Novel Approaches to Identify Biomechanisms in Systemic Sclerosis

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Declaration

I, Henry W. López, confirm that the work presented in this thesis is my own. Where information has been delivered from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Systemic sclerosis is a severe connective tissue disease in which inflammation and autoimmunity are associated with progressive tissue remodeling and fibrosis of the skin and internal organs. Complex genetic backgrounds contribute to susceptibility and the disease can be triggered by environmental factors. It is proposed that based on the genetic susceptibility an immune inflammatory disease microenvironment is initiated leading to overexpression of cytokines and growth factors and the development of a fibrotic disease process.

Analysis of copy number variation in candidate genes was performed using DNA from patients and controls. This identified a possible association between disease susceptibility and one candidate factor, *LEPREL1*, a prolyl 3-hydroxylase involved in collagen alignment in the endoplasmic reticulum. Deletion of the *LEPREL1* gene led to resistance to dermal fibrosis in mice, whereas levels of the encoded enzyme were increased in disease fibroblasts, all consistent with an important role in the fibrotic process.

Furthermore, profiling of tissue fluid from the dermal lesions revealed the presence of an inflammatory, pro-fibrotic microenvironment. When candidate factors present in the tissue fluid (e.g. PDGF), were applied to fibroblasts on aligned collagen matrices, fibroblast orientation and migration was enhanced, modeling the effect on spread of the disease. In contrast, the use of inhibitors (e.g. heparin, imatinib), particularly in combination, attenuated fibroblast alignment and migration.

Finally, since this disease has proved resistant to current non-specific therapies, a novel anti-inflammatory peptide was evaluated using a mouse model of systemic sclerosis-like inflammation and fibrosis. Treatment with the peptide suppressed the pattern of inflammatory changes seen in this model of systemic sclerosis, and significantly reduced tissue fibrosis and the replacement of the normal tissue architecture with scar tissue. This approach using anti-inflammatory peptides could be potentially relevant for the treatment of individuals with systemic sclerosis in order to attenuate the pathological inflammatory fibrotic process.

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I thank Dr. Richard Stratton, Dr. David Abraham and Dr. George Martin, for their wise counsel during the course of this doctoral undertaking. Their knowledge of systemic sclerosis, fibrosis and experimental approaches has been of great value to me. These honorable men have inspired many people from many countries and continue to do so. They have contributed immensely to understanding autoimmunity and fibrotic disease in the belief that their work here will aid in a cure for systemic sclerosis, a complex and devastating disease. Their combined added value cannot be measured by standard means, but the foundations they laid for us to build on, are solid and will endure. I would also like to thank my wife, Christina de López for her patience and enduring support and sleepless nights as I worked on this wonderful project. I would also like to thank UCL for this great opportunity.

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List of Abbreviations

αSMA	alpha smooth muscle actin
ACA	anti-centromere antibodies
ARA	anti-RNA polymerase antibodies
ATA	anti-topoisomerase antibodies
Bleo	bleomycin
BMP	bone morphogenetic protein
bp	base pair
CNV	copy number variation
COL1A2	collagen type I, alpha 2
CTGF	connective tissue growth factor
dcSSc	diffuse cutaneous systemic sclerosis
DKO	double knockout (mouse)
DMEM	Dulbecco's modified Eagle's medium
EC	endothelial cell
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EndMT	endothelial-mesenchymal transition
ERK	extracellular signal regulated kinase
ET	endothelin
FCS	foetal calf serum
FGF	fibroblast growth factor
GCID	gene card ID
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage stimulating factor
GWAS	genone-wide association study
H&E	haematoxylin and eosin
HIF	hypoxia inducible factor
HRP	horseradish peroxidase
IFN	interferon
IL	interleukin
IPF	idiopathic pulmonary fibrosis

JNK	c-Jun N-terminal kinase
KO	knockout (mouse)
IRF	interferon regulatory factor
LacZ	β galactosidase marker gene
LAP	latency associated peptide
LTBP	latent TGFβ binding protein
lcSSc	limited cutaneous systemic sclerosis
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRSS	modified Rodnan skin score
NAC	N-acetyl cysteine
NO	nitric oxide
NOS	nitric oxide synthase
OD	optical density
P3H	prolyl 3-hydroxylase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PHT	pulmonary hypertension
P/S	penicillin and streptomycin
qPCR	quantitative polymerase chain reaction
PSR	picrosirus red
RCS	rat chondrosarcoma cell
ROS	reactive oxygen species
SD	standard deviation
SELDI	surface enhanced laser desorption/ionisation
SEM	standard error of the mean
SKO	single knockout
SMAD	mothers against decapentaplegic homolog
SNP	single nucleotide polymorphism

SSc	systemic sclerosis, scleroderma
STAT4	signal transducer and activator of transcription 4
TβR	TGFβ receptor
TGFβ	transforming growth factor beta
Th	T helper
TLR	toll-like receptor
Tm	meltpoint temperature for amplican
TNFα	tumour necrosis factor alpha
Tsk	tight skin mouse
U3RNP	anti-fibrillarin
VEGF	vascular endothelial growth factor
VWF	Von Willebrand factor

Chapter 1. Introduction

1. Biomechanisms in systemic sclerosis: overview and current concepts

1.1 Systemic sclerosis: overview of disease manifestation and treatments

Systemic sclerosis (scleroderma, SSc) is a disease identified clinically by the presence of skin fibrosis, and associated with autoimmunity, vascular damage, and progressive fibrosis of internal organs (Varga and Abraham, 2007, Gabrielli et al., 2009, Allanore et al., 2015). It is not known how the autoimmunity, vasculopathy and fibrosis are linked, but vascular dysfunction and autoimmunity are present at the earliest stages of the disease supporting the idea that these are early initiating events, whereas fibrosis progresses throughout and typically damages organ function in later stages (Kahaleh and LeRoy, 1999, Nihtyanova et al., 2014).

SSc affects 100-250 persons per million of population, and varies in presentation from relatively mild peripheral skin involvement to widespread skin fibrosis involving most areas of the body, causing life-threatening organ fibrosis in the most severe cases (Mayes et al., 2003, Allcock et al., 2004, Andreasson et al., 2014). SSc is more prevalent amongst females, with a female to male ratio of more than 4:1, and has a typical age of onset in the 4th decade (Mayes et al., 2003). Some familial cases are seen, but the disease has also been linked to industrial exposure, and viral infections, suggesting that environmental factors as well as the genetic background have a role in the pathogenesis (Stephens et al., 1994, Arnett et al., 2001).

In 1980 the American College of Rheumatology proposed a set of criteria for the diagnosis of SSc (Masi et al., 1980). At that stage the diagnosis depended on the presence of skin thickening proximal to the metacarpophalangeal joints, which was the major criterion, or at least two of the following minor criteria; 1) sclerodactyly, 2) pitting scars of the fingertips, or loss of finger pad substance, or 3) bibasal pulmonary fibrosis. These criteria were revised and updated in 2013 as American College of Rheumatology/European League against Rheumatology SSc classification criteria (Table 1.1) (van den Hoogen et al., 2013a) leading to improved diagnostic sensitivity (Pope and Johnson, 2015). These criteria have been

used at the Royal Free Centre for Rheumatology, for classification of patients as SSc.

Current medical management of SSc includes use of vasodilator therapies aimed at reducing vasoconstriction and vascular proliferation, as well as the use of immunosuppressive regimens repurposed from organ transplantation medicine, or from the treatment of other autoimmune diseases (Del Papa and Zaccara, 2015). However current treatment is of only modest efficacy, in many cases failing to prevent progressive fibrosis or vasculopathy, and in general there is an unmet need for truly effective disease-modifying therapies for SSc (Denton and Ong, 2013). An improved understanding of the disease's aetiology and the biomechanisms involved would enable specific targeted therapies for this disfiguring, painful and life-threatening disease.

1.2 Systemic sclerosis: a historical perspective

Hippocrates described individuals whose clinical presentation resembled today's SSc patients (David, 1981). Subsequently a painting of Archangel Raphael and Bishop Francisco Domonte by Murillo in 1680 A.D. showed what appear to be telangiectasia (macroscopically visible dilated skin vessels common to scleroderma) on the face and hands of the bishop (Dequeker et al., 1995). However the first accurate description of SSc is attributed to Carlo Curzio of Rome, who described the condition in more detail in 1753 (Capusan, 1972). The strong association between SSc and Raynaud's phenomenon was first noted by Maurice Raynaud in 1865. The first description of major visceral involvement appeared in 1863 when Auspitz observed a young male who developed headache, loss of vision, generalized convulsions and died 2 years after the onset of SSc, the post mortem showing shrunken fibrotic kidneys (Auspitz, 1863), but a clear understanding of the systemic nature of the disease was not apparent until the middle of the twentieth century (Moore and Sheehan, 1952, Goetz, 1951). More recently authors have emphasized the ubiquitous nature of the severe vascular pathology associated with SSc (D'Angelo et al., 1969), as well as the frequent involvement of internal organs (Black, 1993).

Item	Sub-items	Score
Skin thickening of the fingers	Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints	9
	Whole finger skin thickening, distal to the metacarpophalangeal joints	4
	Puffy fingers	2
Fingertip lesions	Ulceration of fingertips	2
	Fingertip pitting scars	3
Presence of telangiectasia		2
Abnormal nail-fold capillaries		2
Pulmonary hypertension and/or interstitial lung disease		2
Raynaud's Phenomenon		3
Presence of scleroderma-related autoantibodies (anticentromere (ACA), anti–topoisomerase-I (ATA), anti–RNA polymerase III (ARA).		3
	Total score: >9= classified as SSc	

Table 1.1 American College of Rheumatology/European League Against Rheumatism systemic sclerosis classification criteria 2013 (van den Hoogen et al., 2013b). In this updated classification, defined clinical and laboratory characteristics are scored. The presence of skin thickening of the fingers spreading proximal to the metacarpophalangeal joints which scores 9, as well as 7 other criteria, each scoring 2-4, are included. A total score of 9 or greater is sufficient for classification as SSc, and now used for inclusion in clinical trials. These criteria have increased sensitivity when compared to the original 1980 criteria, particularly for patients with mild limited skin involvement but with other highly specific findings such as the presence of hallmark auto-antibodies. Pulmonary hypertension is defined as pulmonary arterial hypertension found on right heart catheter. Interstitial lung disease is defined as lung fibrosis mainly at the bases, seen on high resolution CT scan or chest x-ray, or detected clinically.

1.3 Systemic sclerosis clinical disease subsets

Disease subsets in SSc have been defined based on the pattern of clinically apparent skin involvement (Figure 1.1) (Table 1.2) (LeRoy et al., 1988). This system of classification is pragmatic to apply, and helps predict the course of the disease, including the likely pattern of internal organ involvement.

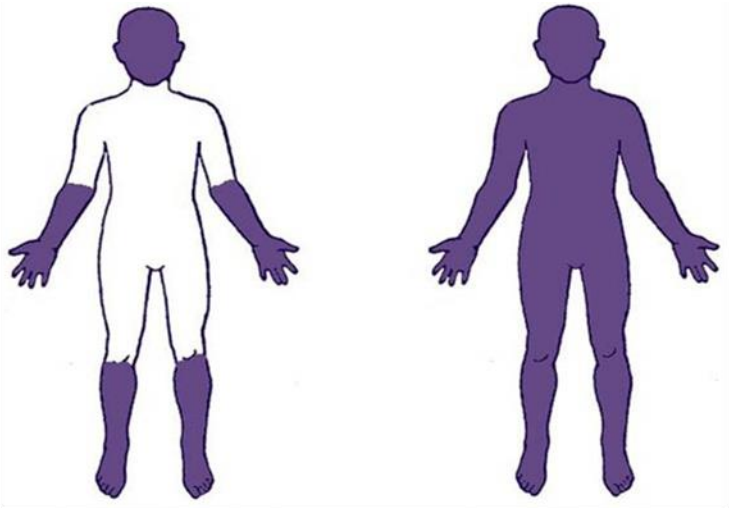


Figure 1.1 Classification of systemic sclerosis subgroups based on the pattern of cutaneous involvement. Patients in whom clinically apparent skin fibrosis (mauve) is restricted to the extremities and the face are classified as lcSSc. Skin involvement spreading proximal to the elbow flexures or extending onto the trunk is classified as dcSSc.

There are two main clinical disease subsets. The first is diffuse cutaneous SSc (dcSSc) in which the fibrotic skin involvement is extensive, spreading proximally to involve the upper arms, the trunk and abdomen (Figure 1.2). This pattern of skin involvement carries a worse prognosis and is more likely to cause associated pulmonary fibrosis or renal involvement, which are major determinants of survival (Penn et al., 2007, Nihtyanova et al., 2014).

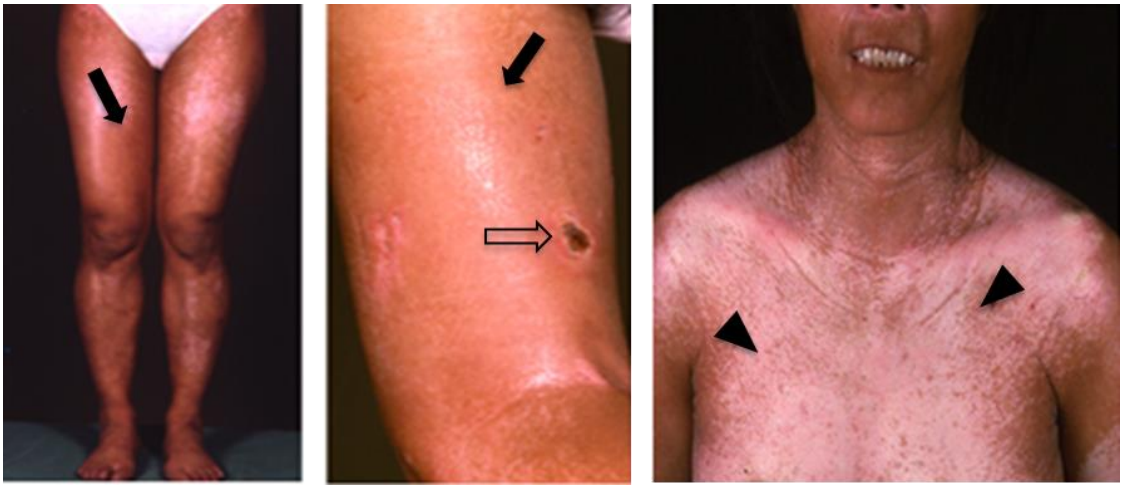


Figure 1.2 Severe diffuse cutaneous systemic sclerosis Severe thickening of skin, proximal to elbows and knees (black arrows), and spreading onto the trunk has occurred. This lady is originally from the Philippines and had café au lait skin. Greatly altered skin pigmentation is also seen in the disease as shown here (black arrowheads indicating areas of both decreased and increased pigmentation). In addition, non-healing skin ulceration has occurred as a further distressing skin complication (black outline arrow).

The other main clinical subgroup is limited cutaneous SSc (lcSSc) in which the skin involvement is less widespread and restricted to the hands and face (Figure 1.3). With this pattern of skin involvement severe vascular complications such as severe Raynaud's phenomenon (Figure 1.4), critical peripheral vascular ischaemia and pulmonary hypertension (PHT) (Figure 1.5) are seen and regarded as the major pattern of organ involvement, whereas pulmonary fibrosis and renal involvement are less common than in dcSSc (Nihtyanova et al., 2014, Steen and Medsger, 2003, Nihtyanova et al., 2008, Marie, 2006). LcSSc is more common than dcSSc by a ratio of 3:1. These two subgroups are well-defined, have some overlap in terms of clinical features and complications, and are likely to have common underlying biomechanisms.

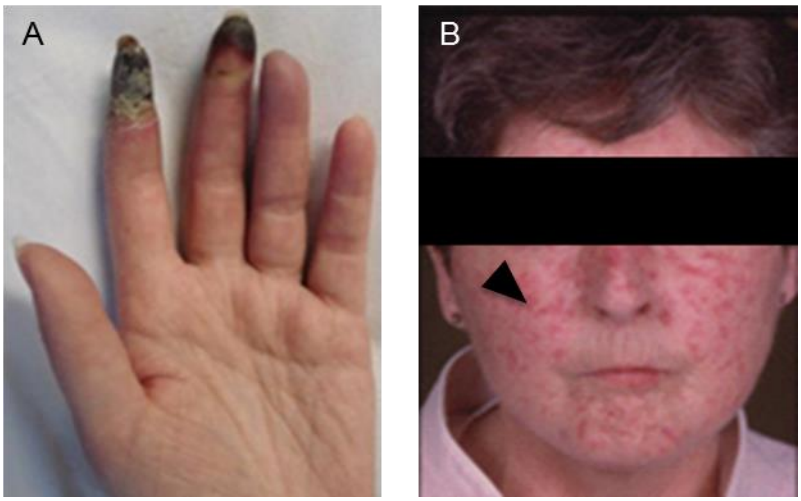


Figure 1.3 Limited cutaneous systemic sclerosis In the limited disease subset skin fibrosis is restricted to hands and face. Vascular changes can be very severe in this subgroup, as in this patient who has suffered critical digital ischaemia (Royal Free Hospital Centre for Rheumatology) (A). A small tight mouth is characteristic due to fibrosis of surrounding skin and deep tissues (B). Vascular damage linked to dysregulated repair leads to vascular abnormalities including telangiectasia (black arrowhead).

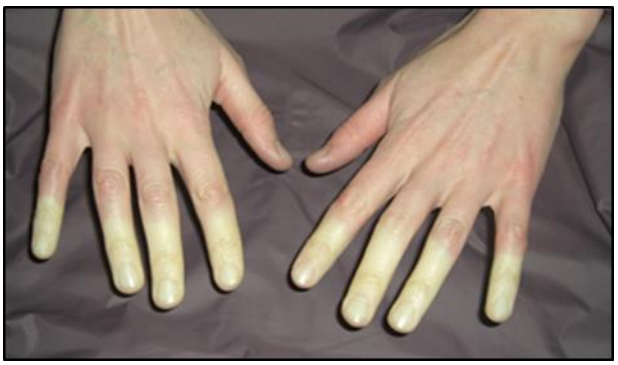


Figure 1.4 Severe Raynaud's phenomenon occurring in a patient with limited cutaneous systemic sclerosis Following exposure to cold there is marked vasoconstriction leading to the pale, poorly perfused digits as shown. Erythema and swelling occur following rewarming. Although Raynaud's phenomenon is a common finding in otherwise healthy young women, in SSc the Raynaud's and underlying vascular pathology can be very severe leading to ischemic tissue damage, pitting of the finger pulp and even loss of digits through necrosis of non-vascularized tissue.

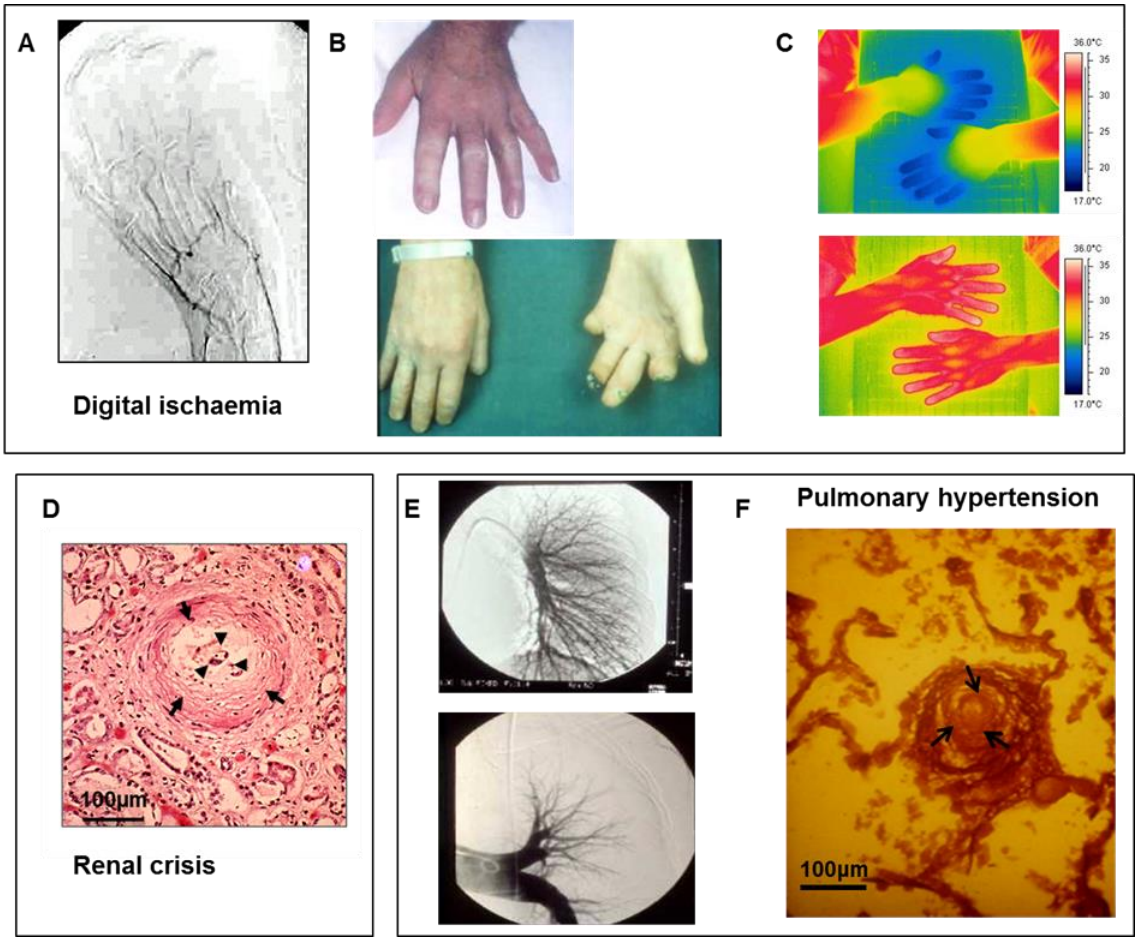


Figure 1.5 Pathologic vascular remodeling in systemic sclerosis leading to major vascular complications A remodeling process of arteries is seen in SSc patients with variable involvement of digital, renal and pulmonary vasculature. Digital arterial remodeling is shown here by angiography (**A**), correlating with degrees of clinical digital ischaemia as Raynaud's phenomenon or critical ischaemia with necrosis (**B**). Thermography shown in (**C**) can be used to quantify digital perfusion, reduced following cooling (upper figure) and normal following rewarming (lower figure) in an SSc patient with digital ischaemia. Renal artery involvement leads to the hypertensive renal crisis. as shown in (**D**) a renal biopsy specimen from an SSc patient with renal crisis showing an artery affected by intimal cell proliferation (arrows) leading to extreme narrowing of the lumen, as well as fibrinoid necrosis (arrowheads). These changes led to accelerated hypertension and acute renal failure, as well as microangiopathic haemolytic anaemia. Small (<200µm) as well as medium size (200-325µm) vessels are usually affected. Pulmonary artery remodeling leading to pulmonary hypertension (PHT) is shown by pulmonary angiogram (**E**) upper figure showing normal pulmonary artery filling, which is markedly attenuated in the peripheral lungs in SSc PHT (lower figure). Post mortem lung biopsy specimen (**F**) showing pulmonary artery occlusion by intimal proliferation (arrows), resembling the vascular changes seen in the renal pathology.

There are additional less common clinical subgroups. A localised cutaneous form, also termed morphea, in which linear cutaneous and subcutaneous involvement is seen (Figure 1.6, Table 1.2), usually without any internal organ involvement and having its onset in childhood in 50% of cases (Careta and Romiti, 2015). This subgroup is rare and may be distinct in terms of underlying mechanisms, since SSc specific autoantibodies are found in only 37-50% of cases and the usual internal organ involvement does not generally occur. Patients in this subgroup were not studied in the current thesis, partly because of the scarcity of cases, and also because of the likely distinct biomechanisms driving the disease process.

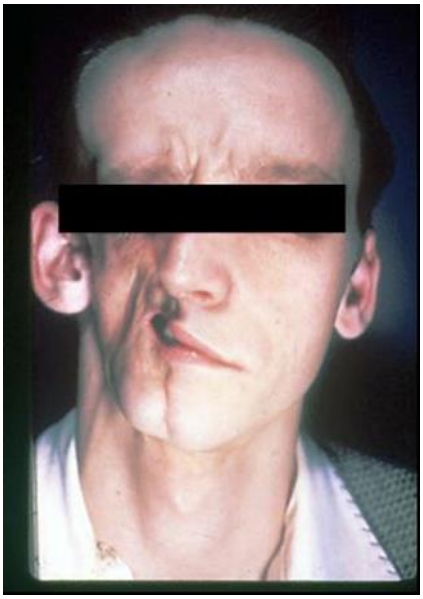


Figure 1.6 Localised scleroderma (morphea) This patient developed linear scarring of the face during childhood, termed “en coup de sabre”, and affecting the growth and development of the underlying connective tissues and facial bones. The majority of localized scleroderma patients do not have positive antinuclear antibodies nor do they typically develop systemic features such as the internal organ involvement or the vascular changes of SSc.

Finally some patients are seen in whom typical vascular or organ based SSc complications occur alongside positive SSc-associated autoantibody testing, but without any clinically apparent cutaneous involvement, and this subgroup are termed SSc sine scleroderma (Rodnan and Fennell, 1962) (Table 1.2). Again this subgroup is rare and atypical, and patients from this subgroup have not been included in the studies presented in this thesis.

SSc subsets	Defining clinical features	Pattern of internal organ involvement	Hallmark autoantibody profile
Diffuse cutaneous systemic sclerosis (dcSSc)	Skin fibrosis spreading proximally to the elbows and involving the trunk.	Tendency to pulmonary fibrosis and scleroderma renal crisis. Oesophageal and gut involvement similar in prevalence to lcSSc	Antinuclear factor present in high titer >90% of cases. ATA, and ARA classical disease-specific antibodies
Limited cutaneous systemic sclerosis (lcSSc)	Skin fibrosis limited to extremities and face.	Pulmonary fibrosis and renal crisis less common than in dcSSc. Peripheral vascular complications and PHT are major complications. PHT main cause of mortality in this subgroup.	Antinuclear factor present in high titre >90% of cases. ACA classical disease-specific antibody.
Localized scleroderma (morphea)	Linear or dermatomal pattern of skin involvement. Predominance of onset in childhood or adolescence. Scarring and growth retardation of underlying tissues.	Internal organ involvement rare. Iritis, seizures, pericarditis reported but infrequent.	Antinuclear factor present in 37-50% of cases. Disease specific antinuclear factors not described.
Systemic sclerosis sine scleroderma (ssSSc)	Absence of skin involvement but with typical organ fibrosis or scleroderma vascular disease, plus SSc-associated autoantibody.	Vascular disease prominent. Pattern of organ involvement resembles lcSSc. Nailfold capillaroscopy or autoantibody profile may clarify diagnosis.	Antinuclear factor profile similar to limited disease.

Table 1.2 Systemic sclerosis disease subsets, definitions, and clinical features This system of classification is based on the clinical pattern of cutaneous involvement is largely pragmatic and gives valuable risk stratification with respect to the likelihood of internal organ involvement. DcSSc and lcSSc are the two major clinical subgroups, and patient materials from these subgroups are studied in this thesis.

1.4 Systemic sclerosis classification by autoantibody subgroup

More than 90% of SSc patients test positive for antinuclear factor autoantibodies. The presence of these autoantibodies, which are in general

SSc specific, also helps classify the patients with regard to the likely clinical subgroup and probable pattern of internal organ involvement. (Hamaguchi, 2010). The two most common SSc-associated antibodies are anti-centromere antibody (ACA) which correlates with limited cutaneous involvement and risk of PHT (Gliddon et al., 2011), and anti-topoisomerase I antibody (ATA) which is associated with diffuse cutaneous involvement and an increased risk of pulmonary fibrosis (Table 1.3) (Hamaguchi, 2010, Hudson et al., 2010, Mierau et al., 2011, Mandai et al., 2012). In addition, anti-RNA polymerase I and III (ARA) is an SSc specific autoantibody, which is very useful clinically as its presence represents a major risk factor for the development of scleroderma renal crisis (SRC) (Bunn et al., 1998, Penn et al., 2007). Interestingly, this autoantibody is also associated with underlying breast cancer in a paraneoplastic form of SSc. In this context it has been shown that somatically acquired mutations in the gene for RNA polymerase occur in the transformed cells, supporting the idea of altered self as a stimulus to the autoantibody production (Joseph et al., 2014).

Further autoantibodies described in the context of SSc include anti-PM/Scl, which is associated with SSc in overlap with inflammatory muscle disease, and anti-U3RNP which is found in severe dcSSc with cardiac and muscle involvement in Afro-Caribbean patients (Table 1.3) (Mierau et al., 2011). Furthermore anti-Th/To is a lcSSc associated autoantibody linked to increased risk of pulmonary fibrosis and PHT in these patients (Hamaguchi, 2010).

Various mechanisms have been proposed in which SSc-related autoantibodies have been linked to pathogenic steps in SSc, which are summarised in Table 1.4. The best established of these putative mechanisms include binding to and activation of endothelial cells via an unknown surface antigen (Carvalho et al., 1996), and activation of target cells including fibroblasts via the PDGF receptor (Baroni et al., 2006). The relative importance of these mechanisms in the overall scheme of SSc pathogenesis is yet to be clearly established.

Scleroderma related antibody	Predominant disease subtype	Clinical phenotype
Anti-centromere (ACA)	lcSSc	Severe Raynaud's, digital ischemia, pulmonary hypertension, improved overall prognosis.
Anti-topoisomerase- I (ATA)	dcSSc	Pulmonary fibrosis, cardiac fibrosis, worse prognosis.
Anti-RNA polymerase I&III (ARA)	dcSSc	Hypertensive renal crisis, severe skin involvement. Association with breast carcinoma at disease onset.
Anti-PM/Scl (polymyositis)	SSc overlap with myositis	Inflammatory myopathy.
Anti-U3RNP (anti-fibrillarin antibody))	dcSSc	Inflammatory myopathy, pulmonary hypertension, cardiac involvement.
Anti-Th/To	lcSSc	Pulmonary fibrosis.

Table 1.3 Hallmark systemic sclerosis-associated autoantibodies used in clinical practice Testing for the above disease specific autoantibodies is routine in clinical practice, helping to confirm the diagnosis of SSc and also useful in risk stratification. Typical clinical disease subgroup associations as well as associated internal organ involvement are shown (Kayser and Fritzler, 2015).

Autoantibody	Proposed mechanism of action in SSc	Reference
Anti-endothelial cell antibody	Activation of endothelial cells, leading to cytokine release, adhesion molecule expression, and enhanced inflammatory cell adhesion	(Carvalho et al., 1996)
Anti-PDGF receptor antibody	Binding to PDGF receptor induces signal transduction and downstream fibroblast activation in SSc patients	(Baroni et al., 2006)
Anti-fibrillin-1 antibody	Increase bioavailability of TGFβ by inducing release from fibrillin-1	(Zhou et al., 2005)
Anti-NAG-2 antibody	Cross reaction with cytomegalovirus peptide. Binding to NAG-2 on endothelial cells and induction of apoptosis	(Lunardi et al., 2000).

Table 1.4 Proposed functional pathogenic autoantibodies in systemic sclerosis Many studies have investigated possible direct pathogenic effects of SSc patients' autoantibodies, and various biomechanisms have been proposed. In some instances (anti-PDGF receptor) there has been lack of reproducibility and currently there is no clear consensus as to the relative importance of these proposed autoantibody dependent mechanisms in the disease, although abnormal B cell function may have a role in some patients (Sakkas and Bogdanos, 2016).

1.5 Systemic sclerosis pathogenesis: the genetic background

Current opinion regarding the aetiology of autoimmune diseases has led to a model in which there is a common genetic background which predisposes to autoimmune inflammatory responses, combined with an initiating insult from the environment, leading to a persistent damaging autoimmune process (Diaz-Gallo and Martin, 2012). In SSc, a body of evidence supports a genetic component to the disease, which in keeping with this model, predisposes to an environment triggered autoimmune inflammatory process.

As mentioned already SSc occurs more frequently in females by a ratio of at least 4:1 consistent with female gender as a major genetic risk factor for the disease (Ranque and Mouthon, 2010). This increased frequency in females might be explained by a number of factors including X-chromosome dosage (Selmi et al., 2006), skewed X-Chromosome inactivation (Invernizzi et al., 2008), and the effect of the estrogen hormonal environment on pathogenic cells (Rubtsov et al., 2010). Work presented in Chapter 3 of this thesis demonstrates a possible genetic variation associated with disease susceptibility in males only.

Racial effects also influence susceptibility to SSc, which is more common among Afro-Caribbean than Caucasians or Japanese (Mayes et al., 2003). Also in Afro-Caribbeans, Hispanics, and Native Americans, patients exhibit a more aggressive disorder with higher morbidity and mortality (Mayes et al., 2003, Reveille, 2003). For example Afro-Caribbeans with SSc are more likely to develop a severe form of interstitial lung disease (Beall et al., 2007).

Also relevant to the question of race, there has been interest in the high prevalence of SSc in Choctaw American Natives, resident in South West Oklahoma, which is some three times higher than other ethnic control populations (Arnett et al., 1996). This cluster of cases of a single ethnicity offered the opportunity to study the disease in individuals of limited genetic heterogeneity, comparing the frequency of polymorphisms in cases versus matched controls from the same ethnic group. Some 20 of these patients were studied further by a genome wide analysis which confirmed specific loci linked to disease susceptibility adjacent to genes encoding secreted protein acidic and rich in cysteine (*SPARC*), major histocompatibility genes on chromosome 6, as

well as topoisomerase (*TOPOI)*, and fibrillin 1 (*FBN1*) genes, all of which are relevant to SSc current pathogenic models. Choktaw SSc patients demonstrated common microsatellite markers in the region of the *SPARC* gene (within 5cM), indicating common ancestory influencing this region of the chromosome. In non-Choktaw patients from several ethnic groups, SNPs in the 3' untranslated region of the *SPARC* gene were shown to be associated with disease susceptibility (Zhou et al., 2003). However, this finding could not be reproduced in a second study, which analysed DNA from UK Caucasians with SSc and was conducted at the Royal Brompton and Royal Free Hospitals, questioning its validity (Lagan et al., 2005). Further investigation of a region on chromosome 15 associated with SSc susceptibility in Choktaws, confirmed *FBN1* as the likely disease-associated locus (Tan et al., 2003). Since fibrillin 1 is a site of latent transforming growth factor beta (TGFβ) sequestration in the deep dermis, it is highly plausible that genetic polymorphisms in this gene alter the susceptibility to SSc through an effect on the bioavailability of TGFβ (Raghunath et al., 1998). It is possible that an environmental insult present in the area of high prevalence in Oklahoma has triggered the disease preferentially in individuals expressing certain disease susceptible haplotypes.

Racial variation in the severity of SSc-related fibrosis may be explained further by altered sensitivity to endogenous anti-fibrotic pathways. For example workers from North Carolina have shown that lung fibroblasts from Black Americans with SSc lung fibrosis are intrinsically resistant to hepatocyte growth factor (HGF)-dependent inhibition of pro-fibrotic responses to TGFβ (Bogatkevich et al., 2007a, Bogatkevich et al., 2007b). These findings could have therapeutic implications since there are currently anti-fibrotic peptides designed to induce signaling downstream of HGF in disease lung fibroblasts (Bogatkevich, presentation SSc Workshop, Lisbon 2016).

Within families the risk of disease concordance for SSc is increased 13-fold above the general population, for first degree relatives, and 15-fold for siblings of patients with SSc (Arnett et al., 2001). In multi-case families, the disease is concordant for the type of SSc autoantibody produced as well as HLA genotype, but not for clinical disease subset (Assassi et al., 2007).

However, it has been estimated that SSc disease heritability is approximately 0.8% (Feghali-Bostwick et al., 2003), and while each of the above observations gives some support to the idea of a genetic predisposition there have been descriptions of important environmental triggers for the disease. Environmental factors generally accepted as having a role in SSc pathogenesis include exposure to fibrosis inducing factors such as silica dust, exposure to organic solvents, as well as viral infections including CMV (Mora, 2009). Some of the clustering within families or within racial groups could be explained by common environmental exposures, or common social or dietary factors.

As with other autoimmune diseases the genetic background to SSc has been investigated using both a candidate gene approach, in which polymorphisms are studied in genes having some *a priori* link to the pathogenesis, and a genome wide association study (GWAS) approach, in which the frequency distribution of a large number of single nucleotide polymorphisms (SNPs) is compared between SSc (typically 2,000-5,000 patients) and healthy controls in an unbiased approach (Gorlova et al., 2011, Allanore et al., 2011, Bossini-Castillo et al., 2012). More recent studies have used next generation sequencing to identify rare genetic variants linked to the disease (Gao et al., 2016, Mak et al., 2016)

In the following sections, genetic factors with an established link to SSc susceptibility will be presented combined with discussion of the implications and possible function of the encoded proteins. Evident limitations to the interpretation of genetic associations are the pleotropic nature of many of the genes, the difficulty in establishing a hierarchy of importance for the genes identified, and the possibility that the gene products are functioning downstream of some initiating environmental influence which has overriding importance.

1.5.1 Pro-fibrotic gene associations with systemic sclerosis

Due to its involvement in fibrosis and tissue repair, connective tissue growth factor (CTGF) has been extensively studied in SSc, where it is believed to act downstream of TGFβ, by enhancing integrin mediated attachment of

fibroblasts to the extracellular matrix and influencing the interaction of TGFβ and BMPs with their receptors (Abreu et al., 2002). Because of this, workers at the Royal Free studied an SNP in the promoter region of CTGF in SSc and control DNA samples and found that a SNP which alters AP-1 transcription factor binding was linked to SSc susceptibility (Fonseca et al., 2007). This finding was replicated in a Japanese population and has been demonstrated to alter basal CTGF levels in fibroblasts (Kawaguchi et al., 2009). Otherwise, surprisingly little has emerged regarding pro-fibrotic factors and their polymorphisms associated with SSc, whereas a wealth of data have been published regarding inflammatory and immunity related genes (see below).

1.5.2 Innate immunity genes linked to systemic sclerosis development

Previously, interferon regulatory genes have been linked to autoimmune disease including SLE. In SSc, interferon regulatory factor 5 (IRF5) gene polymorphisms were significantly linked to SSc development (rs20046040 and rs377385-rs2004640-rs1095421) to SSc development overall, as well as diffuse SSc subtype and lung fibrosis in SSc (Dieude et al., 2009a). This has also been shown in Japanese patients (Ito et al., 2009). In the first Caucasian GWAS in SSc (2,296 SSc patients and 5,171 controls) rs1048863 in IRF5 was revealed as the strongest SNP association with SSc development except for HLA genes (Radstake et al., 2010), a finding which was subsequently confirmed in a European GWAS study (Allanore et al., 2011).

In addition, innate immune regulatory gene MIF (macrophage migration inhibitory factor, (Gregersen and Bucala, 2003)) has significant SSc associations via polymorphisms in the promoter region (Wu et al., 2006). MIF is a pro-inflammatory cytokine which binds to CD74 on other immune cells to trigger an acute inflammatory response. The high expression levels of this haplotype of MIF, is linked to lcSSc development (Wu et al., 2006).

ITGAM encodes an alpha-integrin (CD11b) which forms part of the αMβ2 integrin, involved in leukocyte adhesion and migration, and polymorphisms in the ITGAM gene have been associated with SLE (Nath et al., 2008) due to the presence of a non-synonymous SNP in exon 3, which affects the structure of

the protein. This SNP was also shown to be associated with SSc overall, as well as to lcSSc (Carmona et al., 2011).

1.5.3 Adaptive immunity genes including the HLA associations in systemic sclerosis

Since SSc is considered to be an autoimmune disease and because of the known autoantibody link, many studies have focused on the HLA genes in SSc (Fanning et al., 1998, Kuwana et al., 1999, Gilchrist et al., 2001, Simeon et al., 2009, Arnett et al., 2010). In general, disease associations have been found for the HLA-DR loci, but not for the class I MHC loci. This would support a role for T helper cell responses governed by interaction with class II and antigen, rather than cytotoxic T cells or other class I restricted responses.

Another general finding has been that HLA haplotypes show the strongest association with autoantibody subsets rather than directly linked to the disease overall or SSc disease subset (Kuwana et al., 1999). The strongest associations have been found for HLA-DRB1*1104 and HLA-DRB1*0701 (Arnett et al., 2010). The best replicated HLA-autoantibody association is between HLA-DQB1*0501/HLA-DRB1*0101 and the ACA antibody (Arnett et al., 2010, Simeon et al., 2009). Also HLA-DRB1*1104 and HLA-DPB1*1301 is linked to the ATA antibody in SSc, and the HLA-DQB1*0302 haplotype is associated with ARA antibodies (Fanning et al., 1998, Gilchrist et al., 2001, Arnett et al., 2010).

Other genetic factors linked to adaptive immunity include STAT4. This signaling molecule has a profound influence on effector T cell differentiation (Lim and Cao, 2006), including Th17/Treg balance, and IL-12 and IL-23 induced T cell differentiation (Toussirot, 2012). The rs7574865 polymorphism in STAT4 is linked to both SSc (Rueda et al., 2009) as well as other autoimmune diseases (Martinez et al., 2008). This SNP is linked to limited subset SSc development in both Caucasian as well as Japanese groups (Rueda et al., 2009, Tsuchiya et al., 2009). The above candidate gene studies were supported by two subsequent GWAS studies in which STAT4 SNPs were amongst the most differentially expressed polymorphisms linked to SSc development (Radstake et al., 2010, Allanore et al., 2011).

1.5.4 Role of B lymphocyte-associated genes in systemic sclerosis

Additionally, two B cell related genes, BANK1 and BLK1 have been linked to SSc development (Dieude et al., 2009b) (Gourh et al., 2010a, Ito et al., 2010). Also the OX40 ligand (OX40L , gene =*TNFSF4*) (Gough and Weinberg, 2009) which influences B-cell proliferation, was studied in SSc and linked via 3 polymorphisms, rs1234314, rs2205960, and rs844648, (Gourh et al., 2010b) supported by (Bossini-Castillo et al., 2011). Also the *TNFAIP3* gene (tumor necrosis factor alpha-induced protein 3) (Tavares et al., 2010) which regulates NFkB pathway and B cell apoptosis has been studied in SSc (Dieude et al., 2010), and the TNFAIP3 rs5029939 SNP was linked to the development of diffuse SSc as well as fibrosing alveolitis, all consistent with a role for B cells in SSc pathogenesis.

1.5.5 Genetic factors influencing T cell receptor signal transduction in systemic sclerosis

Further support for the role of effector T cells in SSc comes from the observation that SNPs in T cell receptor signaling molecules and regulators are linked to SSc disease susceptibility. *PTPN22* which encodes LYN (Bottini et al., 2006), a phosphatase regulating T cell receptor signaling, was found to be associated with SSc via a meta-analysis of several studies of the R620W (C1858T, rs2476601) polymorphism. The T variant is linked to SSc (Lee et al., 2012).

Furthermore GWAS studies have shown, with an unbiased approach, that CD247, the T cell receptor complex zeta chain, is SSc associated (Radstake et al., 2010, Dieude et al., 2011), and this is considered one of the strongest disease associations for SSc development as a whole. This finding supports the idea that antigen specific T cell responses have a role in the pathogenesis of SSc.

The various SNPs associated with SSc and SSc subgroups are summarized in Figure 1.7. Strengths of the SNP genetic approach are that large numbers of samples can be rapidly analysed using the SNP genetics chip arrays, to give statistically meaningful results. Limitations of the approach are that some SNPs are not included in these arrays, and important associations with rare haplotypes may be missed because of the small numbers of positive

hits obtained in SSc or controls. One further limitation is due to the large number of comparisons being made between the SNP frequency profiles, with greater than 1 million SNPs being compared. This leads to a multiple comparisons problem when comparing the SNP profile of healthy controls and SSc patients, such that a P value of less than 10^{-9} is required to reach significance, requiring a large sample size in a rare disease. This barrier could be overcome by studying forms of genetic variations present at lower frequency than the large numbers of SNPs present in the genome.

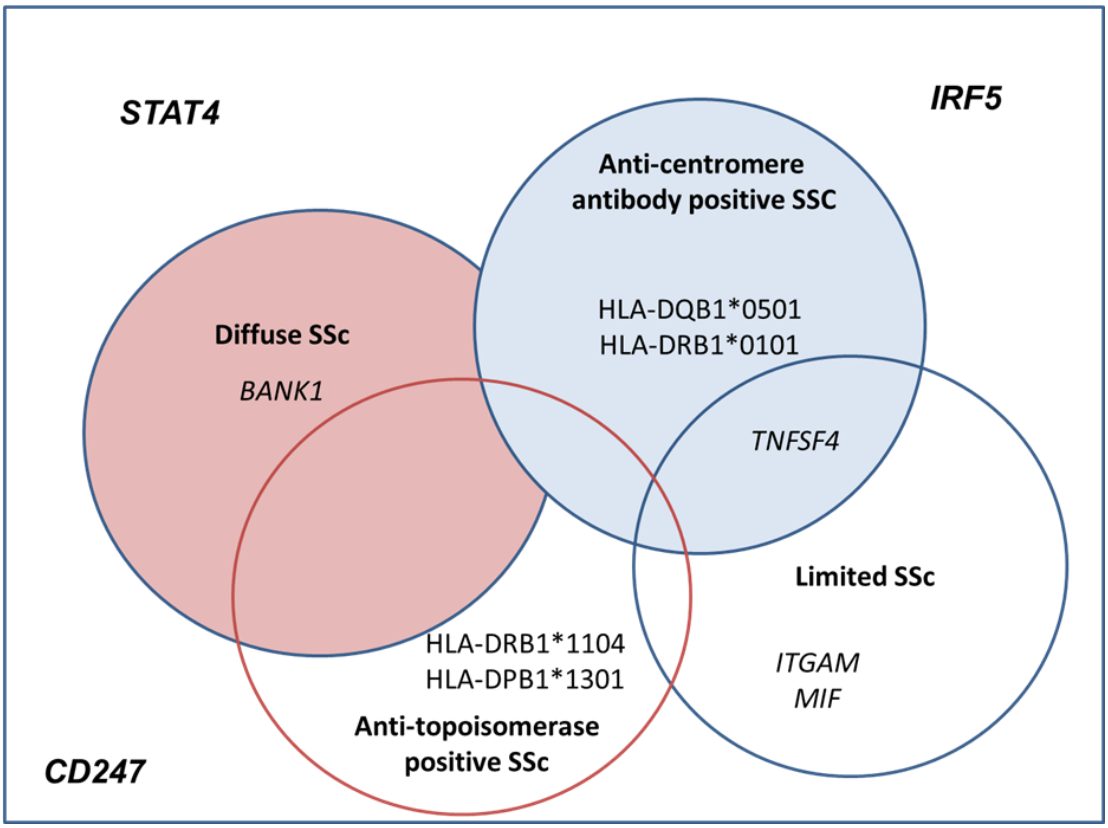


Figure 1.7 Summary of candidate gene and GWAS analysis of systemic sclerosis Genes with established disease associated SNPs are shown. The strongest associations for overall SSc development are with those factors shown in bold italic, *STAT4*, *CD247*, and *IRF5*. HLA haplotypes are more strongly associated with autoantibody defined subsets rather than the disease overall as shown. *ITGAM* and *MIF* SNPs are linked to limited disease subset whereas *BANK1* SNPs associate with diffuse disease (Dieude et al., 2009b).

1.5.6 Potential role of copy number variation

The second most common form of genetic variation which has emerged more recently but has been extensively studied over the last decade is termed copy number variation (CNV) (Sebat et al., 2004, Redon et al., 2006). CNVs are defined as genetic variants involving segments of the genome of at least 1000

base pairs. These include deletions, inversions, or insertions leading to one or more CNVs in certain genes (Figure 1.8). In an apparently healthy individual 1000s of such variations are seen in the germ line DNA, with possible other CNVs acquired in differentiated cells second to environmental stress, ageing, or a failure in DNA repair. The study of CNVs in SSc could lead to new insights into the disease and would provide additional non-overlapping information about genetic susceptibility, which could be used to complement the SNP based approach. CNV analysis has the additional advantage that there are fewer CNVs than SNPs (thousands as opposed to millions of comparisons), so that smaller sample sizes can be used.

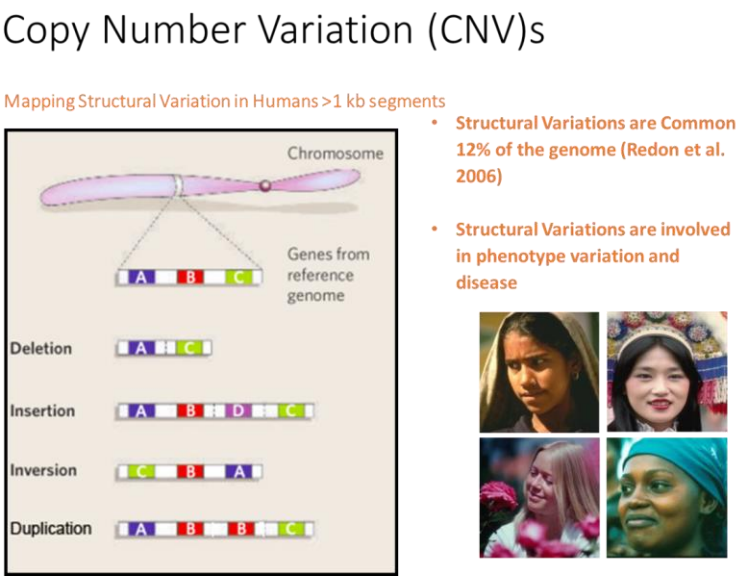


Figure 1.8 Copy number variation (CNV) explanation Genetic variants of greater than 1000 base pairs are frequent within the genome of healthy individuals, including deletions, insertions, reversals of the sequence, and duplications leading to multiple copy numbers. CNVs account for common variants, for example the ability to detect odors, (Veerappa et al., 2013), other inherited traits, and can confer susceptibility or resistance to various diseases (Zarrei et al., 2015).

0.5% of the genome differs in CNV between two randomly selected individuals (Sebat et al., 2004). Large CNVs were found to occur non-randomly through the genome affecting less than 5% of the genomic DNA in a study that included 270 individuals (McCarroll et al., 2008). These findings raise important questions. Are some segments of the genome more mutable than other segments? Why are CNVs so prevalent? Do they confer some survival advantage, for example resistance to infection or improved wound healing?

CNVs can occur in the form of deletions, where a large segment of genomic DNA is lost, insertions where a segment is inserted, duplications where a segment is duplicated, or inversions where the sequence is inverted (Figure 1.8). CNVs can affect genes or non-coding regulatory elements. They may become more prevalent with the passage of generations due to some advantageous survival effect, or more often become less prevalent or disappear if they are deleterious or non-beneficial.

Increased copy number may influence gene dosage causing increased transcription, if the whole gene and regulatory elements are duplicated, ultimately leading to enhanced protein levels. In addition, other genes in the vicinity may be enhanced in expression due to the effects of an upstream CNV. Alternatively if a gene is only partially duplicated this may suppress gene expression by insertion of a transcription termination signal (Henrichsen et al., 2009b, Cahan et al., 2009).

In general, CNV influenced genes are expressed in a limited number of tissues, whereas genes unaffected by CNV are more likely to be widely expressed housekeeping genes. Examples of genes known to be affected by CNVs include olfactory receptors, β-defensins, and immunoglobulins (Hasin et al., 2008, Hollox, 2008, Veerappa et al., 2013, Abdelmotelb et al., 2014). CNVs rarely include evolutionary conserved genes which are stable and without CNVs, having evolved over 10s of millions of years.

CNVs are believed to be formed by the mechanism of non-allelic homologous recombination followed by segmental duplications (Stankiewicz and Lupski, 2002), or through insertion of Alu short interspersed nuclear elements (Wang and Huang, 2014). In addition, non-homologous end joining can be the mechanism responsible for CNV formation (Linardopoulou et al., 2001).

Limitations to the current understanding of CNVs include lack of knowledge of the true allelic frequency of many CNVs within the healthy population. Also, the extent in terms of length and breakpoint for individual CNVs is unknown. The 1000 genomes project, in which 1200 individuals' genomes will be fully sequenced, will help to answer these questions (http://1000genomes.org). It will also help to answer the question regarding certain regions of the genome being more prone to variation.

Positive selection for advantageous genes whose expression is increased by CNV can operate over a few generations to increase their occurrence in a population. An example of an advantageous CNV is the increased copy number of CCL3L1, which confers resistance to HIV (Paximadis et al., 2013). CNVs affecting fertility or fecundity would also be positively selected. In addition, outside of germ line DNA, CNVs may be acquired by cells in the mature individual, which confer cell survival advantage as in some tumours where there is clonal expansion of cells with certain growth promoting CNVs. It is possible that in fibrotic conditions such as SSc, the overgrowth of myofibroblasts could include oligoclonal expansion based on acquired CNVs. This could be addressed by whole genome sequencing of lesional fibroblasts versus fibroblasts taken from the uninvolved skin.

Nguyen et al have asked whether CNVs present in the human genome have largely been affected by positive selection (Nguyen et al., 2008). If positive selection is influencing CNV then they should be enriched in encoding sequences when compared to the genomic DNA as a whole. Secondly any genes included in these sequences, if they are subject to positive selection, should show evidence of protein selection (increased ratio of non-synonymous to synonymous polymorphisms). In fact both are true and high gene density is a feature of CNVs. Also, increased non-synonymous polymorphisms, indicating protein selection, are a feature of CNVs (60% above background genomic DNA rate) (Perry et al., 2008).

In this thesis, CNVs in a number of candidate genes were studied for linkage to SSc disease susceptibility. Gene associated candidate CNVs were selected on the basis of having some *a priori* pathogenic link to SSc, based on the current understanding of the disease process. CNVs in genes relevant to extracellular matrix, female hormonal influence, and vascular biology were selected for study. One CNV in the promoter region in a gene involved in collagen alignment in the endoplasmic reticulum of fibroblasts (prolyl 3-hydroxylase 2, *LEPREL1*) was found to have a possible association with risk of SSc development in males. The findings are presented in Chapter 3 of this thesis. Protein levels of the encoded enzyme were studied in SSc and control fibroblasts. In addition, the importance of the gene *in vivo* is studied using the bleomycin model of dermal fibrosis in a *LEPREL1* null mouse. The bleomycin

model, which is widely used as a model of SSc fibrosis, as well as other animal models of fibrosis relevant to SSc, are discussed below in section 1.14.

1.6 Systemic sclerosis pathogenesis: the role of environmental factors

Because of epidemiological evidence linking the disease to certain occupations and environmental exposures, many studies have attempted to define important environmental factors which trigger SSc (Marie and Gehanno, 2015). It is believed that the artist Paul Klee developed SSc as a result of exposure to solvent chemicals used in oil painting, and that Klee developed severe diffuse form of the disease and eventually died as a result of renal crisis (Suter, 2014). Also there are several SSc-like industrial syndromes including silica dust induced SSc seen in miners and linked to diffuse cutaneous pattern, and ATA positive SSc with lung fibrosis (Freire et al., 2015) (Marie et al., 2015), and also polyvinyl chloride exposure-related SSc-like syndrome characterised by Raynaud's and acroosteolysis (resorption of the terminal phalanges) with sclerodematous changes in the backs of the hands and dorsal forearm areas, usually auto-antibody negative and seen in autoclave cleaners (Black et al., 1983, Ostlere et al., 1992) .

More systematic studies in which environmental exposure of SSc patients and controls was studied, have confirmed disease association with exposure to organic solvents including trichloroethylene used as an industrial degreasing agent in lathes and other machine tools (Nietert et al., 1998, Marie and Gehanno, 2015). Of interest, in model systems, trichloroethylene has been shown to induce oxidative stress in keratinocytes and to promote innate immune responses in the exposed skin of experimental rodents (Zhu et al., 2005). It is possible that the disease is triggered by environmental exposures inducing oxidant stress in epithelial cell layers for external agents affecting the surface layer, or else in endothelial layers, when environmental agents have become systemically absorbed and are blood borne.

It is therefore possible that environment has an important role in triggering the onset of SSc in some patients. However, the majority of patients do not give any clear history of industrial solvent or other known exposure. The influence of environmental exposure in the disease may be through interaction

with the patient's genetic background, or else there may be complex stoichastic interactions such as environmental agent exposure during episodes of severe Raynaud's phenomenon, which would couple oxidant stress with reduced tissue perfusion. The possible role of oxidant stress in SSc is discussed further below.

1.7 Systemic sclerosis pathogenesis: role of reactive oxygen species

It is possible that overproduction of reactive oxygen species (ROS) has a role in the initiation of vascular injury and in the persistence of abnormal fibroblast activation in SSc, (Gabrielli et al., 2009). Unlike other autoimmune conditions where overproduction of ROS is by white blood cells, in SSc the overproduction is seen in cells of mesenchymal origin, including fibroblasts, endothelial cells, as well as vascular smooth muscle cells. Fibroblasts cultured from SSc patients continue to overproduce ROS through several cycles of passage even when cultured under serum and growth factor free conditions (Sambo et al., 2001). ROS production depends on Ras/MEK/ERK signalling which has been shown to be persistently induced in SSc, and is itself induced by ROS producing a persistent feed forward loop (Gabrielli et al., 2012). In addition the ROS overproduction results in DNA damage and provokes autocrine Wnt signalling which further increases the growth and proliferation of SSc fibroblasts (Svegliati et al., 2014). In one centre SSc patients' autoantibodies were shown to induce ROS production in fibroblasts via binding to the PDGF receptor and inducing downstream signalling events (Baroni et al., 2006). However, this has not been reproduced in other centres and its overall importance in SSc remains to be confirmed.

A difficulty which arises when considering SSc pathogenesis is how to put into context the multiple various mechanisms that have been proposed and demonstrated. For example the phenomena relating to overproduction of ROS could be a central driving mechanism or alternatively a downstream epiphenomenon. To complicate matters further this is a heterogeneous disease and certain mechanisms may be of central importance in only a subgroup of SSc patients.

Possible approaches to addressing these difficulties comes from the study of mouse models, as in (Luchetti et al., 2016), or in tissue culture models systems, as in (Dooley et al., 2010). However, clearer answers may now come from the use of specific targeted biologic therapies, applied in subgroups of patients where the relevant biomechanism can be shown to be highly active. An example of this has been the studies of potent anti-TGFβ therapies which have shown trends towards benefit in well-defined SSc patient groups (Rice et al., 2015).

The anti-oxidant supplement N-acetyl cysteine has been assessed in a retrospective and non-controlled trial, showing possible benefit in SSc lung fibrosis (Rosato et al., 2011). Topical antioxidant vitamin E has been shown in a small controlled trial to improve digital ulcers in SSc (Rosato et al., 2011). Also, the synthetic antioxidant drug probucol was shown in a controlled trial to have a modest benefit against severity and frequency of Raynaud's attacks in SSc patients, and to reduce lipoprotein oxidation (Denton et al., 1999).

Taken collectively, these data support some role for ROS in SSc aetiology, but definitive clinical trials using highly effective antioxidants or drugs targeting the ERK or Wnt pathways in order to reduce the responses to ROS, are yet to be taken forward.

1.8 Systemic sclerosis pathogenesis: understanding the role of growth factors and cytokines

The growth factors and cytokines contributing to the development of SSc and the skin injury that characterises it have been extensively studied. TGFβ is present in fibrotic lesions (Gay et al., 1989, Gruschwitz et al., 1990), and is considered to be the strongest signal for scarring and fibrosis and highly relevant to skin fibrosis in SSc (Sargent et al., 2010). TGFβ is a multifunctional growth factor present in mammalians as 3 isoforms, TGFβ1-3, with highly similar protein structures(Kondaiah et al., 1990). Despite structural similarities, TGFβ ligands have distinct affinities for TGF-β receptors (Cheifetz et al., 1990). The three TGFβ ligands are produced by a number of different cell types, and the production of all three occurs during development, although TGFβ1 is the

predominant type in adults. Each TGFβ isoform has specific function *in vivo*, as demonstrated by gene deletion in mice. *Tgfb1* KO mice develop problems in utero, due to abnormal vasculature as well as haematopoietic failure, and when surviving to maturity develop an inflammatory wasting syndrome (Shull et al., 1992). The TGFβ2 KO mice have skeletal, cardiovascular as well as visual problems (Sanford et al., 1997). Furthermore, *Tgfb3* KO are born with cleft palates and fail to suckle, but are otherwise normal developmentally (Proetzel et al., 1995). Similar to their distinct roles in development, TGFβ ligands have differing effects in tissue repair. TGFβ1 is the most abundant form in adult tissues and is highly elevated in wounds, promoting myofibroblast growth and inducing collagen and other extracellular matrix components. However in embryonic wounds TGFβ3 is the most strongly induced form and TGFβ1 is present only at low levels. The addition of TGFβ3 to adult wounds in rodents has been reported to promote scar-free healing (Ferguson and O'Kane, 2004). However, recombinant TGFβ3 failed to improve scar quality in a phase III clinical trial, possibly through variation between batches of the therapeutic (Little et al., 2012). Because of its abundance in adult tissues and wounds TGFβ1 is the most widely used in tissue culture models of SSc like fibrotic responses. In animal models neutralising anti-TGFβ1/2 reduces cutaneous scarring (Shah et al., 1995). Anti-TGFβ1-3 had differential effects depending on the stage of wound healing. When applied to early phase wounds this antibody delayed healing, whereas application to middle phase wounds the treatment reduced hypertrophic scarring (Lu et al., 2005).

TGFβ1-3 are synthesised as proproteins which are then cleaved within cells to release the propeptide from the growth factor. Unusually, the propeptide has a high affinity for the growth factor, so that the propeptide, called latency associated peptide (LAP), binds as a dimer around TGFβ and these proteins are released as a complex (Miyazono et al., 1988). This protein complex is called the small latent complex and is usually bound by a disulphide bond to a further protein member of the latent TGFβ binding protein family (LTBP1-4) to form the large latent complex (Taipale et al., 1994, Rifkin, 2005). LTBPs bind to fibrillin-1, anchoring the large latent complex to the ECM, leading to a reservoir of latent TGFβ in the extracellular environment (Figure 1.9) (Rifkin, 2005, Taipale et al., 1994).

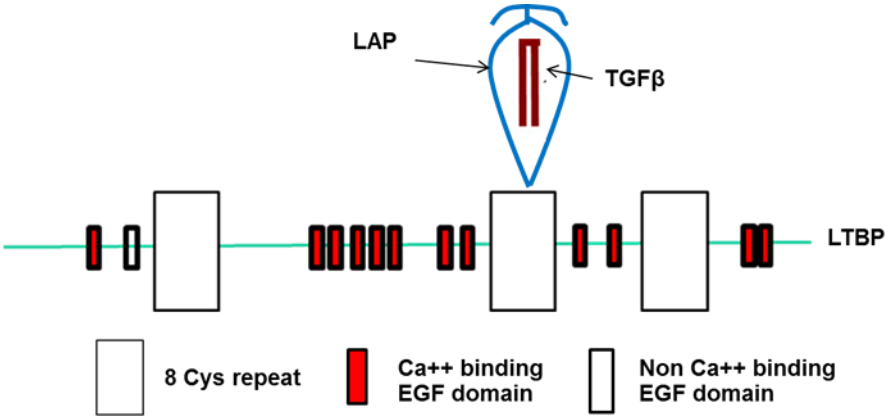


Figure 1.9 TGFβ is present within a large latent complex bound to the ECM TGFβ is synthesized as a proprotein, cleaved intracellularly to yield the LAP and the mature growth factor. The LAP associated TGFβ becomes co-valently bound to a larger protein, LTBP(1-4) and then secreted as a large latent complex, which binds to fibrillin-1 in the ECM.

TGFβ is released from this latent form in the ECM by the action of proteases, αV integrins, low pH, ROS as well as by blood coagulation factor plasmin (Lyons et al., 1988, Munger et al., 1998, Annes et al., 2003) and binds to its receptors TβRI and II, leading to initiation of signal transduction that involves cytoplasmic to nuclear translocation of members of the SMAD family of gene regulatory proteins, which bind to DNA and activate the transcription of target genes (Massague, 1998). TGFβ also activates the mitogen activated protein kinase (MAPK) family (Massague, 2012). Both P38 MAPK and c-Jun N-terminal kinase (JNK) can activate SMAD3 (Roberts, 1999). Decreased Type I collagen expression has generally been demonstrated with p38 MAPK inhibition (Roberts, 1999, Massague, 2012). In a further MAPK pathway, extracellular signal regulated kinase (ERK), provides feedback inhibition of SMAD signal transduction and SMAD1 effects on transcription (Dennler et al., 1998). The impact of ERK on collagen gene transcription is cell type-specific, with ERK activation causing increased collagen production in some cells and decreased production in others (Song et al., 1998).

