCR thermal cycler (Bio-Rad,UK) using Mesa green mastermix (Eurogentec, Southhampton, UK). cDNA samples were diluted 1:20, 1:100 and 1:500 in nuclease free water and 10µl of each sample added to a 96 well plate. 15µl of reaction mastermix was added to each well, this contained 100pM of primer (0.225µl of each forward and reverse primer) 12µl of Mesa green and 2µl of nuclease free water. The plate was sealed with thermal film and loaded into the thermal cycler see table 2.3 for cycling conditions.

Table 2.3 qPCR cycling conditions

Step	Temp (^{o}C)	Duration (mins)
1. Enzyme activation	95	10
2. Denaturation	95	0.25
3. Annealing and Elongation	60	1
4. Read Plate		
5. Amplification	Repeat steps 2-4 39 times	
6. Melt curve generation	60-90 increase	Read every 0.3 ^{o}C

Gene expression was determined relative to a standard sample and normalised to an average of the housekeeping genes Gapdh and Ywhaz

Table 2.4 Primer sequences

Target Gene	Forward primer sequence	Reverse primer sequence
Fbn-1	CAGTGCATTAACACAACGC	TTCTTGCAGGTCCCATTTCC
FBN-1	CAGGACAGGCCCATGTTTTAC	GCACAGCAGAGCGTTTTTGT
Eln	TGTCCCACTGGGTTATCCCAT	CAGCTACTCCATAGGGCAATTT
ERα	AATAGCCCTGCCTTGTCCTT	ATAGATCATGGGCGGTTCAG
ERβ	TGTGTGTGAAGGCCATGATT	GTGTCAGCTTCCGGGCTACTC
Gapdh	TGCCACTCAGAAGACTGTGG	GGATGCAGGGATGATGTTCT
GAPDH	TGCACCACCAACTGAGC	GGCATGGACTGTGGTCATGAG
Ywhaz	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA

2.5 Protein analysis

2.5.1 Western blot

Protein separation was performed by SDS Polyacrylamide gel electrophoresis using 10% gel. Separating gels were made with a stacking gel on top (See appendix). Samples of cell culture media or extracted protein were mixed with 6µl of laemmli buffer (Bio-Rad, UK) containing 5% β-mercaptoethanol (Bio Rad, UK) and incubated at 100^{o}C for 5 minutes to denature the protein then placed onto ice. The gels were placed in the running tank with TGS buffer (see appendix) and samples were loaded into the gel wells along with pre stained all blue standards (Bio-Rad). The gels were run at 90V for 2 hours with an ice pack to keep the temperature down. Proteins were then transferred onto a PVDF membrane (SLS,UK) in transfer buffer (See appendix) at 100V for 90 minutes. The membrane was then incubated overnight in 5% TBS-Tween milk at 4^{o}C to block none specific binding sites. The membrane was washed in TBS-Tween for 15 minutes then incubated with primary antibody for 1 hour. The membrane was then washed 4x15 minutes with TBS-Tween and incubated with HRP-linked secondary antibody for 1 hour. The membrane was washed again as above then incubated for 5 minutes with ECL detection reagent (GE Healthcare, UK). Membranes were then developed on autoradiography film (Kodak). The pixel intensity from scanned images was measured using image J software (Image J, National Institutes of Health, USA) to give an estimate of the quantity of the protein present.

Table 2.5 Western blot antibody dilutions

Antibody	Working Dilution	Manufacturer	Host	Secondary (Amersham)
Elastin	1:200	Santa Cruz (SC-17580)	Mouse	1:5000
β-actin	1:5000	Sigma (A5441)	Mouse	1:5000

2.5.2 Extraction of cell deposited ECM for mass spectrometry

Cells were cultured as described in section 2.3.1 following 8 days of culture the media was removed and cells washed with 2mls of warm PBS. Then 500µl of ExNa buffer (see appendix) was added to the first of duplicate wells and incubated at 37^{o}C for 20 minutes. The same 500µl was added to the second duplicate well and incubated at 37^{o}C for 20 minutes to combine the extracts together. Samples were then frozen at -20 until preparation for mass spectrometry.

The following day 50µl of extracted ECM was added to 150ul of digest buffer in a tube containing immobilized trypsin beads (Perfinity Biosciences) and shaken overnight at 1400 rpm 37^{o}C.

The following day 4µl of 500mM DTT was added to the tubes and shaken at 1400rpm at 60^{o}C for 10 minutes to reduce the disulphide bonds. Then 12µl of 500mM iodoacetamide and shaken at 1400rpm at 25^{o}C for 30 minutes in the dark to oxidise the reduced bonds. Additional DTT was then added to quench this reaction.

Tubes were centrifuged at 13000rpm for 10 minutes to separate from the beads and supernatant transferred to a LoBind tube (Eppendorf) the solution was then acidified with 5 µl of 10% trifluoroacetic acid to make the extract water soluble.

Ethyl acetate (200µl) was added to each tube and vortexed for 1 minute then the tubes were centrifuged at 13000rpm for 1 minute. The organic molecules and surfacants partition into the top phase this was removed and the process repeated 2 more times with additional 200µl of Ethyl acetate. The samples were then dried down in a speed-vac before addition of 200µl of injection solution (See appendix).

100µl of POROS R3 beads were added to the wells of a 96 well filtration membrane plate with a collection plate below and centrifuged for 1 minute at 200xg to leave dry beads. The beads were then re-suspended in 50µl of 50% acetonitrile elution solution in water and the plate centrifuged again to dry the beads this was repeated. The beads were then re-suspended in 0.1% trifluoroacetic acid to wash the beads and centrifuged again to remove the liquid this was repeated.

100µl of sample was added to the beads and the beads re-suspended and centrifuged this was repeated until all the sample had been added and centrifuged through leaving the protein bound to the beads. The beads were then re-suspended in 0.1% trifluoroacetic and centrifuged to wash x2. The collection plate was washed with 50% acetonitrile prior to addition of 50µl

elution solution to the beads the plate was centrifuged then another 50µl of elution solution was added and centrifuge again. The eluted solution was transferred from the collection plate into mass spectrometry vials and dried down in the speed vac. The samples were resuspended in 6µl of injection solution and mixed. The peptide concentration was measured on the direct detect spectrometer and adjusted to 0.3mg/ml for injection on the LC column.

2.6 Mechanical Testing

2.6.1 Tension testing to failure

Ventral skin 1cm wide was excised from relevant experimental mouse groups and kept in PBS on ice. The end of the skin strip was placed into sandpaper to provide grip and clamped into the Instron 3344 (Instron, USA) fitted with a 100N load cell. The skin length was input into the Bluehills lite software (Instron,USA) then samples were loaded at a constant rate of 20mm/min until failure (I.e. the skin completely broke).

2.6.2 Cyclical loading and stress relaxation

Skin 1cm wide was excised from relevant experimental mouse groups and kept in PBS on ice. The end of the skin was placed into sandpaper to provide grip and clamped in the instron 5943 10N load cell (Instron, USA) and submerged in a PBS bath. The skin was loaded to cyclically loaded to 1N and unloaded at a rate of 10mm/min for 5 cycles and then loaded to 1 N and held at a constant strain and the load recorded as the tissue relaxed.

2.7 Statistical Analysis

One way ANOVA with Tukeys post hoc test (Graph pad prism software, USA) was used to compare the experimental groups (Intact/ Ovx and Ovx+E) in Chapter 3 (Young/ Ovx / Aged) Chapter 4 and (Intact / Ovx+Vehicle / Ovx+E / Ovx+PPT /Ovx+DPN). Comparison of 2 groups used a Mann-Whitney-U test to determine statistical significance (Graph pad prism software, USA).

Chapter 3

Results

"Estrogen mediates remodelling of the dermal elastic fibre system and hypodermal adipocytes"

CS was involved in experimental design and carried out experiments analysed results and prepared manuscript JS carried out mass spectrometry DH was involved in methodology for mechanical testing BD was involved in mechanical testing experimental design EE involved in manuscript preparation MH and MS were involved in experimental design and manuscript preparation.

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3 Estrogen mediates remodelling of the dermal elastic fibre system and hypodermal adipocytes

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Running head: Estrogen and skin homeostasis

3.1 Abstract

Age-related remodelling of the dermal extracellular matrix is thought to be a key cause of skin fragility in the elderly population. The peri-menopausal period in females is also associated with rapid development of an age-like phenotype which can be reversed by hormone replacement therapy. Thus suggesting a direct link between circulating hormone levels and tissue ageing. Whilst attention has primarily focused on estrogen deprivation as a mediator of collagen fibril abundance and structure, the influence of estrogen on the elastic fibre system remains poorly defined. As components of this system are both sensitive biomarkers of ageing and key players in tissue homeostasis we hypothesed that estrogen is a direct mediator of elastic fibre network proteostasis.

Using the overiectomised (Ovx) mouse surgical menopause model we demonstrate that an acute, 7 week period of Ovx leads to major changes in the gross mechanical properties of excised skin, significantly reducing both tensile strength and Youngs' modulus (by 41.5% and 38% respectively). Systemic replacement of 17beta-estradiol to physiological levels fully protects against these mechanical changes. Moreover, this acute ovariectomy period revealed differential effects of hormone deprivation on dermal structural networks; significantly decreasing dermal elastic fibre abundance without discernible effect on collagen fibril organisation or abundance. Specific elastic fibre proteolysis may be driven by ECM protease activity, or a consequence of significant adipocyte hypertrophy. We also show that, conversely, 17beta-estradiol supplementation in Ovx mice protects the elastic fibre system and induces cultured human dermal fibroblasts to selectively upregulate the synthesis of tropoelastin, fibrillin-1 and associated elastic fibre-associated proteins (including EMILINs and fibulins). These data suggest that the elastic fibre system may be a major target in estrogen-deprived tissues and that pharmacological intervention to modulate adipocyte phenotype may slow the acute effects of menopause and potentially the chronic effects of ageing in skin.

3.2 Introduction

As a consequence of increasing life expectancy the average woman now spends in excess of 30 years in a state of systemic estrogen deficiency. The onset of menopause and the subsequent loss of circulating sex steroid hormones, including estrogen, has profound effects on tissue homeostasis and function in disparate organs including the blood vessels, lungs, bone and skin (Tutino et al., 2015, Glassberg et al., 2014, Most et al., 1995, Tsukahara et al., 2004). In the skin, ageing and estrogen deprivation are associated with compositional and structural remodelling of the dermal extracellular matrix (ECM) and as a consequence, a loss of tissue resilience and increased tissue fragility (Escoffier et al., 1989, Thurstan et al., 2012, Verdier-Sevrain et al., 2006). These functional changes are thought to directly increase vulnerability of the elderly population to skin injuries, such as skin tears and pressure ulcers, which severely impact on individual wellbeing (Baranoski, 2000, Farage et al., 2009) .

As yet, the pathological mechanisms which drive this loss of mechanical fidelity are poorly defined. Of the three skin layers (the epidermis, dermis and hypodermis) it is the ECM-rich dermis which is usually considered the principle mediator of mechanical properties. The most abundant protein in the dermis is type I collagen, which forms a “basket-weave” of tensile strength-conferring fibril bundles (Osman et al., 2013, Graham et al., 2010). There is a strong correlation between: i) the loss and structural remodelling of dermal collagen fibrils and chronic estrogen deprivation as a consequence of either menopause (in humans) or ovariectomy (Ovx: in animal models) and ii) collagen deposition following estrogen supplementation (Brincat et al., 1987, Brincat et al., 1985, Sauerbronn et al., 2000). In order to further characterise the mechanistic links between estrogen deprivation and dermal matrix remodelling it has proven necessary to use Ovx as an acute model of estrogen deprivation, thereby avoiding the confounding factors associated with chronological ageing (El-Domyati et al., 2002, Naylor et al., 2011).

In rodents surgical Ovx has been shown to successfully model many aspects of human menopause including osteoporosis, neurodegeneration and cardiac dysfunction (He et al., 2011, Huang and Zhang, 2010, Cavasin et al., 2003). In ovine skin Ovx promotes structural remodelling of cutaneous collagen fibrils whilst in rodents surgically induced estrogen deprivation impairs skin recoil and accelerates many of the manifestations of UVR mediated photo-damage (Fang et al., 2012, Tsukahara et al., 2004, Tsukahara et al., 2001). Notably, in the latter study, Tsukahara and colleagues demonstrate that Ovx also induces a loss of elastic recoil in murine skin.

Compared with fibrillar collagens, elastic fibres occupy only a small fraction of dermal volume. Their composition varies with dermal depth, but the large diameter elastic fibres in the reticular

dermis are comprised of a cross-linked elastin core surrounded by an outer mantle of fibrillin microfibrils (Kielty et al., 2002) which in turn bind proteins including MAGP-1, and members of the emilin and fibulin families (Bressan et al., 1993, Gibson et al., 1989, Kielty et al., 2002). The link between circulating estrogen and elastic fibre remodelling remains poorly understood. However, it is becoming increasingly clear that fibrillin microfibrils play important roles in mediating, not only tissue mechanics, but also tissue homeostasis as a consequence of their ability to sequester extracellular cytokines (Ramirez and Rifkin, 2009). Further experimental evidence suggests that the amino acid composition of the fibrillins and other elastic fibre associated components, particularly their relatively high Cys, Trp and Tyr contents, predisposes them to oxidative damage arising from UVR exposure, smoking and/or raised BMI (Sherratt, 2009, Hibbert et al., 2015, Ezure and Amano, 2015, Just et al., 2007). Suggesting the elastic fibre proteins are likely to be highly affected by age associated alterations in their environment such as loss of circulating estrogen which acts as both a direct anti-oxidant and induces antioxidant enzymes (Baeza et al., 2010, Bottai et al., 2013).

Therefore in this study, we have tested the hypotheses that: i) estrogen mediates elastic fibre proteostasis and skin homeostasis in a murine model of menopause and ii) Human dermal fibroblasts are responsive to 17beta-estadiol treatment with respect to the elastic fibre proteins

3.3 Methods

Reagents, tissue samples and cultured cells

All reagents were obtained from Sigma-Aldrich Co. Ltd (Poole, UK) or BDH Ltd (Poole, UK), unless otherwise specified. Procedures involving mice accorded to the UK Animals (Scientific Procedures) Act 1986 under UK home office licence (40/3713). All mice used in this study were wild type C57/BL6 females (Envigo, UK) aged 7 weeks at the start of the experimental period. Primary dermal fibroblasts were derived from female abdominal human skin and maintained in phenol red-free DMEM supplemented with L-glutamine, 10% charcoal-stripped foetal calf serum and penicillin-streptomycin.

Ovariectomy and estrogen supplementation

Estrogen deprivation was induced in five mice (Ovx group) by bilateral ovariectomy as previously described (Emmerson et al., 2012). Briefly, the mice were anaesthetised using a mixture of oxygen, nitrous oxide and isofluorane. Following ventral laparotomy, ovaries were located via

exposure of the uterus and removed using sterile scissors. The body wall and skin were closed using sutures and buprenorphine (0.1mg/kg) administered as analgesia. Uterine atrophy was used to confirm estrogen deprivation at the time of tissue collection (Zhao et al., 2012) with uterine weight maintained by estrogen replacement (Ingberg., 2012) . The potential protective effects of exogenous estrogen in skin were characterised in a further five mice which underwent Ovx but were also treated with a slow-release subcutaneous 17beta-estradiol replacement pellet (0.1mg -60 day release) (Innovative research of America, Florida, USA) inserted at the base of the neck (Ovx+E group). A final group of five mice served as age-matched controls with intact ovaries (Intact group). Mice were collected 7 weeks post ovx.

Mechanical characterisation of murine skin

The effects of Ovx and 17beta-estradiol supplementation on the tensile strength and stiffness of mouse skin were determined by stretching 10mm wide x 30mm long strips of ventral skin from each of the experimental groups (Intact, Ovx and Ovx+E n=5) using an instron 3344 100N load cell (Instron, USA). The tissue was loaded to failure at a rate of 20mm/min. The effects of estrogen deprivation on skin viscoelasticity were characterised by testing the stress relaxation behaviour of ventral skin in a PBS bath using an Instron 5943 10N load cell (Instron, USA). Skin from Ovx and intact control groups (n=5) was cyclically loaded to 1N at a rate of 10mm/min 5 times to precondition the tissue then loaded to 1N and held at a constant strain for 160 seconds. All data was collected and analysed using Bluehill software (Instron, USA).

Histological characterisation of murine skin: collagen and elastic fibre abundance, organisation and depth of subcutaneous fat.

Six µm thick histological sections were prepared from formalin-fixed, paraffin-embedded dorsal skin from the Ovx, Ovx+E and intact (n=5) groups described above. For each stain an average measure was taken from 5 fields of view from one section per mouse to cover the full section. Elastic fibres were stained using Gomori's aldehyde fuchsin (Gomori, 1950), and visualised by bright-field optical microscopy (Nikon eclipse E600 microscope/SPOT camera). Total tissue collagen was assessed by staining with Masson's trichrome and visualised using a microscope. Masson’s trichrome is reported to stain amorphous collagen (Kim et al., 2012) but the most abundant dermal collagen, collagen I, only confers mechanical strength to tissues when assembled into fibrils. We have previously shown that staining with picosirius red (PSR) and illumination by crossed polarised light (Leica DMRB) selectively visualises organised fibrillar

collagen (McConnell et al., 2016). In this study, we stained mouse skin sections with PSR and visualised collagen birefringence using a Leica DMRB microscope. The proportion of tissue area occupied by elastic fibre, amorphous collagen and organised fibrillar collagen was semi-quantitatively assessed using ImageJ 1.46r software as previously described (Graham et al., 2011, McConnell et al., 2016). The ratio of thick and thin collagen fibrils was semi-quantitatively measured using the colour deconvolution plugin to split colour channels, a threshold applied and measured as percentage area (Cooper et al., 2015) (Image J, National Institutes of Health, USA). Finally the collagen orientation (also known as coherency) in the PSR/polarised light images was assessed as previously described using a well-established methodology (Rezakhaniha et al., 2012, McConnell et al., 2016)

***In situ* gelatinase activity in cryo preserved skin sections**

Most ECM-proteases also act as gelatinases (Chakraborti et al., 2003). In order to quantify and localise the effects of Ovx and Ovx+E on mouse skin we used *in situ* gelatin zymography (Akhtar et al., 2014). Cryo-preserved skin in OCT was sectioned to a nominal thickness of 10 µm thick (Leica CM3050 cryostat). DQ gelatin (Sigma,UK) solution (1mg/ml Dq gelatin in low gelling temperature agarose) containing DAPI (1µg/ml) was pipetted over the section and coverslipped. Sections were incubated for 18 hours at 4 ^{o}C and images captured using an Olympus BX51 microscope/ CoolsnapES camera/ Metavue software (Molecular devices). Fluorescence was quantified as previously described using image J 1.46r software (National Institutes of Health, USA) (Akhtar et al., 2014).

***In vivo* expression of murine elastic fibre genes**

RNA was extracted from snap frozen skin to assess the RNA expression levels of elastic fibre components elastin and fibrillin-1 following a standard protocol (Life Technologies UK). Briefly tissue was homogenised in Trizol reagent (Life Technologies) and extracted in chloroform then purified using the RNA purelink kit (Life Technologies UK). Subsequently cDNA was transcribed from RNA (Reverse Transcriptase kit, Promega, Madison USA) using Reverse Transcriptase (Roche, UK) and quantitative PCR was carried out on a Opticon quantitative PCR thermal cycler (Bio-Rad, UK). cDNA was diluted over 3 orders of magnitude and expression ratios normalised to housekeeping genes *Gapdh* (fwd-tgccactcagaagactgtgg rev-ggatgcagggatgatgttct) and *Ywhaz* (fwd- ttgagcagaagacggaaggt rev- gaagcattggggatcaagaa)

ECM gene expression by cultured human cells

Human dermal fibroblasts plated in 12 well plates at a seeding density of $0.17x10^{-6}$ cells per well were allowed to adhere overnight in a CO_2 incubator at 37oC. In n=3 wells 17beta-estradiol was added to the media to a final concentration of 10^{-7}M. Cells were harvested 6 or 24 hrs post-treatment by removal of the media and addition of TRIzol reagent (Life Technologies, UK). RNA was extracted using the RNA Purelink kit (Life Technologies, UK),cDNA synthesis and qPCR were carried out as described above for fibrillin-1 *FBN-1* (fwd-caggacaggcccatgttttac rev-gcacagcagagcgtttttgt) and housekeeping genes *GAPDH (fwd-* tgcaccaccaactgagc *Rev-*ggcatggactgtggtcatgag) *and YWAHZ* (fwd-acttttggtacattgtggcttcaa rev- ccgccaggacaaaccagtat)

Protein synthesis by cultured human fibroblasts: mass spectrometry and Western blotting

Dermal fibroblasts were plated into 2 x 6 well plates at a seeding density of 50 % confluence and allowed to adhere overnight. Fresh media was added on alternate days and half of the samples (6 wells) were supplemented with 17beta-estradiol into the media at a final concentration of 1µM. Cell matrix was extracted after 8 days culture using 2M NaCL 25mm ammonium bicarbonate and 25mM dithiothretol (DTT). The extracts were digested overnight using immobilized-trypsin beads (Perfinity Biosciences) in 1mM calcium chloride and 25mM ammonium bicarbonate. Further reduction and subsequent alkylation was carried out using 10mM DTT followed by 30mM iodoacetamide. The samples were acidified with 0.4% trifluoracetic acid. Samples were cleaned by biphasic extraction by vortexing centrifugation with ethyl acetate. Samples were subsequently dried down in a speed-vac. Peptide desalting was carried out using PORPS R3 beads (Thermo fisher scientific) washed in trifluroacetic acid and eluted in acetonitrile. Samples were dried down in speed vac and resuspended in acetonitrile trifluoroacetic acid in water and stored at 4 ^{o}C prior to analysis by mass spectrometry.

The digested samples were analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Peptide mixtures were separated using a gradient from 92% A (0.1% FA in water) and 8% B (0.1% FA in acetonitrile) to 33% B, in 104 min at 300 nL min^{-1}, using a 75 mm x 250 µm i.d. 1.7 µM CSH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependant analysis. Data produced were searched using Mascot (Matrix Science UK), against the Uniprot database with taxonomy of human selected. Data were validated using Scaffold (Proteome Software, Portland, OR).

The influence of estrogen on tropoelastin synthesis by human dermal fibroblasts was also characterised immunologically by Western blotting. HDFs (n=3) were treated with fresh 17beta-estradiol supplmented media (1uM) on day 1, 3, 7 and 10. The media of control cells was also changed at the same time points. After 14 days, media was collected and added to lamelli sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol (Bio-Rad). Media samples were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk 0.1% tween (Sigma, UK) prior to incubation with primary antibodies to elastin or β actin and detection with peroxidase labelled secondary antibodies (GE Healthcare,UK) and ECL plus (GE Healthcare,UK). Primary antibodies used were against Elastin (Santa-Cruz, raised in mouse at 1:500, dilution) and β-actin (Sigma, raised in mouse at 1:5000 dilution).). The pixel intensity from scanned images was measured using image J software (Image J, National Institutes of Health, USA) to give an estimate of the quantity of the protein present.

3.4 Results

Estrogen modulates skin tensile strength, stiffness and viscoelasticity

As both menopause and Ovx are associated with profound structural and mechanical remodelling of skin (Sumino et al., 2004, Bolognia et al., 1989, Fang et al., 2012, Tsukahara et al., 2004) we first assessed the effects of acute estrogen deprivation, and the protective effects of estrogen in the form of 17beta-estradiol supplementation, on the tensile strength and stiffness of skin. Strips of ventral skin were loaded to failure at a uniform extension rate (Figure 3.1A). Breaking stress (and therefore tensile strength) was significantly lower in Ovx mouse skin compared to control skin (41.5%). 17beta-estradiol replacement (Ovx+E) fully restored breaking stress to pre-Ovx levels (43.9% vs Ovx) (Figure 3.1B). Skin stiffness (measured as Young's modulus, which quantifies resistance to deformation) can be calculated from the linear portion of the stress/strain curve where the tissue behaves in a Hookean manner (Figure 3.1 A). Young's modulus was significantly reduced in Ovx mouse skin, compared with control (44.5%) and again restored in Ovx+E (39%) tissues (Figure 3.1C). Collectively, these data demonstrate that estrogen has major effects on both the tensile strength and stiffness of skin over an acute time period.

