EXPRESSION AND REGULATION OF MONOCYTE CHEMOATTRACTANT PROTEIN-3 (MCP-3) IN FIBROSIS

VOON H. ONG

A thesis submitted for the degree of Doctor of Philosophy at
University College London

Centre for Rheumatology and Connective Tissue Diseases
Royal Free and University College Medical School
University College London

2009

Statement of Contribution

I, Voon Hong Ong, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Dr Voon H. Ong

Abstract

Systemic sclerosis is a multisystem connective tissue disease characterised by skin thickening and widespread, but variable, visceral fibrosis. The aetiopathogenesis is likely to involve immunological activation and microvascular dysfunction leading to excessive accumulation of extracellular matrix (ECM) with increased production of collagen type I in lesional tissues. This implies a dysregulated repair process probably as a consequence of aberrant crosstalk between fibroblasts and inflammatory cells. It has been proposed that a hierarchical cascade of soluble mediators in which initial induction of proinflammatory cytokines expressed by the inflammatory infiltrate may lead to expression of profibrotic mediators including TGFβ. A salient feature of the inflammatory response is directional migration of leucocytes into subendothelial tissues orchestrated by chemokines in a spatially and temporally-regulated multistep process. Work described in this thesis explores the expression of chemokine, monocyte chemoattractant protein-3 (MCP-3/CCL7) in SSc and in murine models for SSc: type 1 tight skin mouse (Tsk1) and a transgenic mouse strain (TβRIIΔk) in which there is fibroblast-directed disruption of TGFβ signalling. The hypothesis that crosstalk between MCP-3 and TGFβ may modulate the signalling response in the fibrotic microenvironment was also explored.

Overexpression of MCP-3 was demonstrated on cDNA expression profiling and protein analysis of neonatal Tsk1 and TβRIIΔk fibroblasts. This was supported by immunohistochemical studies on dermal tissues. Similar upregulation dermal patterns of MCP-3 protein expression were observed in the early stage of diffuse cutaneous SSc. Activation of collagen reporter genes by MCP-3 in transgenic mouse fibroblasts and wildtype neonatal mouse fibroblasts harbouring proα2(I)collagen promoter reporter gene constructs is mediated via sequences within the proximal promoter and is partly dependent on TGFβ. This coinduction between the two factors in the fibrotic response is also demonstrated by activation of TGFβ signalling pathways by MCP-3 leading to type I collagen secretion. In addition, MCP-3 gene expression is stimulated by TGFβ.

Comparison of downstream signalling pathways that regulate collagen gene activation by both cytokines confirms the central role of MAPK pathway activation in mediating the effects of both factors. An additive effect of these two agonists was demonstrated for key TGFβ-regulated genes on comparative microarray analysis.

Overall, these results demonstrate that overexpression of MCP-3 is a key biochemical feature of early stage SSc and murine models of SSc, and suggest a novel role for this chemokine as a profibrotic mediator in addition to its role in regulating leucocyte recruitment. Furthermore, there is a potentially important interplay between MCP-3 and TGFβ in modulation of the signalling response in the fibrotic microenvironment.

Acknowledgements

I would like to thank my supervisors Professors Christopher Denton, David Abraham and Carol Black for giving me the opportunity to work in a fascinating and challenging aspect of medical research of Systemic Sclerosis. I would like to thank in particular Chris for his endless support and encouragement.

The assistance of many other staff members, without whom my work will not come into fruition, is also gratefully acknowledged. Particular thanks are expressed to Dr Shi-Wen Xu for his tireless assistance with fibroblast cultures, Dr Gisela Lindahl for her valuable insight on cloning, Dr Alan Holmes, Dr Maresa Carulli, Lowri Evans and Korsa Khan for their helpful guidance and practical assistance. The MCP-3 promoter contained within the pUC19 plasmid cloning vector was kindly provided by Professor Van Damme, Rega Institute, Belgium.

I would like to acknowledge funding support through grants from the Wellcome Trust and the Raynaud's and Scleroderma Association. Finally, this thesis would not have been possible without the love and support from my family and close friends, JF, Julian and Duke.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 6

Chapter 7

List of Tables

Abbreviations

ACA	Anti-centromere antibodies
ACE	Angiotensin converting enzyme
ALK	Activin receptor-like kinase
ARA	American College of Rheumatology
ATA	Anti-toposisomerase antibodies
µl	Microlitres, volume
Ab	Antibody
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BMP	Bone morphogenetic protein
BMPR2	Bone morphogenetic protein receptor type 2
°C	Degrees Celsius, temperature
CO_2	Carbon dioxide
Col1	Collagen, type 1
CM	Conditioned media
CMV	Cytomegalovirus
CTGF	Connective tissue growth factor
DARC	Duffy Antigen Receptor for Chemokine
DcSSc	Diffuse cutaneous systemic sclerosis
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EMT	Epithelial-mesenchymal transition
ET-1	Endothelin-1
FCS	Fetal calf serum
FGF7	Keratinocyte growth factor
Fbn1	Fibrillin1
FN	Fibronectin
FVC	Forced vital capacity
h	Hour, time
GAG	glycosaminoglycans
GPCR	G-protein-coupled-receptors
GVHD	Graft versus host disease
HGF	Hepatocyte Growth Factor
HLA	Human leucocyte antigen
HRP	Horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
ICAM-1	Inter-Cellular Adhesion Molecule 1

IIP	Idiopathic interstitial pneumonia
ILD	Interstitial lung disease
IGFBP	Insulin-like growth factor binding protein
IL-1	Interleukin-1
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IFNγ	Interferon-gamma
kd	Kilodaltons, weight
KGF	Keratinocyte growth factor
KL-6	Klebs von den Lungen-6
LAP	Latency-associated peptide
LB medium	Luria-Bertani medium
LcSSc	Limited cutaneous systemic sclerosis
LDL	Low-density lipoprotein
LFA-1	Lymphocyte function-associated antigen
LLC	Large latent complex
LPS	Lipopolysaccharide
LTBP	Latent TGF-β-binding protein
M	Molar
MAGP-2	microfibril-associated glycoprotein 2
MAPK	Mitogen-activated protein kinases
MCS	Multiple cloning site
MHC	Major histocompatibility complex
min	Minute, time
MIP-1α	Macrophage Inflammatory Protein 1α
ml	Millilitre, volume
MMP	Matrix metalloproteinase
MCP-1	Monocyte chemoattractant protein-1
MCP-3	Monocyte chemoattractant protein-3
mRNA	Messenger RNA
mRSS	Modified Rodnan skin score
MSC	Mesenchymal stem cell
mTOR	mammalian target of rapamycin
ng	Nanogram, weight
NSF	Nephrogenic systemic fibrosis
PAH	Pulmonary arterial hypertension
PAI	Plasminogen activator inhibitor
PAP	Pulmonary arterial pressure
PI3-kinase	Phosphatidylinositol-3-OH kinase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphoinositol-3-kinase
PKB	Protein kinase B

PMA	Phorbol 12-myristate 13-acetate
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RDU	Relative density units
RNA	Ribonucleic acid
RNP	Ribonuclear protein
ROS	Reactive oxygen species
rpm	Revolutions per minute, speed
R-Smad	Receptor-regulated Smad
SARA	Smad anchor for receptor activation
SBE	Smad-binding element
SD	Standard Deviation
SDF-1	Stromal-cell derived factor-1
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
sec	second, time
SiO_2	Silica dioxide
SLC	Small latent complex
SLE	Systemic lupus erythematosus
SLRPs	Small leucine-rich family of proteogylcans
Smad	A merger of Sma from *Caenorhabditis elegans* and Mad from *Drosophila mothers against decapentaplegic*
SNP	Single nucleotide polymorphism
SPARC	Secreted protein, acidic and rich in cysteine
SRC	Scleroderma renal crisis
SSc	Systemic sclerosis
SSc-ILD	Systemic sclerosis associated interstitial lung disease
TAK1	TGFβ activated kinase
TBS	Tris buffered saline
TE buffer	Tris EDTA buffer
TGFβ	Transforming growth factor-beta
TGFβR2	TGFβ-receptor II
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
Tsk1/+	Tight skin mouse
TSP1	Thrombospondin1
UIP	Usual Interstitial Pneumonia
V	Voltage, power
VEGF	Vascular endothelial growth factor

PUBLICATIONS ARISING FROM WORK INCLUDED IN THIS THESIS

Papers

VH Ong, LA Evans, SW Xu, IB Fisher, V Rajkumar, DJ Abraham, CM Black, CP Denton. Monocyte chemoattractant protein 3 as a mediator of fibrosis: Overexpression in systemic sclerosis and the type 1 tight-skin mouse. **Arthritis Rheum.** 2003 Jul;48(7):1979-91.

CP Denton, B Zheng, LA Evans, X Shi-wen, VH Ong, I Fisher, K Lazaridis, DJ. Abraham, CM Black, B de Crombrugghe. Fibroblast-specific expression of a kinase-deficient type II TGFβ receptor leads to paradoxical activation of TGFβ signalling pathways with fibrosis in transgenic mice. **J Biol Chem**. 2003 Jul 4;278(27):25109-19.

CP Denton, GE Lindahl, K Khan, SW Xu, VH Ong, NJ Gaspar, K Lazaridis, DE Edwards, A Leask, EA Renzoni, GB Gharios, DJ Abraham, CM Black. Activation of key profibrotic mechanisms in transgenic fibroblasts expressing kinase-deficient type II TGFβ receptor (TβRIIΔk). **J Biol Chem.** 2005 Apr 22;280(16):16053-65.

Management of systemic sclerosis. VH Ong, G Brough, CP Denton. **Clin Med.** 2005 May-Jun;5(3):214-9.

MT Carulli, VH Ong, M Ponticos, SW Xu, DJ Abraham, CM Black, CP Denton. Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts: evidence for autocrine regulation of myofibroblast differentiation. **Arthritis Rheum.** 2005 Dec;52(12):3772-82.

VH Ong, M Carulli, SW Xu, K Khan, G Lindahl, DJ Abraham, CP Denton. Cross-talk between MCP-3 and TGFβ promotes fibroblast collagen biosynthesis. **Exp Cell Res.** 2008 Nov 12. [Epub ahead of print]

Book Chapter

V Ong, CP Denton. Neurologic Complications of Systemic Sclerosis. The Neurologic Involvement in Systemic Autoimmune Disorders, **Handbook of Systemic Autoimmune Diseases.** Series Editors: Ronald A Asherson, Doruk Erkan, 2004

Presentations at International and National Conferences

Gene expression profiling identifies MCP-3 as a potential mediator of fibrosis. Plenary session at the Annual General Meeting British Society of Rheumatology, Brighton 23rd April 2001

Expression of MCP-3 in fibrosis. Runner-up in the President's Prize presentation for Clinical Immunology and Allergy, Royal Society of Medicine 10th June 2002

Expression and Function of MCP-3 in Scleroderma. Awarded Best Abstract at the Medical Research Society/Academy of Medical Sciences meeting for clinician scientists in training 5th February 2003

The role of MCP-3 in Scleroderma. Oral presentation at the Annual General Meeting British Society of Rheumatology, Manchester 1st April 2003

Expression and Regulation of MCP-3 in Fibrosis. Oral presentation at Graduate Presentation Day, Department of Medicine, Royal Free Hospital. 23rd July 2004

Synergy between MCP-3 and TGFβ in promoting fibrosis. Oral presentation at the Annual General Meeting British Society of Rheumatology, Birmingham 21st April 2005

Fibroblast-specific perturbation of TGFβ signalling in transgenic mice in European Platform for Novel Treatment for Scleroderma and Fibrotic Diseases and Planning of European 6th Framework Programme Project. Pamplona, Spain May 26 – 27th 2005

VH Ong, M Carulli, GE Lindahl, SW Xu, DJ Abraham, CM Black, CP Denton. Expression and Regulation of MCP-3 in Scleroderma. **8th International Workshop on Scleroderma Research**, Trinity College, Cambridge. 31st July – 4th August 2004

VH Ong, M Carulli, GE Lindahl, SW Xu, DJ Abraham, CM Black, CP Denton. Multiple Potential Levels of Cross-Talk between Monocyte Chemoattractant Protein-3 (MCP-3) and TGFβ1 in Fibrosis **Arthritis Rheum** 2004;1654

CP Denton, GE Lindahl, VH. Ong, SW Xu, E Renzoni, D Edwards, DJ. Abraham, CM. Black**.** A Kinase-deficient Type II TGFβ Receptor Induces a Scleroderma-like Phenotype in Transgenic Mice Through Metalloproteinase Dependent Activation of Latent TGFβ **Arthritis Rheum** 2004;1672

CP Denton, GE Lindahl, VH. Ong, SW Xu, E Renzoni, DJ. Abraham, CM. Black Gene expression profiling delineates constitutively activated targets of TGFβ in a transgenic model of scleroderma. **Rheumatology** Apr 2004; **43**: ii96 - ii101.

MT Carulli, VH. Ong, DJ Abraham, M Ponticos, L Shand, CM Black, CP Denton Upregulation of MCP-1 ligand-receptor axis in early diffuse systemic sclerosis. **Rheumatology** Apr 2004;43: ii23-ii25

MT Carulli, VH. Ong, DJ Abraham, CM Black, CP Denton. Evaluation of CCL2 as a potential biomarker of pulmonary vascular disease in limited cutaneous systemic sclerosis. **Rheumatology** Apr 2005; 44 : i128 – i133

MT Carulli, VH. Ong, DJ Abraham, G Coghlan, CM Black, CP Denton Evaluation of CCL2 as a Potential Biomarker of Pulmonary Vascular disease in Limited Cutaneous Systemic Sclerosis. **Arthritis Rheum** 2004; 1062

VH Ong, MT Carulli, DJ Abraham, G Lindahl, CM Black and CP Denton. Cross-talk between MCP-3 and TGFβ in driving fibrosis **APLAR Journal of Rheumatology** Aug 2006;9(Supp 1).

CHAPTER 1: INTRODUCTION

1.1 Systemic Sclerosis: An Overview

1.1.1 Epidemiology

The 1980 Systemic Sclerosis (SSc) classification criteria have greatly facilitated epidemiological studies by providing a common definition for this disease(Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980). Earlier studies did not have this benefit and therefore, it was difficult to make direct comparisons between pre- and post- 1980 studies. In addition, the 1980 classification scheme is likely to miss 10% of individuals who have limited disease and most studies are hospital-based.

Overall SSc should be considered uncommon rather than rare, with a prevalence of 31 per million (Silman & Black, 1988) and an incidence of 3.7 per million per year. Recent population studies suggest that SSc occurs more frequently in the US than in UK with a prevalence estimated to be 242 cases per million adults with an annual incidence rate of 19.3 new cases per million per year but this has remained relatively stable(Mayes *et al.*, 2003). Women are affected more frequently than men and this is reflected in the UK Central Registry with Royal Free Hospital, with over 1500 cases with a female:male ratio of 5:1. The factors responsible for this are not apparent. Limited cutaneous SSc (lcSSc) is approximately twice as common as the diffuse subtype.

Overall survival has improved over the past few decades with a mean survival of 12 years from diagnosis, with 78% at 5 years and 55% at 10 years(Mayes *et al.*, 2003). This may be related to earlier diagnosis and recognition of mild limited cases which may prejudice studies in the direction of increased survival. In addition, it is likely that increased sensitivity of antibody detection leading to the diagnosis of milder forms of SSc (with better outcome), which may previously not have been recognised may contribute to improvement in survival. Nonetheless, advances in treatment, most notably the use of angiotensin converting enzyme (ACE) inhibitors in renal crisis have led to a real decrease in the mortality from hypertensive renal disease from 25% to 10%. The mortality rate is partially

dependent on organ involvement and pulmonary disease including pulmonary fibrosis and pulmonary hypertension has now emerged as a major cause of death. Among patients with severe ventilatory restriction (forced vital capacity <50% of predicted) as a result of SSc-related interstitial lung disease mortality approaches 42% within 10 yrs after the onset of the disease(Tashkin *et al.*, 2006). In general patient survival in lcSSc is greater than that in diffuse cutaneous SSc (dcSSc). A Swedish series with 250 patients demonstrated standardized mortality ratios (95% Confidence Interval) of 3.72 (2.41–5.32) in lcSSc and 6.06 (4.09–9.02) in dcSSc(Hesselstrand *et al.*, 1998). In lcSSc, most deaths are from the usual causes such as cancer, heart disease and old age. Of those who die of SSc-related deaths, 50% die of pulmonary hypertension and 25% die of pulmonary fibrosis. In the entire SSc population, 50% die of SSc-related deaths die of pulmonary disease. Cardiac disease is also correlated with a poorer outcome. Gastrointestinal involvement contributes to the morbidity of the disease but the extent of this contribution to mortality is less clear.

1.1.2 Classification criteria for SSc and subgroups

The word scleroderma is derived from Greek roots literally meaning, hard (*skleros*) skin (*derma*). The spectrum of scleroderma encompasses Raynaud's phenomenon, localised subtypes of skin fibrosis and the clinically important systemic sclerosis which shares the three key aspects of inflammatory, vascular and fibrotic pathogenic mechanisms. Although the basic principles of classification of scleroderma spectrum diseases remain, the traditional boundaries between the different clinical subgroups of the scleroderma spectrum are being broadened. It is now appreciated that there is a major distinction between patients with skin-based sclerosis, localised forms of SSc and those in whom there is visceral involvement, termed systemic sclerosis. The current classification of the scleroderma spectrum is shown in **Table 1.1**. There is a subset of patients with the clinical features of isolated Raynaud's phenomenon with evidence of microvasculopathy based upon nailfold capillaroscopy or have serum autoantibodies against nuclear antigens. Several studies have demonstrated that these patients with autoimmune Raynaud's phenomenon have a 10-15% likelihood of developing a defined connective tissue disease(Spencer-Green, 1998). It has been suggested that the presence of Raynaud's with scleroderma-related capillaroscopic findings and/or SSc specific antibodies against hallmark antigens identifies a group of

patients who may later develop features of limited cutaneous systemic sclerosis. Based upon the absence of skin changes at presentation, the term ‘limited systemic sclerosis’ has been proposed for this group(LeRoy & Medsger, Jr., 2001). In addition, there is a small number of patients with vascular symptoms and SSc-specific antibodies who develop major organ-based complications in the absence of significant skin sclerosis; these cases are designated systemic sclerosis sine scleroderma(Poormoghim *et al.*, 2000). Based on clinical characteristics of the disease(LeRoy *et al.*, 1988), systemic sclerosis was subdivided into limited and diffuse cutaneous scleroderma (lcSSc and dcSSc respectively). This system is now widely accepted and it proposes that the extent of skin involvement defines the disease subset in systemic sclerosis. Thus, those with sclerosis of skin proximal to the neck, elbows and knees have dcSSc, but those with distal involvement to these areas are designated lcSSc.

Subsets	Key Clinical Features
Raynaud's Phenomenon	
Primary Raynaud's Phenomenon	Vasospasm predominantly affects the extremities without any features of connective tissue disease
Autoimmune Raynaud's Phenomenon	Vasospasm of the extremities in the presence of antinuclear antibodies
Systemic forms	
Limited Systemic Sclerosis	Absence of clinical skin sclerosis with specific SSc-associated antibodies
Limited Cutaneous Systemic Sclerosis	Skin sclerosis restricted to extremities, face and neck. Prominent vascular features
Diffuse Cutaneous Systemic Sclerosis	Extensive skin sclerosis proximal to elbows and knees
Systemic sclerosis sine scleroderma	Visceral features of SSc (especially gut and lung fibrosis) and Raynaud's phenomenon. Skin sclerosis absent.
Scleroderma overlap syndromes	Coexistent with features of other autoimmune diseases including SLE, rheumatoid arthritis, dermatomyositis or Sjogren's Syndrome
Localised forms	
Localised Morphoea	One or more circumscribed patches of sclerotic skin. Indurated inflammatory margin indicative of active progression
Generalised Morphoea	Multiple areas of indurated sclerotic skin, often on trunk and limbs with similar clinical and histological features as above, but more extensive
Linear Scleroderma	One or more elongated sclerotic areas of skin typically asymmetrical and orientated along the affected limb. Commonest form of childhood onset scleroderma and associated with growth impairment of affected extremity
En coup de sabre	Linear sclerotic lesion, usually childhood onset, involved the skin and underlying tissues. Often lesions involve the scalp or face.

Table 1.1 Classification of the Scleroderma spectrum of disorders

1.1.3 Clinical features

1.1.3.1 Skin involvement

The extent of skin involvement is the single major criterion for the subclassification of SSc into its two principal subsets – diffuse (dcSSc) and limited (lcSSc) SSc. This subclassification is closely related to the time of onset, pace of development and patterns of internal organ involvement and is accordingly strongly linked to survival.

The skin changes appear to evolve in sequential stages:
1. Early progression with tissue and systemic immune activation and inflammatory changes
2. Plateau or stabilization with reduced local and systemic resolution,
3. Late improvement with postinflammatory and post-fibrotic atrophy and remodelling of previously fibrosed tissue.

To estimate the degree of skin thickening, modified Rodnan skin scoring (mRSS) system is widely used in which 17 body areas are examined by clinical palpation and scored based on skin thickness on a 4-point ordinal scale (0=normal thickness, 1=mild thickening, 2=moderate thickness and 3=severe thickness) as shown in **Figure 1.1**. There is a strong correlation between mRSS skin scores and skin biopsy weights and histological extent of fibrosis, supporting the usefulness of the skin scores in differentiating SSc diseases subtypes(Verrecchia *et al.*, 2007;Furst *et al.*, 1998) (**Figure 1.2**). Shand et. al. demonstrated that most dcSSc patients develops the maximal skin score early(Shand *et al.*, 2007). It is recognised that worsening skin scores are associated with a higher risk of renal involvement (DeMarco *et al.*, 2002;Steen *et al.*, 1984;Steen & Medsger, Jr., 2001)and death and that improving the skin score is associated with better functional capacity and survival(Clements *et al.*, 2000). In addition, subclassification of patients with early dcSSc according to the longitudinal skin score across time may help to define subsets of patients with distinct morbidity and mortality(Shand *et al.*, 2007).

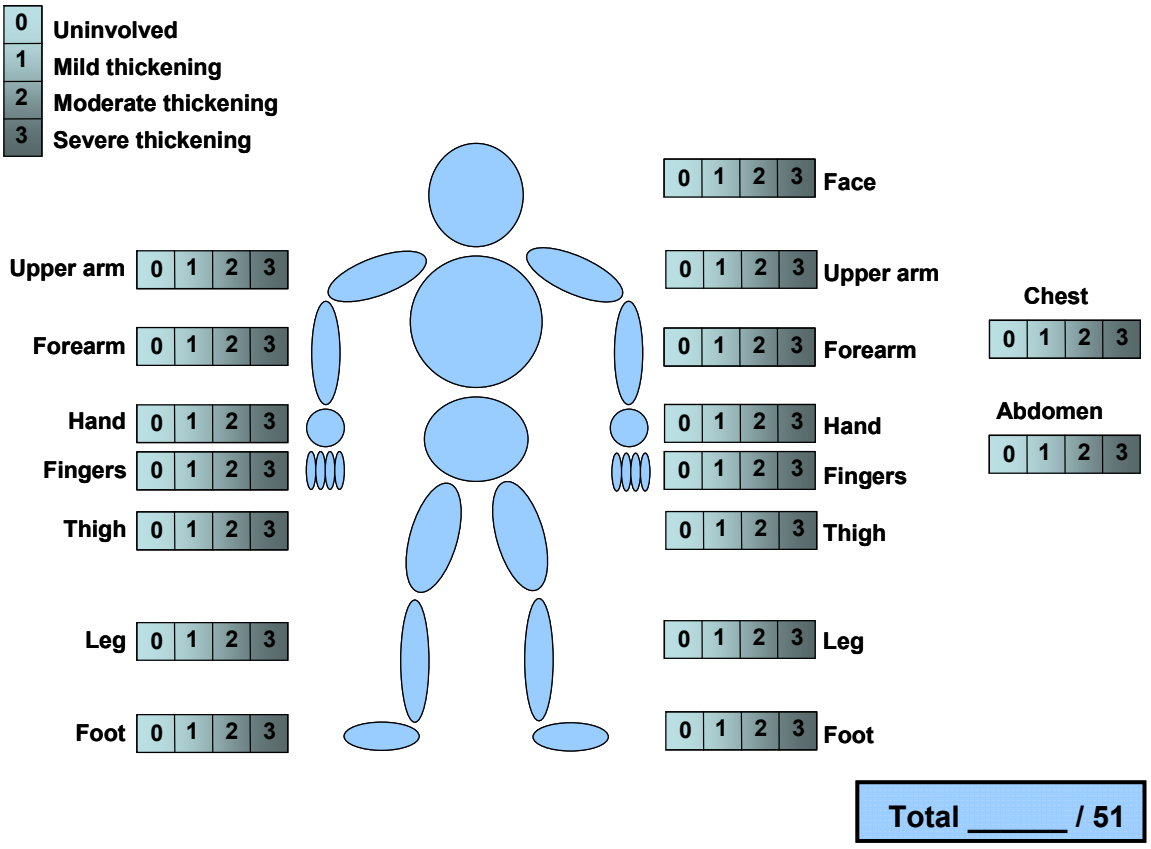


Figure 1.1. Modified Rodnan Skin Score. Semiquantitative estimates by clinical palpation of the extent and severity of SSc skin change.

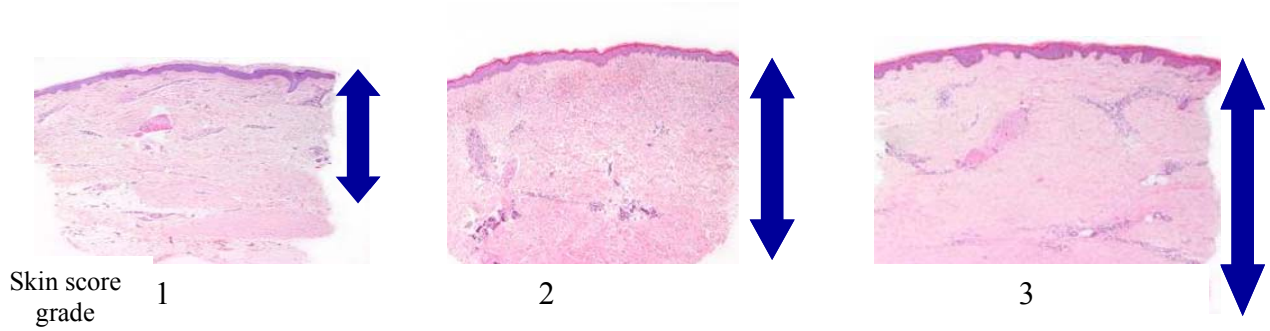


Figure 1.2 Change in mRSS correlates with change in the histological extent of dermal fibrosis, indicating that skin score reflects the underlying pathology of SSc. Dermal fibrosis is graded from mild to severe: mild sclerosis indicates less than 25% sclerosis with focal dense deep dermal fibrosis, and in severe dermal fibrosis, the reticular dermis is replaced by dense compact collagen without normal fascicular bundles or dermal appendages. Low power full thickness biopsies taken from dcSSc skin sites with different skin score grades. Original magnification x2.5. H & E stain.