Gene expression profiling of the lesional skin of a subset of patients with diffuse cutaneous SSc displays a “TGFβ responsive gene signature” (Milano et al., 2008, Whitfield et al., 2003). These patients may have a more severe disease, with more widespread skin lesions and greater risk of lung involvement, than those without this pattern of gene expression. Excessive

TGFβ activity in SSc suggests this disorder as a candidate for anti-TGFβ therapy. Of note, The Royal Free Hospital ran a clinical trial in SSc patients with an antibody neutralizing TGFβ, but it did not prove positive (Denton et al., 2007). However the antibody used in this trial was of low affinity for TGFβ1, and a more recent trial using a highly potent anti-TGFβ1-3, Fresolimumab, has shown a trend towards improved clinical skin involvement and reduction in biomarkers based on gene expression profiling (see below section on SSc therapy) (Rice et al., 2015). Although TGFβ stimulates fibroblasts in tissue culture to synthesize collagen and other extracellular matrix components, the exact sequence of events leading to the induction and perpetuation of fibrosis in patients has not been firmly established.

Other studies suggest that TGFβ also leads to the induction of the pro-fibrotic cytokine, CTGF, which appears to stimulate the overproduction of collagen when present with TGFβ, and is linked by genetic variation to SSc development (see above, section 1.5.1) (Chujo et al., 2005, Fonseca et al., 2007).

CTGF is a cysteine-rich peptide that promotes proliferation, collagen synthesis, and chemotaxis by mesenchymal cells. CTGF is over-expressed in a variety of fibrotic disorders, presumably secondary to the activation and production of TGFβ. CTGF is a secreted matricellular protein that regulates cellular proliferation and has been strongly implicated in the regulation of angiogenesis and extracellular matrix remodeling (Friedrichsen et al., 2003, Ivkovic et al., 2003). A recent *in vivo* mouse model, in which CTGF was overexpressed in mesenchymal cells, clearly demonstrates that multiple organ fibrosis can be induced by CTGF alone and independent of TGFβ activation (Sonnylal et al., 2010). Transgenic mice expressing high levels of CTGF in mesenchymal cells display characteristics of sustained multi-organ fibrosis resembling key features of SSc (Sonnylal et al., 2010). It should be noted that others have published that CTGF is elevated in established fibrotic lesions in SSc (Igarashi et al., 1995b). Thus, CTGF acts further downstream from, as well as together with, TGFβ to stimulate the fibrotic process. These agents appear to be involved in SSc-associated fibrosis, and to act as key cytokines in SSc related fibrosis (Denton and Abraham, 2001b).

In addition to having activity in the acute phase response, the important pro-inflammatory cytokine IL-6 plays a significant role in driving chronic inflammation, autoimmunity, endothelial cell dysfunction and fibrogenesis. IL-6 is a pro-fibrotic cytokine that is elevated in SSc (Feghali et al., 1992, Sato et al., 2001, Matsushita et al., 2006, Khan et al., 2012), and induces collagen I expression. It has recently been shown in normal skin fibroblasts that exposure to IL-6 plus soluble IL-6 receptor leads to enhanced collagen I production, via STAT3 phosphorylation and a protein intermediary Gremlin-1, which increases SMAD3 signaling downstream of TGFβ in these cells (O'Reilly et al., 2014).

An additional pro-inflammatory cytokine, TNFα, has a central role in the initial host response to infections and in the pathogenesis of various systemic immune-mediated diseases (Hehlgans and Pfeffer, 2005). Serum levels of TNFα are elevated in patients with SSc and favor the development of pulmonary fibrosis and pulmonary arterial hypertension (Kantor et al., 1992, Hasegawa et al., 1997, Pehlivan et al., 2012). Inflammatory arthritis can occur in patients with SSc. Infliximab and etanercept, which were designed to be TNFα inhibitors, may improve the inflammatory arthritis and disability in SSc (Alexis and Strober, 2005, Lam et al., 2007). TNFα inhibitors reduce systemic inflammation and improve endothelial cell function, and thereby could decrease the risk of progression of pulmonary arterial hypertension and acute cardiovascular and/or cerebrovascular events (Alexis and Strober, 2005, Murdaca et al., 2014). Randomized placebo controlled trials with TNFα inhibitors in patients with SSc would be helpful in confirming the potential role of these agents in the treatment of SSc.

When comparing TNFα levels in the circulation, SSc patients had higher levels compared to the control group (Pehlivan et al., 2012). In contrast to control fibroblasts, SSc fibroblasts secrete IL-6 under the influence of TNFα or interferon-gamma (IFNγ), contributing further to the inflammatory process (Antonelli et al., 2011).

In Chapter 4 of this thesis, tissue fluid sampled by a suction blister method from the involved forearm skin of SSc patients is profiled for the presence of growth factors and cytokines potentially involved in the disease process. A broad screening is performed using a 41 factor Multiplex analysis,

in order to test whether increased levels of inflammatory and pro-fibrotic factors are found in the disease environment.

1.9 Altered T-cell function in systemic sclerosis

Experimental support for activity of a T cell-mediated immune mechanism in SSc has also been found, including increased IL-2 production. The IL-2 and IL-21 loci have been associated with several autoimmune diseases, and influence the genetic susceptibility to SSc (Diaz-Gallo et al., 2013). The proliferative response to IL-2 was enhanced in SSc lymphocytes compared to matched control lymphocytes (Kahaleh and Yin, 1992). Significantly elevated numbers of high-affinity IL-2 receptors were noted on SSc cells compared to control cells. Furthermore there was an amplification of IL-2 binding in SSc cells mediated by IL-6.

IFNγ is a pleiotropic homodimeric Th1 cytokine, produced predominantly by activated inflammatory cells, which has been used effectively as a therapeutic in viral, immunological and malignant diseases (Borden et al., 2007). IFNγ has several potential anti-fibrotic actions, including inhibition of fibroblast proliferation and collagen deposition, promotion of fibroblast apoptosis, and inhibition of the production and action of the fibrogenic cytokine, TGFβ (Duncan and Berman, 1985, Gurujeyalakshmi and Giri, 1995b, Wynn et al., 2004). The anti-fibrotic effect of IFNγ has been demonstrated in animal models (Oldroyd et al., 1999) and in patients with SSc as well as with idiopathic pulmonary fibrosis (Polisson et al., 1996, Ziesche et al., 1999). Although IFNγ (Interferon gamma-1b in this case) has been therapeutically evaluated in clinical trials involving patients suffering from renal, liver, or idiopathic pulmonary fibrosis (Bouros et al., 2006, Knight et al., 2007), it has proven to be a clinical failure due to limited efficacy and various unfavorable adverse effects, which include flu-like symptoms, fatigue and provocation of autoimmune reactions (Bouros et al., 2006, King Jr et al., 2009).

T-helper cells positive for interleukin 17A (IL-17A), which have the properties of both innate and adaptive immune cells, are increased in the peripheral blood and target organs of patients with SSc (Kurasawa et al., 2000, Radstake et al., 2009), such as skin, where the cells are present in both the

superficial and deep dermis (Truchetet et al., 2013). Additionally, SSc patients with greater severity of skin fibrosis had a higher frequency of IL-17A+ cells in their skin. Levels of IL-17A are increased in synovial fluid from individuals with SSc (Truchetet et al., 2013). However, IL-17A exerts an inhibitory influence on myofibroblast differentiation from tissue-resident fibroblasts (Brembilla et al., 2013). These results suggest that although increased in SSc, IL-17A exerts a negative-regulatory influence on the development of dermal fibrosis, and is no longer considered a potential target for therapy against skin fibrosis in the disease.

The levels of IL-17 as well as other T lymphocyte derived cytokines are analysed as part of the dermal interstitial fluid and plasma profiling presented in Chapter 4 of this thesis.

1.10 Systemic sclerosis pathogenesis: paracrine signaling promoting fibrosis

In normal wound healing a number of pro-fibrotic signals feed into the tissue repair mechanisms including stimuli provided by paracrine signaling from a range of cells including epithelial cells, endothelial cells, perivascular cells, as well as immune inflammatory cells infiltrating the wound (Diegelmann and Evans, 2004). These paracrine effects have been studied in SSc, and shown to have a role (Aden et al., 2010, Good et al., 2015). For example, endothelial cell damage is believed to be an early pathogenic change in SSc (Kahaleh and LeRoy, 1999) which subsequently leads to the release of fibroblast activating factors from endothelial cells including ET-1 and ROS, as well as promoting immune cell adhesion and recruitment into the lesions (Abraham et al., 1997, Vancheeswaran et al., 1994). It is believed that some of the responses of endothelial cells have an important contribution to the downstream fibrosis in the dermis and internal organs. Also, pericytes around damaged microvessels have been shown to migrate into the dermis where they interact with fibroblasts, again enhancing the fibrotic responses (Rajkumar et al., 1999).

Studies from the Royal Free Hospital have also confirmed epithelial cell damage and activation in early SSc which in turn promotes local dermal fibroblast activation as well as the production of inflammatory (S100A9, IL-1α)

and pro-fibrotic (CTGF) factors contributing to the disease (Nikitorowicz-Buniak et al., 2014). Partially evoked epithelial to mesenchymal transition (EMT) may also feed into the dermal fibroblast activation (Nikitorowicz-Buniak et al., 2015). Since both endothelial and epithelial cell layers are specifically adapted to sense and respond to environmental factors via toll-like receptors and other innate mechanisms, it is possible that SSc pathology is initiated in these layers following environmental exposure, and then paracrine signaling spreads to the deeper fibroblast layers initiating the fibrosis seen.

1.11 Systemic sclerosis pathogenesis: increased mechanical stress in the fibrotic tissue maintains myofibroblast activation

By the time patients are seen and assessed in the clinic, they often have extensive skin changes. The skin becomes tight and oedematous, and movements are restricted by increased mechanical stress in the involved tissues. The physical properties of SSc involved skin have been studied in a number of publications (Balbir-Gurman et al., 2002) . The Young's modulus of SSc involved forearm skin has been measured at 50-80 kPa compared to 4-12 kPa for aged matched healthy controls (Sackson, 2013).

Attempts to model the increased stiffness in tissue culture systems have shown a very marked increase in myofibroblast differentiation and activation when cultured on stiff versus soft matrix. α-SMA induction, CTGF release and type I collagen production are all dependent on the mechanical stress induced transcription factor MRTF-A, which translocates to the nucleus in cells undergoing mechanical stress (Shiwen et al., 2015, Luchsinger et al., 2011). It is highly likely that these responses to stiff tissue maintain the disease process and impart resistance to therapy in the disease. It might be possible to uncouple fibroblasts and other cells from the effects of mechanical stress by use of the MRTF-A inhibitors such as CCG-1423 and related compounds (Hayashi et al., 2014).

1.12 Systemic sclerosis pathogenesis: epigenetic changes may lead to persistent myofibroblast activation

Epigenetic traits have been defined as heritable traits not encoded by the DNA gene sequence. Examples include CpG methylation which can modulate gene expression and can be passed on to daughter cells. Also modifications to histone tails greatly influence the 3-dimensional status of chromatin making some areas inaccessible to RNA polymerases whereas other regions are maintained in an open readable conformation. It is highly likely that complex disorders such as SSc are dependent on epigenetic changes which occur in the disease tissues (Altorok et al., 2014).

The SSc fibroblasts show stable and persistent abnormalities in cell culture including the production of greater amounts of collagen I, CTGF and α-SMA than normal cells, and these differences are maintained into high cell passage number (Fleischmajer et al., 1981, LeRoy, 1974, Shi-Wen et al., 2007). This persistence of the pathogenic phenotype is most simply explained by epigenetic changes, such as methylation pattern being passed from parent to daughter cells through several passages of cell culture.

More detailed studies have been published in the field of fibrosis in general showing that epigenetic changes have a role in renal fibrosis where the ERK regulatory protein RASAL-1 has been shown to be silenced by CpG methylation resulting in restrained ERK signaling in myofibroblasts (Bechtel et al., 2010). In addition, cancer associated fibroblasts have been studied in detail for epigenetic changes which maintain the invasive properties of these cells which support cancer cell invasion and metastasis (Albrengues et al., 2015).

Some attempts have been made to determine the epigenetic changes in activated endothelial cells in SSc, showing reversal of phenotypic changes with the inhibitor of DNA methylation azacytidine (Wang and Kahaleh, 2013). However it would be of interest to fully profile the epigenetic changes in SSc myofibroblasts, and possibly study the effects of therapeutic inhibitors in patients or model systems.

1.13 Systemic sclerosis pathogenesis: current model

As discussed above SSc appears to be a multifactorial and heterogeneous, disease that has been studied by many small research groups worldwide. An attempt has been made to combine the various lines of evidence presented, leading to a complex model of the disease process (Figure 1.10). This represents a personal interpretation and other researchers in the field would place greater or lesser importance to some of the biomechanisms shown.

However, since large systematically designed GWAS studies have confirmed that the genetic background is linked to autoimmunity and inflammation, these mechanisms are shown as key initiators. Also, as there is robust evidence for an environmental trigger to the disease, it is proposed in this current model that environmental insults initiate innate responses in the epithelial and endothelial cell layers, which are specifically adapted to sense and respond to environmental factors. Furthermore the complex network of downstream biomechanisms leading to a persistent myofibroblast activation, including cytokine and growth factor induction, paracrine signaling, ROS production, as well as the enhancing nature of increased mechanical stress and epigenetic changes in myofibroblasts, are shown. It seems likely that the relative importance of each of the many biomechanisms varies considerably between patients, accounting for the heterogeneous disease seen.

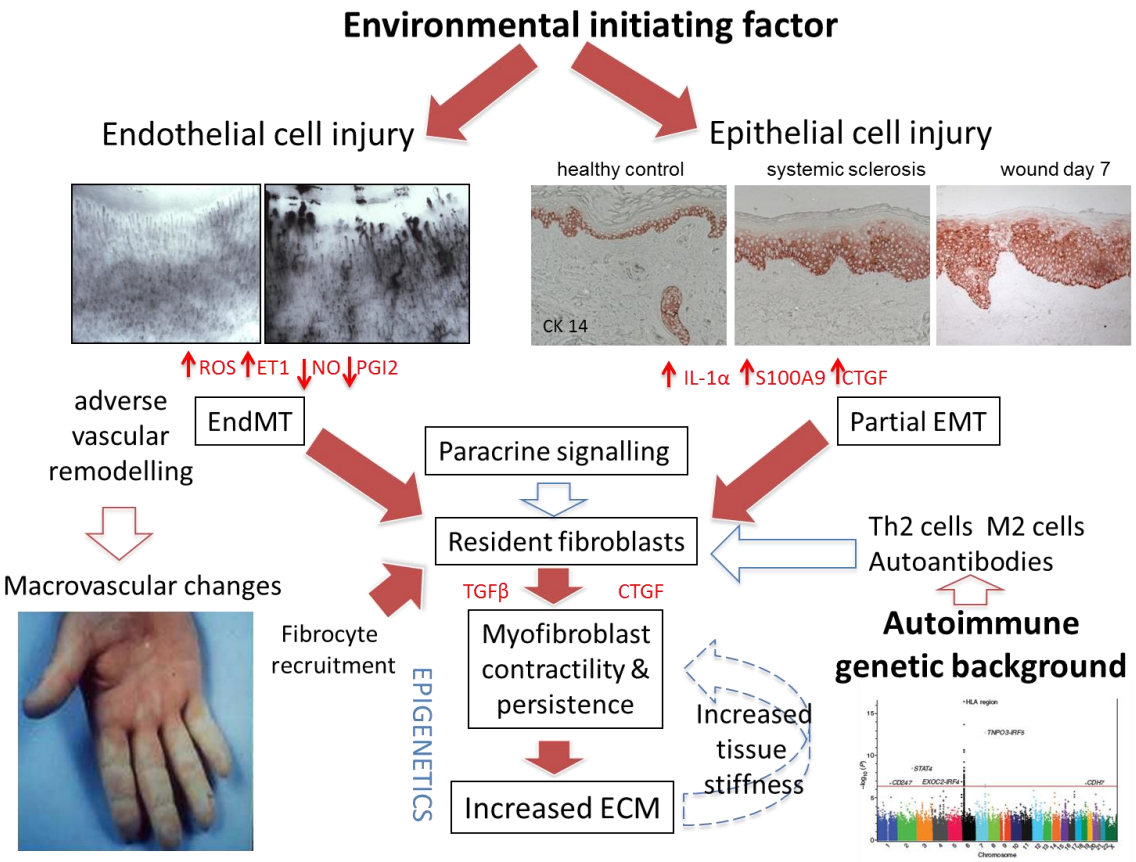


Figure 1.10 Putative biomechanisms contributing to systemic sclerosis pathogenesis Environmental insults including organic solvent exposure, vinyl chloride exposure, silica, as well as viral illness, are believed to initiate the disease process. It is proposed that epithelial cells and endothelial cells, which are specifically adapted to respond to environmental damage, become activated in the early stages of SSc as important initiating events. Downstream paracrine signaling from endothelial cells to fibroblasts includes increased endothelin-1 (ET-1) as well as loss of nitric oxide (NO), and prostacyclin (PGI2). In addition activated epithelial cells release IL-1α, CTGF, and S100A9. EndMT as well as partial EMT contributes to the activated fibroblast pool. Circulating mononuclear cell fibroblast precursors (fibrocytes) are also recruited into the lesions and contribute to the pool of resident fibroblasts. The genetic background based on GWAS and candidate gene analysis indicates an autoimmune genetic background leading to enhanced innate and adaptive responses. Type 2 T helper cells (Th2) as well as alternatively activated macrophages (M2) may feed into the fibroblast activation. Elevated TGFβ in the lesions induces myofibroblast differentiation leading to cells which persistently over secrete extracellular matrix (ECM), and contract the tissue leading to increased stiffness. Epigenetic changes, feed-forward paracrine loops, and increased stiffness of the tissue lead to persistence of the myofibroblast phenotype. Patient to patient variation in the relative contribution of each of the above mechanisms may account for the heterogeneous nature of the disease.

1.14 Modelling systemic sclerosis *in vivo*

As described in this Chapter, SSc has 3 main pathologic elements; 1) microvascular injury, 2) a marked inflammatory immune response, and 3) progressive myofibroblast activation leading to tissue fibrosis (Abraham and Varga, 2005, Gabrielli et al., 2009). One further approach to investigate the pathogenesis of SSc and to determine the relative importance of candidate genes, or to investigate the effect of targeting genes, proteins or signaling pathways therapeutically, is by the use of animal models of the disease. A number of such models have been developed, each of which recapitulates certain elements of the disease process. Their relevant strengths and weaknesses are discussed in the following sections and selected examples are summarised in Table 1.5.

Galen of Pergamon, a Greek philosopher and physician who lived in the 2nd century AD, is credited as having accomplished much in medicine and considered as the founder of experimental physiology. Most of his work was based on the studies he did on non-human primates and pigs. Although his direct extrapolation into human physiology initiated many incorrect conclusions, his approach has been much improved over the centuries (Guthrie, 1945). In the early and middle 1800s large scale use of animals in research began to rapidly advance the knowledge of physiology. Much of this work was being performed in Germany and France. Since those early days, experimenting on live animals has been paramount and essential in biomedical research. This approach has advanced research disciplines in pharmacology, genetics, immunology, medical devices and other areas. Animal models fail to duplicate all clinical manifestations seen in human diseases. While there are many complementary physiological similarities between rodents and humans, the animal models often fail to reproduce the exact clinical and histopathologic features of the human disorders. However, there are both genetic and chemically induced models of SSc that have and continue to elucidate components of the disease, associated with particular dysregulated pathways. These models allow the investigation of novel therapeutic compounds and also gene editing tools.

However, animal models of SSc segregate into two main groups; 1) those in which the disease-causing phenotype results from a genetic mutation such as tight skin 1 (Tsk1) and tight skin 2 (Tsk2), or University of California at Davis line 200 (UCD-200)/206 chickens, and 2) those in which the pathologic changes are induced in normal animals by exogenous factors such as the bleomycin models of lung and skin fibrosis induced by administration of a toxic agent. A number of such models have been developed, and the phenotypic changes plus relevant strengths and weaknesses are discussed in the following sections.

1.14.1 The tight skin models of systemic sclerosis

The Tsk1 murine model is a spontaneous autosomal dominant mutation consisting of a large in-frame intragenic duplication of the fibrillin-1 gene (*FBN1*) from exons 17 to 40 (Siracusa et al., 1996). Fibrillin-1 is a component of connective tissue microfibrils, which controls in part the bioavailability of the profibrotic molecule TGFβ via interaction with the latent TGFβ binding proteins 1 and 4. Altered fibrillin-1 binds more TGFβ than normal fibrillin, supporting the conclusion that elevation of the TGFβ profibrotic cascade results from the accumulation of TGFβ by the mutant fibrillin molecule. GeneChip array analysis of 6-week old Tsk1 mice revealed the upregulation in collagen, bone morphogenic protein (BMP), secreted frizzled-related protein (SFRP4), connective tissue growth factor (CTGF), and Wnt signaling proteins (implicated in the regulation of cell fate and integration of signals from other pathways such as TGFβ, fibroblast growth factor (FGF), and BMP) (Bayle et al., 2008). The fibrosis in the Tsk1 mouse is found in the deep fascia corresponding to the sites of TGFβ sequestration by the altered fibrillin. These changes contrast with the areas of active fibrosis in SSc which are sub-epidermal and deep dermal. Also there is no vascular component to the model, which limits its role as an exact model of SSc, rather it is seen as a model in which to test the effects of therapies or other genetic modifications on TGFβ-induced skin fibrosis.

The Tsk2 mutation is a chemically (ethylnitrosourea) induced autosomal dominant mutation in mouse chromosome 1. Although this produces similar phenotypic skin changes to Tsk1, the histologic changes are closer to that of

human SSc with dermal fibrosis and adipose tissue loss. Similar to Tsk1, mice spontaneously develop skin fibrosis at 2-3 weeks. Furthermore, there are positive antinuclear antibodies in 88% of Tsk2 mice compared with 10% of wild type (WT) controls, and, as in SSc, ATA, ACA, and ARA autoantibodies are seen (Gentiletti et al., 2005). Vessels appear thickened in Tsk2 but still lack the frank vasculopathy of SSc. Elevated steady state levels of collagen type 1 α1 (*COL1A1*) mRNA are seen with transcriptional activation of *COL1A* and collagen type 3 α1 (*COL3A1*) (Christner et al., 1998), as well as inflammatory cell infiltration of the subcutaneous adipose tissue.

1.14.2 Mutant fibrillin transgene models (stiff skin syndrome mice)

Recently, families were reported with stiff skin syndrome, inherited in an autosomal dominant fashion and caused by mutations in the integrin binding domain, Arg-Gly-Asp of fibrillin-1 (Loeys et al., 2010). Several transgenic mice with mutant fibrillin-1 genes have been produced, expressing a variety of phenotypic characteristics. Transgenic mice with cutaneous thickening and ATA antibodies were produced by insertion of a mutated *FBN1* gene, carrying the Tsk1 *FBN1* sequence (Saito et al., 2000). A conditional mutant-fibrillin transgene was involved in the best model, in which embryonic fibroblast culture fibrillin fibers had a distorted structure with increased collagen deposition, despite a lack of change in *COL1A1* mRNA or procollagen production. Instead, elevated levels of microfibril-associated glycoprotein 2, which also stimulates elastic fiber assembly, were detected.

These mice are considered to be most relevant as a model of the congenital stiff skin syndrome, rather than SSc itself. They may be suitable for testing anti-fibrotic therapies aimed at collagen hypersecretion, and to develop therapeutic strategies for the stiff skin syndrome itself, rather than anti-inflammatory therapies for SSc as studied in this thesis.

1.14.3 Conditional constitutively active TGF-β receptor 1 transgenic mouse

A knock-in murine strain has been developed, in which a constitutively active mutation of the activin receptor-like kinase 5 TGFβ type I receptor

(TβR1ca) was inserted downstream of a transcription stop cassette flanked by two loxP sites, targeted to the ROSA26 gene locus promoter. This mouse strain was bred with a transgenic mouse bearing a tamoxifen inducible Cre (CreER) driven by the *COL1A2* promoter. Five days of tamoxifen injection to the offspring caused excision of the stop cassette and led to fibroblast-directed expression of the mutant TβR1ca gene. The transgenic animals recapitulated many of the clinical skin features of SSc (including progressive dermal fibrosis, loss of adipose tissue, and collagen accumulation in small pulmonary vessels). Fibroblasts from these mice showed ligand-independent SMAD2/3 phosphorylation and nuclear accumulation, increased expression of downstream targets of TGFβ (CTGF, tissue inhibitor of metalloproteinase-1, type 1 collagen, fibronectin, plasminogen activator inhibitor-1, and secreted protein, acidic and rich in cysteines, SPARC). p38 and mitogen-activated protein (MAP) kinase pathway activity was also detected. TGFβ signal dysregulation, even without initial inflammation, produced the SSc phenotype in this model (Sonnylal et al., 2007).

While this model is based on a well-characterized disease mechanism and shows development of an SSc phenotype purely by TGFβ signal dysregulation, some histologic changes differ from those seen in human SSc. These mice may not be an optimal model for the vascular changes in SSc, for example, although this aspect of SSc was not specifically studied in the current thesis. Also, whereas these TβR1ca mice display fibrotic pathologies characteristic of human SSc, inflammation and autoimmunity aspects of the human disease are not major features. Because of these limitations this model was not suitable for use in the current thesis where the inflammatory bleomycin induced model was applied to mice of differing genetic background, and used for testing an anti-inflammatory therapeutic.

Taken together, this work developed a novel transgenic mouse model in which the TGFβ signaling pathway can be activated in fibroblasts in the mouse. This model would provide an excellent system for testing potential therapies targeting pathways downstream of TGFβ. The same approach but with different target genes may in the future be used to test other very specific hypotheses

1.14.4 Type 2 TGF-β receptor mutant transgenic mouse

A model involving fibroblast-restricted expression of a mutant "kinase-dead" type 2 TGFβ receptor (TβRIIδk) also produced fibrosis, despite being a reportedly dominant negative inhibitor of TGFβ signaling in cultured fibroblasts (Denton et al., 2003). Plasminogen activator inhibitor-1, connective tissue growth factor, SMAD3, SMAD4, and SMAD7 were upregulated in these mice, and SMAD2/3 phosphorylation was increased in transgenic fibroblasts. This model shows the influence of even small alterations in TGFβ signaling can produce a fibrotic phenotype. The TβRIIδk model provides a detailed *in vivo* view of TGFβ fibroblast signaling, and is a valuable model with which to study fibrosis in SSc. However, molecular mechanisms altered in this model are incompletely understood. Inflammation, and autoimmunity characteristic of human SSc are not noted in this model, but a pulmonary vasculopathy has been described (Derrett-Smith et al., 2013). Breeding and genotyping TβRIIδk mice also require additional resources and technical skills, and for the studies presented in this thesis would requiring a breeding strategy against the polymorphic genes associated with SSc development.

1.14.5 Relaxin-null mouse model

The hormone peptide relaxin can block TGFβ activity in normal and SSc skin fibroblasts, reducing collagen production and fibrosis. Relaxin-null mice develop spontaneous skin and lung fibrosis, which is moderated by relaxin administration (Samuel et al., 2005). Collagen production is higher in relaxin-null fibroblasts than WT. However, none of the vascular or immunologic features of SSc are evident.

1.14.6 The Fli1$^{-/-}$ mouse

Friend leukemia integration-1 (Fli1), a member of the Ets family of transcription factors, represses transcription of collagen genes via an Sp1-dependent pathway (Czuwara-Ladykowska et al., 2001). Fli1$^{-/-}$ mouse embryonic fibroblasts had significantly increased collagen type I mRNA and protein levels, and cultured SSc fibroblasts and SSc skin in vivo show

downregulation of Fli1 expression levels (Kubo et al., 2003). This reduction of Fli1 is associated with increased CTGF and decreased MMP1 (Nakerakanti et al., 2006) and may be caused by Fli1 degradation when TGFβ initiates a PCAF-dependent acetylation of Fli1 (Asano et al., 2007).

1.14.7 The Fra-2 transgenic mouse

The Fos-related antigen-2 (Fra-2) transgenic mouse model displays both pro-fibrotic and vascular characteristics of human systemic sclerosis. Fra-2 belongs to the activator protein-1 family of transcription factors, which are implicated in cell proliferation, inflammation, and wound healing (Wagner and Eferl, 2005). Transgenic mice expressing Fra-2 under control of the ubiquitous class I MHC antigen H-2Kb promoter exhibit Fra-2 overexpression and develop widespread inflammation and fibrosis, largely in the dermis and lungs (Eferl et al., 2008). Starting at the age of 12 weeks, activated fibroblasts and increased numbers of myofibroblasts release excessive amounts of collagens into the dermis and the lung parenchyma (Reich et al., 2010). It has also been noted that Fra-2 transgenic mice develop severe vascular remodeling of pulmonary arteries and non-specific interstitial pneumonia-like lung disease (Greenblatt et al., 2012). This model integrates the characteristic vascular and fibrotic manifestations of human SSc, without the apparent involvement of autoimmune phenomena. The disease course closely resembles that of human scleroderma, but further characterisation is needed.

1.14.8 The Caveolin $1^{-/-}$ mice

Caveolin 1-dependent internalization of TGFβRs reduces TGFβ signaling through increased receptor complex degradation. Radiation-induced lung fibrosis is associated with down-regulation of caveolin 1 in the alveolar epithelium, which preceded the onset of radiation-induced lung fibrosis (Kasper et al., 1997). Caveolin1 KO mice (caveolin $1^{-/-}$) produced a systemic fibrotic disease affecting the lungs and other organs (Drab et al., 2001, Razani et al., 2001). At the age of 12 weeks, caveolin $1^{-/-}$ mice show profound alterations in the alveolar septae of the lungs, with replacement of the normal alveolar bilayer lining by abundant ECM. Alveolar septae thicken due to hypercellularity and

increased deposition of ECM. These mice also develop skin fibrosis with a marked increase in collagen deposition. As caveolin 1 expression is down-regulated in patients with SSc (Galdo et al., 2008), the caveolin $1^{-/-}$ mouse model may serve as an interesting model with which to study skin and lung manifestations in the pathogenesis of SSc.

1.14.9 Bleomycin-induced fibrosis

Based on the development of pulmonary fibrosis in cancer patients treated with bleomycin, this pharmaceutical agent has been used to produce fibrosis in mice (Yagoda et al., 1972). Lung fibrosis is induced with a single intratracheal administration, while skin fibrosis is produced by repeated low dose subcutaneous injections over 3 or more weeks (Yamamoto et al., 1999a). In the cutaneous model marked macrophage infiltration is seen by day 3, and progressive fibrosis is apparent by day 14. On day 21 histologic changes showing epidermal hypertrophy, dermal fibrosis, and an accumulation of myofibroblasts, collagen and dense extracellular matrix material, and adipose atrophy are seen, resembling SSc skin pathology. Biopsy of lesional skin shows upregulated TGFβ, and increased nuclear phosphorylated SMAD2/3 in fibroblasts (Takagawa et al., 2003), and upregulated CTGF (Mori et al., 2008), all consistent with an SSc-like process.

In common with the current model of SSc the bleomycin mouse model represents an environment initiated inflammatory process, followed by excessive tissue repair, leading to fibrosis. As with the human disease, the genetic background of the mouse affects susceptibility to the fibrotic process (Ruzehaji et al., 2015). In many mouse studies the genetic background has been varied in order to examine the role of candidate factors and signaling pathways in SSc-like fibrosis, or to test potential therapeutic strategies for SSc(Yamamoto et al., 1999a, Daniels et al., 2004, Wu et al., 2009, Liu et al., 2009, Wilson et al., 2010, Beyer et al., 2012).

However, there are some limitations to bleomycin induced fibrosis as a model of SSc. For example, the typical vascular effects that develop prior to fibrosis in human SSc are not observed in a generalized pattern in the bleomycin model. Also, the bleomycin model responds to anti-inflammatory

therapies that are less effective in the human disease, possibly because the human pathology is more complex and heterogenous. In addition, TGFβ stimulated collagen production is upregulated in this model via SMAD-independent signal transduction with chronic upregulation of the early growth response factor (Egr)-1 in bleomycin-treated mice, indicating some variation from the pathogenesis of SSc (Chen et al., 2006).

1.14.10 Hypochlorous acid models

A newer chemically-induced model of scleroderma has been developed in mice using daily intradermal injections of a solution generating hypochlorous acid (HOCl) (Batteux et al., 2011). In this HOCl-model release of ROS is followed by fibrosis, inflammation, autoimmunity and vasculopathy. This method is now emerging as a highly useful model of the ROS induced component of SSc pathology which is favoured by some groups as having an important contribution to the disease process (Maria et al., 2016, Gabrielli et al., 2009). However, there is less experience with this as a model for SSc than with the more widely used bleomycin injury, and the overall importance of ROS in SSc is yet to be fully elucidated. Therefore use of the bleomycin model was favoured in the current thesis.

1.14.11 Sclerodermatous graft-versus-host disease

The sclerodermatous graft-versus-host mouse model is produced by the transplantation of immunologically incompatible spleen and bone marrow cells. Irradiated female Balb/c mice receive donor cells from male B10.D2 mice, so that donor cells can be identified using Y chromosome specific sequences. These strains differ at minor histocompatibility loci leading to a sequence of immune activation and subsequent fibrosis. Elevated levels of mRNA for Th1 and Th2 cytokines, TGFβ, MCP-1, macrophage inflammatory protein-1α and RANTES (regulated on activation, normal T Expressed, and secreted) are found, and the tissue is infiltrated by donor T cells and monocytes/macrophages (Zhang et al., 2002). Skin and lung fibrosis then develop at 14–21 days post-transplantation, along with elevated type I collagen RNA synthesis. Anti-TGFβ

antibodies were shown to be effective in blocking this sequence of events, showing the key role of TGFβ in this fibrotic process. Downregulation of TNFα was shown to be important factor in the development of sclerodermatous changes in this model (Askew et al., 2007). Both graft-versus-host scleroderma model mice, and an inflammatory subset of scleroderma patients showed IL-13 cytokine pathway activation. Host dermal myeloid cells and graft T cells were identified as sources of IL-13 in this model (Greenblatt et al., 2012).

A similar model, avoiding the need for irradiation, has also been produced using the Rag 2 mouse (Ruzek et al., 2004), which is immune deficient secondary to the loss of ability to rearrange immunoglobulin and T cell receptor genes. The donor cells can therefore be injected into non-irradiated mice. A similar skin phenotype was observed between weeks 3 and 5, as well as fibrosis and vascular endothelial changes in the kidney, but without the lung pathology.

Both of the above GVHD models are similar to human SSc in their systemic inflammation and tissue fibrosis, resembling the inflammatory stages of diffuse SSc. Their use is well understood and relatively straight-forward. However, working with hematopoietic cells may require sophisticated technical skills, and both models require special facilities. There may be problems with the handling and use of immunocompromised mice, either from irradiation or genetic susceptibility to infection.

1.14.12 The MRL/lpr IFN-γ receptor deficient mouse

The basic MRL/lpr mouse model produces autoimmune antibodies and develops arthritis, vasculitis, cutaneous lesions, and lethal glomerulonephritis. MRL/lpr mice with deficiency of the IFN-γ receptor (MRL/lprgammaR$^{-/-}$) do not show the glomerulonephritis, but instead have a vasculopathy characterized by intimal thickening plus accumulation of collagen, and have been proposed as a model of SSc (Le Hir et al., 1999). In the MRL/lprgammaR$^{-/-}$ animals mononuclear cell infiltrates were frequently seen in skin, lungs and kidneys, and they occurred also in liver, salivary glands and heart. Activated macrophages were seen in perivascular locations. In infiltrated areas abnormal accumulation of bundles of collagen was seen. In the lungs, and occasionally in other organs,

small and middle-sized arteries and veins showed intimal proliferation, resulting in a narrowed lumen. Alveolitis was widespread. Mononuclear cell infiltrates and excessive production of collagen in the skin and several visceral organs, thickening of vascular intima and autoantibodies seen in this model are reminiscent of human SSc. However the typical autoantibodies seen in this model are cryoglobulins, rather than the classical hallmark autoantibodies of SSc. However, taken together, the findings support this model as being a useful representation of certain aspects of SSc, particularly the perivascular inflammation accompanied by fibrosis, and further assessment of this model is justified.

1.14.13 The UCD-200 chicken

One to 2 weeks after hatching, University of California at Davis line 200 (UCD-200)/206 chickens develop microvascular occlusions, followed by prominent perivascular lymphocytic infiltration involving the skin and viscera erythema, edema, and necrosis of the comb, analogous to the vascular dysfunction in SSc which are usually the initial manifestations, with subsequent skin fibrosis and thickening. Similar to human SSc, these animals develop autoantibodies (anticardiolipin, antiendothelial cell, and antinuclear antibodies plus rheumatoid factors). In the UCD-200 chicken, the subsequent inflammatory phase is composed of T cell receptor (TCR) γ/δ+/CD3+/major histocompatibility complex (MHC) class II T cells in the stratum papillare and TCR α/β+/CD3 +/CD4+/MHC class II + T cells in the deeper dermis (Gruschwitz et al., 1991). Finally, skin thickening is caused by excessive accumulation of collagen types I, III, and VI although no mutation of the collagen genes is evident on restriction fragment length polymorphism studies (Sgonc, 1999). Another related line, UCD-206 chickens closely resemble chickens from the UCD-200 line, but more severe disease manifestations may develop (Wick et al., 2006). This model has several aspects which recapitulate SSc disease pathology including the vascular initiation and subsequent fibrosis. However many researchers would prefer to work with mice which also permits breeding strategies with other genetically modified animals to test the role of candidate genes. In addition many other research tools such as monoclonal antibodies, as well as protein

and gene arrays are available for mouse and humans but not for chicken models.

1.14.14 Selection of the bleomycin mouse model for use in the current thesis

Modelling the SSc disease process is highly complex and challenging and no truly ideal model has emerged. However, the bleomycin induced skin models have been used extensively to reproduce inflammation-dependent fibrosis, and are widely published and accepted as valid models of the SSc disease process (Gurujeyalakshmi and Giri, 1995b, Wilson et al., 2010, Wu et al., 2009, Yamamoto and Nishioka, 2005, Yoshizaki et al., 2010, Liang et al., 2016, Ruzehaji et al., 2015).

The bleomycin induced models reproduce innate inflammation, skin and lung fibrosis, and autoantibody production in an SSc-like pattern. The models are technically simple to apply, and the genetic background of the mice can be varied. The vascular pathology of SSc is not systemically reproduced but in the present thesis the SSc vascular pathology was not specifically studied. Because of these factors the bleomycin models were selected for use by the author in this thesis.

In the current thesis these models are used to test the importance of a candidate gene, in the SSc-like fibrotic process, applying bleomycin skin injury to WT and KO mice, and comparing the histologic as well as systemic inflammatory responses. Furthermore, the author assesses the efficacy of a therapeutic anti-inflammatory peptide in the bleomycin skin and lung fibrosis models, as a proof of concept for the use of anti-inflammatory therapies in SSc fibrosis. The bleomycin model is further validated by profiling the growth factor and cytokine responses both in dermal and pulmonary bleomycin injury, and drawing comparison with the pattern of cytokine and growth factor induction seen in SSc plasma and tissue fluid. .

It is possible that other models, as discussed above, could equally well be used to pursue these aims. For example the hypochlorous mouse model described above reproduces inflammation, fibrosis, and SSc-like autoimmunity, but there is much less experience with its use and at the time of the experimental work presented here, was only just emerging as a useful model.

Overall the bleomycin models were felt to be the most appropriate for modelling the SSc disease process and are used in Chapters 3 and 6 of this thesis.

Mouse model	Mechanism	Pathological fibrosis	Vascular changes	Autoantibody	Strengths vs limitations
Bleomycin-induced fibrosis	Clastogen-induced necrosis of cells at exposure site	Skin and lung fibrosis, inflammatory cell infiltrate	Thickening of deep dermal vessels local to injection site	ATA, anti-U1RNP, anti-dsDNA	Well established, technically simple, applicable to mice of varying genetic backgrounds
Tsk1 mouse	Mutation in *fibrillin1* gene. Increased bioavailability of TGFβ	Deep dermis and fascial fibrosis Emphysema in lungs	Right ventricular hypertrophy	ATA, ARA	Well established model of TGFβ induced fibrosis. Non-inflammatory, no lung fibrosis. Skin pathology different to SSc
Tsk2 mouse	Chromosome 1 mutation	Dermal fibrosis, inflammatory infiltrate	Some vascular thickening seen	ATA, ACA, ARA	Not well established Mechanism not fully defined
Fra2 transgenic mouse	Overexpression of AP-1 transcription factor	Skin and lung fibrosis	Vascular remodeling in lung	Not reported	Strong lung phenotype, pulmonary vascular involvement prominent Mice become sick at young age Not widely used
Constitutively active TGF-β receptor 1 transgenic mouse	Conditional overexpression of active activin receptor-like kinase	Skin fibrosis, loss of adipose tissue, no inflammatory changes reported	Vascular hypertrophy in lungs and kidney	Not reported	Recapitulates TGFβ induced fibroblast activation. Non-inflammatory, no autoantibody production
TBRIIδk mice	Dysregulated TGFβ signaling	Skin and lung fibrosis, non-inflammatory	Pulmonary vasculopathy	Not reported	Mechanism of TGFβ overactivity poorly defined, non-inflammatory, no autoantibody

Table 1.5 Selected examples of mouse models of systemic sclerosis Underlying mechanisms, pathologic changes and autoimmune features plus strengths and weakness as models of SSc pathology, are shown.

1.15 Treating systemic sclerosis

Despite advances in diagnosis and treatment, SSc death rates remain high, the highest of any of the connective tissue disorders. This is particularly so in patients with rapidly progressing dcSSc and those with lung involvement (Fett, 2013, Nihtyanova et al., 2014).

The approach to therapy in SSc reflects the three main pathologic changes, so that treatments are introduced to ameliorate 1) the vasculopathy, 2) the inflammatory autoimmune process, and 3) the fibrotic response, the latter being the most challenging component of the disease to treat. Since the relative contribution of each of these pathologic processes to an individual patient's burden of disease is highly variable, treatment is tailored to the individual's need.

In treating the vascular dysfunction in SSc there is an attempt to inhibit the adverse vascular remodeling and also to reverse the vasoconstriction seen. Peripheral vascular involvement manifesting as Raynaud's phenomenon is the most common complication of SSc, occurring in >90% of patients (LeRoy et al., 1988). In the most severe cases the peripheral vascular involvement leads to digital ulcer formation, and one of the aims of therapy is to prevent this complication. Calcium channel blockers are widely used in the treatment of Raynaud's since they reduce vasoconstriction by vascular smooth muscle cells, and have been shown in a meta-analysis to have a modest benefit in SSc patients reducing the frequency and severity of Raynaud's attacks (mean reduction of 8 attacks in 2 weeks, severity of Raynaud's reduced by 35% (Thompson et al., 2001). The European League Against Rheumatism recommends use of the calcium channel antagonist nifedipine as first line therapy for Raynaud's in SSc. Where this is ineffective clinicians are recommended to add the use of intravenous Iloprost, a prostacyclin derivative with vasodilator as well as anti-platelet effects (Kowal-Bielecka et al., 2009). Other commonly used therapies for Raynaud's in SSc include the serotonin reuptake inhibitor fluoxetine, and the angiotensin receptor blocker losartan (communication from clinicians Royal Free Hospital), both of which have moderate benefit (Dziadzio et al., 1999, Coleiro et al., 2001).

Vascular remodeling commonly affects the pulmonary vasculature in SSc leading to PHT, a progressive and life-threatening complication. Three classes

of drugs have demonstrated efficacy against PHT in SSc; prostacyclin analogues including iloprost, and selexipag, phosphodiesterase inhibitors such as sildenafil, and endothelin receptor antagonists including bosentan and ambrisentan (Mathai et al., 2007, Sitbon et al., 2015, Galie et al., 2015). It is possible that these drugs work via a combination of vasodilation as well as anti-proliferative effect on the vascular smooth muscle cells responsible for adverse remodeling. These agents have been shown to improve symptoms in SSc PHT, typically reducing New York Heart Association class of breathlessness by I unit (improvement from breathlessness at rest to breathlessness on exertion being typical), and improving exercise capacity as measured by 6 minute walk time. Overall the survival for SSc patients with PHT has improved but patients remain symptomatic and dependent on these costly and invasive treatments, and may still progress to end stage PHT with heart failure (Williams et al., 2006).

Therapies designed to alleviate autoimmune mediated inflammation are also often used, particularly in early dcSSc, where there is believed to be an active inflammatory immune infiltrate into the involved tissues. A number of disease modifying immunosuppressive drugs used in other rheumatic diseases have been trialed in SSc, with variable benefit. Methotrexate, which inhibits dihydrofolate reductase and also influences JAK/STAT signaling, has shown some weak efficacy against clinical skin involvement in early dcSSc (van den Hoogen et al., 1996, Pope et al., 2001) .

Cyclophosphamide, a chelating agent used as a therapy in vasculitis and lymphoma, has also been assessed in SSc and shown efficacy against lung fibrosis and also has a modest benefit against skin score (Hoyles et al., 2006, Tashkin et al., 2007). However cyclophosphamide causes significant toxicity including a major risk of infection, as well as bone marrow suppression. The benefits seen were modest and not maintained long term after cessation of therapy.

Also mycophenolate mofetil, an inhibitor of de novo purine synthesis, has been studied in SSc most notably as a therapy for interstitial lung involvement. In a current study Scleroderma Lung Study II (clinicaltrialsgov /ct2 / show /NCT0088312), high dose mycophenolate therapy given for 2 years was compared with oral cyclophosphamide for 1 year followed by placebo. Both therapies were associated with modest improvement in lung function, and

adverse effects such as leukopenia or thrombocytopenia were less frequent in the mycophenolate treated group (reported in the plenary session at ACR Annual Meeting, San Francisco, 2015 (Tashkin et al., 2016)). A number of observational studies also support efficacy of mycophenolate against both skin and lung involvement in SSc (Omair et al., 2015). Common side effects with mycophenolate include gastrointestinal upset as well as opportunistic infections.

In addition, anti-TNFα monoclonals have been used in SSc patients and may benefit inflammatory joint symptoms, but not skin or organ based fibrosis (Phumethum et al., 2011). In the modern era specific targeted biologic therapies have transformed the care of rheumatology patients in general, leading to remission in many cases, but this transformation is yet to be seen in SSc. Biologic therapies which have been utilized in SSc include rituximab, an anti-CD20 monoclonal which effectively depletes B cells. In SSc rituximab has been used in a number of non-controlled open label trials. Rituximab effectively depleted B cells from the involved skin in dcSSc in one study but was not associated with improvement in clinical skin score (Lafyatis et al., 2009). A retrospective European registry based analysis of 63 SSc patients treated with rituximab versus 25 controls showed improvement in skin involvement and lack of progression of interstitial lung disease in rituximab treated patients (Jordan et al., 2015). However there are many examples where agents assessed in open labelled or retrospective studies have “shown promise” against SSc, but failed in controlled trials, and there is a well-known tendency for the clinical skin involvement to peak and then improve during the first 2-3 years of disease, giving the impression of improvement in open label studies.

Specific therapies which may target the fibrotic process in SSc have now begun to emerge. Tyrosine kinase inhibitors have been proposed as therapeutics in SSc because of the likely importance of receptor tyrosine kinases in signal transduction in the disease-associated fibroblasts, for example the PDGF receptor, as well as the c-Abl tyrosine kinase which is involved in TGFβ signal transduction (Hinchcliff et al., 2012). Imatinib, which inhibits both PDGF receptor and c-Abl tyrosine kinases, initially showed promise in observational studies, but failed to demonstrate efficacy in a multi-centre controlled study (Prey et al., 2012). Imatinib therapy was in general poorly tolerated in SSc, leading to oedema, and severe gastrointestinal side effects,

which have limited its use (Pope et al., 2011). However in lower doses imatinib can be tolerated in SSc as a long term maintenance therapy, and are associated with stabilization of lung involvement, but ineffective against skin disease (Fraticelli et al., 2014).

Researchers at the Royal Free have found that IL-6 has a central role in SSc pathogenesis in a subgroup of patients with severe inflammatory features suggestive of an IL-6 driven biologic effect, such as anaemia and high CRP (Khan et al., 2012). It appears that in this subgroup IL-6 is contributing to both inflammation and fibrosis, and therefore it was proposed to target IL-6 with more specific therapy. In fact, antagonizing IL-6 by tociluzimab therapy, a monoclonal antibody which blocks the soluble IL-6 receptor, has led to a non-significant trend towards improvement in SSc skin disease when given over a 24 week period, and was a safe and well-tolerated treatment (Khanna et al., 2016b). However, in long term use, tociliuzimab is expensive and experience from use in rheumatoid arthritis has shown its use to be linked to increased risk of infection, and worsening lipid profile. It remains to be seen whether tocilizumab will become adopted as a mainstream therapy for SSc especially as the trend towards improvement did not reach statistical significance, and because of the high cost of this medication in the current environment of constrained health budgets.

As already discussed TGFβ has a central role in fibrosis in general and is believed to be a key inducer of fibroblast activation in SSc, and attempts have been made to specifically inhibit this pathway. Initially a low affinity anti-TGFβ, CAT-192, failed to show any efficacy in a control trial (Denton et al., 2007). However this antibody only targets the TGFβ1 isoform, and even then has low affinity for the growth factor. More recently a high affinity antibody targeting all three isoforms of TGFβ has been studied and shown to reduce skin score and inhibit biomarker expression in the skin of patients with early diffuse SSc, and holds promise as a specific anti-fibrotic therapy for the disease (Rice et al., 2015). However, a large placebo controlled multi-centre study is required to replicate this preliminary study in order to confirm efficacy, and also to give reassurance that toxicities such as loss of epithelial cell growth restriction as well as antagonism of the anti-inflammatory role of TGFβ, do not lead to severe long term adverse effects.

A further specific approach to target the fibrosis in SSc would be to directly inhibit the biochemical mechanisms leading to mature cross linked fibrillary collagen synthesis. Possible targets include the prolyl 3-hydroxylase enzymes which modify collagen in the endoplasmic reticulum prior to triple helical molecular formation, as well as enzymes such as lysyl oxidase or tissue transglutaminase, which are involved in cross linking of collagen in the extracellular environment leading to stable fibrillary collagen in the extracellular matrix (Johnson et al., 1999, Hudson et al., 2015, Cox et al., 2013). One of these mechanisms is explored further in this thesis, where in Chapter 3 a prolyl 3-hydroxylase *LEPREL1* is studied for association by genetic variation with SSc susceptibility, and the *LEPREL1* KO mouse is studied in a bleomycin skin fibrosis model.

Taking an overview of the above studies and data relating to therapy of SSc, the picture that emerges is of a resistant disease that responds only moderately to current therapies, which are often associated with toxicity. None of the therapies discussed has been shown to produce a lasting remission in SSc, and in general patients continue to suffer intrusive and disabling symptoms, and remain dependent on long term therapy and follow up. There is a large unmet need for a truly effective disease-modifying treatment for SSc which would produce a sustained remission and allow patients to return to work and full activity.

1.16 Overall hypothesis and summary of aims

Based on the above extensive body of work from many Centres studying this severe disease a picture emerges of SSc as a polygenic and heterogenous disorder. Multiple genetic polymorphisms have been shown to contribute to susceptibility, varying with ethnicity of the patient groups. Environmental triggers have been established but can only be clearly determined in a minority of cases. The role for autoimmunity is supported not only by the genetic polymorphisms which are located in innate and adaptive immunity genes, but also by the consistent presence of disease-specific autoantibodies, as well as tissue infiltration by T lymphocytes, alternatively activated macrophages and other immune cells. Furthermore inflammatory changes are evident both

clinically and based on profiling of plasma and conditioned media of disease cells. All of the above contribute to a progressive fibrotic process which spreads through involved tissues in the skin and internal organs. There is a very widely published literature in which animal models of the disease are studied with each model system having strengths and weaknesses, the bleomycin induced fibrosis model being a widely used and validated model of the inflammatory fibrotic process. Finally, treating this disease has proved very challenging. Strategies targeting pathogenic molecular pathways identified by rigorously performed *in vitro* studies as well as in genetically modified mouse models, have often met with failure at the stage of controlled clinical trials in SSc. Possible explanations include the heterogenous nature of SSc, the advanced and possibly irreversible nature of the tissue remodeling at the stage of clinical diagnosis, as well as possible redundancy of the targeted pathways.

With the above in mind, the overall ambition of this thesis is to identify novel biomechanisms contributing to this resistant disease in order to identify further targets for specific therapies. The studies presented using genetic analyses, profiling of tissue fluid from the lesions as well as model systems to test the following hypothesis.

1.16.1 Thesis hypothesis

The above information has led to the hypothesis that SSc patients have genetic susceptibility to the disease that manifests itself by perturbations in cytokine profiles within lesional tissue that promote fibrogenesis.

To test this overall hypothesis each of the thesis chapters will address smaller sub-hypotheses

CNVs in one or more candidate genes increase susceptibility to SSc.

The concentrations of cytokines in blister fluid will differ between SSc and healthy controls.

Candidate factors present at increased concentration in the lesions promote fibroblast migration leading to spread of the disease process.

Therapeutic anti-inflammatory peptides will have an inhibitory effect against SSc-pattern fibrosis in vivo.

1.16.2 The aims of the thesis

To test these hypotheses the work will have the following aims:

1) To identify genetic variants associated with SSc development using a genetics platform which assays CNVs present in the first intron of candidate genes using utilized patient and control DNA from the Royal Free Hospital's cell bank.
2) To use Multiplex profiling of growth factors and cytokines, present in SSc lesions, to gain insight into the local biomechanisms.
3) To use novel patterned collagen tissue culture chips in order to study the migration of normal and SSc fibroblasts to determine if the cells are more mobile and invasive. Also, to use these assays to test factors for their ability to stimulate or inhibit SSc fibroblasts migration.
4) To investigate the effect of a therapeutic anti-inflammatory peptide in mouse models of SSc skin and lung fibrosis.

Chapter 2. Materials and Methods

2.1 Clinical definitions used for the group of patients studied

All SSc patients and healthy controls studied in this thesis were seen and assessed at The Centre for Rheumatology and Connective Tissue Diseases, Royal Free Hospital Campus, UCL. SSc was defined according to internationally-agreed guidelines. Initially, when the Royal Free Centre for Rheumatology was established, the American College of Rheumatology criteria were used (Masi et al., 1980), and then subsequently from 2013 updated criteria proposed by the European League Against Rheumatism were used, for classification of patients as having SSc (van den Hoogen et al., 2013b). The extent and severity of cutaneous involvement was measured using the modified Rodnan Skin Score (mRSS), a validated clinical assessment which is considered the gold standard for assessment of cutaneous involvement (Clements et al., 1995). Skin thickness is scored 0-3 (0=no involvement, 1=possible thickening, 2=moderate thickening, 3=severe thickening) at 17 body sites to give a maximum score of 51.

Clinical material used in this study was collected at the Centre for Rheumatology by the medical team during admissions, clinic visits and day case attendances. Specifically dermal blister fluid was collected by Dr Kristina Clark, a clinical research fellow, skin biopsy material was collected by Dr Richard Stratton and the junior medical team, DNA samples were collected over a number of years initially by Professor Dame Carol Black, and then by clinical staff working in outpatients. Ethical committee approval for these studies was obtained from the NHS Health Research Authority, NRES Committee London-Hampstead, Research Ethics Committee London Centre, “Elucidating the Pathogenesis of systemic sclerosis by studying the skin, tissue and blood samples from patients and healthy volunteers”, (see Appendix for Ethical approval letter) NRES and R&D reference number 6398. All patients included received an information booklet and gave written informed consent using a specific consent form (see Appendix for information sheet and consent form). Clinical data was stored on a secure server held at the Royal Free Hospital site.

2.2 Culture of human fibroblasts from systemic sclerosis patients and healthy controls

Skin biopsy material was obtained by 4 mm punch biopsy taken from the involved anterior forearm skin of patients with dcSSc, within the first 2 years of disease. Biopsies were taken prior to the commencement of immunosuppressive or corticosteroid treatments. Biopsy material was also derived from the anterior forearm of healthy controls. In addition lung biopsy material was obtained from SSc patients undergoing open lung biopsy as an assessment for pulmonary fibrosis, and from controls undergoing diagnostic lung biopsy.

Tissue samples were cut into small pieces with a scalpel in a 25 mm sterile tissue culture dish, and then maintained as adherent cultures in DMEM with 10% FCS and Penicillin 100U/ml with Streptomycin 100µg/ml. Explants were cultured for a further 72 hours and media exchanged, and then cultured further for 9-15 days exchanging media every 72 hours to derive primary fibroblast cultures. Cells were examined by microscopy every 24 hours. Once confluent fibroblasts were detached from the culture plates by treatment with warmed 0.05%trypsin/0.02%EDTA and sub-cultured into 3 X 25 ml tissue culture flasks. Further subcultures were established in 75 ml flasks also in DMEM with 10% FCS and Penicillin/Streptomycin, and the fibroblasts were used for experiments at passage 3-5.

2.3 Western blot analysis

For measurement of cellular LEPREL1 protein, SSc and control skin fibroblasts at passage 3-5 were transferred to 6 well plates and grown to near confluence, and then cultured for a further 24 hours in low serum conditons with or without the additon of TGFβ 4 ng/ml. For Western blot analysis samples were lysed with 200 µL of RIPA buffer per well, protein concentration measured by BCA asay, after which the lysates were heated for 5 minutes with 5x Laemmli buffer at 95°C. GAPDH was used as the loading control, to demonstrate equal loading of wells.

Samples were loaded onto 4–12% Tris-glycine gels (Invitrogen, Paisley, United Kingdom) at 10µg per channel and run with broad-range protein molecular weight markers. Proteins were electrophoretically transferred to

nitrocellulose filters (Hybond-C extra; Amersham Pharmacia Biotech), which were then left in PBS with 5% milk protein and 0.05% Tween20 (blocking buffer) for 2 hours to block nonspecific binding. The nitrocellulose filters were incubated for 1 hour using the following primary antibodies: rabbit anti-human LEPREL1 (P3H2) polyclonal (Abcam, ab157507, rabbit polyclonal), anti-GAPDH (Abcam, ab8245, mouse monoclonal) diluted 1/1000 and 1/25,000 respectively in blocking buffer. The nitrocellulose transfers were washed three times in PBS with 0.05% Tween20 (washing buffer), incubated for 1 hour with HRP labelled species-specific secondary antibody in blocking buffer, washed again three times with wash buiffer, before staining with chemiluminescent substrate (Amersham Pharmacia Biotech) for 1.5 minutes, and then developed against photographic film (Hyperfilm ECL; Amersham Pharmacia Biotech).

2.4 Blood sampling and preparation for analysis of CNV in SSc

2.4.1 Safety

Since blood is a potential biohazard, a lab coat, gloves and goggles were worn throughout the processing. Used blood tubes, gloves and other solid waste were placed in a clear plastic autoclavable bag. Any spillages were cleaned immediately with bleach, Hycolin or Virkon. Liquid waste was poured into a plastic bottle containing Hycolin or Virkon, where it remained for a minimum of 2 hours before disposal.

2.4.2 Preparation of samples; blood collection

As described above, written informed consent was obtained from all patients and healthy controls included in the study. The phlebotomist provided three tubes of blood from each donor. Two purple topped tubes containing EDTA anticoagulant were used to prepare plasma, as well as DNA from the white blood cells (buffy coat). One red topped tube without anticoagulant was used to extract serum only.

2.4.3 Processing of blood tubes

The blood tubes were centrifuged at 1710g for 10 minutes at 4^{o} C. Four PT4 tubes (tubes with DNA binding buffer) were labelled - two for plasma and two for serum. Using a sterile plastic Pasteur pipette, the plasma was carefully transferred from the purple topped EDTA tubes and the serum from the no anticoagulant red topped tubes, each into the appropriate PT4 tubes, and frozen. The pellet in the tube of coagulated blood (red top) was discarded.

Using a sterile plastic Pasteur pipette, the buffy coat (thin whitish layer) was carefully transferred from the top of the red blood cells in the EDTA tubes, to a 15 mL centrifuge tube. Transferring an appreciable amount of the red cell layer was avoided, so the subsequent lysis step would be effective. The transferred volume was generally 1 mL maximum at this point. Red Cell Lysis Buffer (RCLB) (made with 0.144M Ammonium chloride, plus 1mM NaHCO3 in 900ml distilled water, pH should be 7.4, then the remaining 100ml of distilled water added) was add to the 14 mL mark, and the tube was placed on a rocker platform at 4^{o}C for 30 minutes. Each tube was centrifuged at 1710g for 10 minutes at 4^{o} C, and the supernatant fluid (lysed red blood cells) was removed by pouring.

RCLB was added to the 14 mL mark, and the tube was placed on a rocker platform at 4^{o}C for 10 minutes. The tube was centrifuged again at 1710g for 10 minutes at 4^{o} C, and the supernatant was removed with a pipette. The white cell pellet should be creamy white, not grey, pink or brown. If the cell pellet was not creamy white, suggestive of red cell contamination, the pellet was washed in RCLB once more by adding RCLB to the 14 mL mark, and the tube placed on a rocker platform at 4^{o}C for 10 minutes. The tube was centrifuged again at 1710 x g for 10 minutes at 4^{o} C, and the supernatant fluid was removed with a pipette. Thorough red cell removal is important due to haemoglobin, which if present would inhibit subsequent PCR reactions.

The tube was centrifuged again at 1710g for 10 minutes at 4^{o} C, and the supernatant was removed with a pipette. The clean pellet was either stored at -80 ^{o}C or used immediately for DNA extraction.

2.4.4 DNA extraction from white blood cells; processing of white cell pellet for DNA extraction

Previously frozen white cell pellets were thawed on the bench before proceeding following the techniques described by Miller, Dykes and Polesky (Miller et al., 1988). Three mL of Nucleic Lysis Buffer (NLB) (10mM Tris-HCl pH 8.2 plus 400 mM NaCl plus 2mMNa_2EDTA pH 8.0) and 200 µL of 10% SDS were added to the white cell pellet and pipetted to disperse the cells. The cell preparation was incubated at 37 ^{o} C for 45-60 minutes, and the tubes were vortexed. Saturated NaCl solution (1 mL/tube) was added, and the tube was shaken vigorously by hand for 15 seconds to mix the contents. The tube was centrifuged at 1710g for 15 minutes at 20^{o}C. The supernatant fluid was transferred to a fresh 15 mL tube, while avoiding disturbance of the protein pellet at the bottom of the tube. Two volumes of absolute alcohol (100% ethanol) were added to the supernatant and the capped tube was inverted several times to mix the contents. Milky white strands of precipitated DNA became visible. The tubes were incubated on the lab bench for 5 min, while the DNA floated to the top of the tube. The DNA was spooled using a 200 µl pipette tip and transferred to a 1.5 mL Eppendorf microfuge tube.

One mL of 70% ethanol was added to wash and desalt the DNA, the tube was centrifuged briefly to pellet the DNA, and the supernatant fluid was removed. One mL of 70% ethanol was added again to wash and desalt the DNA. The tube was again centrifuged briefly to pellet the DNA, and as much of the liquid was removed as possible. The uncapped tube was rested on its side on the bench for the DNA to dry. When the pellet started to become transparent at the edges, the DNA was dissolved in 200 µl of nuclease-free water. The tubes were rotated at 4^{o}C overnight to fully dissolve the DNA, which was mixed thoroughly by tapping or pipetting up and down. The DNA concentration and purity were checked using the Nanodrop spectrophotometer. The DNA was stored at -80 ^{o} C.

2.5 Localization of CNVs within the first intron of target genes

The localization of CNVs including the *LEPREL 1* gene sequence, was initiated after careful selection of candidate CNVs. qPCR assays were designed

to screen and then to validate the presence of the candidate CNVs in the study cohort. This required the selection of the appropriate DNA sequences within the CNV for the design of PCR primers and later Taqman probes.

The first step was to locate the candidate CNVs within the genes within which they reside. The online methods used are summarised in Figure 2.1 and shown in detail in the Appendix. The initial resource used was an online database known as the "Database of Genomic Variants" (DGV, http://dgv.tcag.ca/dgv/app/home). From the home page, the CNV search was initiated by entering the gene name. As an example, the candidate gene "*LEPREL 1*", (a post-translational modifier of procollagen chains into the triple helical procollgen molecule) was screened by typing the name of the gene into the search box. It is important to make note of the genome assembly used to conduct the search, as the coordinates are specific to this build (version of database). Using coordinates retrieved from this search within a different assembly will result in retrieving sequence from the wrong genomic location. Clicking on the search button brought up a genomic map of the results. Similar procedures were followed for all CNVs analysed in this study. The top track shows the gene of interest and underneath it the known CNVs are stacked. Scrolling over the CNV produced a dialog box with a list of identifying information about the track including the Variant Accession ID, the study where the CNV was identified, and coordinates. Additional details were available by clicking on the CNV. The CNV with the sequence most frequently reproduced among those listed was selected as the *LEPREL1* CNV sequence of interest. After scrolling down to the bottom of the page, the link to the UCSC genome browser website (Hg 19 is build 37 in this case) was clicked to navigate back to that website. A map was revealed for the genomic information about the sequence that lies between the coordinates. "Repeat Masker" track within this map was used to screen the sequence for regions of highly repetitive sequence. Regions that contain highly repetitive and/or highly similar sequences are not appropriate for primer design. Highly unique sequences are required so that the primers will bind only to the specific location within the CNV. The entry "Repeating Elements by Repeat Masker" is shown to the right of the "RepeatMasker" entry near the bottom of the left side of the page. The masked

out black band to the right of this entry indicates a region of repetitive sequence within the CNV sequence that was screened.