Biological tissues behave in a viscoelastic manner displaying complex mechanical properties. To provide additional insight into these characteristics we next assessed the stress relaxation properties of ventral skin from ovariectomised (Ovx) versus intact (Int) mice. Skin strips were preconditioned by cyclically pre-loading to 1N for 5 cycles in order to negate the Mullins effect (Munoz et al., 2008) (Figure 3.1D). Stress relaxation analysis revealed that skin from

ovariectomised mice relaxed (that is lost tension) more quickly than corresponding control (Int) skin (Figure 3.1E), quantified as a significant alteration in the non-liner regression one phase decay time constant tau (Figure 3.1 F) p< 0.05 n=6. Collectively, these data indicate acute estrogen deprivation has a major impact on the mechanical properties of skin leaving skin weaker and more easily deformed.

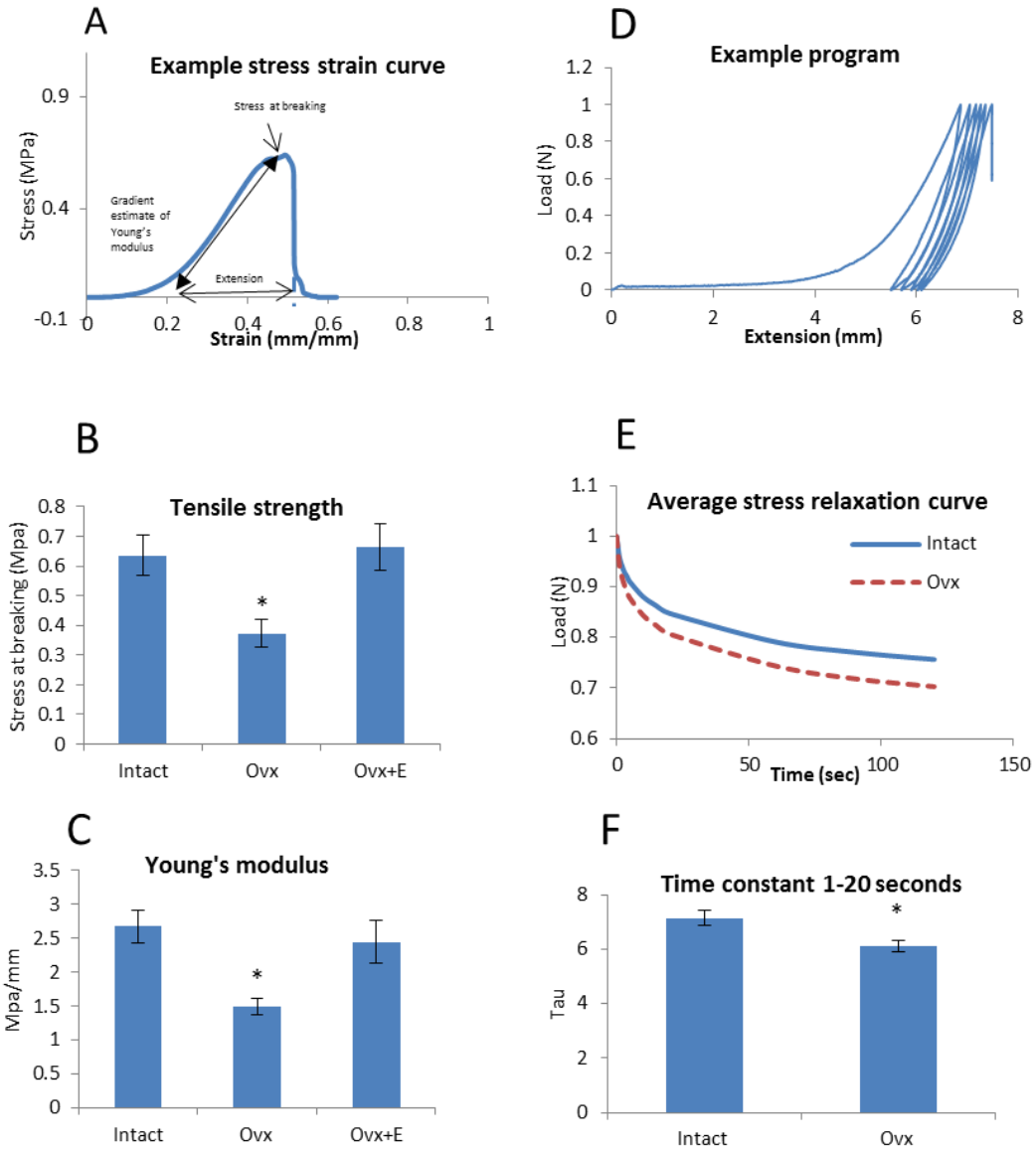

Figure 3.1 The mechanical properties of skin. (A) The tensile strength of the tissue is characterised by the strain (extension) at breaking, whilst the stiffness (Young's modulus) of a tissue can be the estimated from the linear section of the curve. (B) The tensile strength of skin was significantly reduced in estrogen deprived mice (Ovx). Estrogen supplementation following surgery (Ovx+E) was protective. (C) Similarly, skin stiffness was significantly reduced in Ovx animals compared with intact controls and this mechanical remodelling was absent in Ovx+E mice. (D) Strips of ventral skin 1cm wide were cyclically loaded to 1N 5 times then held at the final strain at 1N and allowed to relax The curves become increasingly repeatable the biggest changes occur in the 1st 2 cycles we allowed 5 preloading cycles to reduce this variation. (E)The Ovariectomised skin relaxes quicker than the intact control meaning it loses tension faster and is therefore less resistant to constant force (F) Calculation of the time constant (Tau) from the non-linear regression one phase decay showed a significant difference in the curves . Data shown Mean +/-SEM N=4-5 *p<0.05

Dermal collagen abundance, organisation and orientation are insensitive to acute estrogen deprivation

As chronic estrogen deprivation has been clearly linked to changes in both the mechanical behaviour of skin and collagen fibril ultrastructure and abundance (Brincat et al., 1987, Brincat et al., 1985, Sauerbronn et al., 2000) we next used histological approaches to quantify the area fractions of amorphous collagen and organised fibrillar collagen across experimental groups. Intriguingly, the mechanical remodelling observed following Ovx was not correlated with total collagen content as measured using Massons trichrome. This staining showed no alterations in collagen abundance between any of the groups (Figure 3.2A). However, this histological stain binds to collagen triple helices and hence is insensitive to collagen type or the organisation of fibrillar collagen monomers into mechanically robust fibrils and fibril bundles (Lapiere et al., 1977, Graham et al., 2010). A complimentary technique, PSR-enhanced collagen birefringence, can specifically detect collagen organised into aligned fibril bundles which in turn have been associated with local mechanical stiffening (McConnell et al., 2016). Estrogen deprivation in this study also had no effect on the abundance (Figure 3.2B) or orientation (Figure 3.2C) of organised collagen as measured by PSR-ECB. Thus acute (7 weeks) estrogen deprivation has no obvious effect on the collagen fibrils and they are therefore not directly correlated in abundance to tensile strength.

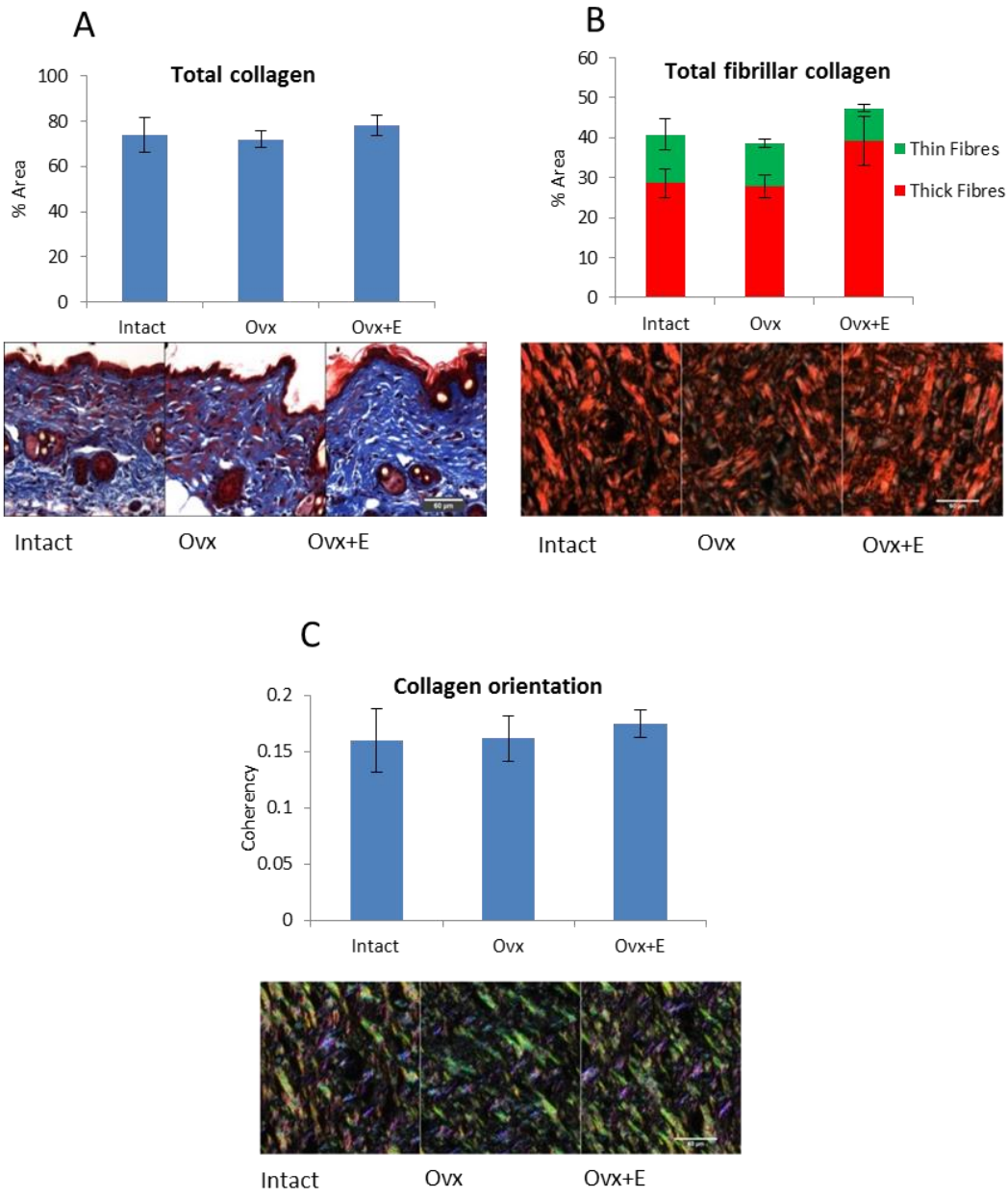

Figure 3.2 Dermal collagen. (A) Total collagen, as visualised by Massons trichrome stain, was unaffected by Ovx or Ovx+E treatment. (B) PSR-enhanced collagen birefringence under polarised light was also estrogen insensitive. (C) Using collagen birefringence it is possible to quantify fibre orientation (coherency). Regions of aligned fibres would appear as similar colours. There was no significant differences in alignment between control, Ovx and Ovx+E mouse skin. Data shown Mean +/-SEM N=5 Scale bar =60µm

Estrogen deficiency profoundly affects both elastic fibre abundance and fibrillin-1 expression in vivo

As the mechanical testing data clearly demonstrated a relationship between circulating estrogen and skin mechanical properties we next characterised the abundance of elastic fibres in mouse skin. Estrogen-mediated changes in the dermal elastic fibre network were characterised using the elastic fibre stain Gamori's aldehyde fuchsin (Figure 3.3A). Ovx induced a significant (34%) reduction in tissue area occupied by elastic fibres compared with intact controls. 17beta-estradiol replacement fully protected against this loss of dermal elastic fibres (Figure 3.3A)**.** The expression of the main micro fibrillar component of elastic fibres, fibrillin-1, was significantly reduced in the skin of Ovx (but not Ovx+E) mice (Figure 3.3B). By contrast expression of the major elastic fibre protein, elastin did not differ between experimental groups (Figure 3.3C). Thus far murine models have provided key insights into the effects of estrogen on skin biology, however it is important to look for cross-species conservation. Therefore we next characterised the effect of estrogen on the synthesis of elastic fibres and other ECM proteins by cultured human dermal fibroblasts.

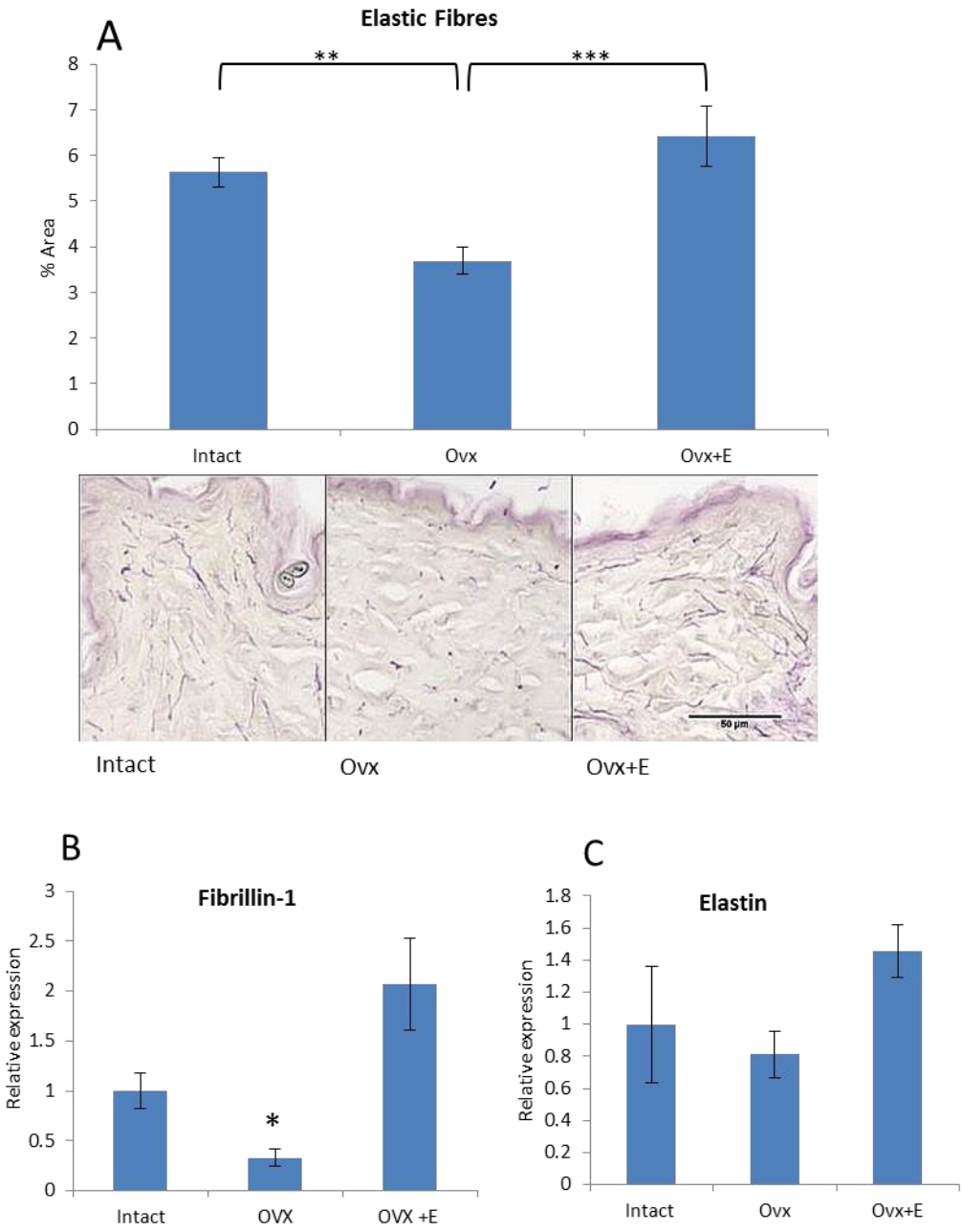

Figure 3.3 Elastic fibre abundance and expression in vivo. (A) Gamori's aldehyde fuchsin staining for tissue elastic fibres elastic fibres. (Elastic fibre appear dark purple). Compared with fibrillar collagens, elastic fibres occupy a much smaller tissue volume (5-6%) in intact control skin. The % area of tissue occupied by elastic fibres was significantly lower in Ovx, not significantly different in Ovx+E animals. (B) Relative expression of fibrillin-1 and elastin in murine skin. Ovx significantly decreased fibrillin-1 gene expression. Ovx mice continued to express higher levels of FBN-1 mRNA. (C) There was no significant effect of either Ovx or Ovx+E on tropoelastin expression. Data shown Mean +/-SEM A N=10 B N=4 **P<0.01 ***P<0.001 * P<0.05 scale bar = 50µm

Estrogen mediates the *in vitro* expression and synthesis of elastic fibre associated proteins by human dermal fibroblasts

To test cell-intrinsic effects of estrogen we moved to cultured primary human fibroblasts. Here treatment with 17beta-estradiol significantly upregulated expression of the key elastic fibre component fibrillin-1 within just 24 hours (Figure 3.4A). To investigate global changes in cell derived matrix proteins as a direct consequence of estrogen treatment we carried out mass spectrometry analysis of cell culture-derived matrix. This analysis reveals 14 matrix proteins were significantly altered by estrogen treatment highlighting the important role of estrogen in the dermal ECM. Four of these proteins (29%) were directly associated with elastic fibre deposition (EMILIN1, EMILIN2, FBN1 and FBLN2) (Figure 3.4B). One key elastic fibre component missing from this list is elastin. However, as elastin is highly crosslinked and extremely difficult to extract for mass spectrometry. We thus used an alternative technique, western blot, to detect the soluble precursor to mature elastin, tropoelastin (Figure 3.4C). Quantification of pixel intensity revealed a strong trend towards increased tropoelastin production following 17beta-estradiol treatment, however this failed to reach statistical significance (Figure 3.4D).

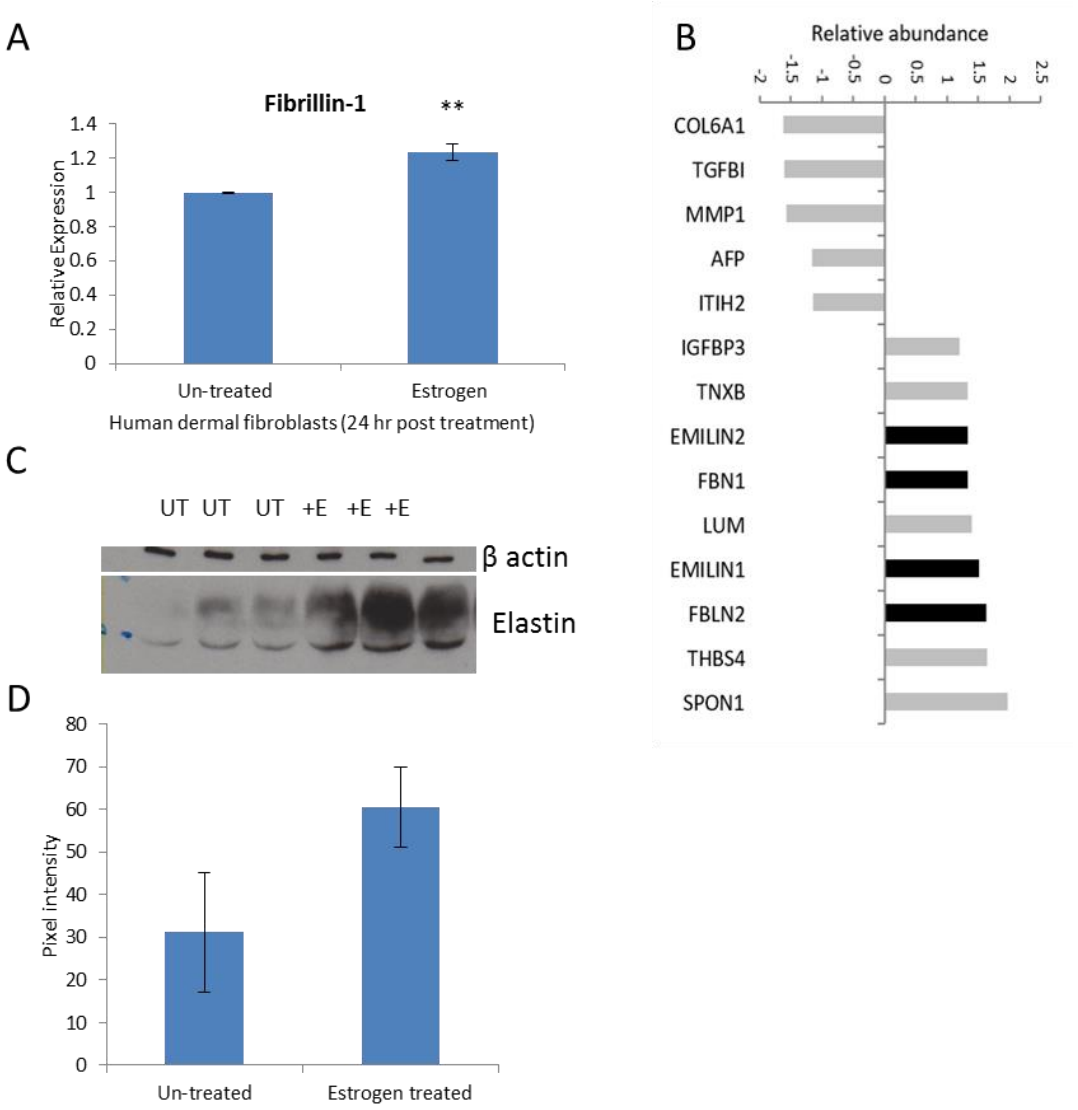

Figure 3.4 - Estrogen treatment induces key elastic fibre proteins in human cell culture

(A) qPCR analysis shows upregulation of fibrillin-1 by estrogen treatment in human dermal fibroblasts (B) Mass spec analysis of cell culture derived matrix revealed that 28% (4 of 14) of all the proteins we identified as significantly altered by estrogen treatment in our samples are proteins directly linked to elastic fibre synthesis shown here as by black bars
(C-D)Tropoelastin (the soluble precursor of mature elastin) is upregulated by estrogen treatment and detected in the cell culture media by western blot UT is untreated cells +E is estrogen treatment at 10^{-7} M (C) and quantified as pixel intensity(D) Data shown mean +/- SEM N=3 ** P<0.01

Loss of elastic fibres correlates with increased dermal gelatinase acitivity and adipocyte hypertrophy

Within tissues, ECM proteins are primarily degraded by either matrix metalloproteinases (MMPs) or cathepsins (Lu et al., 2011). Specifically, elastin and/or fibrillin act as substrates for MMP-2, -3, -7-, -9, -10, -12, -13 and -14 and cathepsin G (Schmelzer et al., 2012, Chakraborti et al., 2003). The activity of both enzyme families is commonly assessed by gelatin zymography. Here we used an *in situ* gelatin zymography approach to both localise and quantify relative gelatinase acitivty in cryo-preserved murine skin sections (Mook et al., 2003). Digital quantification reveals that dermal gelatinase acitivity was raised three weeks post-Ovx (Figure 3.5A) however by the seven week timepoint this difference was lost (Data not shown) with 17beta-estradiol supplementation fully protecting against this increase in proteolytic activity (Figure 3.5A). Increased gelatinase activity in estrogen deprived dermis could be driven by dermal fibroblasts (Philips and Devaney, 2003) epidermal macrophage (Campbell et al., 2010) or hypodermal adipocytes (Ezure and Amano, 2015). Given the known association between increased subcutaneous fat, adipokine-mediated local protease activity and elastic fibre remodelling we characterised the effects of Ovx and Ovx+E on hypodermal remodelling. Ovx induced a significant 237% increase in the depth of the hypodermis (Figure 3.5B) and increased adipocyte size (Figure 3.5C) which was inhibited by 17beta-estradiol supplementation. This considerable increase in adipose tissue could be a driver of the observed elastic fibre degradation in the Ovx group.