1.1.3.2 Vascular involvement

The pathological changes in the vascular tree range from endothelial activation with increased expression of adhesion molecules to capillary necrosis and intimal proliferation of arterioles to occlusion of blood vessels. Various circulating soluble markers reflecting the endothelial injury are overexpressed and these include inter-cellular adhesion molecule 1 (ICAM-1), endothelin-1 (ET-1), nitric oxide, Von Willebrand factor and antiendothelial cell antibodies(Wigley, 2009). Among these mediators, endothelin represents the most potent vasoconstrictor and is additionally considered to be an important mediator in vessel remodelling ultimately leading to major changes in cellular and tissue architecture in SSc. ET-1 was shown to be associated with high levels of soluble ICAM-1 and Von Willebrand factor in the blood of SSc patients(Kahaleh, 1991;Kadono *et al.*, 1995;Vancheeswaran *et al.*, 1994). Elevated levels of soluble ICAM-1 from serum of diffuse SSc patients were shown to correlate with pulmonary fibrosis and overall disease severity(Ihn *et al.*, 1997). Raised sera ET-1 levels were demonstrated to be inversely correlated with diffusing capacity on lung function(Becvar *et al.*, 2005). In contrast, altered circulating levels of NO and production in SSc have been reported(Matucci & Kahaleh, 2002;Dooley *et al.*, 2006). The presence of anti-endothelial cell antibodies may also contribute to endothelial cell injury and apoptosis(Bordron *et al.*, 1998). Whilst there is strong evidence of vascular pertubation from studies of peripheral blood in SSc, little is known about the ability of these biomarkers to predict vascular outcomes. Progressive reduction of the size of microvascular beds in particular the arteriolar segments of the capillary beds in association with reduction of organ blood flow ultimately leads to organ ischaemia. This would encompass the clinical manifestations of Raynaud's phenomenon, digital ulcers and telangiectasia.

Such generalised endothelial dysfunction has been shown to predict future cardiovascular events in many clinical situations via the development of macrovascular atherosclerosis over time(Bolad & Delafontaine, 2005), and it has been reported in some studies that cardiovascular deaths contribute to 20–30% of all premature deaths in SSc(Hesselstrand *et al.*, 1998;Bryan *et al.*, 1996;Jacobsen *et al.*, 1998). In these studies, the macrovascular cause of death was either cardiac, cerebral or limb ischaemia in approximately one-third of the

cases. Apart from autopsy studies, atherosclerosis was also demonstrated on arteriography and non-invasive vascular assessments with carotid duplex scanning and measurement of ankle brachial blood pressure index(Belch *et al.*, 1993;Dick *et al.*, 2001). Arterial occlusion is common particularly in the ulnar arteries in patients with lcSSc and in association with anti-centromere antibodies.

1.1.3.3 Gastrointestinal involvement

Gastrointestinal involvement is second in frequency to the skin with oesophageal dysmotility and its associated complications occurring in 75-90% of patients, small bowel involvement in 40-70%, colon involvement in 20-50% and anorectal involvement in 50-70%. The underlying pathophysiology involves dysmotility of smooth muscles of the tract and the earliest defect may lie in the cholinergic nerves which supply the muscle rather than a primary muscular defect. Such dysmotility problems are likely to increase with frequency and severity with the duration of the disease. Hypomotility in the oesophagus leads to delayed transit down the oesophagus and in a reduced lower oesophageal sphincter pressures. The repeated bathing of the distal oesophagus by hydrochloric acid may result in erosive oesophagitis and stricture. In the small bowel, stagnation of food flow allows secondary colonic bacterial to migrate upstream into the small intestine, where the bacteria breakdown bile acids necessary to absorb fats. Failure of fat absorption leads to malabsorption, weight loss and diarrhoea, and in severe cases, pseudo-obstruction. Large bowel involvement encompasses prolonged transit time leading to constipation, hypotonic internal and external sphincter of the anorectum with faecal incontinence.

1.1.3.4 Pulmonary Involvement

The two major features of lung involvement are interstitial lung disease (ILD) and pulmonary hypertension (PAH), the latter occurring either as an isolated event or secondary to advanced ILD.

SSc is one of the few connective tissue diseases is associated with pulmonary parenchymal involvement which contributes directly to about 30% of deaths in SSc. Although scleroderma-associated ILD (SSc-ILD) is frequently reported in most autopsy cases,

clinically significant SSc-ILD occurs in less than 50% of SSc patients. ILD is characterised by early alveolar inflammation evolving slowly or rapidly to pulmonary fibrosis. The major symptoms may occur at a relatively late stage and these include dyspnoea, cough with the characteristic physical sign of fine inspiratory crepitations over the lung bases.

Chronic inflammatory response to an unknown injury with immunological activation is believed to underlie the pathogenesis of SSc-ILD. However, experimental evidence points towards that lung inflammation per se is not sufficient to cause pulmonary fibrosis. Mice deficient in αvβ6 integrin, a ligand that binds to and activates latent TGFβ, develop exaggerated inflammation but are protected from fibrosis after bleomycin treatment(Munger *et al.*, 1999). In a recent study, transgenic mice developed exaggerated pulmonary fibrosis after bleomycin treatment, independently of neutrophilic inflammation(Hoyles *et al.*, 2008). Other pathogenic mechanisms have therefore been postulated. For example, activation of coagulation pathway as a consequence of endothelial cellular injury generates thrombin release that in turn induces myofibroblast differentiation, activates endothelial cells and releases profibrotic factors, including TGFβ. Apart from endothelial involvement, epithelial cell injury, like in renal fibrosis, may also increase myofibroblast numbers via epithelial mesenchymal transition (EMT)(Willis *et al.*, 2006), promoted by TGFβ and ET-1 expression(Jain *et al.*, 2007). Focal pulmonary inflammation may play a role in stimulating chemokine-mediated fibrocyte chemotaxis. Experimental evidence reveals that in response to chemokines including CXCL12, circulating fibrocytes, which are circulating progenitors of fibroblasts implicated in wound healing and fibrosis, may traffic to the lungs in a murine model of bleomycin-induced lung fibrosis. In this model, treatment with neutralising anti-CXCL12 antibodies abrogated intrapulmonary fibrocyte recruitment and attenuated lung fibrosis(Phillips *et al.*, 2004). These findings were replicated in a recent paper in humans with idiopathic lung fibrosis. Andersson-Sjoland et al. reported that CXCL12 were expressed by alveolar epithelial cells and increased numbers of fibrocytes were stained with combination of CXCL12 and mesenchymal markers (procollagen-I and prolyl-4-hydroxylase) compared with combinations using CD34 or CD45RO with mesenchymal markers(Andersson-Sjoland *et al.*, 2008).

Similar to the changes observed in the skin of SSc, other pathogenic mechanisms including interaction between inflammatory infiltrate and fibroblasts driven by cytokines and chemokines may regulate the fibrotic response. Inflammatory cells including neutrophils, eosinophils, macrophages, and lymphocytes are present in the bronchoalveolar lavage (BAL) fluid of SSc-ILD patients(Bolster *et al.*, 1997). Fibroblasts explanted from SSc-ILD lungs are phenotypically different from control lung fibroblasts and similar to the dermal SSc fibroblasts in that they demonstrate a degree of heterogeneity(Varga & Abraham, 2007). Serum levels of collagen metabolites including N-terminal propeptide of type III procollagen levels, cross-linked carboxyterminal telopeptide of collagen I are associated with ILD. Other potential lung specific markers for ILD include KL-6, a glycoprotein expressed by alveolar type II epithelial cells and the surfactant proteins A and D which are also secreted by Clara cells.

Despite the multiplicity of mechanisms described above, no major therapeutic advances have been achieved in SSc-ILD in recent years. Compared to idiopathic lung fibrosis, SSc-ILD deteriorates insidiously and even when low-grade inflammation drives disease progression, lung involvement is predominantly fibrotic. This is also mirrored in the infrequent histological changes with predominantly inflammatory abnormalities at lung biopsy, being present in less than 20% of cases. Therefore, prevention of disease progression appears to be the most realistic therapeutic goal with immunological modulation. This is emphasised in the outcome of two major prospective, randomised, placebo-controlled trials on the use of cyclophosphamide in SSc-ILD(Hoyles *et al.*, 2006;Tashkin *et al.*, 2006). Although the results suggests that there was a treatment effect on forced vital capacity (FVC), this effect was lost a year following completion of treatment. In addition, the treatment effect was due to a selective decline in FVC in the placebo group in patients with more extensive disease on HRCT in which, paradoxically, inflammation is a minor histological component.

Unlike SSc-ILD whereby stability of extensive disease is deemed as therapeutic success, there have been significant advances in the management of PAH. PAH is defined as a mean

pulmonary arterial pressure (PAP) above 25 mmHg at rest or above 30 mmHg during exercise, with normal pulmonary artery wedge pressure. Recent studies using right heart catheterisation to confirm the diagnosis suggest that the prevalence of PAH in SSc is estimated to be 10-15% with a five-year cumulative survival of 10% compared with 80% in those without PAH(Mukerjee *et al.*, 2003). Indolent exertional dyspnoea is the major symptom, with disease progression characterised by impaired exercise tolerance and fatigue. Whilst the aetiology of PAH in SSc is likely to be complex and the nature of the initial lesion is unclear, a plausible sequence of events would involve a predisposed individual, including genetic predispositions as a result of interaction of both protective and predisposing alleles at several loci. In recent years, genetic studies have significantly increased the understanding of the molecular basis of PAH with identification of mutations in the bone morphogenetic protein receptor-2 (BMPR2) encoding a TGF-β receptor, in about 60% of cases of familial PAH and 25% of sporadic PAH(Lane *et al.*, 2000;Newman *et al.*, 2001). However, most individuals who have PAH do not have an identifiable mutation. So far, these heterozygous germline mutations in BMPR2 have not been described in SSc-APH. Morse et al and Tew et al did not detect BMPR2 mutations in a cohort of SSc patients although the small size of the patient groups is a potential limitation of these studies(Morse *et al.*, 2002;Tew *et al.*, 2002). Nonetheless, there is a growing evidence to support potentially systemic alterations in TGFβ family ligand-receptor axis and the widespread vasculopathy with SSc. Vasoconstriction, vascular remodelling and thrombosis are hallmark pathogenic processes in PAH(Humbert *et al.*, 2004). Endothelial cells activation markers including thrombomodulin and endothelin-1 may reflect endothelial dysfunction have been described in SSc-PAH suggesting that early endothelial pathology may be important(Stratton *et al.*, 2000;Stratton *et al.*, 1998). Histologically, the end-stage lesion of SSc-PAH is microvascular luminal obliteration/attenuation with medial and adventitial fibrosis and proliferative lesions and intimal hyperplasia. It is noteworthy that some of these characteristic changes are also observed in the vascular changes in digital arteries and hypertensive renal crisis consistent with generalised endothelial cell activation and damage in SSc. The existing therapeutic options for PAH address these different pathobiological pathways underlying this condition. The currently licensed

therapeutic agents include prostanoids, ET-1 receptor antagonists and phosphodiesterase type 5 inhibitors.

1.1.3.5 Renal Involvement

Scleroderma renal crisis represents an important link between the fibrotic aspect of SSc and vascular injury. Despite the introduction of ACE inhibitors that led to an impressive improvement in survival from 10% at one year to 65% at five years, mortality as high as 30% at 3 years has been reported in particular in those requiring permanent dialysis. Apart from the accelerated phase hypertension and progressive renal impairment, the presence of hypertensive retinopathy and encephalopathy indicates systemic extrarenal endothelial dysfunction. Recent data also demonstrated upregulation of endothelin-1 and endothelin B receptors in renal tissues in addition to a recently described association between endothelin B receptor polymorphism and renal crisis suggesting a role of this signalling system in the pathogenesis of renal crisis(Kobayashi *et al.*, 1999;Fonseca *et al.*, 2006). Renal biopsy characteristically shows changes in the small interlobular and arcuate arteries. The earliest change is intimal oedema followed by intense proliferation of intimal cells and the production of mucinous ground substance composed of glycoprotein and mucopolysaccharide. Notably, vascular changes of mucoid intimal thickening and thrombosis are associated with poor outcome with half of those with these changes require permanent dialysis compared with 13% of those without.

1.1.3.6 Cardiac Involvement

SSc heart involvement may be either primary or secondary to lung/renal involvement. The pathological hallmark is myocardial fibrosis which affects both ventricles in a patchy distribution in the absence of any significant coronary artery disease. This may occur as a consequence of recurrent ischaemia, coexisting myositis or chronic inflammation leading to fibrosis. Clinically, this is manifested as diastolic dysfunction. Dysrhythmias and conduction disturbances are considered a hallmark for cardiac involvement, reflecting the underlying autonomic dysfunction. Cardiac involvement remains among the top predictors of mortality in SSc(Wynn *et al.*, 1985;Steen & Medsger, Jr., 2000). Recent data from the Canadian registry suggested that any left heart involvement was associated with an

increased risk of death with cardiac involvement in 67% of those deceased compared with only 17% in those living(Al-Dhaher *et al.*, 2008).

1.1.4 Current Concepts of Scleroderma Pathogenesis

Although the exact mechanisms involved in the pathogenesis of SSc remain unclear, there are three key features responsible for the major clinical and pathological manifestations of the disease (**Figure 1.3**):

1. Functional and structural vasculopathy with endothelial dysfunction, intimal proliferation and vascular obliteration as an early feature of the disease,
2. Humoral and cellular immunity dysregulation with inflammatory changes. This involves T-cell activation, autoantibody production and perivascular mononuclear infiltrate in the inflamed skin.
3. Fibrotic phase with increased synthesis and deposition of matrix proteins form the ultimate basis for tissue and organ dysfunction.

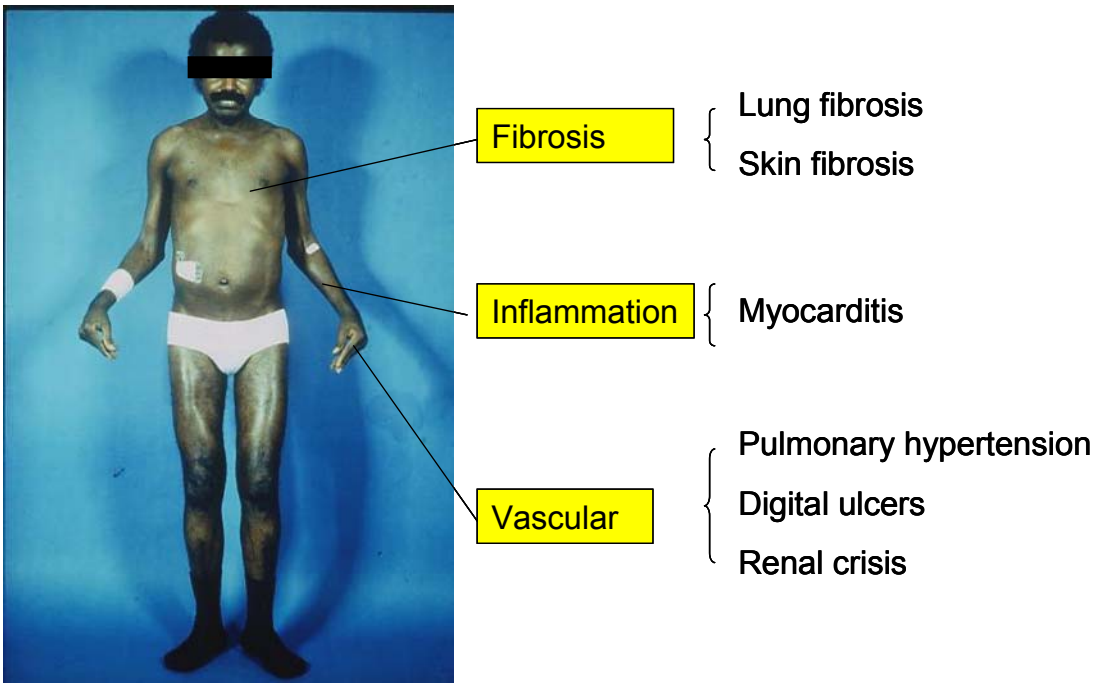


Figure 1.3 Clinical and pathological correlates in Systemic Sclerosis

1.1.4.1 Genetics and Environment

There is growing evidence for genetic contribution in the pathogenesis of SSc. Family history and ethnicity are two major factors in determining the genetic effects involved in SSc. Compared to an estimated population risk of 0.026%, SSc appears to occur in 1.6% of families of SSc cases in three separate cohorts(Arnett *et al.*, 2001). The familial relative risk was approximately 15-fold higher for siblings and 13-fold higher for first-degree relatives. Further studies on multicase SSc families and monozygotic twins describe concordant SSc-specific autoantibodies(Cepeda & Reveille, 2004) associated with a high frequency of other autoimmune diseases such as primary biliary disease and Sjogren's Syndrome(Bennett, 1990). There are also differences in prevalence and clinical features among different ethnic groups of the greater relative of dcSSc in the African and American Indians populations (Tager & Tikly, 1999)and also among the Chowtaw Indians(Kuwana *et al.*, 1999).The genetic contribution in *in vitro* fibroblast activation was demonstrated in gene expression microarray profiles of cultured fibroblasts from SSc twin subjects. Candidate gene association studies have identified several key genes in SSc: growth factors (endothelin), ECM components (TGFβ1, connective tissue growth factor [CTGF], secreted protein, acidic and rich in cysteine [SPARC]), allograft inflammatory factor-1, protein tyrosine phosphatase non-receptor 22, cytokines (macrophage migration inhibitory factor, tumour necrosis factor-alpha [TNFα], IL-1α and IL-1β) and chemokines (MCP-1/CCL2, RANTES/CCL5, MIP-1α/CCL3, IL-8/CXCL8)(Agarwal *et al.*, 2008).

Stratification of patients based on autoantibody profiles reveals strong associations with HLA polymorphisms (**Table 1.2**). For example, an association of HLA DRB1*1302, DQB1*0604/0605 haplotypes were found with anti-U3RNP positive patients, who are more often male African Americans, and the HLA DRB1*0301 haplotype has been shown to be associated with anti-PM-Scl antibody positivity in patients who are nearly exclusively white. However, many of these results have not been corroborated by replication studies with larger and well-characterised cohort of patients. For example, the association of HLA DQB1*0201 in patients with anti-RNA polymerase I, II and/or III antibodies has not been

observed in other studies(Fanning *et al.*, 1998). In addition, it is likely that the multitude of biological signalling pathways involved in SSc suggests a complex regulatory network of genes in the development of the disease. In contrast to candidate-gene approach, genome-wide approaches used in studies of complex diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis will allow simultaneous identification of novel genes in this disease. Taken together, it would appear that genetic factors might provide a permissive environment that allows either the initiation of the disease process or its progression and development of distinct clinical manifestations.

In addition, another possible mechanism that may explain the increased prevalence of SSc in females compared to males is the microchimerism hypothesis that postulates activation of engrafted fetal cells may lead to a graft-vs-host reaction towards the mother or foetus (Jimenez & Artlett, 2005). Although the pathological effect of such cells is unknown, microchimeric cells were found in the cellular infiltrate of sclerodermatous lesions(Sawaya *et al.*, 2004). Circulating levels of CD4+ and CD8+ T cells were also found significantly higher in SSc patients than in controls. This is akin to the microchimeric CD4+ and CD8+ T cells detected in the lung tissues from patients with hypersensitivity pneumonitis(Bustos *et al.*, 2007). Furthermore, patients with diffuse SSc have significantly more CD4+ microchimeric T cells than controls(Randone *et al.*, 2008). In those with circulating microchimeric T cells, the endothelium represents an allotypic stimulus to those cells and this may mimic the same pathway transplanted T cells follow in graft-versus-host disease.

A variety of chemical and environmental agents have been associated with SSc and SSc-like conditions for nearly a century. The earliest description was the manifestation of scleroderma in five stonemasons exposed to silica and scleroderma-like cutaneous manifestations described in association with vinyl chloride and organic solvent exposure in the 1950s(Nietert & Silver, 2000). Exposure to silica dust (silicon dioxide SiO_2) among coal and gold miners and related occupations confers a high risk of development of SSc, and it has been estimated that the incidence of scleroderma is increased at 81.8 per million in silica exposed black South African gold miners compared to 3.4 per million in the general black South African population(Cowie, 1987). Another study has estimated the

relative risk of developing scleroderma following silica exposure is 25 and may be up to 39 times higher in patients with radiologically documented silicosis(Haustein *et al.*, 1990). Serologically, antibodies to DNA topoisomerase I (anti-Scl 70) are found in the majority of silica associated scleroderma patients(McHugh *et al.*, 1994). The list of chemicals and environmental agents continue to grow considerably and more recently, a rapidly progressive form of fibrosing disease, nephrogenic systemic fibrosis (NSF) has been described in individuals with renal impairment, usually in those requiring dialysis(Cowper *et al.*, 2007). Free, unchelated gadolinium is highly toxic and it is detected in the affected skin of patients with NSF, in some cases for months following its administration(High *et al.*, 2007). Importantly, despite the link with renal disease, the fibrosing process may be evident within muscles, myocardium, lungs and testes(Ting *et al.*, 2003). Interestingly, apart from the striking fibrogenic response with appearances similar to those observed in scleromyxedema, abnormal vasculopathy is observed with increased thickness of the adventitial layer of small and medium-sized arterioles(Mendoza *et al.*, 2006). Presence of dermal CD34+ staining fibroblast-like epitheliod or stellate cells suggests that these cells may be circulating fibrocytes and may result from active chemotaxis from the bone marrow. Indirect evidence to support this is the association of erythropoietin use in these patients, which has the ability to mobilise haematopoietic progenitors including mesenchymal precursors from the bone marrow, but its role in the pathogenesis of NSF is unclear(Swaminathan *et al.*, 2006).

There is some evidence from animal models of fibrosis to implicate infectious agents in the development and progression of the pathological features of SSc. Whilst the infection rates compared to control populations do not differ significantly, increased antibody titres, a preponderance of specific strains in SSc and indirect evidence of molecular mimicry inducing autoimmune responses suggest that infections present as potential environmental triggers for SSc. Herpesviruses, in particular cytomegalovirus (CMV) has been demonstrated to induce neointimal lesions in the vascular wall, a feature shared by SSc in immunosuppressed mice although the experimental lesions were only apparent in the large vessels compared to predominant microvascular abnormalities in SSc(Hamamdzic *et al.*, 2001). Recently, infection with gammaherpesvirus in contrast to adenovirus, may

exacerbate pre-existing lung fibrosis in a model of fluorescein isothiocyanate–induced pulmonary fibrosis in mice. This is due in part to upregulation of chemokines (MCP-1 and CCL12, a murine chemokine with homology to human MCP-1), leading to a profibrotic environment with increased fibrocyte recruitment and collagen deposition(McMillan *et al.*, 2008). Other viruses that have been investigated in the context of SSc pathogenecity include parvovirus B19 and Hepatitis G(Seemayer *et al.*, 2001;Ferri *et al.*, 2005). While it is clear that infections alone do not exclusively lead to development of SSc, it is likely that the interaction between host factors and infections determine the susceptibility to disease in response to these triggers.

Antibody	**HLA**	**Ethnic group**
ATA	DRB1*1104 DPB1*1104	White
	DQB1*0301 DPB1*1301	African Americans
	DRB1*1502 DQB1*0601 DPB1*0901	Japanese
	DRB1*1602 DQB1*0301 DPB1*1301	Choctaw
ACA	DQB1*0501	
U3-RNP	DRB1*1302 DQB1*0604/0605	
PM-Scl	DRB1*0301	

Table 1.2 Genetic associations based on autoantibody subsets in SSc. ATA: Anti-topoisomerase I antibody, ACA: Anti-centromere antibody.

1.1.4.2 Vasculopathy

Vascular disease is an early and possibly primary disease component in SSc(Kahaleh *et al.*, 1979). Studies with nailfold capillaroscopy in patients and histopathological analysis of vessels within lesional tissue indicate that vascular damage occurs before fibrosis. For example, Raynaud's disease precedes the other disease manifestations as a result of dysregulation in blood vessel constriction and dilatation (vasomotor regulation), arising from altered production of, and responsiveness to, key vasodilatory (nitric oxide) and vasoconstrictive (endothelins) factors. Other important clinical manifestations of vascular abnormality include digital ulcers, pulmonary hypertension, gastrointestinal vascular ectasia, cutaneous telangiectasia and renal crisis. Triggered by inflammatory cytokines, endothelial cell–specific autoantibodies, vasculotropic viruses, or reactive oxygen radicals generated during ischaemia/reperfusion, endothelial injury results in increased microvessel permeability, enhanced trans-endothelial leucocyte migration and activation of coagulation and fibrinolytic cascades(Cerinic *et al.*, 2003). Vascular endothelial injury predominantly affects the microcirculation and arterioles with most marked changes occurring over the arteriolar segments of capillary beds and these morphological changes are therefore easily demonstrated on nailfold capillaroscopy.

These events lead to vascular remodelling, with hypertrophy of the intimal and medial layers and adventitial fibrosis resulting in progressive luminal narrowing and obliteration(Kahaleh, 2008). Studies in the University of California Davis (UCD)-200 chickens model for SSc, have also highlighted the role of endothelial dysfunction as an early acute disease feature. The microvascular alterations include impaired vessel response to vasodilators, and circulating anti-endothelial cell antibodies provoke endothelial cell injury, a primary event in the vascular pathogenesis of SSc(Sgonc *et al.*, 1996). However, the avian model does not recapitulate capillary rarefaction.

Combined with endothelial cell apoptosis, the process progresses over time to culminate in a striking absence of blood vessels in lesional skin biopsies and on angiograms of patients with late stage disease. Loss of microvasculature is associated with tissue hypoxia, which normally induces strong expression of vascular endothelial growth factor (VEGF) and its

receptors(Mackiewicz *et al.*, 2002). In addition to pro-angiogenic factors, there is a paradoxical switch to anti-angiogenic drive in the face of tissue hypoxia resulting in progressive disappearance of blood vessels. To support this, SSc patients with more advanced disease have fewer circulating bone marrow-derived CD34+ endothelial progenitor cells when compared with patients with less advanced disease, as well as impaired differentiation into mature endothelial cells(Del *et al.*, 2006;Cipriani *et al.*, 2007). These studies suggest that abnormalities in the bone marrow mononuclear cell responsiveness and function may contribute to some of the dysregulated angiogenesis and tissue repair in SSc.