The next step in this process was retrieval of the actual DNA sequence to use in designing the primers/amplicon. Navigation to “View” on the toolbar was the next step, followed by selection of “DNA” in the “View” menu. This "DNA" page lists the coordinates for retrieval of the DNA sequence. Under “Sequence Formatting Options,” the choice of the manner of masking the repetitive elements in the sequence was selected. Here we are choosing to mask them by labeling all of the repetitive elements as “NNNN” within the DNA sequence. Clicking “get DNA” pulled up the DNA sequence in FASTA format. The sequence was copied and pasted into the same file with the CNV data.

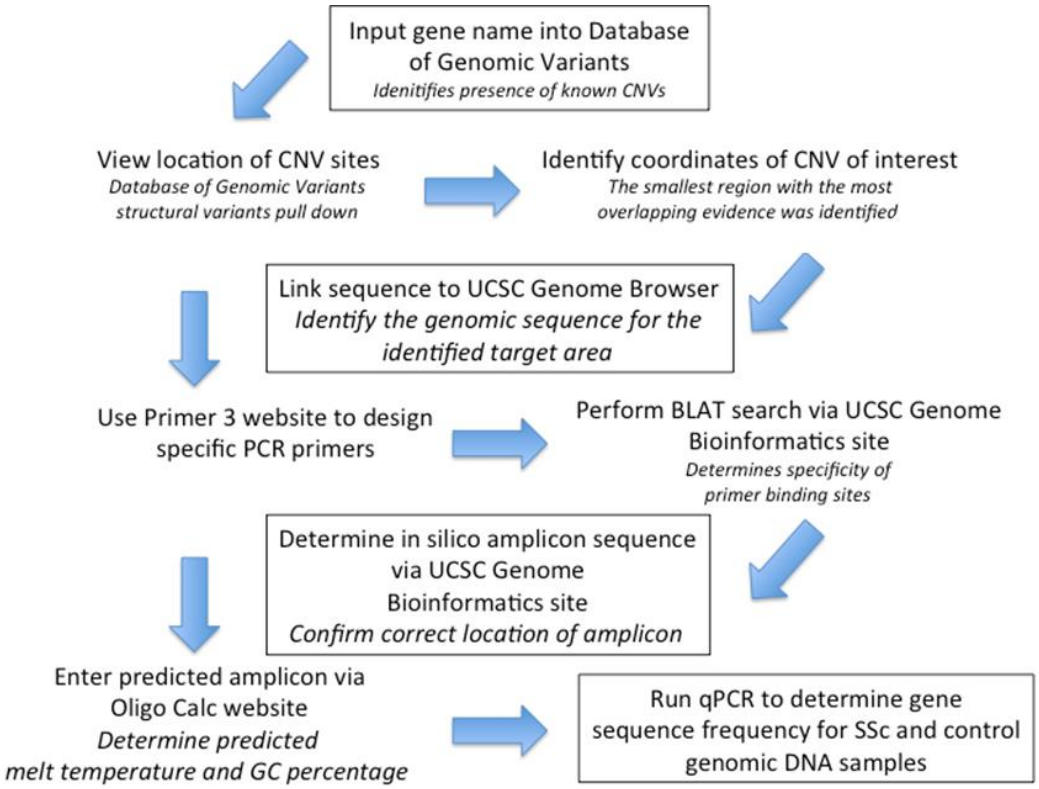


Figure 2.1 Use of online genomic database tools to identify CNVs in candidate genes and to design primers for qPCR assays Genes of interest, for example LEPREL1 were entered into the Database of Genomic Variants (DGV) and the coordinates of suitable CNVs present in the first intron sequence were identified. Primers for qPCR were determined using the Primer 3 website and the predicted meltpoint temperature and CG percentage determined prior to assaying the SSC and control genomic DNA. BLAT=BLAST-like alignment tool (more detailed illustration of the methodology used is given in the Appendix).

Once the DNA sequence was retrieved, PCR primers and probes to amplify the CNV could be designed. Primer 3, an online primer design tool, was used to design the primers/probes. First, the DNA sequence that was retrieved from the genome database was pasted into the sequence window in Primer 3 and the boxes corresponding to: “pick left primer”, “pick hybridization probe” and “pick right primer” were checked. Primer 3 output provided a default of 5 primer pairs based on the selected parameters. A pair of primers was selected to screen for sequence specificity within the human genome. With the pair of primers selected, the UCSC Genome Bioinformatics page (**http://genome.ucsc.edu/cgi-bin/hgBlat?command=start**) was revisited to determine primer specificity. “Blat” in the top toolbar was clicked, and “human” in the Genome menu was selected, making certain to select the correct genome assembly. The left primer sequence was pasted into the window, and the “submit” button was clicked. Once it was determined that the primer/probe sequences were specific for the appropriate genomic location, an *in silico* PCR search was conducted to obtain the entire amplicon sequence. This sequence was viewed in the genome browser. This was accomplished by clicking on “PCR” in the toolbar, making certain that the appropriate genome and assembly were selected. The forward and reverse primer sequences were pasted into the boxes. The “submit” button was clicked. The search returned the PCR primers and PCR amplicon along with the melting temperature (the temperature at which 50% of the base pairs of a DNA duplex are separated, Tm) of the primers.

When conducting qPCR assays using SYBR green as the detection reagent (Figure 2.2), it is imperative to determine whether the fluorescent amplification signal is specific to the PCR product, since SYBR green will bind to the minor groove of any double-stranded DNA that is amplified in the reaction. Determining specificity requires running a melt curve analysis. The PCR amplicon melts at a temperature specific to size and GC content. A characteristic peak at the amplicon melt temperature ™ distinguishes it from other products such as primer-dimers, which melt at different temperatures (Figure 2.3). To do this, the Tm of your product must be determined so that it can be distinguished from any other nonspecific products, including primer dimers. Oligo Calc, an online tool

(http://www.basic.northwestern.edu/biotools/OligoCalc.html), calculates the Tm and GC% of the amplicon. To use the Oligo Calc online tool to calculate the amplicon Tm, the amplicon sequence was pasted into the appropriate window and the “calculate” button was clicked. The output includes the Tm and GC content (%) of the amplicon. This information was saved in a file for reference when running SYBR green melt curve analysis.

2.6 Measuring copy number by PCR

2.6.1 qPCR assays

After the PCR assay was designed, the designed primers were ordered from Life Technologies (Invitrogen Value Oligos, Cat# A15612). Reagents for qPCR (Taq polymerase, dNTPs, appropriate buffer, fluorescent binding agent or fluorescently labeled probe/s) were also purchased. Using extracted DNA, the target sequences were amplified using the qPCR reaction. The resulting data were analyzed and the CNV genotype for unknown samples was determined.

2.6.2 Analyzing qPCR data using the SYBR Green screening assay

In the initial sample screen, primer pairs were selected and the DNA sequences were amplified with PCR. SYBR Green, a dye that binds to the minor grooves of dsDNA, was used to detect the amplified material. The SYBR Green fluorescent signal is proportional to the amount of amplified material in the PCR reaction.

Figure 2.2 shows a plot of fluorescent SYBR Green amplification signals as a function of PCR cycle threshold (Ct). In the initial screen SYBR Green assays were used to determine if the copy number variant was present in the samples. It is reasonable to assume that those samples that amplified contained at least a single copy of the insertion, whereas those that did not amplify contained a deletion. However, a melt curve analysis must be performed before this assumption could be made to be certain that non-specific amplification of other genetic material was not taking place.

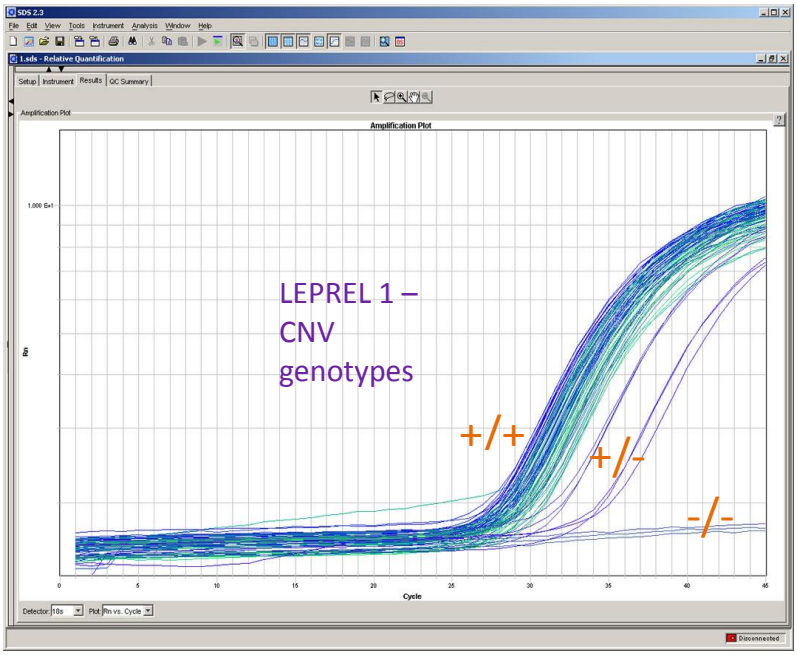


Figure 2.2 Analysis of qPCR data using the SYBR Green Screening Assay - SYBR Amplification Plots When screening for CNVs, SYBR Amplification Plots were used to determine if a particular CNV is represented within a sample cohort. In this instance the *LEPREL1* genotyping is shown, with clear amplification at low cyle number for homozygous +/+ samples, intermediate threshold cycle number for +/- heterozygous samples, and flat curves for samples containing homozygous -/- deletions of the CNV.

2.6.3 Analyzing qPCR data - SYBR Green melt curve plots

Since SYBR Green will bind to any double-stranded material, a melt curve was always run to ensure that the amplification was specific for the target amplicon. Using the Amplicon Tm acquired with the Oligo Calc tool, the melt curve was assessed to determine that the correct product had been amplified and that no additional double-stranded material (such as primer-dimers) were being amplified. In the illustration shown in Figure 2.3, the Tm of the amplified sequence was specific for the calculated Tm (80°C) of the amplicon and there were no additional peaks at other temperatures. It was concluded that those samples showing amplification were either homo- or heterozygous for the insertion (+/+ or +/-) and that those samples showing no amplification were homozygous for the deletion.

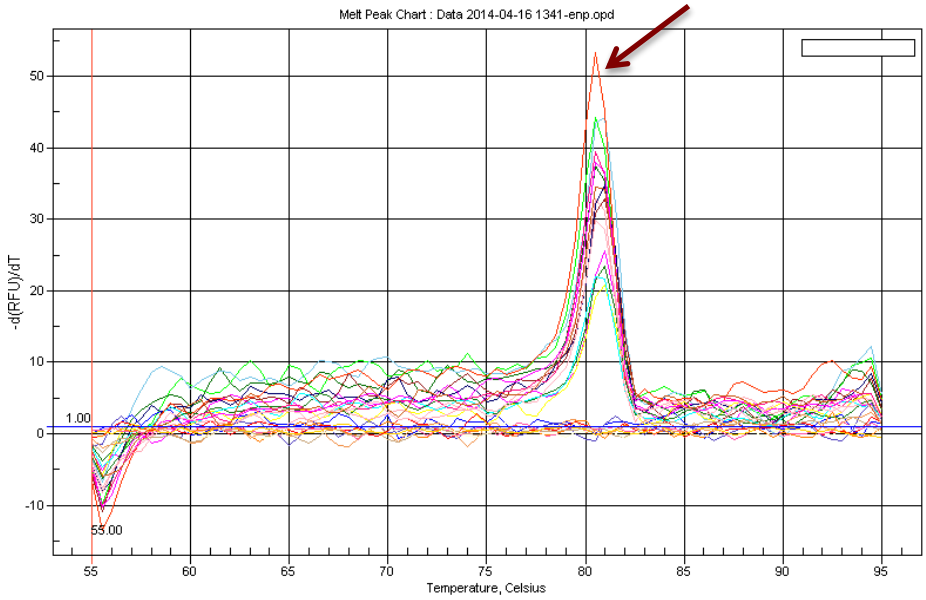


Figure 2.3 Analyzing qPCR data using SYBR Green Melt Curve Plots

The amplicon Tm indicated by the arrow was calculated to be 80°C. Observing a single peak indicates a specific product for the qPCR reaction and absence of primer-dimers or other off target products.

2.6.4 Analyzing qPCR data - Taqman Assays

After initial screening of CNVs using the SYBR Green method, Taqman assays were used to validate and accurately quantitate the number of copies in each sample. Fluorescently bound labels on taqman probes only fluoresce after the primers have bound to the amplicon sequence, extension has begun and the fluorescent reporter dye bound to the Taqman probe is cleaved by the taq polymerase, which separates it from the quencher (Figure 2.4). This renders the resulting amplification signal highly specific for the amplicon. The specificity of the amplification signal allows not only validation for the presence of the CNV within the sample cohort, but also quantitation of the actual number of copies within each patient sample using a method known as the ddCt or comparative Ct method (Livak and Schmittgen, 2001).

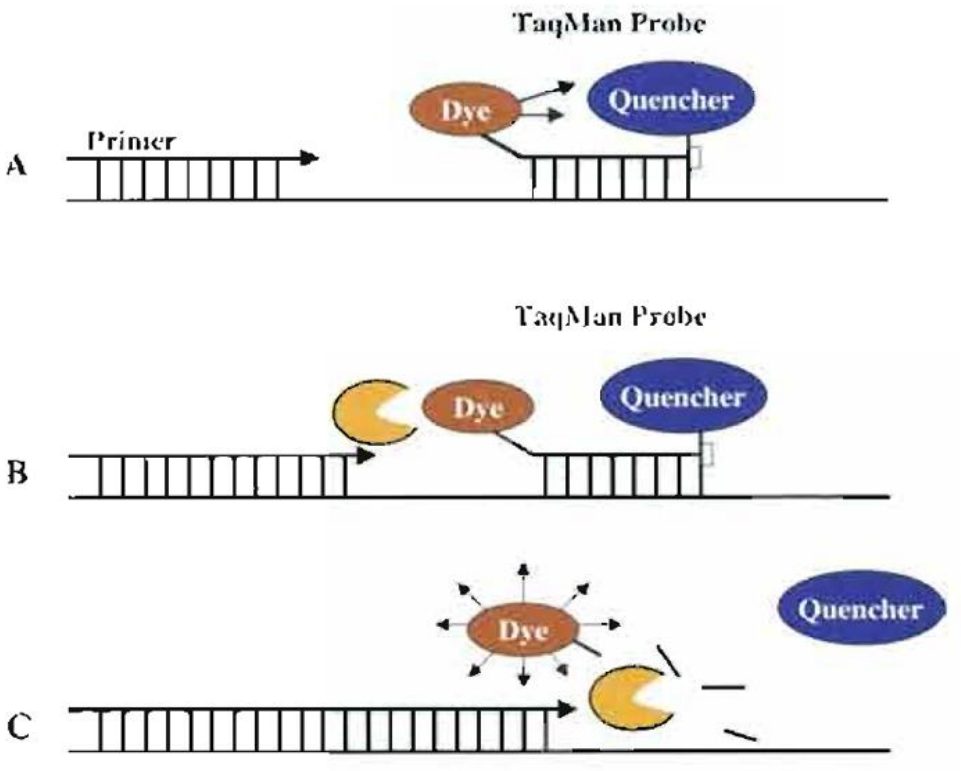


Figure 2.4 Analyzing qPCR data - Taqman Assays In the Taqman Assay, Fluorescently bound labels on Taqman probes do not fluoresce due to the Quencher (A). The Taq polymerase initiates extension and progresses along the sample sequence moving closer to the Taqman probe (B). The fluorescent reporter dye bound to the Taqman probe is cleaved by the taq polymerase, which separates it from the quencher, rendering the resulting amplification signal highly specific for the amplicon (C).

2.6.5 Analyzing qPCR data - ddCt method

By relative comparison to a reference sample with known copy numbers of both the gene target and a gene of known copy number in all samples, it is possible to quantitate the unknown number of target gene copies in a sample. In this case the gene was *LEPREL1* and each of the samples differed in copy number (Sample 1 – 2 copies of *LEPREL1*, Sample2 – 1 copy of *LEPREL1* and Sample3 – 0 copies of *LEPREL1*) (Table 2.1). Using the comparative Ct method (ddCt), the copy number can be quantified and the genotype definitely identified for each sample. In this case, *LEPREL1* CNV genotypes are identifies as +/+, +/- or -/-.

Sample	**Reference gene (*FOXP3*) Ct**	**Target gene (*LEPREL1)* Ct**	**ΔΔCt**	**2^- ΔΔCt**
Reference sample	24 (single copy)	24 (single copy)	0	1
Sample 1 (*LEPREL1* CNV +/+)	24 (single copy)	23 (two copies)	-1	2
Sample 2 (*LEPREL1* CNV +/-)	24 (single copy)	24 (single copy)	0	1
Sample 3 (*LEPREL1* -/-)	24 (single copy)	No product (no copy)	∞	0

Table 2.1 Analysis of qPCR data using the ddCt method Threshold cycle number (Ct) during qPCR was assayed for a reference sample and for each patient and healthy control sample. *LEPREL1* CNV copy number was compared against the reference gene (*FOXP3*). *LEPREL1* CNV copy number was calculated as shown. ΔΔCt =ΔdCt (Test sample) – ΔCt (Reference sample), 2^-ΔΔCt =2 to the power – ΔΔCt.

2.6.6 Genotyping the CNVs: cell lysis, genomic DNA isolation and purification from fibroblasts

Cells were harvested as described above. A total of 5 x 10^6 cells/sample was resuspended in 200 μl of 1x PBS. Cells suspended in PBS were transferred to a 1.5 mL tube containing 20 μl proteinase K. RNase A (20 μL) was added to the cells, which was mixed by vortexing and incubated at room temperature for 2 minutes. Following incubation, 200 μL PureLink Genomic Lysis/Binding Buffer was added to each sample, which was vortexed. Samples were placed into a 55 ºC water bath for 10 minutes to digest proteins. Following incubation, 200 μL of absolute ETOH was added to the lysate and mixed by vortexing. The lysate was added to a PureLink Spin Column and centrifuged at 10,000g for 1 min. The column was placed into a new collection tube, 500 μl of Wash Buffer 1 was added, and the column was centrifuged at 10,000g for 1 minute. The column was placed into a new collection tube, 500 μl of Wash Buffer 2 was added, and the column was centrifuged at maximal speed for 3 minutes. The column was placed into a new 1.5 mL collection tube and 75 μl of PureLink Genomic Elution Buffer was added. The column was incubated for 1

minute at room temperature, and centrifuged at maximal speed for 1.5 min. This step was repeated using the same Elution Buffer for maximum genomic DNA (gDNA) yield. All procedures and reagents were from the Invitrogen PureLink Genomic DNA Mini Kit; Life Technologies Cat #: K1820-00.

2.6.7 qPCR primers

The *LEPREL1* Intron 1 CNV primer sequences, identified via the methodology summarized in Figure 2.1, were:

LEPREL1-TMF	GCTTGCGAGGTTCCAGAGAT	189738996-189739015 forward primer
LEPREL1-TMR	GTGCTCATTTCTCCACCAGACTAA	189739039-189739062 reverse primer
LEPREL1-TMP	TTTCACCCTTTATGCAGATGT	189739017-189739037 hybridisation probe

The *LEPREL1* CNV studied is nsv514192 (Campbell et al., 2011), and the sequence is shown below. Highly repetitive sequences are masked by the software and denoted by NNNN.... Since LEPREL1 is encoded on the reverse strand the qPCR primers are homologous to the forward strand sequences as underlined in red, and the hybridisation probe targets the intervening sequence in blue.

ATCCCTATATCCACAGATGTCTGGATGCTGATGACTGTCCCTTTATCCCT
ATATCCATAGATGTCTGGATGCTCATTATCGTTTCTATTACCATTAATTT
TGCACATTTTAATAGTAAGAAGACAGATGANNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCATTACCTAGAAATTAAAA
ANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCTCACAAACCCACAATTG
ATGCTTCTGTAATTATTTCTATTTAAGTATGTCTTGTCTCCATCCTGATG
ACAAGGACTTGCCATATGTTTCATGTTATCTCAAGTGCTTCAGAAGAGTA
GGCATTCAGTGGATATAAATAATAATGATGAGAATAAAATTTAACATACC
TTACCCCATGAATACAATTCTGCGAGTAGTCATCATTGCCAGCAGGTGGG
GGCACAAACCTGAAGACTTTAATTAGAAAGGTTCATAGTTGGTCTACTGC
TGAAATATGGTGAAAAGCTATCAAATTAGTTCAAAGTTTCTTCCATTATC
TGTGGTGGCTGCCACCTTCAGAAAGGCTAAGGCGTTCAACACCAGGTTTC
TGGCAAAACAGAGACTGTAGGTTTCAGGAAACCTACACCAGGTATCTGAA
CAGCACTGGATATGAAATCAGGAATAAGTCTTAGCACACGAATGTGTGAT
CTTGCTAATGTAAACGTCAAAGGATTTAGAAAAAAGTAAAATGAATTAGA
ATTTTTCTTTTGCGAAAGAGTGGAAGAGTTGTCTCAATAACCAAGATTGC
CTTCTCTTTTCCATCTTAATCCAAACATGTTTTTCTTCTTTTTAAAAAAA
TAAAGCAGCTTACTGGATTCCCAAATATAGTATTAATATCAGTGGTAACT
TCAAAGAAGCCACGATCAGTTCTTTAAGGACTTTCCCTCCTAGCATCTTT
TTTATGGCTCAATAGGCCTGACCTCCAGGTCTCATCTTAAAAGTCAGAGC
TTTCCCCTTTCATGAAAGTCTCCTATGTCTCATCAGTGCCAGATACACAG
CAAAGTTAAATAGGGTCACAGTTTAGCGTGGGTCTCTAGGGTGAAGCCCA
TCCAGGGGTGTGGTGATTTTTCCTATGCATGGTCTGGCTGGGCGGCTGCT
ACCCACACGTTGATTACTTTAAGCCTGCTGAAGTGCTTGAAGCCTAACTG
GCCTTTTAGAAAGGCTTCCTAAGGAGTCCTTTTCCAAGGAGAGGTGGCAT
CTGTCAAAAATGTCAAACAAAAGAAAGCAATGTCTACAAAATCCCTATTA

AAATATTGGGGTAAAAGCTGGATGGCTATGNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTGGAAAAATCTGAGACCC
CAGGACCCTAAGTAACCATCTTTGCCTTACACCAGGCTAGAGTCCTCTAG
ATGTTTATTCTAAATAATGCCAACTACAGCCATCTGGAAACCAATATTTA
CAAAGCAGCCACCATGAACTGTTTCCAATAATCAGAAACTTGGTCTGAAA
AATTAATAAAACAACAAAAAGTGCTTGCGAGGTTCCAGAGATTTTTCACC
CTTTATGCAGATGTTTTAGTCTGGTGGAGAAATGAGCACAATCAGTGGTT
CCCTTTCTTATGCATCAATGTGGCTCATTAAAACAATAATTTGCCTTTGT
CTCTATTTTTTGTCATTTCCTTAAGATTGAAAAAGTAAAATTCAATTATT
AAACTATTTCAGTTTGTTTTTAAAGTCTTCCTTTTTTCAATTTTTTGTTC
TGTAACAAATAGATTTTCAACAATGTTATAATGCATAAATTCTAGCCAAA
TATATTTTTTCTTTTATAATTTCCTTTTTTAAAAAAGTAACTTTCTCTTC
TCTCCAAATATTTCATCCCTAAATTATGTAAACTTTTCTCTTTCAGTTTT
AAAAAATAAAATCCATACTTCTGGCTTAAATTGCACACATTTGTAATATT
CATACCAACAGATGCCTATCCAGCTAGCTGTACATCTGCCTACNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNTTCCCAGTTNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNTACTAAATTTGTAAATTCTGGATTTCGTTAGACAACTATGTACAA
TATTATATGATAGTGTGTTTCTCTAATGTAACTGACATTACAGGACAGTT
CTTAAAAGCTGTCAGTGATACTTTATTGCCTNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
AATAATGTTCTTCTAGGAATGTACAATGACATTAGCCTCTAAAGAGTATT
GAGAGTATCTTGGTTACAAATGACTAACCAGACTTCTCCCAATAACTGAG
AAACAAATTTTACCATTGATTTATTTTTCTTATAGTAAACATTAAAGAGC
ATCTCACAAATAATATTCCATATAATAAANNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTATAAATAATGAAGTGA
AAGTCTCCTTTAGGTATTTTATACAGAATAAACTTGTATATACTTTTTTA
TTTAACATATTATCCTCCAAGAAACATTATTTGTTAGTTAAATGCACATA
CACAAATTTCTCTATTTGCAA

Primers were purchased from Life Technologies (Invitrogen Value Oligos, Cat# A15612) in lyophilized form and reconstituted in nuclease-free water to 100 uM stock solutions. The primer was serially diluted to three concentrations (300, 600, 900 nM) to titrate the primer concentration. The specific details of the other primers used are not given, as they represent important non-disclosed intellectual property, forming the basis of the author's investigation of potential candidate factors in other complex human disorders.

2.6.8 Analyzing qPCR data: statistical methods used

In CNV analysis, test statistics are used to determine the likelihood of an association between the presence of the CNV and the disease or phenotypic trait. The null hypothesis (no association between CNV and the disease trait) can be tested by Pearson's chi-squared test, based on the criterion that a given system of deviations from the probable in the case of a correlated system of

variables is such that it can be reasonably supposed to have arisen from random sampling. Using this method a contingency table can be created for each genotype (-/-, -/+. +/+) of the CNV against presence or absence of SSc disease (Table 2.2). Chi-squared is calculated as: $Chi^2=Sum\ (O-E)^2/E$ where O is the observed value and E is the expected value, with degrees of freedom equal to (number of rows-1)x(number of columns-1), and then compared against a frequency distribution (Table 2.2).

Observed

	-/-	-/+	+/+
SSc	1	20	26
Healthy	9	20	20

Expected

	-/-	-/+	+/+
SSc	4.9	19.6	22.5
Healthy	5.1	20.4	23.5

Table 2.2 Pearson's chi-squared test applied to CNV genotype in systemic sclerosis The observed genotype frequency for *the LEPREL1* CNV in males can be compared against the expected frequency via contingency tables and then subject to Pearsons' Chi-squared test. In this instance Chi-squared=7.1 with 2 degrees of freedom, p=0.028 when tested against the Chi-squared distribution)

However, some have advocated that in these analyses, inaccuracy occurs when the expected value is small and at times equal to zero. It may therefore be more accurate to use Fisher's exact test as the test statistic in CNV analysis (Fisher, 1934), although this has been questioned, some claiming that this test should hardly be used (Lydersen et al., 2009). In the present thesis the observed genotype frequencies were compared against the expected frequency using Chi-squared analaysis, and then corrected for multiple comparisons (see below).

An additional statistical problem arises in the CNV studies as presented in this thesis. Multiple comparisons are being made, and this needs to be taken into account during the statistical analysis. Specifically, 24 CNVs are assayed and analysed for both males and females with and without SSc. Because of

this use of the standard α value of <0.05 would likely result in false positive results with a frequency of 24 x 0.05=1.2, ie about 1 type I error would occur for each set of analysis .

To correct for this bias, adjustment procedures have been developed. The traditional approach to this problem has been to control the probability of making even 1 type I error, which is termed control of the *family-wise error rate,* by statisticians. The well-known method used is the Bonferroni correction which corrects the α value for the number of factors assayed, in this instance reducing the α value to 0.05/24=0.002, and this method was used here in the CNV analysis presented in Chapter 3. This method is considered conservative because it is based on a strict criterion, which controls the probability of making at least one false positive error across all tests. False negatives (Type-II errors) will likely be generated as the number of tests increase, therefore, other methods have developed to adjust for multiple testing, for example in GWAS where many thousands of comparisons are being made. Benjamini and Hochberg introduced a method to control the false discovery rate (FDR) which is less stringent than the Bonferroni correction, and this has been used in multiple genomics studies where very large numbers of comparisons are being made (Benjamini et al., 2001). In these studies, FDR control is used to adjust for multiple hypothesis testing (Table 2.3).

		Test result		
		P non significant	P significant	
True state	Nul hypothesis is true	U (true negative)	V (type I error)	m_o
	Nul hypothesis not true	T (type II error)	S (true positive)	$m-m_o$
Sum		m-R	R=V+S	m

Table 2.3 Use of methods controlling false discovery rate (FDR) in testing multiple comparisons Traditional methods control the family wise error rate (FWER) which is the probability of V≥1. Less stringent methods control the FDR which is the expected value of V/R as defined in the above table.

For example, in a genomics study finding 40 positive statistically significant hits, but with FDR of 2 may still be considered of interest, possibly giving a useful picture of the overall disease process. However a study with 4 positive hits and FDR of 2 may be considered not useful in terms of giving an overview of meaningful differences between groups.

In addition odds ratios (ORs) were calculated for the allele frequencies against development of SSc. For example for the *LEPREL1* CNV in males

Allele frequency

	CNV-	CNV+
Healthy	a	b
SSc	c	d

OR was calculated as= (a/b)/(c/d)
95% confidence intervals were calculated as
exp (nl (OR)-1.96xSqrt(1/a+1/b+1/c+1/d)) for lower confidence limit (LCL) and
exp (nl (OR)+1.96xSqrt(1/a+1/b+1/c+1/d)) for upper confidence limit (UCL)
In this instance for LEPREL1 alleles in males OR=2.1 with 95% confidence intervals of 1.1-3.9).

A review of the above commonly used methods in genetic case-control studies has been published by Clarke in 2011 (Clarke et al., 2011)

In fact none of the CNVs studied in the current thesis provided statistically significant differences between SSc and controls when multiple comparisons were taken into account by the above methods. However since *LEPREL1* had the strongest p value for the simple Chi-squared analysis, and an odds ratio which did not cross 1.0, and because of interest in the likely biologic relevance of this gene to fibrosis, this factor was studied further in tissue culture and mouse biology experiments.

2.7 Measurement of cytokines in blister fluid sampled from the lesions: patients studied

Patients included in the studies were all under the care of the Royal Free Hospital Centre for Rheumatology and Connective Tissue Diseases classified as SSc as defined in section 2.1. Clinical parameters and autoantibody profiling of the patients included are shown in Tables 2.4 and 2.5.

2.7.1 Collection of skin blister fluid

Samples of dermal interstitial fluid were taken from the anterior forearm using a suction blister (Ventipress, Upsala) by the technique as described previously (Kiistala, 1968, Sondergaard et al., 1998) (Figure 2.5). Under a negative pressure of 275–325 mmHg for 2–3 hours, small (~8 mm diameter) blisters were raised on the involved forearm of SSc patients and on the forearm skin of healthy controls, after which 100-250 µL of the interstitial fluid was available by sampling using a 23G needle and 1.5 ml syringe. This is a standard technique used in dermatological research for the collection of dermal interstitial fluid. Previous studies have suggested that the barrier function of the capillary basement membrane is preserved during suction blister formation and that the fluid collected is representative of dermal interstitial fluid, not merely an exudate of plasma proteins drawn from the microcirculation (Vermeer et al., 1979, Rossing and Worm, 1981). Plasma samples were obtained for each case, as close to the time of blister sampling as feasible. Plasma was stored frozen at -80°C in 25 µL aliquots for later analysis.

Following written consent clinical details on the patients were collected, including type of disease, gender, age, date of disease onset, antibody status, current treatment, prior treatment, organ involvement, most recent FVC and FEV1, recent creatinine, recent estimated pulmonary artery pressure on echocardiogram, (Tables 2.4 and 2.5). All patient details and clinical paramters were stored on a secure internal server within the Royal Free Hospital, Centre for Rheumatology and Connective Tissue Diseases, to ensure patient confidentiality.

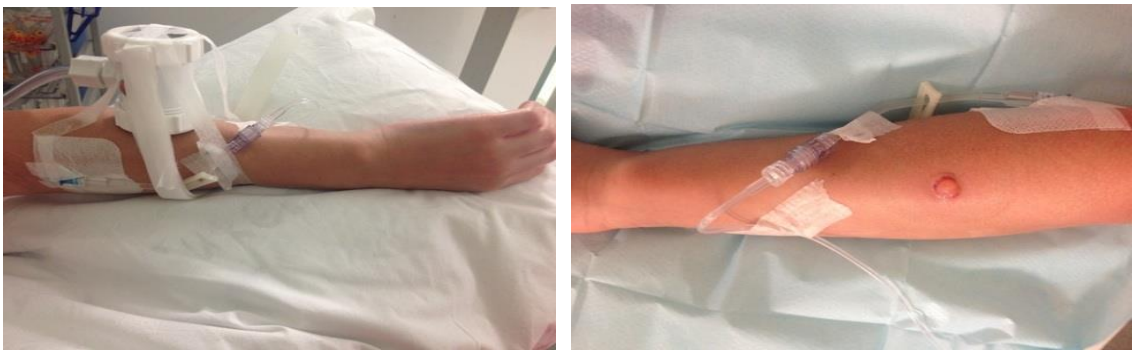


Figure 2.5 Suction blister method Forearm skin was subjected to negative pressure via a suction blister device. After 3 hours fluid filled blisters formed (Kiistala, 1968) and the dermal blister fluid was collected and used for analysis of cytokines and other potential markers of disease activity.

Disease Characteristics	**SSc**	**HCs**
	n=26	n=10
Age (years, mean +/-sd)	55 +/- 10	51 +/- 14
Female Sex n(%)	18 (69)	9 (90)
DcSSc - n(%)	20 (77)	
Disease Duration – yr	9 +/- 7.9	
Duration of Raynaud's	10 +/- 1.6	
mRSS (mean +/- SD)	18.3 +/- 11.24	
Organ Inv (%)		
Oesophageal	69.2	
Other GI	46.2	
Lung	42.3	
Muscle	15.4	
Joint	11.5	
Renal	3.8	
Cardiac	7.7	

Table 2.4 Summary demographic and clinical features of patients and controls included in suction blister method Healthy controls (HCs) included 7 individuals with primary Raynauds, and 3 apparently healthy individuals.

Sample label	mRSS	ANA	SSc antibody	Lung	GI	Immunosupression
P1	15	>1/100	Neg	Yes	Yes	Pred +MTX
P2	6	>1/1000	ACA	No	Yes	none
P3	38	>1/1000	ACA	No	No	MMF+Pred
P4	35	>1:1000	ATA+Ro	Yes	No	Pred and MMF
P5	34	>1:1000,	ATA	Yes	Yes	MTX +Pred-+Cyclo
P6	24	>1:100	ARA	No	No	HCQ
P7	18	>1:1000	ACA	No	No	none
P8	22	>1:100	ATA	Yes	No	MMF
P9	24	>1:1000	ATA	No	No	HCQ
P10	24	>1:1000	ACA	No	No	none
P12	28	>1:100	U3RNP	Yes	No	HCQ+previous Campath1
P13	27	>1:1000	Neg	No	Yes	MMF + Pred
SSC2	4	>1/1000	ACA	No	Yes	Nil
SSC3	8	>1/1000	ATA	No	No	HCQ, previous MMF
SSC4	2	>1/1000	Neg	Yes	Yes	Aza, Pred , Previous Gamma IFN, Cyclo
SSC5	15	>1/1000,	ARA	No	No	MTX + Pred. Previous MMF
SSC6	30	>1/1000	ATA	Yes	No	MMF +Topical imiquimod, Aza and Cyclo
SSC7	10	>1/1000	ATA	Yes	No	MMF and Pred, Previous Cyclo
SSC8	24	>1/1000	ARA	No	Yes	MMF +Pred, +HCQ ,Previous Cyclo
SSC9	9	>1/1000	Neg	Yes	Yes	HCQ 400 mg OD
SSC10	30	>1/640	ATA	No	Yes	Nil past or present
SSC11	28	>1/100	Neg	Yes	No	Aza + Pred. Previous- MTX, MMF, ciclosporin
SSC12	6	>1/1000	Ro + ATA	Yes	Yes	MMF, HCQ, Pred
SSC13	4	>1/1000		No	Yes	Nil past or present
SSC14	12	>1/1000		No	No	IVIG 6 weekly, pred. Previousl MMF
SSC15	6	>1/1000		Yes	Yes	MTX 15 mg, Prednisolone 7.5mg

Table 2.5 SSc patients in dermal blister fluid study - skin score, autoantibody profile, internal organ involvement, prior treatment (Pred=prednisolone, MMF=mycophenolate,MTX=methotrexate,HCQ=hydroxychloroquine,Cyclo=cyclophosphamide,Aza=azathioprine,GI=gastrointestinal, Lung=pulmonary fibrosis).

2.7.2 Luminex® assay procedure for sample analysis of blister fluid and plasma

Both the plasma and interstitial fluid samples were analyzed using the Luminex® bead array for inflammatory cytokines, chemokines, and growth factors, including factors previously implicated in SSc pathogenesis (41 factors were analyzed, see below). The EMD Millipore (Billerica, MA) MILLIPLEX® MAP Human Cytokine / Chemokine panel was used in the Luminex® bead assay for inflammatory cytokines, chemokines, and growth factors. Based on the Luminex® Corporation (Austin TX) xMAP® technology, this panel contains 41-plex premixed beads. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes coated with a specific capture antibody. After an analyte from a test sample captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres are allowed to pass rapidly through a laser, which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay, based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. The kit was used for the simultaneous quantification of the following 41 human cytokines and chemokines: EGF, Eotaxin, FIT-3L, Fractalkine, GRO, G-CSF, FGF-2, GM-CSF, IFNα2, IFNγ, IL-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9 IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IP-10, MCP-3, MDC, MCP-1, MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF-α, TNFα, TNFβ, and VEGF.

To use the 41-plex premixed kit, RANTES, PDGF-AA and PDGF-AB/BB beads were added to the 38-plex premixed bead bottle. Neat plasma or dermal fluid samples were used for measuring the 38 cytokines. With RANTES, PDGF-AA, PDGF-AB/BB assays, the samples were diluted 1:100 in the assay buffer and a standard curve with assay buffer matrix was used.

The Human Cytokine Standard was reconstituted to give a 10,000 pg/mL concentration of standard for all analytes.

In brief the procedure was as follows: the wells were washed with 200 µL/well Wash Buffer. To appropriate wells, 25 µL of standard or control, assay buffer (background and sample wells), appropriate matrix solution (to background, standards, and control wells), and samples (to sample wells, assayed in duplicate) were added. Beads (25 µL) were added to each well and incubated overnight at 4°C or 2 hours at room temperature with shaking. Well contents were removed and washed twice with 200 µL Wash Buffer. Detection antibodies were added (25 µL per well) and incubation occurred for 1 hour at room temperature. Streptavidin-phycoerythrin (25 µL per well) were added and incubation proceeded for 30 minutes at room temperature. Well contents was removed and washed twice with 200 µL wash buffer, 150 µL of sheath fluid or drive fluid was added per well, and the plates were read on a Luminex® (100 µL, 50 beads per bead set).

2.7.3 Blister fluid multiplex data statistical analysis

Further to the discussion of the multiple comparsions in statistical analysis, the measurements of 41 proteins in 26 SSc patients and 10 healthy controls raises this problem to a further level. Permutation analysis has been developed to analyse this kind of profiling data, mainly used in genomic arrays (de Winter, 2009). This method was used here, and the sample data generated in the XPonent® software was imported into the Excel SAM add-on and the Student's t test compared by permutation analysis.

Where samples were undetectable below the threshold, a value equal to the lower limit of detection was assigned, and where sample concentrations were greater than the range available for analysis, they were assigned the upper limit value of the range. Pearson's correlation coefficient was used to assess any correlation between serum and interstitial fluid cytokine and growth factor levels.

2.7.4 Network of potential protein interactions

Using STRING 9.1 database (http://string91.embl.de/), networks of potential protein interactions were created using the inflammatory proteins that were found to be significantly raised in the hierarchical clustering from our analysis, and expanded to include downstream targets.

2.7.5 Heat maps for blister fluid and plasma samples from systemic sclerosis patients

Measurements of cytokine and growth factor levels in blister fluid and plasma by Luminex® were log transformed to normalise the data (original data in pg/ml transformed to natural log e). Data were then expressed as standard errors above and below the mean value. The data was saved as a tab chart and uploaded into the NIH CimMiner heatmap software to generate the heatmap images. Unbiased clustering was performed using correlation, and Pearson coefficient settings.

2.8 Methods for assessing fibroblast migration

2.8.1 Preparation of chips for fibroblast migration studies

The method for the study of fibroblast migration involves the use of glass slides (8 x 15mm) coated with bovine type I collagen, developed by Fibralign Corporation (Sunnyvale, CA), which can be purchased from Advanced Biomatrix (San Diego, CA www.advancedbiomatrix.com). Collagen I was applied to the chips in various patterns including a woven randomly orientated pattern, and wavy aligned collagen fibril pattern (Figure 2.6).

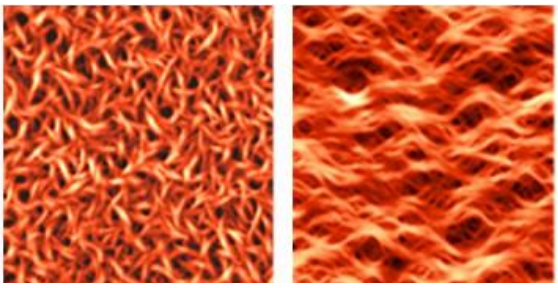


Figure 2.6 Atomic force microscopy image of Fibralign collagen chips Woven dermis like pattern (left image) and aligned pattern (right image) are shown.

Prior to their use in the study described in this thesis, a variety of methods were tested by the author, in order to prepare the collagen substrates for tissue culture. The final protocol used in this thesis is as follows.

The collagen-coated chips were equilibrated in Ca^{2+} and Mg^{2+} free PBS for 5 minutes at room temperature to achieve an equilibrium between the acids and conjugate bases in the buffer and achieve resistance to pH change. The chips were washed in deionized H_2O for 10 seconds, transferred into 70% ETOH for 1 hour in order to sterilise the chip, and dried by leaning on the side of the tissue culture well. The chips were then equilibrated in DMEM culture medium at room temperature for 1 hour before allowing them to dry again for 15 minutes, prior to application of the cells for culture.

Skin and lung fibroblasts, to be used in these studies, were cultured to confluence in 75 ml tissue culture flasks in DMEM plus 10% FCS and then detached using 6 mL of trypsin/EDTA for each flask. Once the cells had detached the trypsin was neutralized by the addition of an equal volume of DMEM with 10% FCS. The cells were centrifuged at 570g for 5 minutes at 20 °C. The total cells (1.5×10^6) were resuspended in 200 µl of serum free DMEM (7500 cells per µl) and applied to the chips (one chip per well of a 6 well tissue culture plate) as 3 µL foci containing 22,500 cells per focus. Culture medium was added adjacent to the chips at 100 µl/well in order to prevent dehydration of the chip, the lid was replaced to prevent media evaporation from the cellular foci, and the cells were allowed to adhere for 45 minutes at room temperature. Finally, the tissue culture dish containing the chip was flooded with 4 mL of 37 °C DMEM with 0.2% or 10% FCS, and cultured further with or without the addition of agonists or inhibitors.

Migration was assayed by Axioscope imaging using an x4 Zeiss objective lens. The area of migration was determined using ImageJ software (NIH http://rsb.info.nih.gov/nih-image/) and normalised against the total area of the culture chip.

2.8.2 CellAlign software

In addition during the initial studies of migration, the orientation of control and SSc lung fibroblasts when migrating on aligned collagen-coated slides was investigated. Alignment was quantified by CellAlign software an automated and adaptable quantification software for cellular alignment analysis from microscopic images.

The input data, which consisted of binary (black and white) images of cell cultures, were prepared from initial Axioscope cell culture images using the public domain NIH ImageJ Image analysis software with Orientation J add in, website as above.

The example of input data and use of the CellAlign software is presented in Figure 2.7.

Binary images transform into a set of isolated (black) clusters (cells) such that each cluster consists of the connected black pixels.

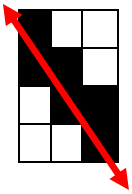
Examples of the connected black pixels are shown below.

Two black pixels A and B are connected if there is a sequence of black pixels such that the first pixel in this sequence is the pixel A, the last pixel in this sequence is the pixel B, and every two sequential pixels have a common boundary. By definition, all black pixels in the cluster are connected and they are not connected with any other black pixels.

The operations on the set of isolated clusters:

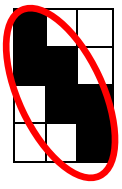
1 Distribution of the areas of the black clusters (area of the cluster is the number of pixels in the cluster), average area of the clusters, standard deviation, colouring clusters according to the distribution (e.g., clusters with largest area – red, clusters with smallest areas – violet).

2 Linear approximation of each black cluster (see example below) and distribution of the cluster orientations. Mean orientation (alignment score), standard deviation, coloring clusters according to the distribution (e.g., clusters with best alignment – red, clusters with poorest alignment – violet).



Linear approximation of the black cluster (cell)

3 Best fit ellipse distribution (see example below), distribution of ellipse areas, distribution of ellipse aspect ratios, distribution of ellipse major axes, average values, standard deviations, coloring according to the distribution.



Minimum ellipse covering the black cluster (cell)

Figure 2.7 Use of CellAlign software to measure orientation of the fibroblasts on Fibralign chips

2.8.3 Scratch wound assay of fibroblast migration

For the scratch migration assay, as described in (Donovan et al., 2013), healthy control and SSc lung fibroblasts were plated and grown to confluence in 6-well plates in DMEM with 10% FCS. The cultures were serum-starved overnight in DMEM with 10 % FCS, and then washed with low serum prior to the assay. Two scratch wounds were induced in each culture using 200 μL micropipette tips, and the cultures were washed twice in PBS. Closure of the scratch wound by fibroblast migration into the wound area was quantified by Axioscope (Carl Zeiss Ltd.) imaging at 24 hours and images of the wound area were measured and analyzed using ImageJ Image analysis software to calculate the area of migration into the scratch wound.

2.9 Methods for *in vivo* model development for testing therapeutic approaches

2.9.1 Mice studied

All mouse studies included in this thesis were performed at Murigenics, Vellejo, California, under approval from the Institutional Animal Care and Use Committee (IACUC Protocol #MG-30, expiration date 17/06/17).

C57BL/6 females were obtained from Charles River Laboratories (Hollister, CA). They were housed in microisolators in a 12:12 light/dark cycle, and were given water and food (standard maintenance rodent chow diet - Teklad 2018) *ad libitum*. Animals were acclimated for 3 days prior to the start of the study, and the mice were 6-7 weeks of age at the initiation of the study.

2.9.2 Bleomycin treated mouse models of fibrosis

The C57BL/6 mice were bred under humane conditions at the MuriGenics facility in San Francisco CA. The standards adhered to were consistent with Institutional Animal Care and Use Committee (IACUC) approved methods in all animal husbandry and experiments for isolation of cells and tissue.

2.9.3 Bleomycin cutaneous model

The bleomycin-induced skin fibrosis mouse model involved daily subcutaneous (SC) bleomycin injections on the flank for 21 days. The injection site was standardized by shaving a small area of the skin of the flank and marking with a henna pencil. Injection was given daily into the center of the marked injection site. Body weights were collected twice each week for the duration of the study. Starting on Day 1, dermal fibrosis was initiated by the first of 30 daily bleomycin injections (BLM; 0.09 units in 100 µL of sterile saline per injection site per mouse, groups 2 and 3).

For studies using the RP 107 anti-inflammatory peptide therapeutic, starting two days before the start of model induction, animals were administered saline or study peptide RP-107 1 mg/kg twice daily, given by subcutaneous injection between the shoulder blades. Saline or study peptide were given twice daily during the 21 day bleomycin treatment period. Animals were euthanized with isoflurane on Day 50, and terminal skin samples (for histology) and blood samples via the retro-orbital sinus (for serum cytokine analysis) were collected.

The extent of skin fibrosis was measured by taking sections of the bleomycin treated area, fixing in formaldehyde and staining histological sections with Masson's trichrome, which stains collagen blue. The thickness of the collagen rich dermal layer of the skin was determined by photographing the histological sections and making measurements using the photographs.

2.9.4 Bleomycin pulmonary model

The bleomycin-induced lung fibrosis mouse model involved the intratracheal instillation into the lungs of bleomycin (Blenoxane, Novaplus, 15 U/vial; VHA Inc., Nippon Kayaku Co., Tokyo, Japan) (0.09 units in 50 µl sterile saline per mouse, groups 2 and 3). The bleomycin solution was administered under anesthesia, using a mixture of 1 mL of ketamine (100 mg/ml) and 1 mL of xylazine (100 mg/ml) plus 4.6 ml of sterile bacteriostatic saline at a dose of 0.1 mL/30 g body weight.

Again in studies with the RP107 anti-inflammatory therapeutic peptide, starting two days before the start of model induction, animals were pretreated with saline or study peptide RP-107 1 mg/kg twice daily by subcutaneous

injection. Saline or subcutaneous study peptide treatments were continued twice daily for 30 days. Animals were euthanized with isoflurane on day 30, and terminal lung samples (for histology) and blood samples via the retro-orbital sinus (for serum cytokine analysis) were collected.

2.9.5 Genotyping the GPVI$^{-/-}$ and LEPREL1$^{-/-}$ GPVI$^{-/-}$ mice

2.9.6 Methods of Breeding GPVI knock-out and GPVI/LEPREL 1 double knock-out mice

GPVI ($^{-/-}$) single knock-out (SKO) and GPVI/LEPREL 1 ($^{-/-}$, $^{-/-}$) double knock-out (DKO) mice were obtained from the laboratory of Hans Peter Bachinger (Shriners Hospital for Children, Portland, OR). At 7-8 weeks of age, each SKO male was bred with 1-3 SKO females and each DKO male was bred with 1-3 DKO females. Because DKO females tended to be less fecund than SKO females, we also bred SKO females to DKO males to produce females with the SKO/HET (GPVI $^{-/-}$, LEPREL 1 $^{-/+}$) genotype. These female SKO/HET mice were then back bred to DKO males to produce 50% SKO/HET and 50% DKO mice. The mice were then ear tagged and tail clipped to determine genotype.

These mice were 8-12 weeks of age when used for experiment. They were group housed in microisolators in a 12/12 light/dark cycle, and were given water and food (standard maintenance rodent chow diet - Teklad 2018) *ad libitum* as approved by IACUC.

2.9.7 Genotyping the *LEPREL1* knockout mice

DNA was extracted from tail biopsies taken from the mice using the Qiagen blood and tissue DNA extraction kit and eluted with 200µl of EB buffer. For PCR reactions Qiagen Master Mix was used, set up as 10 µl reactions as follows:1 µl of each primer (10 – 25 pmol), 3 µl of DNA, 0-2 µl of water and 5 µl of MasterMix. PCR conditions were optimized by using positive controls and titrating the amount of genomic DNA to find the optimum template concentration. Subsequent to completion of the PCR the product is run on agarose gels. Standard DNA ladders/molecular weight size markers are run on

the gels to determine the corresponding size of the PCR products. The reaction products were run on a gel and imaged.

The *LEPREL1* gene silencing in the KO mice is due to the insertion of a neomycin resistance cassette into the second exon of the gene (Pokidysheva et al., 2014). This presence of this insertion was used to genotype the mice for *LEPREL1*, using qPCR primers upstream and downstream of the insertion as well as a primer targeting the neomycin cassette. For *LEPREL1*- the forward primer-5' TCCACTGTATGCTTTAGCTC 3', and reverse primer-5' GTCATGTTGTTCTCGTCTAC 3' were used, and for the neomycin probe 5' CGAAGGAGCAAAGCTGCTAT 3'. This gives 2 potential products on PCR, a heavy band for KO, and a lower MW band for WT, so that both bands are seen for heterozygous mice (Figure 2.8).

For GPVI genotyping the following primers were used; forward primer-5' CTGTAGCTGTTTTCAGACACACC3' reverse primer-5'CCATCACCTCTTTCTGGTTAC 3'. These primers give a band of 381bp for WT and no band for KO. GAPDH was used as an internal positive control. Standard commercially available primers are used for assessing GAPDH.

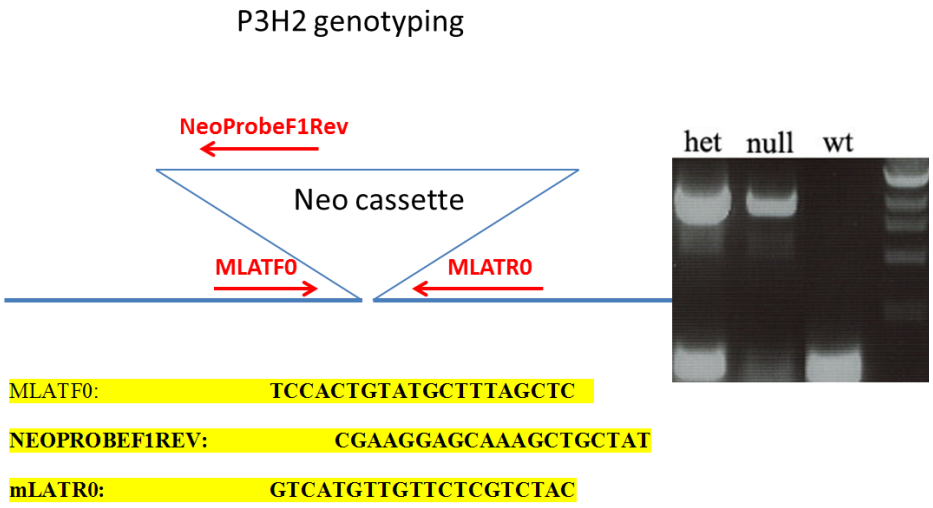


Figure 2.8 *LEPREL1* genotyping DNA was purified from tail biopsies using Qiagen Blood and Tissue DNA extraction kit, eluted with 200 µl of EB buffer. 3 µl of these DNA samples were used per qPCR reaction. For the WT reaction primers designated MLATF0 and MLATR0 were used. A third primer was added complimentary to the neomycin cassette sequence (NeoprobeF1Rev) known to be inserted in the exon 2 of null mice. As shown the PCR reaction gives one short product for WT DNA, one long product for the null DNA, and two bands for the heterozygous DNA.

2.9.8 Bleomycin study protocol with KO mice

The study included the following treatment groups: Group 1. Saline control C57BL/6 background WT mice (n=5), Group 2. bleomycin treated C57BL/6 background WT mice (n=7), Group 3. Bleomycin treated *GPVI* SKO (n=7), and Group 4. Bleomycin treated LEPREL1 and *GPVI* DKO mice (n=7).

Mice were shaved in the lower back quadrant and then a small circle was drawn with henna stain or a felt pen, where mice were injected subcutaneously daily for 21 days with 50 µL of bleomycin at a concentration of 0.56 mg/mL.

Mice were weighed once per week till day 21, the end of the study. Hang time was determined as a measure of endurance and strength with each mouse tested 3 consecutive times and the average was taken. Photographs of the injected areas were taken weekly for 3 weeks. Mice were weighed weekly. On day 30 the mice were sacrificed with CO_2, blood was collected (via cardiac puncture) and the injected cutaneous region was excised, formalin fixed and stained by Masson's trichrome. Plasma was analyzed for IL-6, IL-12p70, TNFα, IL-10, by a limited Multiplex assay, and TGFβ and PDGF-BB by ELISA.

Chapter 3. Investigating Biomechanisms in Systemic Sclerosis by Copy Number Variation Analysis of Candidate Genes

3.1 Introduction

3.1.1 The challenge of identifying genetic elements linked to systemic sclerosis pathogenesis

SSc is a complex disease which results from multiple contributing mechanisms, including injury to vascular tissue, autoimmune effects, inflammatory involvement, as well as age dependence. The course of the disease paints a picture of a complex syndrome that occurs within an individual based on their genetic background, but triggered by external factors. Identifying genes within pathways linked to the causative biomechanisms may lead to the discovery of new diagnostic biomarkers, the development of new therapies, or the identification of those patients who would be the most suited candidates for specific treatments.

SSc is likely to be a polygenic disorder (Bhattacharyya et al., 2012a), and the risk of acquiring SSc is some 13-fold higher in first degree relatives as concluded from a study carried out on three USA cohorts (Arnett et al., 2010). However, there are no known markers sufficient to reliably predict disease susceptibility (Bhattacharyya et al., 2012b), or truly effective therapies (Gabrielli et al., 2009). Since susceptibility markers can be causally related to the disease, corresponding genes and their products potentially represent new drug targets. Thus, advances made in searches for new susceptibility markers may not only lead to better disease diagnosis, but to new therapies as well.

The recent explosion of GWAS was anticipated to bring a stream of clinically valid genetic markers for polygenic diseases including SSc. GWAS have identified previously unknown susceptibility loci, helping to identify novel genes and gene regulatory regions which may be linked to a disease. However, replicating the findings relating to the chromosomal regions showing significance in the analyses of GWAS has proven difficult due to the heterogeneity of both the disease and the various diverse populations in which the disease occurs. The results have been below expectations, few new genetic

markers have emerged, and results with many initially identified markers failed to be duplicated in validation studies (Craddock et al., 2010a).

Finding genetic markers for susceptibility to SSc requires the identification of genomic regions harboring genes or gene regulatory regions involved in the disease process. GWAS involves the rapid screening of genetic markers, most often single nucleotide polymorphisms (SNPs), but sometimes also including copy number variations (CNV mutations), across the genomes of many different groups of people or cohorts to find disease associations.

Since the GWAS approach relies on broad genomic analyses the technique does not require the selection of gene candidates through *a priori* knowledge of how a gene could be involved in the disease. In contrast, candidate gene methods rely on the careful selection of gene candidates based upon some prior understanding of their function as it could relate to the disorder. GWAS provides broader genomic coverage and may help to identify loci with previously unidentified functions. However, large scans such as these are more difficult to parse and have the potential to produce large numbers of false positives, thus requiring a very stringent significance criterion (GWAS level of significance, $p<5 \times 10^{-8}$). The candidate gene method requires more upfront time investment in literature reviews, database queries, or other research based tools to identify functionally relevant gene candidates and will not include any genomic regions with either unidentified functions or functions not thought to be important in the disease being investigated. The advantage of the candidate gene approach is the ability to focus and “home in” on genes that are more likely to be involved in the disease process.

Despite the broad number of genomic regions scanned in GWAS, only a handful of genetic loci have been identified and replicated across SSc studies. To date histocompatibility (HLA/MHC) genetic susceptibility regions show the strongest linkage to SSc, yet even these loci do not always replicate. This is probably due to the heterogeneous nature of SSc, the varied ethnicity of the represented cohorts within each of the study samples, and the relative rarity of SSc (Castelino and Varga, 2013).

The aetiology of SSc is likely to be both multigenic and multi-factorial (Gorlova et al., 2011). While the clinical manifestations tend to cluster to specific subgroups, these populations often are made up of cohorts sharing a

particular environment and are ethnically homogenous. For example, afflicted people within multi-case SSc families tend to be concordant for SSc-specific autoantibodies and HLA haplotypes (Assassi et al., 2007). Also, certain SSc clinical features and the presence of disease-specific autoantibodies vary between different countries and ethnicities (Steen, 2008, Nietert et al., 2006). The aggregation of genes within homogeneous groups could explain why loci only meet a level of significance when specific genetically similar cohorts are represented in sufficient numbers within a study.

One approach that helps circumvent the issue of divergent linkage results between studies is meta-analysis (Gorlova et al., 2011, Martin et al., 2013, Lopez-Isac et al., 2016). Meta-analysis involves combining a number of studies and then stratifying and re-analyzing the data by factors such as clinical features, presence of specific autoantibodies and disease overlap (for instance, with fibrosing autoimmune conditions) (Varga and Hinchcliff, 2014). This type of analysis has a number of benefits: meta-analysis increases the sample size and number of SSc cohorts, thereby more accurately representing the disease population and increasing the chances of achieving significance. Meta-analysis ensures that the same criteria are being used to define and stratify subgroups. Most importantly, meta-analysis contributes to the mapping of genes and gene/environment interactions that can yield a number of complex phenotypes proving difficult to detect within smaller isolated studies. In an example of this approach a recent study combined SSc patients, rheumatoid patients, and control data in a meta-analysis and identified the importance of an IL-12 pathway gene in the pathogenesis of both rheumatoid and SSc (Lopez-Isac et al., 2016).

Taken together, GWAS studies indicate that molecular and clinical classification may allow us to distinguish and stratify patients into subgroups to develop targeted interventions, if the proper level of resolution is applied. Genetic polymorphisms tend to cluster within discrete subgroups that display common clinical and autoantibody profiles. However, most studies lack sufficient statistical power due to the limited sample sizes of the represented cohorts, and this challenge is a proven barrier to defining the genetic contributions to the disease. Meta-analysis can help by increasing and diversifying the sample pool within a GWAS. However, it remains difficult to

identify loci with reproducible associations, and often the functions of the identified loci are elusive.

Applying what has been learned through GWAS, the logical next steps involve larger cohorts to discover variants with lower penetrance. A further step is to use already identified susceptibility loci of known functional significance to select specific genes for higher resolution genotyping using SNP and/or CNV markers and to characterize the expression of genes within these loci using RT-PCR, and to search for associated increased or decreased levels of key biomarkers such as those downstream of estrogens or via the TGFβ pathway.

3.1.2 Examining copy number variants in systemic sclerosis

Alternatively, examining CNVs in gene and gene regulatory regions already identified as having potential relevance to SSc could help narrow the focus to a more workable number of targets. CNVs are a consequence of the multiplication of a genomic segment, amplifying the amount of genomic sequence, a deletion resulting in a reduction in the amount of genomic sequence or an inversion or translocation from one genomic chromosomal location to another (see a description of CNVs, Figure 3.1, and their significance, Figure 3.2).

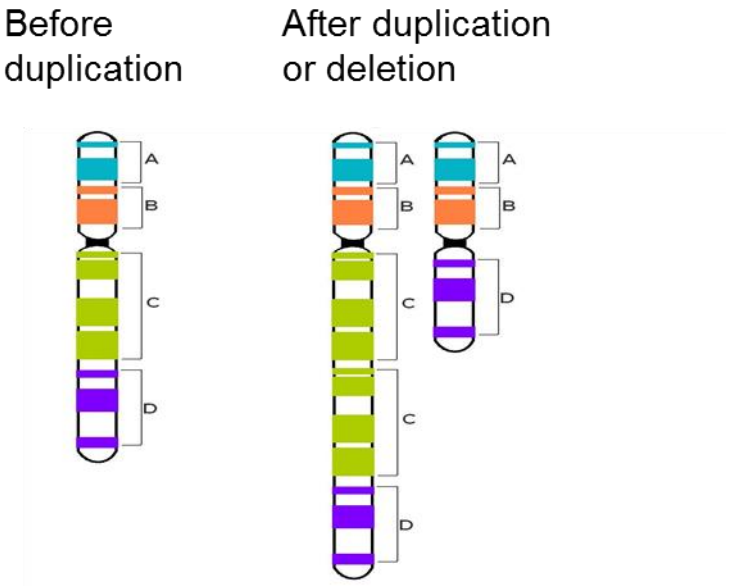


Figure 3.1. Copy number variation CNVs correspond to deletions or duplications of genomic segments greater than 1 kb. These variations in the genomic sequence are common and may be linked to complex disorders and traits. Adapted from (Redon et al., 2006).

Significance of some CNVs

- **CCL3L1**----------**Deleted** (higher risk of HIV infection)
- **CCL3L1**----------**Duplication** (lower risk of HIV infection)
  Competitive binding to HIV co-receptor CCR5
  (Carpenter et al . 2012 Functional effects of CCL3L1 copy Number (Genes Immun 13. 374-9)
- **FCGR3B**----------**Low copy number** (risk for glomerulonephritis)
  Altman TJ et al. 2006 Copy number polymorphism in FCGR3 Predisposes to glomerulonephritis in rats and humans. Nature 439:851-855)

Figure 3.2 Examples of clinically important copy number variations Chemokine (C-C motif) ligand 3-like 1 (CCL3L1) binds to the CCR5, a co-receptor of HIV, thus inhibiting HIV penetrance. A deleted CNV of Fc fragment of IgG, low affinity lllb receptor (FCGR3B) has been associated with glomerulonephritis.

The Human Genome Project which began in 1990, led to a working draft of the human genome sequence that was announced in 2000, and a more complete version published in 2003 (Collins et al., 2003, Schmutz et al., 2004). In 2004, Lee and colleagues reported that approximately 12% of the human genome contained structural variations among healthy individuals, estimating that 0.4% of the genomes of unrelated individuals differ in frequency and location of DNA CNVs (Iafrate et al., 2004). CNVs are large segments of DNA, ranging from 1 KB to a megabase in size (Stankiewicz and Lupski, 2010). CNVs could be the consequence of duplication (increasing the normal number of these DNA sequences) or deletions (resulting in reduced sequence number), as well as inversions or translocations (Stankiewicz and Lupski, 2010). CNVs are found in genes, gene fragments, and intergenic regions (Conrad et al., 2010). It is estimated that the human genome contains between 5,000 and 10,000 CNVs (Conrad et al., 2010).