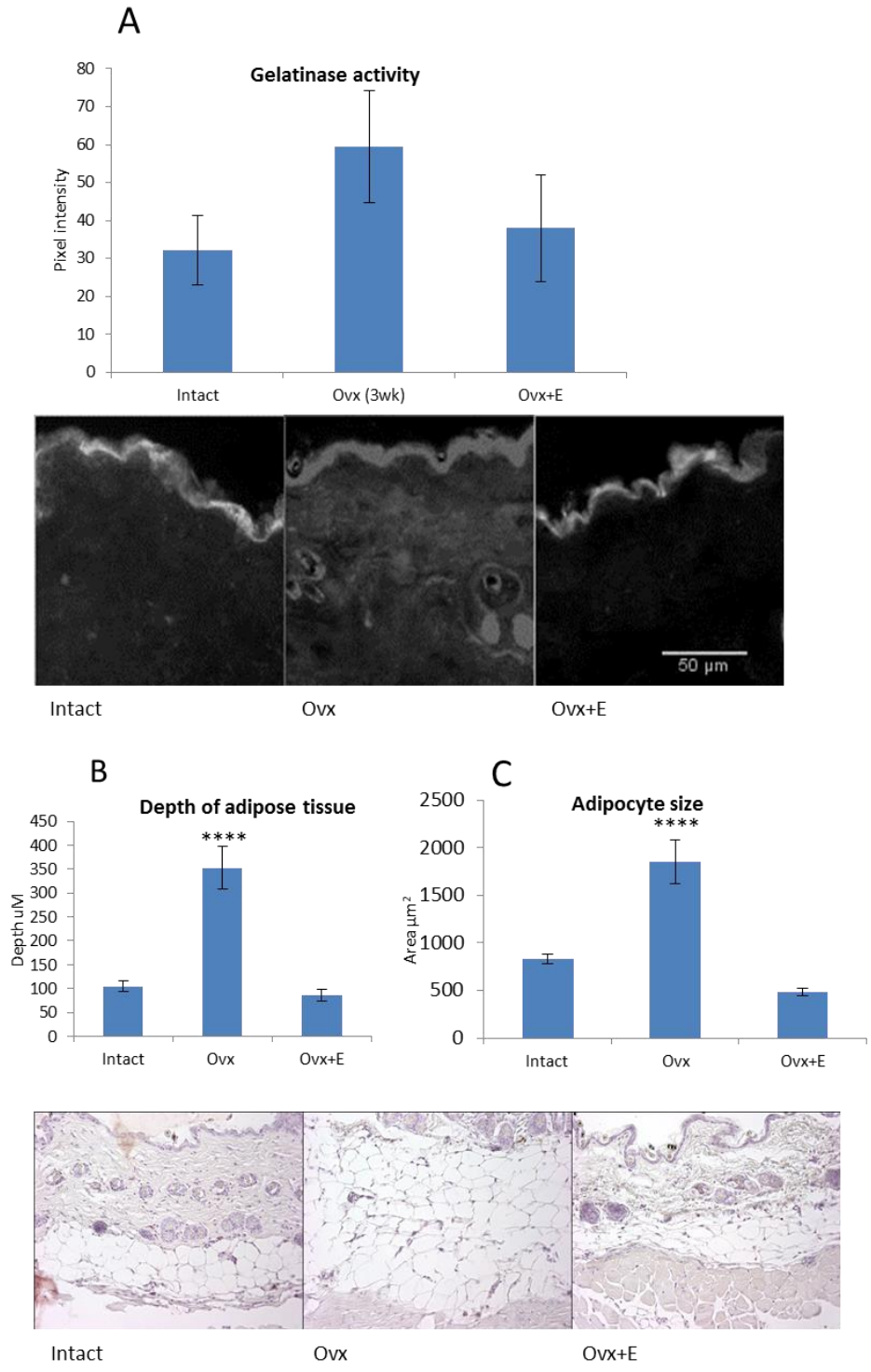

Figure 3.5 Ovariectomy significantly increases adipose tissue and collagenase activity.

(A) Following 3 weeks of ovariectomy we see an increase in collagenase activity in the dermis by in situ zymography Here breakdown of fluorescein-labelled gelatin by photolytic digestion leads to bight florescence seen here as increase in brightness. (B) Seven weeks of ovariectomy leads to a 237% increase in the depth of adipose tissue and an increase in the size of the individual adipocytes (C) Data mean +/- SEM N= 5 ****P<0.0001

3.5 Discussion

This study set out to explore the relationship between estrogen-mediated alterations in skin structure and changes in skin mechanical properties. Our data reveal that estrogen deprivation profoundly alters the mechanical properties of murine skin over an acute, seven week time period. In the same period we see a significant loss of elastic fibres yet little observable effect on fibrillar collagen. This rapid elastic fibre remodelling in estrogen-deprived tissue correlates with substantial adipocyte hypertrophy which likely drives an observed increase in gelatinase activity. In line with a direct effect of estrogen deprivation we find that 17beta-estradiol supplementation is able to induce key elastic fibre components in both murine tissue and human dermal fibroblasts.

The first major finding from our study is that the mechanical properties of murine skin dramatically alter following Ovx. Indeed, estrogen deficiency led to substantially reduced tensile strength and a higher susceptibility to deformation under load, measured as a decreased Young's modulus. That exogenous 17beta-estradiol replacement completely protected against these changes, confirms estrogen's important role in maintaining skin strength and elastic properties This study builds upon non-quantitative data from Tsukahara *et al* suggesting a loss of elastic recoil in Ovx mice by an observed prolonged skin tenting after pinching (Tsukahara et al., 2004). A longer term, 6 month, study in rats also reported an Ovx induced loss of tensile strength and corresponding loss of collagen (Polito et al., 2012). In the human setting Pierard *et al* report increased skin extensibility in the peri-menopausal period (Pierard et al., 1995) which corresponds to our reported reduced Young's modulus on Ovx mice. There is surprisingly little data available on the mechanics of post-menopausal skin, however studies of aged human skin are conflicting, reporting the Young's modulus to increase (Agache et al., 1980), decrease (Boyer et al., 2009) or remain unchanged (Pierard and Lapiere, 1977, Leveque et al., 1980). Here variations in methodology make comparison and interpretation of the literature difficult.

A general, non-skin specific, consensus is that age leads to tissue stiffening (higher young's modulus) (Sherratt, 2013) which is opposite to our findings in the Ovx model. The skin, like many biological tissues, is structurally complex displaying material properties that are viscoelastic with a nonlinear response (Fung, 1993). Viscoelastic materials combine the laws of both elastic materials which respond in a liner fashion, loosing little energy when deformed, and viscous materials which respond in a time-dependent manner to strain, with energy loss, and plastic deformation (Silver et al., 2001). Here we have employed stress relaxation as a simple measure to explore these time dependent aspects of mechanical behaviour. We find that ovariectomy alters the stress relaxation response, accelerating the rate at which skin relaxes versus the intact

control mice. These complex mechanical properties suggest the tissue is less able to withstand constant force and would therefore appear more lax.

Taken together, these mechanical findings suggest that estrogen deficiency leads to an acute remodelling of cutaneous ECM, however, in humans other age-related factors or compensatory mechanisms (e.g. direct protein-crosslinking, or accumulation of AGE) may play a dominant role in determining mechanical properties. The Ovx mouse phenotype does appear to model some aspects of aged human skin which becomes weaker and more lax (Calleja-Agius et al., 2007) suggesting that age associated estrogen deficiency, may be a key contributor of age associated alterations in mechanical function of skin. However, in addition to estrogen deficiency aged human skin experiences a lifetime of UV exposure. UV dramatically alters the mechanical properties leading to stiffened tissues and disordered elastic fibre proteins (Alpermann and Vogel, 1978, Watson et al., 1999). Importantly, here skin becomes an accessible model tissue to explore estrogen related changes that are almost certainly common to other tissues in the body without the compounding UV associated contribution which is unique to human skin (Castelo-Branco et al., 1994, Castanedo-Cazares and Rodriguez-Leyva, 2015). Indeed, parallel changes in the mechanical properties of tissues such as the cardiovascular and respiratory systems will have very serious health consequences (Janssens et al., 1999, Lakatta and Levy, 2003, Graham et al., 2011).

At the molecular level a number of human studies have attributed age-associated changes in the appearance of skin, at least in part to a loss of fibrillar collagen (Calleja-Agius et al., 2007, El-Domyati et al., 2002, Verdier-Sevrain et al., 2006), with inferred effects on tensile strength (Oxlund, 1986). We therefore predicted altered abundance and/or orientation of dermal collagen fibrils in our Ovx mouse model. In fact, collagen remained unchanged after 7 weeks of Ovx, and we instead observed a major reduction in the dermal elastic fibre network, accompanied by reduced gene expression of the key elastic fibre component *fibrillin-1*. The rapidity of this loss was particularly surprising, challenging the long standing view that elastic fibres are subject to very little turnover (Ashcroft et al., 1997, Sherratt, 2009, Davidson et al., 1986). We note that in an ovine model, ovariectomised for a much longer period of 2 years, alterations in dermal collagen have been reported (Fang et al., 2012). However, our data now suggest that elastic fibres are highly susceptible to rapid remodelling. Indeed, elastic fibre alterations are prime candidates for mediating the major changes in skin mechanical properties observed in our model. Extrapolation to human would suggest that elastic fibres will be particularly important in early skin changes following menopause.

The mechanism(s) by which elastic fibres are remodelled in acute estrogen deprivation remain to be elucidated. There are potential anabolic or catabolic mechanisms, and the process is likely to be multifactorial with a balance between degradation and synthesis. A recent study by Ezure *et al* draws a clear link between BMI and elastic fibre degradation, with dermal elastic fibres decreased in females with high BMI (Ezure and Amano, 2015). To test this association as a potential mechanism for estrogen effects we quantified sub-cutaneous fat in our Ovx mice. Interestingly, adiposity was notably increased in Ovx skin. This would suggest adipocyte mediated gelatinase production as a likely contributing factor in the Ovx elastic fibre degradation. To test this further we measured local gelatinase levels in tissue from an earlier time point (3 weeks post Ovx). We observed strongly induced gelatinase activity, which with a wide range of protein targets (Chakraborti et al., 2003) implies a more catabolic environment early following estrogen deprivation. An alternative mechanism at play may be oxidative damage, as hypertrophic adipocytes have been positively correlated with increased oxidative stress (Matsumoto et al., 2014). Watson *et al.* (2014) suggest that the unusually high level of cysteine and methionine residues in fibrillin-1 microfibrils increase susceptibility to oxidative damage (Watson et al., 2014). Further, Hibbert *et al.* (2015) propose a sacrificial role to protect other oxidative damage-sensitive proteins (Hibbert et al., 2015). Estrogen is itself known to have anti-oxidative properties both directly and via induction of antioxidant enzymes (Emmerson and Hardman, 2012), so it is perhaps unsurprising that we see such improvements in the presence of estrogen in our study and this may indeed be a newly identified role for estrogen as an antioxidant. Another key consideration is the natural hair cycling which also has dramatic effects on the dermal adipose. The growth phase, known as anagen, leads to a large increase in both number and size of the adipocytes in the skin (Rivera-Gonzalez et al., 2014). For this study the influence of hair cycling was limited as much as practical by ensuring the mice were in the same hair cycle stage at the beginning of the experimental period and excluding data from animals in anagen at the time of collection. Additionally however the problem is exacerbated by the link between hair cycle and estrogen signalling, with ovariectomy known to induce anagen (Chanda, 2000) it is therefore likely these animals may have entered an additional round of hair cycling with the associated increase in adipose tissue which may have further exacerbated adipose linked elastic fibre degradation.

Our *in vivo* data are also consistent with estrogen directly inducing synthesis of new elastic fibres. This scenario clearly challenges the current dogma that elastic fibres are only laid down during development (Reviewed in Sherratt, 2009). To test this observation we used mass spectrometry to quantitatively analyse the *in vitro* deposited matrix from isolated human dermal fibroblasts directly treated with 17beta-estradiol. We found numerous ECM proteins

were directly induced or repressed following, estrogen treatment, with four identified proteins directly linked to elastic fibre deposition. Fibrillin-1 provides the initial scaffold onto which the elastin localises as the elastic fibre builds (Kielty et al., 2002). The protein, EMILIN-1, direclty localises to the microfibril-elastin interface and has been suggested to play important roles in the regulating the deposition of tropoelastin onto the the microfibril scaffold (Bressan et al., 1993). Also induced is EMILIN-2 a less well-characterised family member which shares 70% similarity to EMILIN-1 (Doliana et al., 2001). Intriguingly, EMILIN-1 and EMILIN-2 can interact in a head to tail arrangement to form larger assemblies (Bot et al., 2015). Thus, co-ordinate induction of both family members by estrogen may be important in the context of elastic fibre assembly. Fibulin-2 is also found at the elastin microfibril interface and in skin is found to co-localise with fibrillin-1 (Reinhardt et al., 1996) suggesting this is also an important protein in the synthesis of mature elastic fibres. Up-regulation of these key proteins involved in the assembly of elastic fibres provides strong evidence that 17beta-estradiol treatment could lead to the formation of new elastic fibres.

Since high levels of crosslinking preclude extraction of the core elastic fibre protein elastin for MS analysis, we instead measured production of the soluble precursor tropoelastin in cell culture media by western blot. Tropoelastin was also strongly upregulated in human dermal fibroblast following 17beta-estradiol treatment, thus further confirming the role of estrogen in elastic fibre remodelling. Taken together, our data strongly suggest that estrogen acts as a potent inducer of cutaneous elastic fibre synthesis in both mouse and human. These data are supported by other human studies reporting increased mRNA for both fibrillin-1 and tropoelastin (Son et al., 2005) or an increase in total elastic fibres (Punnonen et al., 1987) following topical estrogen treatment.

In addition to their role in skin, elastic fibres are critical for normal function of many tissues including both the respiratory and cardiovascular systems. In these tissues rapid loss of elastic fibres following menopause would contribute to serious health issues in post-menopausal women. It is therefore likely that compensatory mechanisms may be at play in postmenopausal women. It is understood that ERs can signal in a ligand independent manner via growth factors such as IGF (Heldring et al., 2007, Emmerson et al., 2012, Aronica and Katzenellenbogen, 1993). This ligand independent signalling may become more prevalent in the postmenopausal period as estrogen itself declines. Indeed, a more detailed understanding of estrogen's dramatic effects on the elastic fibre system is essential to develop new pharmacological interventions based on the manipulation of ER signalling. The therapeutic use of estrogen has been controversial, since the Million Women and Women's Health Initiative studies drew links between HRT and certain cancers over 10 years ago (Banks et al., 2003, Beral et al., 2007), However, the skin offers a

unique opportunity for topical estrogen treatment, avoiding potential systemic side effects. Studies are now needed to determine the relative role of the estrogen receptors, ERα and ERβ. Indeed, in the context of cutaneous wound repair we have previously demonstrated the therapeutic benefit of ERβ selective activation (Hardman et al., 2008, Campbell et al., 2010, Emmerson et al., 2009). Further studies investigating the effect of selective estrogen signalling on tissue structure and mechanical properties should in the future allow a more targeted approach to treat a range of diseases caused by menopause-associated tissue dysfunction.

Overall we have demonstrated acute estrogen deprivation leads to severe alterations to the mechanical properties of the skin leaving it both weaker and more lax. There is a dramatic reduction in the volume of elastic fibres correlated with significant subcutaneous adipose hypertrophy and increased gelatinase activity. Additionally we have demonstrated human dermal fibroblasts to be highly responsive to estrogen treatment up-regulating a number of ECM proteins especially those related to elastic fibre formation. Summarised in figure 6

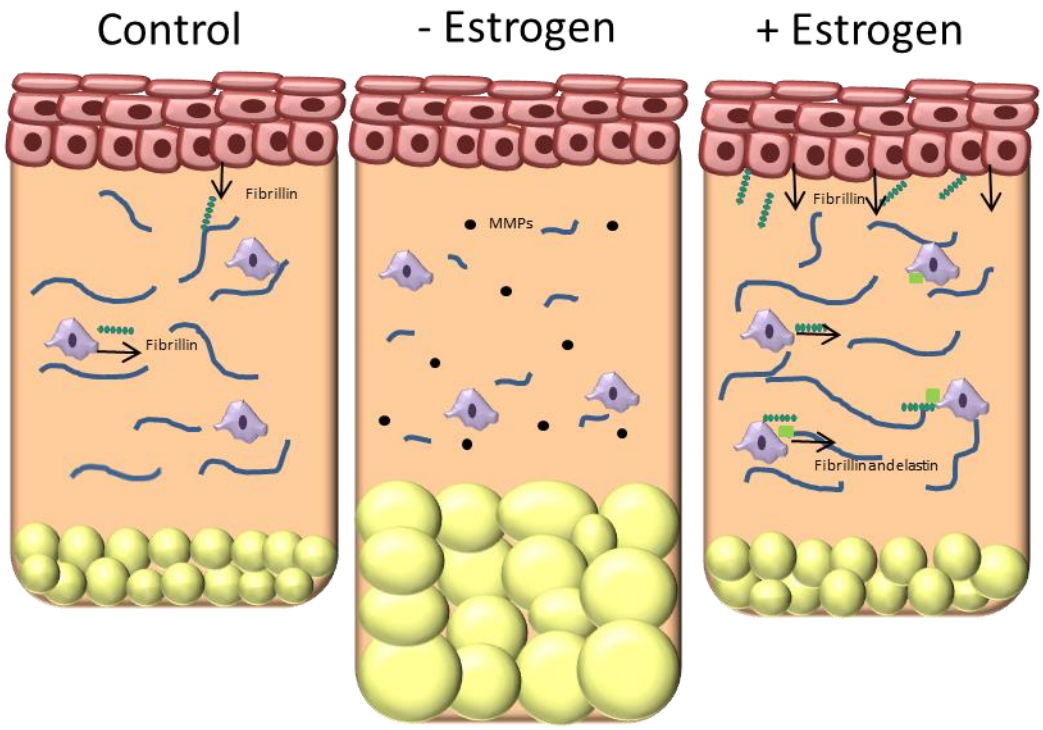

Figure 3.6 Schematic representation of skin following estrogen deprivation and estrogen replacement. Estrogen deprivation leads to an increase in subcutanous adipose tissue and MMP activity causing degradation of elastic fibres along with suppression of normal fibrillin expression. Estrogen treatment leads to upregulation of expression of fibrillin and elastin in fibroblasts.

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Chapter 4
Results

"A combined mechanical and proteomic study of estrogen-deficiency as a model of skin ageing"

Contributions: CS was involved in experimental design and carried out experiments analysed results and prepared manuscript JS carried out mass spectrometry DH was involved in methodology for mechanical testing BD was involved in mechanical testing experimental design EE involved in manuscript preparation MH and MS were involved in experimental design and manuscript preparation.

Prepared for submission to: Disease models and mechanisms

4 A combined mechanical and proteomic study of estrogen-deficiency as a model of skin ageing

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4.1 Abstract

Age related changes in tissue mechanical properties can have serious health consequences leading to a loss of function. Crucial for this study is the observation that the dramatic reduction in estrogen at time of menopause leads to a rapid increase in the signs of skin ageing. Using the ovariectomised mouse and chronologically aged mouse we have studied both the mechanical properties and proteome of the skin to assess if estrogen deprivation is a key driver of ageing.

We find that age and estrogen deprivation affects skin mechanical properties in contrasting ways, with age leading to tissue with increasing tensile strength and stiffness and estrogen deprivation causing a reduction in tensile strength and stiffness. However these two states have similar effects on dermal proteome, showing a trend towards a general loss of proteins with the exception of fibrillar collagen. Additionally we find that estrogen deprivation has more dramatic effects on the proteome than ageing, leading to statistically significant alterations in a number of proteins, particularly elastic fibre related proteins. Intriguingly these results suggest that the proteomic composition of the dermis does not directly affect the mechanical function.

4.2 Introduction

As the average age of the population increases it is becoming increasingly important to understand how ageing affects tissue structure. In general, ageing is associated with tissue atrophy (Fenske and Lober, 1986), chronic low level inflammation (Franceschi and Campisi, 2014) and loss of normal function. In humans, intrinsically aged (i.e. UV radiation-protected) skin is characterised by flattening of the dermal epidermal junction, thinning of the epidermis and loss of ECM proteins from the dermis. This dermal remodelling may be due to: i) an imbalance of normal homeostatic processes (which in turn may be caused by loss of dermal fibroblasts (Fenske and Lober, 1986) and/or the promotion of a catabolic phenotype (Varani et al., 2000)) or ii) the accumulation of damage by structural ECM proteins (Sherratt, 2009). In contrast to intracellular proteins which are rapidly recycled, and protected by antioxidant and damage detection mechanisms, many ECM proteins must function for decades in proteolytic environments (Shapiro et al., 1991, Sherratt, 2009).

Remodelling of the dermal ECM is thought to drive alterations in the mechanical properties of skin (Daly and Odland, 1979), including increased stiffness and reduced resilience and tensile strength. As a consequence aged skin is more fragile and susceptible to wounding, which in addition to other compounding age associated factors leads to a much higher incidence of chronic wounds in the elderly (Sgonc and Gruber, 2013, Sen et al., 2009) incurring a healthcare burden of up to £5.1bn annually in the United Kingdom (Guest et al., 2015). In addition to the need to understand and prevent skin ageing this organ may also serve as a model for age-related remodelling of other human ECM rich tissues such as the arteries and lungs (Sherratt, 2009, Akhtar et al., 2011).

The causative mechanisms of dermal remodelling in skin include accumulation of damage to both DNA and proteins via exposure to external factors, such as ultraviolet radiation (UVR) which in turn can generate reactive oxygen species (ROS) (Scharffetter-Kochanek et al., 1997). Although many anatomical skin sites are, in general, UV protected, these regions will be exposed to endogenous factors such as ROS generated through aerobic metabolism (Cooke et al., 2003) and to the dysregulation of cell-mediated proteolysis (via the expression of matrix metalloproteinases (MMPs) (Haorah et al., 2007, Pimienta and Pascual, 2007) and their inhibitors (TIMPS)(West et al., 1989)). These latter pathways may be mediated by the accumulation of senescent cells which exhibit a senescence associated secretory phenotype (SASP) (Campisi, 2012). Crucially, in the period immediately following menopause women report a rapid onset of the signs of skin ageing, a phenomenon which can be prevented with hormone replacement therapy (Brincat, 2000).

Estrogen deprivation may contribute to the visible signs of skin ageing as a consequence of its innate antioxidant properties (Baeza et al., 2010) or due to its ability to mediate the expression of endogenous antioxidants (Borras et al., 2005). There is compelling evidence which demonstrates that estrogen can protect both keratinocytes and fibroblasts from oxidative damage (Bottai et al., 2013). Estrogen can also influence protease activity, by inhibition of MMP-1, -2, -8, -9 and -13 expression (Son et al., 2005, Pirila et al., 2002, Pirila et al., 2001) and promotion of TIMP expression (Chen et al., 2003). Finally, estrogen can influence cell senescence (Olovnikov, 1996). Once thought to be quiescent it is now known that senescent cells secrete a wide variety of cytokines, chemokines and proteases, which in turn affect the surrounding tissue (Campisi, 2012). Estrogen can prevent cellular senescence in a dose dependent manner and reduce telomere shortening via increase in telomerase in endothelial progenitor cells (Imanishi et al., 2005).

Given the experimental evidence which suggests that acute estrogen deprivation induces compositional, structural and mechanical remodelling in skin, in this study we used gross mechanical testing and proteomic analyses to test the hypothesis that acute ovariectomy (and hence estrogen deprivation) can model the compositional, structural and mechanical consequences of skin ageing in young mice.