1.1.4.3 Immunological Dysregulation

The presence of specific autoantibodies is one of the most common manifestations of SSc and autoantibody production as a result of activation of the humoral immune system is associated with different clinical manifestations of the disease (**Table 1.3**). However, it is not generally accepted that these antibodies are not directly involved in the clinical manifestations of the disease and their titres do not correlate with disease activity or clinical severity. The presence of prominent inflammatory mononuclear infiltrate in the skin and lungs of active SSc is well recognized with macrophages and CD14+ T cells in these early infiltrate(Kraling *et al.*, 1995;Prescott *et al.*, 1992). In the later stages of the disease, T cells predominate with CD4 cells expressing the activation marker Class II MHC antigen DR(Chizzolini, 2008). The expanded T cell populations in affected SSc tissues may release cytokines and chemokines, which initiate and perpetuate the fibrotic process as well as the endothelial and vascular alterations. These stimulated T cells produce more TNFα, IL-1 and IL-2 than healthy controls and the serum concentrations of IL-2, IL-4, IL-6 and IL-8 and soluble IL-2 receptors are elevated(Chizzolini, 2008;Sakkas *et al.*, 2006;Bruns *et al.*, 1994;Sato *et al.*, 2001). However, this finding is not replicated in a recent study which did not demonstrate a strong signature from CD3+ T cells or other CD20+ B cells on immunohistochemical analysis of SSc biopsy specimens(Gardner *et al.*, 2006). SSc patients also demonstrate intrinsic B cell abnormalities. Analysis of gene expression using DNA microarrays has revealed upregulation of genes related to B cells in SSc lesional skin(Whitfield *et al.*, 2003). In addition, peripheral B cell homeostasis and subsets are

altered with expansion of naïve B cells and diminished memory B cells which are chronically activated in vivo, possibly due to CD19 overexpression(Sato *et al.*, 2004). The latter is a key cell-surface signal transduction molecule of B cells and transgenic mice that overexpress this positive regulator lose tolerance and generate autoantibodies spontaneously(Tedder *et al.*, 1997). Similarly, B cells from a tight-skin mouse show augmented CD19 signalling and chronic B cell activation, CD19 loss results in elimination of autoantibody production which is associated with improvement in skin fibrosis(Saito *et al.*, 2002) and a parallel decrease in IL-6 production by B cells. This would suggest that augmented cytokine production by B cells might be important in induction of skin sclerosis.

Antibody	Usual subset[a]	Predominant clinical features
Anti-centromere (ACA)	Limited	Pulmonary arterial hypertension (PAH), small and large bowel involvement late in disease
Anti-Th/To	Limited	Early interstitial lung disease (ILD), PAH, Small bowel disease
Anti-topoisomerase I (Scl 70)	Diffuse	Early ILD, diffuse skin involvement
Anti-RNA polymerase III	Diffuse	Severe cutaneous disease, renal crisis
Anti-U3-RNP	Diffuse	Myositis, cardiac disease, PAH, small bowel involvement, peripheral neuropathy
Anti-PM-Scl	Limited	Myositis, cardiac disease, renal crisis, ILD
Anti-mitochondrial	Limited	Associated with primary biliary cirrhosis
Anti-Ku	Limited	Myositis, arthritis with lack of vascular features
Anti-nRNP	Limited	Overlap myositis and lupus
Anti-U1-RNP	Limited	Myositis, cardiac disease, renal crisis, ILD

Table 1.3 Hallmark autoantibodies in SSc and their clinical associations.
[a]Any antibody may occur in either subset but clinical associations persist (Madani *et al.*, 2008).

1.1.4.4 Fibrosis

Fibrosis is the pathological hallmark in SSc. Critical for wound repair, and integrity of connective tissue matrix, fibroblasts are mesenchymally derived spindle-shaped cells that synthesise the major interstitial fibrillar collagens that provide structure to organs and tissues of the body(Chang *et al.*, 2002). Uncontrolled production of collagens and other ECM components by fibroblasts in the skin, lungs and other organs leads to excess connective tissue accumulation. A unique feature of fibroblasts explanted from lesional SSc tissues is the retention of an abnormal phenotype, characterised by enhanced ECM synthesis and the expression of the myofibroblast marker, α-smooth muscle actin (α-SMA). Myofibroblasts are highly contractile and are essential for tissue contraction which precedes tissue atrophy and organ failure and α-SMA plays a major role in effecting these contractile properties. Myofibroblasts are also a major, yet transient cell population within the granulation tissue during normal tissue repair in wound healing(Tomasek *et al.*, 2002).

Recently, it has been recognised that not all fibroblasts are the same and that myofibroblasts in SSc might arise from multiple cellular compartments, all contributing to fibrogenesis. This heterogeneity appears to be even more profound within inflamed tissues and during wound repair and fibrogenesis(Postlethwaite *et al.*, 2004).
As demonstrated in **Figure 1.4**, interplay among the three pathological processes initiates and sustains progressive tissue damage in SSc. The increased production of ECM which underlies the fibrotic response in SSc also serves as the major reservoir for secreted growth factors including TGFβ and CTGF which together with the connective tissue compartment provide the cues that control differentiation, proliferation, function and survival of resident cells.

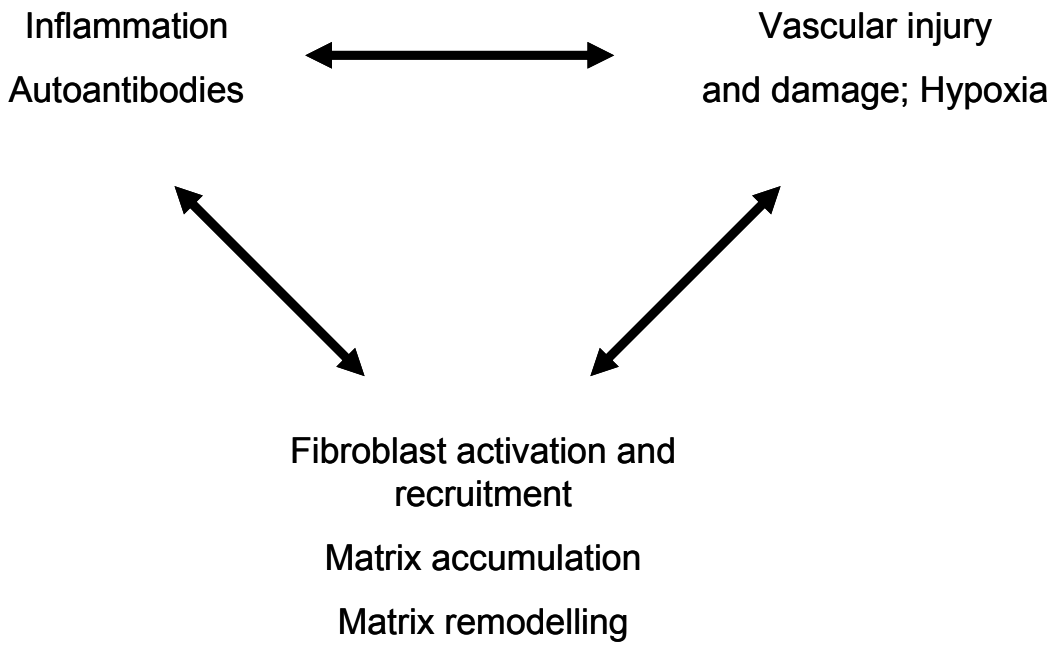


Figure 1.4 The pathogenic triad of SSc. The pathogenesis of SSc involves autoimmunity, vasculopathy and fibrosis. Both autoimmunity and vascular damage precede the onset and contribute to the fibrogenesis. Vascular injury and fibrosis enhance and maintain the chronic autoimmunity and inflammation (Varga & Trojanowska, 2008).

1.1.5 Animal models in Scleroderma

Animal models have been developed to study the evolution of different pathological responses in SSc but none of them exhibit all of the cardinal manifestations of the disease However, these models do offer valuable research opportunities for the elucidation of key cells, mediators and signalling pathways that are relevant in human SSc. Additionally, they provide important preclinical models for the testing of hypotheses as well as potential therapies. These can be classified into naturally occurring models, immunologically mediated and genetically determined mutant strains **(Table 1.4)**.

1.1.5.1 Naturally-occurring models

The tight skin mouse phenotype occurs as a result of reduplication of a central portion of the fibrillin-1 (Fbn1) gene (exons 17-40)(Bona *et al.*, 1997). Biochemical characterisation confirmed many of the key pathological features of SSc including extracellular matrix gene overproduction with excessive accumulation of collagen in the skin, lung and heart (Green *et al.*, 1976). In addition, these mice display humoral immune alterations with presence of autoantibodies against topoisomerase I and centromeres in the plasma. There are now increasing evidence to suggest that fibrillin-1 has a key role in regulating TGFβ activation. Fibrillin-1 is a member of a family of matrix-binding proteins that sequester the large latent TGFβ complexes. Fibrillin-1 deficiency causes excessive amounts of active TGFβ to be liberated from the matrix. The precise mechanisms by which fibrillin-1 controls TGFβ activation remains unclear but it has been shown that fibrillin-1 interacts with the latent TGFβ binding proteins in a tissue specific fashion(Isogai *et al.*, 2003).

The Tsk2/+ is a mutant that appeared in the offspring of a 101/H mouse after the administration of the mutagenic agent ethylnitrosourea (Christner *et al.*, 1995). The Tsk2 mutation is also inherited as an autosomal dominant trait and the mutation has been mapped on chromosome 1. While homozygous (Tsk2/Tsk2) mice have not been reported and are believed to die in utero, heterozygous animals (Tsk2/+) develop skin fibrosis that is apparent as tightness of the skin in the interscapular region at 2-3 weeks of age. Cultured

dermal fibroblasts from Tsk2/+ mice have an elevated steady state level of α1(I) and α1(III) procollagen mRNA. Transcription factors Sp1 and nuclear factor 1 (NF-1) or their homologs have been implicated in the upregulation of transcriptional activity of the Col1a1 promoter in Tsk2/+ fibroblasts. More recently, it has been demonstrated that Tsk2/+ dermal fibrosis is associated with an elevation of collagen transgene expression at the cellular level as well as an increase in the total number of cells actively expressing the transgene, these findings are consistent with elevated Col1a1 transcription(Barisic-Dujmovic *et al.*, 2008). While the molecular defect in the Tsk mutation is known, the genetic locus that is dysfunctional in the Tsk2/+ and the molecular basis of the genetic defect are unknown.

The UCD-200 chicken develops vascular occlusion, severe perivascular lymphocytic infiltration of the skin and viscera, fibrosis of the skin and internal organs, anti-nuclear antibodies, anti-cardiolipin antibodies, rheumatoid factor and polyarthritis (Gruschwitz *et al.*, 1991;van de & Gershwin, 1985). Immuno-histochemical analysis of UCD 200 chicken skin sections show that the apoptosis of endothelial cells is an early event in pathogenesis preceding the mononuclear perivascular infiltration and collagen deposition (Nguyen *et al.*, 2000). More recent observations suggest endothelial induced apoptosis in this model is mediated by anti-endothelial cell antibodies (Worda *et al.*, 2003). This supports the hypothesis that auto-antibodies to intra-cellular proteins and endothelial cell apoptosis are primary events in the disease process. Apoptotic endothelial cells from fibrotic human skin have been detected in the early inflammatory disease stages of SSc (Sgonc *et al.*, 2000). The UCD 200 appears to be good model for the early vascular aspects of the SSc disease process and supports the theory of vascular damage being an early event in disease progression.

1.1.5.2 Induced animal models

Bleomycin-induced pulmonary and dermal fibrosis have been established as an experimental model for SSc. Inflammatory changes and fibrosis are noted to occur by first week with altered activation of Smad signalling, possibly via TGFβ even after the inflammation has resolved. In the latter model of dermal fibrosis, overexpression of MCP-1 and CCR2 were demonstrated in mononuclear cells at early inflammatory stages and in

fibroblasts at the later sclerotic stage in the skin(Yamamoto & Nishioka, 2003). The same group of investigators also suggested that altered apoptosis signals involving differential temporal expression of Fas/FasL system and Caspase-3 activation to be important in the pathogenesis of bleomycin induced SSc. Induction of dermal sclerosis was demonstrated with repeated subcutaneous injections of bleomycin at week 4.This was supported on histopathological and biochemical analysis and it was noted that the sclerosis varied in its intensity among the various murine strains (Yamamoto & Nishioka, 2005). In this model, immunohistological analysis showed that TGFβ was detected on the infiltrating cells, which were predominantly composed of macrophages as well as fibroblasts at sclerotic stages. TGFβ1 and TGFβ2 mRNA were also detected on the lesional skin. Rat lung fibroblast cultures treated with bleomycin results in elevated TGFβ and it was shown that TGFβ mediates the fibrotic effect of bleomycin at the transcriptional level and that the TGFβ response element is required for bleomycin stimulation of the proα1(I) collagen promoter. However, the skin sclerosis observed in this model is transient in response to the subcutaneous injections and occurs only in animals with a susceptible genetic background, and the effect diminishes once the injurious stimulus is removed. It has also been recently reported, using the bleomycin-induced lung fibrosis, that chemokine, CXCL12 helped to recruit and localised fibrocytes to the injured lungs. In addition, the administration of anti-CXCL12 antibody reduced the accumulation of lung fibrocytes and collagen(Phillips *et al.*, 2004). These fibrocytes have also been identified in SSc skin biopsies and it is probable that similar mechanisms may operate in driving recruitment of fibrocytes to the affected skin (Aiba *et al.*, 1994).

In the chronic graft vs. host disease (GVHD), which occurs across minor histocompatibility barriers, severe cutaneous fibrosis is observed with loss of dermal fat, atrophy of dermal appendages, mast cell depletion and mononuclear cell infiltration (Claman *et al.*, 1985;Jaffee & Claman, 1983) and it has been suggested that SSc may be a form of chronic GVHD. Mice that harboured microchimeric cells from previous pregnancies when injected with vinyl chloride, showed remarkable histopathological changes with cutaneous inflammatory changes and fibrosis(Christner *et al.*, 2000;Atamas *et al.*, 2003). Murine Scl-GVHD model was produced by transplanting B10.D2 bone marrow and spleen cells into

BALB/c mice after lethal gamma irradiation of recipients (McCormick *et al.*, 1999). In this model, skin fibrosis is preceded by mononuclear cell infiltration and elevation of TGFβ and chemokines including MCP-1, CCL3 and CCL5(Zhang *et al.*, 2002). Scl-GVHD mice exhibited profound cutaneous thickening and pulmonary fibrosis by 21 days after bone marrow transplantation. However, the severity of the phenotype is variable and is generally self-limiting. In contrast, a modified model of GVHD SSc with transfer of donor spleen cells from wild-type B10.D2 mice into RAG2-deficient mice demonstrates the key aspects of the human disease(Ruzek *et al.*, 2004). The results were similar to a graft-versus-host model using irradiated mice with skin and lung fibrosis. Renal, gastrointestinal and hepatic fibrosis was also demonstrated. Overexpression of both type VII and type III collagens in the skin and type III collagen in the kidneys was also observed. Vasoconstriction of dermal and renal blood vessels with overexpression of ET-1 was also reported. This was associated with altered immune response with ANA and Scl-70 antibodies detected in a majority of the mice.

1.1.5.3 Genetically engineered mice

The advent of transgenic and knockout technologies has enabled the engineering of novel murine strains. These approaches are increasingly used in SSc research to address the pathogenetic roles of candidate molecules and pathways. Transgenic mice represent genetically modified mice in which the DNA has been altered using genetic engineering techniques. Using recombinant DNA technology, DNA molecules from a foreign source are combined into one molecule to create a new set of an expression casette which represents a DNA construct from which expression of one or more exogenous genes is driven. This DNA is then microinjected into fertilised oocytes or embryonic stem cells and transferred into foster mothers generating modified or novel genes. The progeny are then backcrossed with wildtype mice to establish lines, or intercrossed to provide transgenic strains with greater transgene dose that can be maintained on double copy or homozygous strains.

Another useful approach in using the concept of transgenesis is to introduce reporter transgenes such as bacterial β-galactosidase (LacZ) or firefly luciferase (Luc) into

established murine models. This may be achieved by backcrossing reporter transgenic strains with the established mutant model strains. Similar results can be obtained with transient transfection methodology in which the reporter transgene is transduced directly into cultured wildtype cells. The reporter transgene serves as a marker for another gene of particular interest and an ideal reporter gene is one that is not endogenously expressed in the cell of interest. Thus, reporter genes that are incorporated within the transgene construct are generally used to determine whether the transgene of interest has been taken up by or expressed in the cell population and they are frequently used as indicators of transcriptional activity in cells. Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transferred into cells. Following transfer, the cells are assayed for the presence of the reporter by directly measuring the enzymatic activity of the reporter protein. Reporter vectors also allow functional identification and characterisation of promoter and enhancer elements because expression of the reporter gene correlates with transcriptional activity of the promoter driving the reporter gene. The promoter-reporter gene fusion is introduced into cultured cells by standard transfection methods or into a germ cell to produce transgenic organisms.

The selective expression of transgenes to specific cell or tissue types arise from experiments aimed at identifying promoter/enhancer elements responsible for the restricted expression and one of the early studies by Storb et al. reported that an immunoglobulin kappa chain gene may be expressed exclusively in B cells(Storb *et al.*, 1984). The use of transgenic mice has recently also led to substantial progress toward understanding collagen gene regulation. Expression patterns of reporter transgenes controlled by different promoter constructs from mouse collagen genes suggests a modular arrangement of regulatory elements, with certain critical sequences directing expression of collagen genes to specific mesenchymal cell types at the appropriate time in embryonic development(Rossert *et al.*, 1995). Reporter transgenes regulated by such *cis*-acting sequences therefore function as differentiation markers for mesenchymal cell lineages. Similar studies have identified a fibroblast-specific transcriptional enhancer located in the far-upstream region of the murine proα2(I) collagen (Col1a2) gene(Bou-Gharios *et al.*, 1996). A 6-kb sequence (-19.5 kb to -13.5 kb upstream of the transcription start site) was shown to endow high-level fibroblast-

specific expression to reporter genes linked to endogenous and heterologous minimal promoters in transgenic mouse embryos(De *et al.*, 2002). These embryonically defined lineage-specific *cis*-acting enhancer elements are therefore useful for directing expression of a gene of interest to a specific cell-type such as fibroblasts. This novel concept is explored further with the transgenic TβRIIΔk murine strain in this thesis and will be discussed in subsequent chapters.

Several transgenic murine models for SSc have been described. The identification of the intragenic duplication of the Fbn-1 gene in the Tsk1/+ mice led to the development of a transgenic mouse expressing a mutated Fbn-1 transcript(Saito *et al.*, 2000). In this study, the DNA fragment containing the mutant Fbn-1 gene was injected into ova obtained from fertilised C57BL/6 mice of which several founders were obtained and crossed with C57BL/6J mice to establish the transgenic lines. These transgenic mice develop autoantibodies including anti-topoisomerase and anti-Fbn1 antibodies as well as cutaneous hyperplasia. Compared to the Tsk mice, these transgenic mice do not develop lung emphysema or cardiac hypertrophy, suggesting that the Tsk phenotype is more complex than initially thought. Similarly, other authors reported that transgenic mice overexpressing CD19 spontaneously develop high titers of topoisomerase I–specific antibodies characteristically associated with SSc, although they do not develop clinical features of the disease(Sato *et al.*, 2000). Recent studies focussing on manipulation of the TGFβ receptors provide solid support for the critical role of the TGFβ pathway in the pathogenesis of fibrosis and vascular damage in SSc. This was demonstrated using a transgenic mice with conditional, fibroblast-specific gene expression of a constitutively active TGFβ receptor type I(Sonnylal *et al.*, 2007). These mice display features similar to SSc including dermal fibrosis, structural vasculopathy and evidence for generalised abnormalities in ECM deposition.

Knockout mice are usually generated by germline deletion of targeted sequence of gene by homologous recombination. Such germline deleted progeny can be backcrossed by background to provide strains deficient in specific gene products. This approach has been a milestone in understanding many mouse models of human diseases and for dissecting

potential pathogenetic mechanisms. Other knockout models with mice lacking the genes encoding relaxin(Samuel *et al.*, 2005), caveolin(Galdo *et al.*, 2008), Thy-1(Hagood *et al.*, 2005) and Fli-1(Kubo *et al.*, 2003) have been studied and they demonstrate certain fibrotic or vascular features of SSc or show enhanced sensitivity to fibrosis induced by bleomycin. Mice with targeted deletion of relaxin developed spontaneous dermal and lung fibrosis but did not demonstrate vascular or immunological features of SSc.

Transgenic and gene deleted murine strains have been invaluable in not only by de novo incorporation of a mutant gene but also by back-crossing established genetically determined mouse models of SSc with other transgenic mice. The latter strategy is illustrated with back-crossing Tsk1 mice with other mutant strains to explore the dependence on an intact immune response repertoire and upon a number of candidate mediators and pathways. Genetic complementation with a collagen-defective transgenic strain for Col5a2 gene was shown to reduce the degree of cutaneous fibrosis in Tsk1 mice(Phelps *et al.*, 1998;Andrikopoulos *et al.*, 1995) and Tsk1 mice transgenic for a T cell receptor Vβ8.2 segment fail to develop cutaneous hyperplasia(Ong *et al.*, 1999), suggesting that the T cells that mediate this aspect of the tight skin syndrome use distinct T cell antigen receptors. In another genetically-modified murine model, back-crossing IFNγ-receptor-deficient mice into lupus-prone MRL/lpr murine strain generated striking histological findings with accumulation of collagen in connective tissue of various organs and a vasculopathy characterised by vascular intimal thickening(Le *et al.*, 1999). In summary, these transgenic and knockout mice provide robust novel experimental tools for advancing future studies in the pathogenesis of SSc.

Model	Aspects of SSc pathogenesis that are reproduced				Key features
	Vascular	**Inflammation**	**Autoimmunity**	**Fibrosis**	
Naturally-occurring					
Tsk1	√	–	√	√	Genetic in-frame duplication of the fibrillin-1 gene, which leads to ECM development. Altered B-cell signalling, due to elevated expression of CD19. Fibrosis abrogated by IL-4 receptor deficiency.
Tsk2	–	√	√	√	Unknown genetic defect leading to tissue fibrosis. Early inflammatory cell infiltrate noted in some studies.
UCD-200	√	√	√	√	Inbred strain of chickens. Prominent vascular involvement with inflammation and widespread fibrosis.
Induced					
Bleomycin	–	√	–	√	Production of reactive oxygen species, endothelial cell damage and adhesion molecule expression. Some apoptosis of mononuclear cells. Severity is mouse strain-dependent. Activation of TGFβ axis
GvHD I (B10.D2 vs Balb/C)	√	√	–	√	Transfer of spleen cells from B10.D2 mice into irradiated Balb/C, causing skin and lung fibrosis and infiltrates of $CD3^+$ T cells, monocytes and macrophages.
GvHD II (B10.D2 vs Rag-$2^{-/-}$)	√	√	√	√	Transfer of spleen cells from B10.D2 mice into RAG-2 null mice induces systemic fibrosis, inflammation and autoantibodies.
Genetically engineered					
MRL/lpr/IFNgR-/-	–	√	√	√	Lupus prone MRL/lpr strain develops dermal and visceral including pulmonary inflammation and fibrosis when lacking IFNγ receptors.
Conditional TGFβRI	√	√	–	√	Fibroblast-restricted conditional expression of TGFβRI induces generalised fibrosis and inflammation.

Table 1.4. Selected murine models for systemic sclerosis (Varga & Abraham, 2007).

1.1.6 Extracellular Matrix in SSc

1.1.6.1 ECM – Composition and Function

The extracellular matrix is comprised of proteoglycans like decorin and fibromodulin; fibrous proteins including collagen, fibrillin and elastin; adhesion molecules like fibronectin and laminin; and different subtypes of matrix metalloproteinases (MMPs). ECM is a complex structure offering a scaffold for cells as well as influencing cellular phenotype and functions, cell migration, shape, proliferation, differentiation and biosynthesis of connective tissue macromolecules. The deposition of ECM is a pivotal event during wound healing as well as within pathological conditions such as keloid formation and tumour progression.

1.1.6.2 Collagen and SSc

Collagens constitute the major fibrous proteins in ECM. According to the primary structure of different α chains and their assembly into collagen molecules, twenty seven different types of collagen, encoded by more than 40 genes, are currently recognised. These include the subfamily of fibrillar collagen (I, II, III, V, XI), network-forming collagen IV gene products, the fibril-associated collagens with interrupted triple-helix (FACIT) molecules, transmembrane collagens, beaded-filament-forming Type VI collagen, anchoring-filament collagen VII and short-chain, hexagonal-lattice-forming collagens VIII and X. Type I collagen is the most fibrous form and comprises close to 84% of the collagen synthesised by fibroblasts. Type I collagen, the main constituent of the ECM, is a triple helix composed of two α-1 and one α -2 chains, the products of COL1A1 and COL1A2 genes. The excessive deposition of Type I collagen leads to skin and internal organ fibrosis. Early studies suggested that patients with early onset SSc demonstrated increased amounts of type III collagen in the lower dermis whereas type I collagen deposition occurs in the later stage of the disease(Fleischmajer *et al.*, 1978;Prockop & Kivirikko, 1995). The type I collagen genes are often used as a surrogate marker for disease activity because the excessive production of extracellular matrix by SSc fibroblasts is due in part to increased transcription of the type I collagen genes. The expression of these genes is regulated at multiple levels by numerous cytokines and transcription factors.

1.1.6.3 Regulation of ECM by cytokines/chemokines in SSc

The regulation of ECM accumulation and collagen gene expression in SSc is tightly controlled. This process is modulated by paracrine/autocrine mediators, cell-to-cell contact, hypoxia and contact with the surrounding ECM. The collagen genes contain key cis-acting regulatory elements with conserved nucleotide sequences that are recognised specifically by DNA-binding transcription factors(Ghosh, 2002). In response to extracellular cues, Sp1, Ets1, Smad3, Egr-1, and CCAAT-binding factor (CBF) stimulate collagen expression. In contrast, Sp3, C/EBP and Fli1 suppress it. Transcription factors may interact with one another, and with non-DNA-binding cofactors, scaffold proteins, and chromatin-modifying enzymes including p300/CBP and histone deacetylases(Ghosh *et al.*, 2007). Alterations in the expression levels, activities or interactions among the various transcription factors and cofactors may contribute to the persistent fibroblast activation in SSc.

At the same time, it is possible that disruption of normal negative regulation of matrix occurs in SSc, leading to tissue fibrosis. Several studies have focused on TNFα, IFNγ and recently Hepatocyte Growth Factor (HGF). HGF was shown to reduce collagen production in SSc fibroblasts by enhancing MMP-1 expression and this is facilitated by increased autocrine TGFβ signalling leading to overexpression of HGF-receptor c-met(Jinnin *et al.*, 2005). Collagen suppression by TNFα is likely to occur via several mechanisms and may involve induction of NFκB which in turn interferes with Sp-1-mediated activation of collagen transcription(Rippe *et al.*, 1999;Jinnin *et al.*, 2005). The mechanism by which IFNγ causes collagen gene suppression may lie in its induction of Interferon Regulatory Factor-1. Transient transfection experiments in human fibroblasts revealed a region between -129 and -109 may mediate IFNγ mediated suppression of the human α1(I) collagen activity(Yuan *et al.*, 1999). Functional impairment of Smad7 has been demonstrated in SSc fibroblasts. Recent studies have also implicated altered expression or dysregulation of other cell-intrinsic endogenous repressors of collagen synthesis including the transcription factors Fli-1 and the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)-γ. The resultant defect in suppression of fibrogenic responses may contribute to failure to limit fibroblast activation, thereby contributing to ECM accumulation and fibrosis.