CNVs have been found to be associated with various diseases (Girirajan et al., 2011, Fanciulli et al., 2010). For example, individuals with fewer copies of the *CCL3L1* gene are at increased risk of HIV infection, because the protein competes with HIV for binding to a cellular receptor which HIV uses to penetrate the cell (Gonzalez et al., 2005). Higher copy numbers of *CCL3L1* are associated with rheumatoid arthritis (McKinney et al., 2008). CNVs have been implicated in many common disorders, such as cancer (Kuiper et al., 2010), Alzheimer's and Parkinson's diseases (Meeus et al., 2012, Kay et al., 2010), as well as SLE (Yang et al., 2007, Garcia-Ortiz et al., 2010, Molokhia et al., 2011, Cheng et al., 2013). CNVs are usually inherited through the germ line, but can also arise *de novo* as mutations within certain cell types (Cooper et al., 2011, Miller et al., 2010).

Identifying amplified or deleted CNVs in SSc could provide insight into the pathogenic mechanisms involved in the initiation, progression and/or gender association of the disorder. For example, McKinney and colleagues (McKinney et al., 2012) demonstrated that a deletion in the gene *FCGR3B*, which encodes a low-affinity Fc receptor 3B (FCGR3B), is a risk factor in SSc. This gene is involved in the recruitment of polymorphonuclear neutrophils to sites of inflammation as well as their activation by IgG, and was originally identified by its role in SLE. This marker, as well as other identified CNV variants, provides

supporting evidence that the CNV approach is a valid method for identifying susceptibility markers in diseases including SSc.

CNVs represent key factors driving diversification and adaptation by both host and pathogens (Samarakoon et al., 2011). Common CNVs can encompass genes that predispose individuals to disease, have a protective effect or are benign in nature (Craddock et al., 2010a). Compared to inherited CNVs, *de novo* CNVs appear to be detrimental more often than favorable (Miller et al., 2010, Cooper et al., 2011). For example, a recent publication, described an inherited CNV duplication resulting in a positive phenotype (Dennis et al., 2012). This particular CNV (*SRGAP2C*) is ancestral in nature and appears to have increased neuronal migration and connections in humans, possibly leading to higher learning capacity (Dennis et al., 2012).

The location of a CNV in relation to a gene and its regulatory elements, as well as its size, is paramount to the likelihood that it will be responsible for modulating genetic expression (Craddock et al., 2010b). Genes with CNVs are expressed at more variable levels than genes without (Henrichsen et al., 2009a, Stranger et al., 2007), and CNVs also may have an impact on the transcriptome as a whole (Kleinjan and van Heyningen, 2005). The simple presence of a structural change at a given position in the human genome may cause perturbations in particular pathways regardless of gene dosage. CNVs may bring about changes in gene expression in a number of ways, for example directly by affecting the transcript gene itself, or indirectly through effects on downstream pathways and regulatory networks (Dermitzakis et al., 2014, Henrichsen et al., 2009a). CNVs in regulatory regions within a gene could potentially cause steric hindrance or other conformational changes affecting the transcriptional apparatus thus inhibiting efficient transcription of the gene (Sexton et al., 2007, Stranger et al., 2007). CNV-induced mechanisms may involve the physical dissociation of the transcription unit from *cis*-acting regulators (Kleinjan and Heyningen, 1998, Kleinjan and van Heyningen, 2005). It seems clear, however, that genes with CNVs are often expressed differently than the genes lacking CNVs. In general, there is a stronger correlation between the presence of CNVs and the expression levels of a gene product than that seen with nearby SNPs (Schlattl et al., 2011).

Notably, CNVs that are located within introns have greater potential to influence gene regulation than intronic SNPs, because the majority of genetic regulation, including the initiation of transcription, hormonal regulation and splicing, is the result of various factors that bind to intronic “motifs” within a nucleotide sequence (Hannenhalli, 2008, Altobelli, 2012). In this kind of binding, a change at a single nucleotide (as with a SNP) is likely less important than complete disappearance or multiplication of a larger region including one or more “motifs” (as with a CNV) (Hannenhalli, 2008).

3.1.3 Selecting CNVs for analysis: focusing on loci within the first intron of candidate genes

Many genomic analyses have identified CNVs utilizing technologies such as qPCR, Next Generation Sequencing and hybridization, and they are described in several databases available on the internet. In selecting certain CNVs for analysis, a list was made of possible genes with CNVs in the first intron. While the published database shows only a limited number of genes to have CNVs in this intron, other possible candidate genes related to fibrosis but lacking intron 1 CNVs were not examined here.

Initially, CNVs were selected for consideration based on their proximity to genes with potential roles in SSc. Since SSc is a relatively rare disorder, the selection criteria were based on the approach used by Conrad (Conrad et al., 2010), in which screening was conducted for a 10% minor allele frequency (MAF), and CNVs meeting these criteria were identified. A component of the algorithm dictated the exclusion of intergenic locations and overlapping loci with excessive segmental duplication. This also helped reduce the total number of about 10,000 known CNVs greater than 1kb to a more comfortable number of CNVs that could be influential in terms of their relation to the gene and the phenotype. This reduction allowed the switch from array technology to TaqMan PCR, the method of choice in CNV quantification used both at the validation step and at the screening step. As part of the algorithm to identify involved CNVs, the initial focus was on CNVs that were located in introns, since such markers have more potential to influence gene regulation than intronic SNPs or CNVs in other locations.

3.1.4 Summary of background leading to the hypothesis

As described above, SSc is a complex disorder in which multiple genetic polymorphisms contribute to susceptibility. CNVs, as a common form of polymorphism, are relatively unstudied in SSc, compared to the more widely studied SNPs. Analysis of CNVs could yield important information regarding susceptibility to SSc, additional to that obtained from SNP analysis. Identifying CNVs associated with risk or protection against SSc could identify new genes and related biomechanisms involved in the SSc disease process. *Therefore, it is hypothesized that certain CNVs, particularly those present in non-coding sequences of potential regulatory importance, affect susceptibility to SSc and therefore differ in their frequency in SSc patients and controls.*

3.1.5 Overall aim of the chapter

In order to test the hypothesis, this Chapter aims to identify genetic variants associated with SSc development using a genetics platform which assays CNVs present in the first intron of candidate genes.

3.2 Methods

3.2.1 Putative systemic sclerosis-related CNVs selected for study

In order to address the above aim, a PCR-based method was used in to identify CNVs in candidate genes using DNA samples from patients and controls. A collaboration established with colleagues at the Royal Free Hospital has permitted the CNV analyses using the SSc patient DNA collections held there at the Centre for Rheumatology. Using PCR, the amounts of patient DNA required are minimal, representing 30 ng of DNA per PCR analysis (see Chapter 2 Materials and Methods). Previously, this methodology for CNV analysis was developed in panels of DNA from patients with Alzheimer's disease and multiple sclerosis, and the PCR assays were found to be reproducible and sensitive, and validated in independent panels of these patients

Patient samples were matched as closely as possible to controls in sex and age. One hundred and five SSc DNA patient samples (47 M, 58 F) and

ninety-six control DNA samples (49 M, 47F) were chosen for survey in our study. In order to reduce heterogeneity, only Caucasian patients from the dcSSc subset were included. Because of the striking sexual dimorphism of the disease, data from males and females will be analysed independently. Promising candidate factors will be studied further by phenotyping of SSc cells, and in mouse model systems.

The final choice of CNVs to be screened was based on the following criteria. CNVs did not include those with any published SNPs known to be associated with SSc risk. No segmented duplications were examined which overlapped with the CNVs of interest. Minor allele frequency was less than or equal to 10%. The CNV selected must have been verified in independent genomic analyses.

The set of CNVs chosen for investigation includes genes that might be involved in proposed biomechanisms central to the aetiology or the progression of SSc based upon some *a priori* knowledge of their function. The following CNVs were selected, based on the known function of the genes as summarised (see below). Gene Card ID of the named gene (GCID http://www.genecards.org/) as well as genomic coordinates are highlighted. CNVs selected based on the methodology summarised in Figure 2.1 are shown.

CREB5

cAMP Responsive Element Binding (CREB) (GCID: GC07P028305). The encoded protein binds to the cyclic AMP (cAMP) response element and activates transcription. CREB proteins are important global gene regulators, and autoantibodies against a CREB-related factor have been observed in SSc patients (Akiyama et al., 2009). Factors that elevate CREB phosphorylation in fibroblasts suppress collagen and CTGF production (Stratton et al., 2002). Chromosome 7: 28,299,321-28,825,894 forward strand. CNV esv2659069 (Cooper et al., 2011).

NDST4

N-Deacetylase/N-Sulfotransferase (GCID: GC04M115748). The encoded protein is an essential bifunctional enzyme that catalyzes both the N-deacetylation and the N-sulfation of glucosamine (GlcNAc) of the

glycosaminoglycan in heparan sulfate. Heparan sulfate is a component of the ECM, and contributes to the fibrosis seen in SSc (Abraham et al., 2007). Chromosome 4: 114,827,763-115,113,876 reverse strand. CNV esv2266453 (Bentley et al., 2008).

CYP3a43

Cytochrome P450, Family 3, Subfamily A, Polypeptide 43 (GCID: GC07P099426). The encoded protein is a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases that catalyze many reactions involved in drug metabolism and the synthesis of cholesterol, steroids, and other lipids. Polymorphisms at cytochrome-encoding loci have been implicated in scleroderma susceptibility (Povey et al., 2001). Chromosome 7: 99,828,013-99,866,102 forward strand. CNV esv3382874 (Abecasis et al., 2010)

SDC2

Syndecan 2 (GCID: GC08P097575). The encoded protein is a transmembrane (type I) heparan sulfate proteoglycan and is a member of the syndecan proteoglycan family. The syndecans mediate cell binding, cell signaling, and cytoskeletal organization, and syndecan acts as a receptor for and is required for internalization of the HIV-1 tat protein. The syndecan-2 protein functions as an integral membrane protein and participates in cell proliferation, cell migration, and cell-matrix interactions via serving as a co-receptor for growth factors. ECM regulation contributes to the fibrosis seen in SSc, and syndecan 2 is differentially expressed in SSc (Abraham et al., 2007). Chromosome 8: 96,493,351-96,611,780 forward strand. CNV dgv12247n54 (Cooper et al., 2011).

ESR1

Estrogen Receptor 1 (GCID: GC06P151980). The encoded protein is an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. Estrogen and its receptors are essential for sexual development and reproductive function, but also play a role in other tissues, such as bone. Estrogen receptors are also involved in pathological processes including breast

cancer, endometrial cancer, and osteoporosis. ESR1 is a target of interest, given the female bias of scleroderma and the effect of estrogen on ECM production in SSc-derived fibroblasts (Soldano et al., 2010). Chromosome 6: 151,656,691-152,129,619 forward strand. CNV nsv349546 (Mills et al., 2011).

SMAD-1

Mothers Against DPP Homolog 1 (Drosophila) GCID: GC04P146402). SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. This protein with other SMADs mediates signaling by TGFβ and bone morphogenetic proteins (BMPs), and is involved in a range of biological activities including cell growth, apoptosis, morphogenesis, development, immune responses and collagen synthesis. In response to TGFβ and BMP ligands, this protein is phosphorylated and activated by the BMP receptor kinase. Specifically, SMAD-1 is a known effector of TGFβ in SSc fibroblasts (Trojanowska, 2009). Chromosome 4: 146,402,951..146,480,32 forward strand. CNV nsv595621 first intron of splice variant (Cooper et al., 2011).

HPSE2

Heparanase 2 GCID: GC10M100208). The encoded protein is a heparin degrading enzyme, an endoglycosidase that degrades heparan sulfate proteoglycans located in the extracellular matrix and on cell surface. This protein may play a role in biological processes involving remodeling of the extracellular matrix during angiogenesis and tumor progression. ECM dysregulation contributes to the fibrosis seen in SSc (Abraham et al., 2007). Chromosome 10: 98,457,077-99,235,862 reverse strand. CNV nsv552043 (Cooper et al., 2011).

TGFBR3

Transforming Growth Factor Beta Receptor III GCID: GC01M092145). The encoded protein is a TGFβ receptor, a membrane proteoglycan that often functions as a co-receptor with other TGFβ receptor superfamily members. Ectodomain shedding produces soluble TGFBR3, which may inhibit TGFβ activity by binding the growth factor. Decreased expression of this receptor has been observed in various cancers. TGFβ is known to contribute to fibrosis and

systemic sclerosis (Jinnin, 2010, Varga and Whitfield, 2009), and TGFβ3 specifically has been implicated in the pathogenesis of scleroderma (Ozbilgin and Inan, 2003). Chromosome 1: 91,680,343-91,906,335 reverse strand. CNV nsv1360 (Kidd et al., 2008)

MIR384

MicroRNA 384 GCID: GC0XM076057). MicroRNAs (miRNAs) are short (20-24 nt) non-coding RNAs that mediate post-transcriptional regulation of gene expression in multicellular organisms by affecting both the stability and translation of specific mRNAs. Recent data suggests that this miRNA is a regulator of cell signaling and apoptosis genes in cardiac tissue (Bao et al., 2013). Chromosome X: 76,919,273-76,919,360 reverse strand. CNV nsv442809 (extends through coding sequence) (McCarroll et al., 2008).

TNFSF13b

Tumor Necrosis Factor (Ligand) Superfamily, Member 13b GCID: GC13P108903). The encoded protein is a cytokine that belongs to the TNF ligand family. This cytokine is a ligand for receptors TNFRSF13B/TACI, TNFRSF17/BCMA, and TNFRSF13C/BAFFR. This cytokine is expressed in cells of the B cell lineage, and acts as a potent B cell activator. TNFSF13b has also been shown to play an important role in the proliferation and differentiation of B cells. Serum levels of this cytokine are elevated in patients with localized scleroderma (Matsushita et al., 2007). Chromosome 13: 108,251,240-108,308,484 forward strand. CNV esv2563318 (McKernan et al., 2009)

ACOT11

Acyl-CoA Thioesterase 11 GCID: **GC01P055007.** This gene encodes a member of the acyl-CoA thioesterase family that catalyse the conversion of activated fatty acids to the corresponding non-esterified fatty acid and coenzyme A. Expression of a mouse homolog in brown adipose tissue is induced by low temperatures and repressed by warm temperatures. Higher levels of expression of the mouse homolog have been found in obesity-resistant mice compared with obesity-prone mice, suggesting a role of acyl-CoA thioesterase11 in obesity. Chromosome 1: 54,542,257-54,639,192 forward strand. . CNV esv3586093 (1000 Genomes Project Consortium -

ftp://ftp.ebi.ac.uk/pub/databases/dgva/estd214_1000_Genomes_Consortium_Phase_3/)

LEPREL1

Leprecan-Like 1, (P3H2) GCID: **GC03M189674.** This gene encodes a member of the prolyl 3-hydroxylase subfamily of 2-oxo-glutarate-dependent dioxygenases. These enzymes play a critical role in collagen chain assembly and stability by catalyzing post-translational 3-hydroxylation of certain proline residues in the protein. Chromosome 3: 189,956,728-190,122,437 reverse strand. nsv514192 (Campbell et al., 2011).

SRY

Sex determining region Y GCID: **GC0YM002698**. Sex determining region of the Y chromosome (*SRY*) is used as a reference control for these studies. *SRY* is located in the distal part of the short arm of the Y chromosome. *SRY* encodes a high mobility group protein which acts as a transcription factor, binding to double stranded DNA and bending the axis. *SRY* is essential for male sex determination in humans and other mammals. *SRY* initiates the gene expression events leading to the formation of the testis from an undifferentiated gonad (reviewed in (Koopman, 1999)). Chromosome Y: 2,786,855-2,787,699 reverse strand. Coding sequence targeted, used as internal control.

FGFR2

Fibroblast Growth Factor Receptor 2 GCID: **GC10M123223.** The protein encoded by this gene is a member of the fibroblast growth factor receptor family, where amino acid sequence are highly conserved between members and throughout evolution. FGF ligands are involved in angiogenesis and raised levels of FGF2 protein were found in SSc blister fluid (see Chapter 4). Chromosome 10: 121,478,334-121,598,458 reverse strand. CNV esv2676868 (Abecasis et al., 2012).

NRG1

Neuregulin 1 GCID: **GC08P031554.** The protein encoded by this gene was originally identified as a 44-kD glycoprotein that interacts with the NEU/ERBB2 receptor tyrosine kinase to increase its phosphorylation on tyrosine residues. This protein is a signaling protein that mediates cell-cell

interactions and plays critical roles in the growth and development of multiple organ systems. Chromosome 8: 31,639,386-32,767,959 forward strand. CNV esv 2167886 (Bentley et al., 2008).

CXCR4

Chemokine (C-X-C Motif) Receptor 4 GCID: **GC02M136871.** This gene encodes a CXC chemokine receptor specific for stromal cell-derived factor-1. Mutations in this gene have been associated with the WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome. Chromosome 2: 136,114,349-136,118,165 reverse strand. CNV nsv523997 (Shaikh et al., 2009)(CNV extends beyond first intron).

SCN3a

Sodium Channel, Voltage-Gated, Type III, Alpha Subunit GCID: **GC02M165908.** Voltage-gated sodium channels are transmembrane glycoprotein complexes composed of a large alpha subunit with 24 transmembrane domains and one or more regulatory beta subunits. They are responsible for the generation and propagation of action potentials in neurons and muscle. Chromosome 2: 165,087,522-165,204,067 reverse strand**.** CNV esv2585640 (McKernan et al., 2009).

OPCML

Opioid Binding Protein/Cell Adhesion Molecule-Like GCID: **GC11M132321.** This gene encodes a member of the IgLON subfamily in the immunoglobulin protein superfamily. The encoded protein is localized in the plasma membrane and may have an accessory role in opioid receptor function. Chromosome 11: 132,414,977-133,532,519 reverse strand. CNV dgv85n21 (Shaikh et al., 2009).

FOXP3

Forkhead Box P3 GCID: **GC0XM049106.** The protein encoded by this gene is a member of the forkhead/winged-helix family of transcriptional regulators, and has a critical role in regulatory T cell differentiation. Defects in this gene are the cause of immunodeficiency, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX), also known as X-linked autoimmunity-

immunodeficiency syndrome. Chromosome X: 49,250,436-49,264,826 reverse strand. CNV nsv528292 (Shaikh et al., 2009).

EV15

Ecotropic Viral Integration Site 5 GCID: **GC01M092974.** The protein encoded by this gene functions as a regulator of cell cycle progression by stabilizing the FBXO5 protein and promoting cyclin-A accumulation during interphase. The protein may play a role in cytokinesis. Chromosome 2: 88,897,232-88,897,784 forward strand. CNV esv1938423 (Bentley et al., 2008).

KCNJ15

Potassium Inwardly-Rectifying Channel, Subfamily J, Member 15 GCID: **GC21P039529.** Potassium channels are present in most mammalian cells, where they participate in a wide range of physiologic responses. The protein encoded by this gene is an integral membrane protein and inward-rectifier type potassium channel. The encoded protein has a greater tendency to allow potassium to flow into a cell rather than out of a cell. Chromosome 21: 38,157,034-38,307,357 forward strand. esv3387523 (Abecasis et al., 2010).

PTPRM

Protein Tyrosine Phosphatase, Receptor Type, M GCID: **GC18P007557.** The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation by cleaving receptor tyrosine phosphate which terminates signaling. Chromosome 18: 7,566,782-8,406,861 forward strand. CNV 576424 (Cooper et al., 2011).

GRIK4

G protein-coupled receptor kinase 4 GCID: **GC04P002967.** This gene encodes a protein that belongs to the glutamate-gated ionic channel family. Glutamate functions as the major excitatory neurotransmitter in the central nervous system through activation of ligand-gated ion channels and G protein-coupled membrane receptors. Chromosome 4: 2,963,608-3,040,747 forward strand. nsv556475 (Cooper et al., 2011).

PLCB1

Phospholipase C, Beta 1 GCID: **GC20P008061.** Phospholipases are expressed ubiquitously and have diverse biological functions including roles in inflammation, cell growth, signaling and death and maintenance of membrane phospholipids. Chromosome 20: 8,077,251-8,968,360 forward strand. nsv517649 (Shaikh et al., 2009).

3.3 Results

3.3.1 Possible association between a CNV in *LEPREL1* and systemic sclerosis disease susceptibility in males

Of the CNVs assessed for linkage, a CNV in *LEPREL1,* nsv514192 (Campbell et al., 2011) demonstrated an association with SSc, but in males only (Tables 3.1a & 3.1b). Homozygosity for the *LEPREL1* first intron CNV (+/+) was associated with increased risk of SSc development in males with an odds ratio (OR) of 2.1 and 95% confidence interval of 1.1-3.9, Chi Square $p<0.029$. When corrected for multiple comparisons this association was no longer statistically significant. However, because of its known biologic role in collagen metabolism including basement membrane collagen IV, as well as a potential role in fibrillary collagen metabolism, *LEPREL1* was considered a promising candidate factor and studied further in tissue culture and in a mouse model (see below).

Also, the *NRG1* CNV assayed was associated with SSc susceptibility in females, with an OR of 1.4. However, in this instance the 95% confidence interval was 0.82-2.5, and also when corrected for multiple comparisons no overall statistical significance was seen. *NRG1* is functionally linked to growth and differentiation of neuronal tissue, epithelial cells, as well as maintenance of cardiomyocytes, all of which are potentially relevant to SSc pathogenesis. However the link to any potential therapeutic approach was thought to be less obvious with this target than with *LEPREL1,* and so *NRG1* was not pursued further in the current thesis, although this could be a possible further study.

Gene	SSc (-/-)	SSc (+/-)	SSc(+/+)	Odds Ratio[1]	OR 95%LCL	OR 95%UCL	p-value	corrected p value
	HC (-/-)	**HC (+/-)**	**HC(+/+)**					
CYP3A	0	1	46	1.9	0.17	22	0.58	NS
	0	2	47					
SCN3a	39	8	0	0.48	0.19	1.2	0.19	NS
	36	10	3					
KCNJ15	1	24	22	1.8	0.98	3.3	0.074	NS
	4	32	13					
PTPRM	0	17	30	0.57	0.25	1.3	0.14	NS
	0	11	38					
PLCB1	38	7	2	0.63	0.28	1.4	0.63	NS
	36	9	4					
NDST4	3	24	20	1.1	0.59	2.0	0.69	NS
	2	29	18					
HPSE2	31	12	4	1.1	0.52	2.1	0.23	NS
	30	18	1					
SMAD-1	0	44	3	1.0	0.57	1.8	0.96	NS
	0	46	3					
SDC2	0	46	1	0.96	0.55	1.7	0.37	NS
	1	45	3					
EV15	1	40	6	0.97	0.55	1.7	0.98	NS
	1	**41**	**7**					
LEPREL1	1	20	26	2.1	1.1	3.9	0.028	NS
	9	20	20					
GRIK4	5	40	2	0.96	0.54	1.7	0.59	NS
	3	45	1					
ACOT	7	29	11	0.58	0.32	1.0	0.11	NS
	3	26	20					
CXCR4	3	44	0	0.96	0.54	1.7	0.61	NS
	2	47	0					
TNFSF13b	1	44	2	1.2	0.67	2.1	0.35	NS
	4	44	1					
CREB5	3	44	0	1.0	0.56	1.8	0.96	NS
	3	46	0					
TGFBR3	12	32	3	0.80	0.45	1.4	0.065	NS
	5	43	1					
MIR384	3	12	32	0.89	0.43	1.8	0.88	NS
	2	13	34					
OPCML	0	47	0	0.96	0.54	1.70	0.32	NS
	0	48	1					
SRY	0	47	0	1	0.57	1.8	1.0	NS
	0	49	0					
FOXP3	0	47	0	0.92	0.52	1.6	0.16	NS
	0	47	2					
ESR1	1	45	1	1.0	0.59	1.8	0.59	NS
	1	48	0					
NRG1	39	8	0	1.4	0.48	4.3	0.51	NS
	43	6	0					

Table 3.1a Ratios of CNV genotypes (deletion/insertion) in a male population Odds Ratio recorded here is the odds of getting SSc (case) vs. not getting SSc (healthy control, HC) when the CNV is present. Internal control testing performed as expected showing that all male patients were SRY $^{+}$. P value is for Chi squared test.

Gene	SSc (-/-)	SSc (+/-)	SSc (+/+)	Odds Ratio	OR 95% LCL	OR 95% UCL	p-value	Corrected p value
	HC (-/-)	HC (+/-)	HC (+/+)					
CYP3A	0	1	57	0.41	0.016	10	0.37	NS
	0	0	47					
SCN3a	44	5	9	0.68	0.36	1.3	0.57	NS
	33	3	11					
KCNJ15	2	14	42	1.0	0.49	2.2	0.68	NS
	3	9	35					
PTPRM	0	4	54	0.30	0.033	2.7	0.25	NS
	0	1	46					
PLCB1	42	5	11	2.1	0.99	4.4	0.29	NS
	40	2	5					
NDST4	0	11	47	1.7	0.72	3.9	0.37	NS
	1	12	34					
HPSE2	35	4	19	0.67	0.39	1.2	0.48	NS
	23	5	19					
SMAD-1	0	1	57	0.41	0.016	10	0.37	NS
	0	0	47					
SDC2	3	37	18	1.3	0.75	2.3	0.091	NS
	1	39	7					
EV15	0	16	42	0.74	0.32	1.7	0.46	NS
	0	10	37					
LEPREL1	5	8	45	0.79	0.36	1.8	0.63	NS
	2	8	37					
GRIK4	3	29	26	1.0	0.57	1.9	0.94	NS
	2	25	20					
ACOT	3	21	34	0.89	0.46	1.7	0.94	NS
	2	16	29					
CXCR4	1	57	0	1.0	0.58	1.7	0.39	NS
	2	44	1					
TNFSF13b	5	38	15	0.77	0.44	1.3	0.32	NS
	1	31	15					
CREB5	6	52	0	0.88	0.51	1.5	0.24	NS
	2	45	0					
TGFBR3	4	31	23	0.93	0.52	1.7	0.79	NS
	4	22	21					
MIR384	0	3	55	0.41	0.041	4.0	0.41	NS
	0	1	46					
OPCML	0	46	12	1.3	0.74	2.2	0.084	NS
	0	43	4					
SRY	58	0	0	0.81	0.016	41	1.0	NS
	47	0	0					
FOXP3	0	0	58	1.2	0.024	63	1.0	NS
	0	0	47					
ESR1	2	46	10	0.90	0.52	1.6	0.82	NS
	1	36	10					
NRG1	3	36	19	1.4	0.82	2.5	0.032	NS
	1	40	6					

Table 3.1b. Ratios of CNV genotypes (deletion/insertion) in a female population Odds Ratio recorded here is the odds of getting SSc (case) vs. not getting SSc (healthy control, HC) when the CNV is present. As expected the females were SRY $^{-}$. P value is for Chi squared test.

Because of the above findings and its known role in collagen metabolism, it was decided to investigate *LEPREL1* further as a candidate factor in SSc. The *LEPREL1* CNV maps to the first intron of a gene located on human chromosome 3 at 3p26.3, which encodes prolyl 3-hydroxylase 2 (P3H2). P3H2 is an enzyme thought to be primarily responsible for the post-translational hydroxylation of certain prolines in the chains of procollagen IV as well as fibrillar collagens, serving as a potential chaperone involved in proper chain alignment to generate the molecule's intact triple helix.

3.3.2 LEPREL1 protein expression in normal and systemic sclerosis fibroblasts

One interesting feature of SSc is that the myofibroblasts, which are a major effector cell population in the disease, are persistently abnormal in tissue culture maintained through a large number of passages (LeRoy, 1974). This may indicate epigenetic changes in the cells or else persistence of some factor responsible for the phenotype. Persistent abnormalities in these cells include elevated levels of extracellular matrix proteins as well as persistent release of growth factors such as CTGF, as well as active signaling via pathways including the MEK/ERK pathway, all of which are believed to have functional significance leading to the excessive scarring and fibrosis (Abraham and Varga, 2005). If the P3H2 enzyme encoded by *LEPREL1* is an important rate-limiting enzyme in collagen synthesis in SSc then it is likely to be elevated in the pathogenic myofibroblasts. Furthermore it would be important to know whether this enzyme is induced in normal fibroblasts under conditions know to elevate collagen synthesis, such as exposure to the pro-fibrotic growth factor TGFβ.

In view of these considerations, and because changes in the expression of *LEPREL1* and the resulting protein, P3H2, could potentially have effects on the synthesis and stability of collagen IV in basement membranes as well as the production of other collagens in dermal fibrosis as well as microvasculature abnormalities, the levels of the P3H2 protein were assessed in both SSc and normal dermal fibroblasts.

Dermal fibroblast samples obtained from 3 normal and 4 SSc donors were cultured at passage 3-5 and transferred to 6 well plates for study. Cell

lysates were analyzed for P3H2 protein content by Western blot methodology as described in Chapter 2. The normal dermal fibroblasts expressed P3H2 at a low level as visualised on the Western blot. In contrast there was a much higher expression by SSc dermal fibroblasts (Figure 3.3).

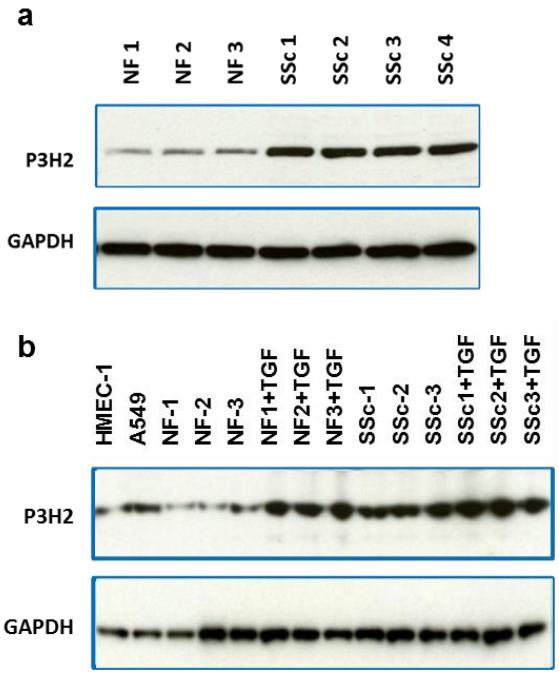


Figure 3.3 Increased expression of LEPREL1 protein (P3H2) in systemic sclerosis dermal fibroblasts and induction by treatment with TGFβ Dermal fibroblasts derived from forearm skin biopsy material of SSc patients (SSc1-4) or from healthy controls (NF1-3), were cultured at passages 3-5 in DMEM with 10% FCS and then switched to serum free DMEM overnight for 16 hours prior to lysis in RIPA buffer for Western blot analysis. P3H2 levels were elevated in SSc skin fibroblasts when compared to controls (**a**). In further experiments SSc and control fibroblasts were cultured as above, and then switched to serum free conditions overnight and cultured further for 24 hours with or without TGFβ1 (4ng/ml). Treatment with TGFβ1 led to the induction of P3H2 in control fibroblasts, up to the levels seen in SSc samples. Treatment of SSc fibroblasts led to only a modest increase in P3H2. Lysates of microvascular cells (HMEC-1) as well as human alveolar epithelial cells (A549) were included for comparison, and both cell lines were found to express the P3H2 enzyme at levels similar to TGFβ treated normal fibroblasts (**b**).

It is accepted that this is a simplistic experiment which, although clear and achievable, has some limitations. For example the genotype of the fibroblasts with respect to the *LEPREL1* CNV status is unknown and the sample size is small. Nonetheless, it does appear that protein levels of the P3H2 enzyme are elevated in SSc fibroblasts. The possible induction of P3H2 by the pro-fibrotic growth factor TGFβ was investigated further.

3.3.3 Effect of TGFβ on LEPREL1 protein expression in normal and systemic sclerosis fibroblasts

The normal dermal fibroblasts expressed the P3H2 at a low level visible on the Western gel plot shown in Figure 3.3, in contrast to robust expression exhibited by SSc dermal fibroblasts. When exposed to the pro-fibrotic cytokine TGFβ, normal dermal fibroblasts displayed a marked increase in P3H2 expression, to a level that appeared to be nearly comparable to that in the SSc dermal fibroblasts without TGFβ stimulation (Figure 3.3b). When incubated with TGFβ, SSc dermal fibroblasts appeared to modestly increase their already high level of P3H2 expression, but only to a level slightly greater than expressed by the stimulated normal dermal fibroblasts. Cultured SSc skin fibroblasts are reported to be less responsive to TGFβ *in vitro* than normal fibroblasts (Kikuchi et al., 1992) possibly resulting from chronic exposure to TGFβ *in vivo*. Considered to be a major factor driving fibrosis (Sargent et al., 2010), TGFβ was used here because the pattern of gene expression produced by normal fibroblasts when stimulated by this cytokine has been shown to resemble that in SSc cells. The finding that TGFβ increased the P3H2 protein from a low level in normal fibroblasts to a level found with unstimulated SSc fibroblasts supports the potential involvement of *LEPREL1* in the signaling pathways involved in SSc.

Because of the likely importance of *LEPREL1* in basement membrane collagen synthesis, microvascular endothelial cells (HMEC-1), as well as bowel cancer epithelial cells (AQ549), were lysed and included in the Western analysis. Both microvascular and epithelial cells were found to express P3H2 protein equivalent to TGFβ1 treated SSc fibroblasts, consistent with some role in epithelial cell basement membrane collagen synthesis, or in epithelial cell-stroma interaction, although this was not investigated further (Figure 3.3b).

3.3.4 Effect of genetic silencing of *LEPREL1* in the bleomycin induced skin fibrosis model

To help understand the role of *LEPREL-1* in fibrosis *in vivo*, it was decided to investigate the responses of a *LEPREL1* KO mouse developed by Hans Peter Bächinger (Shriner's Hospital, Portland, OR,) to the fibrosing agent bleomycin. However, he has shown that mice homozygous for *LEPREL1* KO allele exhibit embryonic lethality (Pokidysheva et al., 2014). In the KO mouse the aberrant collagen IV interacts with glycoproteins on maternal platelets (GPVl) inducing a general thrombosis which kills the embryo. To rescue the phenotype, Dr Bachinger bred and established a colony of double *LEPREL1* -/- *GPVI*-/- DKO, and single *GPVI*-/- SKO mice to study the phenotype that could be contributed by *LEPREL1* and to control for the *GPVl* deletion required for fetal viability. Because of compensatory mechanisms, it is often the case that KO mice do not seem to show abnormal physiological phenotypes until they are challenged, and both the SKO and DKO mice appear phenotypically normal.

It was decided to investigate the role of *LEPREL1* in fibrosis using the bleomycin model of cutaneous fibrosis. Mice were in three groups; WT, *GPVI*-/- SKO and *GPVI*-/-*LEPREL1*-/- DKO and were given daily subcutaneous injections of bleomycin for 21 days and then euthanised on day 30 for analysis of skin by histology and plasma by Multiplex analysis, the comparison between DKO and SKO being the important determinant of the effect of silencing *LEPREL1*. Mice were shaved in the lower back quadrant and then a henna marker was used to draw a small circle was drawn, where mice were injected subcutaneously daily for 21 days with bleomycin. Photographs of the injected areas were taken weekly for 3 weeks. On day 30, the mice were euthanised with CO_2, blood collected (via cardiac puncture) and the injected cutaneous region was excised, and formalin fixed. Sections of the bleomycin treated area of skin were stained with Masson's trichrome staining, or picrosirius red stain (PSR). A full blood count (CBC) was obtained (Heska HemaTrue™), as well as plasma for IL-6, IL-12p70, TNFα, IL-10, as well as TGFβ and PDGF-BB analysis.

By day 9 of the study, the animals injected with bleomycin showed signs of inflammation at the injection site. Lesions had appeared at the injection sites and were much more severe in the DKO group. The lesion became more

severe throughout the study, with the DKO showing a greater lesion size and surrounding tissue inflammation by day 21. In contrast, the skin of control mice injected with bleomycin did not break down and instead became more thickened and fibrotic.

Examination of histologic sections stained by Masson's trichrome for n=3 mice per group, revealed as expected greatly increased dermal thickness in the bleomycin treated WT mice compared to treatment naïve controls. This was also seen in the SKO mice. However in the DKO mice the response to bleomycin was attenuated and dermal thickening did not occur (Figure 3.4).

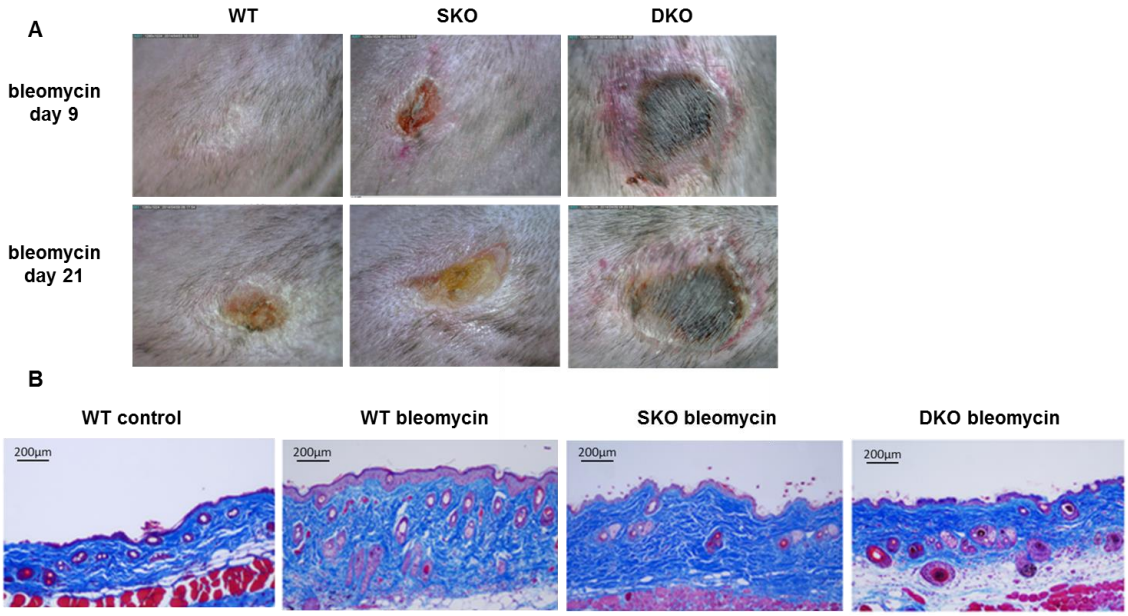


Figure 3.4 Effect of LEPREL1 gene silencing in bleomycin skin fibrosis model WT, or GPVI silenced SKO, or else GPVI and LEPREL1 DKO mice were subject to the bleomycin skin fibrosis model, by daily subcutaneous injection of bleomycin. Macroscopic appearance of the bleomycin treated skin is shown in (**A**). A non-healing erythematous and ulcerated lesion was seen to form in the DKO but not in WT or SKO controls, present on days 9 and 21.
Trichrome staining was performed on the wound tissue sampled on day 30 from n=3 mice per group, and representative images are shown in (**B)**. As expected bleomycin treatment led to thickening of the collagen staining dermal layer and in the WT, which was also seen in the SKO. However in the DKO there was no thickening of the dermis after the bleomycin treatment. Measurements of dermal thickness and statistical analysis is shown below in Tables 3.2 and 3.3.

For the purposes of quantitation and statistical anlysis, dermal thickness, as defined by the blue staining collagen rich dermis seen on the sections shown in Figure 3.4, was measured at 4 points for each section studied. Mean dermal thickness for each treatment group was then compared by ANOVA to establish whether the means of any group differed from the overall population , followed by Tukey's test for specific comparison between groups. This analysis confirmed significant thickening in the bleomycin treated WT and SKO mice, and significant attenuation of this response in the DKO mice (Tables 3.2 & 3.3, Figure 3.5).

Mean dermal thickness (µm)	WT control	Bleo WT	Bleo SKO	Bleo DKO
Mouse 1	360	773	523	385
Mouse 2	328	748	763	118
Mouse 3	355	945	658	395
Overall mean (µm)	**348**	**822**	**648**	**299**
SEM	10	62	69	91

	Dermal thickness				
ANOVA summary					
F	14.5				
P value	**0.0013**				
P value summary	**				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.8448				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	556000	3	185000	F (3, 8) = 14.51	P = 0.0013
Residual (within columns)	102000	8	12800		
Total	659000	11			

Table 3.2 ANOVA of mean dermal thickness in WT control, WT bleomycin treated, SKO bleomycin treated, and DKO bleomycin treated mice

Dermal thickness was measured at 4 points of a representative section for each mouse studied (n=3 per group) and expressed as mean dermal thickness. Overall means for each treatment were obtained (upper Table). ANOVA was used to test for differences between groups, showing a p value of 0.0013 (lower Table). Since ANOVA indicated significant differences between groups, Tukey's post hoc analysis was performed to compare the means of each group (see Table 3.3, below)

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
WT vs. Bleo WT	-474.2	-769.8 to -178.6	Yes	**	0.0039
WT vs. Bleo SKO	-300.0	-595.6 to -4.402	Yes	*	0.0468
WT vs. Bleo DKO	48.33	-247.3 to 343.9	No	ns	0.9510
Bleo WT vs. Bleo SKO	174.2	-121.4 to 469.8	No	ns	0.3050
Bleo WT vs. Bleo DKO	522.5	226.9 to 818.1	Yes	**	0.0021
Bleo SKO vs. Bleo DKO	348.3	52.74 to 643.9	Yes	*	0.0226

Table 3.3 Tukey's multiple comparisons test applied to dermal thickness measurements

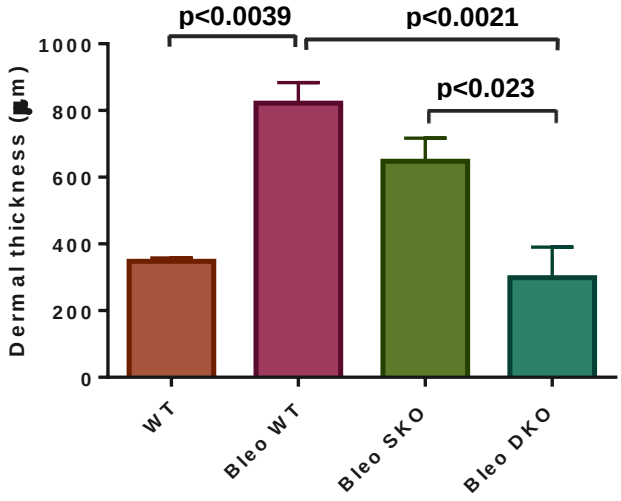


Figure 3.5 LEPREL1 deletion reduces bleomycin induced dermal thickening Mice were euthanised on day 30 and bleomycin induced dermal wounds were excised and subject to Masson's trichrome stain. Dermal thickness was measured at 4 points across each biopsy and the mean dermal thickness compared by ANOVA and Tukey's test. As expected bleomycin treatment led to increased dermal thickness in WT mice. Dermal thickening in response to bleomycin was significantly reduced in DKO mice compared to both WT and SKO animals (all n=3). The difference between the DKO and SKO indicating an effect due to genetic silencing of *LEPREL1*.

3.3.5 Effect of genetic silencing of *LEPREL1* on bleomycin induced skin fibrosis: measurement of plasma cytokines and growth factors

Furthermore, plasma samples were taken on termination of study from WT, SKO and DKO animals following bleomycin treatment and from untreated littermates (n=6-12 mice per group). Inflammatory cytokines IL-6, IL-12p70, TNFα, as well as anti-inflammatory IL-10 were assayed by a limited Multiplex. Pro-fibrotic growth factors TGFβ1 and PDGF-bb were assayed by separate ELISA analysis.

In the WT mice, bleomycin treatment was associated, as would be predicted, with trends towards increased levels of inflammatory cytokines (all P= not significant), as well as significant increases in both TGFβ and PDGF-bb (Figure 3.6). Both SKO and DKO mice had elevated levels of inflammatory cytokines and growth factors under basal conditions, as well as induction following bleomycin treatment. These results suggest that the GPVI deletion somehow alters basal inflammation. Also, they indicate that LEPREL1 deletion does not inhibit the inflammatory response to injury. Similarly the TGFβ and PDGF-bb levels were raised under basal conditions in both SKO and DKO mice, and induction of both TGFβ and PDGF-bb were attenuated in these mice. Overall the DKO mice had the lowest levels of PDGF-bb following belomycin iinjury but this was not statistically significant when compared to SKO mice (Figure 3.6).

Overall these data do not show a clear effect from LEPREL1 deletion in terms of cytokine or growth factor production during bleomycin skin injury. However, the demonstrated effect on tissue remodeling shown in Figure 3.4 was investigated further, by analysis of PSR stained tissue.

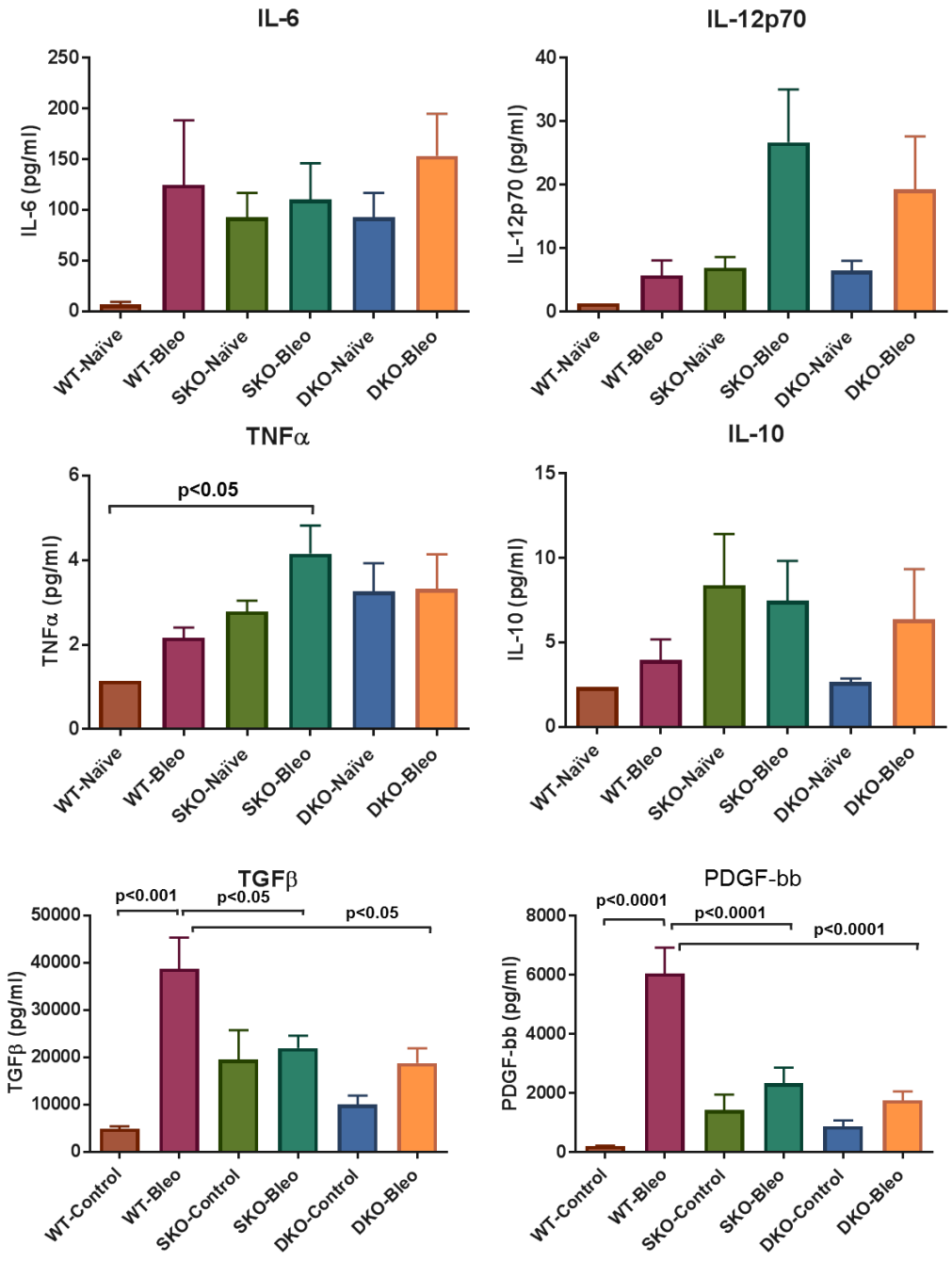


Figure 3.6 Plasma cytokine and growth factor levels following bleomycin induced skin fibrosis: day 30 WT, SKO, or DKO, mice were injected daily with bleomycin for 21 days and then euthanised on day 30, and blood sampled by cardiac puncture and plasma separated in EDTA. 6-12 mice were included per treatment group. Cytokines were assayed by Luminex array (IL-6, IL-12p70, TNFα, IL-10). Pro-fibrotic growth factors TGFβ and PDGF-BB were assayed separately by ELISA. Means were compared by ANOVA followed by Tukey's test to account for multiple comparisons. Significant differences are shown with p values. 6-12 mice per treatment group were studied (Statistical analysis and individual measurements are shown in the Appendix).

3.3.6 Effect of genetic silencing of *LEPREL1* on bleomycin induced skin fibrosis: use of picrosirius red stain to identify cross-linked collagen

After the mice were euthanised on day 30, sections of wound tissue were formalin fixed and then sectioned for staining by PSR as a marker for mature cross-linked collagen, and then imaged at x4 using the Zeiss system, and multiple images merged to show the whole wound and adjacent tissue. As expected, the dermis and deep fascia of WT wounds stained strongly with PSR and gave a red coloured birefringence under polarised light, also seen in the SKO. However in the DKO mice the red staining was reduced and under polarised light the collagen appeared green-gold rather than red in birefringence, consistent with impaired collagen deposition (Figures 3.7). An attempt was made to quantify the red birefringence by importing images into ImageJ using the RGD channel settings to quantify red staining. This analysis indicated slightly increased red birefringence in the SKO sections and significantly reduced birefringence in the DKO sections (Figure 3.8).

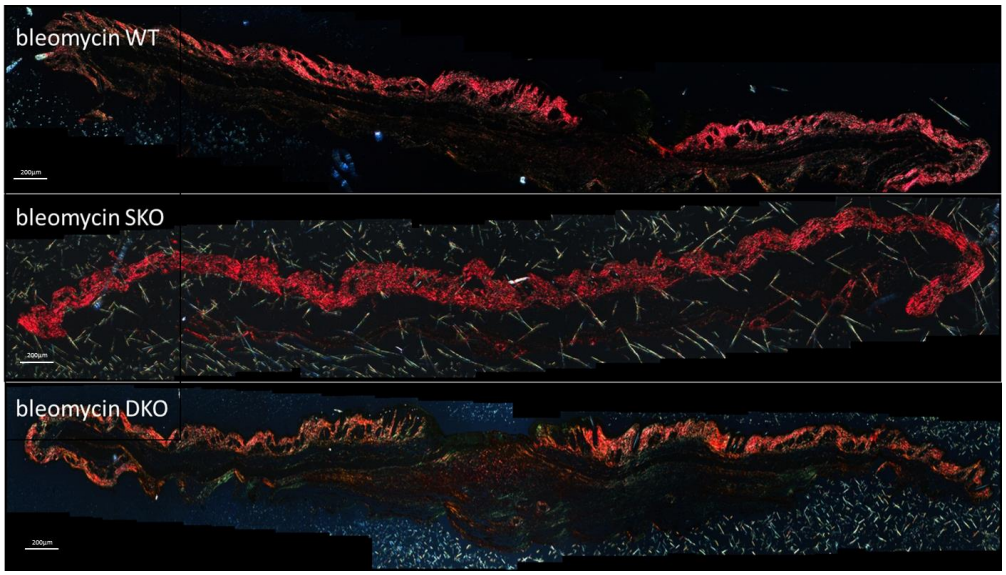


Figure 3.7 Skin biopsy material from Bleomycin injury model in LEPREL1 knockout: picrosirius red stain assessed by polarised light microscopy WT control, SKO, and DKO mice were euthanised on day 30, bleomycin wounded dermal areas were extracted, formalin fixed and stained with PSR (n=3 mice per treatment group). Images were taken with the Zeiss system using a 4x objective with polarised light conditions, and then multiple images per section were merged using Photoelements software to give a panoramic view of the bleomycin wound area. The red staining, which indicates mature collagen fibers as seen under polarised light, appears abundant in WT and SKO, but markedly reduced in DKO, which stained green/golden, indicating immature non cross-linked collagen.

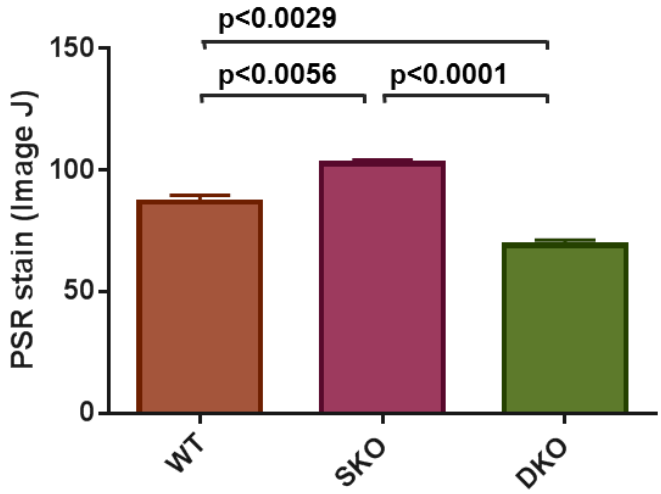


Figure 3.8 Quantitation of picrosirius red birefringence in WT, SKO, and DKO bleomycin treated mice day 30 Polarised light images of WT, SKO, and DKO sections (n=3 mice per group) stained for PSR were captured using the Zeiss system with a 4x objective. Images were imported as JPEGs into ImageJ software and converted to RGB images. Red polaroid intensity was quantified in the G channel images by setting the threshold for red intensity. Four rectangular fields per image were quantified for each section as shown. Means were compared by ANOVA which was significant at $p<0.05$, and then Tukey's test for multiple comparisons (see Appendix for full data and statistical analysis). Mean birefringence was increased in SKO mice when compared to WT controls. In the DKO mice, PSR birefringence was significantly reduced when compared to booth the WT and the SKO mice.

3.4 Discussion

3.4.1 *LEPREL1* association with systemic sclerosis

In this Chapter, data has been presented showing that a CNV contained in the first intron of the *LEPREL1* gene may confer a greater risk of acquiring SSc within a male population, as the presence of this CNV is more common in affected than non-affected males. Although SSc is more prevalent in women than men, the CNV in *LEPREL1* was not linked statistically to SSc in female patients. It is possible that oestrogens bypass the CNV genetic element by action on oestrogen responsive elements in the adjacent sequences. Consistent with this possibility, others (Soldano et al., 2010) (Aida-Yasuoka et al., 2013) have shown that estrogen stimulates matrix synthesis including collagen and the EDA form of fibronectin in fibroblasts.

Insertions and deletions are fine-scale genetic structural element changes in the genome. In this study a CNV insertion was found in the *LEPREL1* gene in 46 out of 47 male SSc patients. However, it also occurred in DNA from normal individuals (40 out of 49 healthy male controls) and thus may sensitize to SSc but not cause the disorder. This CNV was found in the first intron of the *LEPREL1* gene and this sequence contains binding sites for SMAD proteins involved in TGFβ signaling (multiple CAGA elements within the CNV sequence, as shown in section 2.6.7). The presence of the CNVs in the first intron of *LEPREL1* may have functional significance, although that has not been investigated in the current thesis, but should be investigated further with reporter constructs and electromotility shift assays. To further validate the findings larger cohorts of SSc and control patients should be investigated utilizing other sequencing technology such as Next Generation Sequencing, and other disorders of fibrosis and autoimmunity should also be studied.

The *LEPREL1* gene encodes the P3H2 protein, an enzyme responsible for the post-translational hydroxylation of certain prolines to form 3-hydroxyproline residues in the chains of procollagen, a process believed to facilitate the formation of the stable triple-helical collagen molecule (Gelse et al., 2003). Current concepts suggest that this enzyme is part of a protein complex that helps to align the helical portions of the collagen molecule within the endoplasmic reticulum, for correct assembly (Pokidysheva et al., 2014).

Data from the studies presented here show elevated levels of the P3H2 protein in SSc fibroblasts when compared to the normal healthy control fibroblasts. The author is not inferring that this change in protein level is directly linked to the CNV polymorphism, since the fibroblasts studied were not genotyped. However, it is proposed that elevated P3H2 protein levels is a property of the disease SSc fibroblasts, which may be highly relevant to other known phenotypic changes such as oversecretion of collagen. The data also show that healthy control fibroblasts exposed to TGFβ expressed elevated levels of P3H2 protein, increased to a similar level to that in SSc fibroblasts under unstimulated conditions. It is possible that P3H2 is a rate limiting enzyme in collagen synthesis by dermal fibrosis, and that following TGFβ treatment the induction of P3H2 is essential for the oversecretion of collagen to occur.

Current data suggests that both the basement membrane collagen as

well as fibrillar collagens are dependent on P3H2 and its over-production might have a dual role in SSc lesions, particularly if this enzyme is rate limiting (Fernandes et al., 2011). Basement membrane damage and its effect on local cell populations (endothelial, epidermal) may contribute to some of the conditions complicit in SSc, including Raynaud's phenomenon and microvascular lesions leading to tissue ischemia, or the major vascular complications such as pulmonary hypertension (see below and Figure 3.9 for an illustration of ECM and associated cells and tissue).

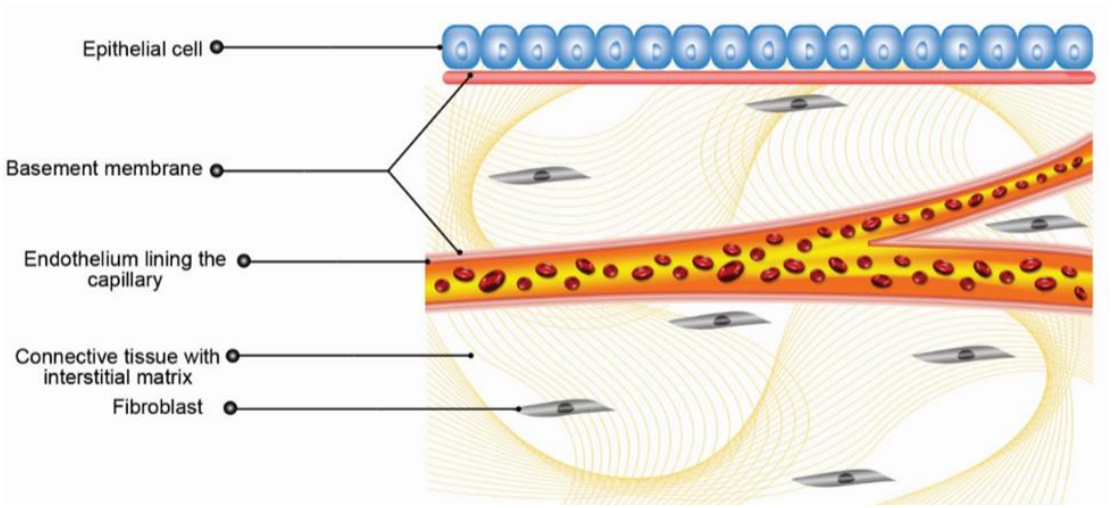


Figure 3.9 Illustration depicting extracellular matrix in relation to epithelium, endothelium and connective tissue Since cellular injury and activation of the epithelial and endothelial cells are seen in early stage SSc, it is possible that the nature of the basement membrane proteins is altered and having an important role in pathogenesis. For example, increased basement membrane permeability would alter paracrine signaling from activated endothelial and epithelial cells to dermal fibroblasts. Also, altered adhesion of these cells to the basement membrane could influence cell fate in processes such as EndMT or EMT.

The involvement of any of the prolyl 3-hydroxylases in human disease was first observed in certain patients with osteogenesis imperfecta, indicating a major developmental abnormality in bone, a tissue with abundant amounts of collagen I (Cabral et al., 2007). Prolyl 3-hydroxylase 1 deficiency caused a recessive metabolic bone disease resembling lethal or severe osteogenesis imperfecta. By contrast *LEPREL1* deficiency in humans is not known to be associated with skeletal abnormalities but is linked to non-syndromic severe myopia (Mordechai et al., 2011). Further, in a *LEPREL1* KO mouse developed

by another group, there were reduced amounts of collagen I and IV in the sclera and lens capsule, which could be responsible for the progressive myopia (Hudson et al., 2015). These mice were not found to have musculoskeletal abnormalities. Tissue repair and fibrosis is yet to be studied in this KO mouse, but that would be an important future aim to corroborate the findings of the work presented in this thesis. It also remains unknown why these mice are viable in contrast to the mouse used in this thesis, for which *LEPREL1* deletion resulted in placental abnormalities and embryonic lethality, corrected by additional deletion of the platelet receptor GPVI.

Most interest in the prolyl 3-hydroxylase enzymes has focused on basement membrane collagen IV, since it has the most 3-hydroxyproline of all collagens. Collagen IV has previously been shown to be a target of autoantibodies in SSc as well as in Goodpasture syndrome, in which the anti-collagen antibodies are key drivers of the disease process leading to bleeding in the lungs and inflammatory lesions in the glomerular basement membranes. When autoantibodies to collagen IV are present or when collagen IV is knocked-out in a mouse model, the stability and organization of the basement membrane is profoundly altered in both structure and function (Mackel et al., 1982). In fact, when one of the collagen IV-encoding genes is knocked out, it is embryonic lethal in mice (Pokidysheva et al., 2014), and in humans, mutations in *COL4A3*, *COL4A4* or *COL4A5* result in Alports syndrome in which basement membrane defects in the glomeruli, vasculature, and cochlea are seen. However, recent studies on P3H2 implicate it in the synthesis of other collagens, particularly by fibroblasts (Fernandes et al., 2011).

Fibroblasts from SSc patients had higher basal levels of P3H2 protein expression than did control cells. Co-incubation of SSc cells with TGFβ also increased the levels of P3H2 when compared to control cells. A proposed mechanism based on these results is shown in Figure 3.10.

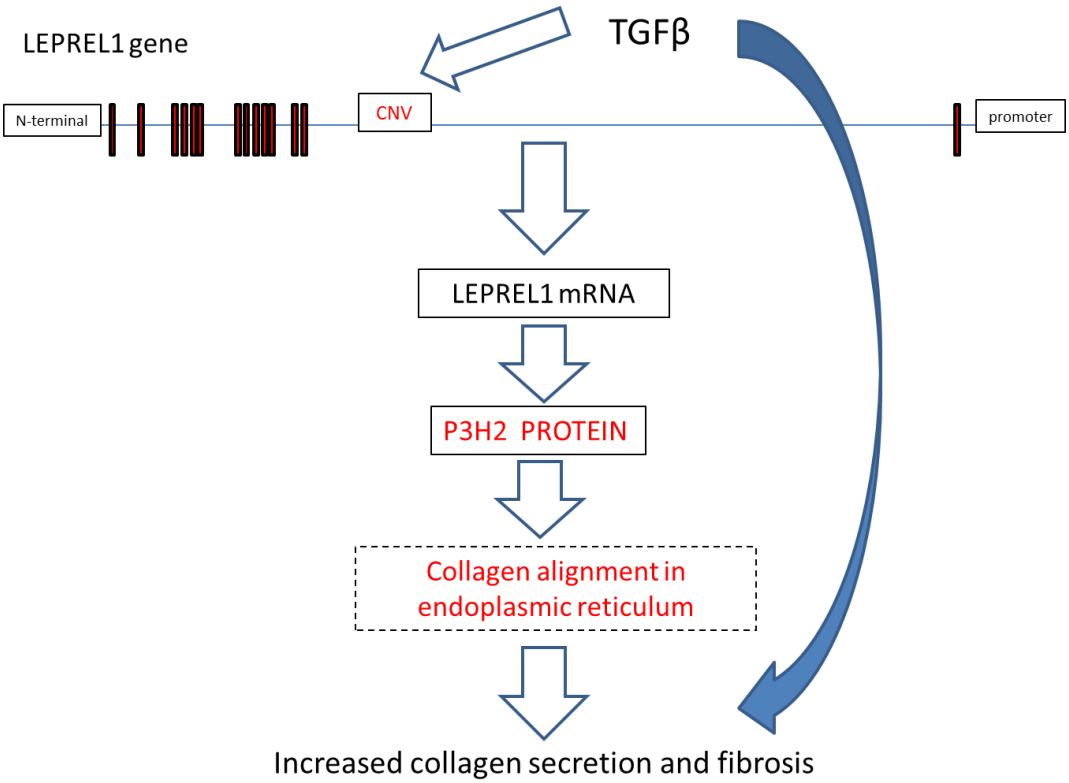


Figure 3.10 Proposed mechanism showing role of CNV in first intron of LEPREL1 gene TGFβ acting via SMAD binding element of a CNV associated sequence in the first intron induces LEPREL1 expression leading to enhanced mRNA, and protein levels of P3H2. The P3H2 enzyme level is coupled to increasing collagen biosynthesis also induced by TGFβ, and is responsible for prolyl 3-hydroxylation of the collagen chain, which permits alignment of the collagen triple helix prior to cross-linking and export of the mature collagen protein.