4.3 Materials and Methods

Aged and ovariectomised mice

All animal experiments were performed under UK home office licence (40/3713) following local ethics committee approval. All experimental mice were wild-type C57/Bl6 females. Otherwise healthy aged mice were sourced at 12, 20 and 24 months of age from Charles River laboratories.

Bilateral ovariectomy (Ovx) was carried out on mice aged 7 weeks as previously described (Emmerson et al., 2012). Briefly, the mice were anaesthetised using a mixture of oxygen, nitrous oxide and isofluorane. A small incision in the skin was made in the lower ventral side of the mouse, followed by an incision in the body wall. The ovaries were located by following the length of the uterus and removed using sharp sterile scissors while gripping the fallopian tubes with forceps. The body wall and skin were closed using sutures and buprenorphine (0.1mg/kg) administered as analgesic. All mice had age matched intact controls (n=5-6 per group for all groups).

Macro-mechanical testing of murine skin

Tensile testing was carried out to measure the breaking stress and stiffness of the skin. Ventral skin was shaved and excised from mice and stored in cold PBS. The excess fat was removed and then the skin was cut into strips 10mm wide 40mm long. These were gripped with sandpaper (5mm at each end) and clamped into an instron 3344 with a 100N load cell (Instron, USA) and loaded to failure are a rate 20mm/min (Cooper et al., 2015). From the stress strain curves generated an estimate of the Young's modulus (stiffness) was calculated as stress/strain at the liner portion of the curve, where stress refers to the force per cross sectional area and strain as a measure of the resultant deformation. The tensile strength was measured as stress at breaking. Stress relaxation data was obtained as a measure of the viscolelastic properties, this was collected using ventral skin as described above on an Instron 5943 with a 10N load cell (Instron, USA), in a PBS water bath. The skin was cyclically loaded to 1N at a rate of 10mm/min 5 times to precondition the tissue then loaded to 1N and held at a constant strain for 160 seconds. All data was collected using Bluehill software (Instron, USA). The average thickness of the samples was measured from histology and found to be unchanged between samples.

Histological and immunochemical analysis of skin structure

Six µm thick histological sections were prepared from formalin-fixed, paraffin embedded tissue. Elastic fibres were stained using Gomori's aldehyde fuchsin (Gomori, 1950), total collagen was stained using masons trichrome, orgainised fibrillar collagen was stained using picrosirius red (McConnell et al., 2016). Immunohistochemistry for Advanced Glycation End products was carried out with AGE antibody (Abcam,UK). Bound primary antibody was detected using Vectastain ABC kit and NovaRed detection kit (Vector Laboratories). Brightfield images were taken on a Nikon eclipse E600 microscope/SPOT camera. Pircosirius red images were captured using crossed polarised light which visualises organised fibrillar collagen (Leica DMRB) (McConnell et al., 2016). All images were analysed using ImageJ 1.46r software (National Institutes of Health, USA). To quantify elastic fibres, images (I.D blinded to measurer) were thresholded and the pixels measured as a % area (Graham et al., 2011). Collagen was measured using the colour deconvolution plugin (G.Landidni) (Image J, National Institutes of Health, USA). Immunohistochemistry was analysed using Image Pro Plus software (Media Cybernetics).

Proteomic characterisation of aged and estrogen deficient skin

Immobilized-trypsin beads (Perfinity Biosciences) were suspended in 150 µL of digest buffer (1.33 mM CaCl2, Sigma, in AB) and 50 µL of dermal ECM extract and shaken overnight at 37 °C. The resulting digest was then reduced (addition of 4 µL x 500 mM DTT in AB; 10 min. shaking at

60 °C) and alkylated (addition of 12 µL x 500 mM iodoacetamide, Sigma, in AB; 30 min. shaking at RT). Peptides were acidified by addition of 5 µL x 10% trifluoroacetic acid (Riedel-de Haën) in water, and cleaned by two-phase extraction (2 x addition of 200 µL ethyl acetate, Sigma, followed by vortexing and aspiration of the organic layer). Peptides were desalted, in accordance with the manufacturer's protocol, using POROS R3 beads (Thermo Fisher) and lyophilized. Peptide concentrations (measured by Direct Detect spectrophotometer, Millipore) in injection buffer (5% HPLC grade acetonitrile, Fisher Scientific, 0.1% trifluoroacetic acid in deionized water) were adjusted to 300ng/µL prior to MS analysis.

Digested samples were analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation) coupled to an Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. Peptide mixtures were separated using a gradient from 92% A (0.1% formic acid, FA, Sigma, in deionized water) and 8% B (0.1% FA in acetonitrile) to 33% B, in 104 min at 300 nL min-1, using a 75 mm x 250 µm inner diameter 1.7 µM CSH C18, analytical column (Waters). Peptides were selected for fragmentation automatically by data dependent analysis. Spectra from multiple samples were aligned using Progenesis QI (Nonlinear Dynamics) and searched using Mascot (Matrix Science UK), against the UniProt human database. The peptide database was modified to search for alkylated cysteine residues (monoisotopic mass change, 57.021 Da), oxidized methionine (15.995 Da), hydroxylation of asparagine, aspartic acid, proline or lysine (15.995 Da) and phosphorylation of serine, tyrosine, threonine, histidine or aspartate (79.966 Da). Peptide detection intensities were exported from Progenesis QI as Excel spreadsheets (Microsoft) for further processing using code written in Matlab (The MathWorks USA**)**

4.4 Results

Ageing and estrogen deprivation alter mechanical properties of murine skin in contrasting ways

We found a strong positive correlation (R^2=0.97) between age and the tensile strength of the skin; by 24 months it was 50% stronger than at 6 weeks. Conversely, ovariectomy led to a 32% reduction in strength after just 7 weeks of estrogen deprivation ($p<0.05$) (Figure 4.1A). This trend was mirrored in the Young's modulus, where increased age led to an increase in Young's modulus showing a positive correlation between age and tissue stiffness (R^2=0.95) , while estrogen deprivation led to a reduction in Young's modulus and was therefore more compliant ($p<0.05$) (Figure 4.1B). Finally the visocoelastic properties were measured via stress relaxation and this also revealed differences, where the aged tissue relaxed slower (or retained tension for longer) than the control skin and Ovx tissue relaxed faster (lost tension more readily) indicative

of tissue being more yielding (Figure 4.1C).These dramatic alterations in mechanical properties suggest ECM proteins must be altered with both age and estrogen deficiency.

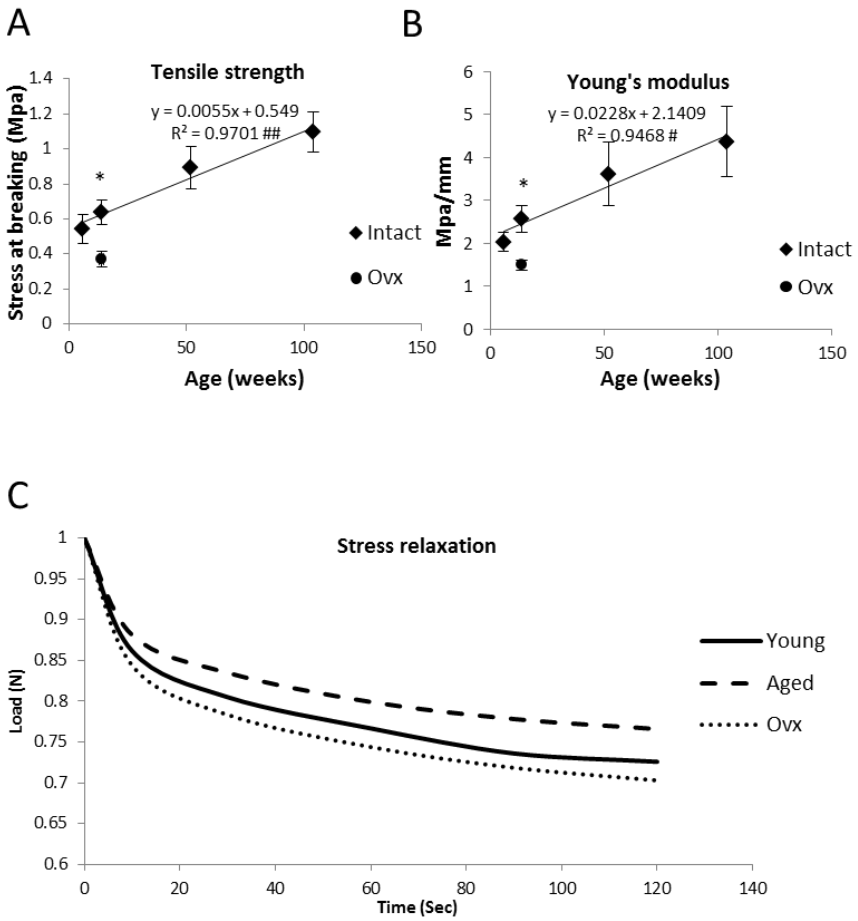


Figure 4.1 Ageing and Ovx alter the mechanical properties of murine skin in contrasting ways

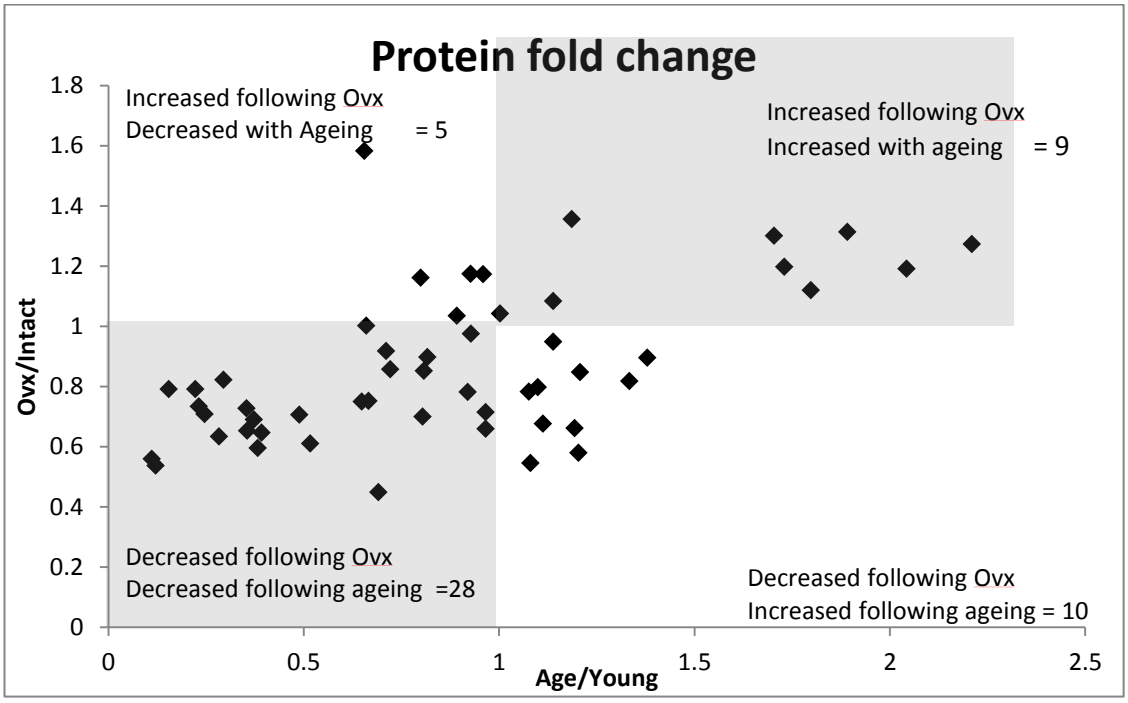
(A) Increasing age leads to a linear increase in the tensile strength of the skin and by 24 months the skin is 50% stronger than at 6 weeks of age. 7 weeks of Ovx has the opposite affect and leads to a loss of tensile strength compared to 14 week age matched control.(B) Age also leads to a linear increase in stiffness measured as by the Young's modulus and Ovx lead s to significant a decrease in the Young's modulus, compared to 14 week age matched control. (C) The viscoelastic properties are also altered in opposing directions in response to ageing or Ovx, with age increasing stress relaxation time and ovx reducing stress relaxation time. * p <0.05 (T-test) # p<0.05 ##P<0.001 (Pearsons R score p value). Data in A and B is presented as mean +/- SEM n=5-6

Estrogen deprivation and age alter the dermal proteome in similar ways

To investigate further we studied the dermal ECM proteome by mass spectrometry analysis. Overall the dermal proteins from mice aged 20 months versus 14 week old mice which had undergone 7 weeks of estrogen deprivation exhibited a similar profile (Figure 4.2A). Many of the proteins (73.4%) were reduced by both age and estrogen deprivation (lower left quadrant), while estrogen deprivation alone led to a reduction in a larger number of proteins (39.5%) than

age alone (34.3%). Interestingly and perhaps surprisingly the fibrillar collagens appeared to be increased in abundance by both ageing and estrogen deprivation (Figure 4.2B; upper right quadrant). Proteins which are differentially altered appear to be mainly basement membrane associated proteins (Figure 4.2C). This family of proteins appeared to be particularly sensitive to estrogen with many more reduced by estrogen deficiency than ageing (lower right quandrant). Small lucine rich proteoglycans SLRPs which are important for the correct organisation of collagen fibrils were reduced by both Ovx and age with the exception of biglycan which is sensitive only to estrogen deprivation (Figure 4.2D). Elastic fibre proteins were the family most reduced by Ovx with ageing reducing both fibrillin-1 and fibrillin-2 but not fibulin-5 (Figure 4.2E). There was no apparent differential expression in the remaining minor collagens between age and estrogen deficiency (Figure 4.2F). The majority of detected signalling proteins were reduced by both states with the exception of serpina1d peptidase inhibitor which was the most upregulated protein detected in the Ovx tissue. Additionally Tgfβi and aebp1 were increased by both Ovx and ageing (Figure 4.2G).

A



	Ovx	Age	Total
Increased	14	19	33
Decreased	38	33	71
Total	52	52	

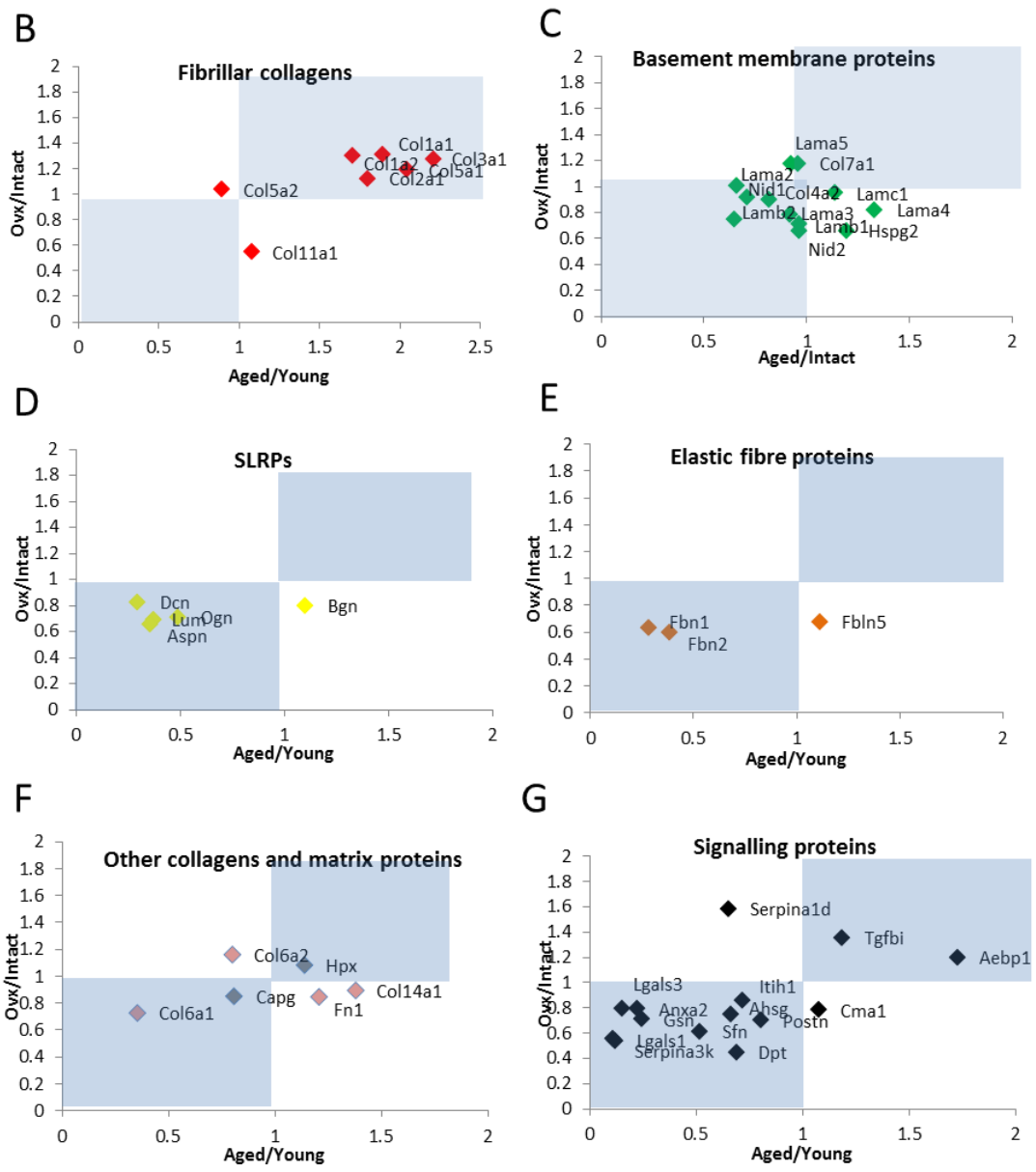


Figure 4.2 Mass spectrometry reveals age and estrogen deprivation alter the dermal proteome in similar ways (A) Mass spectrometry revels that the majority (73.84%) of dermal proteins are reduced by both ageing and ovariectomy and that ageing and Ovx have similar effects on the dermal proteome. (B) The majority of fibrillar collagens are increased (in abundance or extractability) by ageing and estrogen deficiency. (C) The basement membrane proteins appear more susceptible to estrogen deficiency than ageing. (D) All detected SLRPs are reduced by Ovx with all but biglycan reduced by ageing. € All detected elastic fibre proteins are reduced by Ovx and 2 of the 3 reduced by ageing. (F) Other matrix proteins appear more effected (reduced) by Ovx than age. (G) The majority of signalling proteins are reduced by both Ovx and ageing however Serpina1d is increased in estrogen deficient tissue alone with tgfbi and Aebp1 increased in both age an Ovx n=3

Seven weeks of estrogen deficiency leads to significant alterations in dermal proteins

Although we saw a clear trend in both estrogen-deficient and aged mice affecting the dermal proteins, Ovx elicited a more consistent response, reflected in the fact that only estrogen deficiency led to statistically significant alterations ($P<0.05$). The proteins most affected were key elastic fibre proteins; with a quarter of proteins detected as being significantly reduced by Ovx directly linked to elastic fibres. The SLRPs were also sensitive to estrogen deficiency and 4 of the 20 detected proteins were members of this family. The only protein significantly increased by estrogen deficiency was collagen 1 (Figure 4.3).

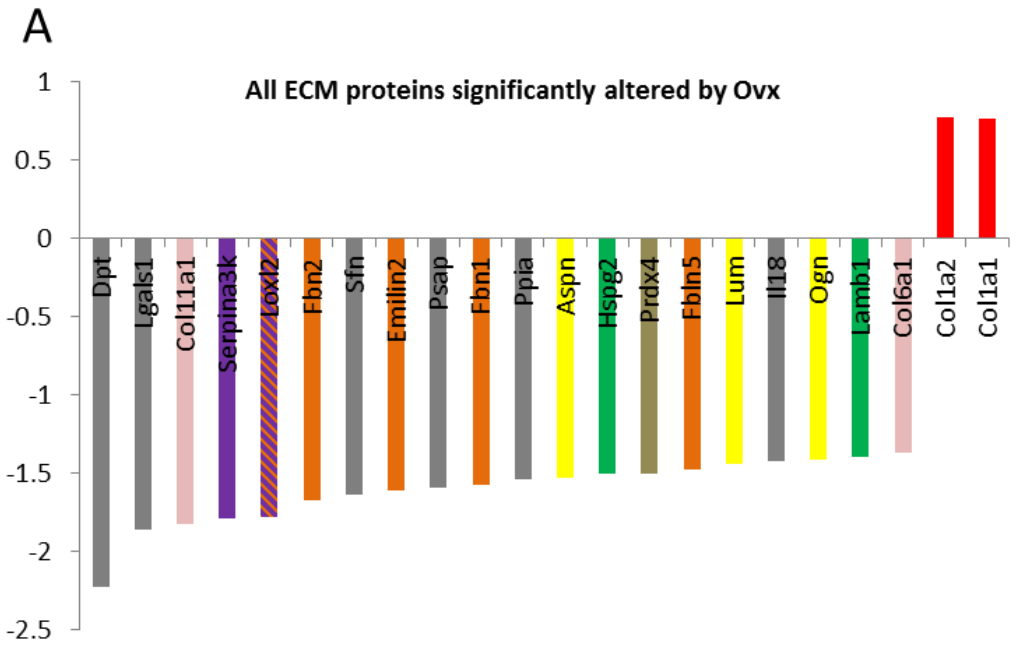


Figure 4.3 Only estrogen deficiency lead to significant alterations in the dermal proteome All the detected ECM proteins significantly altered ($P<0.05$) by estrogen deprivation. The most significantly affected family is the elastic fibre proteins (orange bars) which represent a quarter of all the significantly reduced proteins. The SLRP's (Yellow bars) are also a highly represented family significantly reduced. The only protein significantly increased following Ovx in collagen I (Red bars)

Alterations in mechanical properties are not due to ECM abundance

The mechanical properties of the skin are attributed to the ECM proteins, the most abundant of which is fibrillar collagen, which is principally responsible for tensile strength and elastic fibres which provide resilience. Mass spectrometry analysis highlighted changes in both these proteins which we investigated histologically. Massons trichorome staining for total collagen revealed no observable differences between the groups (Figure 4.4A). Next we stained for the elastic fibres and found they showed a trend towards reduction with chronological age and by 20 months were 14% lower than the 14 week old mice. However estrogen deprivation had a statistically

significant ($p<0.001$) effect reducing the elastic fibres to 39% compared to young intact controls (Figure 4.4B). The same trend was mirrored in the elastic fibre protein fibrillin-1 which is also only significantly reduced by Ovx not ageing ($p<0.05$) (Figure 4.4C). We found there is no correlation between the tensile strength of the skin and collagen content (Figure 4.4D). Similarly we also found no correlation between total collagen content and Young's modulus (Figure 4.4E) or elastic fibre content and Young's modulus (Figure 4.4F). As the alterations in the mechanical properties do not correlate with protein abundance we considered there may be differences in the collagen fibrils.