Several chemokines are involved in the fibrotic processes by upregulating production of collagen. MCP-1 increases collagen expression in dermal and lung fibroblasts probably via autocrine TGFβ effect(Gharaee-Kermani *et al.*, 1996). This is consistent with the results from another study using a renal model of fibrosis induced by a unilateral ureteral obstruction in CCR2 gene-targeted mice in which transcripts and protein of MCP-1, transforming growth factor-β, and type I collagen were decreased compared to wildtype mice(Kitagawa *et al.*, 2004). Receptor blockade against CCR1, which binds to several MCP chemokines including MCP-3, has been shown to reduce collagen and smooth muscle actin expression expression, in addition to abrogation of inflammatory cell infiltrate in several animal models of fibrosis(Anders *et al.*, 2002;Anders *et al.*, 2004;Eis *et al.*, 2004). Recent data showed that CCL18 stimulate collagen mRNA and protein production by dermal and lung fibroblasts through activation of transcription factors Sp1 and c-Myb(Atamas *et al.*, 2003). This is in contrast to a study which revealed that systemic administration of CXCL11 reduced pulmonary collagen deposition, procollagen gene expression and extracellular matrix deposition in the lung of bleomycin-treated mice(Burdick *et al.*, 2005). The authors reported that this effect is related to the reduction in angiogenic activity with decrease in number of endothelial cells with no direct functional effect on lung fibroblasts or leucocyte populations. Recent reports have suggested in addition to local tissue fibroblasts responsible for fibroproliferative process, circulating cells of haematopoietic origin or fibrocytes have been shown to express ECM proteins, such as pro-Col I and pro–Col III, and migrate in response to chemokines such as CXCL12 and traffic to the lungs in a murine model of bleomycin-induced pulmonary fibrosis(Phillips *et al.*, 2004).

There is increasing evidence for the role of Akt in regulation of fibrotic response(Wu *et al.*, 2007;Vittal *et al.*, 2005;Somanath *et al.*, 2008). Akt is a key enzyme in signal transduction pathways involved in cell survival, glycogen synthesis, cell-cycle progression, angiogenesis and migration. The three Akt isoforms are ubiquitously expressed and are likely to have overlapping functions. Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K) by growth factors, stimulators of G-protein-coupled receptors (GPCR) and

integrins. Following PI3K activation, Akt is recruited at the plasma membrane via binding of phosphoinositol lipids to the Akt pleckstrin homology domain. TGFβ increases Akt phosphorylation in dermal fibroblasts and Akt is constitutively phosphorylated in SSc biopsies suggesting an important role for Akt activation in collagen synthesis in SSc fibroblasts(Jun *et al.*, 2005). More recently, Akt activation has been shown to mediate the downregulation of the collagen protease MMP1(Bujor *et al.*, 2008).

1.1.6.4 Fibroblasts in SSc

Mesenchymal fibroblasts that contribute to excessive tissue repair or fibrogenesis (the production of ECM, notably collagen type I, and the development of contractile myofibroblasts) in SSc can be derived from at least three types of progenitor populations. In addition to resident connective tissue fibroblasts which may be activated directly along the myofibroblast lineage as discussed in **1.1.6.3**, fibrocytes or mesenchymal stem cells from the bone marrow and circulation may be recruited, in response to chemokines, into the extravascular tissues at sites of injury(Varga & Abraham, 2007). Fibrocytes are CD34+ bone marrow-derived mesenchymal cells normally present in small numbers in the peripheral blood. They express CD14+ and chemokine receptors, CCR3, CCR5 and CXCR4 that facilitates trafficking into specific tissues including fibrotic lesional tissues whereby they undergo specialisation into fibroblasts and myofibroblasts, losing the CD14 and CD34 markers in the process(Quan *et al.*, 2006). Other studies have also identified multipotent monocyte-derived mesenchymal progenitor cells in peripheral blood(Gomer, 2008).

Other resident cell populations, such as epithelial cells, might also potentially serve as alternative sources of fibroblast-like cells. EMT is a well characterised phenomenon involved in the generation of mesenchymal cells in several models of renal fibrosis and Crohn's disease (Bataille *et al.*, 2008;Baum *et al.*, 2008). Another potential fibroblast progenitor in SSc is the pericyte. Being integral cells of microvessels, pericytes maintain microvascular integrity and function, both regulating, and being regulated by, the endothelium. In response to endothelial cell injury, pericytes are activated under the influence of endothelin-1 (ET-1) or platelet-derived growth factors (PDGFs) and are able to

express a distinct splice variant of fibronectin (ED-A or FN), which, in the presence of TGFβ, is crucial for myofibroblast differentiation. Transition of vascular pericytes into myofibroblast-like cells not only contributes directly to fibrosis but also promotes the expansion and differentiation of resident 'immature' fibroblasts into myofibroblasts(Rajkumar *et al.*, 1999). Therefore, the origins of the myofibroblasts in SSc also appear diverse and the relative commitment of mesenchymal cells towards this lineage will determine which cell populations predominate in fibrosis in SSc.

1.2 Chemokines

1.2.1 Classification

Chemokines constitute a family of 42 small, heparin-binding mainly secreted proteins, smaller than cytokines with a molecular weight of 8-14 kDa which chemoattract cells by engaging 19 transmembrane heptathelial GPCRs (**Figure 1.5**). Induction of chemotaxis depends predominantly on coupling to pertussis toxin-sensitive Giα proteins, but some chemokine receptors couple to additional G proteins. Chemokines were originally identified by their chemotactic activity on bone marrow-derived cells as key mediators in recruitment of innate immune cells and effector T cells to inflammatory sites. Approximately 80% of these proteins have from 66 to 78 amino acids in their mature form. The remainder are larger with additional amino acids occurring upstream of the protein core or as part of an extended C-terminal segment. They played a key role in leucocyte trafficking (**Figure 1.6**), recruiting and recirculation. In addition, they are also important in many pathophysiological processes including allergic responses, infectious and autoimmune diseases, lymphoid trafficking (Luther & Cyster, 2001), lymphoid organ development, angiogenesis (Belperio *et al.*, 2000), inflammation, tumour growth and haemapoietic development (Youn *et al.*, 2000).

They are characterised by a conserved protein scaffold that is strictly dependent on two conserved disulfide bonds connecting NH_2-terminal cysteine residues, whose relative position determines the identification of four subfamilies. The majority of chemokines fall into CC (CCL1-28), CXC (CXCL1-16) subfamilies, while the C family contains only 2 members (XCL1 and XCL2) and CX_3C only 1 member (CX_3CL1) (Murphy *et al.*,

2000;Murphy, 2002) as shown in **Table 1.5.** In the CXC chemokine subfamily the cysteine residues are separated by a single intervening amino acid, while in the CC chemokine subfamily, the first two cysteine residues are in adjacent position. The C subfamily presents a single cysteine residue, and CX_3C chemokines, with three amino acid groups separating the cysteine tandem. These subfamilies have also different location in the genome: most CC chemokines are coded by a large multigenic cluster on chromosome 17q11.2, while most of the CXC chemokines are coded by two large multigenic clusters on chromosome 4q21. C and CX_3C chemokines are encoded by single genes located on chromosome 1q23, and 16q13 for the CXC chemokine.

This group of chemoattractants continues to grow and chemokines were identified in cell-culture media by classical protein chemistry, followed by cDNA cloning and over last few years, through broad-based searches from sequence homology in expressed sequence tag databases. The chemokines are identified with their original names derived from their function or from the cell type that produced them as well as the new systematic classification (Zlotnik & Yoshie, 2000). In analogy to their ligands, four subfamilies of receptors distinguished: 10 for CC chemokines (CCR1 to 10), 6 for CXC chemokines (CXCR1 to 6), and 1 for C chemokines and CX3C chemokines (XCR1 and CX3CR1 respectively). They are identified through the cloning and characterisation of numerous "orphan" receptors.

One of the key *in vitro* characteristic features of chemokines is that several chemokines bind to more than one receptor and the majority of chemokine receptors have multiple possible ligands, although not shared between CXC and CC subfamilies. This pleiotropic feature, characteristic of the chemokine family, is responsible for their redundant action on target cells, which accounts for the biochemical robustness of the chemokine system. For example, in animal model of allergic airway responses, the production of chemokines is organised in a coordinated fashion that is dependent upon multiple chemokines for the recruitment and activation of different cell populations at distinct stages of disease evolution. Similarly, in relapsing remitting experimental autoimmune encephalomyelitis, the rodent model for multiple sclerosis, it was shown that CCL3/MIP-1α is involved in the

onset of the disease, whereas CCL2/MCP-1 has a key role in relapses(Kennedy *et al.*, 1998). Temporal expression of chemokines has also been demonstrated in animal models of arthritis: MCP-1 appeared to be involved in the later course of rat adjuvant-induced arthritis, a model for rheumatoid arthritis(Kasama *et al.*, 1995). Another group of investigators observed elevated CCL5/RANTES and CCL3/MIP-1α production in murine collagen-induced arthritis compared with that in normal mice (Thornton *et al.*, 1999).

Determining the time course of chemokine expression during these inflammatory diseases should aid in pinpointing therapeutic intervention points in disease.
In addition, on the level of chemokine receptor-ligand interaction much promiscuity can be found as many chemokines and chemokine receptors show cross reactivity. A single cell may produce multiple different chemokines that have the same function. This particular type of redundancy is termed polyspeirism (Mantovani, 1999). Similar feature has been observed with other cytokine networks and provides a certain degree of robustness as alterations of the individual components will not greatly influence immune function. Taken together, redundancy, promiscuity and polyspeirism protect the chemokine network from excessive modulation. Although the *in vitro* evidence suggests that the chemokine network exhibits much redundancy, several chemokine actions *in vivo* are not at all redundant. Studies in chemokine receptor knockout animals suggest that several apparently redundant chemokine receptor knockout reveal altered immune responses (Carter, 2002). From this, it appeared that the chemokine network was less redundant as initially assumed and that several factors contribute to fine-tuning of the chemokine response(Devalaraja & Richmond, 1999).

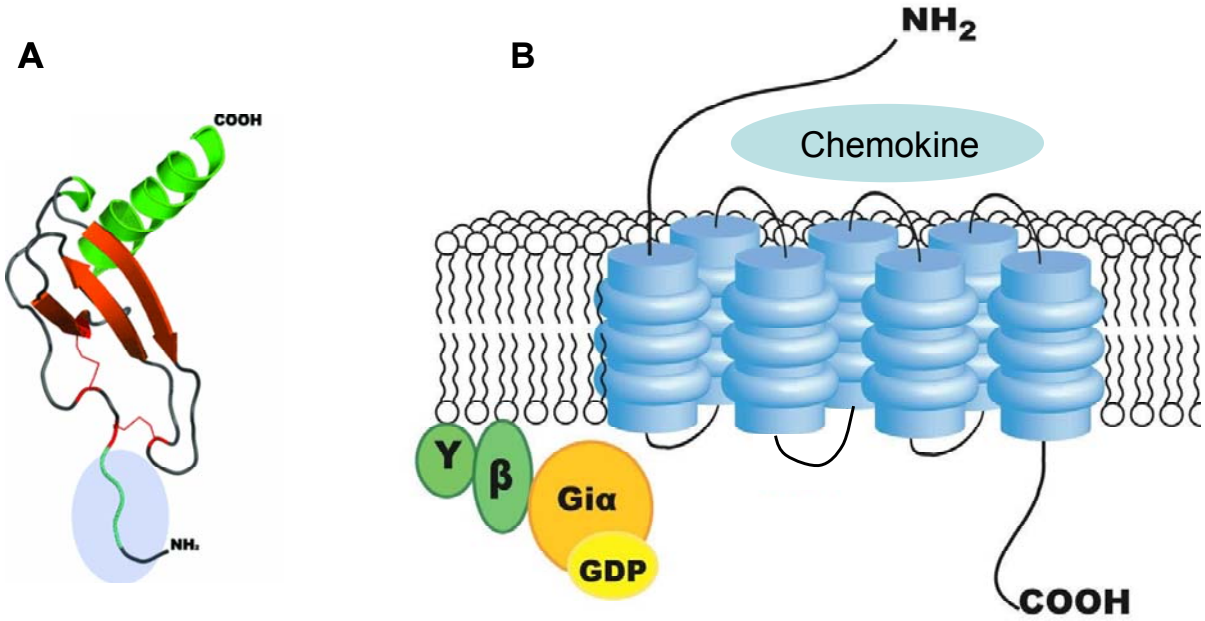


Figure 1.5 Chemokines adopt a characteristic fold that consists of an N-terminal unstructured domain that is critical for signalling, a three-stranded β-sheet connected by loops and turns, and a C-terminal helix as shown in **A** in which the highlighted region of N-terminal signalling domain is postulated to insert into the helical bundle of the receptor, shown in **B**. Chemokine receptors are seven-transmembrane molecules coupled to the heterotrimeric G proteins comprised of three subunits:α, β and γ. The β- and γ-subunits are assembled into γβ-dimers that act as functional units (O'Hayre *et al.*, 2008).

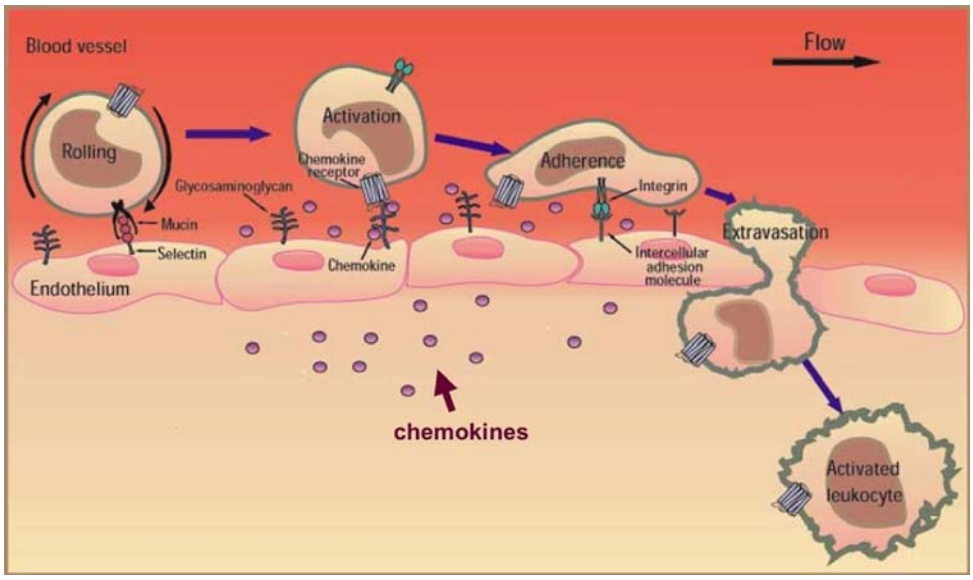


Figure 1.6 Schematic representation of model of chemokine involvement in leucocyte trafficking into discrete stages. The initial stage involves rolling of leucocytes along the vessel wall through transient interactions between specific selectin proteins and their carbohydrate ligands. The next stage involves activation of the leucocyte resulting in a firm adhesion to the endothelial surface mediated by integrin molecules. The final stage involves extravasation of the leucocyte which includes crawling along the endothelium, diapedesis and migration into the interstitial space (Allen *et al.*, 2007).

Systematic Names	Original Names	Receptors
CC family		
CCL1	I-309	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1α, LD78α	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR2, CCR5
mCCL6	C10	CCR1
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2	CCR3
mCCL9	MIP-1γ	CCR1
mCCL10	MIP-1γ	CCR1
CCL11	Eotaxin-1	CCR3
mCCL12	MCP-5	CCR2
hCCL13	MCP-4	CCR2, CCR3
hCCL14	HCC-1	CCR1
hCCL15	HCC-2, Lkn-1, MIP-1δ	CCR1, CCR2
CCL16	HCC-4, LEC	CCR1, CCR2, CCR5
CCL17	TARC	CCR4
hCCL18	PARC, DC-CK1, AMAC	Unknown
CCL19	ELC, MIP-3β, exodus-3	CCR7
CCL20	LARC, MIP-3α, exodus-1	CCR6
CCL21	SLC, 6Ckine, exodus-2	CCR7
CCL22	MDC, STCP-1	CCR4
hCCL23	MPIF-1, CKβ8	CCR1
hCCL24	Eotaxin-2, MPIF-2	CCR3
CCL25	TECK	CCR9
hCCL26	Eotaxin-3	CCR3
CCL27	CTACK-ILC	CCR10
CCL28	MEC	CCR3, CCR10

Systematic Names	Original Names	Receptors
CXC family		
CXCL1	GROα	CXCR1, CXCR2
CXCL2	GROβ, MIP-2	CXCR2
CXCL3	GROγ	CXCR2
CXCL4	PF4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
hCXCL7	NAP-2	CXCR2
hCXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3A/B
CXCL10	IP-10	CXCR3A/B
CXCL11	I-TAC	CXCR3A/B
CXCL12	SDF-1α/β	CXCR4, CXCR7
CXCL13	BCA-1	CXCR5
CXCL14	BRAK	Unknown
mCXCL15	Lungkine	Unknown
CXCL16	SR-PSOX	CXCR6
C family		
XCL1	Lymphotactin α, SCM-1α	CXR1
hXCL2	SCM-1β	CXR1
CX3C family		
CX_3CL1	Fractalkine	CX_3CR1

Table 1.5 The chemokine system. The ligands are identified with one old acronym and the current nomenclature, in which the first part of the name identifies the family and L stands for ligand, followed by a progressive number. Chemokines present only in humans or mice are prefixed with ‘h’ or ‘m’ respectively.

1.2.2 Chemokines receptors and Signalling

One of the key features of chemokines which distinguishes them from other cytokines is that they are the only members of the cytokine family that act on the receptor superfamily of GPCRs. These represent single polypeptide chains with three extracellular and three intracellular loops with an acidic N-terminal extracellular domain involved in ligand binding, and a serine/threonine-rich intracellular C-terminal domain. The external interface contributes to the ligand-recognition specificity, while conserved transmembrane sequences, the cytoplasmic loops and the C-terminal domain are involved in receptor signalling and internalisation.

Like other GPCR agonists including bacteria-derived formyl-peptides, the complement fragments C5a and C3a, bioactive lipids (sphingosine, leukotrienes and platelet-activating factor), activation of G_i PCR when bound to chemokines requires their functional coupling to the heteromeric G_i proteins (Thelen, 2001;Gilman, 1987). After ligand binding the G_i protein rapidly exchanges GDP for GTP, and the GTP-loaded Gα and βγ subunit dissociate from the receptor to activate downstream effectors. Pertussis toxin treatment causes uncoupling of the G_i protein from the receptor (Kurose *et al.*, 1983;Van Dop *et al.*, 1984) and abolishes most receptor-mediated responses, but does not prevent receptor phosphorylation and internalisation (Amara *et al.*, 1997;Giannini & Boulay, 1995). The latter are now considered to be the main mechanisms for termination of receptor-mediated responses (Haribabu *et al.*, 1997;Aragay *et al.*, 1998). Pharmacological inhibitors have been used to investigate the regulation of chemokine-mediated activities such as chemotaxis of monocytes. Downstream of G proteins, chemokines induce activation of kinases, including PI3K and extracellular signal regulated kinase and p38 kinase (Tilton *et al.*, 2000;Jones *et al.*, 1995). The free Gβγ activates the small G protein Ras which in turn directly activates PI3K, promoting conversion of the membrane phospholipid PIP_2 into PIP_3. Once generated, PIP_3 will then recruit proteins including Akt/PKB to the plasma membrane. This step is required for chemotaxis including gradient sensing and cell polarisation for neutrophils and fibroblasts(Haugh *et al.*, 2000;Wang *et al.*, 2002).

It is also increasingly recognised that there is a subfamily of chemokine receptors that binds the chemokines but do not facilitate cellular migration or activate conventional signalling pathways(Comerford *et al.*, 2007). This characteristic is unique to several receptor members including Duffy Antigen Receptor for Chemokines (DARC), D6 and CCX-CKC. In these 'silent receptors', there is no conserved DRY motif with the triplet amino acid sequence (aspartic acid, arginine and tyrosine) within the second intracellular loop which is necessary required for receptor signalling. For example, DARC which is expressed on vascular endothelial cells of postcapillary venules and veins in many organs including skin shares 40% similarity with chemokine receptors and is able to bind to the pro-inflammatory chemokines in both CC and CXC chemokines (**Figure 1.7**). In vitro data reveals that DARC is able to internalise the ligands upon chemokine binding but it does not support ligand-induced signalling or migration(Mantovani *et al.*, 2006;Nibbs *et al.*, 2003). Similarly, D6 which are selective for CC chemokines and are expressed by lymphatic endothelial cells in different organs including the skin does not demonstrate sustained signalling and functional activities that are typically observed after chemokine receptor signalling(Mantovani *et al.*, 2007). These studies support that such receptors may function as a sink/reservoir or decoy for chemokines by facilitating transfer of chemokines across cellular barriers and present them to leucocytes. However, there is evidence to suggest that members of this subfamily such as D6 may function in vivo by clearing chemokines from the tissue microcirculation acting as negative regulator of inflammatory response(Borroni *et al.*, 2006). Chemokines are thus rapidly dissociated and degraded in intravesical, leaving the chemokine receptor free to recycle to the cell surface. However, it remains unclear if these decoy receptors indeed transduces any other signal in association with internalisation such as non-G protein mediated pathways.

Apart from the cognate GPCR as the main receptor, the chemokine ligand also binds to the glycosaminoglycans(GAG) present on the cell surface or ECM. While the localisation of receptor binding site is highly conserved, there are four possible GAG binding clusters on the chemokine and these may or may not overlap with the receptor binding domain. Thus, GAG may potentially control receptor selectivity. Among these molecules, heparin sulphate

was thought to provide the substratum to which chemokines bind to create a high local concentration of chemokine. In vivo experiment with peritoneal recruitment assay with engineered mutants of MCP-1/CCR2 confirms that engagement with GAGs is necessary to elicit cell migration(Proudfoot *et al.*, 2003).

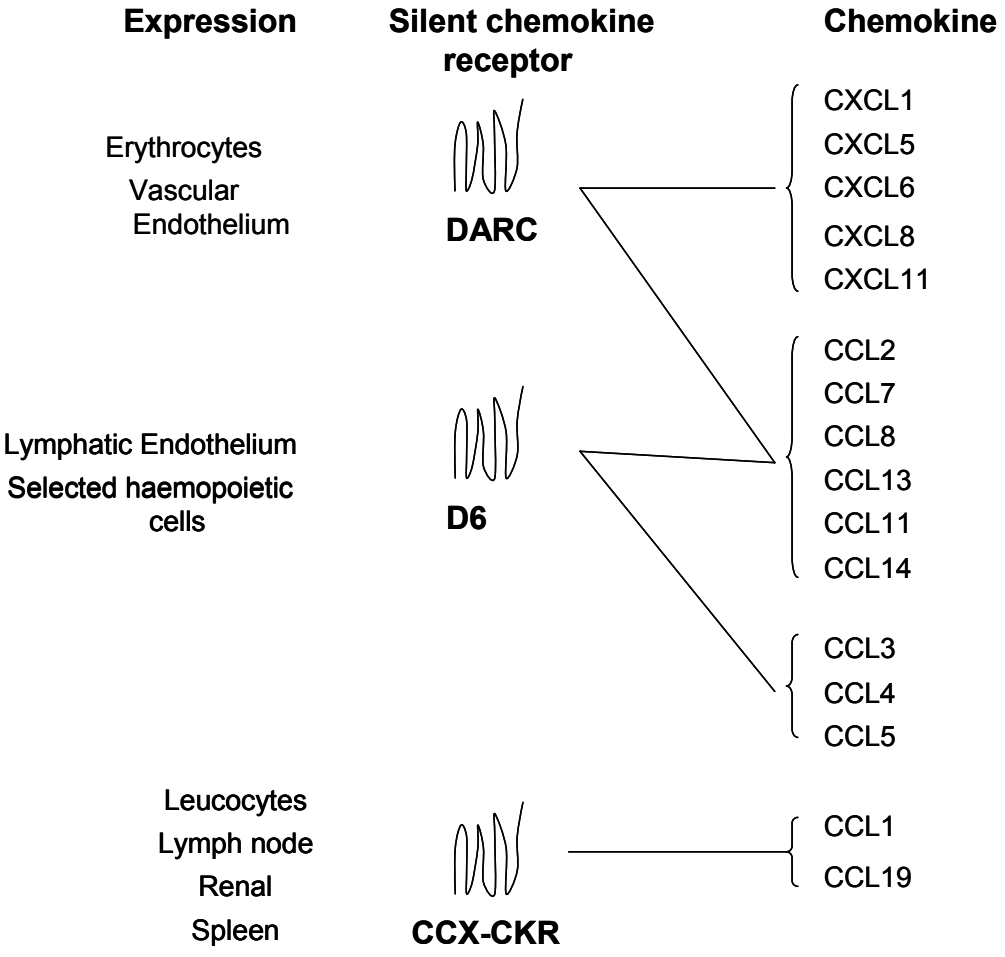


Figure 1.7 Silent chemokine receptors ligand specificity and tissue/cell-type distribution. DARC and D6 are mainly expressed on the endothelium; DARC is also expressed by erythrocytes. CCX-CKR is expressed by various tissues.

1.2.3 Chemokines and Autoimmune Diseases

The cellular infiltrate of immune cells and proinflammatory mediators in SSc forms a complicated network between fibroblasts and immune cells via cell-cell communications. A growing body of evidence points towards the role of chemokine-driven networks in leucocyte activation in autoimmune diseases including SSc. Chemokines mediate leucocyte-endothelium interaction and transmigration at multiple steps and this is accompanied by an inflammatory response with various cell types releasing inflammatory mediators to promote the recruitment of inflammatory cells. For example, MCP-1, CCL11 and CCL17 are critical in T cell polarisation and recruitment into the lesional skin and MCP-1 skews T cell differentiation toward Th2 phenotype. These chemokines also recruit macrophages, which in turn release fibrogenic mediators such as TGFβ and PDGF. Activation of mast cells by MCP-1 and CCL5 releases cytokines and growth factors that are capable of activating fibroblasts or endothelial cells. Moreover, these cellular infiltrate also expresses chemokines, which may play a part in the induction of fibrosis.