Genotyping for the CNV in the first intron sequence of *LEPREL1* could be helpful in determining a person's potential to develop fibrosis in patients with associated conditions as well as those receiving radiation and chemotherapy. Women have a greater predisposition than men (4:1) for developing SSc, which is usually attributed to the effects of oestrogen. However, 5 out of 58 female SSc patients studied had homozygous deletion of the CNV, questioning the importance of this CNV in females. It is possible that in females, oestrogens or other hormonal factor bypass the effect of the CNV in fibroblasts.

Further studies should assess the course of the disorder in such individuals with SSc despite deletion at the *LEPREL1* CNV. Further female stratification in *LEPREL1* and estrogen receptor 1 (*ESR1*) or other hormonal genes might reveal subpopulations with different pathological and predisposition to SSc in response to aberrant modifications in sex hormones.

Such individuals might be tested for their response to the various TGFβ antibodies now in clinical trials. It is not surprising that distinct genomic factors may be acting in men versus women.

3.4.2 Role of prolyl hydroxylases in human physiology and disease

The prolyl hydroxylase family of gene products includes a family of proteins having evolved different collagen substrate sites and functions, as well as other members, which regulate the activity of different proteins, such as the hypoxia inducible nuclear factors. There are some hundred 4-hydroxyprolines in each chain of collagen 1, which have been shown to stabilize the collagen triple helix. The prolyl 4-hydroxylase enzyme acts on nascent collagen chains within the endoplasmic reticulum, but when the triple helix forms, further hydroxylation ceases. Prolyl 4-hydroxylase recognizes the sequence –gly-X-pro-gly-, which occurs regularly, and hydroxylates prolines preceding glycine utilizing iron, ascorbic acid, alpha-ketoglutarate and oxygen as cofactors. In contrast, there is usually only one 3-hydroxyproline in each chain of type 1 collagen located near the carboxyl end of the helical domain.

The enzyme prolyl 3-hydroxylase-1 (P3H1) is present in a complex with CRTAP and cyclophilin, which is responsible for converting one proline to 3-hydroxyproline in each chain of type I collagen. The enzyme hydroxylates a proline following a glycine. Current concepts suggest that the complex acts as a chaperone aiding the alignment of the collagen chains in forming the triple helix. In phenotyping a CRTAP knock-out mouse, it was determined the 3-hydroxyproline was missing in type I collagen α-chains (Pokidysheva et al., 2014). This resulted in the discovery of P3H1 gene mutations linked to inherited osteogenesis imperfecta, also known as brittle bone disease. Since this discovery, a number of 3-hydroxyproline sites have been identified in various collagens.

Collagen IV, the basement membrane collagen, contains many more 3-hydroxyprolines than the fibrillar collagens. Non-helical segments interrupt the helix in type IV collagen, which may explain why it requires more 3-hydroxyprolines to achieve proper alignment. The presence of more collagen IV

in fibrosis would change the proportion of the constituents in the basement membrane and its properties.

At least four mutations in the *P3H1* gene have been identified in people with a rare, severe form of osteogenesis imperfecta classified as type VIII. These mutations prevent cells from producing functional prolyl 3-hydroxylase. Without this enzyme, certain forms of collagen are not modified through proline 3-hydroxylation. The altered collagen molecules are incorrectly folded, and some abnormal collagen is secreted from cells more slowly than usual. These collagen defects weaken connective tissues, resulting in extremely slow growth and thin, brittle bones that may fracture before birth. Because bone contains abundant amounts of collagen 1, prolyl 3-hydroxylase may be rate limiting in bone.

It is notable that the CNVs in *LEPREL1* studied in this Chapter, contain SMAD-binding nucleotide sequences, which could explain their function. Mutations in *LEPREL1* or members of its complex could slow helix formation by collagen as well as secretion. Thus, it is reasonable to hypothesize that this reaction may be rate-limiting in collagen synthesis particularly when the synthesis of procollagen chains is stimulated in fibrosis by TGFβ.

The protein encoded by the *LEPREL1* gene, P3H2, is highly expressed in kidney, placenta, and lung in cells producing collagen IV (Hudson and Eyre, 2013). Changes in the expression of the *LEPREL1* gene and the subsequent translation of the P3H2 protein may also lead to the destabilization of collagen IV which contains many more 3- hydroxyproline residues than fiber forming collagens. Basement membranes separate epithelial and endothelial cells from the underlying mesenchymal tissue, and are a vital component of the dermal-epidermal junction as well as the microvasculature. It is important to the regulation of cellular behavior, not just a structural feature of tissues. It is responsible for mediating the compartmentalization of tissues, sending signals to epithelial cells about the external milieu and acting as a barrier to the passage of cells and proteins (Hasegawa et al., 2007, Konomi et al., 1984).

Collagen IV, through its functional and structural influence on blood vessels, may provide information to endothelial cells and pericytes concerning the existing environment of the ECM, as well as serving as a permeability barrier around blood vessels and capillaries (Abreu-Velez and Howard, 2012).

Because collagen IV serves to stabilize the basement membrane, a reduction in the bioavailability of mature collagen IV could serve to destabilize both the basement membrane and endothelial cells, causing or contributing to microvascular involvement in the disease. When a collagen IV-encoding gene is knocked-out in mouse embryos, the structural integrity and internal organization of the vascular basement membrane is perturbed, leading to amorphous deposition of the ECM, loss of cell contact, and detachment from the underlying cell layer with irregular folding (Poschl et al., 2004). Additionally, autoantibodies to collagen IV are documented in SSc tissues (Riente et al., 1995), possibly due to abnormal folding exposing otherwise cryptic epitopes.

Basement membranes are composed of collagen IV, laminin, and proteoglycan (Rosenbloom and Jiménez, 2008, Jimenez et al., 1996). Excess collagen deposited in the ECM and within the basement membrane often damages the microvasculature and causes fibrosis. Vascular alterations appear to arise in the early stages of fibrosis and correlate with accumulations of ECM proteins and abnormal numbers of transdifferentiated myofibroblasts (Beon et al., 2004).

Structural abnormalities and fibrosis in particular, are a cause for concern in lung tissues because oxygen diffuses across the basement membranes of the alveoli to be picked up by the hemoglobin in red blood cells for circulation throughout the vascular system. Extensive damage to the basement membrane may contribute to pulmonary fibrosis, which is a major complication seen in up to 75% of patients with SSc (Poschl et al., 2004). This condition can lead to respiratory failure or pulmonary hypertension, and is one of the leading causes of SSc-related morbidity and mortality.

In SSc tissues (Hoyland et al., 1993), endothelial cell damage, reduction in number of vessels and vascular basement membrane thickening may all lead to changes in surrounding tissues associated with development of fibrosis. Pathology studies have demonstrated that minor structural differences in collagen IV can lead to distinct clinically discrete diseases (Abreu-Velez and Howard, 2012). Collagen IV in mammals is derived from six genetically distinct alpha chain polypeptides (alpha 1-6) giving rise to 3 distinct collagen IV molecules. The NC-1 domain of collagen IV is considered important for chain selection.

To investigate whether there were significant changes to the structure of type IV collagens during the progression of SSc, two monoclonal antibodies against different conformational epitopes within the alpha 1 and alpha 2 helical domains were used to probe skin tissues in immunohistological studies (Hoyland et al., 1993). Polyclonal antibodies to all species of collagen IV were also used. Monoclonal antibodies to specific alpha chain epitopes were only found to bind to normal skin and early SSc skin tissues, but not to skin samples from patients exhibiting the more advanced forms of the disease. However, polyclonal antibodies were found to bind to skin tissue at all stages of the disease. This suggests that epitopes of collagen IV may be made inaccessible to these monoclonal antibodies due to conformational modifications that occur as the disease progresses.

P3H2 could be hypothesized to contribute to the fibrotic process by stabilizing abnormal and potentially pathological late stage SSc collagens in the basement membrane or elsewhere, especially when the protein is expressed at high levels. Rat chondrosarcoma cell line (RCS) cells were shown to have additional 3-hydroxylation sites in fibrillar collagens due to the high level of P3H2 expression (Abreu-Velez and Howard, 2012, Fernandes et al., 2011). The progressively higher 3-hydroxylation levels of Pro-470, Pro-707 and Pro-944 substrate binding sites in RCS collagen alpha 1 (II) chains as well as the 3-hydroxylation of GPP4 repeats at the C-terminus in fibrillar collagens are not observed in cells with lower levels of P3H2 expression, and might be a consequence of the overexpression of P3H2 in these cells. The *LEPREL1* first intron CNV may potentially cause changes to the level of *LEPREL1* transcription or to the distribution of *LEPREL1* isoforms, influencing both the number and types of collagens that are 3-hydroxylated during SSc. It would be of interest to analyze collagen from fibrotic lesions in scleroderma and measure its 3-hydroxyproline content in future studies.

The fibrotic network and ECM are part of a highly complex interactive milieu. A single perturbation within related pathways could hypothetically stimulate compensatory mechanisms that may trigger changes in the regulation of fibrosis and the deposition of collagens and other ECM components. The activation of the fibroblast may be an attempt to compensate for the damage by

patching the unstable regions with other collagens. These collagen deposits may contribute to the extensive fibrosis seen in SSc, causing damage to the thin basement membrane and leaky capillaries.

3.4.3 Possible functional significance linking the *LEPREL1* CNV to disease phenotype

A change in the expression of *LEPREL1* within cells could be caused by a genetic mutation, such as a CNV, and/or the modulation of expression through epigenetic control. It is interesting that the CNV in *LEPREL1* exists within the gene's first intron. There is a substantial amount of evidence to suggest that elements for gene regulation are contained within the first intron, particularly in collagen genes. For example, *COL1A1* contains regulatory elements within the first intron and there is speculation that the interaction between upstream promoter sequences and intronic regulatory elements may be essential to the transcriptional regulation of its expression (Bornstein and Sage, 1980, Bornstein et al., 1987). It has been demonstrated that the presence of intronic sequences in a gene construct containing 804 bp of *COL1A1* promoter markedly enhanced transcription of a reporter gene in fibroblasts (Bornstein et al., 1988, Sherwood and Bornstein, 1990). *LEPREL1* transcription levels may differ due to sequence changes that are the result of the CNV deletion/insertion in Intron 1 that change the efficiency of transcription.

Transcription factors may also find enhancer elements in the first intron. An interaction in the formation of a nuclear protein-mediated loop structure between the promoter and the enhancer of the gene was shown to be essential for the regulation of transcription of the type II collagen gene (SAVAGNER et al., 2009). This could be a model for how CNVs, such as this *LEPREL1* CNV, function as susceptibility factors in complex diseases. CNVs may selectively impact how and to what extent a gene is transcribed, but this effect on transcription may only be actualized when particular environmental and/or epigenetic triggers are available.

It is possible that the relationship between sequence copy number of regulatory elements and the activity of specific trans-acting factors ultimately determines the transcriptional activity of collagen genes in a given cell. If normal fibroblasts do differ by *LEPREL1* CNV genotype, expression differences

between normal and SSc may be the result of differences in gene dosage, altering the amount of P3H2 protein translated and increasing or decreasing the amount of 3-hydroxylation of fibrillar and basement membrane collagens. It can be speculated that, given the right intracellular environmental conditions, this variation may contribute to the disease course of SSc. It could possibly act by promoting the production, stabilization and deposition of abnormal collagens or, alternatively, by altering and destabilizing the basement membrane, weakening this structure and leading to alterations in surrounding epidermal or microvascular cells.

In related work, P3H2 expression is down-regulated in breast cancer by epigenetically controlled abnormal CpG methylation (Shah et al., 2009), although not to the author's knowledge in other epithelial cancers. The CpG effect on P3H2 production demonstrates that *LEPREL1* expression can be regulated through epigenetic events possibly influenced by the environment. Environmental contaminants, such as organic solvents, are potentially implicated as epigenetic modifying factors in SSc (Povey et al., 2001).

3.4.4 Discussion: effects of genetic silencing of *LEPREL1* in bleomycin induced skin fibrosis

In these studies, it was shown that repeated dermal bleomycin injection activates an inflammatory response as indicated by a trend towards elevated serum cytokine levels. This was followed by a fibrotic reaction in the WT and *GPVI* SKO controls. However, this fibrotic reaction was attenuated in the *LEPREL1/ GPVI* double KO mice, and the failure to repair was associated with a breakdown of the skin. These results support the concept that *LEPREL1* has a role in influencing inflammation induced collagen synthesis and repair.

In order to assess mature collagen deposition in the skin of these animals PSR staining combined with polarized light microscopy was used. It was found that the amount of red staining was decreased in the DKO mice compared to both SKO and WT animals. The red stain is believed to represent mature collagen fibers, and the findings support the mechanistic link between *LEPREL1* expression and mature collagen deposition in the dermis.

3.4.5 Summary of findings and future studies

In work described in this chapter, the contribution of the genetic component to SSc was investigated. CNV analysis was conducted a possible association between a CNV in *LEPREL1* and SSc disease development in males, was identified. A possible role in SSc of the *LEPREL1* encoded protein P3H2 was tested by analysis of this enzyme in patients' fibroblasts. Expression was low in the unstimulated normal skin fibroblast cultures, but the P3H2 level was much higher in unstimulated SSc fibroblast cultures. Normal skin fibroblasts stimulated with the pro-fibrotic cytokine TGFβ increased P3H2 protein content to a high level similar to that of the unstimulated SSc lung fibroblasts. In the Western Blot studies, P3H2 synthesis is near maximal in the scleroderma cells and only marginally elevated by TGFβ.

The candidate and associates plan to both expand the genotyping sample size when possible, and extend the studies to additional fibrotic conditions such as radiotherapy induced fibrosis, diabetic nephropathy or other. In addition the functional significance of the LEPREL1 CNV should be explored using reporter gene constructs, transfection experiments into SSc and control cells, and EMSA assays to determine which transcription factors bind to the CNV sequence. These collective efforts should allow acquisition of sufficient information, and statistical power to better understand the role *LEPREL1* plays in the fibrotic process, and whether expression differences that result from genetic or epigenetic sources are involved in the establishment of SSc.

In the future, there is hope as well for the development of simpler assays for these CNVs in *LEPREL1* and the assay of the P3H2 protein that will allow pre-identification of individuals who are susceptible to SSc and those who would be responders to tailored pharmacogenetic therapies. Additional surveys being conducted at the Royal Free Hospital, UK, will use information on SNPs located within the *LEPREL1* gene. Also, publication of these findings would encourage other centres for SSc treatment and research to repeat and extend these studies.

3.4.6 GWAS and CNVs - next steps and applications

GWAS studies may allow stratification of patients into subgroups based on the presence of polymorphisms, to receive targeted interventions, if the proper level of resolution is applied. Polymorphisms tends to cluster within discrete subgroups that display common clinical and autoimmune profiles. However, most studies lack sufficient statistical power due to the limited sample sizes of the represented cohorts, and this challenge is also a proven barrier to defining the genetic contributions to the disease. Meta-analysis can help by increasing and diversifying the sample pool within a GWAS. However, it remains difficult to identify loci with reproducible associations, and often the functions of the identified loci are themselves elusive.

Applying what has been learned through CNVs and GWAS, the logical next steps involve identifying the underlying causal variants and the genes involved, and then determining the mechanistic link to disease via the resulting protein products. The first step is to use larger cohorts to discover variants with lower penetrance. The second step is to use already identified susceptibility loci of known functional significance to select specific genes for higher resolution genotyping using SNP and/or CNV markers. The third step is to characterize the functional significance of these polymorphisms and the proteins encoded and influenced.

Since P3H2 is instrumental in the stabilization of collagen I and IV it might be a good therapeutic target in fibrosis. The enzyme represents a viable drugable target in that the inhibition of related enzyme such as prolyl 4-hydroxylases has proven feasible.

A large number of compounds, in some cases analogues of alpha ketoglutarate, chelators of iron or proline analogues, have been tested as inhibitors of prolyl 4-hydroxylase and the hypoxic induced factor (HIF) prolyl hydroxylase. Some compounds are in phase 3 clinical trials (FG-4592, (Roxadustat), FibroGen). A similar approach could be undertaken here with the expectation that selective inhibitors can be developed to reduce the elevation of this enzyme in scleroderma patients, since the substrate is distinct from that recognized by the prolyl 4-hydroxylaes.

In the next Chapter of this thesis a direct approach is used with SSc patients to investigate and characterize the localized milieu of SSc lesions, in

which the pro-fibrotic responses are being induced. Fluid derived by suction blisters on SSc lesions is assayed for the content of a wide range of mediators. The suction blister technique allows sampling of the lesional environment with access to a variety of cytokines, chemokines and other factors that may contribute to the fibrotic process. This approach offers a localized view of the fibrotic process as well as access to the lesions that could potentially be utilized to assess the response to therapeutic treatment.

Chapter 4. Cytokines in Fluid Drawn from Systemic Sclerosis Lesions

4.1 Introduction

4.1.1 Biomarkers in systemic sclerosis

Clinical trials in SSc have been hampered by the lack of reproducible biomarkers that can be used as surrogate measures of therapeutic responses. At present, studies focus on the mRSS, a clinically assessed measure of skin thickness that is subject to fairly wide inter-observer variation, as the main outcome measure in clinical trials (Clements et al., 1995). A highly sensitive and reproducible biomarker assay would be useful and would enable the identification of effective therapeutics, particularly in subsets of patients. Such a marker should be able to be assayed at various clinical centres, correlated with disease severity and outcome, and be linked to important components of the pathogenesis of the disease.

Numerous soluble factors have been assayed in SSc patients' plasma, including collagen fragments, profibrotic growth factors such as TGFβ, ET-1 and CTGF, and factors linked to vascular damage including Von Willebrand Factor (VWF), e-selectin and other soluble adhesion molecules (Denton et al., 1995, Dziadzio et al., 2005, Kuryliszyn-Moskal et al., 2005, Lakota et al., 2012, Leask, 2011, Man et al., 2012, Nagy and Czirjak, 2005, Scheja et al., 2001, Sondergaard et al., 1998). In general, these have shown significant elevation in SSc sera but poor performance as biomarkers, with major overlaps between disease and control groups, lack of sensitivity as well as a lack of correlation with disease progression.

More recently the labs of Whitfield, Lafayatis (Boston University), as well as in the Centre for Rheumatology and Connective Tissue Diseases, Royal Free Hospital have focused more on the patterns of gene expression that have been identified using RNA extracted from skin biopsy material of SSc patients and controls (Hinchcliff et al., 2013, Pendergrass et al., 2012). Various signatures have been identified and now categorized as fibroproliferative, inflammatory, limited and normal-like (Whitfield et al., 2003). It may be possible

to combine gene expression markers identified by PCR with protein biomarkers and to use these as composite markers of disease activity.

4.1.2 Dermal blister fluid soluble factor analysis in systemic sclerosis

Colleagues at the Royal Free Hospital/UCL and others utilize a vacuum device over the surface of the lesion to form a blister through which blister fluid can be obtained by needle and syringe for assaying (Kiistala, 1968, Vermeer et al., 1979, Rossing and Worm, 1981, Søndergaard et al., 1997, Follin and Dahlgren, 2007, Davidsson et al., 2013). As such the suction blister technique is a widely used method in dermatology research for obtaining dermal interstitial fluid, is reproducible, and has been used previously in studies of soluble factors in the dermis of SSc patients (Kiistala, 1968, Vermeer et al., 1979, Rossing and Worm, 1981). Furthermore, fluid drawn from the lesions should provide information about activity in the lesions, and this could be used to follow the progression of the disease or response to therapy at the lesion site.

The availability of broad screening protein assays such as the Luminex Multiplex system presents an opportunity to profile the cytokines and growth factors present within SSc lesions and gain further insight into the biomechanisms acting within the disease microenvironment. *Based on this approach the hypothesis of this Chapter is that cytokines and growth factors of pathogenic significance will be differentially expressed in tissue fluid derived from SSc patients' skin lesions and healthy controls.* This hypothesis will be tested under the following aim.

4.1.3 Overall aim of the chapter

To use Multiplex profiling of growth factors and cytokines present in SSc lesions, to gain insight into the local biomechanisms. In order to pursue this aim the suction blister methodology will be used to extract fluid from the fibrotic forearm lesions, as well as from the forearm skin of healthy controls. Luminex® Multiplex assays will be performed to determine the levels of various candidate growth factors and cytokines in the dermal fluid from SSc patients and controls. SSc patients from various stages of the disease will be studied. Furthermore hierarchical clustering analysis will be used to group together patients with

similar trends in growth factor and cytokine expression, or to look for correlation between pattern of cytokine and clinical parameters such as clinical subgroup, autoantibody production, and disease duration. Clustering will also be performed in terms of the growth factors and cytokines to see if functionally related proteins cluster together. In addition String software will be used to try to model the network of protein:protein interactions in the SSc tissue fluid.

4.2 Results

4.2.1 Multiplex analysis of the dermal blister fluid

In total, 26 patients with SSc were studied, as well as 10 healthy controls (HC). The mean age of the SSc patients was 55 years +/- 10 (SD) years. Eight SSc subjects were male, and 18 were female. Twenty SSc patients were classified as DcSSc, and 6 had LcSSc. The mean duration of disease, defined by the first non-Raynaud's manifestation of SSc, was 9 years +/- 7.84 (SD), with a range of 1 to 33 years. Five patients had early disease, defined as disease duration of <2 years. The mean mRSS of patients was 18.3 +/- 11.2 (SD). The mean age of SSc patients included was 55 +/- 10 (SD) years, and the mean age of control subjects was 51 years +/- 14 (SD). SSc patients were mainly female with 18 females and 8 males, and in the healthy controls 9 were female, and 1 was male (for clinical features and autoantibody profile see Chapter 2, Tables 2.4 & 2.5).

The results of the interstitial fluid from the Luminex® array can be seen in Table 4.1. This assay identified 7 cytokines whose mean concentrations were significantly higher in the blister fluid of the SSc patient cohort than in the healthy control group blister fluid. These factors included inflammatory cytokines IL-6 and IL-15, the chemokine MCP3, vascular growth factors such as FGF2 and profibrotic growth factors PDGF-AA, as well as effector T cell associated cytokines IL-17A and IFNγ. Among these factors, IL-17A and IFNγ levels displayed the greatest proportional margin of increase in SSc blister fluid over the level in blister fluid of healthy controls. A figure summarising these results as a bar chart, for ease of visual comparison of the relative levels is shown (Figure 4.1).

Correlation was sought between protein levels and clinical parameters. Of these comparisons only IL-6 levels within the blister fluid were found to correlate significantly with skin score (r^2=0.202, p=0.024). MCP3 concentrations in the blister fluid correlated with disease duration, its concentration increasing with disease duration (r^2=0.295, p=0.005). In addition, some factors were specific to the SSc dermal blister fluid samples and were not detectable in healthy control samples. IL-17 was only detectable in dcSSc (5 of 19), and in 0 of 6 lcSSc and 0 of 10 HC. IL-6 showed a trend towards increased concentrations in dcSSc compared to lcSSc, but this was not statistically significant (mean=71.7 pg/mL in dcSSc, 32.3 pg/mL in lcSSc, p=0.07). MCP3 also showed a similar trend within the dermal blister fluid with increased concentrations in dcSSc compared to lcSSc, but again this was not statistically significant (mean=7.93 pg/mL in dcSSc, 6.09 pg/mL in lcSSc, p=0.36).

Biologic function		Mean HC	SEM	Mean SSc	SEM	P
Innate immunity	IFNα2	5.7	1.17	6.83	0.63	0.41
	IL-1a	97.1	29.7	85.0	19.2	0.73
	IL-1B	0.2	0.2	0.7	0.41	0.28
	IL-1Rα	1120	245	105	227	0.86
	IL-6	17.9	6.86	77.2	20.5	0.01
	IL-12p40	5.59	1.81	7.72	3.31	0.57
	IL-12p70	1.73	0.36	1.86	0.24	0.77
	IL-15	4.12	0.42	7.38	0.98	0.004
	IP-10	922	156	1770	456	0.08
	TNF-α	39.4	10.9	49.4	15.5	0.6
Adaptive immunity	IFNγ	0.13	0.13	1.59	0.48	0.006
	IL-2	0.37	0.37	1.03	0.54	0.31
	IL-3	0.13	0.13	0	0	0.34
	IL-4	2.3	1.06	4.04	2.01	0.44
	IL-5	0.53	0.44	0.4	0.16	0.79
	IL-7	4.48	0.57	6.16	0.69	0.07
	IL-9	0.22	0.22	0.39	0.24	0.60
	IL-10	30.0	5.99	33.0	4.15	0.69
	IL-13	2.05	1.1	2.03	0.77	0.99
	IL-17A	0	0	0.61	0.27	0.03
	sCD40L	380	86.7	362	49.9	0.86
	TNFβ	0	0	0.47	0.33	0.16
Chemokines	Eotaxin	37.8	5.85	36.7	3.08	0.86
	Fractalkine	53.4	14.1	49.4	9.04	0.81
	GRO	144	42.1	173	24.1	0.55
	IL-8	128	41.1	79.1	15.1	0.29
	MCP-1	1860	732	1320	324	0.51
	MCP-3	3.45	1	8.59	1.56	0.008
	MDC	1090	191	764	75.1	0.14
	MIP-1a	32.5	12.1	15.0	4.46	0.20
	MIP-1b	66.6	20.9	38.6	8.16	0.24
	RANTES	114	44.4	186	90.9	0.47
Growth factors	EGF	0.96	2.08	3.41	1.22	0.56
	Flt-3L	65.7	12.1	60.4	10.7	0.74
	GCSF	10.5	3.33	9.85	2.29	0.87
	GMCSF	6.19	2.03	4.77	0.96	0.54
	PDGF-AA	7.54	0.97	16.4	4.28	0.05
	PDFG-BB	0.64	0.43	20.0	14.1	0.18
	TGFα	7.81	0.91	7.1	0.55	0.51
Angiogenesis	FGF-2	13.9	1.14	19.9	2.2	0.02
	VEGF	13.5	3.15	21.7	5.21	0.18

Table 4.1 Growth factor and cytokine profiling of systemic sclerosis and control blister fluid samples Levels of the growth factors and cytokines are shown in pg/ml. Data for SSc patients and controls were compared using permutation analysis - SAM for Excel, combined with student's t test. Significant results are shown in red.

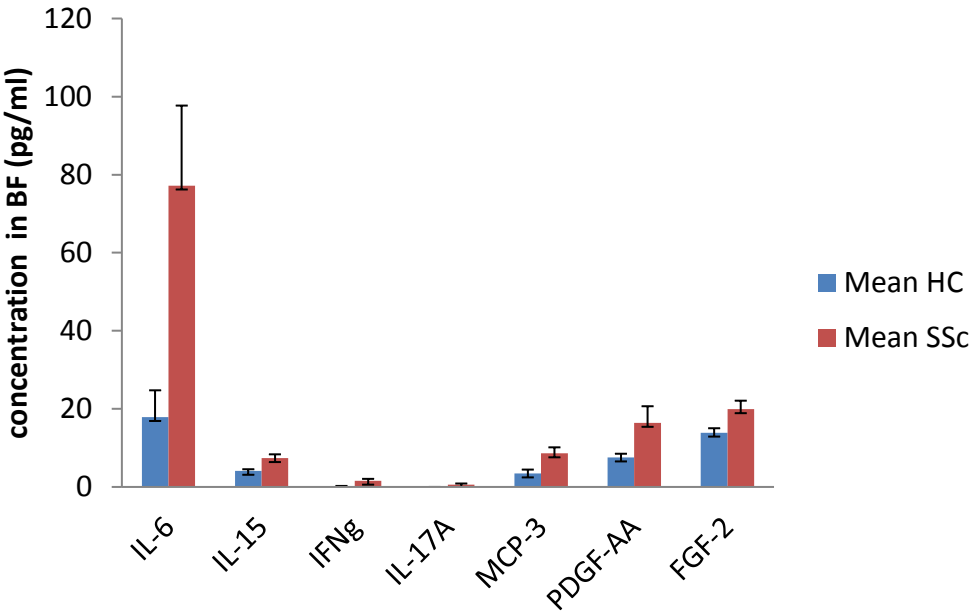


Figure 4.1 Elevated growth factors and cytokines in systemic sclerosis blister fluid samples Dermal blister fluid (BF) was sampled from 26 SSc patients and 10 controls and assayed for 41 protein growth factors and cytokines. Factors significantly elevated ($p<0.05$) in SSc BF are shown. The striking elevation and predominance of the inflammatory cytokine IL-6 in the lesions is shown as well as the pro-fibrotic PDGF-AA and the angiogenic factor FGF-2.

4.2.2 Multiplex analysis of the plasma samples

Plasma samples were available from 19 of 26 SSc patients, and 8 of 10 HCs, and these were included in the analysis. The results from the Luminex® array of plasma are shown in Table 4.2. Twelve of the factors studied were significantly elevated in SSc compared to HC plasma. The proteins that were significantly elevated ($p<0.05$) in the plasma, also elevated in blister fluid, were MCP3, IL-15 and IL-6. The other significantly elevated proteins in plasma were IL-1RA, IL-1a, IL-12p40, GM-CSF, IL-2, IL-4, IL-9, Flt-3L and VEGF (all $p<0.05$, summarized in Table 4.2 and presented graphically in Figures 4.2).

Of interest, Th_2 cytokines IL-4, 5, and 13, were only detectable in SSc plasma, however of these only IL-4 was significantly elevated in SSc plasma compared to HCs due to variation among samples. IL-6 was detectable only in SSc plasma, and not in HCs plasma samples.

Nineteen of 26 SSc patients had adequate samples of plasma and blister fluid for paired analysis. When comparing the paired plasma samples with the

Luminex® array profiling of the dermal blister fluid from those with SSc, the only significant correlation was in MCP3 levels (r^2=0.56, p=0.013). Other pro-inflammatory cytokines IL-6 (r^2=0.083, p=0.23), IL-17 (r^2=0.02, p=0.56), and growth factors VEGF (r^2=0.03, p=0.47), PDGF (r^2=0.08, p=0.22) showed no correlation between concentrations in the plasma samples and in the dermal blister fluid. This supports the idea that analysing the dermal interstitial reveals the local inflammatory profile, not evident in the analysis of the plasma samples.

The healthy controls showed greater correlation between the Luminex® array results from the dermal blister fluid and plasma samples, with many reaching significance. These included MCP-3 (r^2=0.68, p<0.001), IL-10 (r^2=0.39, p=0.005), FGF-2 (r^2=0.58, p<0.001), IL-1Rα (r^2=0.37, p=0.006), EGF (r^2=0.22, p=0.043). Hierarchical clustering of plasma and blister fluid samples is shown in Figures 4.3a and 4.3b.

Biologic function		Mean HC	SEM	Mean SSc	SEM	P
Innate	IFNα2	24.5	4.89	30.6	3.73	0.34
immunity	IL-1a	2.25	1.10	13.4	5.25	0.05
	IL-1B	0.22	0.22	1.09	0.45	0.10
	IL-1RA	38.2	6.65	62.9	7.97	0.03
	IL-6	0.00	0.00	1.59	0.70	0.04
	IL-12p40	2.05	1.34	17.6	4.98	0.01
	IL-12p70	5.03	1.16	6.00	0.73	0.49
	IL-15	0.00	0.00	1.31	0.53	0.02
	IP-10	333	66.0	416	39.6	0.30
	TNF-α	5.34	1.02	7.25	0.63	0.13
Adaptive	IFNγ	4.38	0.81	6.19	1.28	0.24
immunity	IL-2	0.24	0.24	1.64	0.60	0.04
	IL-3	0.40	0.19	0.65	0.18	0.35
	IL-4	0.00	0.00	3.90	1.75	0.04
	IL-5	0.00	0.00	1.44	1.22	0.25
	IL-7	2.54	0.37	3.07	0.41	0.35
	IL-9	0.00	0.00	0.61	0.29	0.05
	IL-10	1.79	0.53	4.10	1.07	0.06
	IL-13	0.00	0.00	2.20	1.08	0.06
	IL-17A	2.06	0.39	2.97	0.51	0.16
	sCD40L	8990	806	8990	560	0.99
	TNFβ	1.13	0.75	4.56	1.76	0.09
Chemokines	Eotaxin	99.5	16.3	106	10.9	0.76
	Fractalkine	54.7	7.26	63.1	4.43	0.34
	GRO	573	56.9	559	64.2	0.87
	IL-8	3.18	0.47	3.95	0.50	0.27
	MCP-1	232	23.6	238	22.0	0.86
	MCP-3	8.54	2.25	16.4	2.65	0.03
	MDC	716	36.0	695	43.4	0.72
	MIP-1a	1.25	0.87	2.79	0.98	0.25
	MIP-1b	23.9	5.83	23.6	2.37	0.96
	RANTES	2270	403	1590	156	0.15
Growth	EGF	88.6	25.2	93.7	18.8	0.87
factors	Flt-3L	0.00	0.00	2.98	1.29	0.03
	GCSF	38.5	2.88	44.4	3.30	0.19
	GMCSF	8.88	1.41	15.8	2.37	0.02
	PDGF-AA	1460	224	1380	191	0.80
	PDFG-BB	3990	575	4860	480	0.26
	TGF-α	0.00	0.00	0.62	0.34	0.08
Angiogenesis	FGF-2	63.3	7.07	81.0	6.25	0.08
	VEGF	79.2	14.6	163	32.8	0.03

Table 4.2 Growth factor and cytokine profiling of systemic sclerosis and control plasma samples. Levels of the growth factors and cytokines are shown in pg/ml. Data for SSc patients and controls were compared using permutation analysis - SAM for Excel, combined with student's t test. Significant results are shown in red.

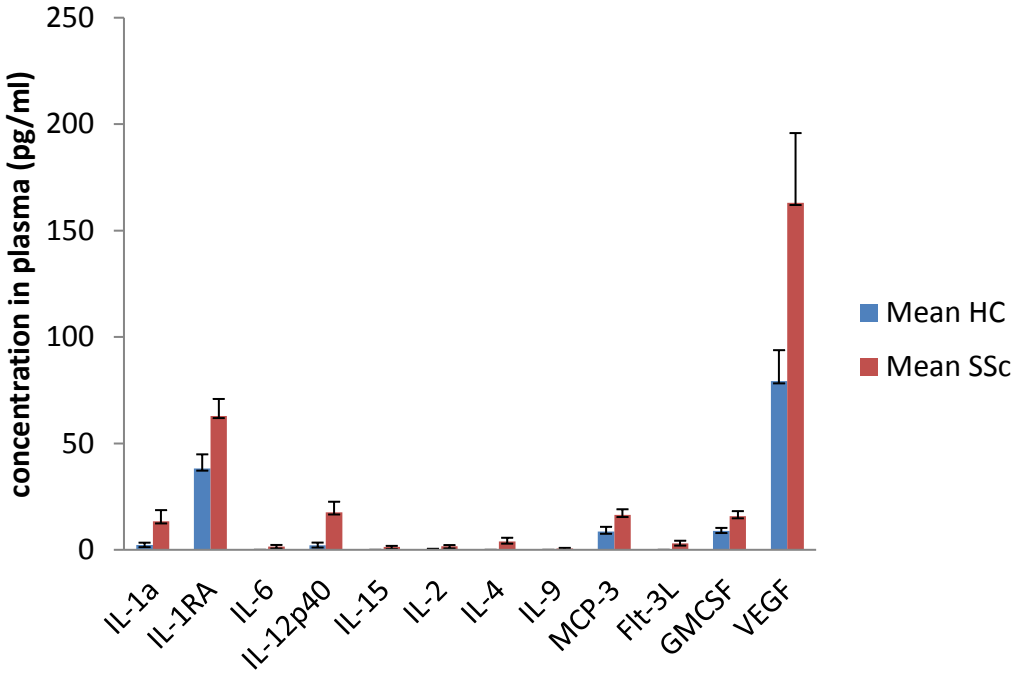


Figure 4.2 Elevated growth factors and cytokines in systemic sclerosis plasma samples Plasma samples were available 19 SSc patients as well as 8 controls, taken synchronous to the BF sampling, and then also assayed for 41 protein growth factors and cytokines. Factors significantly elevated ($p<0.05$) in SSc plasma are shown. This illustrates the excess of IL-1RA as a regulatory factor compared to IL-1a, as well as the high levels of IL-6, IL-12p40, as well as VEGF in SSc plasma.

4.2.3 Hierarchical clustering

In order to look for possible co-expression of related growth factors and cytokines, and also to see if patients from related clinical or autoantibody subgroups would group together, Hierarchical clustering was performed in 2 dimensions to cluster cosegregating proteins and patients in an unbiased fashion. Protein concentrations were expressed as ratios of the mean value, log transformed and then expressed as standard deviations above or below the mean value. Heat maps were then constructed for blister fluid and plasma Luminex® results using CIMminer NIH software. The results are shown in Figures 4.3a for blister fluid and Figure 4.3b for the plasma measurements. The lengths of the dendrograms are inversely proportional to the degree of correlation. The resulting dendrograms show clustering of the patients studied into 3 groups (Figure 4.3a). Patient group 1 was characterized as IL-6, IL-10,

TNF-α, and IL-1α high (innate inflammatory, IL-6 associated, early dcSSc patients), Group 2 by increased concentrations of IFNγ, IL-2, IL-4, IL-5, MCP-3, IL12p40, IL12p70 (IFNγ Th cell group, late stage dcSSc), and Group 3 by low levels of cytokines and chemokines (quiescent. lcSSc or mild diffuse).

The clinical and laboratory characteristics of the patient groups clustering in the BF analysis are presented in Table 4.3, showing Group 1 to be early dcSSc with higher skin scores, Group 2 to be late stage dcSSc, and Group 3 to contain lcSSc, or dcSSc with low skin score. There was no apparent difference in antibody profiles, pattern of internal organ involvement or immunotherapy between the three groups.

Plasma hierarchical clustering did not resemble the pattern obtained with blister fluid (Figure 4.3b). In general meaningful clustering of patients into well-defined clinical subgroups was not seen with the plasma hierarchical analysis.

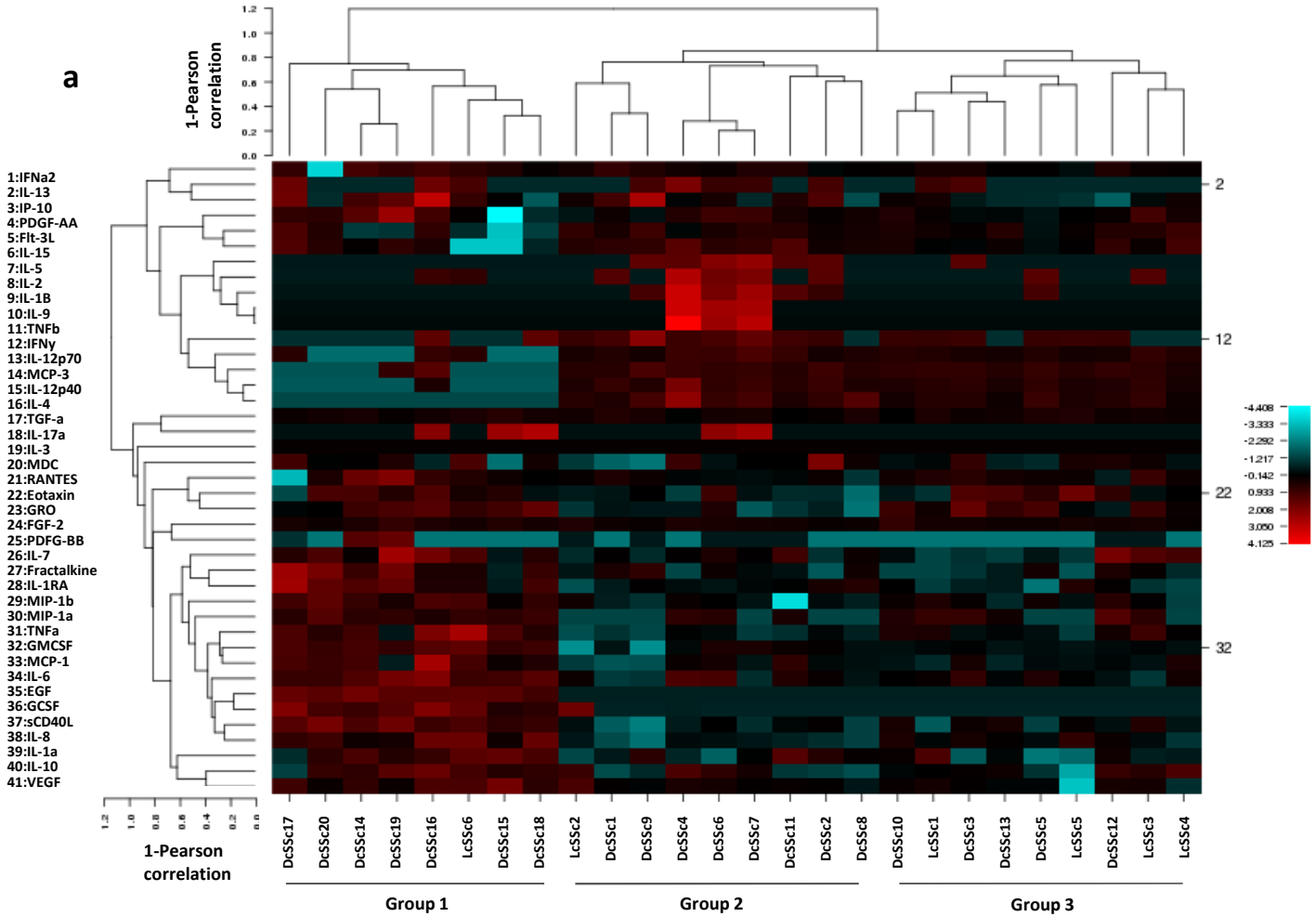


Figure 4.3a. Heat map of blister fluid samples of systemic sclerosis patients characterized as lcSSc or dcSSc Hierarchical clustering was performed using the NIH CIMminer software. Cosegregating growth factors or patients are clustered together, the length of the dendrogram being inversely proportional to the strength of correlation.

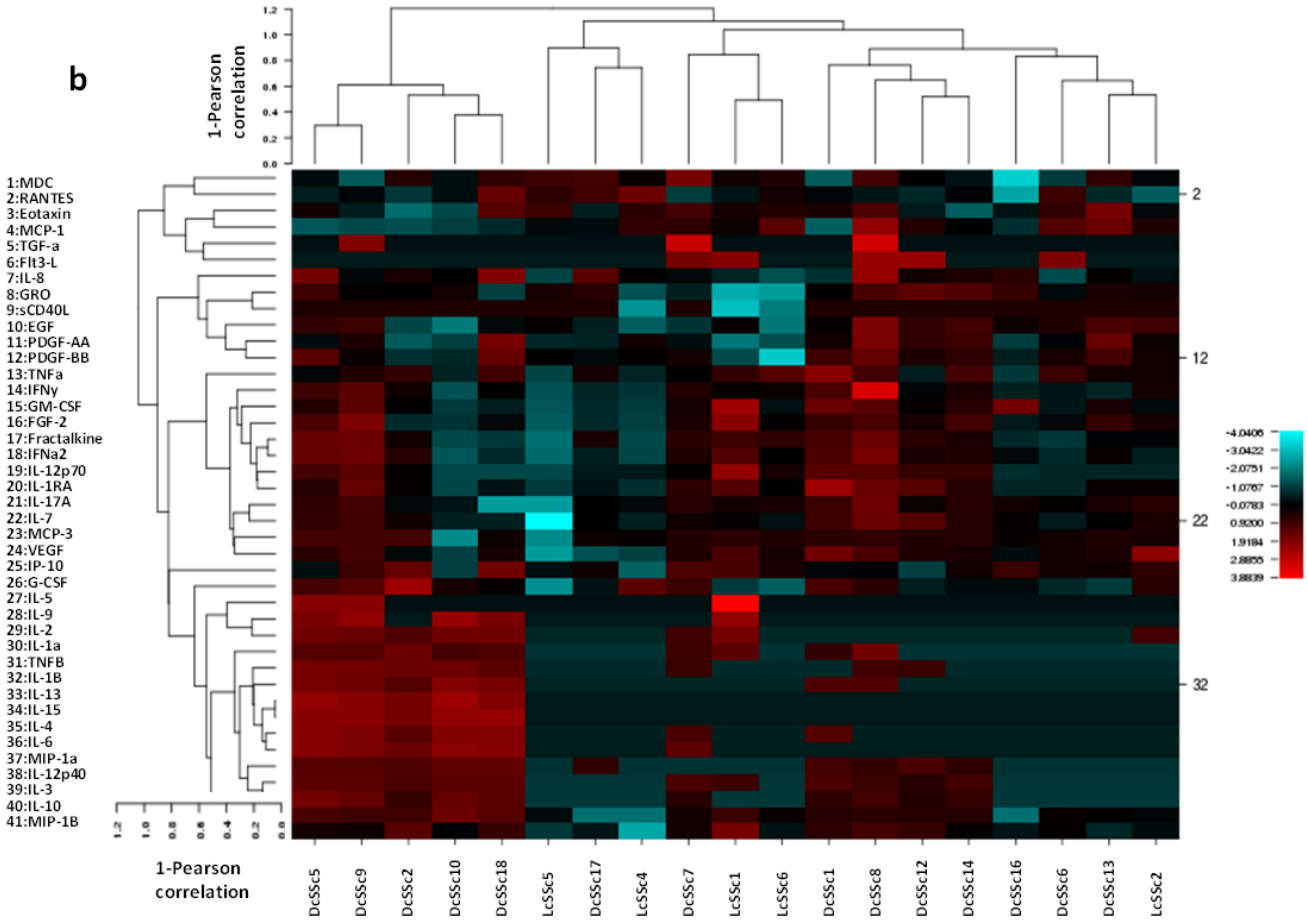


Figure 4.3b. Heat map of plasma samples from systemic sclerosis patients characterized as lcSSc or dcSSc. Hierarchical clustering was performed using the NIH CIMminer software. Cosegregating growth factors or patients are clustered together, the length of the dendrogram being inversely proportional to the strength of correlation.

Heat map cluster	DcSSc/LcSSc	Disease duration (years)	Skin score	Antibody	Current steroid
Group 1	DcSSc17	8	24	3, 2	n
IL-6	DcSSc20	1	27	4,0	y
	DcSSc14	1	38	1,0	y
	DcSSc19	5	28	3,3	n
	DcSSc16	2	34	2,1	y
	LcSSc6	3	6	1,0	n
	DcSSc15	5	35	2,1+5	y
	DcSSc18	15	24	2,1	n
Mean (SEM)		5 (1.7)	27 (3.5)		
Group 2	LcSSc2	23	6	3,1+5	y
IFNγ	DcSSc1	10	16	5,4+5	y
	DcSSc9	33	28	4,0	y
	DcSSc4	11	30	2,1	n
	DcSSc6	6	24	3,2	y
	DcSSc7	13	9	4,0	n
	DcSSc11	2	34	2,1	y
	DcSSc2	8	8	2,1	n
	DcSSc8	20	30	2+4,1	n
Mean (SEM)		14 (3.2)	21 (3.6)		
Group 3	DcSSc10	3	12	4,0	y
Quiescent	LcSSc1	6	4	1,0	n
	DcSSc3	8	15	3,2	y
	DcSSc13	2	12	3+6,0	y
	DcSSc5	20	10	2,1	y
	LcSSc5	12	15	3,0	n
	DcSSc12	10	14	2,1	n
	LcSSc3	3	6	1,0	n
	LcSSc4	4	6	3+4,0	y
Mean (SEM)		7.5 (1.9)	10 (1.4)		

Table 4.3 Patients grouped according to blister fluid heirarchial clustering. Patients in Group 1 (IL6 high, innate inflammatory) were more likely to have early diffuse SSc with high skin score, Group 2 (IFNγ high, effector T cell) patients were mainly late stage diffuse SSc, Group 3 (quiescent pattern) were lcSSc or dcSSc with low skin scores. **P=0.0017, *P=0.025 versus quiescent Group3, Antibody profile first number immunofluorescence pattern, 1= Centromere, 2= homogenous, 3= fine speckled, 4= nucleolar, 5= coarse speckled, 6= cytoplasmic pattern of, second number extractable nuclear antigen, 0= none, 1= Scl70, 2= RNA polymerase, 3= U3RNP, 4= nRNP, 5= ro, 6= la. Therapy: aza= azathioprine, cyc= cyclophosphamide, cic= cyclosporine, hcq= hydroxychloroquine, ivig= intravenous immunoglobulin, mmf= mycophenolate, mtx= methotrexate. ANOVA showed significant difference for disease duration between groups (p=0.042), and skin score (p=0.003). Scheffe post hoc analysis, showed higher skin score in Group 1 (p=0.005), otherwise P NS.

4.2.4 Network analysis of the multiplex data

Potential protein: protein interactions within the disease microenvironment were explored using STRING 9.1(http://string91.embl.de/software). Proteins elevated in each clinical group were entered into the STRING database and a network of potential protein interactions generated. Group 1 is characterized by IL-6 innate inflammatory protein pattern, with IL-6 occupying a central node with multiple downstream protein interactions (Figure 4.4a). In Group 2, IFNγ appears as a central cytokine with multiple related immune and inflammatory proteins (Figure 4.4b). In general Group 3 was quiescent without elevated protein factors so no String diagram is shown for this group.

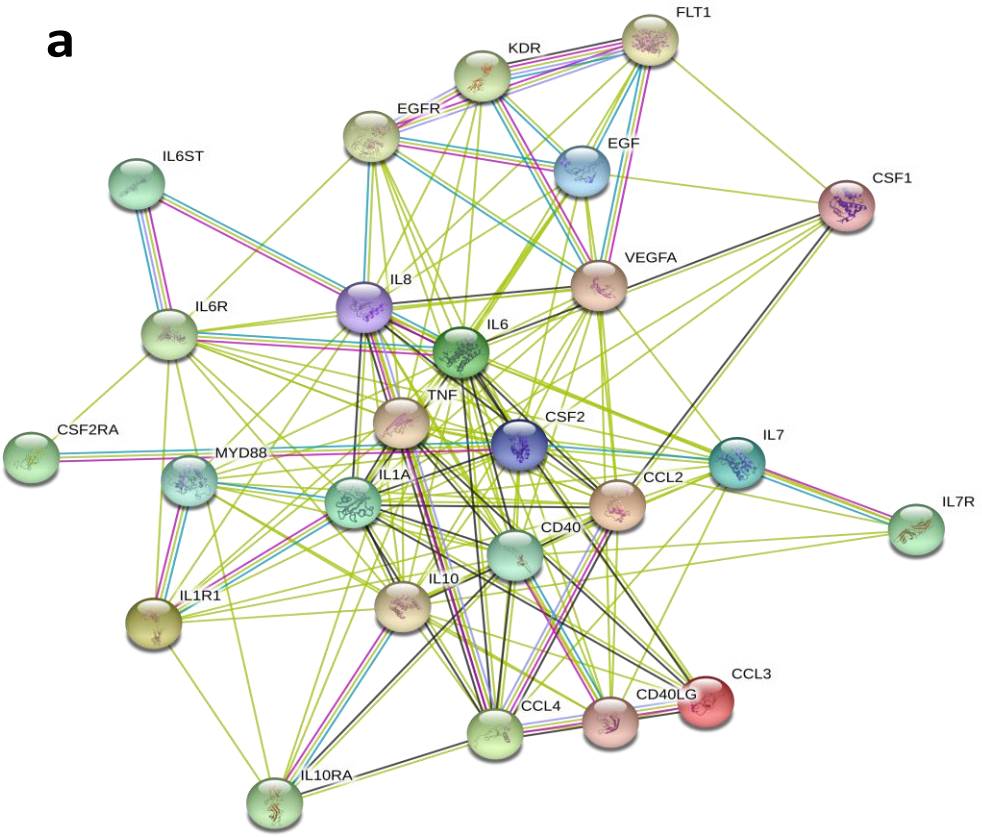


Figure 4.4a Network analysis of blister fluid cytokine levels from systemic sclerosis patients – network of proteins detected as increased in the dermal blister fluid of Group 1 patients. Using STRING 9.1 database, a network of potential protein interactions was detected in SSc dermal blister fluid clustered into Group 2. An IL-6 centred inflammatory and innate protein network predominates in this subgroup. (Light green lines indicate association by text mining, pink by experiment, black by co-expression, dark green by neighborhood).

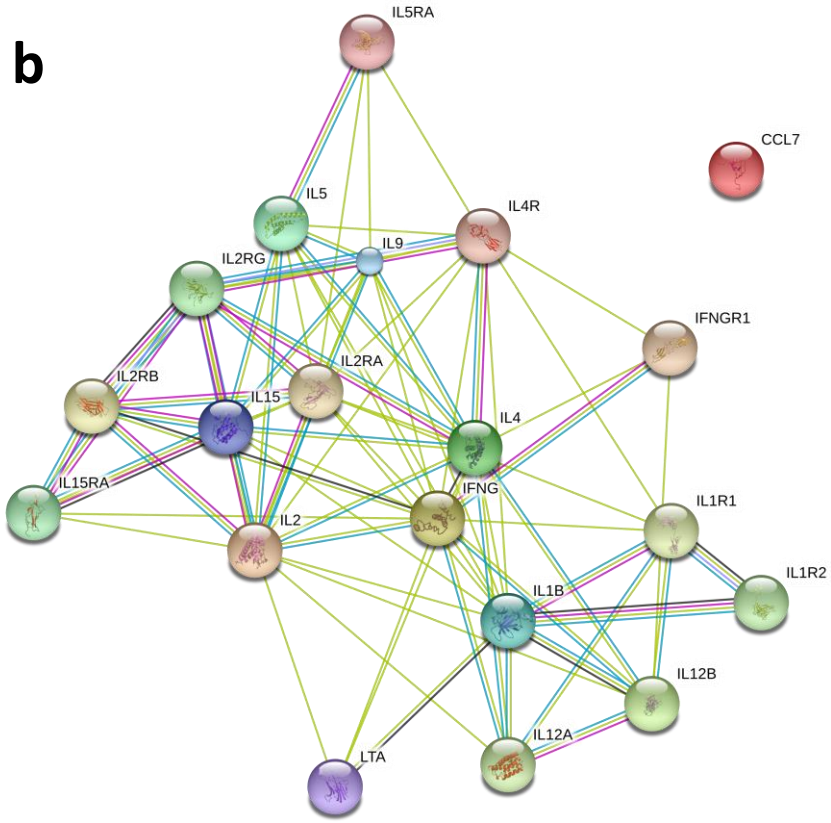


Figure 4.4b. Network analysis of blister fluid cytokine levels from systemic sclerosis patients - network of proteins detected as increased in the dermal blister fluid of Group 2 patients Using STRING 9.1 database, a network of potential protein interactions was detected as increased in SSc dermal blister fluid clustered into Group 2. An IFNγ and effector T-lymphocyte associated protein network predominates in this subgroup. (Light green lines indicate association by text mining, pink by experiment, black by co-expression, dark green by neighborhood).

4.3 Discussion

4.3.1 Use of blister fluid to assess disease activity

Methods used to diagnose and judge progression in patients with SSc include the mRSS, that encompasses a simple assessment of the ability to “pinch the skin” into a fold. Other measurements that have been proposed include measuring autoantibodies and other relevant biomarkers in serum, or profiling of gene expression patterns in RNA extracted from skin biopsy material, but these have generally not found acceptance in routine clinical practice. Others have used blister fluid to assess disease activity in SSc (Sondergaard et al., 1998). In general, previous studies have found that

collagen markers and markers of fibrosis were elevated in the blister fluid from SSc patients (Stratton et al., 2001, Dziadzio et al., 2005). Thus analysis of blister fluid offers a way to determine the active biologic processes in the involved skin, and serve as surrogate markers to assess therapeutic responses.

In the present Chapter, Millipore's Luminex® 41 Multiplex apparatus was used to profile cytokines and growth factors present in the blister fluid. Of the 41 cytokine/chemokines measured, two inflammatory cytokines, IL-6 and IL-17 were highly elevated; angiogenic factors FGF-2 and PDGF were also elevated in SSc patients. PDGF is also a potent, chemoattractant, motility and proliferative factor for fibroblasts (Seppä et al., 1982, Pierce et al., 1991).

Translational medicine, in particular biomarkers, holds great therapeutic possibilities. Under normal conditions, inflammation is regulated and does not persist. However, depending on the nature of the initiating process and the genetic makeup of the individual, the process may become prolonged and excessive, destroying normal tissue architecture and replacing it with scar tissue, defined as excessive connective tissue (collagen and other extracellular molecules), and a loss of normal tissue function. Fibrosis is foremost among the common sequelae of inflammatory conditions affecting the lungs, skin, heart, bone marrow and other tissues (Takahara et al., 2004). While many drugs and other substances have anti-inflammatory activity, they are often not effective in fibrosis generally or SSc (Ong and Denton, 2010). Thus there is a need for more targeted therapeutics able to regulate and reduce acute and chronic inflammation.

IL-6 is most notably associated with provoking an inflammatory response and activation of fibroblasts. In rodent bleomycin studies, as a model of SSc, IL-6R blockade has ameliorated the inflammatory and fibrotic effects induced by bleomycin (Kitaba et al., 2012). This has resulted in the first clinical trial to treat SSc with a specific IL-6 receptor antibody, tocilizumab, A multicenter phase II trial including the Royal Free Hospital, has recently been published showing a non-significant trend to improvement in skin score in SSc patients who received the drug (Khanna et al., 2016a). It should be noted that the cellular source of elevated IL-6 in the sera and blister fluid of SSc patients is unknown.

An increased concentration of the neutrophil chemoattractant IL-8 has been reported in SSc serum, although not corroborated here. IL-8 and IL-6 are

overexpressed in the lesional skin of patients with SSc. Different patterns of expression are apparent, with IL-8 overexpression being associated with early disease (<1 yr), unlike IL-6 overexpression that is associated with later disease (Koch et al., 1993). Stimulated and unstimulated fibroblasts isolated from SSc lesional skin produce increased amounts of IL-8, which may be involved in promoting localized intralesional soluble IL-6R release by neutrophils, sensitizing target cells to the effects of IL-6 (Kadono *et al*., 1998). IL-6 is known to function in propagating chronic inflammation, and may do so as well in SSc.

Of interest also, a number of angiogenic or predominantly vascular related factors were studied and found altered in SSc. Vascular injury is manifested in SSc early in the disease, even prior to the initiation of fibrosis (Prescott et al., 1992, Blann et al., 1993, LeRoy, 1996). SSc is characterized by a widespread microangiopathy comprised of reduced capillary density and irregular chaotic architecture that results in chronic tissue hypoxia (Distler et al., 2004). Hypoxic conditions in SSc notwithstanding, however, there appears to be insufficient compensative angiogenesis (LeRoy, 1996, Distler et al., 2004, Hummers et al., 2009). This is the result of, and may play a part in, the imbalance between angiogenic and angiostatic factors contributing to the pathogenic mechanisms of SSc (Distler et al., 2002, Distler et al., 2006, Manetti et al., 2010). Among angiogenic factors, the most important are VEGF and its receptors, FGF-2, PDGF, TGFβ, FGF-2, angiopoietin 1 (Ang-1), stromal cell-derived factor 1 (SDF-1/CXCL12), ET-1, MCP1, urokinase type plasminogen activator receptors (uPAR) and kallikreins, and vascular adhesion molecules. On the other hand, angiostatic factors include: endostatin, angiostatin, thrombospodin-1 (TSP-1) and angiopoietin 2 (Ang-2) (Liakouli et al., 2011, Riccieri et al., 2011). Our knowledge concerning the dysregulation of angiogenic homeostasis in SSc is incomplete and needs further research.

FGF-2, a key hypoxia-induced angiogenic factor present in the basement membranes of blood vessels and normally associated with increased angiogenesis, was elevated in the SSc blister samples. In fact, there is an interesting paradox in SSc that multiple angiogenic factors are increased and yet there is a failure of vascular repair, and normal angiogenesis is not seen. There is, however, a chaotic dysregulated angiogenesis resulting in

telangiectasia, gastric vascular ectasia, and adverse vascular remodeling in general, possibly the response to an excess of these angiogenic factors.

Previously serum levels of FGF-2 were shown to be elevated in nearly half (31 of 74) of the subset of patients with SSc, in a study of collagen diseases (Kadono et al., 1996). Because FGF-2 expression at the site of injury (*i.e.*, skin in SSc) was viewed as being more relevant, the expression of FGF-2 in the skin biopsies of patients with SSc was determined, and compared to the levels in serum (Lawrence et al., 2006). The skin biopsies from patients showed increased expression of FGF-2 in the basal layer of the epidermis and dermis (periappendageal, perivascular, matrix tissue) as compared to normal tissues. Overexpression of FGF-2 in SSc patients' skin with normal serum levels was taken to suggest that FGF-2 acts locally within the fibrotic lesions (Lawrence et al., 2006).

Peripheral blood mononuclear cells (PBMC) from SSc patients with both early and established disease spontaneously released significantly greater amounts of VEGF as compared to PBMC from controls (Bielecki et al., 2011), consistent with the results presented in this chapter showing elevated plasma VEGF. PBMC from SSc patients produce increased amounts of VEGF in the early stage of disease. This early production of VEGF contributes to the imbalance in the profile of proangiogenic mediators produced by PBMC in SSc that could contribute to the pathogenesis of SSc. Intriguingly, a transition from proangiogenic to antiangiogenic VEGF isoforms may play a crucial role in the insufficient angiogenic response in chronic ischemia in SSc (Manetti et al., 2011).

In an investigation of the possible role of angiogenesis imbalance in the pathogenesis of SSc, a significant increase in the mean plasma levels of the anti-angiogenic factor endostatin (derived from collagen 18) in SSc patients compared to controls has been reported (Farouk et al., 2013). Serum endostatin was significantly increased in late compared to early stages of disease. There was also a significant positive correlation between endostatin serum levels and histopathologic skin thickness score. The authors concluded that the angiogenic inhibitor endostatin is induced in SSc, and reduces any reparative or beneficial effect of VEGF in late stages of SSc. Further, the increased serum endostatin they observed was associated with the severity of

skin sclerosis, favoring disease progression, combined with failure of angiogensis.

Furthermore, vasculogenesis is also impaired, further supporting an imbalance between vascular growth and inhibitory factors (Distler et al., 2002, Distler et al., 2006, Manetti et al., 2010).

Of note also were the elevations of PDGF-AA, and trend towards elevation of PDGF-BB in the blister fluid. PDGF has potent activity in stimulating the migration and proliferation of fibroblasts, and could be an important factor in driving the disorder. It is notable that there are a number of Inhibitors of the PDGF receptor kinases and their further assessment in SSc may be warranted. Because of these findings PDGF and several inhibitors are studied in the following Chapter for their capacity to stimulate or block SSc fibroblast migration.

Within the skin and elsewhere, endothelial cells produce PDGF which could contribute to vascular pathogenesis and also stimulate the proliferation and migration of fibroblasts contributing to the development and progression of SSc lesions. Along with CTGF and TGFβ, fibroblast growth factors (FGFs) are important fibrogenic cytokines (Strehlow and Korn, 1998), with bFGF (FGF-2, FGF2) considered to be the most potent. After secretion into the local milieu, FGF-2 remains sequestered in the extracellular matrix (Dinbergs et al., 1996) and are less likely to be found in the circulation. FGF-2, is a potent mitogen for cells of mesodermal and neuroectodermal origin, such as fibroblasts and endothelial cells (Allouche, 1995).

Of note PDGF has also been shown to play a role in animal models of fibrosis (Bonner, 2004). In addition to inhibiting the c-Abl kinase, imatinib also inhibits PDGF receptor kinase, and is effective in preventing both bleomycin- and radiation-induced lung fibrosis (Daniels et al., 2004, Abdollahi et al., 2005).

PDGF regulates the level of the b-FGF (FGF-2) receptor, FGF-2 modulates the expression of the PDGF receptor, and this receptor regulates the level of the receptor for FGF-2 (Ichiki et al., 1995, Kikuchi et al., 1992). Basic FGF also mediates the mitogenic effect of PDGF on vascular smooth muscle cells (Millette et al., 2005). There is thus evidence suggesting that FGF-2 and PDGF can act synergistically in an autocrine and paracrine manner. TGFβ, the

importance of which in SSc was described above, enhances the expression of PDGF and PDGF receptors on fibroblasts. Basic FGF may act subsequent to the effects of TGFβ and PDGF in the perpetuation of the abnormal phenotype characteristic of SSc fibroblasts.

One possible refinement to the approach presented in this Chapter is the possible inclusion of some direct fibrosis markers in the blister fluid analysis, which could be performed by ELISA synchronous to the Multiplex growth factor and cytokine measurements. Previously it was found that levels of CTGF, a pro-fibrotic growth factor, were greatly elevated in blister fluid samples from patients with both diffuse and limited SSc when compared with healthy controls (Dziadzio et al., 2005). The elevation of CTGF in these samples is consistent with previous work showing increased levels of CTGF by immunohistochemistry in skin biopsy material from SSc patients (Igarashi et al., 1995a). The candidate and colleagues believe that the elevated levels of CTGF are due to increased transcriptional activity of the CTGF gene and increased synthesis of CTGF, because the candidate's colleagues showed previously by differential array analysis that CTGF mRNA levels are elevated in involved skin in scleroderma (Shi-wen et al., 2000).