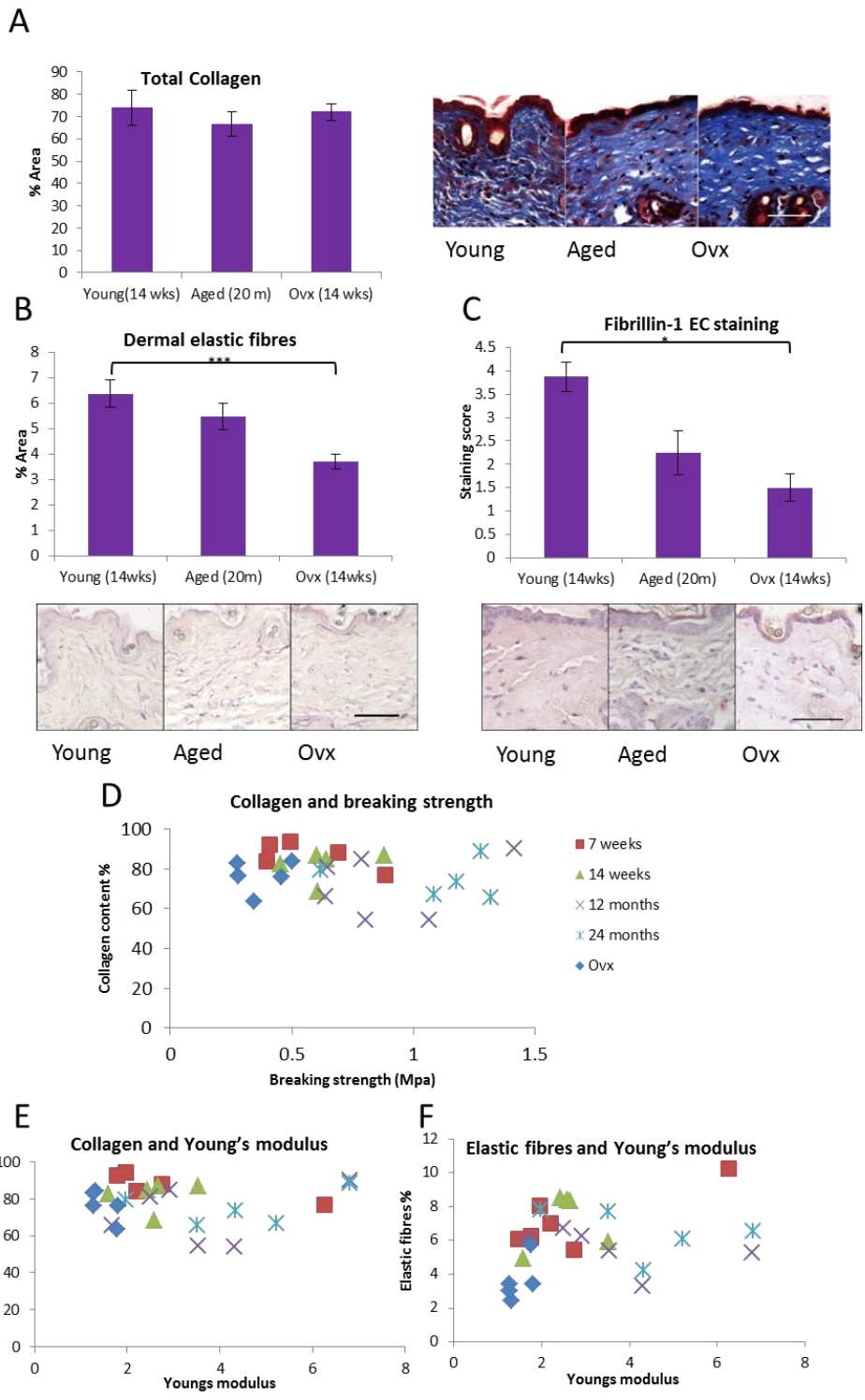


Figure 4.4 ECM abundance does not correlate with mechanical properties (A) Total dermal collagen is not altered by ageing or Ovx. (B) 20 months of ageing leads to a small reduction in elastic fibres of 14% however 7 weeks of estrogen deprivation leads to a reduction of 42% of the dermal elastic fibres. (C) The same pattern is observed in the elastic fibre component fibrillin-1 where estrogen deficiency leads to a significant loss. (D) Collagen abundance does not correlate with breaking strength. (E) Collagen abundance does not correlate with Young's modulus (tissue stiffness). Elastic fibres do not correlate with Young's modulus (F) n=5 * $p<0.05$ ***$p<0.001$ scale bar = 60µm data is presented as mean +/- SEM

Alterations in Mechanical properties may be due to crosslinking

As the mechanical properties show no correlation to the abundance of ECM proteins we considered if the observed changes were due to organisation, orientation or modification of the collagen fibrils. To address this we used picrosirruis red staining, which stains specifically for organised fibrillar collagen, however we did not see obvious observable alterations in abundance or composition of the collagen fibres following age or Ovx (Figure 4.5A) Additionally we did not see alterations in the orientation of the collagen fibrils measured as the coherency (Figure 4.5B). However we found that only age led to an increased level of AGEs (Figure 4.5C). Suggesting the age associated increase in strength and stiffness was related to an increase in protein crosslinks not to protein abundance

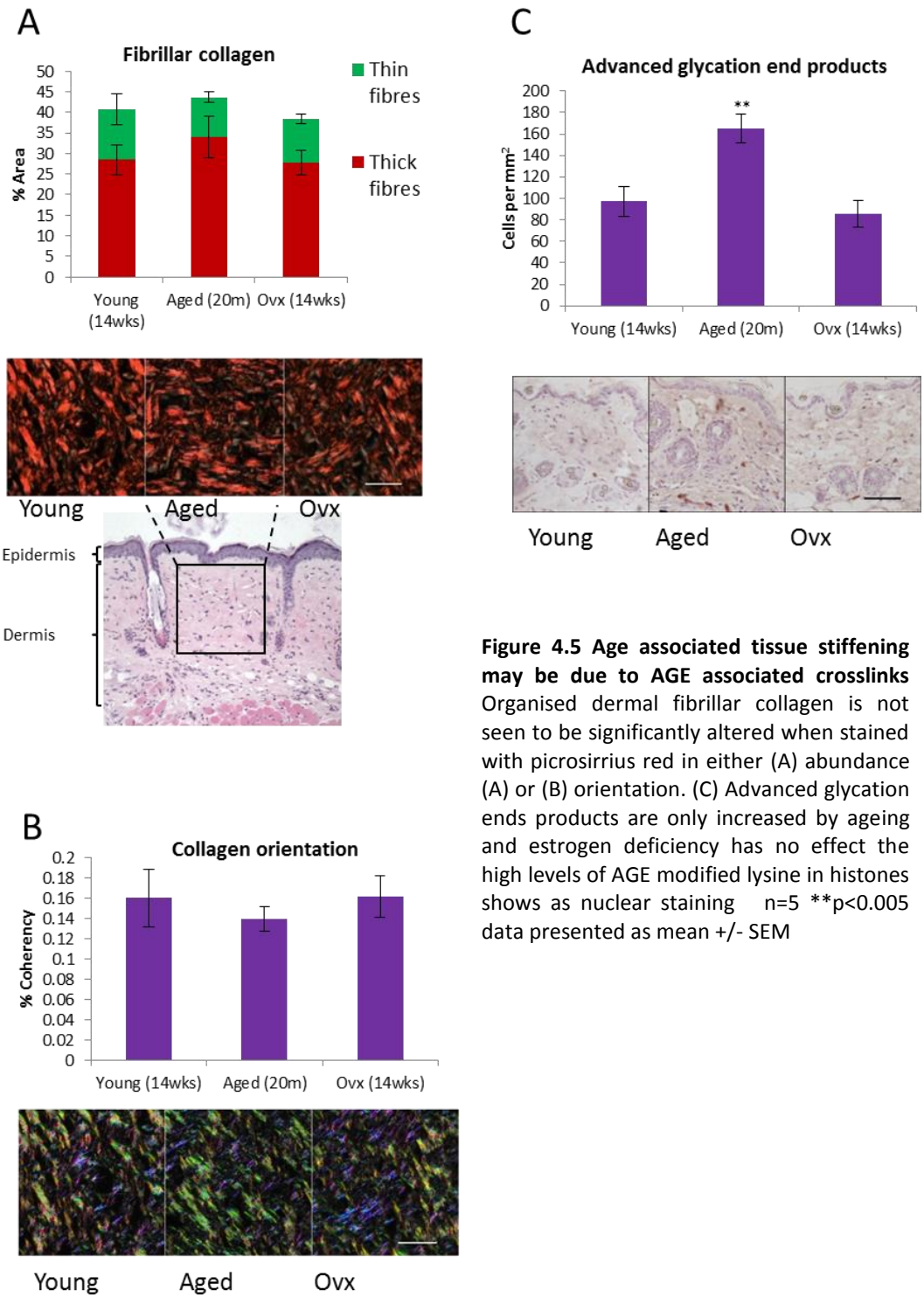


Figure 4.5 Age associated tissue stiffening may be due to AGE associated crosslinks Organised dermal fibrillar collagen is not seen to be significantly altered when stained with picrosirrius red in either (A) abundance (A) or (B) orientation. (C) Advanced glycation ends products are only increased by ageing and estrogen deficiency has no effect the high levels of AGE modified lysine in histones shows as nuclear staining n=5 **p<0.005 data presented as mean +/- SEM

4.5 Discussion

Here we aim to investigate if estrogen deprivation is a key driver of skin ageing in postmenopausal women through changes in the ECM, since such changes in the ECM and mechanical properties of the tissue are the causative factors in the development of the characteristic signs associated with ageing skin.

Mechanical changes

We find that in the mouse model natural chronological ageing and estrogen deprivation have opposing effects in all the measured mechanical parameters, with age leading to tissue which is higher in tensile strength and stiffness as well as altered in its viscoeleastic properties, measured by an increase in stress relaxation time. Although estrogen deprivation also leads to striking alterations in the mechanical properties the changes are opposite to those with advanced age, leading to tissue which is weaker and more compliant with a faster stress relaxation time. In human skin torsion testing in vivo has shown the Young's modulus increases with age (Agache et al., 1980) however dynamic indentation reported the opposite (Boyer et al., 2009) though little has been reported on age related changes *ex vivo*. However, the reported literature varies greatly due to inconsistency and difficulties which arise from measuring mechanical properties *in vivo* (Reviewed in Moronkeji and Akhtar, 2015). Crucially, we find that in the mouse the Young's modulus increases with age, in line with the widely accepted view that age leads to tissue stiffening, indicative of similarities between murine and human skin. However estrogen deficiency rapidly leads to skin which is both weaker and more compliant modelling the effects associated with advanced human ageing where the skin is lax, fragile and more susceptible to skin tears particularly in aged females (El-Domyati et al., 2002, Kennedy and Kerse, 2011).

Proteomic analysis

One of the key questions we wish to address is if the dermal ECM proteome is affected in similar ways by estrogen deficiency and chronological ageing. We carried out mass spectrometry analysis of whole dermis samples from aged and ovariectomised young mice, and compared to young intact controls. This revealed a clear trend with the majority of ECM proteins decreased by both ageing and Ovx. However the analysis also revealed proteins which are reduced following ovariectomy but not altered by ageing which could hold key insights into the differences we observe in the mechanical properties of these 2 groups. The proteins were divided into families and discussed below

Fibrillar collagens and SLRPs

Surprisingly, by mass spectrometry analysis, we find the major fibrillar collagens to be increased in both the aged and estrogen-deprived states. However both ageing and Ovx have a detrimental effect on the SLRPs lumican, decorin, asporin and mimecan, with biglycan seemingly only affected by Ovx. The SLRPs have important roles in collagen fibrillgenisis, controlling the correct formation and spacing of the collagen fibres. Alterations in the correct formation of collagen will alter the mechanical properties of the tissue. In vitro lumican knockout mice and decorin knockout mice show skin fragility due to alterations in the spacing, size and formation of collagen fibrils (Chakravarti et al., 1998, Danielson et al., 1997). Additionally SLRPs are also able to protect collagen fibrils from proteolysis (Geng et al., 2006) therefore making the collagen more susceptible to degradation and in this case the extraction technique used for mass spectrometry. This may explain why we observe an apparent increase in the fibrillar collagens through mass spectrometry which is not reflected in the histology.

Whereas all of the SLRPs were reduced by Ovx, biglycan remained unaltered in aged tissue. There is evidence that decorin and biglycan can interact with the same binding site on collagen I (Schonherr et al., 1995) and in a decorin knockout mouse biglycan can functionally compensate during collagen fibrillogenesis in the cornea (Zhang et al., 2009), suggesting that aged tissue may be able to maintain healthier fibrillogenesis through the maintenance of biglycan.

Elastic fibre proteins

Elastic fibres are made principally of an elastin rich core surrounded by an outer mantle of fibrillin microfibrils. The elastin core is so highly cross-linked it is virtually impossible to extract from tissue therefore the key proteins we would expect to represent the elastic fibres are the fibrillins 1 and 2. We find that both these are reduced by Ovx and ageing. Fibullin-5 is essential for formation arrangement and stabilisation of new elastic fibres, demonstrated by evidence that mutation leads to the disease Cutis laxia (Loeys et al., 2002). Importantly, in our study this protein was found to be reduced only by estrogen-deprivation, and not by age. This could present a key reason the elastic fibres are more severely affected by estrogen deprivation than age. In the Ovx group the elastic fibre proteins proved to be the family most detrimentally affected by estrogen deprivation and this was also reflected in histological analysis.

Age and Ovx have differential effects on basement membrane proteins

The basement membrane proteins are seemingly more affected by estrogen deprivation than ageing. One of the proteins most differentially affected is HSPG2, alternatively named perlecan. This protein is important for maintaining basement membrane integrity especially under mechanical stress (Costell et al., 1999) and may be a contributing factor to the loss of tensile strength associated with Ovx. Oh et al (Oh et al., 2011) find perlecan to be reduced only in aged females, and not aged males. This, together with our findings, suggests perlecan to be particularly sensitive to a reduction in circulating estrogen.

Signalling Proteins

The only signalling protein which was found to be increased in the estrogen-deprived dermis was Serpina1d, a serine peptidase inhibitor whose key role is inhibiting elastase activity. The fact that the ECM protein family most reduced by the Ovx state is the elastic fibre proteins already suggests there is deregulation of elastic fibre homeostasis. Serpina1d both regulates and is regulated by elastase (Ingunuity software) suggesting that by this time point (7 weeks post-Ovx) a large increase in elastase activity, which has led to the dramatic loss of elastic fibres, has induced its own inhibitor.

The signalling protein found to be highest in the aged group is the peptidase Aebp1 Adipocyte enhancer binding protein1, known to have roles in adipogenesis. Aebp1 also causes upregulation of macrophages (Majdalawieh et al., 2007) which may contribute to the chronic inflammation state associated with ageing as well as the increased adipose tissue observed in both aged and estrogen deficient tissue.

In estrogen-deprived skin down regulation of the protease inhibitor serpina3k could leave the matrix more susceptible to degradation by proteases. Further to this serpina3K also has roles in protecting against oxidative stress and it is known that fibrillin-1, a key component of elastic fibres, is particularly susceptible to oxidative damage due to its unusually high cysteine and methionine residues (Watson et al., 2014, Hibbert et al., 2015). This may be further exacerbated by the down-regulation of perioxiredoxin 4, which is an antioxidant enzyme, leading to the huge loss of fibrillin-1 we observe in the estrogen-deprived tissue. Deregulation of elastic fibre proteins can also lead to aberrant TGF-β signalling as fibrillin-1 sequesters and inactivates TGF-β allowing fine tuning (Kaartinen and Warburton, 2003)

Histological analysis

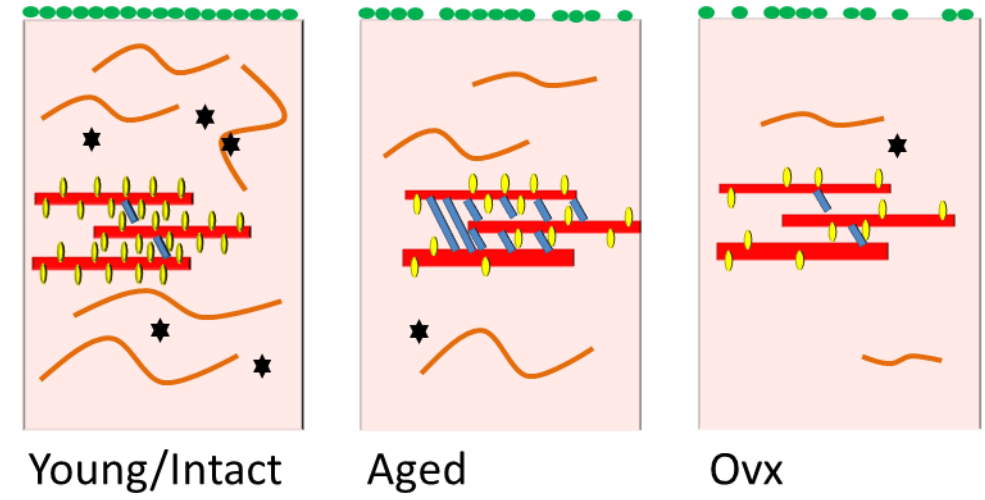
It is widely accepted that the mechanical strength of tissue can be attributed to the fibrillar collagen; we therefore expected that age would lead to increased dermal fibrillar collagen and Ovx would lead to a loss. However histological analysis revealed little differences in either the abundance of ordered fibrillar collagen or the orientation of the fibrils. Additionally a second key contributor to the mechanical function of tissue is the elastic fibres and again we observed similarities not differences in the effects of estrogen deprivation and age on these protein assemblies, with both leading to a reduction in abundance. One key difference we did observe however is the presence of advanced glycation end products (AGEs), which were only increased with ageing. These form when proteins are exposed to sugars and non-enzymatic glycosylation occurs, primary in lysine residues, forming early glycation products (Ansari, 2011). These modified proteins undergo a number of further reactions leading to AGEs such as glucosepane (Sell,2005). This AGE is one of the major contributors to collagen crosslinking forming covalent bonds between lysine and arginine sidechains both within molecules and between different molecules (Gautieri, 2014), with excessive crosslinking of collagen leading to increased stiffness (Verzijl et al., 2002). AGE related crosslinks differ from lysyl oxidase (LOX) derived crosslinks which are enzymatically driven by LOX enzymes. These catalyse the formation of reactive aldehydes from lysine residues within the collagen or elastin fibril to form intra molecular crosslinks to stabilise the structure (Csiszar, 2001) however LOX activity is also found to be increased in photoaged skin which may further contribute to age associated tissue stiffening (Langton, 2016). The lack of correlation observed between protein abundance and mechanical properties suggests that post transcriptional modification could be a major contributor to the observed alterations in mechanical properties.

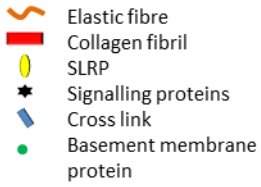
Overall conclusions

From this study we determine that ageing leads to tissue which is both stronger and stiffer perhaps due to post-translational modifications of proteins such as crosslinks formed between AGE's, with an overall general loss of ECM dermal proteins. Particularly susceptible to ageing are the elastic fibre proteins and SLRP's perhaps leaving the fibrillar collagens more vulnerable to degradation and extraction, as these were the only family of proteins increased by ageing. Interestingly very few proteins were reduced by ageing alone and not by estrogen deficiency and it appears that estrogen deprivation and ageing have similar effects on the dermal ECM proteins, generally driving a loss. However, the ECM proteins seem even more susceptible to degradation following estrogen deprivation than ageing supporting the theory that estrogen

deficiency is a key driver of ageing. From a mechanical point of view the loss of proteins following Ovx leads to tissue which is both weaker and more compliant and lax possibly in part due to a down regulation of all the detected SLRPs leading to dis regulation of collagen fibrillogenesis the opposite of ageing where excessive crosslinking may lead to stiffened tissue. This suggests that the overall abundance of major ECM proteins is not a predictor of mechanical properties of skin and that both smaller proteins and post translational modifications have major roles to play. Estrogen deprivation has incredibly rapid effects on the ECM proteins suggesting prompt intervention is required at the time of menopause to maintain both the protein homeostasis and tissue mechanical properties. Further work to establish if these effects are permanent or reversible would be advantageous to development of clinical intervention.

We conclude that Ovariectomy has rapid effects on the dermal ECM proteins and is a good model of the compositional remodelling associated with ageing skin. However it has proved to be a poor model of mechanical ageing. Further to this it also suggests that composition of ECM is not a good predictor of mechanical properties.





	Age	Estrogen deficiency
Tensile strength	↑	↓
Stiffness	↑	↓
Stress relaxation (time to relax)	↑	↓
Extractable Collagen	↑	↑
Extractable Elastic fibre	↓	↓
SLRPs	↓	↓
Signalling proteins	↓	↓
Basement membrane	↓	↓

Figure 4.6 Summary schematic. (A) Age and estrogen deficiency affect the dermal proteome in similar ways however estrogen deficiency leads to a loss of a greater number of proteins in each represented family (SLRPS(yellow), Elastic fibres (Orange), Signalling proteins (black) and basement membrane proteins (green)). We propose that the loss of SLRPs leads to disrupted collagen fibrillogenesis and leaves the fibrils more susceptible to degradation (and extraction) and the estrogen deficient skin weaker, however increased crosslinking of the collagen fibrils with age causes an increase in stiffness and tensile strength. (B) A summary of overall findings from the mechanical testing of breaking strength, stiffness(Young's modulus) and viscoelastic properties (stress-relaxation) and the proteomic analysis by mass spectrometry where direction and size of arrows represent findings.

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Chapter 5
Results

"Signalling through estrogen receptor alpha has an acute effect on elastic fibres"

Contributions: CS was involved in experimental design and carried out experiments, analysed results and prepared manuscript MS was involved in experimental design EE was involved in manuscript preparation MH was involved in surgical procedure experimental design and manuscript preparation.

5 Signalling through estrogen receptor alpha has an acute effect on elastic fibres

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5.1 Abstract

The elastic fibre network endows tissues, such as those which make-up skin lungs and cardiovascular system, the ability to stretch and recoil and is crucial to maintain normal mechanical function. The effect of estrogen deprivation on the elastic fibre system is becoming increasingly apparent with a loss of circulating estrogen correlated with a dramatic loss of cutaneous elastic fibres. This wide acting steroid hormone is known to signal via two distinct receptors, ERα and ERβ, which are differentially expressed. This study aimed to give a first insight into the role of the ER's in maintenance of elastic fibres utilising specific ER agonists PPT and DPN. There was an initial role for ERα in the maintenance of elastic fibres during estrogen deprivation, with a corresponding up-regulation of the elastic fibre scaffold protein fibrillin-1 and down-regulation of hypodermal adipose tissue. Conversely ERβ stimulation during wound healing delayed elastic fibre deposition into the wound bed. An additional insight from ER α-/- and ERβ -/- mice revealed reduced elastic fibres in ERα-/- mice supporting the involvement of ERα signalling in correct elastic fibre deposition.

5.2 Introduction

Following menopause, which sees the cessation of ovarian function, estrogen levels are dramatically reduced. This loss of estrogen correlates with a rapid increase in the signs of skin ageing, which can be prevented using hormone replacement therapy (Verdier-Sevrain et al., 2006, Bolognia et al., 1989). Studies from numerous groups over several decades have linked estrogen deprivation to profound effects on skin physiology, including loss of elasticity, increased wrinkling, skin thinning and loss of hydration (Brincat et al., 1985, Sumino et al., 2004). Much of this previous work has focussed on collagen as the primary mediator of the signs of skin ageing (Sauerbronn et al., 2000, Brincat et al., 1987). However, we have recently demonstrated that estrogen deprivation also leads to severe alterations in the dermal elastic fibre mediated mechanical properties of murine skin (Chapter 3).

Since estrogen signals via multiple receptors/co-factors and estrogen signalling is complex, many uncertainties remain around how estrogen elicits these positive effects. Estrogen is known to signal via at least four different mechanisms, involving genomic and non-genomic pathways. The most widely explored "canonical" pathway involves two distinct estrogen receptors, termed ERα and ERβ, which dimerise to regulate transcription of a wide range of genes (Heldring et al., 2007). The estrogen receptors are expressed in a wide range of tissues with differential expression of ERα and ERβ (Dahlman-Wright et al., 2006). Both estrogen receptors are reportedly expressed in human skin however a detailed study of the distribution of ERs in human scalp reported ERβ to be more widely expressed in a number of regions, including the epidermis, dermal fibroblasts and the blood vessels with ERα only localised in the sebaceous gland (Thornton et al., 2003a, Thornton et al., 2003b). Cultured human fibroblasts express both ER's with ERβ up-regulated in response to estradiol (Haczynski et al., 2004, Haczynski et al., 2002). Human keratinocytes have also been reported to express both ER's however it is ERβ which is thought to be the dominant receptor in human keratinocytes (Thornton et al., 2003a, Verdier-Sevrain et al., 2004). A study of estrogen receptor expression in mouse skin found expression of both receptors in fibroblasts and keratinocytes (Campbell et al., 2010). Differential expression is also reported in human immune cells with ERα dominating in CD4+T cells and ERβ more prevalent in B-cells (Phiel et al., 2005). In the mouse wound model, ERβ has been shown to be important for re-epithelialisation (Campbell et al., 2010) with ERα having anti-inflammatory effects pushing macrophages into the pro-healing, alternatively activated state (Campbell et al., 2014) .