As discussed in **1.1.4.1**, patients with SSc have a strong genetic predisposition. Previous studies have identified a variety of genetic polymorphisms in the genes encoding chemokines, and have found significant associations of these polymorphisms with the risk of SSc (**Table 1.6**). A small study of 18 patients with SSc identified an association of the -2518 GG genotype of MCP-1 with SSc. This polymorphism lies within the promoter region and is also associated with increased MCP-1 production in vitro(Karrer *et al.*, 2005). However, this was not replicated in other European and Far East patient populations(Carulli *et al.*, 2008;Lee *et al.*, 2007;Navratilova *et al.*, 2008). Lee reported that the two genetic polymorphisms (non-polymorphic +113C/T and +459C/T) for CCL3 were not implicated in the development of SSc. However, in a Caucasian study group, 2 polymorphisms occurring close to each other in the CXCR-2 gene (+785 C/T and +1208 T/C) were associated with SSc, independent of the presence of lung fibrosis(Renzoni *et al.*, 2000). 2 coding polymorphisms for CX3CR1, the key receptor for fractalkine was associated with SSc-associated pulmonary arterial hypertension(Marasini *et al.*, 2005). In addition, a significant gene-gene interaction between polymorphisms in the genes CXCL8 -353 A/T

and CCL5 -403 G/A, and both of these were associated with an increased risk of SSc. This data is interesting in that it suggests the importance of potential gene-gene interactions in SSc. However, there was no association demonstrated with polymorphisms for CCR2 and CCL5(Lee *et al.*, 2007). Interestingly, NSF as described in Section **1.1.4**, has been shown to be associated with upregulation of dermal MCP-2/CCL8 on immunofluorescence analysis. In addition, in vitro activation of macrophages treated with gadolinium also resulted in transcript overexpression for MCP-2/CCL8, MCP-1, CXCL10 and CXCL11. These strongly suggest the role of chemokines in SSc and SSc-like syndromes.

Gene	SSc	Control	Populations	Year	Reference
MCP-1 (MCP-1)	18	139	Germany	2005	(Karrer *et al.*, 2005)
MCP-1 (MCP-1)	99	199	Korea	2007	(Lee *et al.*, 2007)
MCP-1 (MCP-1)	94	102	UK	2008	(Carulli *et al.*, 2008)
MCP-1 (MCP-1)	46	449	Slovak	2008	(Navratilova *et al.*, 2008)
CCL3 (MIP-1α)	99	199	Korea	2007	(Lee *et al.*, 2007)
CCL5 (RANTES)	99	199	Korea	2007	(Lee *et al.*, 2007)
CCR2	99	199	Korea	2007	(Lee *et al.*, 2007)
CCR5	99	199	Korea	2007	(Lee *et al.*, 2007)
CCR7	100	40	Germany	2007	(Kahlmann *et al.*, 2007)
CX3CR1	76	204	Italy	2005	(Marasini *et al.*, 2005)
CXCL8 (IL-8)	128	194	UK	2000	(Renzoni *et al.*, 2000)
CXCL8 (IL-8)	99	199	Korea	2007	(Lee *et al.*, 2007)
CXCR1 (IL-8R1)	128	194	UK	2000	(Renzoni *et al.*, 2000)
CXCR1 (IL-8R1)	99	199	Korea	2007	(Lee *et al.*, 2007)
CXCR2 (IL-8R2)	128	194	UK	2000	(Renzoni *et al.*, 2000)

Table 1.6 Candidate chemokine genes associated with SSc susceptibility.

Studies in gene-targeted mice provided evidence for a non-redundant role for chemokines in leucocyte recruitment associated with inflammatory response. For example, CXCR2-/- mice demonstrated that a significant role of CXCL8 in sustaining neutrophils-mediated inflammatory disease, suggesting that CXCL-8-CXCR1/2 axis resulted in almost complete protection from multiple inflammatory challenges(Harada *et al.*, 1994). Further studies support a non-redundant role of chemokine system in autoimmune diseases including rheumatoid arthritis(Koch, 2005), Sjogren's Syndrome(Petrek *et al.*, 2002), sarcoidosis(Kolek *et al.*, 2002), autoimmune thyroiditis(Chen *et al.*, 2005), multiple sclerosis(Tanuma *et al.*, 2006), psoriasis(Nickoloff *et al.*, 2007) and autoimmune uveoretinitis(Takeuchi *et al.*, 2005). In addition, activity of these diseases is related to presence and levels of the chemokines. For example, patients with rheumatoid arthritis show a correlation between clinical disease activity and MCP-1 and CCL5 levels, and the effect of methotrexate treatment on radiological erosions may be predicted by serum levels of CCL5 and CXCL1(Boiardi *et al.*, 1999). Similarly, raised sera levels of CXCL10 have been associated with disease activity in SLE, and CCR4 and its ligand CCL17 have been

described to play a role in leucocytes recruitment in cutaneous lupus erythematosus(Wenzel *et al.*, 2005).

In addition to autoimmune diseases, chemokines and their receptors have been described to be involved in different aspects of tumourogenesis and these can be divided into three board categories: providing directional cues for migration or metastases, shaping the tumour microenvironment and providing survival and growth signals. In numerous cancers, malignant cells demonstrate aberrant expression of chemokine receptors relative to their normal counterparts notably CXCR4, CCR7 and CCR10(O'Hayre *et al.*, 2008). CXCR4 expression reflects tumour progression, and it is also associated with lymph node metastasis in oral squamous cell carcinoma, human nasopharyngeal carcinoma, pancreatic cancer, non-small lung cancer and human colorectal cancer. The altered chemokine/receptor expression may be related to the tumour microenvironment and altered signalling in the cancer cell itself. Antibodies targeted against CXCR4 have been demonstrated to abrogate metastasis in a murine model of breast cancer.

The altered tumour microenvironment represent a state of chronic inflammation with the presence of tumour cells, development of aberrant vascular network and the persistence of inflammatory mediators: all these factors may help in the progression of cancer.
In addition to facilitating migration from the primary tumour to a metastatic site, chemokine receptor signalling may also provide a survival advantage. Chemokines are ideal migratory signals to control the homing of lymphocytes to protective niches in the bone marrow and lymph nodes, and they may also promote survival of cancer cells. The molecular strategies for survival and growth are often the result of utilising and reprogramming of existing physiological pathways.

1.2.4 Chemokines/Chemokines receptors as targets for therapies

Evidence for the role that many chemokines and receptors play in the pathogenesis of different acute and chronic inflammatory diseases is rapidly increasing(Anders *et al.*, 2002;Horuk *et al.*, 2001), and there is now increasing interest in targeted therapeutics against chemokines and their receptors. The first pharmacological approach was based on the inhibition of chemokine production through classical anti-inflammatory agents. For example, IL-8 and MCP-1 production were blocked by cyclosporin and FK506. The apparent redundancy of the chemokine network and the lack of fidelity many receptors display for their ligands may render targeting chemokines difficult and may potentially lead to lack of specificity and side effects. However, results from research using knockout mice, specific monoclonal antibodies and receptor antagonists have largely allayed such fears. For example, studies in CCR2 knockout mice suggested a potential role for this receptor in both inflammation and fibrosis since mice lacking CCR2 display impaired mononuclear cell recruitment to sites of inflammation (Kuziel *et al.*, 1997)and are resistant to experimental fibrosis(Moore *et al.*, 2001). This in vivo results indicate that a nonredundant role of individual chemokines in specific pathological circumstances. This would suggest that spatial and temporal regulation might be more functionally relevant than would be expected by extrapolating from in vitro results and suggest a role for chemokines in modulating other aspects of the inflammatory response beyond simple leucocyte recruitment including cross talk with other cytokines and growth factors.

Other studies have demonstrated the success of targeting key proteins such as the antibody neutralisation of the TNFα in autoimmune diseases. There are several ways in which the chemokine network may be targeted for therapeutic interventions (**Figure 1.8**). As for other cytokines, blocking antibodies interfering with the chemokine binding to the receptors have also been developed. However, the need for parenteral administration is a major limitation to widespread clinical use of such agents. As discussed in the following Section **1.2.5**, chemokine variants with N-terminal processing are one of the main chemokine inactivation mechanisms and several N-truncated chemokines have been reported. Modification of the structure of CCL5 with an additional methionine reside at the N-terminal, Met-RANTES retains receptor binding but fails to induce receptor activation. (Ponath *et al.*,

1996;Proudfoot *et al.*, 1996). This analogue has been shown to have profound effects on several models of inflammation including Th-1-mediated inflammatory disorders such as murine collagen-induced arthritis(Ponath *et al.*, 1996) and organ-transplant rejection(Grone *et al.*, 1999) as well as those mediated by Th2 proinflammatory cytokines such as allergic lung airway inflammation (Gonzalo *et al.*, 1998). The CCR1, 3 and 5 antagonist, Met-RANTES has been shown to antagonise effector functions of human eosinophils stimulated with MCP-3 with inhibition of actin polymerisation and intracellular calcium flux transients and release of reactive oxygen species (Chvatchko *et al.*, 2003;Elsner *et al.*, 1997;Elsner *et al.*, 1999). The latter processes have been observed to occur in SSc (Gabrielli *et al.*, 2008) and chemokine inhibition is potentially useful in this aspect of the disease.

The most frequently targeting approach is small molecules that prevent the interaction of the chemokine ligand with its receptor, which is the basis for numerous drug discovery programs in the pharmaceutical industry. The first small molecule targeting the chemokine system was approved for the prevention of HIV infectivity: maraviroc, a small molecule targeting CCR5 developed by Pfizer. Biologicals in the guise of neutralising antibodies are also being developed although to date there are no positive trial outcomes. However neutralising antibodies have been used extensively in the validation of the chemokine system in animal models of disease.

Among the chemokine receptor antagonists that are currently in trials (**Table 1.7**), a recent trial with a human blocking antibody against CCR2, MLN1202 in patients with rheumatoid arthritis reduced the levels of free CCR2 on synovial monocytes but it did not alter the clinical course(Vergunst *et al.*, 2008). In this study, however, there was no analysis on chemokine expression on the fibroblasts which are likely to be the key effector cells in fibrotic diseases. CCR1 antagonists are currently studied in clinical trials for treatment of multiple sclerosis and CCR5 antagonists that block HIV entry into cells are being used in advanced clinical trials as adjuvant treatment for AIDS. Small molecule inhibitors of CX3CR1 are being used in phase I/II trials for psoriasis and rheumatoid arthritis, and CXCR4 antagonists are being evaluated in rheumatoid arthritis and cancer. More recently, a novel compound which selectively targets the production of MCPs including MCP-3,

bindarit, an indazolic derivative was described(Mirolo *et al.*, 2008). It was demonstrated to interfere with monocyte recruitment in several in vivo experimental models of inflammation for pancreatitis, arthritis and lupus nephritis.

Whilst chemokine and receptor inhibition remains a promising area and an unexplored approach, the possible adverse effects of such therapy need careful consideration. Experimental studies with CCR1(-/-) mice have indicated that such approach may not only affect the recruitment phase of the inflammatory cellular infiltrate but also the effector phase of the pathological processes, and therefore may exacerbate or provoke the underlying disease(Topham *et al.*, 1999).

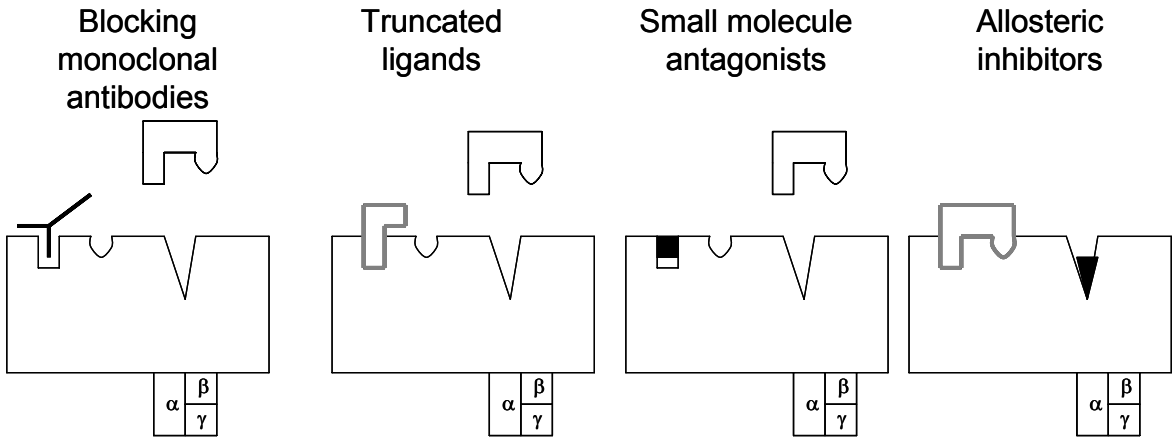


Figure 1.8 Pharmacological inhibition of the chemokine system. Different pharmacological strategies are used to interfere with the chemokine receptor signalling. Blocking monoclonal antibodies sterically impair ligand binding (**A**). N-terminal truncated chemokines bind the receptor and compete with the biologically active full length ligand (**B**). Small molecule competitive antagonists compete with the chemokine for the binding site of the receptor (**C**). In all these cases, the ligand recognition is impaired. Receptor signalling may also be blocked by non-competitive inhibitors (**D**), which binds the receptor in an allosteric site preventing receptor conformational change and activation without interfering with chemokine binding.

Target	Compound and Company	Diseases	Reference
CCR1	BX471(Berlex)	MS, Renal fibrosis(Phase II)	(Wells *et al.*, 2006)
CCR2	INCB-3284 (Incyte)	RA(Phase I)	(Brodmerkel *et al.*, 2005)
	MLN1202 (Millennium)	RA(Phase II)	(Vergunst *et al.*, 2008)
CCR3	766994 (GSK)	Asthma (Phase II)	(Allen *et al.*, 2007)
CCR5	Sch-D (Schering-Plough) TAK-799 (Takeda)	AIDS, Cancer (Phase II)	(Allen *et al.*, 2007)
CXCR2	656933 (GSK)	COPD (Phase I)	(Mirzadegan *et al.*, 2000)
CXCR3	T-487 (Amgen)	MS, RA (Phase II)	(Allen *et al.*, 2007)
CXCR4	AMD3100 (Anormed)	AIDS, Cancer (Phase IV)	(Ribeiro & Horuk, 2005)

Table 1.7 Chemokine receptor antagonists currently in trial. This list is not exhaustive. MS, Multiple sclerosis; RA, Rheumatoid arthritis; COPD, Chronic Obstructive Airways Disease.

1.2.5 Chemokines and Matrix Metalloproteinases (MMP)

The MMPs are a group of zinc-dependent proteinases that are critical for ECM degradation and remodelling. They facilitate tissue degradation required for leucocyte infiltration during inflammation and tumour cell escape during metastases.

Chemokines and MMPs have an interdependent relationship, since they control the function of one another. The action of MMPs releases GAGs that in turn will influence the nature of the interaction between chemokines and their receptors. In addition, cleavage of the ECM may allow the release of deposits of chemokines. For example, in a mouse model of neutrophil-mediated lung injury in response to bleomycin, MMP-7 has been shown to mediate the release of CXC chemokine associated with syndecan-1 and helping to recruit neutrophils into the alveolar space(Li *et al.*, 2002). MCP-1 induces the expression of MMP-12 by monocytes/macrophages and it also upregulates MMP-1, MMP-2 and TIMP-1 expression in human dermal fibroblast at transcriptional level (Yamamoto *et al.*, 2000;Wu *et al.*, 2000). To extend this observation, recent study with MCP-1-/- mice when treated with subcutaneous injections of bleomycin, demonstrated increased expression of MMP-13, TIMP-2, and TIMP-3 levels(Ferreira *et al.*, 2006). This supports the crucial role of both

MMPs and TIMPs in the maintenance of the normal balance between ECM synthesis and degradation. This equilibrium is thought to be disrupted in fibrosis with decreased levels of MMPs and increased levels of TIMPs to create an environment that favours collagen accumulation. It has also been shown that regulation of transepithelial chemokine gradient including MCP-3 is dependent on MMP9 in a mouse lung model of allergy resulting in altered inflammatory cell recruitment in bronchoalveolar lavage of affected mice(Corry *et al.*, 2004).

Conversely, proteolysis of chemokines within their N-terminal signalling domains by MMPs would allow fine-tuning of chemokine activity; examples include the MCPs, CXCL11, CXCL8 and CXCL12. In the case of the MCPs, the cleavage products have impaired activity in chemotaxis assay and reduced calcium signalling activity. In contrast, posttranslational modifications may be mandatory for receptor signalling or results in enhanced biological activity. For example, the cleavage of the six or seven N-terminal residues of CXCL8 by MMP-9 increases the activity of this chemokine(Van Den Steen *et al.*, 2003). Truncated CCR1 ligands have been detected at high levels in synovial fluids from subjects with inflammatory joint disease and these chemokines may mediate the inflammatory responses(Berahovich *et al.*, 2005). Similarly, NH2-terminal truncation of natural MCP-3 between the fourth and fifth amino acid with MMP-1,-2,-3,-13 and –14, will result in reduced chemotactic and calcium signalling but retained its receptor binding properties which essentially acts a as a chemotaxis antagonist(McQuibban *et al.*, 2002). In contrast to MCP-3, the other MCPs are only cleaved by MMP-1 and MMP-3. Unlike the critical chemokine processing at the NH2 terminal leading to altered receptor-binding and activity, very few chemokines undergo processing at the COOH-terminal or other internal sites, and most of them have little or no consequence on activity. Overall, the ability of MMPs to dramatically alter the function of a given chemokine makes the full significance of their presence in the microenvironment difficult to define.

1.2.6 MCP-3

MCP-3 was identified in 1992 in the supernatant of stimulated cultured osteosarcoma (MG-63) cells as an 11 kDa protein(Van Damme *et al.*, 1992). It shares a high sequence

homology with MCP-1 (74%) and MCP-2 (58%). The cDNA and gene encoding human MCP-3 were cloned on the basis of the amino acid sequence determined for the purified MCP-3 protein. The cDNA for the putative murine homologue of human MCP-3 was isolated by different groups and was named MARC (mast cell activation-related chemokine), FIC (fibroblast-inducible cytokine) or murine MCP-3. Human and murine MCP-3 show 59% homology at the protein level.

The gene encoding human MCP-3 is located on chromosome 17q11.2-12. clustered with the other CC chemokine genes. It comprises three exons and two introns, with the first exon containing the 5' untranslated region and a sequence coding for the signal peptide and the first two amino acids of the mature protein. The second exon encoded 3 to 42 of the mature protein whereas the third exon consists of the coding sequence for the COOH-terminal region of the protein and the 3'untranslated region. A polyadenylation signal and several destabilising AT-rich sequences have been described in the 3'untranslated region of the human MCP-3 gene. The TATA-box present in the MCP-3 promoter region controls the rate of basal transcriptional activity and is characteristic of rapidly transcribed genes. Other regulatory elements including a CAAT-box, an AP-1 like sequence and an Ets-like motif have been described(Van *et al.*, 1999). Human MCP-3 is synthesised as a precursor protein of 99 amino acids, including an aminoterminal hydrophobic signal sequence(Van *et al.*, 1999). Cleavage of the signal peptide from the precursor protein yields a mature protein of 76 amino acids (**Figure 1.9**). The primary structures of MCP-3 and the related CC chemokines are shown in (**Figure 1.10**).

Other than stimulated osteosarcoma cells, several other tumour cell lines have been demonstrated to express MCP-3 mRNA **(Table 1.8)**. These include promonocytic U937 cells in which MCP-3 mRNA was found to be transiently induced by phorbol 12-myristate 13-acetate (PMA). In addition, MCP-3 mRNA was also expressed in the myelomonocytic leukaemia THP-1 cell line after stimulation with PMA or lipopolysaccharide (LPS). Low levels of MCP-3 transcripts were expressed by monocytes when activated by IL-1β, TNF-α or IFN-γ. This was suppressed by anti-inflammatory cytokines IL-4, IL-10 or IL-13. Peripheral blood mononuclear cells from lung transplant recipients were shown to express

high levels of MCP-3 protein and this may suggest that these patients are likely to progress to transplant rejection (Hodge *et al.*, 2005). Secretion of MCP-3 protein by mononuclear leucocytes was also demonstrated after stimulation with IL-1β, IFN-α, IFN-β and measles virus.

Synergistic induction of MCP-3 protein was observed in fibroblasts stimulated with a combination of IL-1β and IFN-γ. Physical stress is another mechanism in which MCP-3 may be induced. Cyclic mechanical stretch significantly elevated MCP-3 secretion from cultured human uterine cervical fibroblast cells. The stretch-induced augmentation of MCP-3 expression was significantly suppressed by an activator protein-1 (AP-1) inhibitor, curcumin(Takemura *et al.*, 2004). Stimulation of human umbilical vein endothelial cells (HUVEC) with IL-1β, IFN-γ or TNF-α resulted in expression of the MCP-3 gene. In airway smooth muscle cells, IL-1β, IFN-γ and TNF-α were found to induce MCP-3 mRNA. Dexamethasone significantly inhibited this cytokine-induced accumulation of MCP-3 mRNA, in contrast to IL-4, IL-10 and IL-13 which had no inhibitory effect. Corticosteroids also reduced the number of MCP-3 mRNA-positive cells in the epithelium and subepithelium in endoscopic biopsy specimens from patients with asthma (Fukakusa *et al.*, 2005). Finally, platelets constitutively expressed low levels of MCP-3 mRNA.

Like other MCP chemokines, MCP-3 recruits and activates mononuclear phagocytes and dendritic cells, and to a lesser degree, T lymphocytes and NK cells and occasionally other cell types. The most sensitive cells are lymphocytes and monocytes. MCP-3 elicits a type 1 T-cell dependent immunity ie Th1 type immune responses. MCP-3 gene transfer in P815 mastocytoma cells elicits tumour rejection by activating type 1 T-cell dependent immunity resulting in reduced tumourigenicity and augmented leucocyte infiltration along with perivascular accumulation of dendritic cells in peritumoural tissue and neutrophil recruitment within the tumour (Fioretti *et al.*, 1998). Recent work has also demonstrated that MCP-3 is regulated by β-catenin and dysregulation of MCP-3 associated with impaired β-catenin expression leads to increased tumourogenesis(Fujita *et al.*, 2000). Oestrogen and progesterone downregulate MCP-3 expression individually or in synergy. MCP-3 induces signalling through three separate chemokine receptors,CCR1, CCR2 and CCR3; although

MCP-3 also binds CCR5 with high affinity this results in no detectable intracellular signal transduction, suggesting that MCP-3 may play a role in regulating its own production(Mueller *et al.*, 2002). It is also noteworthy that CCL5 through its interaction with CCR5 is a major stimulus for the production of MCP-3.

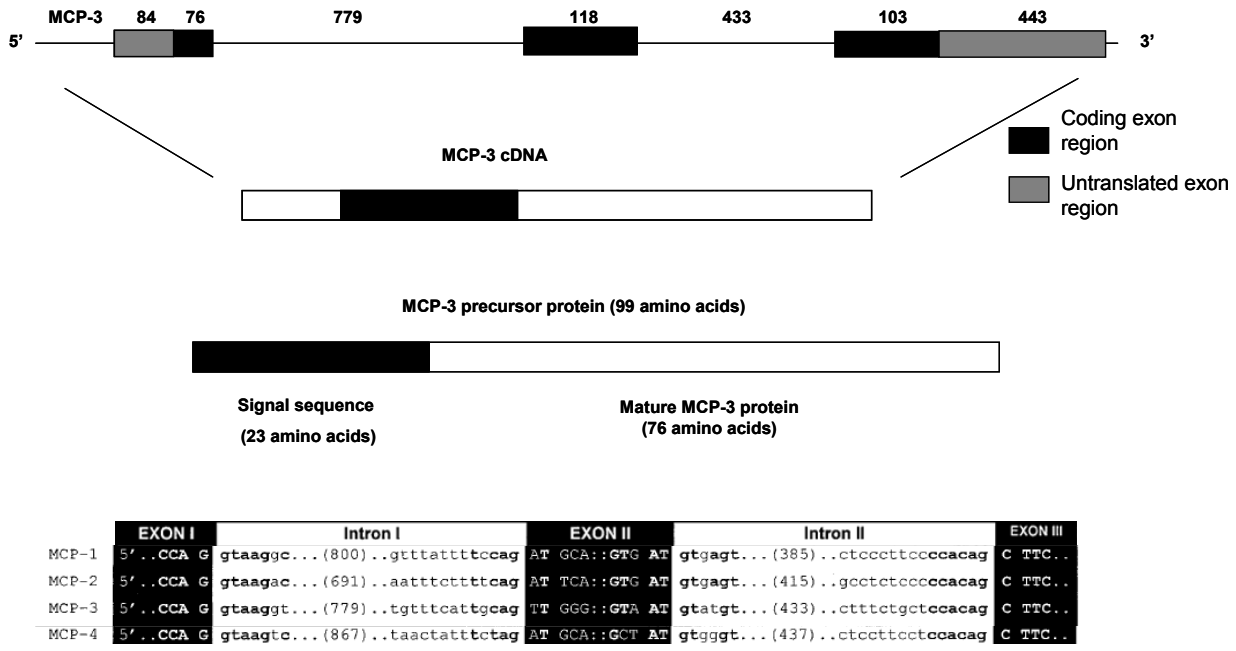


Figure 1.9 Organisation of the human MCP-3 gene, cDNA and protein. The shaded and empty boxes in the top panel indicate translated and untranslated regions of the exon sequences respectively. The intron sequences are indicated as horizontal lines. Length of segments are in bp.
Exon/intron boundaries of the MCP-3 gene in comparison to the CC-chemokines MCP-1, MCP-2 and MCP-4. Nucleotides within exons are represented by capital letters. The size of the introns (number of nucleotides) is indicated.

```
                1    5    10   15   20   25   30   35   40   45   50   55
hMCP-1      QPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAKEICADPKQKW
hMCP-2      QPDSVSIPITCCFNVINRKIPIQRLESYTRITNIQCPKEAVIFKTKRGKEVCADPKERW
hMCP-3      QPVGINTSTTCCYRFINKKIPKQRLESYRRTTSSHCPREAVIFKTKLDKEICADPTQKW
hMCP-4      QPDALNVPSTCCFTFSSKKISLQRLKSY VITTSRCPQKAVIFRTKLGKEICADPKEKW

                60   65   70   75
hMCP-1      VQDSMDHLDKQTQTPKT
hMCP-2      VRDSMKHLDQIFQNLKP
hMCP-3      VQDFMKHLDKKTQTPKL
hMCP-4      VQNYMKHLGRKAHTLKT
```

Figure 1.10 Primary structures of human MCP-3 compared to MCP-1, MCP-2 and MCP-4**.** The amino acid sequences are aligned with the conserved cysteine residues underlined (Murakami *et al.*, 1997).