The usefulness of the blister technique for testing responsiveness to therapy was shown in the publication by Stratton *et al*. (stratton, 2001). The dermal blister fluid was taken from SSc skin of patients before and after treatment with Iloprost. The blister fluid was tested for the content of mediators, allowing comparison to be made with control samples. In this study treatment with Iloprost reduced the CTGF content of the blister fluid, which was much greater in SSc patients than in normal controls.

Also, the studies in this Chapter, measured adaptive immune, T lymphocyte-derived cytokines in the blister fluid samples. The IL-2 level of the SSc blister fluid trended towards being greater in SSc than the control, but this did not reach statistical significance and was not present at such a high level as IL-6. The same trend was observed with IL-17A, and IFNγ levels being much higher than the control level. Also in plasma analysis presented here the Th2 cytokines IL-4, 5 and 13 were found to be present in SSc but not controls. The bias toward a Th2 response in SSc patients (and in bleomycin-treated mice) may reflect a systemic orientation not fully reflected in the localized milieu of

SSc-affected skin. This conclusion is clearly supported by the increase in Th2 cytokines previously reported in SSc patients, and in the plasma, skin and lungs of mice in the bleomycin mouse model of fibrosis (Chizzolini et al., 2011, Fichtner-Feigl et al., 2006, Kaviratne et al., 2004a, Kitaba et al., 2012, Truchetet et al., 2011, Yoshizaki et al., 2010). This result raises the question of the relevance of sampling SSc patients systemically (plasma) vs. localized (skin blister fluid from a delimited location). Whereas serum would reflect systemic, body-wide effects, the skin blister fluid is from a more spatially restricted location that would be expected to reflect local activities and effects, and may be considered to be more relevant to the skin lesions in SSc.

In the context of interest here, the interplay between Th1 and Th2 cytokines results in promotion of inflammation in SSc patients. These subsets exert opposing effects in regulating tissue remodeling and fibrogenesis. On one hand, IFNγ suppresses fibroblast activity and extracellular matrix protein production (Duncan and Berman, 1989). On the other hand, Th2 cytokines activate fibroblasts and collagen production directly or indirectly by inducing secretion of profibrotic cytokines such as TGFβ (Gurujeyalakshmi and Giri, 1995a). It appears that a complex network of cellular and humoral mediators exerting profibrotic activity develops within SSc patients' target tissues (Varga and Abraham, 2007). The overall bias toward a Th2 response leads to a Th2 cytokine increase in SSc, as well as in the serum, skin and lungs of bleomycin-treated fibrotic mice (Chizzolini et al., 2011, Fichtner-Feigl et al., 2006, Kaviratne et al., 2004a, Kitaba et al., 2012, Truchetet et al., 2011, Yoshizaki et al., 2010). It follows that cytokine levels can be used as prognostic markers in SSc patients, or as diagnostic indicators of the effectiveness of therapeutics on the Th1/Th2/Th17/Treg balance (Baraut et al., 2010).

Alternatively activated macrophages, which function in the resolution of inflammation and promotion of wound healing, are found in SSc skin biopsies. Moreover, phenotypic markers of alternatively activated macrophages ($CD204^+$ and $CD163^+$) are elevated on $CD14^+$ cells in SSc (Higashi-Kuwata et al., 2009). These alternatively activated macrophages secrete factors that promote differentiation of skin-infiltrating naïve T cells to Th2 cells.

Vessel permeability and injury in SSc appears to coincide with the infiltration of, T cells and macrophages into perivascular areas (Kräling et al.,

1995, Kahaleh and LeRoy, 1999, York et al., 2007). There is a strong correlation between immune cell infiltration and the severity and progression of SSc (Roumm et al., 1984). Vascular injury is believed to be the initial mechanism initiating pathogenesis in patients with SSc (Jun et al., 2003, Denton and Abraham, 2001a), resulting in vascular inflammation and ultimately fibrosis.

Immune cell recruitment to peripheral tissues operates primarily within chemokine gradients. There are a number of chemokines shown to have upregulated expression in SSc skin, including CXCL9 (MIG) and CXCL10 (IP-10), which recruit T cells (Groom and Luster, 2011, Rabquer et al., 2011), CCL2 (MCP1) that recruits macrophages (Distler et al., 2001, Yamamoto et al., 2001) and other chemokines such as CCL5 (RANTES), MCP3, and CCL20 involved in recruiting T cells and macrophages and other cells (Van Damme et al., 1992, Ong et al., 2003, Distler et al., 1999, Tao et al., 2011). Of these, MCP3 was shown to be increased in SSc blister fluid and plasma in the current study. These and other chemokines have the potential for a complex regulation and mediation of cell infiltration in SSc.

Considerable redundancy of these and other chemokines, lymphokines, angiogenic and vascular factors, proinflammatory mediators, profibrotic factors and growth factors indicates the potential for a complex regulation and mediation of cell activation and infiltration in the SSc microenvironment, and demands a more comprehensive understanding of the role of these numerous soluble factors in the processes leading to the fibrotic state in SSc. Effective therapies may require a combination of cytokine inhibitors.

Overall these results show that this dermal blister fluid harvesting and analysis technique with SSc patients can provide results that are very useful for characterization of parameters of the local disease process. This approach of using blister fluid to sample skin lesions may be more robust than certain other local skin sampling methods, and offers a variety of possible analyses. These include histopathology of sampled skin, assaying sampled skin by PCR for RNA expression, analysis of cytokine, chemokine or antibody content of the fluid, and direct analysis of transmigratory leucocytes. In addition to the research perspective, it can be readily concluded that this technique using biological samples from SSc patients, along with current and future assays, can also be

used to evaluate responses to therapeutic treatment in a noninvasive manner. The candidate is confident that both of these approaches can contribute to advancement of the understanding and treatment of SSc patients.

4.3.2 Summary perspectives

As described in this chapter, fluid was drawn from dermal blisters formed on the surface of SSc lesions by a vacuum device, and the fluid was assayed for mediator content. The information on mediator content available from analysis of dermal blister fluid, and the lack of correlation of cytokine content between serum samples and blister fluid supports the use of the latter approach with SSc patients. The dermal blister fluid method provides access to mediators that may be driving the local fibrotic process, as well as providing a minimally invasive method that could potentially be utilized to assess the local response in the lesion to therapeutic treatment. Both of these approaches with blister fluid can contribute to advancement of the understanding of SSc, identification of therapeutic targets and ultimately to development and use of new treatments for SSc patients.

Moving to Chapter 5, the transition is made from describing SSc patient-derived lesional dermal fluid samples containing bioactive components, to the study of fibroblast cells derived from SSc patients, and a view of their migratory behavior when cultured with bioactive factors like those present in the lesional fluid. As one example, high levels of the fibroblast chemoattractant PDGF were found in the studies in the current Chapter to be present in the blister fluid from SSc patients. Based on this observation, studies described in Chapter 5 evaluate PDGF as well as imatinib (Gleevec), an inhibitor of the PDGF receptor, as modulators of the migration of fibroblasts growing on aligned collagen chips. This approach enables the evaluation of therapeutic agents in the migration assay utilizing SSc fibroblasts, and tests whether fibroblast migration assays could inform clinical practice concerning agents that may be beneficial in treating SSc.

Chapter 5 Assessing the Migration of Normal and Systemic Sclerosis Fibroblasts Using Aligned Collagen Matrices

5.1 Introduction

5.1.1 Spread of the pathologic process in systemic sclerosis

In severe dcSSc, the fibrotic skin lesions spread from the hands and the forearms, onto the upper arms and eventually involve the skin of the thorax and abdomen (Haustein, 2002, Varga and Trojanowska, 2008). These changes are the result of a pathologic process that begins in the perivascular dermis of peripheral tissues, and then spreads to become more generalised in the skin, and also to involve the internal organs (Varga and Trojanowska, 2008). With internal organ involvement, as seen in the lungs for example, fibrotic lesions initially develop as localised lesions in the lung subpleural basal areas, but then spread more extensively to involve most of the lung tissue, in the most severe cases (Minai et al., 1998, Haustein, 2002, Lam et al., 2011). Models of the disease pathogenesis in SSc should account for the spreading of the fibrotic process through the skin and affected internal organs. The spread of fibrotic lesions is an important component of SSc, and offers a potential target for therapies (Distler and Distler, 2008, Tyndall et al., 2009, Asano, 2010, Hunzelmann and Krieg, 2010, Leask, 2010, Leask, 2012, Bhattacharyya et al., 2012b).

5.1.2 Fibroblast migration in systemic sclerosis

The origin of the fibroblasts within fibrotic tissues is not well defined. It is generally believed that the matrix-producing cells in fibrotic lesions are derived from a number of sources, including resident fibroblasts, epithelial cells undergoing EMT, perivascular cells, endothelial cells undergoing EndMT, and blood derived monocytes (fibrocytes). The candidate offers the hypothesis that the recruitment of fibroblasts and fibroblast precursors into the fibrotic skin lesions is promoted by soluble chemotactic factors produced within the active skin lesions ((Trojanowska and Varga, 2007, Gilbane et al., 2013). The approach taken to test this hypothesis was to study the migratory and invasive activity underlying SSc and control fibroblasts, and to use this information to identify targets that might be employed to inhibit the spread of cells in the skin,

and/or prevent spreading of fibrosis to organ systems (Denton and Ong, 2013). In this Chapter, studies are undertaken to investigate SSc and control fibroblast migration, mechanisms suggested to contribute to SSc fibrotic lesion development and spread. As an initial step in research into this hypothesis, the candidate and colleagues found that PDGF, a known chemoattractant for fibroblasts, is present at an increased level in SSc blister fluid (see Chapter 4) (Gay et al., 1989, Trojanowska, 2008).

Fibroblast migration has an important role in other forms of human pathology such as other types of fibrosis and cancer invasion. For example, the invasion of fibroblasts into surrounding healthy tissues promotes the spread of fibrosis and facilitates the invasion of malignant epithelial cells (Tejada et al., 2006, Lederle et al., 2006, Pietras et al., 2008). Working with a biotechnology company, Fibralign Corporation, which developed a process to coat slides with aligned type I bovine collagen fibers, a method for the study of fibroblast migration was developed. This study employs sterile glass slides coated with collagen fibrils either present in a linear or in a woven, nonlinear pattern on the slide. These slides measure approximately 8 by 15 mm and 1 mm thick. These collagen-coated slides can now be purchased from Advanced Biomatrix in San Diego California, USA.

5.1.3 Evaluation of fibroblast migration on collagen in order to model invasiveness

Since invasion of disease fibroblasts into surrounding tissues may be an important factor leading to spread of the disease, *it is hypothesised that SSc fibroblasts may be intrinsically more invasive than controls, or that increased invasiveness is due to altered levels of candidate factors (eg PDGF) in the SSc lesions.* In order to test this hypothesis, a comparison is made between normal and SSc-derived fibroblast migration, with or without additional PDGF, or in the presence of potential inhibitors.

5.1.4 Overall aim of the chapter

To use novel patterned collagen tissue culture chips to study the migration of normal and SSc fibroblasts in order to determine if the disease cells are more mobile and invasive. Also, to use these assays to test factors for their ability to stimulate or inhibit SSc fibroblasts migration.

In order to address these aims fibroblasts will be cultured from healthy skin and SSc lesions, as well as from control and SSc lung biopsy material, and plated onto patterned collagen-coated slides in order to examine basal and growth factor stimulated migration, and to assess agents that might prevent fibroblast migration.

5.2 Results

5.2.1 Preliminary migration experiments using HFF cells cultured on aligned and woven Fibralign collagen chips

For preliminary experiments, human foreskin derived fibroblasts (HFF cells) obtained commercially were studied. On aligned collagen substrates HFF appeared to align and migrate in the presence of 10% FCS but not in media with 0.2% FCS. No alignment or migration was observed with the woven substrates even with 10% FCS in the media (Figure 5.1).

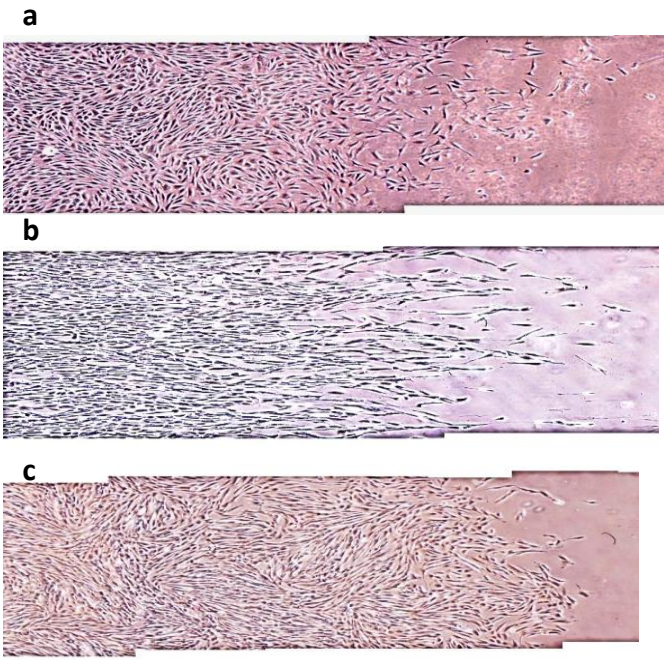


Figure 5.1 HFF cells align and migrate on the aligned collagen chips dependent on the presence of serum

In preliminary experiments commercially obtained HFF cells were cultured on aligned and woven collagen substrates in low serum (0.2% FCS) or high serum (10% FCS) conditions. No alignment or migration was seen with aligned substrate in the presence of low serum (a). However when FCS 10% was added to the media cells were seen to align and migrate (64 hour time point shown) (b). Further experiments were performed on woven collagen and no alignment or migration was seen on these substrates even when 10% FCS was added (c).

5.2.2 Lung fibroblasts align and migrate on aligned collagen dependent on serum and PDGF

Further experiments were then performed to compare the migration of primary fibroblasts derived from tissue biopsies of SSc patients and healthy controls on aligned or woven pattern collagen substrates. The aligned collagen employed is more highly ordered than dermis-like collagen. The collagen fibrils are visibly directional, in a helix-like, wavy manner. The woven collagen substrate is decidedly non-directional with a random appearance. Using these collagen substrates, dermal fibroblasts remained in a non-migrating cellular focus on the woven pattern and did not migrate in response to either serum or the known migration enhancer PDGF-BB. In contrast, lung fibroblasts elongated and orientated in parallel to the aligned pattern collagen matrices, and migrated dependent on the presence of serum or PDGF-BB. SSc lung but not dermal fibroblasts were also found in preliminary experiments to migrate on aligned collagen but could not be stimulated to migrate on woven collagen. Further experiments were performed with SSc and healthy control lung fibroblast lines to compare the extent of migration, in order to investigate whether SSc cells are intrinsically more invasive. However, as for normal cells migration was consistently achieved by culturing fibroblasts on aligned collagen fibers and was dependent on the presence of FCS 10%. The area of migration by SSc cells at 24 hours was similar to that seen with control cells (Figure 5.2).

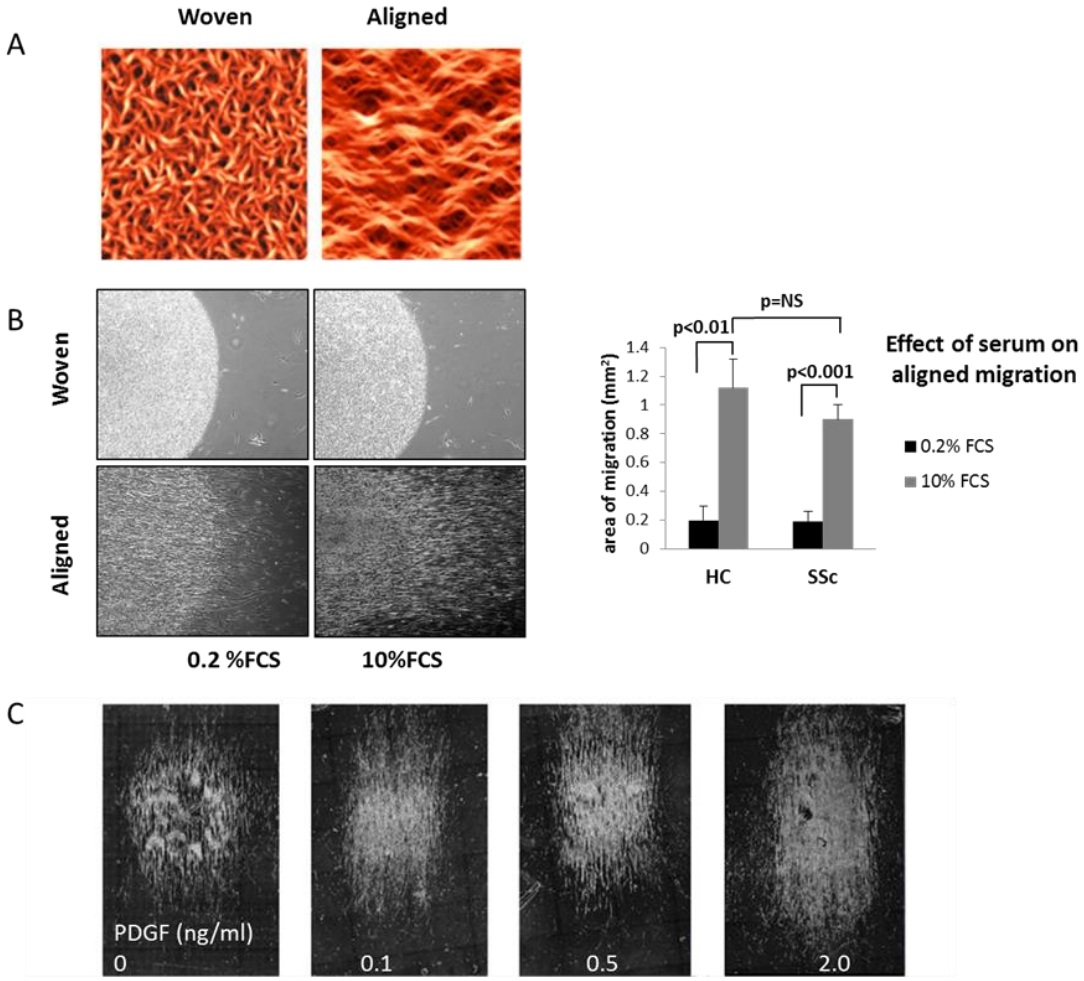


Figure 5.2 Migration of lung fibroblasts cultured on aligned collagen: effect of the additon of serum or PDGF to the media Experiments were performed with normal lung fibroblasts cultured on woven and aligned chips (A) in DMEM with 0.2% or 10% FCS. No migration was seen with cells cultured on woven collagen even with the addition of 10% FCS, but cells were seen to align and migrate on aligned collagen, dependent on the addition of 10% FCS (24 hour time-point shown, X 4 Axioscope). The area of migration was similar for SSc and control lung fibroblasts (B). Normal lung fibroblasts were maintained on aligned collagen with or withut the additon of PDGF-BB in 0.2% FCS media. PDGF-BB 2 ng/ml enhanced migration as shown at the 48 hour time point (X2.5 Axioscope) (C).

5.2.3 Altered orientation of systemic sclerosis lung fibroblasts when cultured on aligned collagen

The orientation of the fibroblasts when migrating on aligned collagen-coated slides was also investigated. Control and SSc lung fibroblast lines (control n=6 replicates, SSc n=11 replicates) were applied as cellular foci to aligned collagen and migration followed over 24 hours. Cell alignment software

(Orientation J) was used to study orientation of the SSc and control cells. Whereas healthy control fibroblasts aligned clustered at the 180° meridian parallel to the aligned fibres, some of the SSc cell lines (5 out of 11 studied) failed to align with the collagen, although overall the area of migration was similar to controls (Figure 5.3).

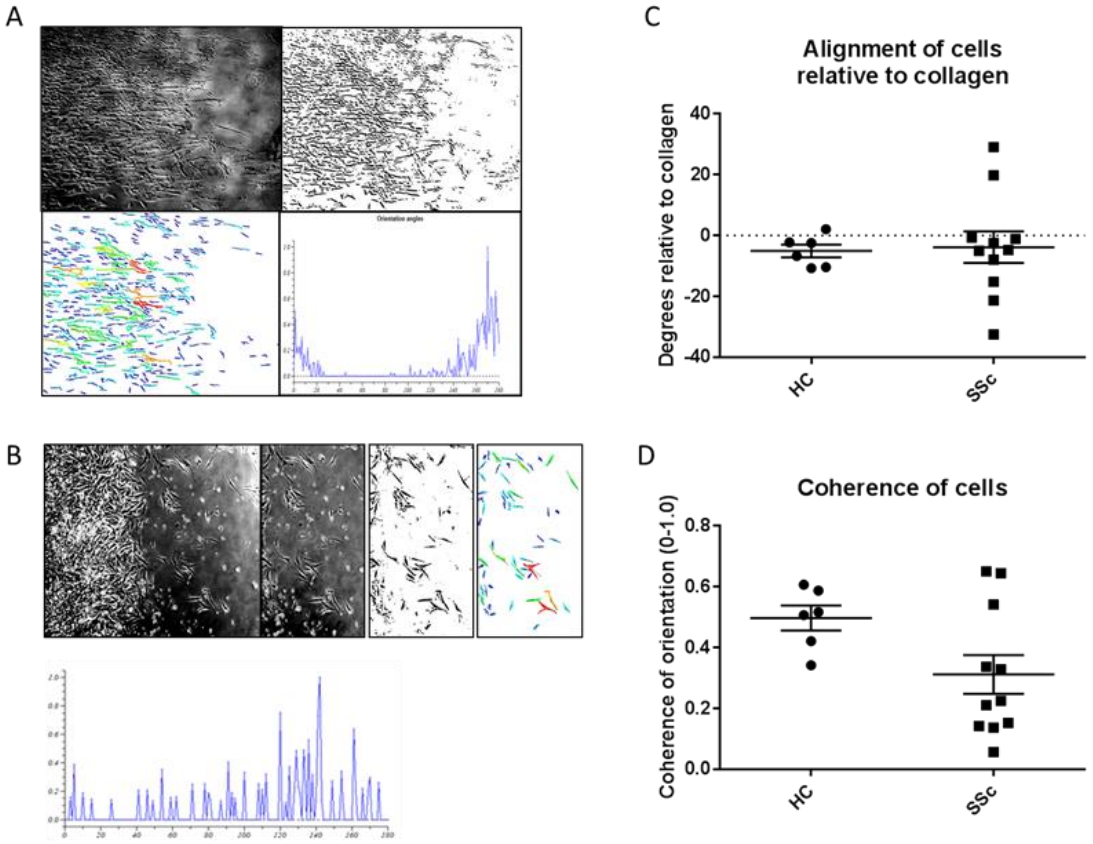


Figure 5.3 Control and systemic sclerosis lung fibroblast alignment analysis Control (A) and SSc (B) lung fibroblast lines were cultured as foci on aligned collagen tissue culture slides and allowed to migrate in DMEM with 10% FCS. Representative analysis steps are shown in sequence from left to right–original image, binary image derived from the selection of cells to analyse, and cluster analysis of the image to arrive at the profile of orientation angles, clustering around the 180 degree median is shown in the graphs. Control cells consistently aligned at 180° on linear collagen culture slides, whereas SSc lines diverged from the collagen alignment by up to 40° (C), and in general there was a lack of coherence of orientation in some but not all of the SSc cell lines (D).

5.2.4 Effect of PDGF antagonists on normal fibroblast migration

The tyrosine kinase inhibitor imatinib (Gleevec) is used in the treatment of a number of cancers, most notably Philadelphia chromosome-positive (Ph^+) chronic myelogenous leukemia (CML). The molecular target of this compound is c-Abl, a tyrosine kinase, as well as the PDGF receptor tyrosine kinase. Imatinib has been in clinical trials in SSc patients, with early trials showing encouraging, if somewhat equivocal results. Pivotal trials turned out to be negative (Prey et al., 2012). However, the activity of this tyrosine kinase inhibitor may be informative as to fibroblast migration and signalling. Potential blocking of migration on the aligned mattrices was assessed using imatinib as an inhibitor of the PDGF receptor tyrosine kinase, and using FCS as a source of PDGF (known concentration in FCS 50 ng/ml). The doses of imatinib tested were 0.0, 0.025, 0.25 and 2.5 µg/mL and the fibroblasts were cultured on the collagen slides for 24 hrs. Whereas the control cells on the unaligned collagen coated slide showed no directional migration, cells on the aligned collagen coated slides displayed directed migration as before. In the portion of the study involving imatinib, the low dose slide looked similar to the no imatinib control, perhaps with slightly less migration toward the border of the slide. At the intermediate concentration (0.25 µg/mL), there was a clear reduction in cell migration and a seeming loss of directionality of the spread cells. At the 2.5 µg/mL level, there was a clear reduction in cell migration and a decrease in the oriented migration of the fibroblasts on the slide (Figure 5.4). Since imatinib inhibits the PDGF receptor, it is likely that it is blocking the ability of the PDGF present in the serum, to stimulate migration.

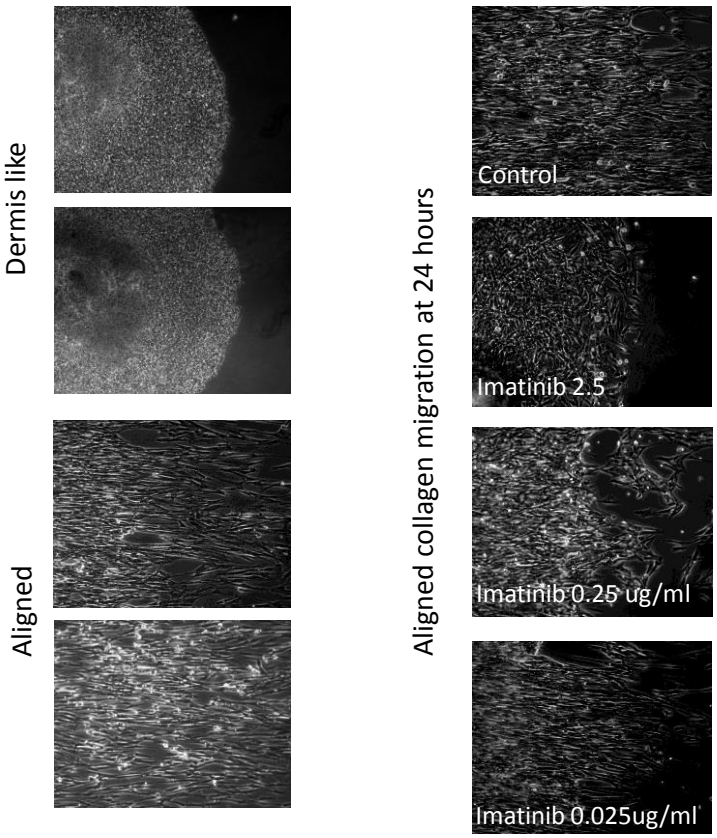


Figure 5.4 Imatinib dose response for inhibition of migration on aligned collagen chips at 24 hours Normal control lung fibroblasts were incubated on unaligned and aligned collagen tissue culture slides and cultured for 24hrs in DMEM with 10%FCS with and without the addition of imatinib 0.025-2.5µg/ml. As expected migration failed to occur on the woven dermis like substrates, but progressed on the aligned collagen. Imatinib at 2.5 fully inhibited, and 0.25 µg/ml partially inhibited the FCS dependent migration, whereas 0.025µg/ml had no effect.

5.2.5 Effect of heparin on fibroblast migration

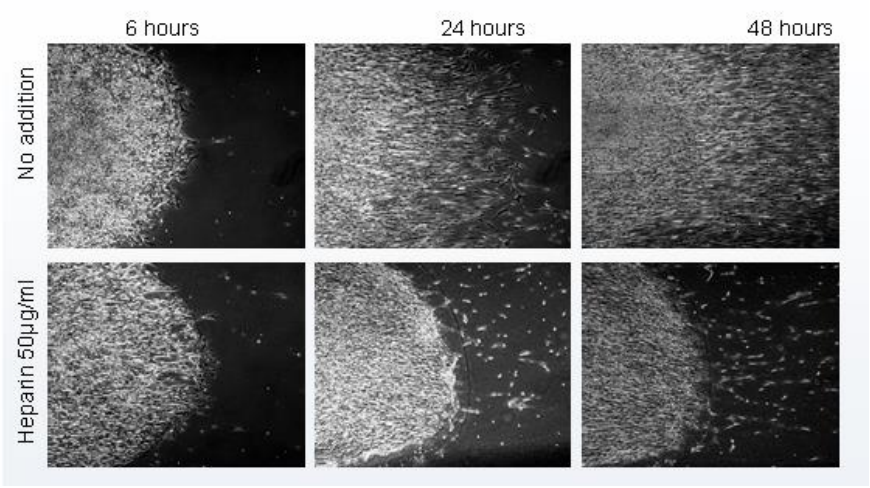
Heparin, a sulfated glycosaminoglycan obtained from various animal organs, has been used clinically as an anticoagulant since the 1940s (Bjork and Lindahl, 1982). For its antithrombotic activity, heparin binds to plasma anti-thrombin with very high affinity, inhibiting a series of coagulation proteins such as thrombin and factor Xa (Church et al., 1989). It also binds to numerous other proteins in a manner directly related to the biofunctionality of its structurally similar relative, heparan sulfate (Capila and Linhardt, 2002). Heparin has the highest negative charge density of any known biological molecule.

Heparin is known to bind to certain growth factors including PDGF and inhibit the activation of the PDGF receptor which utilize heparan sulfate as a co-

receptor to stabilize growth factor-receptor interactions (Kuo et al., 2010). Because of these effects, and because it is a relatively safe licensed drug which could be used in SSc patients, it was investigated here as a possible inhibitor of fibroblast migration.

The effect of heparin on the directed migration of SSc fibroblasts was tested, again using 10% FCS as the stimulus. SSc fibroblasts were incubated on aligned collagen-coated slides in the presence of 50μg/mL heparin and then imaged at 6, 24, and 48 hrs. To quantitate migration, ImageJ software was used to measure the area of migrating cells at the various timepoints. Heparin at 50μg/mL was found to largely abolish fibroblast migration on the collagen chips when evaluated at 24 and 48 hours which reached statistical significance (Figure 5.5). At 6 hrs, there was a slight difference in the two groups with the heparin slide appearing very similar to the control, but showing a slightly larger advancing edge of migration to the right. After 24 hours, there are considerably more cells that have migrated toward the right side of the control slide and have done so in a directional manner. In contrast, the cells on the heparin slide showed little migration. There are a few fibroblasts that have migrated to the right beyond the cell mass, but this number is minimal to the extent that single cells are easily observed. By 48 hours, the edge of the migrating cell mass has reached the right extent of the control slide, the directionality of the migration was maintained, and the slide was filled with migrating fibroblasts. With heparin at 48 hrs, there was little difference from the 12 and 24 hr. time points, although again individual fibroblasts are conspicuous on the right side.

These results show clearly that heparin inhibits the directional migration of SSc fibroblasts on aligned collagen when stimulated using 10% FCS.



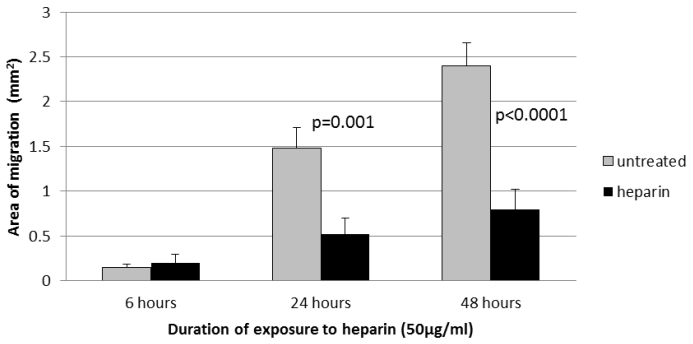
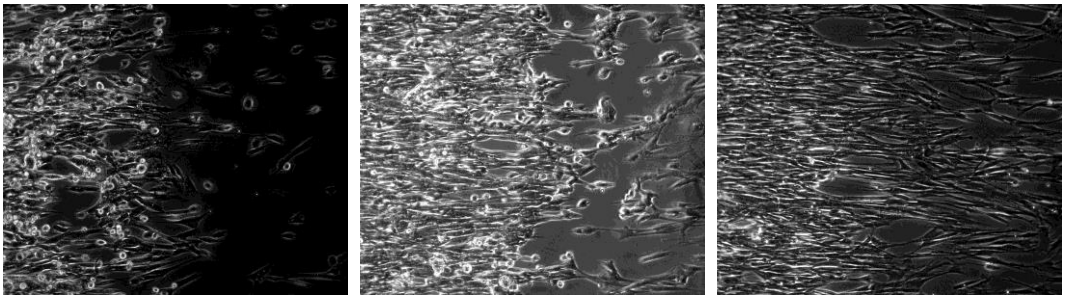


Figure 5.5 Heparin inhibits systemic sclerosis fibroblast migration on aligned collagen SSc lung fibroblasts were cultured on aligned collagen in DMEM with 10% FCS in order to stimulate migration, with or without the addition of heparin at 50 µg/mL. The migrating field of cells was quantified by ImageJ software at 6, 24 and 48 hours, by outlining the migrating field of cells using the freedrawing tool, and then measuring the area callibrated to the known dimensions of the 4x axioscope field. The addition of heparin inhibited cellular migration at 24hours ($p=0.001$) and 48 hours ($p<0.0001$).

5.2.6 Effect of the endosome inhibitor chloroquine on aligned migration of systemic sclerosis and control lung fibroblasts

During these studies with fibroblast migration the candidate proposed that the attachment and alignment of the lung fibroblasts was likely to involve integrin receptors on the cell surface, with continual internalisation and recycling of cell surface integrins to the leading edge of the migrating cells. Chloroquine accumulates in lysosomes and is a known inhibitor of integrin recycling(Cho, 2008). Chloroquine at 100µM fully inhibited and, at 10 µM, partially inhibited fibroblast migration, suggesting that the endosomal

trafficking of integrins may be involved in the migration by fibroblasts (Figure 5.6).



Chloroquine 100μM **10μM** **control**

Figure 5.6 Effect of chloroquine on normal lung fibroblast migration at 24 hours Normal lung fibroblasts were cultured as cellular foci on aligned collagen matrices and migration induced by culture with DMEM supplemented with 10% FCS for 24 hours (n=3 control fibroblast lines). Controls with no chloroquine migrated as shown in the right hand Figure, whereas cells treated with 100μM chloroquine failed to migrate, and cells treated with 10μM showed some inhibition.

5.2.7 Investigating possible synergy between imatinib and heparin as inhibitors of migration

The candidate tested whether combining heparin, which binds to and inhibits PDGF activity and imatinib, which blocks PDGF receptor signal transduction, would together lead to enhanced inhibition of migration. The impact of combination treatment using heparin plus imatinib on the directed migration of healthy and SSc lung fibroblasts was tested using 10% FCS as the stimulus. Control and SSc lung fibroblasts (both n=4 cell lines) were incubated on aligned collagen-coated slides imaged at 12, 24, and 36 hrs. Heparin (50μg/mL) or imatinib (2.5μg/mL), or both factors were added to the cultures. The area of migration for the healthy control and SSc lung fibroblasts was measured using ImageJ, and the results are shown in Figures 5.7a, b &c. Representative photomicrographs are shown in Figure 5.7d. The overall rate of migration was similar between SSc and control lung fibroblasts stimulated by DMEM with 10% FCS (controls, Figures 5.7a, b, c). However, there was a clear inhibitory effect of heparin on migration which reached statistical significance at the 24 and 36 hour time points in control lung fibroblasts, and at 24 hours in SSc lung fibroblasts. Imatinib, as expected based on the experiments described

above, suppressed migration reaching statistical significance at 24 hours in both control and scleroderma fibroblasts. The heparin/ imatinib combination showed no synergy in the control cultures, but there appeared to be a reduction of SSc fibroblast migration with the combination which was greater than observed with either treatment alone and was statistically significant at 24 hours (Figure 5.7c). Thus, the combination of 50 μg/mL of heparin and 2.5 μg/mL of Imatinib shows possible synergy leading to greater inhibition of SSc lung fibroblast migration on aligned collagen chips.

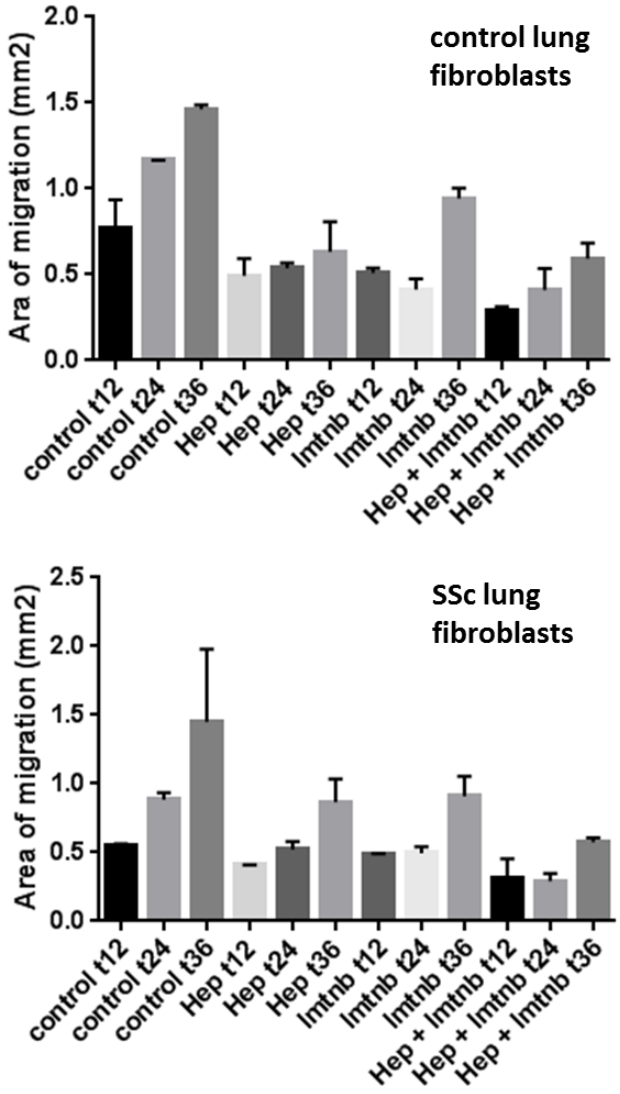
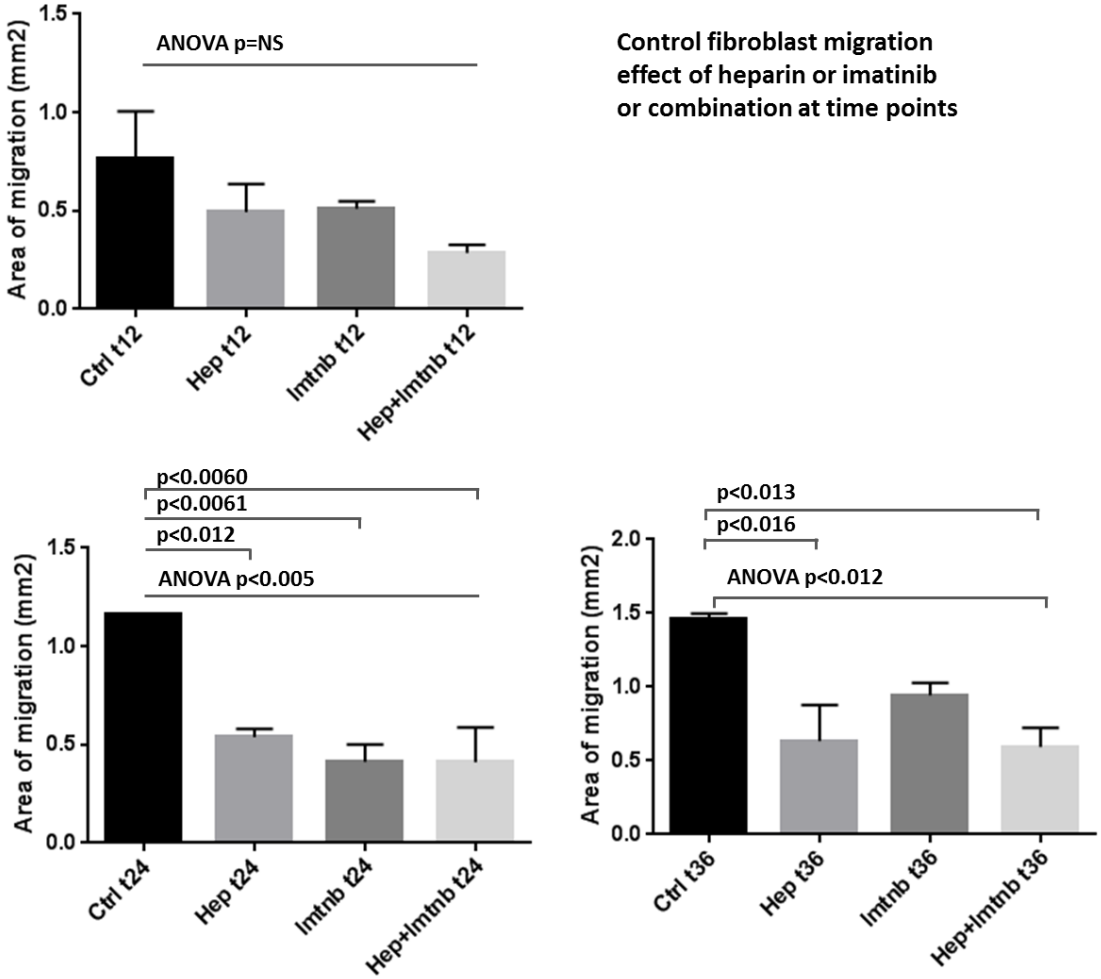
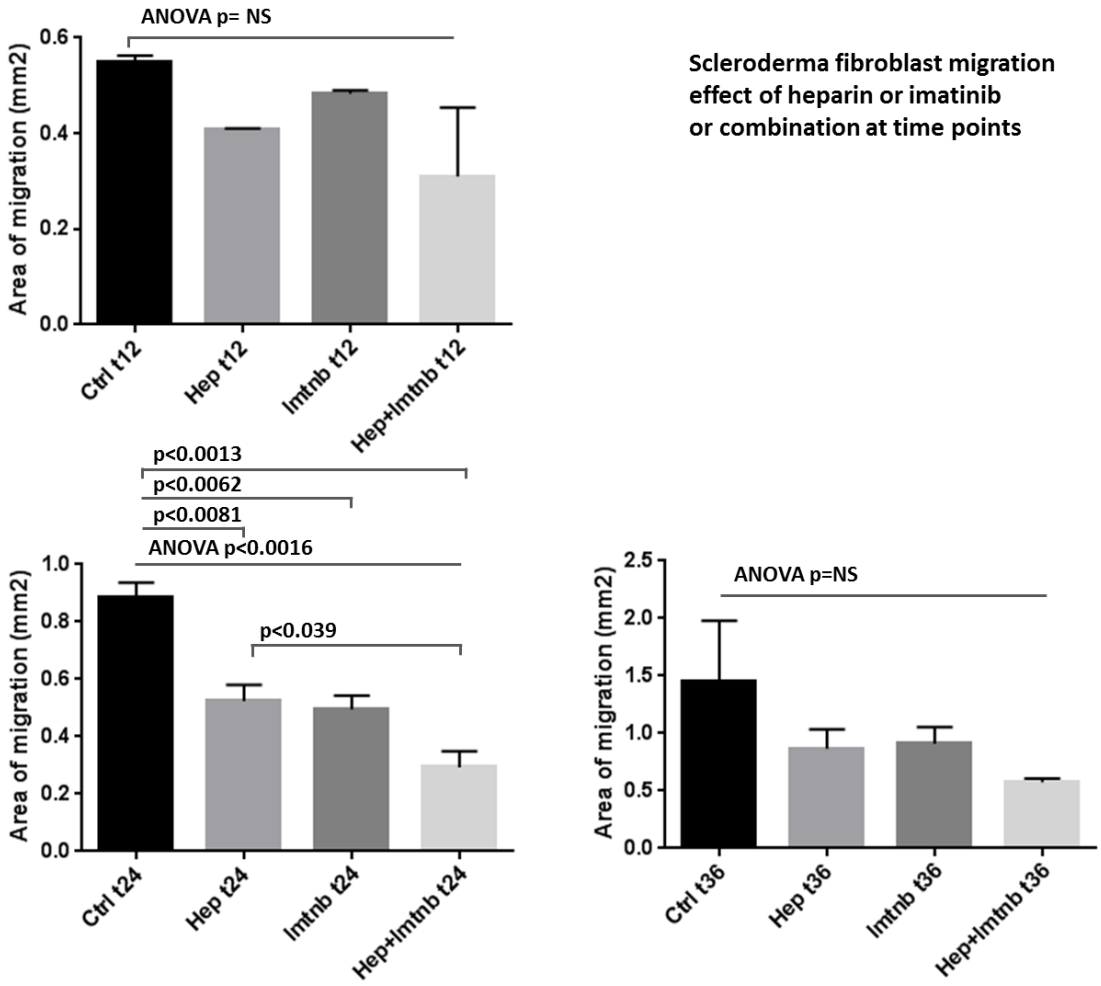


Figure 5.7a Investigating possible synergy between heparin and imatinib as inhibitors of control and systemic sclerosis lung fibroblast migration Healthy control and SSc lung fibroblasts were stimulated to migrate on aligned matrices with DMEM plus 10% FCS for 12, 24 or 36 hrs. and the area of migration was determined. Heparin (50μg/mL), Imatinib (2.5μg/ml) or both together (H plus I) was added at baseline. Summary data shown the mean area of migration trended towards being lower for combination therapy at each time point.



Figures 5.7b. Investigating possible synergy between heparin and imatinib as inhibitors of control and systemic sclerosis lung fibroblast migration: statistical analysis for control cells Mean area of migration at 12, 24, and 36 hour (t12, t24, t36) time point for each of the 4 treatment groups were compared by ANOVA. Where ANOVA was significant, Tukey's test was used for comparison between groups. ANOVA reached significance at 24 and 36 hour time points in control fibroblast migration indicating some difference between the means overall. At 24 hours all treatments reduced migration when compared to controls, and at 36 hours heparin and combination treatment further suppressed migration (*=p<0.05).



Figures 5.7c. Investigating possible synergy between heparin and imatinib as inhibitors of control and systemic sclerosis lung fibroblast migration: statistical analysis for SSc cells Mean values for area of migration at each time point for each of the 4 treatment groups were compared by ANOVA. Where ANOVA was significant, Tukeys test was used for multiple comparisons between groups. ANOVA reached significance at 24 hour time point only. At this timepoint all treatments reduced migration when compare to controls. The combination of heparin and imatinib reduced migration more than heparin treatment alone.

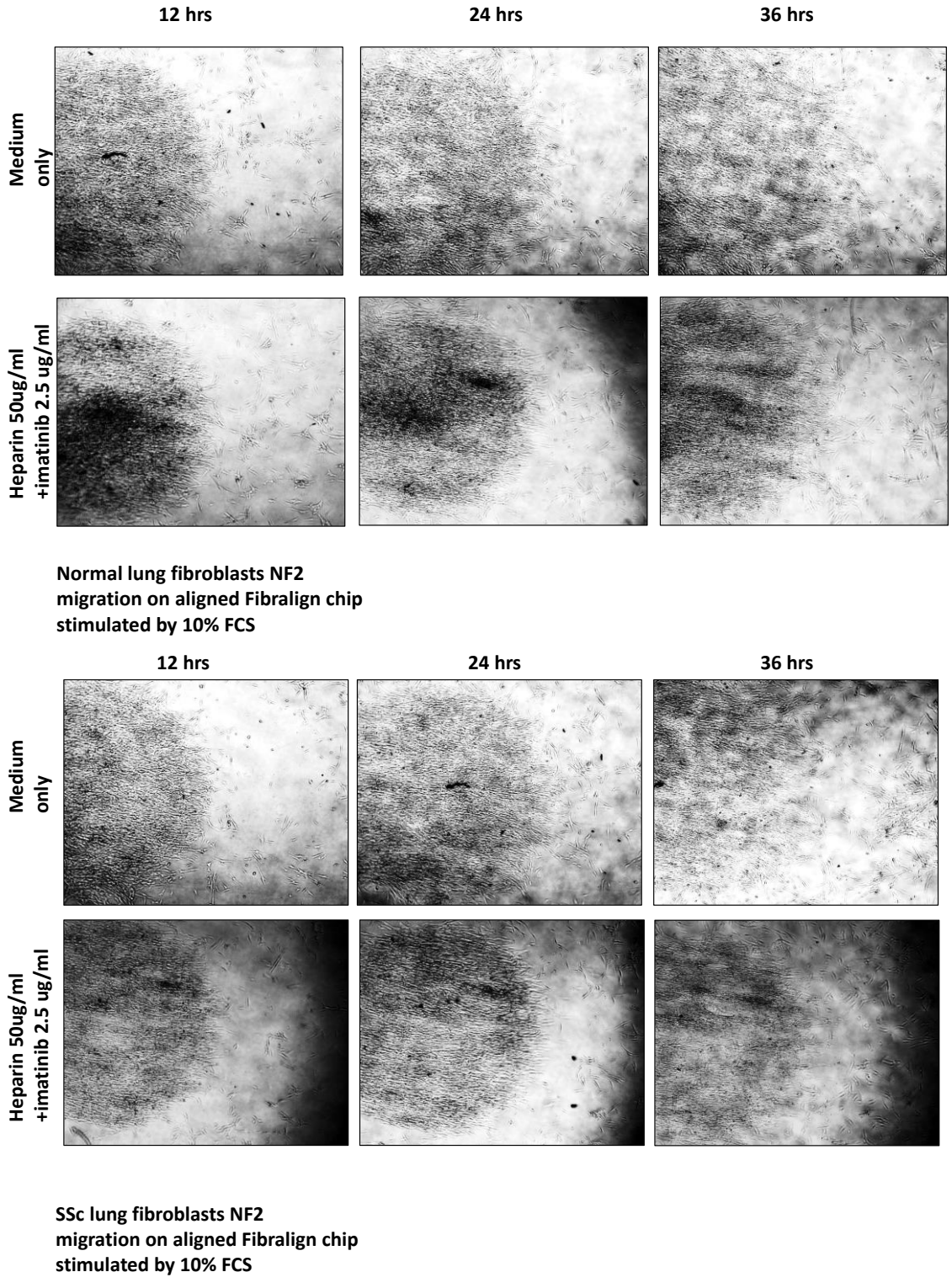


Figure 5.7d Investigating possible synergy between heparin and imatinib as inhibitors of control and systemic sclerosis lung fibroblast migration: representative images SSc and control lung fibroblasts were stimulated to migrate on aligned matrices by the addition of DMEM with 10% FCS. The inhibitory effect of additional heparin 50µg/ml plus imatinib 2.5 µg/ml is shown.

An additional experiment was performed using an alternative and commonly used migration model; the scratch wound migration model ((Donovan et al., 2013). In this model workers at the Royal Free had found that 2% FCS optimally stimulates active invasion of the scratch wound which can be blocked with antagonists (thesis of Adrian Gilbane). The effects of imatinib with or without heparin were tested in SSc and control lung fibroblasts in a scratch wound assay performed on 12 well plates. In some but not all experiments inhibition of migration was seen with imatinib 2.5 µg/ml, but there was no additional or synergistic effect with the addition of heparin 0.5-50µg/ml (Figure 5.8).

It is accepted by the author that there are important differences between this assay and the aligned collagen migration assay, including the dose of FCS used (2% versus 10%), and the underlying substrate, which is the glass with secreted endogenous collagen in the case of the scratch migration, versus patterned bovine collagen in the case of the aligned collagen chips. However, this study illustrates the point that the aligned collagen assay may detect inhibitory effects not seen with the more conventional scratch assay.

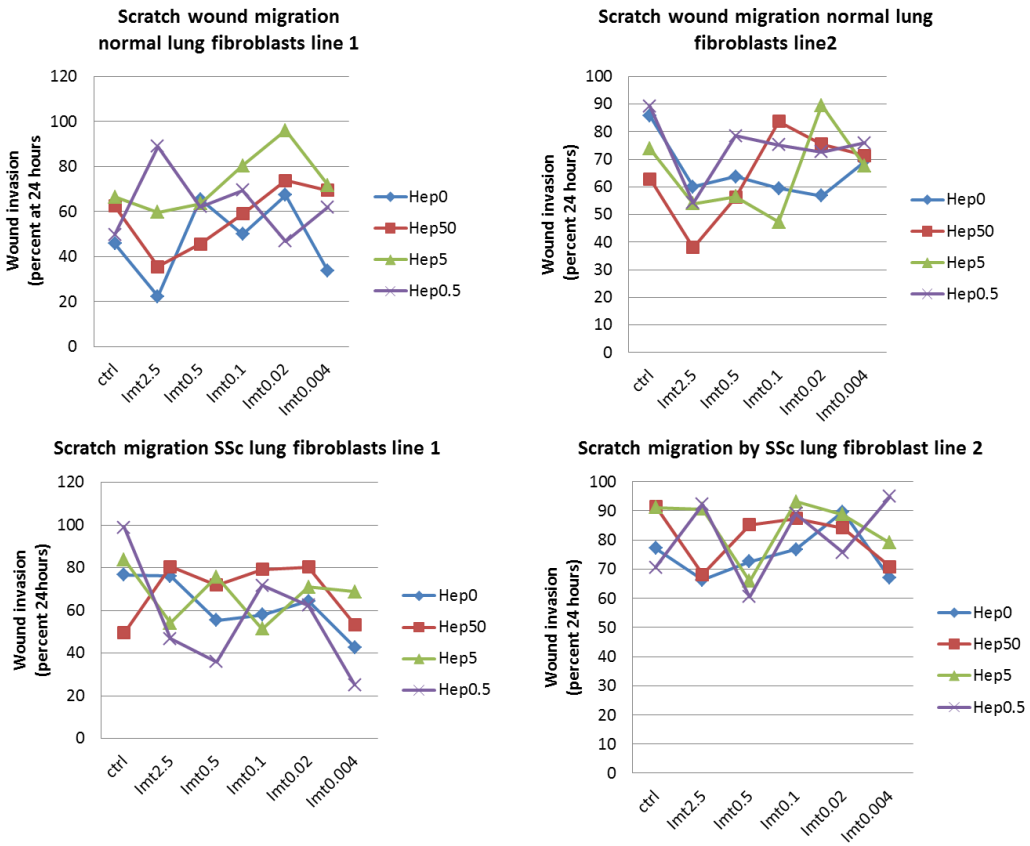


Figure 5.8 Effect of Imatinib and heparin on SSc in a scratch wound migration assay SSc and control lung fibroblasts were cultured on 12 well plates in DMEM with 10%FCS and then switched to 2% FCS at the start of the experiment. A scratch wound was induced using a 200µl pipette tip at t=0 and then the area of wound invasion was quantified by axioscope imaging of the wound at 24 hours, and the area of invasion, expressed as percent, measured using ImageJ image analysis. Whilst imatinib at 2.5µg/ml appeared to exert an inhibitory effect on migration, the addition of heparin up to 50µg/ml had no effect on migration and failed to synergise with the imatinib.

5.3 Discussion

One of the most alarming aspects of SSc is the way it spreads with time leaving dense stiff skin. In the more severe forms of SSc, lesions are also seen to spread to internal organs, in particular the intestine, lungs, heart and kidneys. This phenotypic migration of fibroblasts is not unlike the behavior of cancer cells.

To study and measure the migration of SSc and healthy human fibroblasts, tissue culture slides coated with bovine type I collagen fibers either

linearly arrayed or in a woven pattern were utilized (Advanced Biomatrix, San Diego California, USA). These collagen matrix-coated slides allow quantitative measurement of fibroblast migration, and can be used to test potential therapies that could block fibroblast migration.

The migratory behavior of fibroblasts derived from both SSc patients and from controls was studied on these specially prepared aligned collagen surfaces. When applied to these coated surfaces, cells align and migrate down but not across the collagen fibers or on the woven collagen substrate, considerably facilitating quantitative assessment of cellular migration. Lung fibroblasts from both SSc and control sources migrate in the presence of FCS and PDGF. Preliminary studies suggest that individual agents as well as combinations of potential therapeutics can be assessed in this system, based on the hypothesis that promising therapeutics can be detected by their activity as inhibitors of fibroblasts migration.

It was previously shown that PDGF enhances fibroblast migration, as well as attracting fibroblasts to sites of injury (Seppa et al., 1982, Pierce et al., 1991), and in Chapter 4, elevated levels of the fibroblast chemoattractant PDGF were described in the blister fluid from SSc patients. PDGF-BB enhanced fibroblast motility on aligned collagen fibers (Li et al., 2004). In this current study of the mechanisms underlying SSc and control fibroblast migration, no fibroblast migration occurred at low serum concentrations. However, linear migration took place with lung fibroblasts cultured on aligned collagen fibers following treatment with 10% serum or PDGF-BB in low serum. Migration was similar in magnitude for SSc and control fibroblasts, although orientation was altered in the SSc cells, and neither cell type migrated on the woven collagen substrates. PDGF-BB enhanced migration. An explanation for enhanced migration of SSc fibroblasts in fibrotic tissue would be a higher concentration of the inducing factor, PDGF in the lesions.

The altered alignment of SSc fibroblast with greater spreading on the aligned collagen fibers found in these studies raises interesting questions. SSc fibroblasts are known to have altered cell surface markers compared to controls including integrins which are important for the cell-ECM interactions. For example, previous studies have demonstrated that SSc dermal fibroblasts have reduced levels of *α1β1* and *α2β1* integrins and increased levels of *αvβ5*.

Although those studies focused mainly on synthetic function of fibroblasts, it may be that altered interaction results in poor alignment (Ivarsson et al., 1993, Kozlowska et al., 1996, Asano et al., 2004). Integrin-ECM interaction is crucial for the development of polarity in fibroblasts as well as inducing cystoskeletal changes. Alteration in these cell surface markers in disease fibroblasts may impair cell-ECM interaction and result in inability to align properly. Moreover, it was shown in this Chapter that the addition of chloroquine, an inhibitor of integrin recycling, prevented fibroblast migration.

In this effort to test the candidate's hypothesis that the recruitment of fibroblasts and fibroblast precursors into the fibrotic lesions is promoted by soluble chemotactic factors produced within the active lesions, an attempt was made to identify targets that could be inhibited to block recruitment of cells into the fibrotic lesions, and/or prevent the spreading of fibrosis to internal organs. The PDGF receptor tyrosine kinase inhibitor, imatinib, is already considered a potential therapeutic in fibrosis. Accordingly, an experiment was designed to determine if imatinib could inhibit the migration of lung fibroblasts on aligned collagen. Similarly another experiment was conducted using serum induced migration on aligned collagen slides plus or minus heparin. Heparin, which binds PDGF as well as other fibroblast-related growth factors in the extracellular environment, strongly inhibited fibroblast migration as did imatinib. The combination of these two agents demonstrated synergy, with an increased inhibition compared to the use of single agents in SSc fibroblasts. These results suggest that the PDGF receptor tyrosine kinase (a target of imatinib) can be viewed as a candidate target to block migration of cells in SSc lesions.

At the time that these studies were underway, the Royal Free Hospital was taking part in a multicenter clinical trial evaluating the therapeutic effects of imatinib on SSc patients (Spiera et al., 2011). At the conclusion of these human studies, it was determined that there was a small sub-population that benefited from the administration of imatinib, but not enough to sanction it as a viable therapeutic agent to treat SSc patients. Further analysis is underway to determine if there are shared characteristics in the responders or in the non-responders to imatinib. In addition, a recent study has been published using low dose imatinib as a treatment for SSc related lung fibrosis. Some 26

patients whose lung disease was non-responsive to cyclophosphamide were recruited in an open label study, leading to stability or improvement of lung function in 12 patients (Fraticelli et al., 2014).

5.4 Summary

In this chapter, the candidate has proposed the hypothesis that the recruitment of fibroblasts and fibroblast precursors into the fibrotic skin lesions is being promoted by soluble chemotactic factors produced within the active skin lesions. The approach taken to test this hypothesis was to study the mechanisms underlying SSc and control fibroblast migration using a novel *in vitro* methodology and to investigate targets that could be inhibited to block recruitment of cells into the fibrotic lesions and/or prevent spreading of fibrosis through organ systems. This *in vitro* investigation of SSc and control fibroblast migration mechanisms suggested an approach to slow down the spread of the SSc fibrotic lesion. A new transition is now made to the use of animal models of fibrosis to be described in Chapter 6, for the assessment of a novel therapeutic peptide agent, which targets the fibrotic process directly. Analysis of possible serum factors that may be involved in mediation of fibrosis will be made in these *in vivo* models, also linking it to the work presented in Chapter 4. Similarities in cytokine elevation between SSc and the fibrosis animal models will be used to help validate the latter for testing potential anti-fibrotic agents and provide a way to predict SSc patient responses to new therapies.

Chapter 6. Modelling Fibrosis *in vivo* for Testing Therapeutic Approaches

6.1 Introduction

6.1.1 Modelling inflammation-dependent fibrosis

Inflammation is normally highly regulated and does not persist as, for example, in normal wound healing. However, depending on the nature of the initiating process and the genetic makeup of the individual, the process may be prolonged and excessive (Diegelmann and Evans, 2004). This leads to the destruction of normal tissue architecture, which is replaced by scar tissue, defined as excessive connective tissue containing collagen and other extracellular molecules. Fibrosis is a common sequel of inflammation affecting the lungs, skin, heart, bone marrow, and other tissues. While many drugs and other substances have anti-inflammatory activities when tested in tissue culture systems *in vitro*, these compounds are not very effective in treating fibrosis *in vivo* either generally or as seen in SSc patients. Thus, there is a need for therapeutics able to regulate and reduce both acute and chronic inflammation to limit the fibrosis associated with SSc and other fibrotic disorders.

There are a large number of publications describing a range of *in vivo* models of SSc and other common forms of fibrosis (e.g., lung, renal, liver, skin fibrosis) each with their own characteristic weaknesses and strengths (reviewed in Chapter 1) (Derrett-Smith et al., 2009). We don't know which models most closely reflect the underlying causes of SSc.

The Tight-Skin Mouse-1 (Tsk1) is a rodent model of fibrosis attributable to a single-gene defect in *fibrillin 1* (Pablos et al., 2004). As the Tight-Skin Mouse name suggests, this animal exhibits extensive depositions of collagen in the skin and visceral organs. The link between the excess collagen appears to be due to a mutated *fibrillin* gene, which produces a matrix associated protein with duplicated TGFβ binding sites that increases the bioavailability of TGFβ in the deep fascia areas, where LAP-associated TGFβ is normally sequestered in healthy uninjured tissues. This model has its limitations; since histological assessment of the skin of the Tsk1 mice shows no difference in dermal thickness when compared to the WT mouse and the excessive collagen is located in subdermal fascia. In addition, this is a non-inflammatory model, and

is therefore thought to best model TGFβ-induced fibrosis. It could be used to test therapies aimed specifically at fibrosis downstream of TGFβ, for example, therapeutics targeting collagen gene induction or collagen crosslinking since both are enhanced by TGFβ.

Bleomycin treatment is also used to induce fibrosis and has been widely studied (Batteux et al., 2011). Bleomycin, a bacteria-derived DNA clastogenic agent, produces cellular injury, activates innate and adaptive immunity, and induces fibrosis with SSc-like pathology. Various authors have evaluated multiple mouse models of cutaneous and pulmonary bleomycin-induced fibrosis (Diegelmann and Evans, 2004). The strength of this model is that bleomycin initiates both inflammation and downstream pro-fibrotic responses as seen in SSc. One possible weakness is the absence of specific vascular injury in this model, which has led some investigators studying vascular injury in SSc to use the University of California at Davis chicken model, in which spontaneous endothelial cell apoptosis occurs along with fibrosis (Sgonc et al., 1996).

However, in the studies presented in this Chapter, there is a focus on understanding the inflammatory cascade that occurs in the bleomycin model, and testing of synthetic peptides designed to inhibit inflammation-induced fibrosis in order to regulate the responses of injured tissues.

6.1.2 Treatment with RP107, a potential therapeutic peptide

Recently, in collaboration with Dr. Jesse Jaynes, from Tuskegee University, a peptide was obtained, RP107, that exhibits potent anti-inflammatory properties. This and other peptides are being characterized for their immune-modulating activities. These molecules are rationally designed synthetic peptides that have structural and chemical homology with domains from natural immunomodulatory proteins (Haney and Hancock, 2013). These are basic peptides with pairs of alternating hydrophobic and hydrophilic amino acids. They resemble a sequence in the antibacterial peptide mellitin consisting of a sequence of 12 amino acids composed of alternating pairs of hydrophilic amino acids (basic) and hydrophobic amino acids (alanine/ phenylalanine). The peptides have been shown in reporter cell assays to prevent nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) from translocating to

the nucleus and binding the NF-κB DNA binding sequence elements within appropriate target gene promoters, thus inhibiting the expression of key pro-inflammatory cytokines. It is believed that these properties account for the biological effects that have been observed in various models.