Mice null for each ER were generated nearly two decades ago and have provided major insight into the role of each receptor in tissue development, homeostasis and pathology (Reviewed in Couse and Korach, 1999). As one might expect both ERβ -/- and ERα -/- mice display a range of

reproductive phenotypes (Couse and Korach, 1999). In addition, ERα -/- mice display decreased bone density (Korach, 1994) and increased adipose deposits (Heine et al., 2000), while ERβ -/- mice exhibit abnormalities in the cardiovascular (Otsuki et al., 2003) and respiratory systems (Morani et al., 2006) along with many other defects. In skin ERα plays a role in the prevention of skin flap necrosis (Toutain et al., 2009) and estrogen signalling through ERβ, but not ERα, accelerates cutaneous wound healing despite a role for both ER's as anti-inflammatory (Campbell et al., 2010). Crucially, the estrogen receptor β agonist DPN accelerates cutaneous healing in an Ovx mouse model (Campbell et al., 2010), while rodent wounds treated with the estrogen receptor α agonist PPT exhibit significantly reduced wound tensile strength compared to placebo-treated Ovx rats (Gal et al., 2010). Of direct relevance skin collagen content is reportedly increased in ERα -/- female mice concurrent with decreased MMP-15 expression, with the opposite reported for ERβ -/- mice (Markiewicz et al., 2013). Additionally ERβ signalling has been shown to reduce MMP1, MMP 13 and cytokine production in UV challenged cultured fibroblasts (Chang et al., 2010).

Direct ER specific effects on the cutaneous elastic-fibre system and/or elastic fibre-mediated skin mechanics have yet to be determined. This is important as controversy continues to surround the direct therapeutic use of estrogen. It would be of important clinical benefit to develop ER signalling targeted interventions for skin ageing. Here, as a first step, we have employed the selective estrogen receptor agonists 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; 70-fold selective affinity for ERβ) and 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) (PPT; 410-fold selective affinity for ERα) to elucidate receptor specific effects on the elastic fibre network in both skin homeostasis and repair.

5.3 Methods

All animal procedures were performed under home office project licence (40/3713) in accordance with the UK animal scientific procedures act 1986 following University of Manchester ethical review. Female wild-type C57BL/6 females (Envigo) were used at 7-8 weeks of age at the beginning of procedures. ERα -/- and ERβ -/- mice have been previously described (Dupont et al., 2000). This study used archived tissue originally described in (Campbell et al.,2010).

Ovariectomy and treatment with estrogen, PPT and DPN

Estrogen deprivation was induced in female mice by bilateral ovariectomy described in (Emmerson et al., 2012). Briefly, mice were anesthetised by inhalation of oxygen, nitrous oxide and isoflourane. Following ventral laparotomy, ovaries were located via exposure of the uterus and removed using sterile scissors. The body wall and skin were closed using sutures and

buprenorphine (0.1mg/kg) administered as analgesia. Uterine atrophy was used to confirm estrogen deprivation at the time of tissue collection (Zhao et al., 2012). Estrogen replacement was via a slow release 17beta-estradiol pellet inserted at the base of the neck (0.1mg - 60 day release) (Innovative research of America, Florida, USA). PPT or DPN at 330µg/kg (in corn oil) were administered s.c. (Campbell et al., 2010) three times per week for two weeks, followed by one time per week (7 week protocol) or three times per week (3 week protocol) and Ovx mice were injected with vehicle control the same time points. Mice were wounded two weeks prior to the end of the experiment with 2 x 1cm full thickness dorsal incisions as described (Emmerson et al., 2009) and allowed to heal by secondary intention for 14 days before sacrifice and analysis. Wounds and normal skin were collected and either fixed in formaldehyde for histology or processed for RNA isolation.

Cell culture conditions

Human dermal fibroblasts were cultured in phenol red-free DMEM (Sigma, UK) supplemented with L-glutamine and 10% charcoal stripped foetal bovine serum (Sigma, UK) to minimise estrogenic activity. Cells were treated with either 1µM PPT or DPN (Tocris, UK).

Histological and immunohistochemical analysis of tissue

Tissue was fixed in 10% formalin saline and embedded into paraffin blocks. Sections 6µm in thickness were stained with Gomori's aldehyde fuchsin to selectively stain elastic fibres (Gomori, 1950) or were subject to immunohistochemical analysis with the following antibodies: anti-fibrillin-1 goat polyclonal, anti-ERα rabbit polyclonal and anti-ERβ rabbit polyclonal (Santa Cruz Biotechnology). Antibodies were detected using the VECTASTAIN ABC kit (Vector laboratories) and NovaRed kit (Vector laboratories) as recommended by the manufacturer. All images were taken using a Nikon eclipse 600 microscope with Spot camera (Image solutions Inc.). Elastic fibre images were analysed using image J 4.6r software (National institutes of Health, USA) to measure the % area occupied as described previously (Graham et al., 2010). Positively stained cell counts were performed using Image Pro Plus software (Media Cybernetics).

Gene expression analysis by quantitative real time PCR

Total RNA was isolated from snap frozen tissue or from cultured fibroblasts. Briefly, tissue samples were homogenised or cells were vortexed in Trizol reagent (Invitrogen, UK) and RNA was isolated in chloroform and purified using the RNA purelink kit (Life Technologies UK), cDNA was transcribed from RNA (Reverse Transcriptase kit, Promega, Madison, USA) and quantitative PCR was carried out on an Opticon quantitative PCR thermal cycler (Bio-Rad, UK) using the SYBR

green core kit (Eurogentec) (Gilliver et al., 2007). cDNA was diluted over 3 orders of magnitude and expression ratios normalised to housekeeping genes *Gapdh* and *Ywhaz*.

Table 5.1 Primer sequences

Target gene	Forward primer	Reverse primer
Gapdh (mouse)	TGCCACTCAGAAGACTGTGG	GGATGCAGGGATGATGTTCT
Ywhaz (mouse)	TTGAGCAGAAGACGGAAGGT	GAAGCATTGGGGATCAAGAA
ERα (mouse)	AATAGCCCTGCCTTGTCCTT	ATAGATCATGGGCGGTTCAG
ERβ (mouse)	TGTGTGTGAAGGCCATGATT	GTGTCAGCTTCCGGGCTACTC
Fbn-1 (mouse)	CAGTGCATTAACACAACGC	TTCTTGCAGGTCCCATTTCC
GAPDH (human)	TGCACCACCAACTGAGC	GGCATGGACTGTGGTCATGAG
YWHAZ (human)	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT
FBN-1 (human)	CAGGACAGGCCCATGTTTTAC	GCACAGCAGAGCGTTTTTGT

Protein analysis of tropoelastin synthesis in cultured human dermal fibroblasts

Human dermal fibroblasts were cultured for 14 days and treated with supplemented media to a final concentration 1μm PPT or DPN (media changed three times per week), alongside an untreated control (n=3). The media was collected on day 14 and added to lamelli sample buffer (Bio-Rad) containing 5% 2-mecaptoethanol (Bio-Rad). The samples were separated by SDS-PAGE, blotted onto nitrocellulose membrane and blocked with 5% non-fat milk 0.1% tween prior to incubation with primary antibody anti-elastin mouse (Santa Cruz Biotechnology). Detection was performed using peroxidase labelled anti-mouse secondary (GE Healthcare, UK) and ECL plus (GE Healthcare, UK).

5.4 Results

Estrogen receptor alpha selectively maintains cutaneous elastic fibres following Ovariectomy

It has previously been demonstrated that in the skin dermal elastic fibres positively correlate with estrogen levels (Chapter 3). To determine if signalling via ERα or ERβ predominates in elastic fibre maintenance, mice underwent Ovx followed by three week treatment with ER agonists (PPT for ERα and DPN for ERβ). Dermal elastic fibre density was measured from histological sections stained with Gomori's aldehyde fushsin (Gomori, 1950). We found that just 3 weeks of estrogen deprivation significantly reduced dermal elastic fibre density (Figure 5.1A and 5.1B). Crucially, this loss of elastic fibres was completely prevented by treatment with either 17beta-estradiol or the ERα agonist PPT. By contrast, treatment with the ERβ agonist DPN did

not significantly alter elastic fibre maintenance (Figure 5.1A). This ERα selective protection is maintained at the gene (Figure 5.1C) and protein (Figure 5.1D-E) expression level for the key elastic fibre component fibrillin 1. Indeed, both 17beta-estradiol and PPT (ERα agonist) initiate strong induction of fibrillin-1 gene expression in ovariectomised mouse skin. This suggests that these treatments may directly induce new fibrillin synthesis. Finally, cellular fibroblast expression of fibrillin-1 protein was also strongly and selectively increased in the skin of PPT treated mice (Figure 5.1D-E). Curiously, in longer term (7 week) estrogen-deficient tissue DPN was also able to protect the elastic fibre network from estrogen deficiency-associated loss (Supplemental figure 5.1).

To confirm this preferential role for ERα in elastic fibre development and homeostasis we turned to ERα -/- and ERβ -/- mice. Examining histological skin tissue revealed a specific and significant reduction in dermal elastic fibres in ERα -/- mice alone. Indeed, ERβ -/- mouse skin appeared phenotypically normal (Figure 5.2).

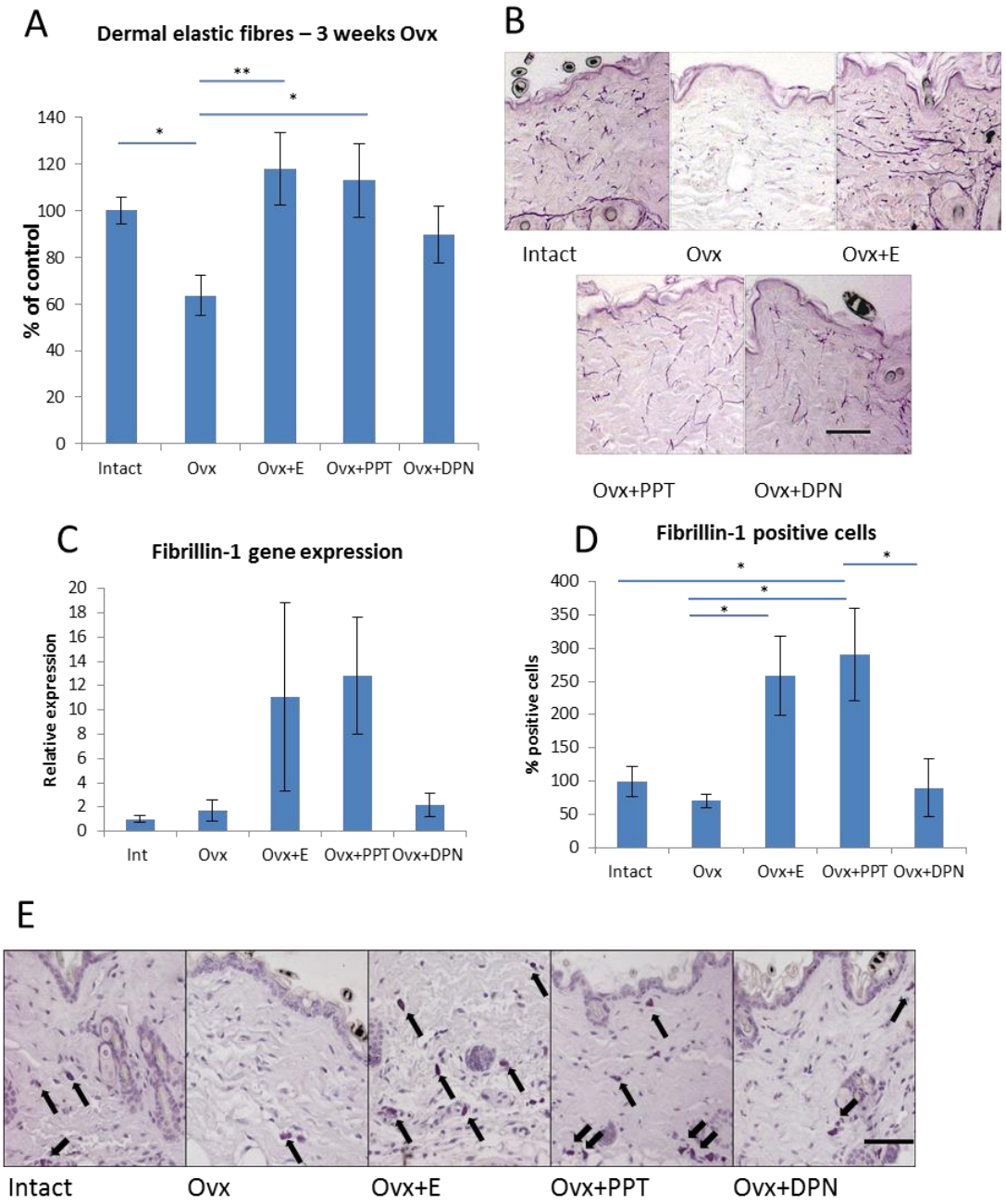

Figure 5.1 Elastic fibres are acutely and selectively induced by ER alpha activation in ovariectomised mice. (A) Relative change in dermal elastic fibre quantity (area) at 3 weeks post Ovariectomy (Ovx) or following co-treatment with 17beta-estradiol (E), ERα agonist (PPT) and ERβ agonist DPN. Data are shown relative to intact (non-ovariectomised) mice. (B) Corresponding representative images of dermal elastic fibre staining with Gamoris aldehyde fuchsin. (C) Gene expression of *fbn1* in whole skin at 3 weeks post Ovx reveals upregulation with E and PPT. (D) Immunohistochemistry reveals dermal cells positive for the elastic fibre protein fibrillin-1 at 3 weeks post Ovx. Treatment with either PPT or E induced dermal positive cells. (E) Corresponding representative images of fibrillin-1 positive cells in each treatment group (arrows indicate positive staining). n=5-6, mean +/- SEM. *p<0.05 **p<0.01, scale bar = 60µm (B) and 40µm.

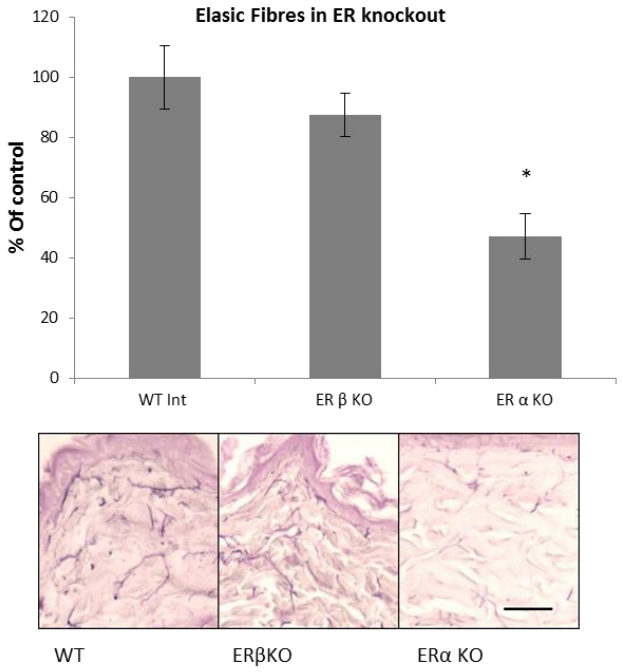

Figure 5.2 Estrogen Receptor alpha KO skin displays reduced elastic fibre density. (A) Dermal area of elastic fibres stained with Gamoris aldehyde fuchsin in ER α and ER β null tissue as a percentage of wild type control. (B) Representative images of elastic fibre staining in ER α and ER β null tissue n = 2 for ERβ knockout, n=5 for WT, n=4 for the ERα knockout Data mean +/- SEM scale bar =40µm

ERβ stimulation delays wound elastic fibre deposition.

In normal skin homeostasis it is difficult to elucidate anabolic versus catabolic effects i.e. is estrogen maintaining elastic fibres by protecting them from degradation or inducing synthesis of new elastic fibres. Evidence presented in figure 5.1 suggested induction of new elastic fibre proteins (Figure 5.1C-E). We therefore investigated this further using an incisional wounding model, where synthesis of new elastic fibres is an integral part of the healing process that should be more easily quantified. A pilot study (data not shown) indicated measurable restoration of elastic fibres at 14 days post wounding. Thus elastic fibre deposition was assessed in mice at this 14 days post wounding time point, treated 17beta-estadiol, PPT or DPN during 3 weeks of Ovx. Restoration of elastic fibres into the healing wound bed was measured in histological sections from the centre of the wound stained with Gamori's aldehyde fuchsin (Figure 5.3A).

Following 3 weeks of estrogen deprivation wound elastic fibre restoration was delayed. This delay was partially restored by treatment with 17beta-estradiol or the ERα agonist PTT. Unexpectedly, treatment with the ERβ agonist DPN led to a delay in elastic fibre formation over and above that observed in Ovx wild type mice (Figure 5.3B). The % area (density) of the newly formed elastic fibres was not significantly altered by any of the treatments (Figure 5.3C). With a longer period of Ovx (7 weeks) the negative effect of DPN treatment was lost (Supplemental

figure 5.2A). Thus, wound deposition largely mirrors findings in the normal skin, with an initial dominance of ERα signalling which is then lost. At the 7 week time point the density of the newly restored elastic fibres are significantly increased by 17β-estridiol and maintained by PPT and DPN (Supplemental figure 5.2B)

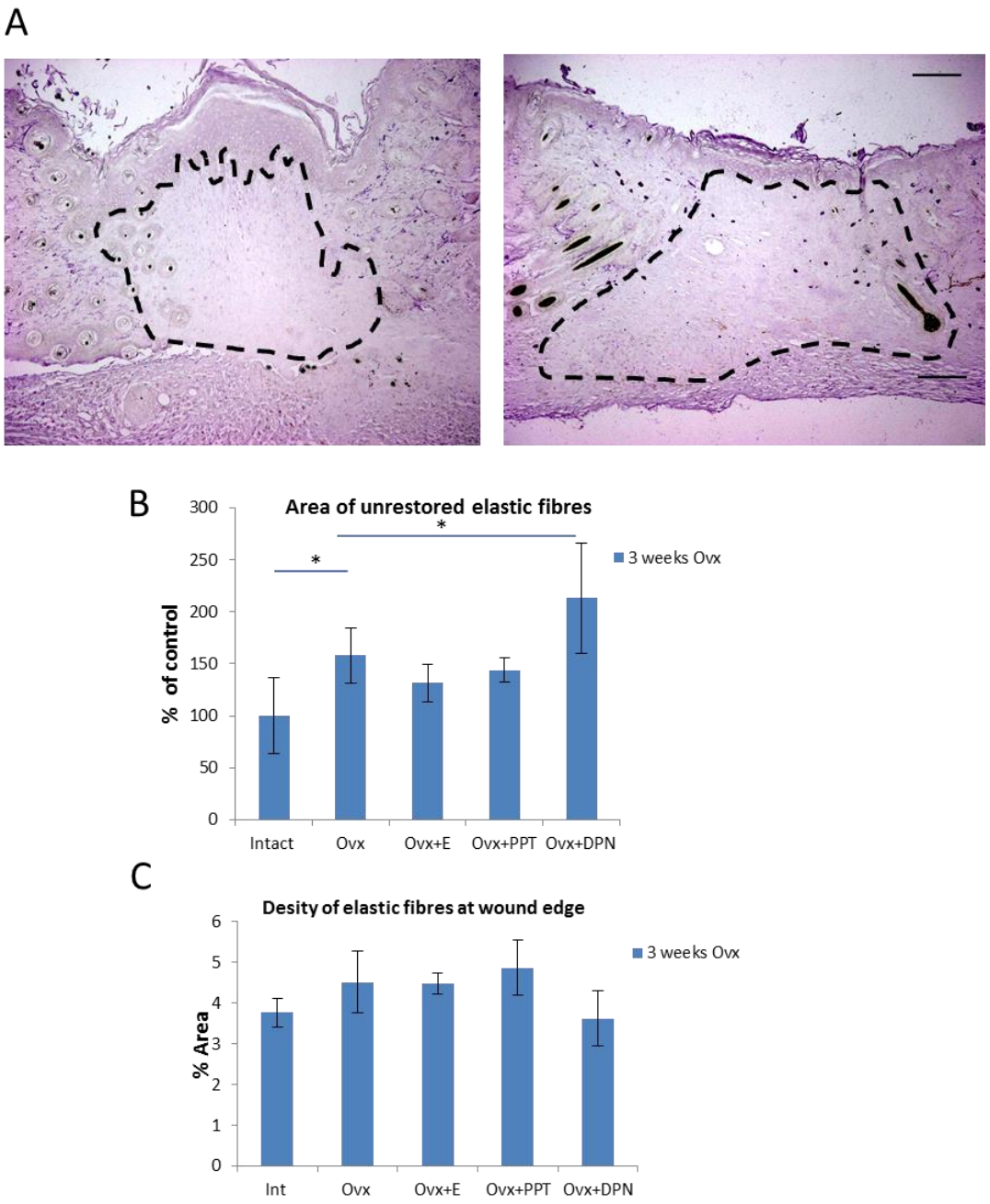

Figure 5.3 ERβ signalling exacerbates delayed wound elastic fibre deposition following ovariectomy. (A) Representative image of what is measured as the area of unrestored elastic fibres (Inside black line). (B) The wound area where elastic fibres have not yet been restored 14 days post wounding in mice which have been Ovx'd for 3 before the end timepoint. Following 3 weeks of estrogen deprivation, treatment with DPN delays the restoration of elastic fibres (C) The percentage area of resorted elastic fibres measured immediately adjacent to the unrestored area. This is unaffected by 3 weeks of Ovx. Data is Mean +/- SEM n=5-6 *p<0.05.

Estrogen receptor expression is altered by treatment with agonists

It has previously been demonstrated that short term (3 day) treatment, in a healing Ovx wound, with either DPN or PPT induces expression of ERβ in the leading edge of the epidermis (Emmerson, 2010). Moreover, the SERM tamoxifen specifically up-regulates ERβ expression in dermal cells *in vitro* (Hardman et al., 2008). However, the effects of longer term agonist treatments on ER expression in normal skin are unknown. Here we demonstrate that ovariectomy plus treatment with PPT and estrogen led to alterations in the expression of the estrogen receptors, namely a large increase at the transcriptional level of ERα and a trend towards increasing ERβ (Figure 5.4A and B), Treatment with PPT also led to an increase in both ERα and ERβ at the protein level, while DPN treatment only increased ERβ expression (Figure 5.4C-F). These effects were less pronounced with longer term estrogen deprivation when the reduced treatment may have been insufficient to maintain induction (Supplemental figure 5.3A and 5.3B).