Cell type	Inducer(+)/Inhibitor(-)		Level	Reference
Monocytes	+	IL-1β; TNFα; IFNγ; LPS;Colony stimulating factor-1	mRNA	(Van *et al.*, 1999;Irvine *et al.*, 2008)
	-	IL-4; IL-10; IL-13	mRNA	(Polentarutti *et al.*, 1997)
Mononuclear cells	+	IL-1β; IFNγ; IFNβ	protein	(Menten *et al.*, 1999)
Fibroblasts	+	IL-1β + IFNγ	protein	(Menten *et al.*, 1999)
Endothelial cells	+	IL-1β; TNFα; IFNγ; LPS	mRNA	(Polentarutti *et al.*, 1997)
Airway smooth muscle cells	+	IL-1β; IFNγ; TNFα	mRNA	(Pype *et al.*, 1999)
	-	Dexamethasone	mRNA	
Platelets		constitutively	mRNA	(Power *et al.*, 1995)
Myelomonocytic leukaemia cells	+	PMA	mRNA	(Van *et al.*, 1999)
Osteosarcoma cells	+	IL-1β	Protein	(Van *et al.*, 1992)
	+	IL-1β; IFNβ; PMA	mRNA	(Van *et al.*, 1999)
Promonocytic leukaemia cells	+	PMA	mRNA	(Minty *et al.*, 1993)

Table 1.8 Cellular sources and regulators of MCP-3. PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide

1.2.7 MCP Chemokines and Diseases

Early studies have indicated a role for the pro-inflammatory MCP family of proteins in macrophage recruitment in inflammatory diseases and tumourogenesis. MCP-1 is produced by numerous tumours including breast, pancreas, lung, cervix, ovary, melanomas, sarcomas and glial cell tumours and by fibroblasts, endothelial cells and macrophages at the tumour site(O'Hayre *et al.*, 2008). Its expression may correlate with the extent of macrophage recruitment and for some cancers, it may be of prognostic value. However, this relationship between chemokines and prognosis is complicated and is dependent on upon the cancer. For example, whilst in melanoma cell lines transfected with high levels of MCP-1 may

promote tumour rejection, higher levels of MCP-1 are associated with increased malignancy in experimental models of mammary adenocarcinomas. In addition, MCP-1 is found in bronchial epithelium of pulmonary fibrosis and asthma patients(Meloni *et al.*, 2004), tuberculous effusions, pleural infections and inflammatory skin diseases including psoriasis and atopic dermatitis (Vestergaard *et al.*, 2004) and also in wound healing which correlates with collagen deposition, fibroblast proliferation and neovascularisation(Gillitzer & Goebeler, 2001;Habibipour *et al.*, 2003).

CCR2, the main receptor for MCP-1, 2 and 3 is associated with cancers of the breast, lung, prostate, glioma, melanoma and multiple myeloma. This receptor may either be directly expressed on cancer cells or function indirectly by recruitment of macrophages and fibroblasts, polarisation, invasion and metastases and angiogenesis. These processes may be driven by MMPs but it is clear that the roles of MMPs are complicated and may play both tumour-promoting and tumour-suppressing roles. For example, MCP-1 induces the expression of MMP-12 by macrophages.

In addition to its major role in inflammatory cellular recruitment to sites of tissue injury, a novel role has been described for MCP-3 as a homing factor for mesenchymal stem cell (MSC) to sites of injury such as infarcted myocardium(Schenk *et al.*, 2007). It is transiently expressed by the injured myocardium and MSC engraftment is associated with recruitment of myofibroblasts and cardiac remodelling. MCP-3 has been implicated in several autoimmune and inflammatory diseases. These include primary biliary cirrhosis (Tsuneyama *et al.*, 2001) and inflammatory bowel disease (Helwig *et al.*, 2000) and it has also recently been described to be elevated in peripheral blood monocytes from lung transplant recipients and persistent elevated levels may suggest early transplant rejection(Hodge *et al.*, 2005). MCP-3 mRNA was significantly elevated in the bronchial mucosa of atopic and non-atopic asthmatics(Powell *et al.*, 1996;Humbert *et al.*, 1997), and elevated MCP-3 expression was associated with increased numbers of bronchial mucosal eosinophils in the atopic asthmatic patients(Powell *et al.*, 1996). Moreover, MCP-3 was significantly increased in bronchoalveolar lavage fluid obtained from asthmatics(Rojas-Ramos *et al.*, 2003), and allergen challenge induced the expression of MCP-3

predominantly in the airway mucosa of experimental asthma epithelium(Stafford *et al.*, 1997). MCP-3 has been demonstrated in the oxidised low-density lipoprotein (LDL) stimulated foam cells, but not that of smooth muscle cells, in atherosclerotic plaques (Jang *et al.*, 2004) and it was suggested that it can be involved in chemotaxis of inflammatory cells into the plaques. Recently, a number of studies have implicated MCP-3 in HIV-1/AIDS pathogenesis. Moreover, the receptors for MCP-3, CCR2 and CCR3 have been demonstrated to bind HIV-1 in vitro but there is little evidence that they serve as HIV-1 coreceptors in vivo. Immature dendritic cells exhibited potent chemotaxis in response to MCP-3(Lin *et al.*, 1998), thus it may affect cellular differentiation and migration. Additionally, MCP-3 has been shown to have both inhibitory(Vicenzi *et al.*, 2000) and stimulatory(Greco *et al.*, 1999) effect on replication in peripheral blood mononuclear cells from HIV-1-infected individuals. Finally, one study has shown that MCP-3 stimulated anti-HIV cytotoxic T cells(Hadida *et al.*, 1998).

A recent study has shown that MCP-3 is highly expressed in biopsies and pulmonary fibroblast lines obtained from patients with usual interstitial pneumonia (UIP) relative to patients without idiopathic interstitial pneumonia (IIP), and that this CC chemokine may have a major role in the progression of fibrosis in this IIP patient group(Choi *et al.*, 2004).

1.3 Transforming Growth Factor-β Superfamily

Transforming Growth Factor-β (TGFβ) belongs to the TGFβ superfamily of peptides whose members include the bone morphogenetic proteins (BMPs), activins, inhibins, TGFβ and growth differentiation factors, glial derived neurotrophic factor and Mullerian inhibiting substance. The TGFβ superfamily is characterised by seven cysteine residues at the carboxyl terminal and conserved in the majority of the family members. Six of these cysteine residues form a characteristic cysteine ‘knot’. Synthesized as large precursor molecules, the superfamily members are proteolytically cleaved to yield the mature active protein. The mature proteins have been shown to mediate many aspects of development as well as play important roles within the adult.

1.3.1 TGFβ

TGFβ is synthesised as prepropolypeptides that are proteolytically processed in the Golgi apparatus to a mature growth factor and its propeptide, also know as the latency associated peptide(LAP). Dimers of mature TGFβ and LAP form a tight complex termed the small latent complex(SLC), that is biologically inactive. The SLC is covalently bound to another protein called latent TGFβ binding protein (LTBP), forming the large latent complex(LLC). The LLC is secreted from cells to the extracellular space, where it binds to the extracellular matrix(Miyazono *et al.*, 1991;Saharinen *et al.*, 1996).

1.3.2 TGFβ signalling pathway

Members of the TGFβ superfamily signal through the sequential activation of 2 distinct serine/threonine kinase cell surface receptors that are expressed on virtually all cell types (**Figure 1.11**). Upon ligand binding, a type II receptor (TβRII) recruits and phosphorylates a type I receptor (TβRI), which is also known as activin receptor-like kinase (ALK). Of the 7 known type I (ALK) receptors, ALK-5 is most specific for TGFβ, whereas the closely related ALK-4 and ALK-7 interact with other members of the TGFβ superfamily(Miyazawa *et al.*, 2002). In addition, ALK-1 was recently shown to function as a type I TGFβ receptor, but it is expressed primarily on endothelial cells or at sites of epithelial-mesenchymal interactions(Oh *et al.*, 2000).

The Smads have been identified as major signalling molecules downstream of activated TβRI(Verrecchia *et al.*, 2006). These highly conserved modular proteins function as signal transducers/transcriptional activators that shuttle between the cytoplasm and the nucleus(Gordon & Blobe, 2008). In response to TGFβ, ALK-5 phosphorylates Smad2 and Smad3 on serine residues, whereas ALK-1 activates Smad1 and Smad5. Activin signals are also transduced by Smad2 and Smad3, but via the ALK-4 and ALK-7 receptors, whereas Smads 1, 5, and 8 are substrates for the bone morphogenetic protein-activated ALKs. In contrast to these receptor-activated Smads (R-Smads) that are phosphorylated by type I TGFβ receptors, Smad4 serves as a Smad cofactor, and Smad7 functions as an inhibitor of TGFβ-Smad signalling. Upon activation, R-Smads interact with Smad4, and the

heteromeric complex is then imported into the nucleus. Within the nucleus, the DNA-bound Smad complex regulates the transcription of target genes directly or in association with transcription factors such as Sp-1 and forkhead activin signal transducer 1 (FAST-1), coactivators such as p300/CREB binding protein, or corepressors such as Ski and SnoN(Moustakas *et al.*, 2001). Inhibitory Smad7 interacts with activated TβRI in competition with R-Smads and enhances receptor ubiquitination and proteasomal degradation in caveolae.

In addition to the canonical Smad pathway, TGFβ also activates alternate signal transduction pathways in a cell type- and context-specific manner. Non-Smad signalling pathways induced by TGFβ in cell lines include protein kinase A, protein kinase C, calmodulin-dependent protein kinase II, the MAP kinases ERK, JNK, and p38, and PI3K(Derynck & Zhang, 2003) and the family of Rho GTPases(Wakefield & Roberts, 2002). By regulating Smad activation or through intracellular cross-talk with the Smad pathway, these kinases can influence the amplitude and duration of Smad-dependent signalling and may directly induce Smad-independent TGFβ responses. The mechanisms linking non-Smad signalling pathways with activated TGFβ receptors are unknown. Furthermore, because most studies examining the cross-talk between ligand-induced Smad and non-Smad signalling pathways have been performed in transformed or immortalised cell lines, the biological consequences for non-Smad signalling in normal fibroblasts and their relevance in the physiological context remain incompletely understood. Studies into the delineation of Smad-dependent versus Smad-independent cellular responses to TGFβ have yielded conflicting results. For example, TGFβ stimulation of fibronectin and α-SMA expression has been shown to be Smad-dependent or Smad-independent in different studies and different cell types(Hu *et al.*, 2003;Isono *et al.*, 2002).

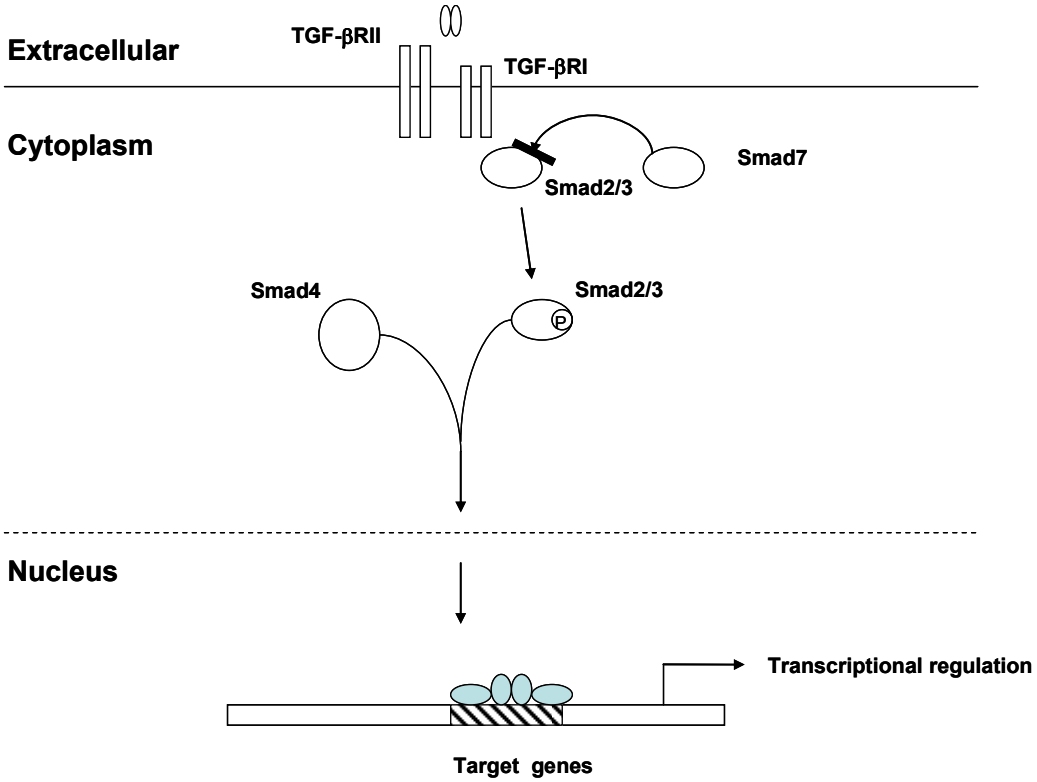


Figure 1.11 TGFβ signalling in fibroblasts via Smad pathway. TGFβ activate cell surface TGFβ-receptor, TβRI and TβRII, resulting in phosphorylation of downstream targets including Smads. Activated Smad2 and Smad3 form a complex with Smad4, and transit from the cytoplasm into the nucleus, bind to conserved DNA sequences and activate or repress transcription.

1.3.3 TGFβ and Fibrosis

Tissue fibrosis represents a state of dysregulated wound healing which failed to terminate and involves activated mesenchymal cells including dermal and lung fibroblasts, hepatic stellate cells and mesangial cells. Fibrosis can therefore be defined as the replacement of the normal connective tissue architecture by disorganised ECM or scar tissue.

Dysregulated expression of or response to TGF-β has been implicated in a variety of disease processes, including autoimmune disease, fibrosis and chronic inflammation. Transgenic and gene-targeted mutant mice have been invaluable for investigating the important roles of the TGFβ family in these diseases. For example, the predominant phenotype of the TGF-β1 knockout mouse suggests that the loss of this gene eliminates a critical regulator of immune and inflammatory responses. These mice show a substantial inflammatory response with a striking infiltration of leucocytes into numerous organs including the heart, lung, liver, salivary gland, pancreas, stomach, and intestine(Kulkarni *et al.*, 1995). This overwhelming tissue inflammation is associated with several spontaneous manifestations of autoimmunity, including circulating antibodies to nuclear antigens, immune complex deposition, and increased expression of both class I and class II MHC antigens(Letterio *et al.*, 1996). This suggests that dysregulation of TGF-β signalling in the TGFβ1 knockout mouse may potentially underlie a number of autoimmune disorders, including multiple sclerosis, experimental autoimmune encephalomyelitis, and inflammatory bowel disease.

Found abundantly in platelets and released from activated macrophages or lymphocytes, TGFβ is a strong chemoattractant for fibroblasts. TGFβ increases the synthesis of ECM, such as collagen type-I and type –III or fibronectin, by fibroblasts, modulates cell-matrix adhesion protein receptors, promotes myofibroblast differentiation and regulates the production of proteins that can modify the ECM by proteolytic mechanisms such as plasminogen activator or procollagenase(Roberts *et al.*, 1992). In addition, TGFβ is capable of stimulating its own synthesis by fibroblasts through autoinduction(Van Obberghen-

Schilling *et al.*, 1988). TGFβ induces rapid fibrosis and angiogenesis when injected subcutaneously into newborn mice(Roberts *et al.*, 1986).

It has been demonstrated that in SSc, increased activity of TGFβ signalling pathways, altered expression of high and low affinity TGFβ receptors and autocrine overproduction of several TGFβ-regulated genes(Ihn, 2008). In addition, several recent studies suggest altered expression of TGFβ pathway signalling intermediates in SSc with basal level and TGFβ-induced expression of Smad7 are selectively decreased, whereas Smad3 expression is increased(Dong *et al.*, 2002). Gene transfer of Smad7 abrogated bleomycin-induced lung fibrosis in mice by intratracheal injection of a recombinant adenovirus containing Smad7 cDNA(Nakao *et al.*, 1999). In bleomycin-treated skin, fibroblasts showed predominantly nuclear localisation of Smad3 and intense staining for phospho-Smad2/3(Takagawa *et al.*, 2003). On the other hand, the expression of Smad7 was downregulated in bleomycin-treated fibroblasts, which may account for sustained activation of TGFβ–Smad signalling in the lesional skin. In addition, gene transfer of Smad7 into unilateral ureteral obstruction-induced renal fibrosis in rats significantly reduced the accumulation of extracellular matrix proteins (Terada *et al.*, 2002). Inhibition of TGFβ signalling by Smad7 overexpression inhibited rat hepatic stellate cells transdifferention and block fibrosis induced by bile duct ligation in rats (Dooley *et al.*, 2003). Gene profiling or polymerase chain reaction-based differential display experiments suggest that the gene expression pattern for SSc is similar to that of TGFβ-activated normal fibroblasts, thus supporting a role for TGFβ in SSc (Yang *et al.*, 2003;Shi-Wen *et al.*, 2000). For example, global analysis of gene expression has revealed that >95% of transcriptional responses induced by TGFβ in mouse embryonic fibroblasts were Smad3-dependent(Yang *et al.*, 2003).

Myofibroblasts are also shown to be more frequent in SSc skin and lung samples and the myofibroblast phenotype is maintained in explanted cultures from SSc skin or lung biopsies. In addition, a number of studies have shown that antagonising TGFβ1 prevents the development of tissue fibrosis(Noble, 2003). Furthermore, targeted overexpression of TGFβ1 has been shown to produce progressive fibrosis(Sime *et al.*, 1997). Studies with rat

models revealed that TGFβ3 is associated with regular wound healing, whereas TGFβ1 is associated with fibrotic wound healing(Shah *et al.*, 1995).

1.3.4 TGFβ and Chemokines Crosstalk in fibrosis

There is now accumulating evidence to suggest that an important regulatory loop between CC chemokines and TGFβ in experimental models of inflammatory diseases such as Thy-1 nephritis(Wolf *et al.*, 2002;Schneider *et al.*, 1999). It has been demonstrated in that TGFβ regulates eotaxin(CCL11) expression by human airway smooth muscle cells in recruitment of eosinophils into the lung in the different phases of allergic asthma(Wenzel *et al.*, 2002). In a murine model of gut fibrosis, direct transfer of MCP-1 by intramural injection of an adenoviral vector was associated with increased TGFβ expression and collagen deposition(Motomura *et al.*, 2006). Recent immunohistochemical studies also showed colocalisation of CCR2 with TGFβ1 positive infiltrating epithelial cells in a renal model of fibrosis. Moreover, the authors reported that diminished CCR2 signalling may down-regulate TGFβ expression, thereby attenuating progressive fibrosis in the diseased kidney(Kitagawa *et al.*, 2004). CCR2 knock-out mice are protected from bleomycin-induced lung fibrosis, and cultured fibroblasts demonstrated reduced TGFβ1-induced αSMA expression and decreased TGFβRII and Smad3 expression. Lower bioactive and total TGFβ levels were detected in fibroblasts from CCR2 knock-out murine model in a recent study(Lee *et al.*, 2006). Similarly, there were reduced TGFβ levels in lesional skin of bleomycin-treated MCP-1 deficient mice(Ferreira *et al.*, 2006). This supports a convergent pathway between MCP-1 and TGFβ(Gharaee-Kermani *et al.*, 2003).

There have been few studies on the regulatory pathways underlying the crosstalk between chemokines and TGFβ. Recently, it has been reported that in lung fibroblasts and glomerular cells, MCP-1 can stimulate TGFβ via transcriptional mechanisms (Gharaee-Kermani *et al.*, 1996;Schneider *et al.*, 1999). This may be in part related to monocyte infiltration mediated by MCP-1. This is further supported by recent evidence that in fibroblasts cultured from humans with UIP, MCP-1 may upregulate expression of TGFβRII and IL-13(Murray *et al.*, 2008). In addition to the fibrotic response, abnormal angiogenesis

has been described in SSc and a recent study supported the role of MCP-1 in facilitating in vitro recruitment of vascular smooth muscle cells towards endothelial cells by TGFβ(Feinberg *et al.*, 2004). It has been recently demonstrated that TGFβ via upregulation of CCR1 in neonatal murine astrocytes facilitates chemotaxis by MCP-3 in a dose-dependent manner(Bandyopadhyay *et al.*, 2007;Han *et al.*, 2000) but it remains unclear if this effect occurs in fibroblasts.

1.4 Role of individual mediators in SSc pathogenesis

The fibrotic process is a final common pathway which involves an intricate interaction and activation of multiple cell types resulting in an increase in ECM of which collagen is the main component. Fibroblasts are often regarded as physiologically inert cells which are concerned with stromal function providing mechanical support and fillers of wounds or otherwise damaged tissues. However, recent studies from numerous laboratories revealed that such understanding of fibroblasts interact with ECM, immune cells and endothelial cells are subject to a range of locally acting cytokines and other soluble mediators including chemokines. Such factors include endothelin, prostanoids, nitric oxide, thrombin and others. This would form an autocrine or paracine loops or networks in SSc as outlined in **Figure 1.12**. Understanding the cytokine/chemokine network which forms an intricate and robust communication between these cells is important in fibrotic diseases including SSc. There are two key ways in which chemokines may promote fibrosis:
a. recruitment and activation of key inflammatory cells to express profibrotic mediators,
b. direct profibrotic effect on fibroblasts.

Circulating levels of cytokines and chemokines have also been measured in systemic sclerosis, and although there is variation between these studies, there is often measurable IL-10 and MCP-1, CXC chemokines including CXCL8, CXCL1 and Il-2, IL-6, SPARC and thrombospondin1 (TSP1). Some of these mediators have been shown to have clinicopathological significance. For example, presence of MCP-1 is correlated with eosinophilic infiltrate in the lung fibrosis of SSc, CXCL1 and CXCL8 with pulmonary involvement(Meloni *et al.*, 2004). Upregulation of fractalkine (CX_3CL1) was associated with digital ischaemia and pulmonary fibrosis (Furuse *et al.*, 2003;Hasegawa *et al.*, 2005).

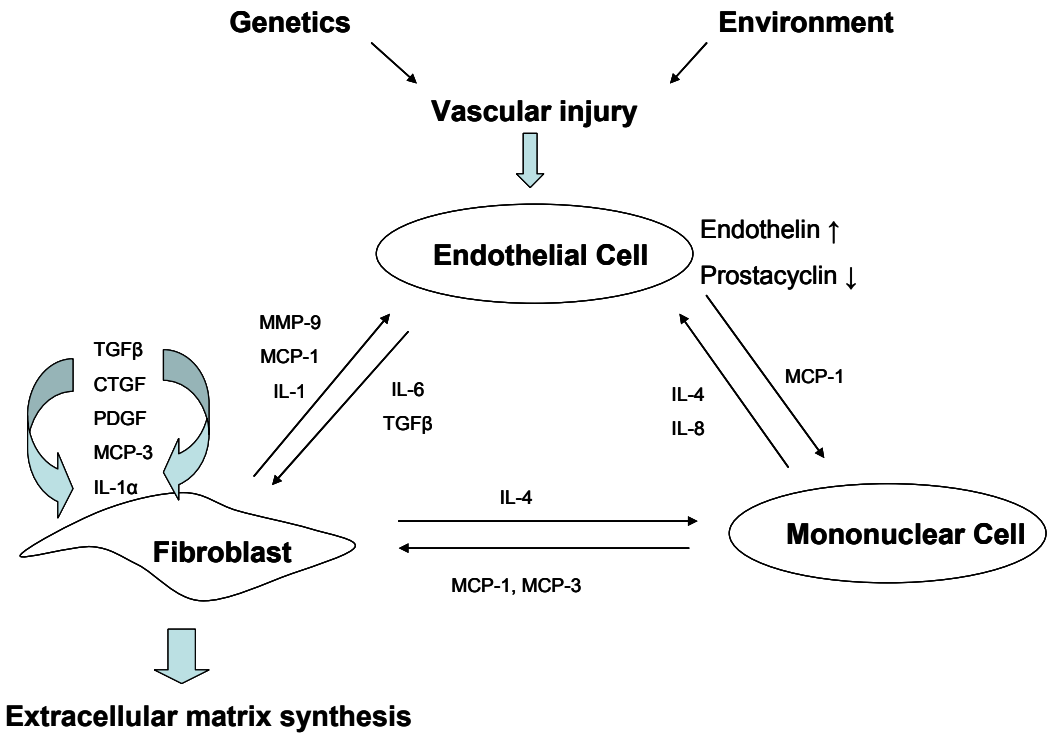


Figure 1.12 Pathophysiology of SSc.
Fibrogenesis characterised by excessive ECM matrix is a major pathological feature of SSc. Distinct genetic and environmental influences including silica exposure may predispose individuals to development of SSc. Tissue damage initially involving the vascular endothelial cells evokes the release of chemokines, endothelins, platelet-derived growth factor and vascular endothelial growth factor; these alter endothelial permeability and promote the recruitment and proliferation of leucocytes. The interactions between endothelial cells, mononuclear cells and fibroblasts via the milieu of cytokines and growth factors result in accumulation of macrophages and neutrophils in the affected tissues. Endothelial-derived chemokines and growth factors recruit and activate mesenchymal progenitor cells and resident fibroblasts. Profibrotic factors including MCP-1, IL-4 and IL-13 secreted by activated mononuclear cells promote fibroblast activation and the synthesis and secretion of ECM.
CTGF, connective tissue growth factor; ECM, extracellular matrix; ET1, endothelin-1; MCP-1, Monocyte chemoattractant protein-1; MMP-9, Matrix metalloproteinase 9; IL, interleukin; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor.

1.4.1 CCN2/CTGF

The matricellular protein connective tissue growth factor (CTGF/CCN2) has emerged as a key pathological mediator in fibrotic diseases. CTGF levels are increased in almost all organ-specific fibroproliferative diseases such as idiopathic pulmonary fibrosis and systemic fibrotic disorders including SSc. Originally identified as a secreted growth factor in the conditioned media of cultured human umbilical vascular endothelial cells (Bradham *et al.*, 1991), CTGF is a 36 to 38 kDa cystein-rich peptide containing 349 amino acid and belongs to the CCN family of matricellular proteins, which consist of six members that shares a similar predicted structure. It has been detected in endothelial cells, fibroblasts, cartilaginous cells, smooth muscle cells and some cancer cell lines. CTGF is induced by TGFβ and modulates fibroblast cell growth and ECM expression.

Depending on the cell type, CTGF has diverse biological functions ranging from regulation of cell growth to tissue remodelling. In earlier studies, CTGF was demonstrated to have mitogenic and chemotactic effects on fibroblasts. Little or no detectable levels of CTGF are observed in normal adult skin tissue. However, during normal wound healing CTGF expression is activated by TGFβ at the wound site and this expression is turned off when the repair process is complete. Recombinant CTGF protein was found to activate DNA synthesis and to upregulate collagen, fibronectin and integrin expression in fibroblasts(Frazier *et al.*, 1996) in addition to promoting fibroblast proliferation, matrix production and granulation tissue formation. The current evidence suggests that whereas TGFβ or CTGF alone may only result in transient fibrosis, the combination of both factors together may result in a sustained fibrotic response.