Based on the above background information it was hypothesized that inflammation and fibrosis are linked in SSc pathologenesis and that treatments effectively suppressing the inflammation will reduce the tissue remodeling and fibrosis seen. In order to test this hypothesis the peptide will be studied in mouse models of SSc-pattern fibrosis, and serum cytokine and MMP measurements as well as histologic analysis of tissue remodeling used as readouts of efficacy.

6.1.3 Overall aim of the chapter

To investigate the effect of a therapeutic anti-inflammatory peptide in models of SSc-like skin and lung fibrosis. In order to achieve this aim the peptide will be administered *in vivo* using both the bleomycin skin model (a model of SSc-like dermal fibrosis) and the bleomycin pulmonary model (a model of SSc lung fibrosis and idiopathic pulmonary fibrosis). In both models, as shown below, histology plus serum levels of inflammatory cytokines TNFα, IL-6, IL-12p70 as well as MMPs 1 and 9, will be used as biomarkers of the inflammatory and tissue remodeling processes induced by bleomycin, in order to assay the biological efficacy of the therapeutic peptide.

6.2 Materials and methods specific to the therapeutic inhibitor studies

6.2.1 The bleomycin skin fibrosis model

In the experiments presented in this Chapter, C57BL/6 female mice were obtained from the Charles River Laboratories, Hollister, California, USA, and used at age 6-7 weeks. A very simple experimental protocol was chosen in order to minimize the numbers of mice needed and to increase the probability of detecting an effect of the therapeutic peptide. Two experimental groups were studied, Group 1 was treated with daily bleomycin for 21 days (n=10), and in Group 2 the bleomycin injections were followed by daily injections of the

therapeutic peptide RP-107 (n=6). Animals were acclimated for 3 days prior to the start of the study, and were housed in microisolators in a 12/12 light/dark cycle. A standard maintenance rodent chow diet, Teklad 2018, was fed to the mice and tap water and food were given *ad libitum.*

This was intended as a proof of concept study and hence designed as a prophylactic study, with the intention of running a full rescue study at a future date.

Starting on Day -2 (i.e., two days before the start of model induction), animals were administered twice daily 0.25 ml. subcutaneous injections of saline between the shoulder blades in Group 1. or with RP107 at 1 mg/kg in Group 2, such that they were pretreated for 48 hours with the potential therapeutic peptide. Then beginning on Day 1, dermal fibrosis was initiated by once daily subcutaneous (SC) injection of bleomycin (0.09 units in 100 µl sterile saline per mouse) in both groups and continued for 21 days. Group 2 received daily SC injection of the therapeutic RP-107 in addition to the bleomycin treatment, whereas Group 1 received saline only in the second daily injection. Animals were euthanized on Day 50, and terminal skin samples (for histology) and blood samples (for plasma cytokine analysis) were collected. Previous unpublished studies have shown that fibrosis continues to develop long after bleomycin dosing has been halted and the author was interested in the prolonged effects of the potentially therapeutic peptide.

6.2.2 Methods-bleomycin pulmonary model

Two days before the start of treatment, animals were administered twice daily 10ml/kg, SC with saline Group 1 or with RP107 1 mg/kg Group 2. N=10 mice were studied per treatment group. On Day 1, animals were anesthetized with a mixture of isoflurane and medical grade oxygen. Pulmonary fibrosis was induced in Groups 1 and 2 by a single intratracheal injection of bleomycin 0.09 units in 50µl sterile saline per mouse. Animals were then treated by daily saline injection in Group 1 and daily therapeutic peptide RP-107 in group 2. Animals were euthanized on Day 30, and terminal lung samples (for histology) were taken from 5 out of the 10 mice per treatment group, and blood samples for

serum cytokine analysis were collected by cardiac puncture for each of the 10 mice per treatment group.

Lung histology was scored according to the modified Ashcroft scale, developed for use in bleomycin lung injury models in rodents (Hubner et al., 2008). This method, which compares the histology against standard images scored 1-8, has been shown to have a mean variability of 0.25 compared to 0.62 for the standard Ashcroft scale, as well as a higher degree of between observer and intra-observer agreement. Sections from the 5 mice per treatment group studied were added to a power point presentation, one section per slide, blinded as to treatment group, and then scored 1-8 by each of 3 observers, using the Ashcroft scale standard images for comparison.

6.3 Results

6.3.1 Effects of RP107 peptide treatment in the bleomycin induced skin fibrosis model

At day 50, mice were euthanised and the site of the bleomycin wound excised for histology. Masson's trichrome stain demonstrated thickening of the collagen rich (blue staining) dermal layer in mice treated with bleomycin compared to uninjured controls (Figure 6.1). However, in the group treated with bleomycin plus the therapeutic RP-107 peptide, the thickening of the dermal layer was reduced ($p<0.0009$), and appeared similar to untreated controls.

The levels of cytokines and matrix metalloproteases were assayed by a limited inflammatory Luminex assay (IL-6, TNFα, IL-12p70), and by ELISA (MMP-1 and 9) of serum derived from blood samples taken at the end of the experiment on day 50. The cytokine levels are shown in Figures 6.1. In the cutaneous model, as expected, treatment with bleomycin was associated with high levels of inflammatory proteins IL-6 and IL-12p70, whereas TNFα levels were only slightly increased compared to previously established untreated control values (untreated control serum cytokine levels established in previous experiments, mean +/- SD; IL-6 6.6 +/- 6.4 pg/ml, IL-12p70 1.35 +/- pg/ml, TNFα 1.1 +/- 0 pg/ml, basal MMP levels not established, see Appendix and Chapter 3). Treatment with the therapeutic peptide was associated with a trend

towards reduction of all cytokines as well as MMPs, and the effect reached statistical significance in the case of IL-6 and IL-12p70. Strikingly, IL-12p70 was undetectable in the plasma samples of mice receiving the therapeutic.

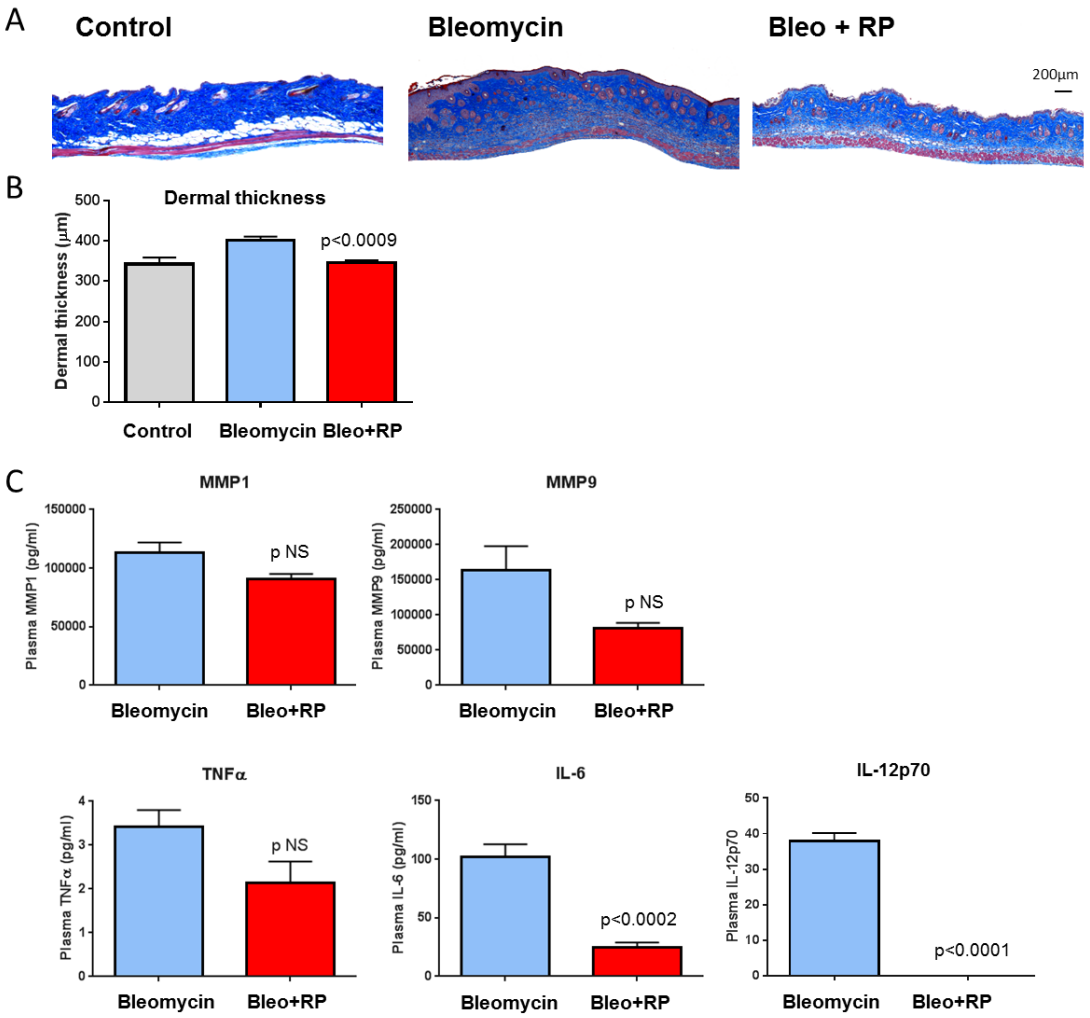


Figure 6.1 Effect of RP-107 in the bleomycin induced dermal fibrosis model C57/BL6 mice were treated by daily bleomycin injection plus saline (n=10 mice) or daily therapeutic peptide RP-107 (n=6 mice), for 21 days, and then euthanized on day 50 for serum and tissue sampling. The blue staining collagen rich dermal layer was thickened and a loss of subcutaneous fat was seen in bleomycin treated mice. Co-treatment with the peptide inhibitor prevented dermal thickening and some preservation of the subcutaneous fat layer was seen (A). When formally measured the reduction in skin thickening with the peptide therapy was confirmed reaching statistical significance, p<0.0009 vs bleomycin treated (B). As expected, inflammatory proteins as well as matrix degrading enzymes were present at high levels following the Bleomycin treatment. There was a trend towards reduction of all factors following treatment with the peptide, which was statistically significant for IL-6 and IL-12p70. Means were compared by Student's t test and p values corrected for multiple comparisons. Individual measurements and statistical analysis is shown in the Appendix. Control basal values were established from previous experiments as follows (mean+/- SD); dermal thickness 343+/-27.6 µm, IL-6 6.6 +/- 6.4 pg/ml, TNFα 1.1 +/- 0 pg/ml (lower limit of detection of assay), IL-12p70 1.35 +/-0 pg/ml (lower limit of detection of assay). Basal MMP levels were not established.

6.3.2 Effects of RP107 peptide treatment in the bleomycin induced lung fibrosis model

Mice were euthanised at day 30, after induction of lung fibrosis with bleomycin. Lungs were removed from 5 mice per group and fixed prior to Masson's trichrome staining. The images of histologic sections were scored by 3 blinded observers, using the modified Ashcroft scale. Histologic analysis of lung tissue showed extensive infiltration and fibrosis in the lung interstitium accompanied by collagen deposition around the airways, in the bleomycin treated animals. Co-treatment with the therapeutic RP-107 peptide reduced the severity of these changes as measured by Ashcroft score. Representative images are shown in Figure 6.2, and the corresponding modified Ashcroft scores are shown in the accompanying Table 6.1. Treatment with the RP 107 peptide reduced the mean Ashcroft scale score from 5.8 (SEM, 0.29), to 2.8 (SEM, 0.53), $p<0.0024$.

In addition, plasma cytokines and MMPs were assayed on day 30. As for the bleomycin skin model, serum levels of IL-6 and IL-12p70 were markedly elevated compared to previously established control values, whereas TNFα was only slightly elevated. The effect of the therapeutic peptide was very striking leading to significant reduction of all factors studied. Again the IL-6 response was greatly attenuated, but the most profound effect was on IL-12 p70 which was undetectable in the plasma of mice receiving the peptide (Figure 6.3).

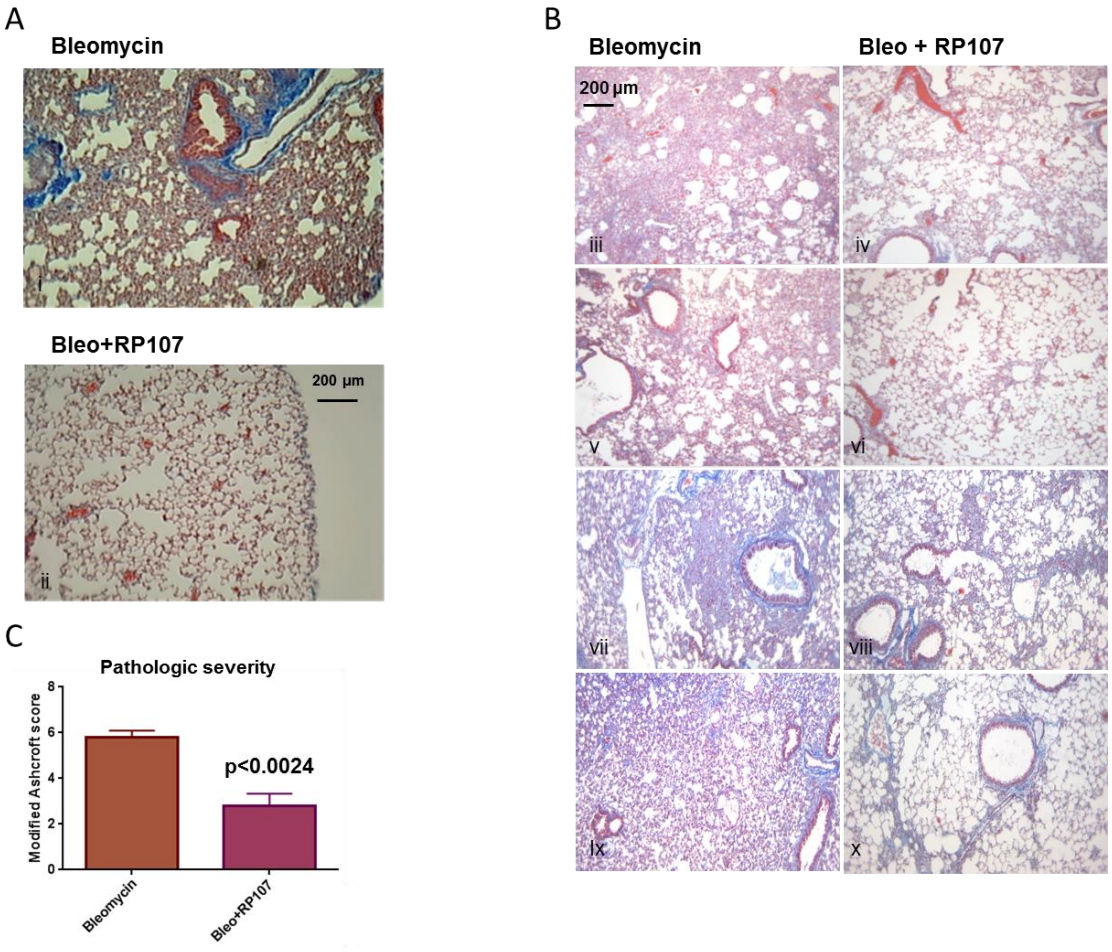


Figure 6.2 Effect of RP-107 in the bleomycin induced lung fibrosis model C57/BL6 mice were treated by a single intrathecal bleomycin instillation and then subsequently given daily subcutaneous injection with saline or daily RP-107 therapeutic peptide, and then euthanized on day 30. The lungs were extracted from n=5 mice per treatment group, and stained by Masson's trichrome stain and imaged (10x Zeiss). In preliminary studies the lungs from the bleomycin treated mice showed interstitial infiltrates and marked fibrotic change plus extensive remodeling of the alveolar structures, whereas treatment with the RP107 peptide greatly attenuated these changes (representative images i and ii shown in (A)). Further sections, iii-x, were analysed from each mouse as shown in (B). Each of the above sections was scored using the modified Ashcroft scale by 3 blinded observers (C). The mean Ashcroft score was reduced by treatment with the RP107 peptide $p<0.0024$. Each individual score is shown in Table 6.1 below.

				Bleo					**Bleo+ RP107**
Scorer/ section	RS	ZT	BAA	Mean score		RS	ZT	BA A	Mean score
Mouse i	6	5	5	5.3	Mouse ii	1	2	1	1.3
Mouse iii	6	6	6	6	Mouse iv	4	4	5	4.3
Mouse v	5	4	6	5	Mouse vi	2	2	3	2.3
Mouse vii	6	6	6	6	Mouse viii	3	4	4	3.7
Mouse ix	6	7	7	6.7	Mouse x	2	3	2	2.3
overall mean				**5.8**					**2.8**
SEM				0.29					0.53
P (t test)									0.0024

Table 6.1 Patholgic severity of bleomycin induced lung fibrosis scored by 3 blinded observers A powerpoint presentation of the histologic lung sections from bleomycin and bleomycin+RP107 treated mice was sent to each of 3 observers blinded as to the treatment group, and including a guide to the modified Ashcroft scoring system. Each section shown in Figure 6.2 was analysed and scored by each observer. There was good concordance between observers with no score differing by more than 1 for each section. The overall mean score was 5.8 (SEM 0.29) in bleomycin treated animals, and 2.8 (SEM 0.53) in animals treated with bleomycin +RP107. Based on these findings a significant effect of the peptide on lung tissue remodeling was demonstrated.

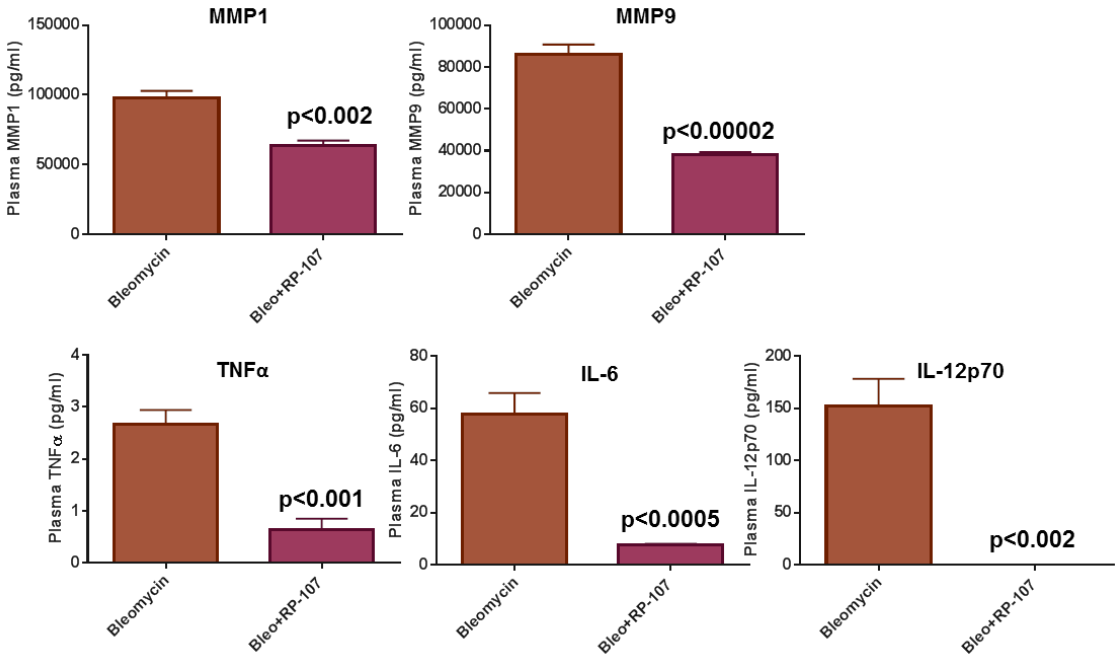


Figure 6.3 Effect of RP107 on cytokine levels in the bleomycin induced lung fibrosis model. Cytokines and MMPs were assayed in serum samples taken from each of the n=10 mice per treatment group on day 30. Bleomycin treatment was associated with high levels of cytokines, which were significantly reduced by treatment with the peptide. There was a statistically significant reduction in serum levels of all factors studied. The relative reduction was greatest for IL-6 and IL-12p70, the latter being undetectable in RP107 treated mice.

6.4 Discussion

Studies were conducted examining the cytokine and growth factor changes as well as the pathologic fibrosis induced in the bleomycin skin and lung fibrosis models, which have often been used to model the fibrosis seen in SSc. In addition, the testing of a novel peptide that shows potential as a therapeutic for fibrosis in SSc, was performed. In these experiments, the peptide was administered subcutaneously to mice two days prior to, and then daily throughout the experiment with bleomycin-induced skin or lung fibrosis, and resulting plasma samples were analyzed for cytokines and MMPs. Using multiplex profiling of the mouse serum, high levels of IL-6, and IL-12p70 were found following bleomycin treatment.

The finding of elevated IL-6 in this model is noteworthy because IL-6 has been implicated in the clinical pathogenesis of SSc, and as shown in Chapter 3, IL-6 is elevated in the blister fluid and plasma of SSc patients compared to

healthy volunteers. Further, other groups have shown elevated IL-6 in the serum of SSc patients, confirming its promise as a therapeutic target (Muangchant and Pope, 2013). A human clinical trial was performed at the Royal Free Hospital (London) using a monoclonal antibody to IL-6R, being associated with a trend toward improvement in SSc skin fibrosis (Khanna et al., 2016b). The correlation of biomarkers seen in humans with those in bleomycin-treated mice adds credence to the use of IL-6 as a therapeutic target and bleomycin-treated animals as a suitable model. The therapeutic RP107 peptide studied greatly reduced the IL-6 levels in both bleomycin models studied, consistent with an important in *vivo* anti-inflammatory effect.

In addition, elevated IL-12p70, as found here in the serum of bleomycin treated animals, has been documented in SSc patients with pulmonary hypertension, and an SNP at the gene encoding the IL-12 receptor has been associated with SSc (Matsushita et al., 2006, Bossini-Castillo et al., 2012). IL-12p40 a constituent of p70, was found in Chapter 4 to be raised in plasma of SSc patients. The therapeutic RP peptide, reduced IL-12 p70 to undetectable levels in both dermal and pulmonary models studied. Since IL-12 is thought of as a predominantly macrophage and dendritic cell-derived inflammatory protein, the results are consistent with an anti-inflammatory, macrophage inhibitory effect of the peptide.

MMPs were also studied. MMPs are a family of zinc dependent peptidases involved in normal development, tissue remodeling, and implicated in fibrosis (Bonnans et al., 2014, Pardo et al., 2016). MMP activity can be specifically inhibited by tissue inhibitors of metalloproteinases (TIMPs), which bind to active MMPs in a 1:1 ratio. MMPs have been extensively investigated in SSc where an imbalance between matrix secretion and degradation is implicated in the fibrotic process (Young-Min et al., 2001). MMP-1, an interstitial collagenase which breaks down the interstitial collagens I, II, and III, is increased at the level of gene expression in SSc fibroblasts from early stage SSc (less than 1 year), but not in medium or late stage disease, whereas TIMP-1 levels remain increased throughout (Kuroda and Shinkai, 1997). Plasma levels of MMP-1 appear normal in SSc, whereas MMP-9, a gelatinase which degrades collagen breakdown products and is also implicated in basement membrane collagen IV degradation, is known to be elevated in SSc plasma and

correlates with the severity of skin involvement and with levels of the pro-fibrotic TGFβ (Kim et al., 2005, Kim et al., 2004).

In this Chapter plasma levels of MMP-1 and 9 were investigated in the bleomycin skin and lung injury models. A limitation of the approach used is that basal levels in synchronous untreated control animals were not established. However, as the main purpose of the experiment was to evaluate differences between bleomycin treated and bleomycin plus therapeutic peptide treated mice, the results still have validity. Similar levels of MMPs were found in both the dermal and lung bleomycin models, with somewhat higher levels of MMP-9 in the dermal model. Treatment with the therapeutic led to a significant reduction in both MMP-1 and 9 in the lung fibrosis model, and a non-significant trend to reduction in the dermal fibrosis model. These data indicate a biologic effect of the therapeutic peptide on the expression levels of these tissue remodeling enzymes, and are consistent with the reduced remodeling and fibrosis seen on histologic analysis. Since MMP-9 degrades basement membrane collagen and is known to be increased in SSc, these effects of the therapeutic could be important clinically, and support the idea of a future clinical trial.

Multiple cell types involved in the inflammatory-fibrotic process could contribute to the plasma MMPs found in these studies, including macrophages, fibroblasts, epithelial cells, and mast cells. The therapeutic peptide, could be acting through suppression of macrophage activity, or modulation of macrophage-fibroblast cross-talk, accounting for the suppression of MMPs. Multiple growth factors and cytokines are known regulators of MMPs, notably IL-1α and TGFβ itself (Gordon et al., 2009). Arguably the reduction in MMPs following treatment with the peptide could be due to reduced inflammation or suppressed secondary TGFβ activation.

Overall, in the pulmonary bleomycin model, the levels of all of the measured cytokines were significantly lower in the RP107-treated than in the saline-treated animals. In the cutaneous-bleomycin model, levels of IL-6, and IL-12p70, were also lowered significantly by the peptide. These results are consistent with the postulated anti-inflammatory activity of the RP107 peptide.

In addition an anti-fibrotic effect is supported by the findings of the histologic analysis, showing reduced skin thickening and reduced interstitial fibrosis in the animal models. A future, more comprehensive study could include biomarkers of collagen synthesis as well as untreated controls and multiple time points, although this would be costly and use more mice.

The bleomycin fibrosis model was chosen for study because it is well defined and established with a large number of publications describing its correlation with SSc (Yamamoto et al., 1999b, Yamamoto and Nishioka, 2005, Wu et al., 2009, Akhmetshina et al., 2009, Svegliati et al., 2014). Rodent bleomycin studies have contributed extensively to the elucidation of the role of pro-fibrotic factors such as TGFβ, as key components of pulmonary fibrosis. However, there are limitations to this model. Bleomycin is an antibiotic-antineoplastic agent that induces intense inflammation that rapidly progress to a fibrotic phenotype, in contrast to SSc patients where the skin changes becomes chronic and slowly progressive over time. That being said, the bleomycin rodent model still stands as the *in vivo* model of choice for the study of inflammation-associated lung and skin fibrosis.

As reported here, the data shows some correlation between the mouse models and SSc as a clinical condition, with regards to the inflammatory factors increased systemically. In addition, the peptide RP107 has been shown to reduce both the inflammatory changes as well as the histological changes of fibrosis in the mouse models. Limitations of the study include the use of a single dose of the therapeutic, which was established previously by the author in other mouse inflammatory and neoplastic models, and also the examination of a single time point for the readouts. However, the data presented support a significant anti-inflammatory as well as anti-remodeling effect of the peptide therapeutic as studied.

Chapter 7. Summary Discussion and Future Work

7.1 Studying biomechanisms in systemic sclerosis: the findings of the thesis

In this thesis a number of approaches were utilised to study the molecular mechanisms involved in SSc, with emphasis on those underlying the development and spread of the fibrotic process. At the onset of this thesis it was hoped that application of these assays would help to define biomechanisms and lead to possible new therapeutic approches to meet the urgent need for effective therapeutics for fibrotic diseases and particularly for SSc. In keeping with this, a potential therapeutic peptide was investigated which showed relevent activities.

There are reasons to believe, based on familial and racial disease prevalence , that genetic factors affect the development of SSc. GWAS studies are ongoing, but to date have identified a limited number of gene targets mainly related to innate and adaptive immunity, which overlap with the genetic associations seen in other inflammatory and autoimmune conditons. In general, these approaches have not indicated novel approaches to therapy. Here using a different approach applied to the Royal Free Hospital's respository of stored DNA samples, a limited number of CNVs in SSc patients' DNA were evaluated.

Initially, a CNV genetics platfrom was used, which had been developed by the author to assess CNV elements in the first intron of a set of target genes chosen because of their possible involvement in complex inflammatory fibrotic disorders. The first introns of genes often contain multiple regulatory elements which enhance the promoter activity of the gene. Using this method, a CNV was identified in the first intron of *LEPREL1* possibly associated with susceptibility to development of SSc in males, whereas none of the other CNVs studied were associated with either disease risk or protection. When the statistical analysis was adjusted for multiple comparisons, the significance of the association between the *LEPREL1* CNV and SSc became non-significant. However, since *LEPREL1* is involved in collagen synthesis, it was decided to pursue this factor further as a possible candidate factor relevent to the disease pathogenesis. In fact, in a subsequent SNP analysis based on much larger

sample size (over 700 SSc samples), studies conducted at the Royal Free Hospital have indicated a highly significant association with an SNP adjacent to the CNV sequence (work of B Abdi Ahmed and R Stratton, data not included here).

The presence of the CNV does not cause SSc, since it also occurred in healthy males and females without SSc. However, P3H2, the product of *LEPREL1*, was found to be overexpressed in cells cultured from SSc skin lessions, and the P3H2 protein level in normal cells was enhanced by TGFβ. The CNVs contain a nucleotide sequence known to be a binding site for the nuclear SMAD factors which are activated by TGFβ.

Given the general role for TGFβ in all the fibrotic processes, people with the CNV may be at risk for fibrosis due to other causes, and it would be important to assess the frequency of the CNV in other common fibrotic disorders. Prolyl 3-hydroxylases are known to have an important function, as inactivating mutations in the gene encoding prolyl 3-hydroxylase 1 (*LEPRE1*) have been identified in certain patients with brittle bone disease (Cabral et al., 2007). While it doesn't appear to be as critical an enzyme for tissues producing less collagen than bone, the development of fibrosis may cause prolyl 3-hydroxylation to be a rate limiting factor. However, it should be noted that SSc describes a set of patients with very diverse presentation from disseminated to localized (morphea) to the sine form without skin firbosis. It is not unlikely that the genetic factors associated with these diverse forms will also be heterogenous.

Furthermore, mice were studied in which the *LEPREL1* gene was silenced in order to investigate the role of this gene in tissue repair and fibrosis *in vivo.* The model utilized was by no means definitive as a KO of the *LEPREL1* gene was embryonic lethal due to the fetal animals developing extensive blood clots by binding the mother's platelets via GPVI on the platelets. Knocking out both *GPVI* and *LEPREL1* produced viable offspring with a resistance to developing fibrosis in the skin when treated with bleomycin, which was not seen in the *GPVI* SKO. This result would appear to confirm the role of *LEPREL1* in dermal fibrosis.

This enzyme could potentially be targeted by developing specific inhibitors or by iron chelators such as deferoxamine, which chelates ferric iron

and is approved for clinical use in iron overload. Ferric iron is an essential cofactor for prolyl 3-hydroxylase. In SSc patients, the iron chelator could deactivate the enzyme and slow the fibrotic process (Vranka et al., 2004). However, inducing iron deficiency of any severity in SSc patients might be expected to have other deleterious effects, and might be dose limiting in any such treatment.

Proline analogues such as cis-4-hydroxy-L-proline and L-azetidine-2-carboxylic acid can also be used to target collagen synthesis in fibroblast cell cultures, and could potentially be used to inhibit the effect of P3H2 on nascent collagen (Tan et al., 1983). Additionally, analogues of α-ketoglutarate, which is also required for enzyme activity, are currently in clinical trials primarily for anemia where it is being used to target the prolyl 4-hydroxylase involved in HIF metabolism. P3H2, has been cloned and expressed and could be used in screening for selective inhibitors. Selective inhibitors are likely to be found as the substrate on which the enzyme acts is distinct from those of other prolyl hydroxylases.

An additional problem in developing effective therapeutics in SSc is the lack of specific biomarkers. In the third chapter of this thesis, Multiplex profiling of body fluids was applied, a method which had been used by the author in animal pharmaceutical studies to profile bioactive factors in plasma and body fluids in order to test the responses to experimental drugs. Royal Free Hospital scientists had previously utilised a vacuum blister approach to study the lesional mileau before and after Iloprost, which is used to treat pulmonary hypertension and Raynaud's in SSc (stratton, 2001), and this method was applied again in the current thesis to study the local biomechanisms. The analyses performed on blister fluid and plasma from patients and controls showed striking differences between the control and SSc blister fluid. Multiplex analyses were not closely correlated between blister fluid and plasma from the patients, suggesting that biomechanisms relating to the local dermal pathology are being assessed using blister fluid. This method could be used to assess the response of the patients' lesions to potential and already approved therapeutics to which some patients respond, or as part of a future therapeutic trial.

To a non-clinician, the spread of fibrosis through the skin and to internal organs seems almost cancer like. It is possible that the SSc cells have become

intrinsically more invasive than healthy cells, or that factors present within the lesions, such as elevated PDGF, as shown in the blister fluid analysis, are acting as chemoattractants and enhancing recruitment of cells. In order to model the spread of the disease process, SSc cells stored at the Royal Free Hospital, were studied in a model of migration on collagen coated chips. On a chip coated with aligned collagen fibers, both control and SSc cells migrated in media containing fetal bovine serum or PDGF, but not in their absence. Neither cell type migrated on woven collagen substrates.

The SSc fibroblasts were not highly invasive and migrated somewhat slower than the control cells. These studies appear to show that the SSc cells are not unusually mobile. Rather, it appears that the spread of the cells is promoted by PDGF, which had been identified as an elevated factor in SSc blister fluid. Thus it would appear that the spread of the SSc fibroblasts was driven by factors present in elevated levels in their lesions. This assay was also used to study the effect of potential inhibitors which act through inhibiton of the PDGF pathway. Both imatinib and heparin inhibited migration of the fibroblasts and when tested together showed synergistic activity.

Finally a study of a potential therapeutic peptide, developed by the author and co-workers, was performed. This peptide was designed to antagonize inflammatory responses in patients with resistant inflammatory disorders, and as a possible therapy in SSc. It was investigated in a mouse model, testing whether the peptide reduces inflammation or fibrosis in models designed to mimic some aspects of the fibrotic inflammatory lesions in SSc. The peptide appeared to be effective in reducing both inflammation and fibrosis.

7.2 Interpreting the thesis in the context of the current understanding of systemic sclerosis

The work presented in this thesis needs to be considered in the context of current understanding of biomechanisms in SSc and fibrosis based on extensive work by many centres worldwide committed to studying the disease. Notably, centers in London, Boston, Pittsburgh, Zurich, and Chicago have well developed programs of research into the disease and produce high quality publications relating to the biomechanisms. The findings of the current thesis are discussed in the context of known biomechanisms based on published

work. The challenges of developing new treatment strategies targeting these biomechanisms are explored further.

7.2.1 The role of genetic variation in the disease

As discussed in the introductory Chapter, SSc is a heterogeneous disease, and multiple genetic variants are thought to have a role. Polymorphisms in several cytokine genes have been investigated for a role in SSc and in other fibrotic diseases, but with conflicting results. The most relevant cytokine for fibrosis is TGFβ. Outside the context of SSc, SNPs found in the coding region of the TGFβ1 gene have been reported to be associated with an increased risk of fibrosis (Awad et al., 1998, Gewaltig et al., 2002, Osterreicher et al., 2005, Tag et al., 2003). This mainly involves a nucleotide in codon 10 and/or 25. Hepatic fibrosis following chronic hepatitis C infection is found more frequently with ^{10}Leu/^{10}Pro and ^{10}Pro/^{10}Pro than ^{10}Leu/^{10}Leu and with ^{25}Arg/^{25}Pro more frequently than ^{25}Arg/^{25}Arg. However, these results are from small studies and other groups have failed to replicate these associations, finding instead that ^{25}Arg/^{25}Arg was associated with an increased risk of hepatic fibrosis following HCV infection (Powell et al., 2000).

Polymorphisms in other cytokines such as TNF-α, IL10, IFN-γ, IL1 receptor antagonist, and chemokine receptors have also been investigated for a role in fibrosis in general. There is also an interest in polymorphisms in the Toll-like receptors (TLR) as factors in fibrosis (Awad et al., 1998). A lack of validation that these polymorphisms have a role in fibrosis is partly due to the small numbers of patients studied, differences in histological scoring, ethnicity, population stratification and uncontrolled variables associated with the disease progression (Hold et al., 2009).

Currently, there is a lack of specific biomarkers that can be used to identify patients who might benefit from personalized therapies. The use of information obtained by genotyping could be a significant help when selecting patient populations for clinical trials. Stratified medicine involves the use of patient-specific features to determine groups of patients who might respond well or badly to a given medication (Hall, 2013). Therefore, genetic information could

help to define populations that would respond to a given treatment, providing a more homogenous patient population for specific trials.

In addition, when considering the treatment options available and the clinical trials that have been conducted, it appears that responsiveness to any treatment regimen by an individual patient will only be predictable with reasonable accuracy by conducting appropriately designed pharmacogenomics studies (Raghu, 2006).

In studies presented in this thesis, it was found that a genomic CNV in *LEPREL1* which encodes P3H2, is a possible candidate for involvement in the pathogenesis of SSc. P3H2 is a critical factor in collagen synthesis by contributing to the formation of 3-hydroxyproline in collagen, and might be a target for SSc diagnosis and therapy. Limiting excessive collagen deposition could be an approach to treating SSc and other forms of fibrosis. Furthermore, since P3H2 has been cloned and expressed, it could be used in screening for novel compounds to be tested as selective inhibitors. It would be interesting to see how the CNV presence or absence correlates with collagen synthesis from isolated fibroblasts, and in patients with the progression of the disease. The *LEPREL1* CNV marker might be used as a biomarker for determining a predisposition of SSc and its progression, as well as in other fibrotic disorders

7.2.2 Understanding the disease microenvironment

One advantage of SSc is that the hallmark severe fibrotic changes occur in the skin which can be sampled directly with minimally invasive methods such as the blister fluid sampling used in Chapter 4. Using this method it is possible to gain some insight into the microenvironment underlying the skin fibrosis. Fibrosis is a pathological feature of most chronic inflammatory diseases and is defined by the excessive accumulation of extracellular matrix (ECM) components, such as collagen and fibronectin, in and around the inflamed or damaged tissue. Fibrosis can affect nearly every organ in the body and there are many triggers that can lead to fibrosis. Some of these are inherited genetic disorders, persistent infections, recurrent exposure to toxins, irritants or smoke, chronic autoimmune inflammation, minor human leukocyte antigen mismatches in transplants, myocardial infarction, high serum cholesterol, obesity, poorly

controlled diabetes and hypertension (Wynn, 2008). The common feature of all fibrotic diseases is the presence of myofibroblasts, the cells involved in excessive matrix deposition (Gabbiani, 2003, Kalluri and Zeisberg, 2006). Therefore, it is critical to understand the mechanism and factors by which fibroblasts are converted into ECM-producing myofibroblasts within the disease microenvironment.

Both innate and adaptive immunity play a role in fibrosis. When a bodily injury occurs, platelets become activated and release TGFβ, which stimulates local fibroblasts to differentiate into ECM-producing myofibroblasts (Barrientos et al., 2008). Although TGFβ is a key driver of fibrosis, its activity appears to be dictated by its cellular source. TGFβ is produced by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells. TGFβ produced by macrophages is involved in wound healing and profibrotic activity, while TGFβ produced by $CD4^+$ T_{reg} cells is anti-inflammatory (Kitani et al., 2003, Kitani and Xu, 2008). However, in the current studies using Multiplex, TGFβ was not assayed as it is not available in the Luminex systems, and this is a limitation of the study. It would be possible in future studies to measure free and LAP-associated TGFβ in the SSc blister fluid by ELISA assays.

In addition to platelets and macrophages, other myeloid-derived cells, such as mast cells, eosinophils and basophils, have been implicated in contributing to the fibrotic microenvironment in multiple organ systems. In a rat study, mast cells appeared to promote fibrosis by recruiting inflammatory leukocytes into the fibrotic lesions, and also by producing pro-fibrotic mediators (Levick et al., 2009). Eosinophils have been associated with the development of pulmonary fibrosis (Humbles et al., 2004) as well as being linked to the activation of myofibroblasts in skin, liver fibrosis and retroperitoneal fibrosis (Levi-Schaffer et al., 1999, Reiman et al., 2006). Basophils have been implicated in the pathogenesis of myelofibrosis and are found in large numbers in patients with interstitial lung disease (Gilbert, 1984). Basophils are also an important source of type 2 cytokines, which suggests that they may serve as a driver of IL-4 and/or IL-13-dependent fibrosis. It is possible to sample the cellular infiltrates in SSc skin via a modified dermal blister technique. If the blister is left in situ for 24 hours, inflammatory cells accumulate in the fluid and can be profiled by FACS.

Cells involved in adaptive immunity have also been implicated is having a role in fibrotic lesions. Th17 cells that express IL-17A appear to be drivers of fibrosis. IL-17A expression has been implicated in pulmonary fibrosis (Wilson et al., 2010), chronic allograft rejection (Faust et al., 2009), fibrosis after orthotropic lung transplantation (Fan et al., 2011), myocardial fibrosis (Feng et al., 2009), and hepatitis-induced hepatic fibrosis (Wang et al., 2011a). Recent studies have shown a link between IL-17A and TGFβ (Wilson et al., 2010). Consequently, therapeutic agents that disrupt IL-17 signaling might prove beneficial in the treatment of fibrosis. In the studies presented in Chapter 4, an elevation of IL-17 was found in SSc patients' blister fluid, confirming altered expression in the disease. However, the absolute levels of IL-17 were low, and present in only a subset of patients. Also, recent studies have questioned the importance of IL-17 as a profibrotic.

Th2-mediated immunity is also a potent driver of fibrosis. IL-13 produced by Th2 cells is the dominant mediator of fibrotic tissue deposition in several models of fibrosis (Chiaramonte et al., 1999). This cytokine may act locally by stimulating production and activation of TGFβ (Lee et al., 2001). IL-13 is known to up-regulate TGFβ1 stimulation of fibrosis through the chemokine CCL2. Therefore, in addition to TGFβ, IL-13 and CCL2 are potential targets for regulation of fibrosis. Several clinical trials are ongoing or have recently been completed using antibodies to IL-13 or CCL2. IL-13 may also directly activate collagen synthesis and the proliferation of fibroblasts, epithelial cells and smooth muscle cells (Lee et al., 2001, Kuperman et al., 2002, Kaviratne et al., 2004b, Fuschiotti et al., 2013). One surprising result of the studies in Chapter 4 is that Th2 cytokines were not in general increased in the blister fluid. However, there was a trend to increased Th2 cytokines in the plasma of patients, and in general Th2 cytokines were not present in healthy plasma and seen only in the patients' samples. It is possible that Th2 cytokines are driving the SSc disease process at sites other than the skin lesions, including the gut and lungs, leading to plasma levels being increased.

The role of T_{reg} cells is still unclear since they can either suppress or promote fibrosis. T_{reg} cells are an important source of TGFβ1, which would suggest that they are involved in driving fibrosis (Estes et al., 2007, Liu et al.,

2010). However, IL-10, a classic T_{reg} product, was not elevated in the blister fluid.

In Chapter 4 of this thesis, elevated growth factors and cytokines were found in human dermal blister fluid (interstitial fluid) obtained from the lesional skin of SSc patients. Elevated pro-inflammatory proteins, such as IL-6, are recognized targets in the treatment of SSc, and neutralizing the copious amounts of these cytokines may help to clarify the importance of their role and identify possible treatments for the disease. Currently at the Royal Free Hospital, London, UK, there is an ongoing clinical trial to treat SSc patients with a monoclonal antibody against IL6R, as a follow up to a published multicenter trial showing a non-significant trend to improvement of skin involvement (Khanna et al., 2016b). IL-6, usually viewed as an inflammatory cytokine, is highly elevated in serum and blister fluid of SSc patients as well in the rodent bleomycin fibrosis models.

In addition, elevated angiogenic factors were identified in the blister fluid (FGF2) and also an earlier study has shown elevation of pro-fibrotic protein CTGF (Dziadzio et al., 2005). These results confirm previous models and ideas about SSc, consistent with an inflammatory fibrotic disease process with dysregulated vascular repair. Furthermore it is proposed that these methods give a picture of the various active components of the local disease process and could be used to identify patients for future personalized therapies.

In future experiments using a delayed sampling technique, it might be possible to identify the cell types that act as drivers of SSc in the localised affected area, since at 24 hours cellular recruitment into the blister has been found to occur, and may prove to be important in addressing targets for treatment and determining the cells involved in the progression of the disease.

7.2.3 Combining therapies to improve efficacy against systemic sclerosis

Dysregulated immune responses, both innate and adaptive, are major contributors to fibrosis. However, cell-intrinsic alterations in fibroblasts and other structural cells also contribute to fibrosis. Therefore, an approach using combination therapies is appropriate and could be developed further. SSc is a multisystem disorder characterized by excessive deposition of collagen and

other extracellular matrix components in the visceral organs as well as the skin. One major manifestation of the disease is interstitial pulmonary fibrosis, or interstitial lung disease (ILD). In a recent study, a combination of imatinib, the tyrosine kinase inhibitor, and cyclophosphamide, an immunosuppressive agent, were used to treat five SSc-ILD patients (Sabnani et al., 2009). The use of imatinib was based mostly on the known anti-proliferative properties of the drug, as well as its role as an inhibitor of PDGF receptor function and its ability *in vitro* to inhibit collagen production by fibroblasts. The combination of imatinib and cyclophosphamide was well tolerated with no major side effects. However, only two patients completed the one year study and only a single patient with mild restrictive lung disease showed clinical improvement. This was the first study known to use a combination of an anti-fibrotic and immunosuppressive drug. More definitive studies are obviously needed. However, this study did show that lower doses of imatinib could be used in combination, which improved tolerability and effectiveness in early stages of the disease.

Other studies have been conducted using a combination of a steroid and cyclophosphamide in Idiopathic Pulmonary Fibrosis (IPF) (Collard et al., 2004). Little improvement was seen in the patients treated with this combination. This result led the investigators to conclude that inflammation only plays a minor role in the progression of IPF as a relatively pure fibrotic disorder.

N-acetylcysteine (NAC) is a precursor to the antioxidant glutathione. Since glutathione is depleted in the lungs of IPF patients, NAC was viewed as a potential therapy for IPF with the hope that repletion of glutathione would restore the natural oxidant/antioxidant balance and prevent the oxidative injury that precedes fibroproliferation which leads to fibrosis (Meltzer and Noble, 2008). In clinical trials, NAC has been administered in combination with a corticosteroid and an immunomodulator. In a small, non-randomized study, this combination therapy resulted in improved lung function (Demedts et al., 2005, Raghu et al., 2012a). However, in larger randomized trials, no benefit was seen with the addition of NAC. In fact, one trial was stopped due to a significant increase in mortality, hospitalizations and serious adverse events, and there was no improvement in pulmonary function in the group receiving the three drugs (Wynn and Ramalingam, 2012).

Nezam Altorok *et al.* (2014) has described epigenetics as “the holy grail of systemic sclerosis.”(Altorok et al., 2014) In terms of genetic contribution to the disease, it has been estimated that disease heritability is approximately 0.8% (Feghali-Bostwick et al., 2003). These findings concur with published research that the etiology of the disease continues to remain elusive. However, recent publications continue to convey supporting evidence for the hypothesis that epigenetics contributes to the devastating pathology seen in SSc patients (Feghali-Bostwick et al., 2003, Luo et al., 2013b, Altorok et al., 2014). Perhaps treatment with compounds such as azacytadine (5-azacytidine), an approved antineoplastic drug known to cause DNA hypomethylation, could be used in combination with anti-inflammatory therapies to treat SSc and other fibrotic diseases. It has been shown that incubation of SSc fibroblasts with azacytidine can “restore collagen expression to control levels” (Dees et al., 2014).

Because of the multiple factors that contribute to fibrosis, an integrated anti-fibrotic strategy that simultaneously targets important inflammatory mediators, pro-fibrotic cytokines, epigenetic alterations plus cell and tissue-intrinsic changes, will probably emerge as the most successful approach to treat this highly complex and difficult-to-manage disorder (Wynn and Ramalingam, 2012). However, more work is needed to determine the right combination of drugs for each fibrotic disease.

SSc is a multifaceted, polygenic disorder and for this reason may require simultaneous treatment of multiple disease targets. Inflammation is a major component of diffuse SSc, therefore treatment with an immunomodulatory peptide such as the one described earlier in this thesis, in combination with diverse agents that target DNA methylation, microRNAs or histone modifications may be warranted. In Chapter 5, the ability of individual as well as combination therapies in blocking the migration of fibroblasts cultured from the lesions of SSc patients was tested. Evidence is presented here for synergy between imatinib and heparin in inhibiting the *in vitro* migration of SSc fibroblasts. It is reasonable to suggest that this combination should be studied in animal models of fibrosis to assess their effectiveness in attenuating the spread of disease, possibly leading to a clinical trial in SSc patients with lung fibrosis.

7.2.4 The challenge of developing new therapies for systemic sclerosis

As a significant unmet need, SSc fibrosis induced downstream of inflammation is a potential target for specific therapies against cytokines or cells involved in the process. Mouse models of pulmonary and cutaneous fibrosis, induced with the antimicrobial/antineoplastic drug bleomycin, were used to study these biomechanisms leading to lung and skin fibrosis. Some of the same elevated cytokines found in this thesis to be increased in the blister fluid and plasma of SSc patients were also found in these bleomycin mouse models, thereby supporting the relevance of the bleomycin model.

A number of anti-inflammatory drugs have been used previously to treat fibrotic diseases including SSc. The classes of drugs include corticosteroids, immunomodulatory agents and cytotoxic drugs. Unfortunately, they have been found to have little or no beneficial effects. Therefore, it is uncertain if the newer classes of anti-inflammatory drugs will prove effective.

Various anti-TNFα antagonists have been tested in a number of experimental models and were resulted in the reduction of fibrosis (Chen et al., 2007, Luo et al., 2013a). However, in an exploratory clinical trial in IPF, the TNFα blocking agent, etanercept, was well tolerated but there were no differences in the predefined endpoints among patients who received etanercept or placebo (Raghu et al., 2008). However, a decreased rate of disease progression was observed in several measures, which the investigators felt warranted further investigation with a larger number of patients.

IL-20 is another pro-inflammatory cytokine that is a potential therapeutic target. It has been shown to activate quiescent rat hepatic stellate cells, up-regulate TGFβ1, TNF-α, and Type I collagen expression, as well as promoting the proliferation and migration of activated hepatic stellate cells. Monoclonal antibodies to IL-20 and the IL-20 receptor inhibited fibrosis in an experimental model of liver fibrosis (Chiu et al., 2014).

Another favored target category is growth factor receptor tyrosine kinases, as already discussed. Studies using tyrosine kinase inhibitors that inhibit signaling of PDGF receptors have been conducted to evaluate their potential in treatment of fibrotic diseases in general as well as SSc (Kay and High, 2008, Richeldi et al., 2011, Daniels et al., 2010).

In Chapter 6, bleomycin-challenged mice were treated with a novel peptide exhibiting immunomodulatory properties. This peptide is a newly developed therapeutic resembling a sequence in the antibacterial peptide mellitin consisting of a sequence of 12 amino acids composed of alternating pairs of hydrophilic amino acids (basic) and hydrophobic amino acids (alanine/ phenylalanine). Optimization of the sequence is in process. This peptide has been demonstrated in *in vitro* using mouse macrophages to have major anti-inflammatory modifying effects and in reporter cells lines to prevent NF-κB from translocating to the nucleus. In Chapter 6, it was demonstrated that this peptide was able to reduce serum levels of IL-6 and particularly IL-12p70 to in the mouse cutaneous and pulmonary bleomycin-induced fibrosis models, indicating an important *in vivo* anti-inflammatory effect. These effects would be consistent with a mechanism of action in which activated macrophages are being suppressed within the inflammatory fibrotic lesions, in particular as IL-12 is considered a macrophage derived factor. In addition, a significant anti-fibrotic effect was demonstrated, since the peptide prevented dermal thickening and lung fibrosis induced in the bleomycin models, consistent with the idea that inflammation is coupled to fibrosis in these models. If preclinical toxicity and pharmacokinetic data are carried through then a possible clinical trial of these peptides in SSc patients can be developed. Challenges would include mode of delivery (local interlesional forearm skin delivery in order to miniumise exposure, and using the opposite forearm skin as control have been proposed), establishing a sensitive readout (dermal blister fluid sampling before and after treatment), as well as patient selection/stratification (those with an active inflammatory profile in terms of dermal blister fluid analysis).

A great deal of effort has been made to investigate the role of fibroblasts, or more importantly myofibroblasts, and their contribution to fibrosis, but little has been done concerning the coordinated activity of fibroblasts and macrophages. Incubating macrophages with IL-4 polarizes them to M2 phenotypes and incubation with IFNγ leads to M1 phenotype polarization (Martinez and Gordon, 2014). M2 cells produce TGFβ and could be important in driving a fibrotic event in SSc (Wermuth and Jimenez, 2015). In cell culture viability studies, M2 cells but not M1 cells died when exposed to the peptide. The p50 component of NF-κB is a driver of M2 polarization and is needed for

their survival (Porta et al., 2009). The therapeutic peptide could be preferentially suppressing this kind of polarized macrophage, and this is being currently assessed.

7.2.5 Improving biomarkers to assist therapeutic development in systemic sclerosis

When future clinical trials are to be undertaken, of the peptide therapeutic or other, then the use of new biomarkers could improve the assessment of outcome measures, reduce the sample size required and reduce the risk of missing a significant biologic response. There are multiple ongoing efforts to define biomarkers and imaging for fibrosis. Historically, fibrosis has been detected by histology (stains, immunohistochemistry) in the affected tissues, but these analyses require collection of tissue samples by invasive biopsies (Wynn, 2008). Hence, biomarkers defined as indicators of disease state that can be identified in biological fluids, are more appealing in terms of accessibility. While blood is more standard, urine is especially appealing for kidney fibrosis (Genovese et al., 2014). The best characterized biomarkers for fibrosis are molecules indicative of excess ECM production, especially those related to collagen (e.g., hydroxyproline or peptides released from procollagen during its deposition). Fibrosis is associated with unbalanced ECM remodelling, resulting in changes in the distribution, quantity, and quality of ECM (Wynn, 2008), which can be assessed using commercially available assays (Bottero et al., 2009, Stevenson et al., 2012).

Recent work has focused on “neo-epitopes” corresponding to pathologic tissue turnover, including a collagen pro-peptide and collagen-III and -V proteolytic degradation products (Vassiliadis et al., 2013, Vassiliadis et al., 2011). Other indicators include CTGF (in the TGFβ pathway) and various chemokines and cytokines which have been found to correlate with fibrosis (Kovalenko et al., 2009). These biomarkers can be detected by ELISA in serum and urine, and have been shown in animal models to correlate with active liver fibrosis (i.e., during induction), but are not useful once the fibroproliferative process is no longer active (Vassiliadis et al., 2011). It is also uncertain whether these ECM markers will display sufficient sensitivity to distinguish different

grades of fibrosis in patients. More promising, though at much earlier stages of development, are other ‘Omics’ markers of fibrosis or susceptibility to fibrotic disease. For instance, genomic analysis has defined several polymorphisms (i.e., SNPs) associated with human and animal fibrosis (Spagnolo et al., 2014). Other ‘Omics’ analyses (e.g., proteomics, metabolomics, RNAomics, epigenomics) have been proposed for the comparison of specimens from normal and fibrotic patients (Karsdal et al., 2014). The “signatures” (e.g., protein “fingerprint”) provided by these analyses are expected to include a range of biomarkers that can be tracked/collated using “big data” analyses (Mas et al., 2009). Many of these biomarkers will not represent obvious connections to fibrosis, but the signature is still expected to be diagnostic for a relevant disease state. Some of these biomarkers also promise to be potential therapeutic targets.

Progress in the field of imaging is more apparent. Various imaging techniques have been proposed for visualizing fibrosis in clinical settings, including ultrasound, MRI, transient elastography to detect tissue stiffness and PET (Bonekamp et al., 2009, Castera et al., 2008, Faria et al., 2009, Win et al., 2012, Wu et al., 2013, Berzigotti and Castera, 2013). At least two “targeting agents” with binding affinity for collagen have been described (Muzard et al., 2009). One such agent (EP-3533) incorporates a Gadolinium contrast moiety, permitting non-invasive detection of liver and lung fibrosis by MRI in animal models (Caravan et al., 2013, Polasek et al., 2012). Separate work identified an optical probe (MMP-P12) that is activated by MMP, permitting non-invasive optical detection of pulmonary fibrosis in live animals in a mouse bleomycin model (Cai et al., 2013). Similarly, PET with probes specific for fibroproliferating tissues (e.g., MMP activity) are being tested for assessing fibrosis (Win et al., 2012, Wu et al., 2013). The challenge with these new imaging techniques will be to validate their clinical accuracy, especially in the context of staging different degrees of fibrosis and in detecting and distinguishing early-stage/mild as opposed to late-stage/chronic fibroses (Bonekamp et al., 2009, Inoue et al., 2011, Maurea et al., 2014)

As discussed above, blister fluid obtained from SSc fibrotic dermal lesions was found to have elevated cytokines. A more global analysis of the

proteins in SSc blister fluid should be made using proteomics, as well as FACS analyses to identify possible cells involved (e.g., M2 macrophages). Additionally, the growing interest in the role of exosomes in disease supports assessment of these structures in blister fluid, which may also guide therapeutic development.

7.2.5 Extending findings in systemic sclerosis to the broader issue of fibrosis in general

Outside of the context of SSc, fibrosis is a very prevalent pathologic change contributing to a number of severe and as yet untreatable conditions. Fibrotic diseases affect a range of organs, including but not limited to liver (cirrhosis), kidney (progressive kidney disease), skin (keloids), lung (pulmonary fibrosis (IPF)), and heart (cardiovascular fibrosis). These disorders account for as much as 45% of deaths in the developed world. Classically, these diseases were considered to be part of acute inflammation, consistent with the efficacy of anti-inflammatory agents in treating fibrotic disease. More recently, fibrosis is considered to be a distinct response associated with chronic inflammation, suggesting a shared mechanism among different fibrotic diseases. This proposed mechanism has been the subject of several recent reviews (Rafii et al., 2013, Rastrick and Birrell, 2014, Rieder, 2013, Wynn, 2008) that are summarized here.

Stimuli inducing inflammation can results from tissue injury including from unknown causes as in IPF, alcohol in cirrhosis, to autoimmunity as in SSc. In response to such inflammatory stimuli, the body engages in a two part repair process, including a regenerative phase in which injured cells are replaced with cells of the same type, and a fibrotic phase in which scar tissue replaces normal tissue. Blocking TGFβ or its signalling is known to reduce fibrosis in various animal models. Notably, progression depends primarily on the TGFβ1 isoform (rather than the β2 or β3 isoforms), with regulation at the level of secretion and activation rather than gene expression. Non-TGFβ pathways (*e.g.*, via viral stimulation, other growth factors, epigenetic changes) have also been implicated in various fibrotic disorders.

Activation of the TGFβ pathway results in chemokine/cytokine secretion and elevated expression of cell surface receptors. These changes induce

fibroplasia in turn by facilitating the development of myofibroblasts. These myofibroblasts can be derived directly from the normal tissue resident fibroblasts, by transitions such as epithelial or endothelial-to-mesenchymal transition as in SSc, or by recruitment of circulating marrow-derived fibroblast precursors as in nephrogenic systemic fibrosis (Wagner et al., 2012). Additionally, myofibroblast development is known to be subject to differential regulation by cells of the immune system including Th1 and Th2 lymphocytes. The myofibroblasts produce excess ECM components, including collagens, growth factors, matrix metalloproteases (MMPs), etc., yielding the fibrotic lesions seen clinically.

Fibrotic diseases represent a loss of function for the original tissue, such as loss of hepatic function with cirrhosis, kidney function in chronic kidney disease and lung function with. Mechanistically, fibrosis is currently considered to be pathological, with the myofibroblasts and fibrotic matrix replacing/displacing the original tissue. Thus, in contrast to the classic image of fibrosis as an adaptive response (*e.g.,* walling off infected tissue), fibrotic disorders might be better considered as direct contributors to the disease state.

To date, evidence for reversibility of fibrosis is ambiguous (Czaja, 2014) and emphasis has instead been placed on ameliorating the existing damage. In part, this bias reflects the fact that most clinical fibrosis is detected at a late stage, as many patients present with symptoms only when organ function is already severely impaired. Consistent with the long-term pathology of fibrosis in the clinic, most animal models do not appear to involve lesions that regress. Only a subset of models (e.g., carbon tetrachloride induction of liver fibrosis (Vassiliadis et al., 2012)) and mouse models of scleroderma (Gerber et al., 2013) appear to reverse spontaneously. This pattern follows from the proposed mechanism of fibrosis, in that an initial stimulus, for example an autoimmune response, infection or chemical insult induces tissue damage and inflammation with subsequent development of myofibroblasts and fibroplasia. Myofibroblasts are terminally differentiated cells that replace/displace the endogenous cells, and generate fibrotic deposits (typically extracellular), which are by definition scar tissues that the body is unable to displace. Thus, correction of fibrosis would require removal of the scar tissue, for example by surgery, and introduction of fresh tissue by organ transplantation. Only recently, with

advances in stem cell biology, have there been indications that tissue might be regrown, dedifferentiated or redifferentiated (Nasir et al., 2013, Schuetze et al., 2014). However, stem cell approaches are still at the research stage and are not yet clinically available (McNulty and Janes, 2012, Zhang and Wang, 2013). Furthermore, any such treatment would require additional techniques for correcting the original stimulus (e.g., curing the original infection, suppressing the original autoimmune defect) and for degrading existing extracellular scar tissue.

There are a number of possible therapeutics that are currently being evaluated as treatments of fibrosis, some of which are growth factors, inhibitors of ECM synthesis, antagonists of intracellular enzymes needed for collagen synthesis, immunomodulators, antioxidants and inhibitors of the coagulation pathway (Wynn and Ramalingam, 2012).

Pirfenidone is an anti-inflammatory and antioxidant that inhibits TGFβ *in vitro* (Walter et al., 2006). It also acts as an anti-fibrotic by directly altering the expression, synthesis and possibly accumulation of collagen, as well as inhibiting the recruitment, proliferation and possibly expression of the ECM-producing cells (Carter, 2011). Pirfenidone was approved in the EU and Japan for the treatment of IPF but is yet to be investigated in SSc. Approval by the FDA was delayed due to perceived lack of efficacy and absence of survival benefits. However, a phase III clinical trial to confirm the treatment effects and safety of pirfenidone in IPF compared with placebo was recently completed, with the drug significantly reducing IPF disease progression and enhancing progression-free survival with a favorable safety profile (King Jr et al., 2014). As a result, pirfenidone was recently approved by the FDA for the treatment of IPF.

A phase I clinical trial has also been recently completed using an anti-TGFβ antibody as a treatment for IPF (Rafii et al., 2013). In addition, Lafayatis' group from Boston have just completed an open label study using a high affinity anti-TGFβ active against all 3 isoforms, fresolimumab, and this has shown a reduction in skin score and in gene expression based biomarkers, although there was no control group (Rice et al., 2015).

However, since TGFβ plays a key role in cellular homeostasis, acting as a tumor suppressor under certain conditions, the direct inhibition of TGFβ could result in very undesirable side effects. The TGFβ activation cascade may be a

more attractive therapeutic target. Partial inhibition of αvβ6 integrin, a key activator of TGFβ, has been shown to prevent bleomycin-induced pulmonary fibrosis without exacerbating inflammation in mice (Rafii et al., 2013). STX-100, a humanized monoclonal antibody against αvβ6 integrin, is currently being evaluated in a randomized, placebo-controlled phase II trial for IPF. Other members of the TGFβ superfamily, such as bone morphogenic protein (BMP), may offer additional therapeutic targets since they are also involved in injury repair and homeostasis (Myllarniemi et al., 2008).

CTGF is another possible target in SSc fibrosis. This matricellular protein is thought to be a central mediator of tissue remodeling and fibrosis. It is induced by TGFβ and mediates some of the pro-fibrotic effects of TGFβ (Mukherjee et al., 2012). In murine models of fibrosis, the administration of a human CTGF antibody, FG-3019, results in reduced histological signs of fibrosis (Wang et al., 2011b). Preliminary safety and efficacy data from a phase II trial with FG-3019 were reported at the European Respiratory Society 2012 conference (Raghu et al., 2012b). The preliminary data suggested an improvement or stability of fibrosis.

The somatostatin analog SOM230 decreased the expression of TGFβ and CTGF in bleomycin-induced lung fibrosis in mice, resulting in an anti-fibrotic effect (Borie et al., 2008) suggesting that somatostatin analogs could be tested as anti-fibrotic therapeutics. In a small non-randomized, open-labeled trial in IPF with octreotide, another somatostatin analog, the rate of decline in pulmonary function was lower in the octreotide-treated subjects when compared to historical controls (Crestani et al., 2012).

Thalidomide is a drug with a tragic history in causing birth defects. However, in revelations of positive effects (Moreira et al., 1993, D'Amato et al., 1994), it has been shown to be an anti-inflammatory drug and to be effective in reducing fibrosis in animal models (Koch, 1985). An open-labeled study to determine the safety, feasibility and efficacy of thalidomide as an anti-fibrotic agent was completed in 2007; however, the results have not been published (Rafii et al., 2013). Thalidomide has been approved for treating multiple myeloma.