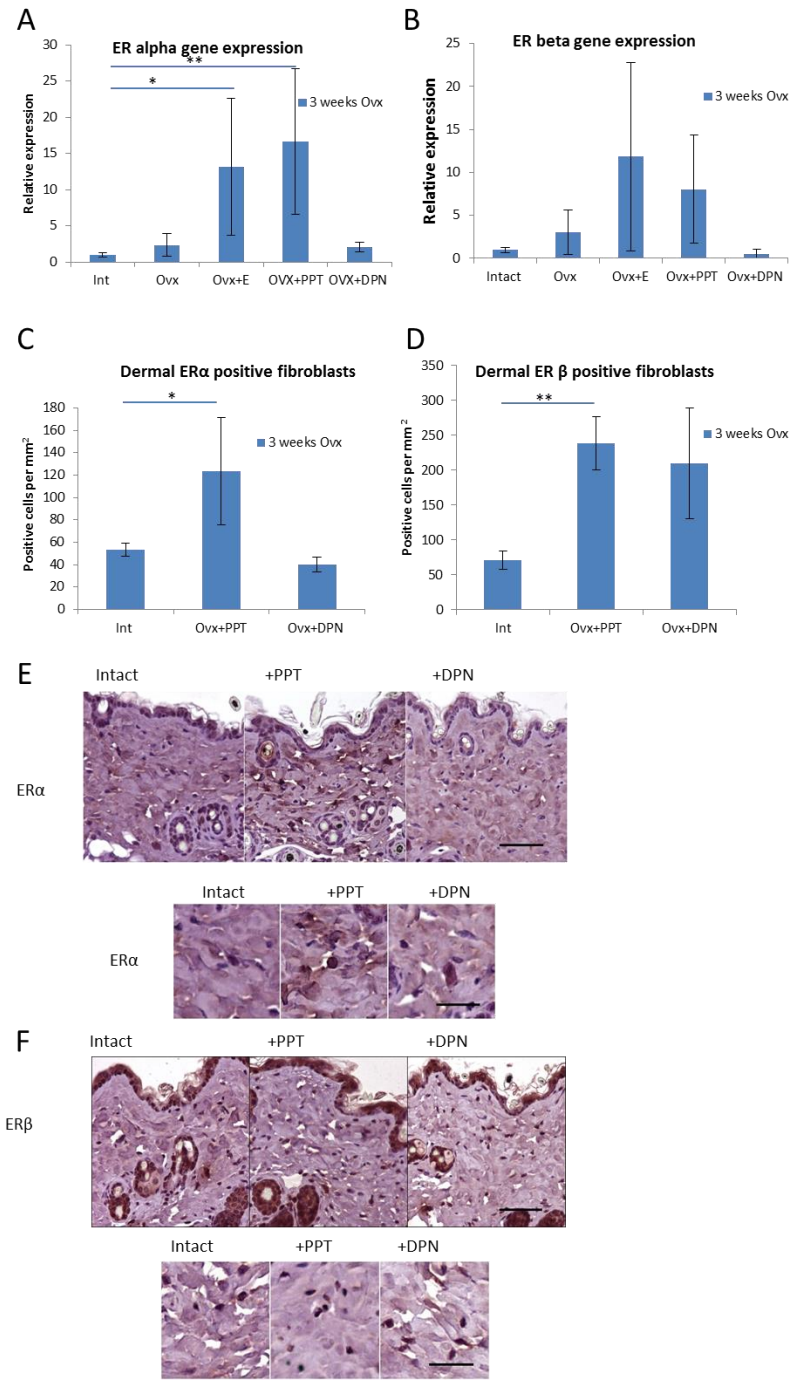

Figure 5.4 Estrogen receptor agonist treatment locally alters estrogen receptor expression. (A, B) Estrogen receptor alpha (*Esr1*) (A) and estrogen receptor beta (*Esr2*) (B) gene expression was measured in the skin of mice following 3 weeks of ovariectomy (Ovx) with or without ER agonist co-treatment. Both receptors were strongly induced by 17beta-estradiol and ERα agonist (PPT) treatment. (C) Parallel immunohistochemical analysis reveals that PPT alone increases the number of ERα positive fibroblasts 3 weeks post-Ovx and (D) By contrast, both PPT and DPN (ERbeta agonist) increased the number of ERβ positive dermal fibroblasts 3 weeks post-Ovx, although DPN treatment was more variable and failed to reach statistical significance. (E,F) Corresponding representative images of ERα and ERβ immunohistochemical localisation. Data shown mean +/-SEM *p<0.05 **p<0.01 n=5-6

Adipose hypertrophy is prevented via ERα signalling

Adipose tissue is emerging as one of the key mediators of elastic fibre homeostasis. It has previously been shown that adipose hypertrophy positively correlates with loss of elastic fibres in the skin (Ezure and Amano, 2015). We therefore explored differences in the subcutanous adipose tissue. We observed significant adipose hypertrophy in Ovx (Figure 5.5A and 5.5B) that was prevented by treatment with either 17beta-estradiol or the ERα agonist PPT. By contrast, treatment with DPN only partially rescued the Ovx-induced increase in adipose thickness (Figure 5.5A). This observed lack of rescue correlates with the lack of restored elastic fibres in the wound bed at 3 weeks post-Ovx. In addition, estrogen supplementation and deprivation (Ovx) are known to alter the hair cycle (unpublished observations and Chanda et al 2000). At 3 weeks post-Ovx 4 of 5 mice in the Ovx group and 3 of 5 in the DPN group were in the anagen stage of the hair cycle. By comparison no mice in the intact or the estrogen treated groups and just 1 in 5 of the PPT group was in anagen. Re-analysis accounting for hair cycle indicates that the cycle stage was only partly responsible for the observed differences (supplemental figure 5.4). Since it is known that there is crosstalk between hair follicles and the underlying subcutaneous adipose tissue (reviewed in Schmidt and Horsley, 2012) it seems likely that the observed number of mice in anagen is due to DPN group being particularly susceptible to fluctuation in adipose depth. Overall our results suggest that the prevention of Ovx-induced adipose hypertrophy is mediated by ER alpha.

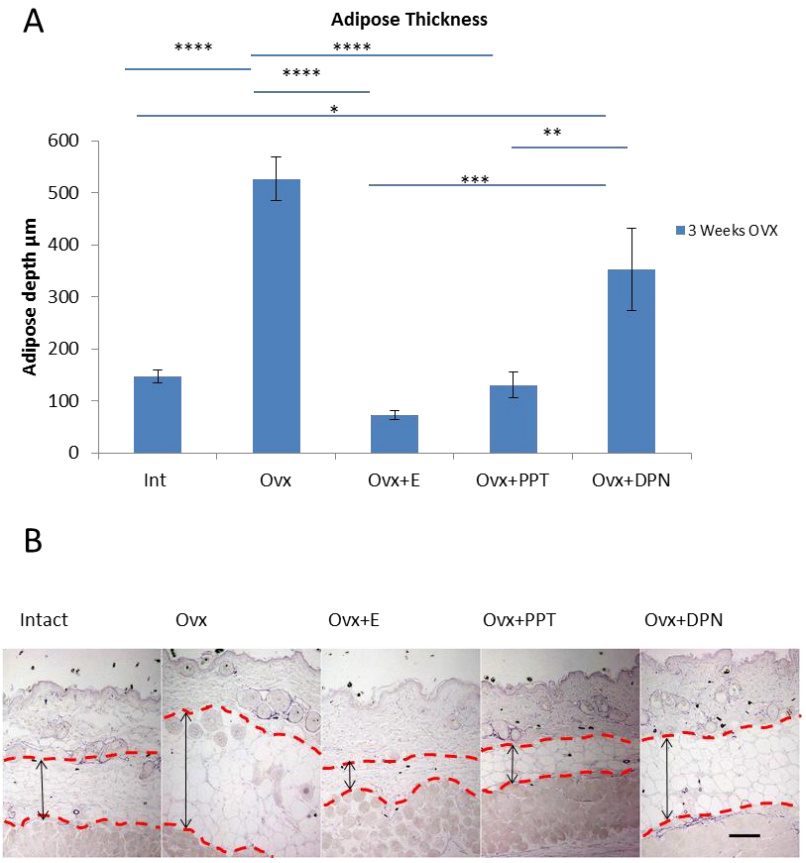

Figure 5.5 . ER α signalling prevents Ovx induced cutaneous adipose hypertrophy. (A) The depth of the adipose layer was measured in full thickness skin histological sections from mice 3 weeks post ovariectomy (OVX). Ovx induced adipose hypertrophy was entirely prevented by either estrogen or PPT treatment. Treatment with DPN failed to prevent adipose hypertrophy (B) Representative images from 3 and 7 weeks post Ovx tissue with treatments highlighting the adipose layer (between red lines) and an indication of the depth (Black arrows) n= 5-6, Scale bar = 100um Data shown mean +/-SEM *P<0.05 **P<0.01 ***P<0.001. ****p<0.0001

ERβ induces key elastic fibre proteins in human dermal fibroblasts *in vitro*

To address cross-species conservation we cultured human dermal fibroblasts and measured the effect of PPT and DPN treatment on induction of elastic fibre proteins. Initially fibroblasts responded equally to treatment with PPT or DPN, inducing expression of fibrillin-1 (Figure 6A). However with longer treatment only DPN significantly induced expression with no increase following PPT treatment (Figure 6B). Secreted tropoelastin the soluble precursor to mature elastin was measured from the media. Both agonists led to an up-regulation of tropoelastin, however, only DPN treatment led to a statistically significant increase (Figure 6C). This data confirms that ER signalling directly influences elastic fibre production in human cells. The data may also correspond to a the apparent dominance of ERβ in human skin fibroblasts (Thornton et al., 2003b) compared to murine skin which appears to have a stronger expression of both ERs (Campbell et al., 2010)

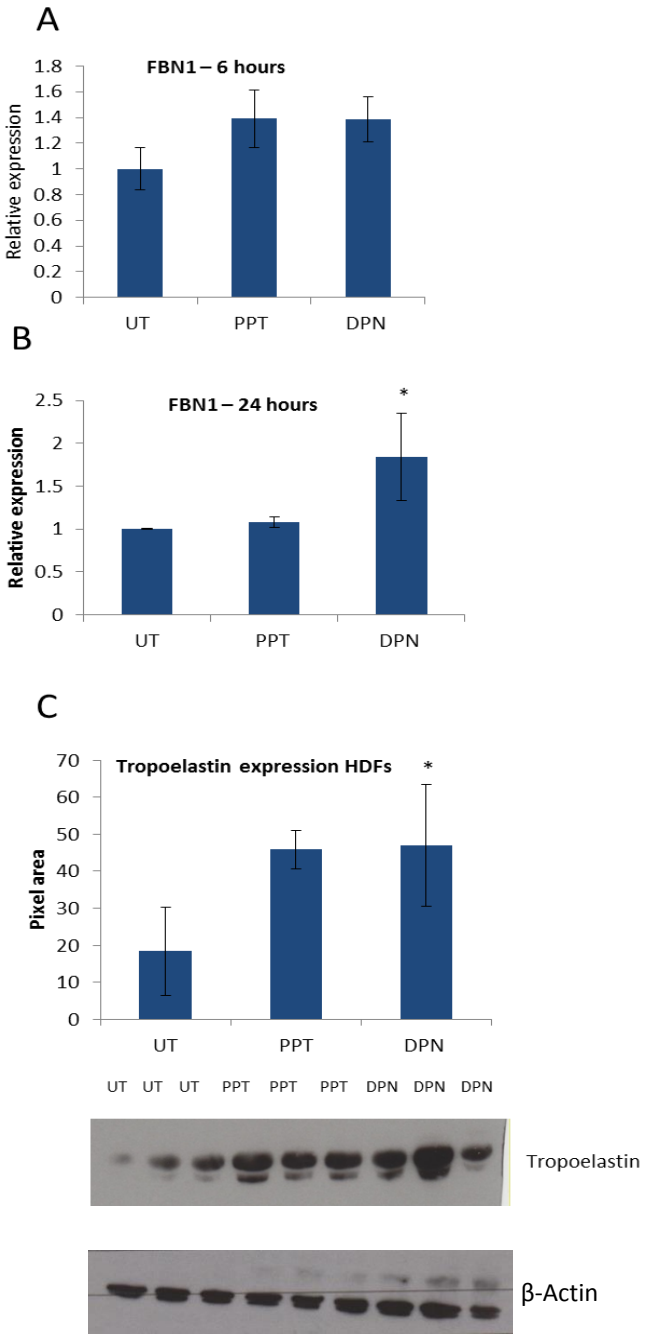

Figure 5.6 Human dermal fibroblasts induce *in vitro* expression of elastic fibre components in response to both ERalpha and ERbeta activation. (A) Monolayer cultured human dermal fibroblasts treated for 6 hours with either ERα agonist (PPT) or ERβ agonist (DPN) showed induced expression of the elastic fibre scaffold component fibrillin-1 (*FBN1*). (B) By 24 hours post-treatment only DPN significantly induced *FBN1* expression. (C) At the protein level both PPT and DPN induced production of the soluble elastin precursor tropoelastin in monolayer cultured human dermal fibroblasts. N=3 *P<0.05

5.5 Discussion

We have previously reported that estrogen deprivation leads to a dramatic loss of dermal elastic fibres in an ovariectomised murine model of menopause. Moreover, this loss can be prevented with estrogen replacement (Chapter 3; Saville et al. in preparation). Here we extend this work with experiments designed to elucidate the relative contribution of ERα versus ERβ signalling in estrogen's protective effects on skin ageing. Our data reveal that agonism of ERα is able to maintain dermal elastic fibres following ovariectomy, closely matching the effects of estrogen. Specifically, the protein fibrillin-1 forms an initial scaffold for the generation of new elastic fibres. Here we report pronounced induction of fibrillin-1 positive fibroblasts with both estrogen and PPT treatment, but not with the ERβ agoinst DPN.

These data are clearly at odds with the concept that elastic fibres undergo minimal turnover in normal skin homeostasis (Davidson et al., 1986, Ramirez and Pereira, 1999, Zhang et al., 1999). To increase turnover and confirm this role we moved to a skin injury model, where new elastic fibre deposition is essential to restore the dermis. Here we find that ERβ agonist treatment actually delays restoration of wound granulation tissue elastic fibres in line with our findings in unwounded skin. A previous study of wound healing identified ERα signalling as important for switching macrophages to the pro-resolutory alternatively activated phenotype (Campbell et al., 2014). Here this may make a more favourable environment for restoration of the matrix in the PPT treated samples. Another possibility is that estrogen signalling is linked to the rate of elastic fibre degradation due to changes in MMP expression or activation, ERβ-/- mice have elevated MMP1 and MMP2 levels along with TIMP2 (Morani et al., 2006) and in an estrogen treated ERβ-/- wound model MMP9 was increased (Campbell et al., 2010) with ERα-/- mice showing decreased MMP-15 levels (Markiewicz et al., 2013). However, this seems at odds with our data but does suggest disregulation of matrix degrading enzymes with ER agonism, with MMPs known to have complex self-regulation (Sternlicht and Werb, 2001). Our data reveal a clear link to alterations in the subcutaneous adipose tissue. There is emerging evidence (Chapter 3 and Ezure and Amano, 2015l) that adipocyte hypertrophy leads to elastic fibre degradation which may be mediated via up regulation of MMP9 (Ezure and Amano, 2015). We find that DPN is far less effective than either PPT or estrogen at preventing Ovx induced dermal adipocyte hypertrophy which may indeed contribute to reduced elastic fibre density. Further evidence from the ERα -/- mouse supports that signalling via this ER is important, but not critical, for correct abundance of the elastic fibres. Thus, other mechanisms must also contribute to induction or protection of elastic fibres.

The observed effects on elastic fibre induction are correlated to an initial specific up-regulation of both ERα and ERβ by ERα agonist treatment alone. Short term (3 day) agonist stimulation has

been previously shown to induce ERβ in keratinocytes (Emmerson, 2010). *In vitro* treatment of fibroblasts with SERMS reveals raloxifen upregulates ERβ however tamoxifen initailly down regulates in the short term and then up regulates ERs with longer term treatment (Haczynski et al., 2004). We also observe temporal effects where the length of treatment varies ER expression (however it should be noted the 7 week dose was also reduced). After longer term (7 weeks) estrogen deprivation the initial differences in PPT and DPN treatment are lost and both are able to partially protect the elastic fibres from Ovx induced degradation in normal skin. A similar effect is observed in the wounds where the DPN induced lag is no longer found. This temporal change in estrogen receptor responsiveness may be due to temporally altered ER expression, with lack of ER induction at the 7 week timepoint.

Overall, stimulation of ERα clearly protects the dermal elastic fibres network from estrogen-deprivation associated loss, with a corresponding protection against adipose hypertrophy. Although the response is species variable, both human dermal fibroblasts *in vitro* and mouse dermal cells *in vivo* are highly sensitive to ER stimulation directly up-regulating key elastic fibre proteins. Of wider relevance, the importance of elastic fibres in healthy ageing is not limited to skin health. They are critical for function of the cardiovascular and respiratory systems, which rely on the resilience conferred by these protein assemblies to function. It is likely that insight provided here in skin will translate to these other tissues, where ER specific stimulation may prove therapeutically effective.

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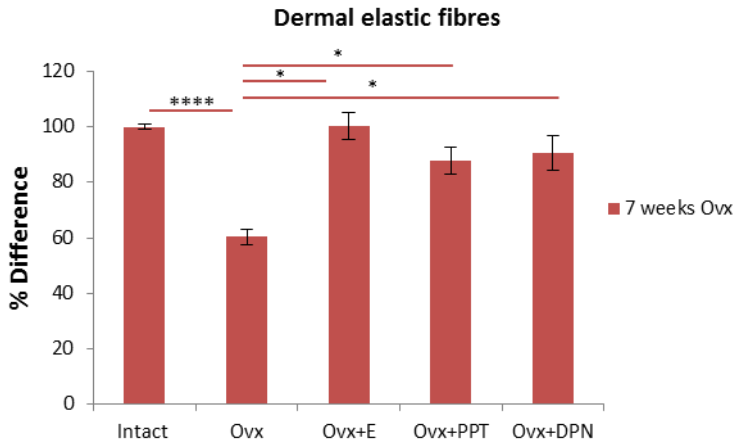
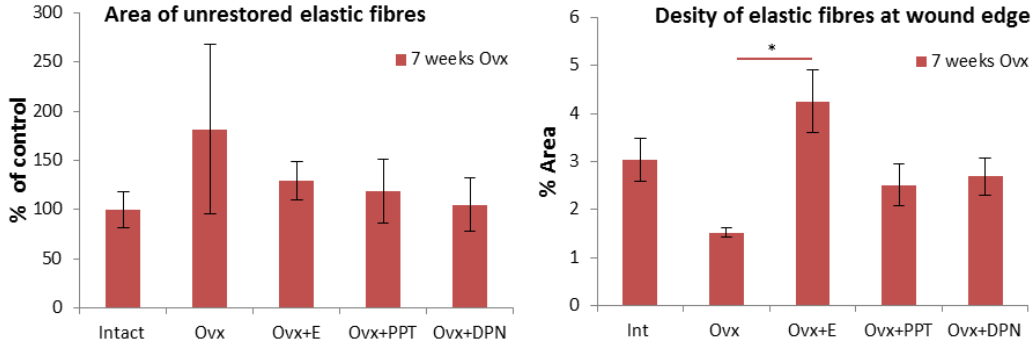
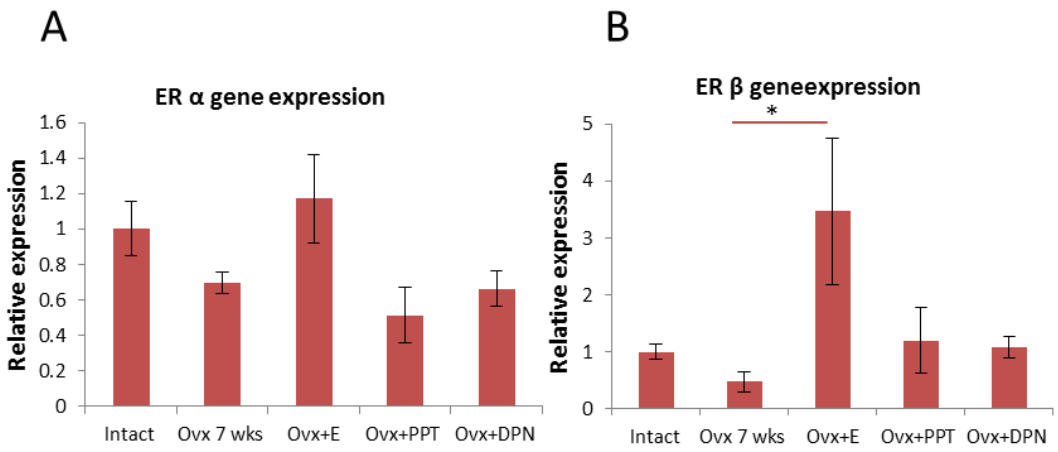
5.7 Supplemental figures



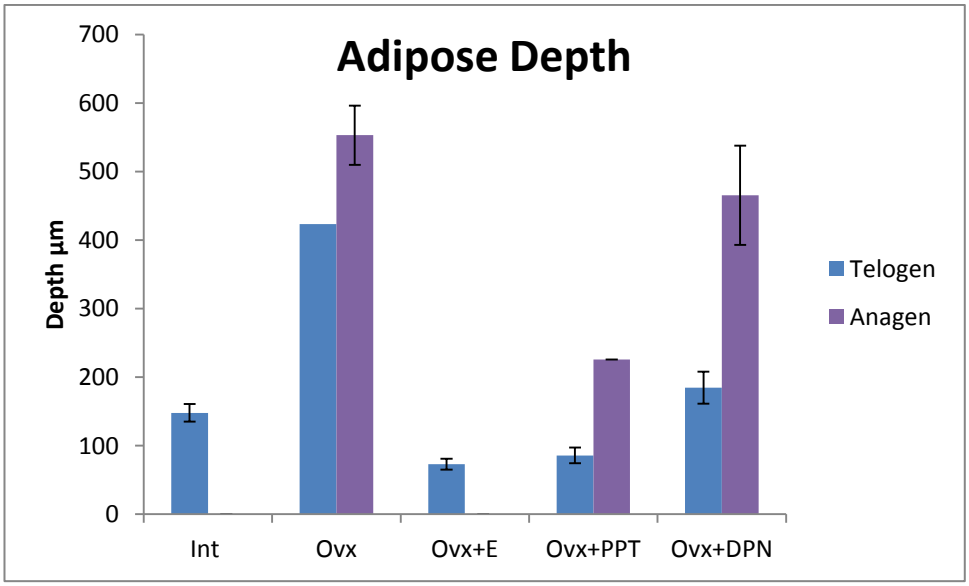
Supplemental figure 5.1 Relative change in dermal elastic fibre quantity (area) at 7 weeks post Ovariectomy (Ovx) or following co-treatment with 17beta-estradiol (E), ERα agonist (PPT) and ERβ agonist DPN. Data are shown relative to intact (non-ovariectomised) mice. n=5-6, mean +/- SEM. *p<0.05



Supplemental figure 5.2 Elastic fibres in healing 14 day wound bed (A) Area of unrestored elastic fibres in 14 day wounds following 7 weeks of ovx (B) the density of restored elastic fibres at the wound edge . n=5-6, mean +/- SEM. *p<0.05



Supplemental figure 5.3 Estrogen receptor expression 7 weeks Ovx (A) Gene expression if estrogen receptor alpha following 7 weeks of Ovx plus treatment with 17beta estradiol (E) ERalpha agoinist PPT or ER beta agoinist DPN (B) Gene expression if estrogen receptor alpha following 7 weeks of Ovx plus treatment with 17beta estradiol (E) ERalpha agoinist PPT or ER beta agoinist DPN . n=5-6, mean +/- SEM.



Supplemental figure 5.4 The effects of haircycle on adipose depth blue bars are samples in telogen only purple bars samples in anagen at 3 weeks post-Ovx n= 5 mice per group. Mice in anagen Int=0, Ovx=4, E=0, PPT=1 DPN=4

Chapter 6

Discussion

6 Discussion

Over the past century average life expectancy in the UK has increased by 34 years for men and 30 years for women and is predicted to continue to rise over the next decades (Hicks, 1999). With the average age of the population now higher than ever there is an urgent need to understand more about healthy ageing.

One of the most obvious external signs of ageing, is the appearance of the skin which becomes increasingly wrinkled with signs of dryness and laxity. Functionally, aged skin becomes weaker and more susceptible to damage. In females one of the key life events which accelerates this ageing phenotype is the menopause (Verdier-Sevrain et al., 2006), which results in a dramatic fall in the levels of circulating estrogen. Skin ageing is therefore correlated with the level of circulating estrogen. Evidence that HRT can reduce these detrimental effects, suggest that this correlation may be due to a causal link (Hall and Phillips, 2005, Brincat, 2000, Brincat et al., 1987).