1.4.2 Platelet-Derived Growth Factor

PDGF is a multifunctional growth factor with mitogenic activity for mesenchymal cells, regulation of matrix metabolism and wound healing, vasoactive and chemotactic properties and production of inflammatory cytokines(Heldin & Westermark, 1999). The PDGF system consists of four polypeptide chains including, PDGF-A and -B, and the newly-discovered PDGF-C and –D chains. They signal through the tyrosine kinase receptors PDGF receptor α(PDGFRα) and receptor β (PDGFRβ). The biologically active PDGF

protein forms disulphide-bonded dimers, including four homodimers PDGF-AA, PDGA-BB, PDGF-CC and PDGF-DD, and one heterodimer, PDGF-AB. PDGF-A and PDGF-B are secreted in their active forms while PDGF-C and -D are secreted in latent forms. Upon receptor binding, overlapping signal transduction pathways including PI3K, Ras-MAPK, Sac family kinases and phospholipase Cγ are activated.

Originally isolated from platelets, PDGF isoforms are also synthesised by fibroblasts, macrophages, epithelial cells and capillary endothelial cells. Overexpression of PDGF has been reported in a number of fibrotic diseases with upregulation of PDGF and PDGFβ receptor in SSc fibroblasts and increased PDGF levels in bronchoalveolar lavage fluid. PDGF-A chain was shown to be elevated in affected skin in SSc(Yamakage *et al.*, 1992). In addition, TGFβ upregulates PDGF-α mRNA and protein levels in SSc fibroblasts, compared to wildtype fibroblasts. In contrast, TGFβ1 and MCP-1 have been shown to upregulate PDGFRβ gene transcript in UIP fibroblasts. More recently, serum autoantibodies directed against the PDGF receptor have been detected in patients with SSc, and are capable of inducing fibroblast activation in vitro(Baroni *et al.*, 2006). However, stimulatory autoantibodies targeted against the PDGF receptor are not specific for SSc, and have also been described in patients with extensive chronic graft-versus-host-disease(Svegliati *et al.*, 2007).

1.4.3 IL-4

CD4+ T-cell clones from SSc skin biopsies have been shown to demonstrate type-2 cytokine profiles. Sera from this group of patients also exhibit increased levels of CD30 expressed on activated type-2 cells. IL-4 is produced by activated memory T-cells and mast cells; both of which have been implicated in SSc. IL-4 is known to promote fibroblast proliferation, gene expression and synthesis of extracellular matrix proteins including collagen and tenascin(Postlethwaite *et al.*, 1992;Makhluf *et al.*, 1996). It also upregulates TIMP-2 in dermal fibroblasts via p38 Mitogen-activated protein kinases (MAPK pathway) (Ihn *et al.*, 2002) and T-cells (Seder *et al.*, 1998). IL-4 is overexpressed in the sera and also by activated peripheral blood mononuclear cells of patients with SSc (Needleman *et al.*, 1992). SSc fibroblasts express more IL-4 receptorα and produce more collagen after IL-4

stimulation (Serpier *et al.*, 1997). In addition to TGFβ, it is also important in the synthesis of α1(I) collagen mRNA and activation of α2(I) collagen promoter in Tsk mice(McGaha *et al.*, 2001). This is demonstrated with targeted mutations in the signalling chain of the IL-4 receptor with abrogation of cutaneous hyperplasia in Tsk mice(Ong *et al.*, 1999). Furthermore, neonatal Tsk mice treated with anti-IL4 antibody prevents skin fibrosis(Ong *et al.*, 1998). In the bleomycin model, IL-4 was found to be detected in the sera and skin of the mice(Yamamoto *et al.*, 1999).

1.4.4 Regulated upon activation, normal T cell expressed and secreted (RANTES/CCL5) and macrophage inflammatory protein-1α/CCL3

Elevated levels of RANTES and MIP-1α were detected in BAL of patients with SSc(Bolster *et al.*, 1997) and in the lungs of patients with idiopathic pulmonary fibrosis and hypersensitivity pneumonitis leading to pulmonary fibrosis(Oshima *et al.*, 1999). Expression of these two chemokines were demonstrated to precede the development of dermal and pulmonary fibrosis in the murine sclerodermatous graft-versus-host disease(Zhang *et al.*, 2002). Abundant RANTES mRNA has been detected in situ in the skin of SSc patients compared to skin of control subjects(Anderegg *et al.*, 2000). However, apart of their role in recruitment of inflammatory cells into tissue, the direct role of these chemokines in fibrosis remains to be clarified.

1.4.5 IL-6

IL-6 is a pleiotropic cytokine with multiple biological effects on immune regulation, haematopoiesis, inflammation and cancer(Kishimoto, 1989), and it has been implicated in SSc pathogenesis(Hasegawa *et al.*, 1999;Kondo *et al.*, 2001;Nishimaki *et al.*, 1999;Sato *et al.*, 2001). Increased induction of IL6 has been observed in SSc fibroblasts (Takemura *et al.*, 1998)and IL-6 stimulated human dermal fibroblasts expressed increased production of collagen and glycosaminoglycans, hyaluronic acid and chondroitin-4/6-sulphates(Duncan & Berman, 1991). There was preferential increase in IL-6 in the sera from patients with diffuse SSc compared to limited SSc and this increase was correlated with pulmonary involvement (fibrosis and hypertension)(Bolster *et al.*, 1997;Hasegawa *et al.*, 1998).

1.4.6 IL-8

This CXC chemokine is overexpressed in the dermis of lesional skin from patients with early dcSSc(Koch *et al.*, 1993) and BAL fluids from affected patients(Bolster *et al.*, 1997). Secreted by the alveolar macrophages and lung fibroblasts in patients with lung fibrosis(Ludwicka-Bradley *et al.*, 2000;Pantelidis *et al.*, 1997), in vitro cultured dermal fibroblasts have been shown to express more IL-8 than normal fibroblasts(Kadono *et al.*, 1998;Ludwicka-Bradley *et al.*, 2000). Two polymorphisms in the CXCR-2 gene have been associated with SSc(Ludwicka-Bradley *et al.*, 2000;Renzoni *et al.*, 2000).

1.4.7 Pulmonary and activation-regulated chemokine

Also known as CCL18, it is highly expressed by lung macrophages. Recent evidence suggests that it induces collagen mRNA and protein production by dermal and lung fibroblasts and this appears to be mediated via ERK kinase signalling pathway(Atamas *et al.*, 2003). Its protein is also elevated in BAL fluids from SSc patients with lung inflammation compared to those without. It is also found in BAL fluids of other fibrotic conditions including hypersensitivity pneumonitis, asthma and sarcoidosis.

1.4.8 Monocyte chemoattractant protein-1 (CCL2/MCP-1)

There is evidence to suggest that the related chemokine, MCP-1 is expressed in the skin and lungs of SSc and pulmonary fibrosis. One of the early studies to demonstrate chemokine overexpression in SSc came from in vitro studies showing that SSc fibroblasts, but not normal fibroblasts, selectively promote leucocyte migration across endothelial cell monolayers in a co-culture experimental system and that this was via an MCP-1 dependent mechanism(Denton *et al.*, 1998). This lends further evidence to altered intracellular interaction between fibroblasts and endothelial cells and that promotion of perivascular inflammatory infiltrates is important in early stage lesional skin in SSc. Further in vitro studies showed that fibroblast lines cultured from SSc skin biopsy specimens display increased expression of MCP-1 compared with normal dermal fibroblast lines. Autoinduction of MCP-1 mRNA has been observed in cultured SSc fibroblasts.

Subsequent studies have also demonstrated that MCP-1 may modulate fibroblastic properties with stimulation of proα1(I) collagen mRNA expression in both rat lung and normal human dermal fibroblast cultures in vitro (Yamamoto & Nishioka, 2003). This effect is mediated in part by stimulating fibroblast production of TGFβ, with TGFβ acting in an autocrine manner to stimulate ECM formation. Immunohistochemical localisation studies revealed expression of MCP-1 in the lesional skin of SSc in particular fibroblasts, keratinocytes, perivascular mononuclear cell infiltrate and endothelial cells. In addition, MCP-1 mRNA is detected in BAL cells from SSc, with greater expression in patients with lung inflammation than those without inflammation and healthy controls. There is parallel increase in MCP-1 protein in the BAL fluids in SSc with active lung inflammation(Luzina *et al.*, 2002). In addition, several studies have described an association between a functional -2518 (A/G) single nucleotide polymorphism (SNP) in the promoter region of MCP-1 and SSc (Fonseca & Denton, 2007)but this was not demonstrated in our larger cohort of Caucasian SSc patients(Carulli *et al.*, 2008).

These findings suggest that MCP-1 may contribute to dermal fibrosis through several mechanisms: stimulation of collagen via TGFβ, the induction of more MCP-1 production by SSc fibroblasts and the recruitment of monocytes to the skin(Distler *et al.*, 2008). MCP-1 has also been implicated in recruitment of monocytes into early atherosclerotic lesions and development of restenosis after coronary artery angioplasty and stenting. $CCR2^{-/-}$ mice show greater reduction in intimal hyperplasia and intima/media ratio than in mice with MCP-1 gene deletion. This would suggest that other chemokines such MCP-3 which activates CCR2 might contribute to intimal changes.

1.4.9 Fractalkine (CX_3CL1)

Fractalkine is strongly expressed on the endothelial cells in the lesional skin and affected lung tissues of SSc. Soluble CX_3CL1 was elevated in the sera of SSc. Its cognate receptor CX_3CR1 are overexpressed on peripheral monocytes/macrophages and T cells in SSc. Accordingly, immunohistochemical studies revealed that the number of monocytes/macrophages expressing this receptor was increased in the lesional skin and

lung tissues from these patients. It is postulated that the ligand-receptor interaction may promote endothelial injury and migration of CX_3CR1 cells into the skin and lung in SSc.

1.4.10 IL-13

Originally described as an 1L-4 like molecule, it is major effector of Th2 inflammation and tissue remodelling(Corry & Kheradmand, 2002;Elias *et al.*, 2003;Wills-Karp, 2004). It is overexpressed in *in vivo* transgenic mouse model of fibrosis, and mice infected with Schistosoma mansoni. In SSc, elevated sera levels of IL-13 stimulates MCP-1 and MCP-3 production *in vivo* in a CCR2-dependent manner(Hasegawa *et al.*, 1997;Zhu *et al.*, 2002). This interaction with chemokine system is further supported by upregulation of IL-13 and its receptor by MCP-1 in fibroblasts from patients with UIP (Murray *et al.*, 2008). In bleomycin-induced lung fibrosis, the IL-13 receptor is reported to mediate the induction of collagen deposition by IL-13 via activation of TGFβ (Fichtner-Feigl *et al.*, 2006). Consistent with this, it has been demonstrated recently compared to non-fibrotic human lung fibroblasts, that fibrotic fibroblasts from patients with UIP, there is induction of TGFβ1 gene expression by IL-13 and IL-13 may upregulate TGFβ receptor(Murray *et al.*, 2008). Similarly, in FITC model of lung fibrosis, the fibrotic response with increased total collagen production is dependent on the expression of IL-13(Kolodsick *et al.*, 2004).

1.5 Hypothesis

The experimental work described in this report addresses the hypothesis that in addition to its key role in regulating leucocyte recruitment that the CC chemokine, MCP-3 has an important role in determining ECM overproduction and fibrosis. It is also likely that this depends on interplay with other key profibrotic mediators including the TGFβ family members. SSc is used as a prototypic fibrotic disease platform for these studies.

To address this hypothesis, the following specific aims have been pursued:

1. Expression of MCP-3 was examined using contemporary analysis of established murine model for SSc, Tsk1 and TβRIIΔk transgenic mice and relating these to key subsets of human SSc skin biopsies.

2. The profibrotic effect of MCP-3 on ECM gene expression was assessed using dermal fibroblasts cultured from transgenic mice harbouring a reporter gene regulated by a fibroblast-specific expression cassette.

3. The regulation of MCP-3 expression by TGFβ was explored using in a series of transfection experiment with MCP-3 promoter. The signalling pathways governing the activation of MCP-3 by TGFβ were investigated using a series of pharmacological inhibitors.

4. Activation of the type I collagen promoter Col1a2 by MCP-3 and the dependence of TGFβ activity have been explored. Responsiveness of MCP-3 promoter and of the proα2(I) collagen promoter to MCP-3 in transient transfection assays in wildtype cultured dermal fibroblasts, and to examine the potential role of TGFβ isoforms as downstream effector of collagen gene activation by MCP-3.

CHAPTER 2: GENERAL METHODS

2.1 Patients, Clinical Details and Dermal Biopsies

2.1.1 Patients

All SSc patients fulfilled the American College of Rheumatology (ARA) preliminary criteria for classification of SSc(Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980) as well as the criteria for SSc as proposed by LeRoy et al(LeRoy *et al.*, 1988). All patients were undergoing treatment at the Royal Free Hospital and controls included healthy volunteers who do not have SSc. Informed consent and ethical approval according to local institutional guidelines were obtained from patients and control subjects.

2.1.2 Clinical details

Patients were classified as early dcSSc (n=14) if within the first two years of disease onset, defined by the appearance of the first non-Raynaud's symptom. Patients with established dcSSc (n=8) had more than two years of disease. The methods used to determine the clinical and serological features of the SSc patients are discussed below together with the demographic features of the SSc and control subjects from whom the biopsies were taken. Major organ involvement was assessed according to standard clinical practice at the Royal Free Hospital, and broadly followed the SSc Severity Score (Medsger, Jr. *et al.*, 1999).

The extent of skin involvement in SSc is a reliable measurement of skin thickening and has been shown to reflect disease activity and associated with visceral involvement (Clements *et al.*, 2000;Gifford, Jr. & Hines, 1960;Barnett & Coventry, 1969;Valentini *et al.*, 2003). Several skin scoring methodologies have been presented including the modified-Rodnan total skin thickness score as discussed in Section **1.1.3.1**, and this has been shown to be at least as reliable for measuring skin thickness in SSc as are the ARA and Ritchie joint tenderness counts for assessing joint disease in rheumatoid arthritis(Clements *et al.*, 1995). The clinical and laboratory parameters used for the assessment of disease included duration of skin and Raynaud's disease, pattern of major internal organ involvement and serology.

These patients' characteristics are summarised in **Table 2.1**. Briefly, oesophageal involvement was determined by history, barium swallow or scintigraphy; lung fibrosis by high resolution CT scanning in the presence of a restrictive pattern of pulmonary function test abnormality; cardiac involvement by altered ECG, impaired ventricular function or pericardial effusion on echocardiography; PAH was determined by elevated pulmonary arterial peak systolic pressure on Doppler-echocardigraphy, other echocardiographic hallmarks of PAH and usually confirmed by right heart catheterisation. Creatinine kinase level elevated more than two fold defined skeletal muscle involvement. Renal involvement was identified by previous SSc renal crisis or significant impairment of creatinine clearance. Patients were treated with vasodilators and in most cases of dcSSc by current immunosuppressive regimen including anti-thymocyte globulin mycophenolate mofetil and cyclophosphamide according to standard protocol (Nihtyanova & Denton, 2008).

	Early dcSSc (n=14)	**Established dcSSc (n=8)**	**lcSSc (n=6)**	**Controls (n=11)**
Gender (M/F)	3/11	2/6	0/6	5/6
Age (years,mean±sd)	49.3 ± 9.9	57.3 ± 12.4	44.3 ± 16.2	45 ± 14.4
Duration of Disease (months,mean±sd)	16.7 ± 4.2	69 ± 51.2	66 ± 58.2	-
Duration of Raynaud symptoms (months,mean±sd)	18.9 ± 6.6	75 ± 53.2	154 ± 49.4	-
Organ Involvement, %				
Oesophageal	71	88	83	
Other gastrointestinal	14	25	33	
Lung	36	50	17	
Muscle	14	13	17	
Joint	14	0	0	
Renal	21	0	17	
Cardiac	7	0	0	
Neurological	0	13	0	
Serology, %	All dcSSc (n=22)		lcSSc (n=6)	
Anti-nuclear	100		100	
Anti-centromere	0		67	
Anti-topoisomerase 1	32		17	
Anti-RNA polymerase I/III	9		0	
Anti nRNP	0		17	
Other	59		0	

Table 2.1 Clinical features of systemic sclerosis patients and healthy controls. Early dcSSc defined by less than 2 years duration.

2.1.3 Dermal biopsies

For experiments described in this thesis, fibroblasts were grown from skin biopsies taken from SSc patients and from healthy volunteers or patients without SSc. For the SSc patients (22 patients with dcSSc and 6 with lcSSc), 3-6 mm punch biopsies were taken from the involved forearm skin on the non-dominant limb. Paired biopsy samples were obtained from patients with early dcSSc (n=14) and those with established dcSSc (n=8). Control biopsies were taken from normal forearm skin from 11 healthy volunteers using the same biopsy techniques as for SSc patients. Biopsies were immediately divided for tissue culture and snap frozen for histological study. Biopsies of early stage dcSSc were taken prior to immunosuppressive treatment. Patients with lcSSc were on vasodilator therapy only at the time of biopsy. Among patients with established dcSSc, 88% (n=7) were taking immunosuppressant therapy. Three patients with lcSSc had pulmonary, renal and muscle involvement requiring low dose corticosteroids and immunosuppression.

2.2 Cell culture

2.2.1 Fibroblast lines

Apart from the SSc dermal biopsies described above, murine fibroblasts derived from several sources were used for the experimental work in this thesis. Tight skin (Tsk/1+), TβRIIΔk and 2kb-LacZ transgenic and control skin dermal fibroblasts were cultured from skin taken from the upper back of same-sex littermate mice. Murine and human fibroblasts were grown by standard explant technique(LeRoy, 1974). Briefly, tissue was dissected en-bloc, subcutaneous fat was removed from the dermal aspect of the skin sample and finely minced under aseptic conditions in a laminar flow cabinet. After 15 min of drying at room temperature the pieces of biopsy were adherent to the tissue culture plastic and then the fibroblasts were grown by explant culture and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin, and 100 mg/ml streptomycin, and maintained in a humidified atmosphere of 5% CO_2 in an incubator (Hera Cell240) at 37^{o}C. At confluence, cells were trypsinized (0.125% trypsin in phosphate buffered Saline (PBS), 0.5 mM EDTA) and passaged. All cultures were inspected at high power using a phase-contrast inverted microscope to confirm absence of epithelial cells and a typical fibroblastic morphology.

Fibroblasts on reaching confluence were routinely re-cultured by diluting in a 1:3 ratio. For experiments, fibroblasts were used at the first or second passage because later-passage cells showed greater interculture variability.

The TβRIIΔk strain was generated and characterised by Professor Christopher Denton at University College London and University of Texas, and has been described previously(Denton *et al.*, 2003). In brief, a fibroblast-specific expression cassette was devised in which a target cDNA can be expressed together with a reporter marker gene (pCD3). The fibroblast-specific expression cassette contains a promoter enhancer fragment subcloned from the upstream and proximal promoter region of the proα2(I)collagen gene. This incorporates a fragment between -19.5 and -13.5kb upstream of the transcription start site linked to an endogenous minimal promoter and drives gene expression at high levels in fibroblasts, but not in other type I collagen-producing cells. Reporter genes linked to this promoter-enhancer show high level fibroblast-specific expression in embryonic development and postnatally. The mouse strain TβRIIΔk was generated by subcloning the cDNA encoding the extracellular and transmembrane portion of the human type II TGFβ receptor into the Sal1 site of the pCD3 expression vector. A bacterial β-galactosidase marker gene (LacZ) was co-expressed from a dicistronic transgene mRNA product via an encephalomyocarditis virus internal ribosome entry site sequence (IRES) (Kim *et al.*, 1992). Founders were previously shown to demonstrate consistent transgene expression in fibroblastic tissues.

In addition, fibroblasts were cultured from a reporter transgenic mouse harbouring the LacZ reporter gene regulated by a central EcoRI fragment of the –19.5 kb to –13.5 kb enhancer described above, (–17.1 kb to –15.1 kb from the transcription start site, 2kb-LacZ) linked to the minimal endogenous COL1A2 promoter. In these mice, basal high-level expression of the reporter gene correlates with expression of that of collagen type I. The cultured fibroblasts from the two transgenic murine strains were used in the functional studies of fibroblasts response to recombinant MCP-3 and TGFβ1 (Chapters 4 and 6). This allowed expression of the reporter transgene to be examined in tissue culture, and allowed the effect of recombinant MCP-3 and TGFβ1 on the level of transgene expression to be assessed. In

addition, tight-skin dermal fibroblasts were cultured from skin extracted from the upper back of same-sex littermate mice at different time-points: 1, 6 and 12 weeks. Selective upregulation of the 2kb-LacZ reporter transgene has been shown in vivo using Tsk1 mice and there are three Smad-binding consensus sequences within this 2kb-transgene, suggesting that there may be a functional TGFβ1 responsiveness for the transgene(Denton *et al.*, 2001). However, this does not completely exclude that the upstream enhancer element may act by amplification of responses mediated via previously described TGFβ1-responsive elements in the proximal promoter of the Col1a2 gene, which include a Smad3-binding consensus sequence at -259 bp upstream of the transcription start site. In addition, human dermal fibroblasts were cultured from dcSSc patients and from age- and site-matched control human skin. This is used for protein analysis for Western blot studies and ELISA (Chapters 3 and 6).

2.2.2 TGF-β, MCP-3 and MCP-1 treatment

Murine recombinant TGF-β1 and murine recombinant chemokines, MCP-3 and MCP-1 (from R&D Systems) were used at 4 ng/ml and 200-400ng/ml respectively, unless otherwise stated. TGFβ, MCP-3 and MCP-1 were added to confluent fibroblast cultures and incubated for 24 hours unless stated otherwise, after which the cell monolayers were washed with fresh media and used immediately in the experiments detailed below.

2.2.3 Transient transfection of promoter reporter constructs

2.2.3.1 Promoter reporter constructs

Apart from using primary cultures of dermal fibroblasts established from transgenic mice, transient transfection was used to assess MCP-3 and TGFβ responsiveness of proα2(I) collagen promoter constructs. The reporter constructs are shown in **Figure 2.1**. These included minimal promoter sequences of mouse or human proα2(I) collagen as well as constructs that incorporate sequences from the far-upstream fibroblast-specific enhancer previously defined in transgenic mice(Bou-Gharios *et al.*, 1996). This element operates as a lineage-specific transcriptional enhancer in vivo and is a target for activation in Tsk1 mice(Denton *et al.*, 2001). Both the human and murine minimal promoter and 2kb-LacZ constructs were transfected into murine control fibroblasts.

To assess TGFβ1 responsiveness to MCP-3 (Chapter 7.4), an additional TGFβ-regulated promoter-reporter construct, 3TP-lux construct, which contains part of the promoter region of the plasminogen activator inhibitor-1 gene and three tandem repeats of AP-1-binding sites of the collagenase 1 gene (provided by Joan Massague, Memorial Sloan-Kettering Cancer Center, New York, New York, USA) is used. To examine the molecular mechanisms underlying the activation of MCP-3 by TGFβ in dermal fibroblasts, a 1kb upstream fragment of the murine MCP-3 promoter was generated from pUC19 plasmid cloning vector containing a 4.7kb MCP-3 promoter(Murakami *et. al.*, 1997). This construct was cloned into the luciferase reporter vector pGL3 (Promega).

For the human proα2(I) collagen gene (COL1A2-LacZ) and murine proα2(I) collagen gene (Col1a2-LacZ), 1 μg of collagen reporter plasmid was cotransfected with 0.1 μg of pCMVluc control plasmid to allow correction of β-galactosidase expression for transfection efficiency. For cells cotransfected with the 1 kb-MCP-3 promoter construct linked to luciferase reporter gene, the cells were cotransfected with a CMV promoter-driven β-galactosidase (0.1 μg/well) expression from which was used to adjust for differences in transfection efficiencies among wells.

2.2.3.2 Transfection methods

Transient transfection of fibroblasts was performed using liposomal formulated, Lipofectamine Plus (Invitrogen) or non-liposomal formulated FuGENE 6 (Roche) transfection reagents. Briefly, fibroblasts were plated at 50-80% confluence in 12-well culture plates in the appropriate growth media prior to the day of experimentation. After 18 hours seeded cells were rinsed with DMEM and maintained in 0.5 ml of DMEM containing 0.2% FCS for 4 hours.

For transfections with FuGENE 6, serum and antibiotic free DMEM was warmed to 37^{o}C and 50 μl per well, was transferred to a microfuge tube and the appropriate volume of FuGENE 6 per transfection added. The tube was mixed by tapping and incubated for 5 min at room temperature. DNA (500 ng per well) was added and the sample incubated at room

temperature for a further 15-30 min. The complex was added drop-wise to its designated well and the plate swirled to ensure even mixing of the complex in all the wells. The cells were returned to the incubator and maintained in a humidified atmosphere containing 5% CO_2 at 37^{o}C. After 8 hours, media was replaced with the DMEM containing 0.2% FCS.

Transient transfection of fibroblasts with Lipofectamine Plus (Invitrogen) was performed in a similar manner to that of FuGENE 6. Briefly, serum and antibiotic free DMEM was warmed to 37^{o}C and 50 µl per well, was transferred to a microfuge tube and DNA (500 ng per well) and 4 µl of Plus reagent (Invitrogen) was added and the 'pre-complex' sample incubated at room temperature for 15 min. To 50 µl of serum and antibiotic free DMEM 2 µl lipofectamine (Invitrogen) reagent per transfection was added. The tube was mixed by gentle tapping and added to the 'pre-complex', mixed and incubated at room temperature for a further 15 min. The lipofectamine/DNA complex was added drop-wise to its designated well and the plate swirled to ensure even mixing of the complex in the wells. The cells were returned to the incubator and maintained in a humidified atmosphere containing 5% CO_2 at 37^{o}C. After 8 hours, media was replaced with the DMEM containing 0.2% FCS and, where appropriate, recombinant TGFβ1 and MCP-3 added for a further 24 hours. Following this, media was collected. The cell monolayer was washed once with PBS and lysed in 120 µl of lysis buffer (Tropix). Both media and lysed monolayers were stored at -20^{o}C. Reporter gene activity was determined as below. All cell mono-layers were assessed for cell viability by visual appraisal.