Other potential therapeutic agents for which clinical trials are being planned or are underway includes an antibody inhibiting the enzyme lysyl

oxidase-like 2 (LOXL2) that cross-links collagen and generates the scaffold upon which fibroblasts grow, tyrosine kinase inhibitors that suppress pro-angiogenic intracellular signaling, angiostatic chemokines and other agents with angiostatic properties such as tetrathiomolybdate and minocycline, inhibitors of MMPs like doxycycline, and angiotensin inhibitors (Rafii et al., 2013). However, the studies of the monoclonal against LOXL2 (Simtuzimab) in IPF have been terminated due to lack of efficacy (http://www.gilead.com/news/press-releases/2016/1/gilead-terminates-phase-2-study-of-simtuzumab-in-patients-with-idiopathic-pulmonary-fibrosis).

Elfibranor (GFT505, Genfit), is a PPARα/δ agonist, which has reached Phase 3 trial level as a treatment for non-alcoholic steatohepatitis and associated fibrosis. Elfibranor benefits insulin resistance in such patients, without causing the weight gain associated with the PPARγ agonists.

Furthermore, apoptosis signal-regulating kinase 1 (ASK1, also known as MAP3K5), has been identified as a target for therapies. ASK1 regulates the downstream stress activated p38 and JNK MAPK pathways. GS-4997, an oral inhibitor of ASK1 has entered into Phase 2 clinical trials for diabetic chronic kidney disease (Lin et al., 2015).

Although there are preclinical data for most of these potential therapeutic agents, and some clinical data, some of the studies to date have been small with few patients. To render them more accessible and relevant, the clinical trials must be designed correctly with an adequate number of patients and the proper control groups.

A potential target described in this thesis is a collagen modifier, *LEPREL1*, with an important role in collagen synthesis. As shown in KO mice for the *LEPREL1* gene, the lack of this gene produces suppression of bleomycin-induced fibrosis. This could be an interesting target for new drug development or as a component of combination therapy with agents such as the novel synthetic peptide with anti-inflammatory properties discussed here. Blister fluid sampled from the skin lesions of SSc patients could be used to determine inflammatory profiles, progression of the disease and response to therapy.

7.3 Final summary of thesis and overall conclusions

A prolyl hydroxylase, *LEPREL1*, has been shown to be possibly associated with the disease through genetic polymorphism and by overexpression of the protein in disease cells. Validation of these findings should be attempted using patient materials from other Centres, and also by studying other fibrotic disorders. Furthermore the functional significance of the structural variant needs to be established. Potentially, inhibitors of the encoded P3H2 enzyme could be developed through high-throughput screening or informed drug design, but challenges to be met include the high cost and long timescale of such a project.

Spread of the disease process has been investigated with particular relevance to the pulmonary involvement. Migration of the SSc cells remains growth factor dependent, and becomes arrested when encountering complex pattern collagen matrix. Also SSc cells were shown to be sensitive to suppression by existing therapies, particularly when used in in combination, although there are reservations about the potential toxicity, in particular the risk of bleeding.

Furthermore, the concept of an inflammatory-dependent fibrotic process in SSc has been investigated using analsysis of tissue fluid taken at various stages of the disease, indicating a pro-fibrotic cytokine network in early stage dcSSc. Consistent with this model, specific anti-inflammatory peptides were shown to reduce downstream tissue remodeling in SSc-like skin and lung fibrosis. More comprehensive assessment in mouse model systems, as well as proof of concept studies using human cells are justified.

The beneficial effects of the anti-inflammatory peptides could be investigated in future clinical trials in SSc if the cost of drug development up to the level of clinically useful compounds can be justified. Additional challenges to be addressed in the therapeutic development include mode of delivery of therapeutic to the lesional tissues, plus the need for sensitive and responsive readout biomarkers. The blister fluid technique could be applied before and after therapy to provide biomarkers.

Based on these data, and when taken in the overall context of known published results, some new insights into the underlying biomechanisms have been suggested and are summarised in Figure 7.1. An attempt has been made

to link each mechanism to a therapeutic strategy in keeping with the initial overall ambition of the thesis.

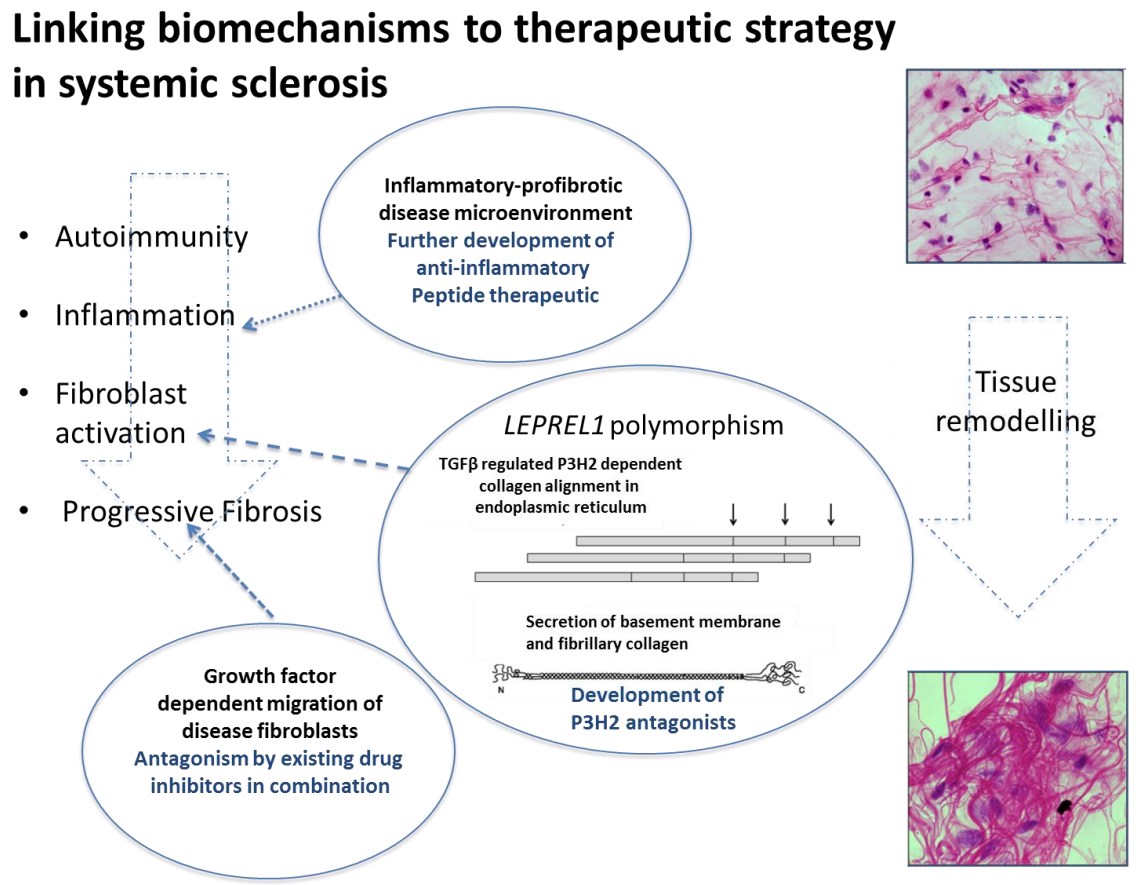


Figure 7.1 Linking biomechanisms to therapeutic approaches in systemic sclerosis Based on the information presented in this thesis, and the more general background information reviewed, the above biomechanisms are proposed. On the left hand side the time course of the disease is illustrated progressing from an autoimmune reaction, leading to inflammation and downstream fibrosis which progresses and spreads. The resulting tissue remodeling is illustrated on the right. An inflammatory pro-fibrotic microenvironment has been confirmed in this thesis, based on profiling of involved skin tissue fluid. Furthermore, anti-inflammatory peptides reversed the fibrosis seen in SSc-pattern mouse models confirming this as a therapeutic approach. A genetic polymorphism in a potential regulatory region of *LEPREL1* has been suggested based on CNV analysis. P3H2 could be a rate limiting enzyme in collagen synthesis in the disease, and is a potential target for small molecule treatments. Finally spread of the fibrotic process has been modelled revealing that the SSc fibroblasts remain growth factor dependent, arrest migration when encountering complex pattern collagen and remain sensitive to drug inhibitors which could be used in combination.

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Appendix

Supplementary methods and data

Supplementary methodology for Chapter 2

Methodology using online databases to identify sites of CNV in the first intron of target genes

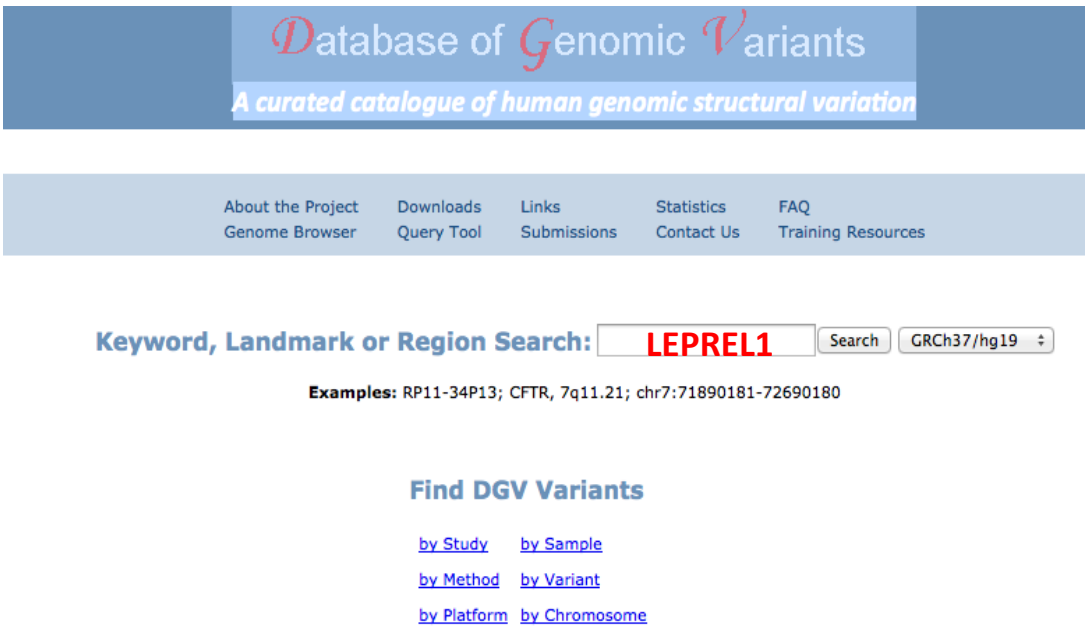

Figure S1 Localization of CNV within *LEPREL 1* This shows the first step in locating the candidate CNV (*LEPREL1* in this case) in the "Database of Genomic Variants."

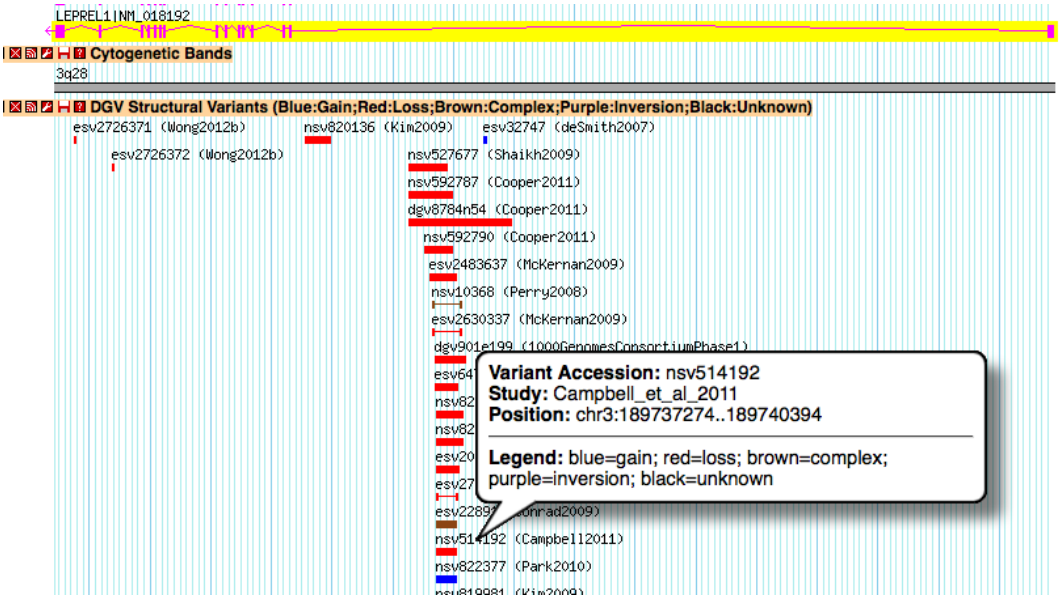

Figure S2 Genomic map of *LEPREL 1* CNVs This shows the second step in locating the candidate CNV in the Database of Genomic Variants, with the gene of interest at the top and the known CNVs listed below it.

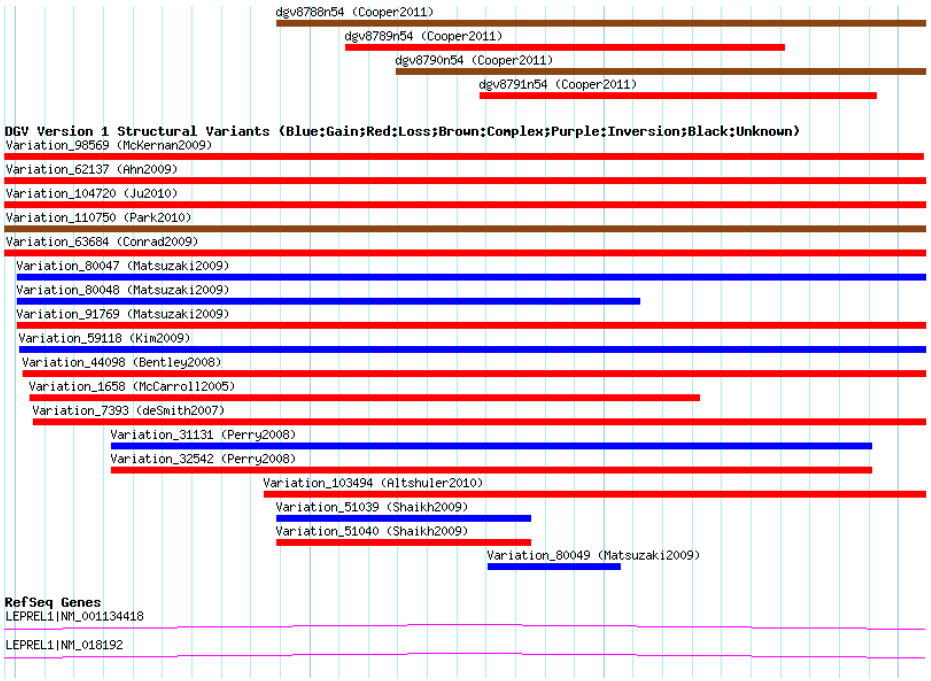

Figure S3 Coordinates of possible CNV sequences for *LEPREL1*

This shows the list of possible CNV sequences for *LEPREL1* revealed in the DGV database. The smallest region with the most overlapping evidence is shown at the bottom.

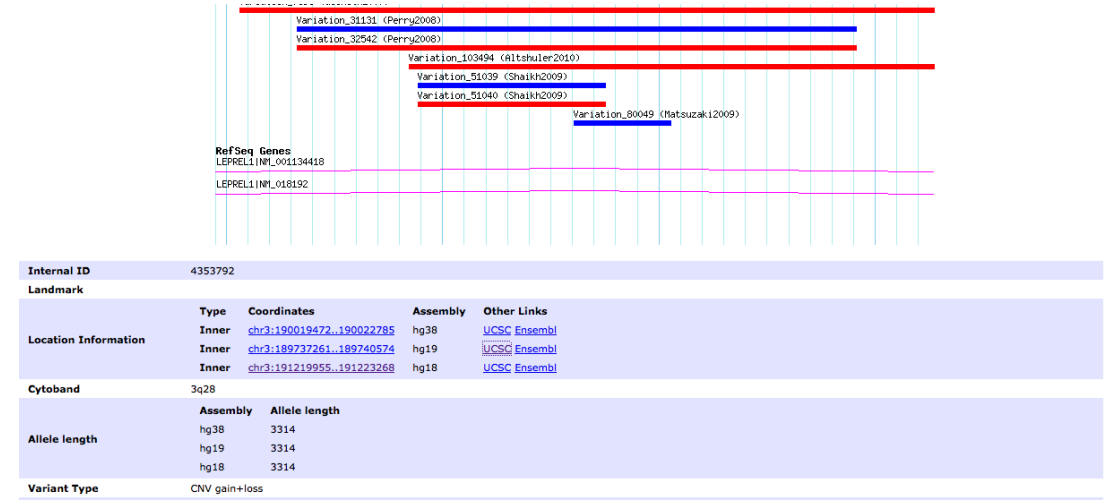

Figure S4 DGV web page showing link to UCSC genome browser website

Figure S5 Screening for repetitive elements A map of the genomic information about the sequence between the coordinates is shown. The "Repeat Masker" detected regions of highly repetitive sequence (repeating elements), as shown toward the bottom.

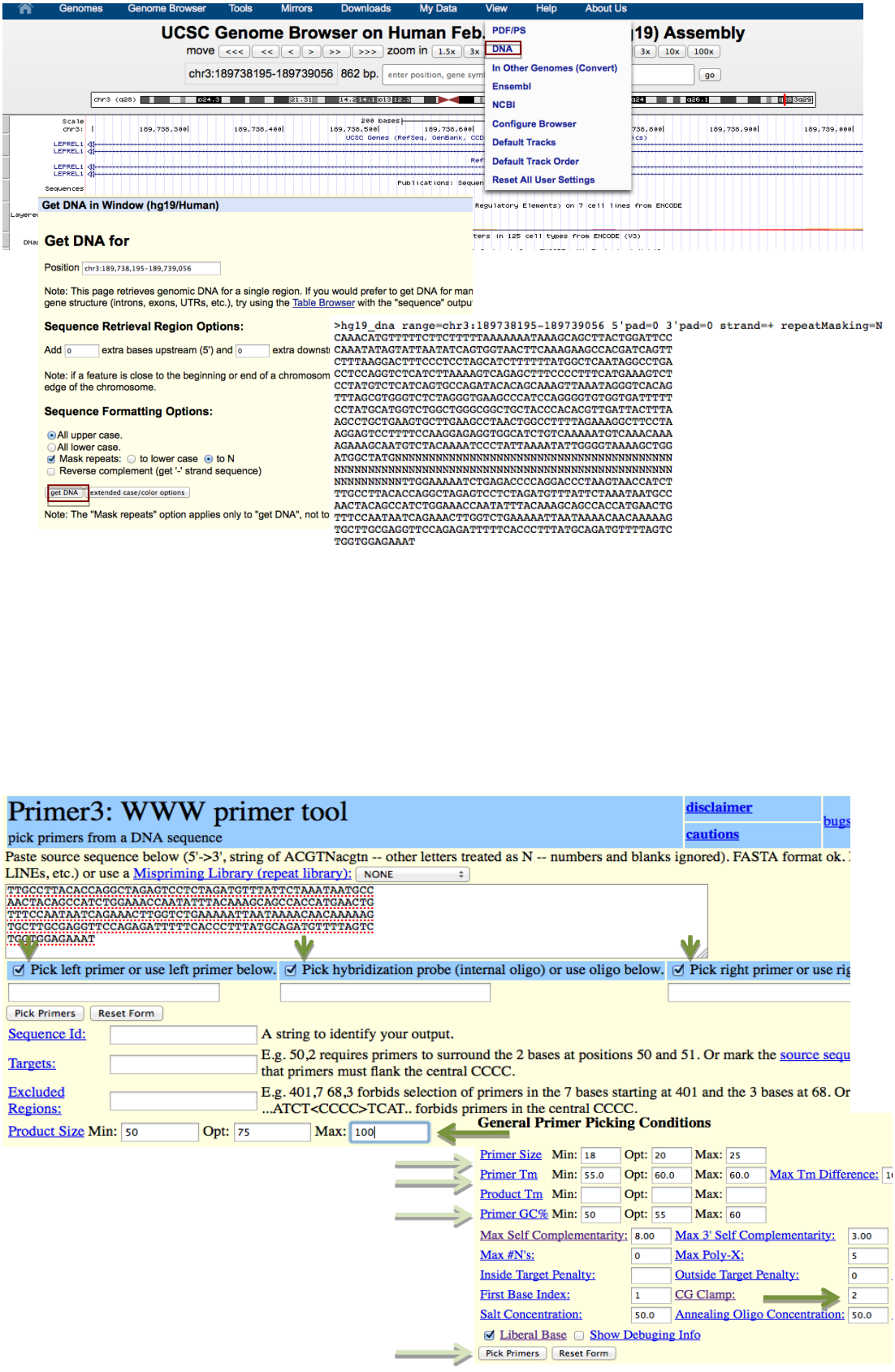

Figure S6 Selecting primers using Primer 3 PCR primers and probes to amplify the CNV were designed with Primer 3.

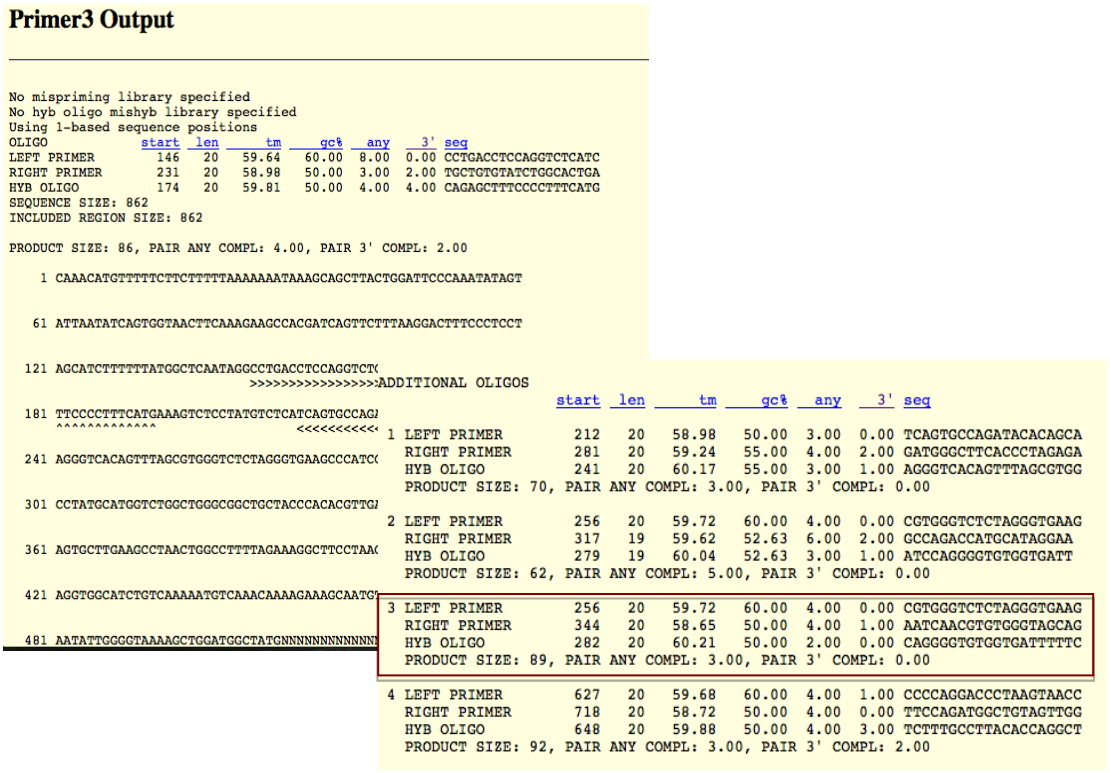

Figure S7 Selection of a primer pair A pair of primers was selected on Primer 3 to screen for sequence specificity within the human genome.

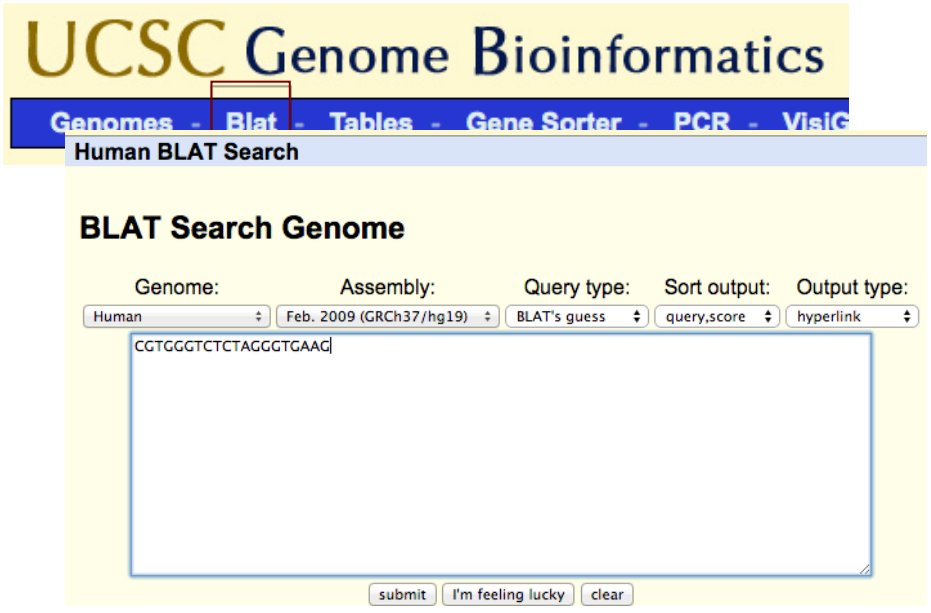

Figure S8 Using BLAT search to determine primer specificity

The BLAST-like alignment tool (BLAT) search on the UCSC Genome Bioinformatics page was used to determine primer specificity. In this instance only the sequence of interest in the *LEPREL1* was identified as homologous with the proposed primer sequence.

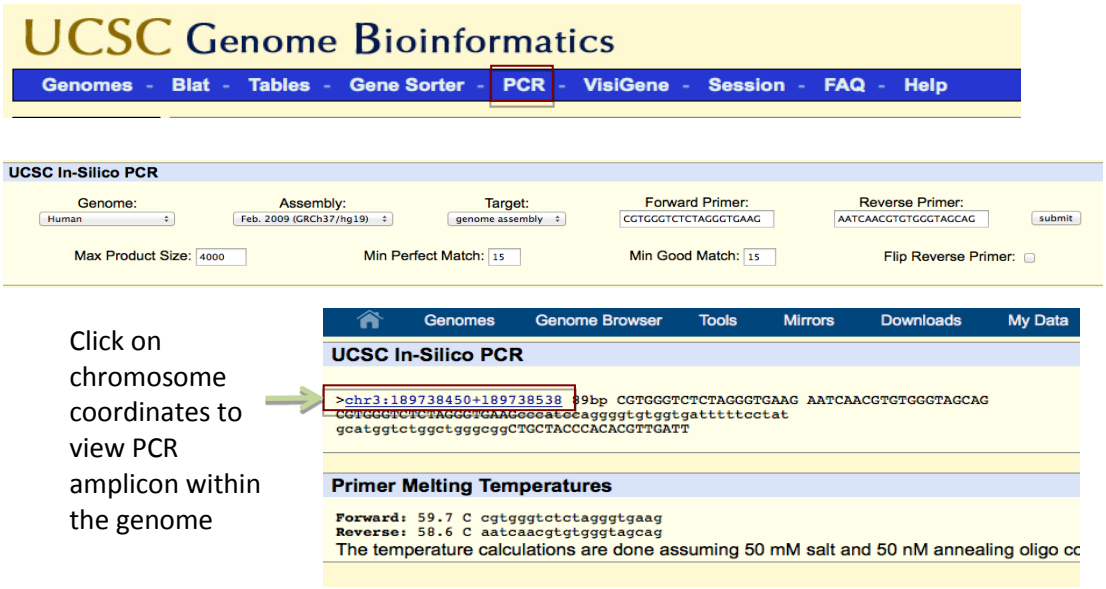

Figure S9 Screening selected primers (In-Silico PCR). Using primer/probe sequences that were specific for the appropriate genomic location, an *in silico* PCR search was conducted. The entire amplicon sequence was viewed in the genome browser along with the Tm of the primers.

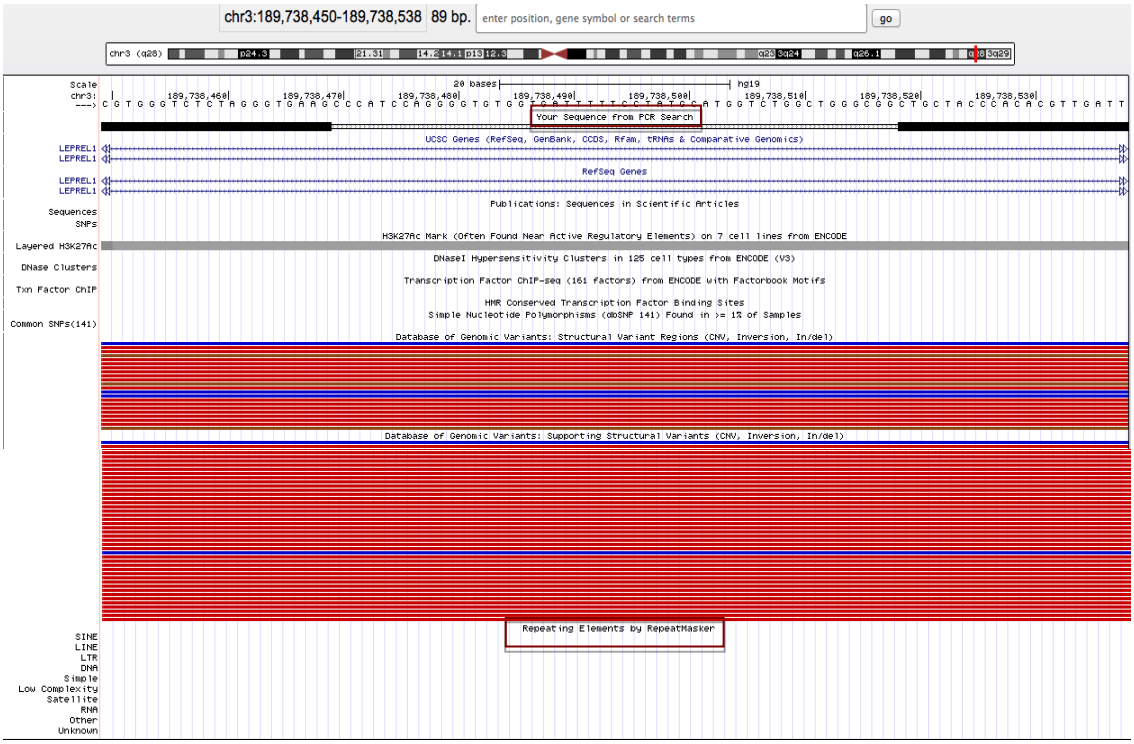

Figure S10 Amplicon sequence viewing in genome browser The amplicon sequence was viewed in the genome browser for inspection of the genomic features within the sequence and assurance that the appropriate genomic location was being amplified.

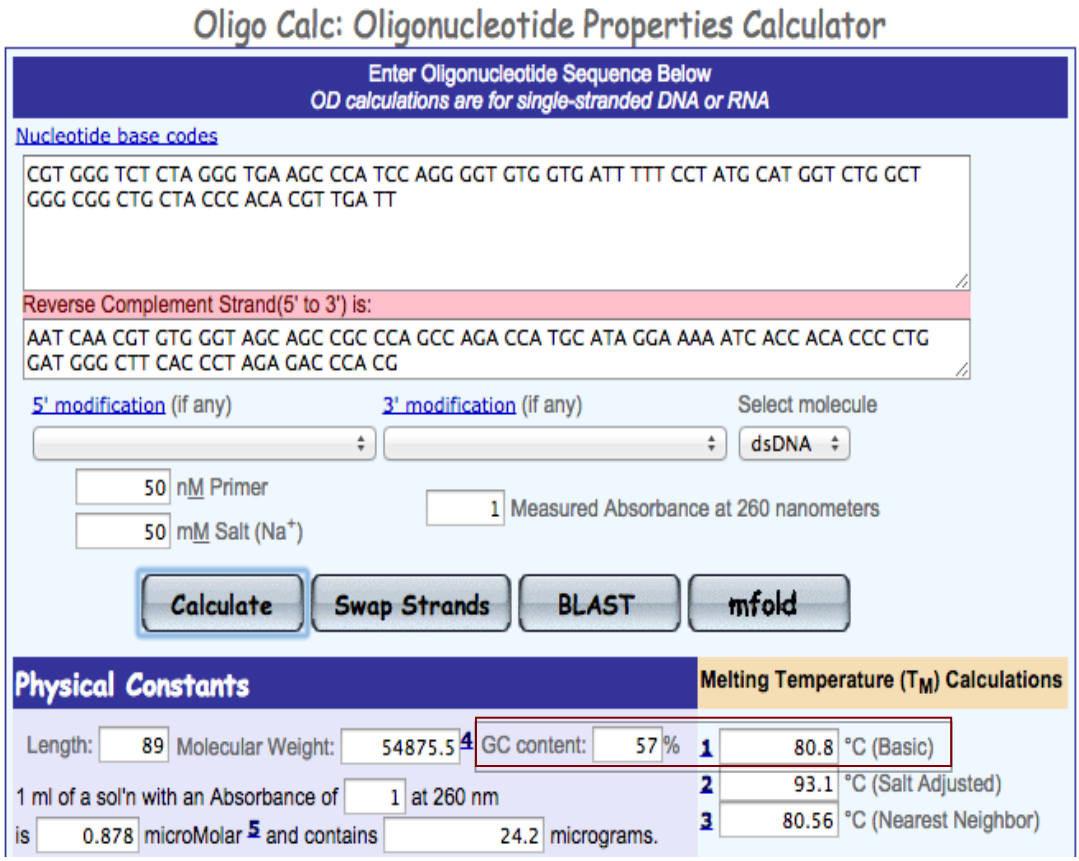

Figure S11 Using Oligo Calc to determine amplicon Tm The amplicon sequence was pasted into Oligo Calc to determine the Tm and GC content (%) of the amplicon, for later use when running SYBR green melt curve analysis.

Supplementary data for Chapter 3

Mice from WT, SKO, and DKO *LEPREL1* genetic background were treated by daily subcutaneous bleomycin injection and then euthanised on day 30. Cytokines were assayed by Luminex, and growth factors by separate ELISA. 6-12 mice were included per treatment group. Data were analysed by ANOVA, and where this indicated a significant difference between groups, Tukey's test was used for multiple comparisons. The statistical analysis is shown below.

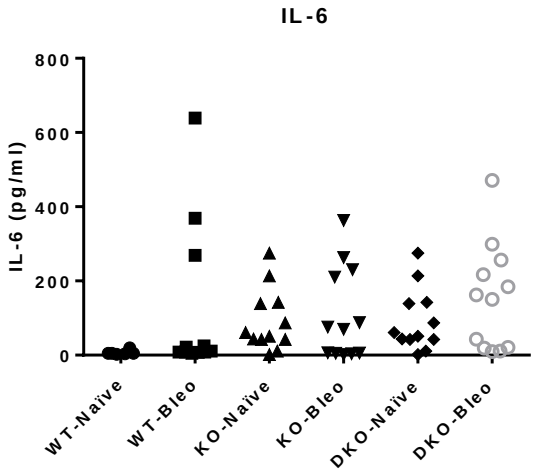

Figure S12 IL-6 levels in WT, SKO, and DKO mice day 30 in bleomycin skin injury model

ANOVA of IL-6 data	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	94625	5	18925	F (5, 59) = 1.117	P = 0.3614
Residual (within columns)	999789	59	16946		
Total	1.094e+006	64			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	65				

Table S1 Statistical analysis of plasma IL-6 levels day30 bleomycin skin injury model ANOVA was performed showing no statistically significant difference between groups.

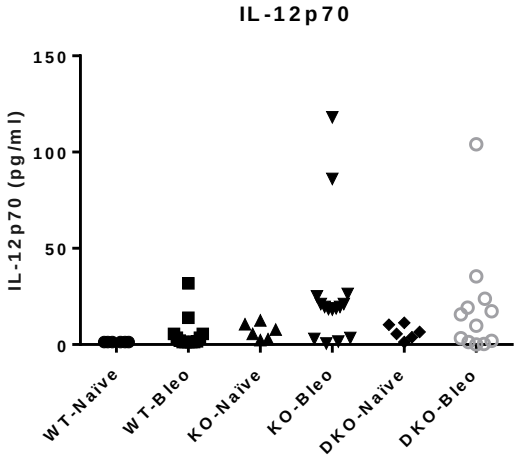

Figure S13 IL-12p70 levels in WT, SKO, and DKO mice day 30 in bleomycin skin injury model

Table Analyzed	IL-12p70				
ANOVA of IL-12p70 data					
F	2.195				
P value	0.0688				
P value summary	ns				
Are differences among means statistically significant? (P < 0.05)	No				
R square	0.1742				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	5246	5	1049	F (5, 52) = 2.195	P = 0.0688
Residual (within columns)	24860	52	478.1		
Total	30106	57			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	58				

Table S2 Statistical analysis of plasma IL-12p70 levels day30 bleomycin skin injury model ANOVA was performed showing no statistically significant difference between groups.

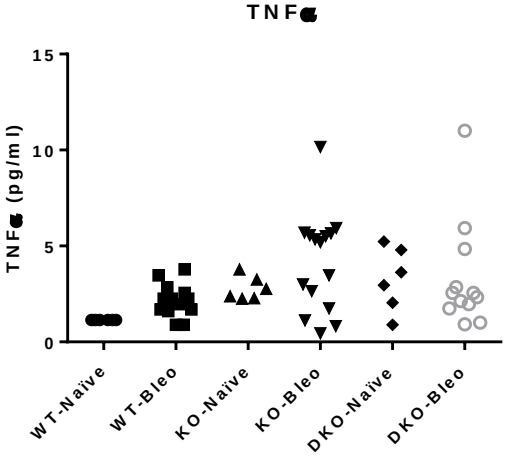

Figure S14 TNFα levels in WT, SKO, and DKO mice day 30 in bleomycin skin injury model

Table Analyzed	TNF				
ANOVA for TNFalpha data					
F	2.590				
P value	0.0364				
P value summary	*				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.1994				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	51.31	5	10.26	F (5, 52) = 2.590	P = 0.0364
Residual (within columns)	206.0	52	3.962		
Total	257.3	57			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	58				

Table S3 Statistical analysis of plasma TNFα levels day30 bleomycin skin injury model ANOVA was performed showing an overall statistically significant difference between groups. Comparisons between groups was performed by Tukey's test (see below).

Number of families	1				
Number of comparisons per family	15				
Alpha	0.05				
Tukey's multiple comparisons test for TNFα data	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
WT-Naïve vs. WT-Bleo	-1.018	-3.925 to 1.888	No	ns	0.9033
WT-Naïve vs. KO-Naïve	-1.638	-5.038 to 1.762	No	ns	0.7115
WT-Naïve vs. KO-Bleo	-3.001	-5.846 to -0.1566	Yes	*	0.0330
WT-Naïve vs. DKO-Naïve	-2.107	-5.507 to 1.293	No	ns	0.4542
WT-Naïve vs. DKO-Bleo	-2.172	-5.116 to 0.7729	No	ns	0.2636
WT-Bleo vs. KO-Naïve	-0.6199	-3.526 to 2.287	No	ns	0.9882
WT-Bleo vs. KO-Bleo	-1.983	-4.214 to 0.2487	No	ns	0.1084
WT-Bleo vs. DKO-Naïve	-1.088	-3.995 to 1.818	No	ns	0.8760
WT-Bleo vs. DKO-Bleo	-1.153	-3.511 to 1.204	No	ns	0.6984
KO-Naïve vs. KO-Bleo	-1.363	-4.208 to 1.482	No	ns	0.7163
KO-Naïve vs. DKO-Naïve	-0.4683	-3.868 to 2.932	No	ns	0.9985
KO-Naïve vs. DKO-Bleo	-0.5333	-3.478 to 2.411	No	ns	0.9944
KO-Bleo vs. DKO-Naïve	0.8947	-1.950 to 3.739	No	ns	0.9368
KO-Bleo vs. DKO-Bleo	0.8297	-1.451 to 3.110	No	ns	0.8886
DKO-Naïve vs. DKO-Bleo	-0.06500	-3.010 to 2.880	No	ns	> 0.9999

Table S4 Further statistical analysis of plasma TNFα levels day30 bleomycin skin injury model Multiples comparisons between groups was performed using Tukey's test, revealing a significant difference between WT controls and bleomycin treated KO.

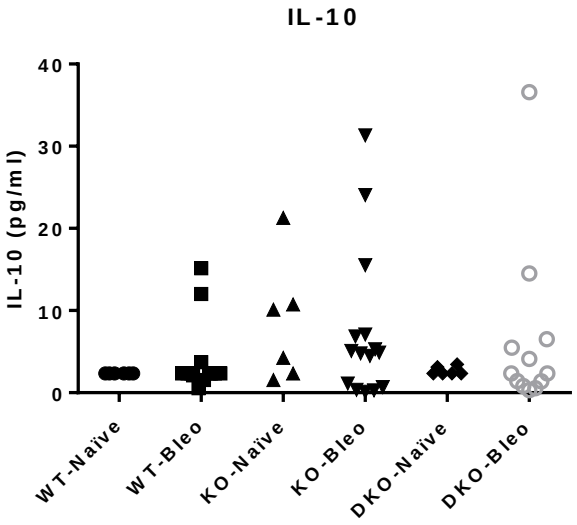

Figure S15 IL-10 levels in WT, SKO, and DKO mice day 30 in bleomycin skin injury model

Table Analyzed	IL-10				
ANOVA for IL-10 data					
F	0.9277				
P value	0.4707				
P value summary	ns				
Are differences among means statistically significant? (P < 0.05)	No				
R square	0.082				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	255.0	5	51.00	F (5, 52) = 0.9277	P = 0.4707
Residual (within columns)	2859	52	54.97		
Total	3114	57			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	58				

Table S5 Statistical analysis of plasma IL-10 levels day30 bleomycin skin injury model
ANOVA was performed showing overall no statistically significant difference between groups.

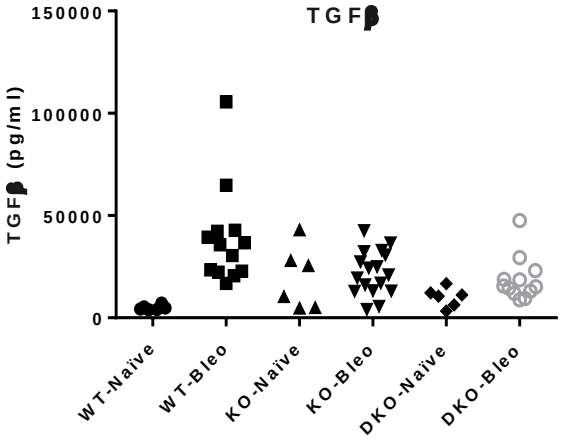

Figure S16 TGFβ levels in WT, SKO, and DKO mice day 30 in bleomycin skin injury model

Table Analyzed	TGFβ				
ANOVA for TGFβ data					
F	6.145				
P value	0.0001				
P value summary	***				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.3626				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	6.412e+009	5	1.282e+009	F (5, 54) = 6.145	P = 0.0001
Residual (within columns)	1.127e+010	54	2.087e+008		
Total	1.768e+010	59			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	60				

Table S6 Statistical analysis of plasma TGFβ levels day30 bleomycin skin injury model ANOVA was performed showing an overall statistically significant difference between groups. Further analysis comparing individual groups was performed, see below.

Number of families	1				
Number of comparisons per family	15				
Alpha	0.05				
Tukey's multiple comparisons test for TGFβ data	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
WT-Naïve vs. WT-Bleo	-33834	-54899 to -12768	Yes	***	0.0002
WT-Naïve vs. KO-Naïve	-14603	-39245 to 10039	No	ns	0.5053
WT-Naïve vs. KO-Bleo	-17069	-37336 to 3198	No	ns	0.1458
WT-Naïve vs. DKO-Naïve	-5133	-29775 to 19509	No	ns	0.9894
WT-Naïve vs. DKO-Bleo	-13901	-35241 to 7440	No	ns	0.3989
WT-Bleo vs. KO-Naïve	19230	-1835 to 40295	No	ns	0.0924
WT-Bleo vs. KO-Bleo	16765	1039 to 32490	Yes	*	0.0303
WT-Bleo vs. DKO-Naïve	28700	7635 to 49765	Yes	**	0.0024
WT-Bleo vs. DKO-Bleo	19933	2847 to 37019	Yes	*	0.0134
KO-Naïve vs. KO-Bleo	-2466	-22733 to 17802	No	ns	0.9992
KO-Naïve vs. DKO-Naïve	9470	-15172 to 34112	No	ns	0.8644
KO-Naïve vs. DKO-Bleo	702.7	-20638 to 22043	No	ns	> 0.9999
KO-Bleo vs. DKO-Naïve	11936	-8332 to 32203	No	ns	0.5123
KO-Bleo vs. DKO-Bleo	3168	-12924 to 19261	No	ns	0.9918
DKO-Naïve vs. DKO-Bleo	-8767	-30108 to 12573	No	ns	0.8282

Table S7 Further statistical analysis of plasma TGFβ levels day30 bleomycin skin injury model Multiples comparisons between groups was performed using Tukey's test, revealing a significant difference between WT controls and bleomycin treated WTs, and between WT treated with bleomycin and SKO and DKO treated with bleomycin, consistent with an effect of LEPREL1 silencing on the fibrotic response to bleomycin.

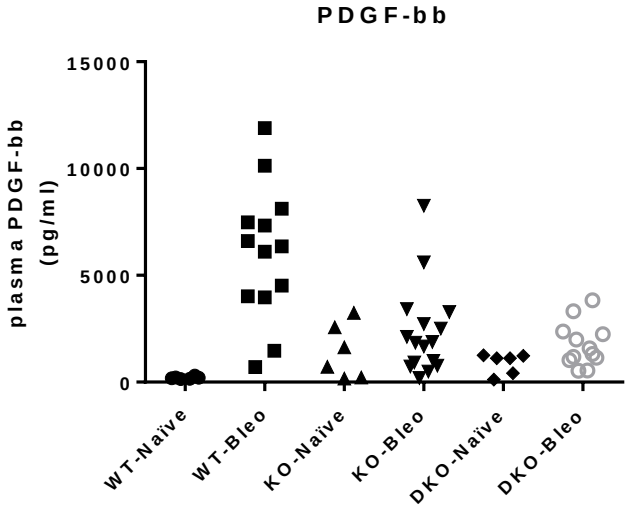

Figure S17 PDGF-bb levels in WT, SKO, and DKO mice day 30 in the bleomycin skin injury model

Table Analyzed	PDGF-bb				
ANOVA for PDGF-bb data					
F	11.58				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.5220				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2.254e+008	5	4.509e+007	F (5, 53) = 11.58	P < 0.0001
Residual (within columns)	2.064e+008	53	3.895e+006		
Total	4.319e+008	58			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	59				

Table S8 Statistical analysis of plasma PDGF-bb levels day30 bleomycin skin injury model ANOVA was performed showing an overall statistically significant difference between groups. Further analysis comparing individual groups was performed, see below.

Number of families	1				
Number of comparisons per family	15				
Alpha	0.05				
Tukey's multiple comparisons test for PDGF-bb data	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
WT-Naïve vs. WT-Bleo	-5854	-8734 to -2974	Yes	****	< 0.0001
WT-Naïve vs. KO-Naïve	-1224	-4593 to 2145	No	ns	0.8894
WT-Naïve vs. KO-Bleo	-2136	-4929 to 657.3	No	ns	0.2284
WT-Naïve vs. DKO-Naïve	-676.9	-4046 to 2692	No	ns	0.9910
WT-Naïve vs. DKO-Bleo	-1555	-4473 to 1362	No	ns	0.6175
WT-Bleo vs. KO-Naïve	4630	1750 to 7510	Yes	***	0.0002
WT-Bleo vs. KO-Bleo	3718	1540 to 5897	Yes	****	< 0.0001
WT-Bleo vs. DKO-Naïve	5177	2297 to 8057	Yes	****	< 0.0001
WT-Bleo vs. DKO-Bleo	4299	1963 to 6635	Yes	****	< 0.0001
KO-Naïve vs. KO-Bleo	-911.9	-3705 to 1881	No	ns	0.9268
KO-Naïve vs. DKO-Naïve	547.2	-2822 to 3916	No	ns	0.9967
KO-Naïve vs. DKO-Bleo	-331.1	-3249 to 2586	No	ns	0.9994
KO-Bleo vs. DKO-Naïve	1459	-1334 to 4252	No	ns	0.6378
KO-Bleo vs. DKO-Bleo	580.8	-1648 to 2809	No	ns	0.9713
DKO-Naïve vs. DKO-Bleo	-878.3	-3796 to 2039	No	ns	0.9473

Table S9 Further statistical analysis of plasma PDGF-bb levels day30 bleomycin skin injury model Multiple comparisons between groups was performed using Tukey's test. Significant differences were seen between PDGF-bb levels in WT controls and WT bleomycin treated mice. Other significant differences include WT bleomycin versus SKO bleomycin and WT bleomycin and DKO bleomycin.

Chapter 3: further supplementary data and analysis

Quantitation of dermal PSR red birefringence in the bleomycin skin fibrosis model: effect of genetic silencing of *LEPREL1*.

Mouse	RGB intensity	Mean	Mouse	RGB intensity	Mean	Mouse	RGB intensity	Mean
WT1	95.5		**SKO1**	98.3		**DKO1**	67.2	
	89.3			96.1			69.8	
	90.0			104.1			66.3	
	86.3	90.3		98.6	99.3		68.6	68.1
WT2	78.2		**SKO2**	106.9		**DKO2**	63.1	
	87.4			109.8			63.8	
	105.2			101.1			71.7	
	84.6	88.9		97.4	103.8		66.3	66.2
WT3	77.1		**SKO3**	94.8		**DKO3**	71.5	
	76.1			102.9			75.4	
	86.2			124.4			72.4	
	86.9	81.6		95.9	104.5		73.4	73.2

Table S10 Measurement of PSR birefringence On day 30 mice were euthanised and skin biopsy material taken at the site of bleomycin injury. Skin was stained by PSR and then imaged under polarized light. Red polarized light intensity was measured using RGB converted images in ImageJ. 4 rectangular 200 x 400µm dermal areas per mouse section were quantified as above**.**

ANOVA summary	
F	58.56
P value	0.0001
P value summary	***
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.9513

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
WT vs. SKO	-15.61	-25.08 to -6.140	Yes	**	0.0056
WT vs. DKO	17.77	8.302 to 27.25	Yes	**	0.0029
SKO vs. DKO	33.39	23.91 to 42.86	Yes	****	< 0.0001

Table S11 Statistical analysis of dermal PSR intensity ANOVA showed a significant difference between groups. Tukeys test confirmed increased PSR red polarized staining in the SKO mice and reduced PSR staining in the DKO.

Chapter 6: supplementary data and analysis

Bleomycin dermal fibrosis model, effect of the therapeutic peptide RP107.

Mouse/ treatment	Dermal thickness Measure 1	Dermal thickness Measure 2	Dermal thickness Measure 3	Mean (µm)	SD	Overall Mean (µm)

Control 1	313	313	333	319	12	**343**
Control 2	413	393	313	373	53	
Control 3	343	353	313	336	21	
Bleomycin 1	403	413	463	426	32	**402**
Bleomycin 2	413	463	413	429	29	
Bleomycin 3	463	413	433	436	25	
Bleomycin 4	363	363	363	363	0	
Bleomycin 5	413	313	413	379	58	
Bleomycin 6	413	363	353	376	32	
Bleomycin 7	423	383	413	406	21	
Bleomycin 8	363	383	383	376	12	
Bleomycin 9	423	413	443	426	15	
Bleomycin 10	413	423	373	403	26	
Bleo+RP 1	333	363	363	353	17	**345**
Bleo+RP 2	323	333	353	336	15	
Bleo+RP 3	363	393	363	373	17	
Bleo+RP 4	333	353	333	339	12	
Bleo+RP 5	333	323	313	323	10	
Bleo+RP 6	363	353	323	346	21	

Table S12 Dermal thickening in the bleomycin induced skin fibrosis model:effect of the RP 107 therapeutic peptide.

Table Analyzed	Peptide bleomycin
ANOVA summary	
F	13.42
P value	0.0004
P value summary	***
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.6266

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant ?	Summary	**Adjusted P Value**
Control vs. Bleomycin	-59.33	-100.6 to -18.10	Yes	**	**0.0051**
Control vs. Bleo+peptide	-2.333	-46.63 to 41.96	No	ns	**0.9899**
Bleomycin vs. Bleo+peptide	57.00	24.65 to 89.35	Yes	***	**0.0009**

Table S13 Statistical analysis of dermal thickness. As expected treatment with bleomycin led to increased dermal thickness (p<0.0051). Treatment with the peptide prevented the bleomycin induced dermal thickening (p<0.0009

Statistical analysis of the cytokine levels in Bleomycin dermal fibrosis model was performed: assaying the effect of the anti-inflammatory peptide.

Cytokine	**IL-6**		**IL-12**		**TNFα**		**MMP1**		**MMP9**	
Treatment	**Bleo**	**Bleo +RP 107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**
Mouse 1	136	41	40	0	5	3	134239	92714	281288	65146
Mouse 2	110	21	38	0	5	3	122973	94316	74487	85145
Mouse 3	158	34	51	0	4	3	142744	73065	303012	81917
Mouse 4	143	18	41	0	4	2	156797	95974	347773	108577
Mouse 5	80	18	35	0	3	0	109341	103491	197467	54738
Mouse 6	110	16	33	0	3	2	91066	84918	73983	90840
Mouse 7	57		29		2		82989		86234	
Mouse 8	82		37		2		100818		121131	
Mouse 9	67		46		3		122151		77283	
Mouse 10	75		30		2		67639		68779	
Mean	**101**	**24**	**38**	**0**	**3**	**2**	**113076**	**90746**	**163144**	**81061**
SD	35	10	7	0	1	1	27965	10516	109824	19059
SEM	11	3	2	0	0.38	0.38	8843	4293	34729	7780
P		**$1.8x10^{-04}$**		**$1.5x10^{-07}$**		**NS**		**NS**		**NS**

Table S14 Levels of cytokines and MMPs in mice treated with subcutaneous Bleomycin with or without therapeutic RP-107 peptide: day 50. Plasma values for each mouse studied are shown, plus means and standard deviations. P values are for Student's t test, which was used because in general the data followed a Gaussian distribution. P values are corrected for multiple comparisons (n=5) by Bonferroni correction.

Bleomycin lung fibrosis model: effect of the RP 107 therapeutic peptide on cytokine and MMP levels

Cytokine	**IL-6**		**IL-12**		**TNFα**		**MMP1**		**MMP9**	
Treatment	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**	**Bleo**	**Bleo+ RP107**
Mouse 1	65	9	260	0	1.6	1.2	93031	60580	84225	41219
Mouse 2	58	8	186	0	3.6	1.4	75735	82199	81877	38287
Mouse 3	56	7	195	0	1.6	0	78522	66745	76409	31123
Mouse 4	49	9	253	0	3.2	0	98014	70267	96546	41218
Mouse 5	95	8	165	0	4	1.2	101012	52352	75217	32159
Mouse 6	101	8	175	0	2.5	0	120092	68685	74128	40645
Mouse 7	54	5	68	0	2.1	0	118241	58981	86248	40581
Mouse 8	53	8	70	0	3.2	1.1	113852	72895	125141	41196
Mouse 9	16	3	0	0	3.2	0	99187	61001	72692	41548
Mouse 10	33	7	147	0	1.6	1.5	79858	45572	86139	32377
Mean	**58**	**7**	**152**	**0**	**2.66**	**0.64**	**97754**	**63928**	**85862**	**38035**
SD	25	2	83	0	0.89	0.68	16289	10542	15598	4350
SEM	1	0.085	3.5	0	0.53	0.21	5700	2279	4936	1532
P		**0.0005**		**0.0014**		**0.001**		**0.0019**		**$1.5x^{10-5}$**

Table S15 Levels of cytokines and MMPs on day 30 in mice treated with intratracheal Bleomycin followed by saline or therapeutic RP-107 peptide. Plasma values for each mouse studied are shown, plus means and standard deviations. P values are for Student's t test, which was used because in general the data followed a normal distribution. P values are corrected for multiple comparisons (n=5) by Bonferroni correction.

NHS
Health Research Authority
NRES Committee London- Hampstead
HRA
Research Ethics Committee (REC) London Centre
Ground Floor
Skipton House
80 London Road
London
SE1 6LH

Tel: 020 797 22580

Favourable opinion of a substantial amendment

30 November 2012

Professor Chris Denton
Department of Rheumatology
Royal Free Hospital
Pond Street
London
NW3 2QG

Dear Professor Denton

Study title:	**Elucidating the pathogenesis of systemic sclerosis by studying the skin, tissue and blood samples from patients and healthy volunteers.**
REC reference:	**6398**
Amendment number:	**Amendment 4**
Amendment date:	**05/Sep/2012**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

No ethical issues.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

[List documents with version numbers and dates]

Document	*Version*	*Date*
[name of document]	[1]	[27 September 2004]
Cover Letter		05/10/2012
Notice of Substantial Amendment	Amendment 4	05/09/2012
Protocol (Track Changed)	4	05/09/2012
Protocol Clean	4	05/09/2012
Patient Information sheet and consent form (Track Changed)	5	05/09/2012
Patient Information sheet and consent form (Clean)	5	05/09/2012

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

6398	**Please quote this number on all correspondence**

Yours sincerely

Stephanie Ellis
Chair
E-mail: NRESCommitee.London-Hampstead@nhs.net

Royal Free Hampstead NHS
NHS Trust

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Dr Geraldine Brough – Associate Specialist

INFORMATION SHEET AND CONSENT FORM FOR STUDY PARTICIPANTS

Elucidating the pathogenesis of systemic sclerosis by studying skin and blood samples from scleroderma patients and healthy volunteers

(Understanding the cause of scleroderma)

Principal Investigator: **Professor C Denton PhD, F.R.C.P**
Co-investigators: **Professor D Abraham PhD**
Dr G Coghlan M.D, F.R.C.P

You are being invited to take part in a research study that may help us understand the cause and mechanisms of scleroderma. Before you decide whether to take part you will need to understand why the research is being carried out and what it will involve. Please take time to read the following information carefully and discuss it with anyone you want. Please ask your Doctor or other study staff about anything in this form, for example any words and expressions that you have questions about or that you do not understand. Do not sign the form unless you are satisfied with the answers to your questions and decide that you want to take part in this study.

What is the purpose of the study?

Scleroderma develops differently in each patient but we do not currently understand why particular patient's progress in different ways. It is thought that there are likely to be many complicated reasons including levels of excess collagen in the body tissues, changes to cells within the lining of the blood vessels and the different types of cells activated by the immune system.

The aim of this research is to understand better the cause and mechanisms underlying the development of scleroderma and related conditions.

Why have I been chosen?

You have been invited to take part because you have scleroderma or a condition related to scleroderma and are being seen in the connective tissue disease clinic at the Royal Free Hospital.
You may also have been invited to take part as a healthy control for this study.

We will be including over 1000 patients with scleroderma, Raynaud's Phenomenon and other diseases related to scleroderma. Up to 300 healthy control subjects will also be taking part.

Do I have to take part?

It is entirely up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time, without giving reason. A decision to withdraw at any time or to take part, will not affect your care in any way. Your study doctors can also withdraw you from the study at any time if it is in your best interest or if you are unable to comply with the requirements of the study. In addition, Your medical team will continue to care for you normally whether you are in the study or not. If you decide to withdraw from the study, any samples or information collected during your participation will be kept on record but no new samples or information will be collected from you.

What will happen to me if I take part?

If you agree to take part you will need to spend up to 45 minutes extra in the clinic on one occasion. This will be in addition to the time you spend in clinic for your routine appointment. You will be asked to give a urine sample, and 40ml sample of blood (about 4 tablespoons). If you have limited scleroderma you will be asked to give one small (4mm) sample of skin tissue (a biopsy). If you have diffuse scleroderma you are asked to give two samples of skin tissue (one from involved, hardened skin and another from uninvolved skin). If you are a healthy volunteer only one biopsy will usually be taken.

One of the blood samples taken will be to look for genes that may tell us why some patients develop scleroderma and why others do not. This sample would be anonymous and could not be traced back to you. Due to the strict laws governing research into genes this sample would be considered a gift from you to the hospital. The samples will be kept for up to 10 years.

You may also be invited to undertake skin blister fluid analysis. This involves using a suction apparatus to raise a small skin blister. The blister fluid can then be removed using a small needle.

What do I have to do?

You can carry on with your daily activities as normal. You should keep the dressing over the biopsy site(s) dry for 3 days. You should continue on your regular medications, unless these are changed as a part of treatment for your scleroderma.

What are the possible disadvantages and risks of taking part?

You may experience some local discomfort whilst blood samples are taken. This may include pain, inflammation, infection or a small bruise at the puncture site. Local anaesthetic will be used during the skin biopsies to minimise discomfort however you may suffer some pain or stinging during the injection of local anaesthetic. The site of the biopsies may also be sore for a few days after the biopsy. There is also a small risk that the biopsy site may become infected.

You have the right to change your mind and withdraw from the study at anytime.

What are the possible benefits of taking part?

There is no guarantee that you will benefit directly from the research study. However information obtained from this research study may contribute to a better understanding of your disease and maybe useful in preventing worsening of your disease or selecting medicines for your future treatment.

What if something goes wrong?

If you have concerns about any aspect of this study, you should ask to speak with the researchers, who will do their best to answer your questions. If you are unhappy and wish to complain formally you can do this through the NHS complaints procedure. Details can be obtained from the Royal Free Hospital.

If you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone else's negligence, then you may have grounds for legal action. Regardless of this if you wish to complain or have concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Your study doctor will inform your GP that you are participating in a research study and we ask for your permission to do this and for your doctor's name and phone number. If your Consultant is not the study doctor we will also keep him/her fully informed.

Will my taking part in this study be kept confidential?

Your name will not be disclosed outside the hospital clinic. If you consent to take part in the research, your trial records may be inspected only be authorized individuals concerned with the trial. Your records are protected by security arrangements and are not accessible to anyone else. They may also be looked at by regulatory authorities to check that the study is being carried out correctly.

Throughout the study you have the right to ask what kind of data is recorded on you. You also have the right to ask for corrections to be done to the records. Furthermore you have the right to ask who keeps the data and who has access to it. If you withdraw your consent you have the right to request that all previously retained identifiable samples will be destroyed and that no new information/data will be collected and added to the existing database.

What will happen to the results of the research study?

This research will allow doctors to identify important factors causing sclerodema. The findings will be made available to scientists and doctors through reports and publications. You will not be identified in any report or publication.

Who is organizing and funding the research?

The Arthritis Research Campaign is funding and sponsoring this study, which is being conducted at the Centre for Rheumatology at the Royal Free Hospital Hampstead NHS Trust London. The study is being run by medical staff at the Royal Free Hospital. No compensation payments will be received by patients for taking part in the study.

Who has reviewed the study?

This study has been reviewed by the Arthritis Research Campaign (UK), the largest rheumatology research charity in the UK.

Additionally the study protocol has been fully reviewed by the Royal Free Hospital Ethics Committee, which is completely independent of the study. The committee includes healthcare professionals as well as non-medical people. All members of the committee are completely independent from anyone organizing the study.

Whom do I contact if I have questions or problems?

If you have questions about this study or if you suffer any side effects as a result of taking part in this trial, please contact medical staff on the numbers below.

Contact Details:

During normal working hours:	Contact the clinical trials nurses on 020 317 7544
Outside working hours in an emergency:	Call the main switchboard on 020 7794 0500 and ask for the Rheumatology doctor on call

If you decide to take part in this study, we would like to thank you for your help. You will be given a copy of this information sheet and the signed consent form to keep.

Thank you for reading this information sheet.

Royal Free Hampstead NHS
NHS Trust

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Christopher Denton – Professor of Rheuamatology
Dr Geraldine Brough – Associate Specialist

Patient Identification Number for this trial:
Hospital number:
Diagnoses:

CONSENT FORM FOR STUDY PARTICIPANTS

Title of Project: **Elucidating the pathogenesis of systemic sclerosis by studying skin and blood samples from scleroderma patients and healthy volunteers**
(Understanding the cause of scleroderma)

Name of Researcher: Professor Denton
Please initial box

1. I confirm that I have read and understand the information sheet. I have had the time to consider it and the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at anytime, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Centre for Rheumatology or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I consent to my G.P being told that I am taking part in this study.

5. I agree to take part in the above study.

_____ _____ _____
Name of Patient Date Signature

_____ _____ _____
Name of Person taking consent Date Signature

Patient Identification Number for this trial:

Royal Free Hampstead NHS
NHS Trust

(2 copies need signing, one to be given to the phlebotomist and one for the patient to keep)

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