When examined histologically, intrinsically aged skin is characterised by dermal atrophy, a reduction in cell numbers and a flattening of the dermal epidermal junction (Gilchrest, 1982). There is reported to be loss of the ECM components such as collagen and elastic fibres (Fenske and Lober, 1986, El-Domyati et al., 2002) an increase in MMP activity and a reduction in fibroblast function (Pimienta and Pascual, 2007, Jenkins, 2002, Langton et al., 2009). Indeed it is the ECM proteins which endow tissues with their mechanical properties, with the fibrillar collagens conferring tensile strength which is complemented by the elastic fibres which allow tissues to passively recoil following deformation (Kielty et al., 2002a). The mechanical properties of tissues are vital for their function, this is especially apparent in elastic tissues such as those which make up the cardiovascular and respiratory systems and Robert *et al* (Robert et al., 2008) went so far as to suggest that it is the elastic fibres which will ultimately determine lifespan. Importantly the skin acts as the external, visual, tissue however due to similarities in the structure of other tissues it is likely that they will also be affected in similar ways.

The key observations that i) ageing leads to alterations in mechanical properties of skin which in turn inducing wrinkling and ii) there is a rapid increase in the signs of skin ageing reported at the time of menopause in women which is reduced by HRT (Verdier-Sevrain et al., 2006, Brincat, 2000, Pierard et al., 1995a) lead us to test the hypothesis that: estrogen deprivation is a key driver of skin ageing leading to alterations in ECM proteins and mechanical function which mimics chronological ageing

6.1 Acute estrogen deficiency leads to rapid loss of dermal elastic fibres chapter 3

There have been a limited number of human studies which have measured elastic fibres in postmenopausal women. A notable study found elastic fibres to be prematurely degraded in a cohort of women in early menopause (Bolognia et al., 1989) there have been two studies of topical estrogen treatment reporting improvements in the elastic fibres. The first found local improvement in the size and concentration of elastic fibres following 3 weeks of treatment (Punnonen et al., 1987) and a second study reports improved morphology following 3 months treatment (Varila et al., 1995). However there have also been studies of longer term (6-12months) systemic estrogen treatment which did not find improvements in the elastic fibres (Sauerbronn et al., 2000, Haapasaari et al., 1997). Non-invasive studies have also been employed to measure skin extensibility as a measure of the elastic properties and report that skin extensibility increases with age and elasticity can be improved with HRT (Pierard et al., 1995a, Henry et al., 1997, Sator et al., 2001)

This conflicting data leaves many unanswered questions in the area of estrogen and the elastic fibres. Chapter 3 sought to determine if acute estrogen deprivation does indeed lead to ECM remodelling and alterations in mechanical properties of the skin, in particular changes in elastic fibres and if the mechanisms driving this are degradative or synthetic.

To address this, the Ovx mouse was used as an animal model of human menopause. Collected tissue was used to measure gross mechanical changes with corresponding histological and molecular analysis. Additionally human dermal fibroblasts were employed to assess the potential of estrogen to induce direct synthesis of ECM components in human cells.

The data presented in this chapter highlights the dramatic effect of estrogen deprivation on the mechanical properties of the skin, particularly the tensile strength, which is significantly reduced, with corresponding alterations in the Young's modulus and viscoelastic properties. The speed and severity of these effects support the reported steep increase in skin extensibility in the perimenopausal period (Pierard et al., 1995a). We have identified that, in contrast to fibrillar collagen, elastic fibres and their component proteins are highly sensitive to estrogen levels with estrogen deprivation leading to a dramatic reduction in elastic fibre abundance. Mechanistically we have identified significant adipose hypertrophy in estrogen deprived tissue as a potential key driver of this loss of elastic fibres. First identified by Ezure *et al* (Ezure and Amano, 2015) as a negative correlation between human BMI and elastic fibres, linked to increased MMP9 from enlarged adipocytes. Additionally we note increased gelatinase activity in the early stages of estrogen deprivation. Taken together this makes an unfavourable

environment for elastic fibres compounded by the fact that estrogen acts to dampen MMP activity (Ashcroft et al., 1999) and oxidative stress (Hernandez et al., 2000) to which microfibrils are highly sensitive (Sherratt et al., 2010, Hibbert et al., 2015) Leading on from the findings that estrogen deprivation leads to reduction in elastic fibres raises the complementary question, which is potentially even more interesting. That is if estrogen treatment is able to induce new elastic fibres. Treatment of human dermal fibroblasts with estrogen leads to induction of many of the proteins involved in the synthesis of new elastic fibres (Fibrillin-1, EMILIN1 and 2, Fibulin-2 and tropoelastin) and suggests estrogen may have the potential to induce deposition of new functional elastic fibres in human skin, an interesting area for further investigation. This evidence challenges a long standing dogma that elastic fibres are laid down during development and persist for a lifetime as a relatively static system and instead paints a picture of a dynamic and highly sensitive protein family. Additionally the use of human cells is important in highlighting the translation of the research from murine to human. If the elastic fibres are indeed able to be restored by estrogen treatment this could have a major impact on maintaining healthy tissues.

6.2 Acute estrogen deficiency is a key driver of age associated dermal protein loss chapter 4

The focus of Chapter 4 was to determine if estrogen deprivation is a key driver of age associated skin phenotype. Studies of human skin and menopause have identified a number of similarities to chronological skin ageing, such as dryness, atrophy, epidermal thinning, loss of collagen and increased wrinkling (Reviewed in Hall and Phillips, 2005). However in human studies it is impossible to distinguish between the effects of age and estrogen deficiency as they occur simultaneously. Direct comparison of chronologically aged and Ovx mouse skin gross mechanics, dermal proteome and histology was used to build a detailed picture of these two states.

Age and estrogen deficiency had opposing effects on the mechanical properties of the skin with age leading to increased tensile strength, Young's modulus and stress relaxation time, whereas estrogen deprivation led to decreased tensile strength, Young's modulus and stress relaxation time. These alterations suggest estrogen deprived skin is less able to withstand tension and more easily damaged, in line with the reported increase in skin slackness post-menopause (Pierard et al., 1995a) however the mechanical data for aged skin in the literature has inconsistencies, due to large variations in methodology (Moronkeji and Akhtar, 2015) it is generally accepted that age leads to tissue stiffening (higher Young's modulus) (Sherratt, 2013). In contrast the effects on the dermal proteome were synonymous between aged and Ovx samples. The majority of proteins were decreased with the notable exception of fibrillar collagens which increased. Only Ovx led to statistically significant alterations and also affected a

larger number of proteins, suggesting this has a greater or more consistent effect than ageing. The elastic fibre and SLRP protein families were particularly sensitive to estrogen deprivation which may have an effect on skin hydration and laxity both of which are reported to be affected post-menopausally (Sator et al., 2001). Histological analysis confirmed more severe loss of the elastic fibres following estrogen deprivation than age, however no alterations were observed in collagen staining, longer term studies do report alterations in collagen with estrogen deprivation (Fang et al., 2012, Polito et al., 2012) suggesting this time point may be too short for collagen alterations and highlighting how rapidly the elastic fibres are affected. As the mechanical properties are principally attributed to the ECM proteins (Muiznieks and Keeley, 2013, Gosline et al., 2002) it was expected that mechanics and protein abundance would correlate, however evidence from this study emphasised that the abundance of ECM proteins is not correlated with the mechanical properties of the tissue. The presence of age associated protein modifications such as advanced glycation end products may explain the differences in the mechanical properties, as they are known to increase collagen stiffness (Verzijl et al., 2002). This data has revealed that acute estrogen deprivation has severe effects on the dermal proteome, more so than chronological ageing. It maybe that in humans there are severe effects in the perimenopausal period and compensatory mechanisms restore the dermal proteins via an alternative pathway, such as ligand independent ER signalling (Emmerson et al., 2012). The study has confirmed that both elastic fibre proteins and SLRP's are highly sensitive to estrogen levels in line with reports from human studies of menopause, which find both loss of elasticity and increased dryness (Verdier-Sevrain et al., 2006, Sator et al., 2001), properties conferred by these two protein families respectively (Chen and Birk, 2013, Kielty et al., 2002a). Surprisingly what was also apparent from this study is how little correlation lies between ECM abundance and mechanical properties.

6.3 Signalling via estrogen receptor alpha may have important roles in the maintenance of elastic fibres during acute estrogen deficiency chapter 5

HRT has been reported to have many positive effects in revealing many of the symptoms of menopause in both the short term and longer term maintenance of the health of many tissues including skin, bone, cardiovascular system (Sator et al., 2001, Mosekilde et al., 2000, Greendale et al., 1996). However it also has negative effects and has been linked to increased risk of breast, ovarian and womb cancer making it a far less appealing option for many women in the long term (Beral, 2003, Beral et al., 2007). It is clear that estrogen signalling plays roles in a huge variety of signalling pathways and has disparate effects in tissues. This drives an urgent need to understand estrogen signalling more fully to allow development of pharmacological intervention in a much more targeted manner. Estrogen is known to signal via 2 distinct receptors ERα and

ERβ (Heldring et al., 2007). These offer the possibility of more targeted treatment and there has been much interest in the development of SERMS (Maximov et al., 2013). Chapter 5 investigated the potential of differential roles for the distinct estrogen receptors in the observed estrogen mediated effects on elastic fibres which emerged in chapter 3. Specific ER agonist (PPT and DPN) were employed in the Ovx mouse to offer a first insight in to the mechanism by which estrogen signalling may contribute to the maintenance or induction of elastic fibres. Additionally the possibility of human translation and the ability of treatments to directly induce elastic fibre proteins in human dermal fibroblasts were explored. Evidence provided in chapter 5 suggests that in the mouse, signalling through ERα may be responsible for up-regulation of elastic fibre proteins and additionally evidence from the ER α null mice suggest signalling via this receptor in necessary for the normal development and/or maintenance of elastic fibres. An additional key finding from this study is that ER α signalling is important in the regulation of adipocytes and sufficient to prevent estrogen deficiency induced adipocyte hypertrophy, which is emerging as a possible mechanism by which elastic fibres are degraded (Ezure and Amano, 2015). Evidence from human cell culture revealed an initial early induction of elastic fibre proteins by both PPT and DPN however at a later time point DPN signalling had a stronger effect. This highlights that signalling via ER's has direct influence on the induction of elastic fibre proteins and suggests there may be dose dependent or biphasic effects with the different treatments. Previously treatments with SERMS have been found to alter ER expression differently dependent on the length of treatment (Haczynski et al., 2004) further highlighting the complexity of estrogen signalling. The dominance of ERβ in human skin may also offer an explanation for the apparent difference in the murine model and human cells (Thornton et al., 2003). Importantly there are direct effects on the fibroblast and elastic fibre proteins which would warrant further study.

Table 6.1 Overall conclusions

		Estrogen +Ve	Estrogen -Ve	Aged
Composition	Elastic Fibres	Slight increase	Large decrease	Slight decrease
	Elastic fibre proteins	Induced	Decreased	Decreased
	Collagen histology	Unchanged	Unchanged	Unchanged
	Collagen Mass spec	-	Increased	Increased
	SLRP's	-	Decreased	Decreased expt (Bgn)
Structure	Epidermal thickness	Unchanged	Unchanged	Unchanged
	Dermis thickness	Unchanged	Unchanged	Unchanged
	Subcutaneous Adipose	Decreased	Large increase	Increased
Mechanics	Breaking Strain	Maintained	Decreased	Increased
	Young's modulus	-	Decreased	Increased
	Stress relaxation time	-	Decreased	Increased
Mechanisms		ERα signalling maintains elastic fibres and decreases adipose	Increased gelatinise activity and adipose hypertrophy	Increased AGE associated crosslinking may affect influence mechanics

6.4 Overall conclusions

At the beginning of this thesis evidence from studies of postmenopausal skin and HRT suggested that estrogen levels correlated to ageing phenotype. (Verdier-Sevrain et al., 2006) With HRT reported to improve age associated epidermal thinning, hydration and collagen content (Hall and Phillips, 2005, Pierard et al., 1995a, Sauerbronn et al., 2000, Brincat et al., 1987). It is known that elasticity of skin can be improved by hormone replacement therapy (Pierard et al., 1995a, Sumino et al., 2004) and a very small study noted deterioration of the elastic fibres in early menopause (Bolognia et al., 1989). Along with collagen and elastic fibres other dermal components such as polysaccharides, hyaluronic acid and sebum are also increased by estrogen (Grosman et al., 1971, Danforth et al., 1974, Callens et al., 1996). Collagen has certainly been the primary focus of many studies, implicated as the main mediator in the development of skin wrinkles and principally responsible for mechanical properties (Verdier-Sevrain et al., 2006, Contet-Audonneau et al., 1999, Bosset et al., 2002). Leaving much less known about the elastic fibres in this context, however an early study related age related mechanical changes to alterations in the elastic fibre network (Daly and Odland, 1979). Mechanistically topical estrogen is able to induce expression of collagen, fibrillin and tropoelastin in human skin as well as

reducing MMP 1 (Son et al., 2005) At a cellular level estrogen can stimulate keratinocyte proliferation, inhibit apoptosis and protect both keratinocytes and fibroblasts from oxidative damage (Kanda and Watanabe, 2004, Kanda and Watanabe, 2003, Bottai et al., 2013). Despite a long history of investigation, a detailed study of the effects of acute estrogen deficiency on the mechanical properties of skin and direct comparison to the ECM proteins to our knowledge had not been carried out.

We have identified that the elastic fibre proteins are among the most sensitive to estrogen levels, being rapidly lost from the dermis following acute estrogen deprivation and highly induced in estrogen treated dermal fibroblasts. Estrogen deficiency initially leads to an up regulation of gelatinase activity at least in the initial 3 week period. This could, in part, be as a consequence of significant subcutaneous adipocyte hypertrophy which has been linked to increased MMP 9 production and elastic fibre degradation (Ezure and Amano, 2015). Crucially we have identified signalling via ERα is able to fully protect against Ovx induced increase in adipose tissue along with induction of fibrillin-1 and protection of elastic fibre homeostasis in Ovx tissue, offering a first insight for development of pharmacological intervention. A study of the dermal proteome during estrogen deficiency and supplementation confirmed the ECM proteins to be highly sensitive to estrogen levels having a greater and more consistent effect than age alone. The SLRP's stood out as particularly estrogen sensitive which may help explain both the alterations in skin hydration reported post menopause and also the alterations in skin collagen which require SLRP's for correct formation and protection (Chakravarti et al., 1998, Danielson et al., 1997, Geng et al., 2006). The study found that both age and estrogen deficiency affected the dermal proteome in a similar ways driving a general loss of proteins with the exception of fibrillar collagen. Intriguingly it also became apparent that the effects on the mechanical properties were opposing in all measured parameters. This not only raised interesting questions about the differences in the two states but also illustrated no correlation between protein abundance and mechanics.

6.5 Impact

The importance of maintaining health in later life has never been more pertinent with people living longer the pressures on the NHS are only set to increase. The possibility of maintaining healthier function of not only the skin but also other elastic fibre rich tissues such as the lungs and cardiovascular system could transform the health of the postmenopausal population dramatically and relieve pressures on the NHS and social care system. The elasticity of the arteries is critical for their function and the largest arteries close to the heart have the highest

amount of elastic fibres. If the dramatic loss of elastic fibers we have observed in the skin following seven weeks of estrogen deprivation is indeed replicated in the blood vessels this would have a huge impact on the function. Evidence from the literature suggests that this is indeed the case with a higher risk of cardiovascular disease post menopause (Gordon et al., 1978, Kannel et al., 1976) which is reduced by HRT (Greendale et al., 1996) . This strengthens the use of the Ovx mouse to model menopause as well as the transferability of discoveries from one elastic rich tissue being applicable to others. As the use of estrogen therapeutically has been linked to an increased risk of certain cancers the development of SERMs is perhaps a more interesting option. Evidence provided in chapter 5 that the elastic fibres can be maintained thorough distinct estrogen receptor signalling provides a promising beginning for future pharmacological developments.

6.6 Effects on original hypothesis

At the beginning of this work the hypothesis was that estrogen deprivation is a key driver of ageing phenotype and would have effects on both the mechanical properties and the ECM proteins in a way consistent with age.

It has been proven that estrogen deprivation indeed has drastic consequences for the ECM proteins, apparently even more detrimental than the effects of ageing and that the elastic fibres are especially susceptible to estrogen levels. The study has highlighted opposing effects of the two states on the mechanical properties of the tissue. This was surprising when the effects on the dermal proteome were so similar, and raised a very interesting theme that the mechanical properties do not correlate with the abundance of either fibrillar collagen or elastic fibres. The data supports the hypothesis that estrogen deprivation is a key driver of age associated ECM loss however this does not model the mechanical effects of ageing

6.7 Further work

This work has provided evidence that the ECM proteins are highly sensitive to estrogen levels especially the elastic fibre proteins however many important questions still remain

What are the mechanisms leading to loss of elastic fibres?

This study has shown potential increase in geleatinase activity in the estrogen deprived tissue may be driving the loss of elastic fibres, however the gelatinases responsible have not been identified. Directed IHC staining and qPCR to identify increases in specific ECM degrading

enzymes MMPs, caspases, elastases could offer a better understanding of the mechanisms of degradation. Adiopocyte hypertrophy has also been highlighted as a potential driver of elastic fibre loss however we have not provided direct evidence for this. Further investigation into this using adipocyte cell culture and also specific harvesting of the adipose tissue for zymography experiments could dissect if estrogen deprived adipocytes are producing increased gelatinases. A third mechanism which may be at play is that of oxidative damage, this could be measured from its consequences such as increased protein carbonyls in Ovx tissue.

Is estrogen and/or PPT treatment able to induce new functional elastic fibres?

This study demonstrated estrogen supplementation is able to protect elastic fibres from Ovx associated loss and also able to induce key elastic fibre proteins. However has not provided direct evidence that estrogen can induce new functional elastic fibres. To address this key question, mice would be Ovx'd for 7 weeks to induce elastic fibre loss and then treated with estrogen or PPT for a period of 4 weeks. Histological analysis along with mechanical testing would be carried out the asses if the elastic fibres had been restored and if the mechanical properties were also equal to control tissue.

What is driving the differences in age and Ovx associated mechanical properties?

Intriguing evidence presented illustrated a lack of correlation between ECM abundance and mechanical properties. A potential explanation for this is the presence of post-translational modifications such as AGE associated cross links. This presents an area for further investigation which could utilise a mass spectrometry approach to identify specific post translational modifications.

Following on from these specific questions investigation into Selective estrogen receptor modulators (SERMS) is an obvious area to develop this research as these are developed to be far more specific than HRT reducing the risks of systemic estrogen signalling (Maximov et al., 2013) and one, raloxifene, has already shown promise in increasing elasticity of forearm skin (Sumino et al., 2009). The area of phytoestrogens, which are plant based compounds which mimic estrogen, is also a very appealing area for further research. Plants such a soybeans are rich in phytoestrogens and it has been suggested that the high levels of soy in Asian diets is linked to the lower rates of cardiovascular disease, bone fracture and menopausal symptoms (Tham et al., 1998). Phytoestogens have also been implicated in the reduction of skin ageing via their antioxidant properties and activation of ERβ (Jackson et al., 2011). The idea of a natural estrogen replacement removes many of the concerns of pharmacological interventions therefore offering

a safer alternative. Suggestion from this study is that induction of the elastic fibres may well be more responsive to signalling though ERα. This could offer the potential for more targeted approach to specific maintenance of elastic tissues.

Another key area for investigation would be to look into the other elastic fibre rich tissues such as the arteries and lungs to assess if these findings are indeed preserved in other tissues. This would both highlight other areas for concern in postmenopausal health and potential areas for development of treatments along with determining the applicability of skin research into other ECM rich tissues and the transferability of findings. Of course the most pressing question in many areas of research is how applicable are the findings from murine studies to human health. It would therefore be of utmost importance to consider utilising human tissues to confirm the findings.

Overall acute estrogen deprivation leads to dramatic loss of ECM proteins especially the elastic fibres. This is likely to be driven by a combination of anabolic and catabolic mechanisms such as heightened gelatinase activity driven by subcutaneous adipocyte hypertrophy and a loss of elastic fibre protein induction which may be maintained via ERα signalling.

6.8 References

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7 Method Appendix

Tissue preparation

Tissue Fixative

Centramide 0.5g

Sodium chloride 9g

Distilled water 920ml

Formaldehyde 40ml

Acetic acid 20ml

Vectabond

Slides incubated in 100% acetone for 5 minutes

Slides transferred to 350ml acetone containing 1 vial of vectabond solution (Vector laboratories, UK)

Histological analysis

Immunohistochemistry

PBS

Phosphate buffer (Sigma) 25ml

Sodium Chloride 6g

Up to 1l with distilled water

Citrate buffer

Citric acid 2.1g

Distilled water 900ml

pH to 6.0

Make up to 1L with distilled water

Blocking Serum (Vector laboratories, UK)

PBS 10 ml

Normal blocking serum 3 drops (Vector laboratories, UK)

Secondary antibody (Vector laboratories, UK)

PBS 10ml

ABC Reagent

PBS 10ml

4 drops of Reagent A (Vector laboratories, UK)

4 drops of Reagent B (Vector laboratories, UK)

Biotinylated antibody 2 drops (Vector laboratories, UK)

Novared (Vector laboratories, UK)

Distilled water 10 ml

Reagent 1 6 drops (Vector laboratories, UK)

Reagent 2 4 drops (Vector laboratories, UK)

Reagent 3 4 drops (Vector laboratories, UK)

Hydrogen peroxide 4 drops (Vector laboratories, UK)

Histological stains

Aldehyde fuchsin

Basic fuchsin 5g

Distilled water 150ml

Ethanol 350ml

Solution mixed and warmed to dissolve then filtered before adding

Hydrochloric acid 5ml

Paraldehyde 10ml

Aniline Blue

Analine Blue 25 g

Glacial acetic acid 20 ml

Distilled water 1L

Biebrich scarlet-acid fuchsin

Brebrich scarlet (1% aqueous) 360ml

Acid fuchsin (1% aqueous) 40ml

Glacial acetic acid 4ml

Weigert Hematoxylin

Solution A

Haemotoxylin 10g

IMS 950ml

Distilled water 50ml

Solution B

Ferric chloride (29% aqueous) 20ml

Distilled water 275 ml

Glacial acetic acid 5ml

Working solution is equal parts A and B

Phosphomolybdic/Phospotungstic Acid solution

Phosphmolybdic acid 25g

Phospotungstic acid 25g

Distilled water 1l

Quantitative Real-time PCR

qPCR Matermix (per reaction)

MESA green mastermix (Eurogentec) 12.5µl

Nuclease free water 2.5µl

Forward primer 0.225µl

Reverse primer 0.225µl

Protein analysis

Western blot

Running buffer

10 x Tris-glycine SDS (Bio-Rad) 100ml

Distilled water 900ml

Separating gel

4 x Tris- HCL pH8.8 3.75ml

10% SDS 150µl

10% Ammonium persulphate (Bio-Rad) 50µl

0.8% Bisacrylamide (National Diagnostics) 5ml

Distilled water 6.25ml

Stacking gel

0.8% Bisacrylamide (National Diagnostics) 0.6ml

4 x Tris- HCL pH6.8 1.25ml

10% SDS 50µl

10% Ammonium persulphate (Bio-Rad) 25µl

TBS-Tween (10X)

Tris base 6g

Sodium chloride 9g

Tween 20 100ml

Up to 1l with distilled water

Used at 1x (100ml in 900ml distilled water)

TBS-Tween Milk

Skim milk powder 5g

1x TBST 100ml

Transfer buffer

10x Tris-Glycine SDS (Bio-Rad) 100ml

Methanol 200ml

Distilled water 700ml

Mass spectrometry

Digest buffer

1mM calcium chloride

25mM ammonium bicarbonate

ExNa extraction buffer

2M NaCl

25mM ammonium bicarbonate

25mM dithiothretol

Injection solution

5% acetonitrile

0.1% trifluroacetic acid in water