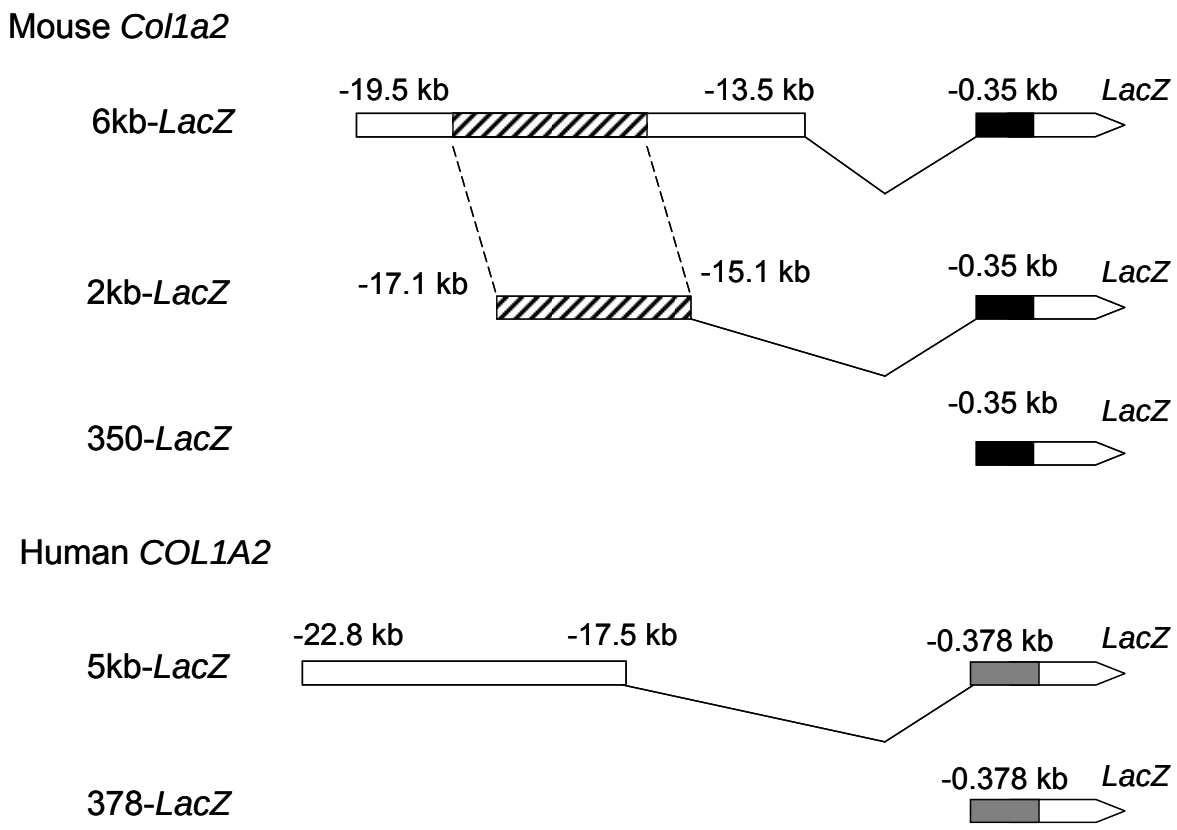


Figure 2.1 Transgene constructs harbouring far-upstream enhancer elements of the mouse or human proα2(I) collagen gene.
The minimal promoter sequences were defined from -354 bp and -378 bp upstream of the transcription start site for the mouse and human pro 2α(I) collagen genes, respectively. Larger constructs incorporated additional sequences from the evolutionarily conserved far-upstream transcriptional enhancer that has previously been shown to be a target for activation in fibrosis. The 6kb-*LacZ* construct includes the region from -19.5 kb to -13.5 kb upstream of the transcription start site, and the 2kb-*LacZ* incorporates 2 kb of the upstream region linked to a minimal murine -354-bp promoter. Construction of the human 5kb-*LacZ* contains sequences -22.8 kb to -17.5 kb, which is linked to a minimal -378-bp promoter.

2.2.3.3 Determination of Luciferase and β-galactosidase reporter gene activity

Expression of luciferase and β-galactosidase was determined using the Dual light system as per the manufacturer's instructions (Applied Biosystems). Briefly, to 10 µl of each lysed cell mono-layer 25 µl of Buffer A, equilibrated to room temperature, was added and the mixture incubated at room temperature. After 5 min, room temperature equilibrated Buffer B containing the galactosidase substrate Galacton-Plus (1:100 Galacton-plus to Buffer B) was added and luciferase signal read for 2 sec. After a further incubation of 30 min, 100 µl

of Accelerator-II was added to each well and galactosidase activity (RLU) from the co-transfected CMV-β-Galactosidase constructs determined for 0.2 sec. All luciferase RLU values were normalised to galactosidase RLU in order to control for transfection efficiencies.

2.2.3.4 Smad3 siRNA transfection studies

RNA interference is used by most eukaryotes in vivo for anti-viral defence and gene regulation and this is a major tool for gene silencing. Enzymatically processed from long double-stranded RNAs, small interfering RNAs (siRNAs) are short RNA duplexes of 19-21 nucleotides with two nucleotide 3 overhangs on each strand. The complex formed by an assembly of these siRNAs suppresses the expression of a target protein by stimulating the specific degradation of the target mRNA homologous to the integral siRNA. The target mRNA is cleaved in the region complementary to the siRNA, with the net result being rapid degradation of the target mRNA and decreased protein expression.

Smad proteins are important downstream effectors of TGFβ signalling and Smad3 is involved in TGFβ and activin-mediated growth modulation. Smad3 siRNA is a pool of 3 target-specific 20-25 nucleotide siRNAs designed to knock down expression of Smad3 protein in murine cells. As a negative control, non-silencing or control siRNA with non-targetting 20-25 nucleotide siRNA is used.

Cells were incubated at 37^0C until 60-80% confluence was attained. A combination of diluted Smad3 siRNA and siRNA transfection reagent in transfection medium were gently mixed in a microfuge tube by pipetting in the absence of serum or antibiotics. This mixture was incubated for 45 minutes at room temperature. For each tube containing the siRNA, the total volume of mixture was made up to 1 ml with transfection medium. This mixture was gently agitated and overlaid onto each well of the cultured murine fibroblasts. The cells were incubated for 5 hours at 37^0C in a CO_2 incubator. Following this, an equal volume of DMEM containing 5% FCS was added and the mixture was incubated overnight. The following day, the medium was aspirated and replaced with fresh DMEM containing 0.2% FCS for 4 hours. Recombinant TGFβ1 and MCP-3 was added overnight.

2.3 Molecular Biology Techniques

Unless stated, all chemical reagents used were molecular biology grade and supplied by Sigma-Aldrich.

2.3.1 Bacterial culture

E. coli were grown in *Luria-Bertani* broth (LB) (Tryptone 10g/l; Yeast extract 5g/l; NaCl 10g/l supplemented with ampicillin at the specified concentration.

2.3.1.1 Strains

DH5α (genotype: F- Φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 PhoA SupE44 thi-1 gyrA96 relA1 λ^-) competent (1x 10^8 /μg DNA) were purchased from Invitrogen.

2.3.1.2 Selection

For experiments with *E. coli* harbouring plasmids encoding the ampicillin resistance gene, transformed cells were plated on LB-agar (LB containing 15% agar [Sigma Aldrich]) containing ampicillin at 100 μg/ml. Selection was maintained during growth in liquid culture by the inclusion of ampicillin at 50μg/ml.

For experiments using Transforming One Shot Competent *E.coli* cells harbouring plasmids encoding the kanamycin resistance gene in the TOPO cloning reaction (Invitrogen), S.O.C. medium containing 1.2 M NaCl and 0.06 $MgCl_2$ was used to adjust the cloning reaction to the recommended final concentration of 200 mM NaCl and 10 mM $MgCl_2$ to maximize the number of colonies on LB plates containing 50 μg/ml kanamycin. Once the correct clone was identified, a glycerol stock of the plasmid was prepared by streaking the original colony on LB plate containing the appropriate antibiotics. A single colony was isolated and inoculated into 1 ml of LB with the antibiotic and 0.85 ml of the culture was mixed with 0.15 ml of sterile glycerol in a cryovial for storage at -80^0C.

2.3.2 Plasmids

2.3.2.1 Cloning vector pGL3

The pGL3 basic cloning vector (Promega) is derived from pUC19, with beta-lactamase gene conferring ampicillin resistance and a ColE1 origin of replication for propagation in *E. coli*. The synthetic multiple cloning site (MCS) contains 8 unique restriction sites. The plasmid also harbours contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity of cis-acting factors such as promoters and enhancers in transfected eukaryotic cells.

Using the following primers, sequencing of the 1kb MCP-3 promoter construct cloned into the pGL3 reporter vector was performed to confirm the correct position of the construct.

5'-CTG CTG GCT GTT GAA GTC –3'

5'-GCC ACC AGG ATT TAA GAC AGT G-3'

5'-CTT TAT GTT TTT GGC GTC TTC CA-3'

5'-CTA GCA AAA TAG GCT GTC CC-3'

2.3.2.2. pCR-Blunt II-TOPO plasmid vector

Zero blunt TOPO PCR Cloning (Invitrogen) provides a one-step cloning strategy to insert directly the blunt-end 1kb-MCP-3 promoter PCR product into pGL3-Basic vector. The pCR-Blunt II-TOPO is supplied linearised with Vaccinia virus DNA topoisomerase I covalently bound to the 3' end of each DNA strand.

Topoisomerase I binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5'-CCCTT in one strand(Shuman, 1991). The energy released from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3'phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase(Shuman, 1994). This reaction provides the mechanism in which PCR products is cloned and transformed into *E. coli* competent cells.

2.3.3 Transformation of competent bacteria with plasmid DNA

Frozen competent cells (Invitrogen) of the *E. coli* strain DH5α were placed on ice until thawed. Plasmid DNA was added to pre-chilled microfuge tubes containing 100 µl of competent cells, gently mixed and left on ice. After 30 min the DNA/competent cells were heat shocked by placing the microfuge tubes in a water bath at 42^{o}C for 60 sec, and subsequently transferred back onto ice for a further 3 min. 900 µl of LB medium was added and incubated for 45 min at 37^{o}C with agitation. The bacterial cells were then pelleted by centrifugation (Heraeus Biofuge Fresco) at 12,000 revolutions per min (rpm) for 30 sec and resuspended in 50 µl of LB media and plated onto selection plates. Plates were dried then inverted and incubated at 37^{o}C overnight(Cohen *et al.*, 1972).

2.3.4 Isolation of plasmid DNA

2.3.4.1 Mini-plasmid preps

Individual colonies were picked from plates and inoculated into 3 ml of LB Ampicillin broth and the plasmid DNA isolated using an alkali lysis procedure. Briefly, 1 ml of medium was transferred to a microfuge tube and the bacteria were pelleted by centrifugation at 13,000 rpm for 1 min and resuspended in 300 µl of resuspension buffer (50mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). The cells were lysed by the addition of an equal volume of lysis buffer (200 mM NaOH; 1% SDS (w/v)) and the solution mixed gently and incubated at room temperature until the solution cleared, whereupon an equal volume of neutralisation buffer (3 M potassium acetate, pH 5.5) was added and the solution agitated strongly. The precipitate of cell debris, chromosomal DNA and SDS was pelleted by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was removed, 0.7 volumes of propan-2-ol added and the plasmid DNA precipitate pelleted by centrifuged at 13,000 rpm for 30 min at 4°C. The pellet was washed with 80% EtOH air dried, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl (pH8.0) 0.1 mM EDTA). All plasmids were validated by diagnostic restriction digestion and agarose gel electrophoresis as described in **2.3.5** and **2.3.6**.

2.3.4.2 Maxi-plasmid preps

100 ml of LB containing 100μg/ml Ampicillin were inoculated with 100μl of mini-prep culture and grown with agitation over night (as above). Plasmid DNA was isolated based on a modified alkaline lysis (Birnboim & Doly, 1979) procedure followed by binding of plasmid DNA to an anion–exchange resin, as per the manufacturer's instructions (Qiagen, UK). In short, the overnight bacterial culture was pelleted by centrifugation at 3000 rpm for 10 min, and the resuspended in 20 ml of resuspension buffer. The cells were lysed by the addition of an equal volume of lysis buffer, and the solution mixed gently at room temperature until the solution cleared, whereupon an equal volume of neutralisation buffer was added and the solution agitated strongly. The precipitate of cell debris, chromosomal DNA and SDS was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was removed and passed over the Qiagen Anion-Exchange resin, the bound plasmid DNA washed with low salt buffer (1 M NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v) and finally eluted in 5 ml space of elution buffer (1.6 M NaCl; 50 mM MOPS, pH 8.5; 15% isopropanol (v/v)). The eluted DNA was precipitated with the addition of 0.7 volumes propan-2-ol, and the DNA pellet washed in 80% EtOH and air dried. The DNA pellet was resuspended in appropriate volume TE buffer and the concentration was determined from the absorbance at A_{260} of the preparation, assuming that a DNA solution of 50 μg/ml in water gives a value of $A_{260} = 1$ using a spectrophotometer (Hitachi U2001). Plasmid DNA was stored at 1 mg/ml in TE, at 4°C (short term) or -20°C (long term).

2.3.5 Restriction enzyme digests

Restriction enzymes were used according to the manufacturer's instructions (Promega). In general, 5μg of plasmid DNA was cut with 50 units of restriction enzyme for 18 h at 37°C in the presence of 150μg/ml bovine serum albumin (BSA) to stabilize the enzyme. Where multiple digests were to be performed, the buffer conditions were selected to be compatible with both enzymes.

2.3.6 Agarose gel electrophoresis of DNA

Gels were prepared by boiling the appropriate mass of low melting point or general purpose agarose in 1x Tris-Acetate-EDTA (TAE; 242 g Tris base, 57.1 ml Acetic acid and 100ml 0.5M EDTA to a total volume of 1 liter with ddH_20 and adjusted to pH to 8.5) cooling to 50°C before adding Ethidium Bromide (EtBr) at 1 µg/ml and then setting this in the chosen gel former; "mini" gels (6.5 x 10 x 1 cm, 15 sample wells). All gels were run in 1xTAE; at 10 volts/cm. DNA fragments were satisfactorily resolved on 1-2% agarose gels.

2.3.7 Detection and isolation of DNA fragments

Ethidium bromide intercalates DNA, and in this state fluoresces when illuminated by ultraviolet (UV) light. DNA was visualised by illuminating the gel with short wave UV light on a transilluminator (Stratagene). When DNA was to be recovered from the gel, a hand-held long wavelength lamp was used to avoid damage to the DNA. DNA fragments, after restriction endonuclease digestion, were excised from the agarose gel and the slice containing the DNA fragment purified as per manufacturer's instructions using QIAquick gel extraction (Qiagen).

2.3.8 Analysis of fibroblast mRNA by Northern Hybridisation

2.3.8.1 RNA extraction from fibroblast monolayers

RNA was extracted from fibroblast monolayers using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, culture medium was aspirated and cells were gently washed with sterile PBS at 4^{o}C. The PBS was removed and 1 ml of TRIzol per 10^6 cells was added to each dish and incubated at room temperature for 10 min to lyse the cells. The lysate was pipetted several times then 1ml was transferred to a 1.5ml RNase free microfuge tube. Upon transfer 0.2 ml of chloroform was added per 1ml. Tubes were shaken vigorously for 15 sec and then incubated at room temperature for a further 2 min. The samples were then centrifuged at 13,000 rpm at 4^{o}C for 15 min. After centrifugation the aqueous phase was transferred to a fresh 1.5ml tube and an equal volume of isopropanol was added to precipitate the RNA. After 5 min incubation the sample was centrifuged at 12,000xg at 4^{o}C for 30 min. The pellet was washed in 80% ethanol and resuspended in an appropriate volume of diethylpyrocarbonate (DEPC) treated water. To confirm integrity a sample of the RNA was subjected to gel electrophoresis.

RNA concentration and purity were measured spectrophotometrically (Hitachi U2001) by determining the absorbance of a diluted aliquot (of 4 µl of sample with 796 µl DEPC treated water in a quartz cuvette) at 260nm. The RNA concentration was determined based on the absorbance co-efficient of an OD_{260nm}=1 equivalent to a RNA solution of 40 µg/ml. The absorbance of the solution at 280nm was also measured and a ratio of OD 260nm:280nm was used as an index of purity.

2.3.8.2 Northern blotting

Electrophoresis of RNA and transfer to Hybond membrane. RNA samples were electrophoretically separated on a 1% (w/v) denaturing formaldehyde-agarose gel. This was prepared by dissolving 1g of molecular biology grade agarose in 66 ml DEPC treated water, adding 20 ml of 5X gel running buffer (0.1M MOPS, 40mM sodium acetate, 5mM EDTA, pH7.0) and then 14 ml 2.2 M formaldehyde to a final gel concentration of 2.2M. Samples were prepared as follows: 10 µg of RNA was added to a microfuge tube and

DEPC-treated water added to a final volume of 4.5 µl to which formaldehyde (3.5 µl), formamide (10 µl) and 5X running buffer (2 µl) were added. This sample mixture was incubated at 65^{o}C for 15 min, and kept on ice before loading. Prior to loading, 2 µl of 6X gel loading buffer (50% glycerol, 1mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to each sample. The gel was pre-run for 5 min at 5V/cm then samples were loaded and electrophoresis performed for 1-2 h at 3-4V/cm.

The agarose gel was rinsed in DEPC-treated water to remove the formaldehyde and soaked in 20X sodium chloride-sodium citrate (SSC) buffer (3.0 M NaCl, 0.3 M Na-citrate*)* for 45 min. The gel was placed on blotting paper, pre-soaked in 20X SSC, hybond membrane placed on top of the gel and a piece of pre-soaked blotting paper placed over the membrane. Multiple (8-10) dry blotting papers were placed on top of the soaked layer, a glass plate was placed over the dry layers and transfer performed for 4-12 h at room temperature. After transfer the RNA was UV cross linked to the hybond membrane (15 sec at 254nm) using a UV stratalinker (Stratagene).

Radiolabelling of cDNA probes. Labelling of DNA fragments specific for conserved region of the pro-α2(I)collagen mRNA was performed using a Megaprime labelling kit (Amersham Life Sciences) according to the manufacturer's instructions. The Cola2(I) probe was a 200bp fragment (PstI-EcoRI) coding for the C-terminal region of the mouse Cola2(I) gene in pBluescript II(Ponticos *et al.*, 2004). In brief, approximately 25 ng of template DNA was added to 5 µl of random primers and boiled for 5 min to denature the DNA. The tube was placed on ice and 10 µl labeling buffer, 5 µl of αP^{32} dATP (250 uCi; 9.25 MBq) and 2 µl of DNA polymerase (Promega) were added. The mixture was then incubated for 30 min at 37^{o}C. The labelling reaction was stopped by addition of 50 µl of 4M ammonium acetate (pH4.5) and the probe purified from unincorporated radiolabel using a microspin G50 column (Amersham Life Sciences) as per manufacturer's instructions. Using this protocol, probes were labelled to a specific activity of at least 10^9 dpm/µg.

Hybridisation. The hybridisation bottle was half-filled with 2X SSC, and the membrane applied to the inside wall of the bottle. The SSC was poured off and replaced with 5ml

Rapid-hyb (Invitrogen) buffer. The membrane was pre-hybridised in a Hybaid oven at 65^{o}C for 15 min. Prior to the addition of the labelled probe to the hybridisation buffer, cDNA was denatured by boiling for 5 min. The labelled probe was added and hybridisation performed at 65^{o}C for 2 h. The membrane was washed twice with 2X SSC at room temperature, twice with 1X SSC at 65^{o}C and then rinsed in 2 X SSC.

Autoradiography and quantitation. The membrane was placed against X-Ray film, and left at -70 ^{o}C between 12-72 h. X-ray films were digitised using a digital camera (UVP v1.0) and UVP Grab software (Synoptics, UK).

2.3.9 Analysis of gene expression by cDNA microarray

In this thesis, murine fibroblastic gene expression analysis was performed using the Atlas mouse cDNA expression array (Version 1.2, ClonTech, Palo Alto, CA).
Total mRNA was prepared from confluent cultures of early passage adult Tsk1 and neonatal wildtype, Tsk1 and TβRIIΔk dermal fibroblasts as described in Section **2.3.8.1**. Briefly, cell monolayer were washed twice with PBS, TRIzol (Invitrogen) lysis reagent added for 10 minutes and then RNA was recovered by alcohol precipitation.

2.3.9.1 Probe labeling, prehybridisation, hybridisation and washing

A radioactively-labelled cDNA probe was synthesised from the sample RNA by reverse transcription using MMLV-reverse transcriptase, α-P^{32}, DTT, dNTPs and a gene specific cDNA synthesis (CDS) primer mix supplied with the array which is enriched for sequences corresponding to the cDNA for the genes on the array, thus ensuring high specificity and increasing the rate of reaction.

This probe was then purified by the Nucleospin Column protocol supplied with the arrays. Prehybridisation of the membranes was carried out using 5ml ExpressHyb^{TM} solution (ClonTech) and denatured sheared salmon testes DNA for two hours at 68^{0}C. After this time, the purified probe was then added directly to the hybridisation solution, and incubated overnight at 68^{0}C with constant rotation. Following this, the arrays were washed in three changes of Wash Solution 1 (2x SSC, 1% SDS) at 68^{0}C for 30 min each, and then washed

in Wash solution 2 (0.5x SSC, 0.5% SDS) at 68^0C for 30 mins. A final 5 min wash at room temperature in 2x SSC was carried out with constant agitation, before the arrays were quickly sealed in plastic wrap. These high-stringency washes ensured removal of all unbound radioactive label, leaving the arrays to be analysed using a phosphoimager screen.

2.3.9.2 Analysis of cDNA microarrays

The phosphoimager images of each filter were obtained using the ImageQuant™ software package and analysed using the ClonTech AtlasImage™ software. Normalisation using the sum global intensities of the arrays was chosen with the local background for each cDNA spot taken into account. A significance level of a 2-fold difference in gene expression was selected so as to decrease biological noise.

2.3.9.3 Stripping of cDNA microarrays

The procedure detailed below allowed the arrays to be stripped of radioactivity and reused at least three times. The arrays were heated to 95-100^0C for 5-10 mins in 0.5% SDS in DEPC-treated water, and then left standing for a further 10mins. The arrays were then rinsed in Wash Solution 1 (2x SSC, 1% SDS), and then quickly wrapped in plastic wrap and stored at -70^0C. At no point were the arrays allowed to even partially dry.

2.3.10 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was used to clone the upstream 1kb promoter for MCP-3 gene contained within the pUC19 plasmid cloning vector which was donated by Professor Van Damme, Belgium. This fragment was amplified by the polymerase chain reaction (PCR). Briefly, DNA (100ng) was mixed with PCR primers (1-3μM), reaction buffer (67mM Tris-Base, 16.6 mM Ammonium Sulphate, 2mM Magnesium Chloride, 0.02% (v/v) Tween20), dNTP (10mM) and *Pfu* DNA polymerase. Samples were overlaid with 10ul of mineral oil (Sigma) in order to avoid evaporation and amplified using the thermal cycler PTC-100 (MJ Research Inc). The resultant DNA fragments were analysed by agarose gel electrophoresis (0.8%) in presence of ethidium bromide (0.08% v/v). The PCR reaction was diluted with Orange G loading solution (2μl) (Ficoll 20% w/v, Orange G 0.25% w/v) and then loaded into the wells of the flat-bed agarose gels. Electrophoresis was

carried out at 100V with TBE buffer (1x, 18 mM Tris-borate and 4mM EDTA) and the migration of DNA fragment was monitored. Following the electrophoresis the gels were visualised under UV light and documented by photography using the UVP camera (Ultra Violet Productions Ltd., UK) Grab-IT annotation image capture system package with Sony digital graphic printer (UP-D860D).

2.4 Protein extraction

2.4.1 Preparation of total protein samples

Fibroblasts were grown to confluence in six-well tissue culture plates in DMEM with 10% fetal calf serum. The medium was replaced with DMEM containing 0.2% FCS for 24 h. Cell monolayers were washed twice in serum-free medium and replaced with DMEM containing 0.2% FCS for 24 h in the presence or absence of recombinant TGF-β1or chemokines, MCP-3 and MCP-1 for a further 24 h. For experiments where collagen type I was to be assessed, 1 ml of media was removed and 300 µl saturated ammonium sulfate added, and the sample incubated at 4^{o}C with rotation overnight. The next day the samples were centrifuged at full speed (13,000 rpm) for 30 min at 4^{o}C, and the pellet was resuspended 50 µl of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4 mM EDTA 1 mM PMSF; 1% NP-40; 1% Sodium deoxycholic acid; 0.1% SDS; 1% protease inhibitor cocktail inhibitor (Boehringer Mannheim). For all cell-associated proteins, cell monolayers were washed twice in ice cold PBS and lysed in 150 µl RIPA buffer. The lysed cells were scraped and DNA sheared by repeated passage of the sample through a 23-gauge needle.

2.4.2 Protein Expression- Western blot analysis

2.4.2.1 Electrophoresis and transfer of protein samples for Western blotting

Protein samples had an appropriate volume of 6x Laemmli sample buffer (0.2 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.004% bromophenol blue) and 1 µl of 14.2 M β-mercaptoethanol, added and were heat denatured at 100^0C for 3 min. The proteins are resolved by passing a current across a polyacrylamide gel, separating protein based on size. 20 µl, containing 10-20 µg of protein were run upon a ready-cast 18% Tris-Glycine gel (Invitrogen) alongside a broad-range protein marker (New England Biotech) at 100V, until the dye front had reached the bottom of the gel (approximately 1 ½ h) in 1x Tris-Glycine Running Buffer (Invitrogen). This was carried out within an Electrophoresis tank (Invitrogen), ensuring that the running buffer (25 mM Tris Base, 192 mM Glycine, 0.1 % SDS, pH 8.3) completely covered the gel at all times. Equal sample loading was ascertained with 0.2% Coomassie Blue (LKB, France), followed by de-staining with a 30% methanol, 10% acetic acid buffer (VWR, UK).

Each gel was removed from the casing and placed within a transfer set-up, using chromatography paper, Hybond C+ membrane (Amersham), and sponge filters. All these components had previously been soaked in 1x transfer buffer (25 mM Tris Base, 192 mM Glycine, 20 % Methanol). The transfer set-up was then carefully placed within a transfer module (Invitrogen) and placed in an electrophoresis tank (Invitrogen), completely submerged in 1x transfer buffer. Overheating during transfer was prevented, by surrounding the transfer module with cold water. The transfer was then allowed to proceed at 30V for approximately 1½ h. After this time, the blue dye from the protein markers could be clearly seen transferred onto the Hybond™ membrane, indicating that transfer had occurred. Each membrane was removed from the transfer apparatus and protein transfer confirmed by staining for 1 min in Ponceaus solution (0.1% Ponceaus S (w/v), 5% acetic acid (v/v)). Membranes were washed in 0.5% PBS-Tween for three washes of 10 min, with constant agitation. For detection of type I collagen samples were resolved on a 6% Tris-Glycine gel (Invitrogen) as described above.

2.4.2.2 Immunoblotting

Immunoblotting was performed by incubating the membranes in PBS-Tween (0.01% (v/v) Tween 20) with 5% non-fat dry milk at 4^{O}C overnight to block non-specific antibody binding. The blocking solution was removed and the primary antibody added at the appropriate concentration to PBS-Tween (0.01% (v/v) Tween 20) with 5% non-fat dry milk and incubated for 2 h at room temperature. The membranes were then washed in PBS-Tween, three times for 15 min and then incubated with the appropriate biotinylated species-specific secondary antibody in PBS-Tween containing 5% milk at room temperature for 1 h. The membranes were washed three times before staining with biotin substrate (Vectastain; Vector Laboratories, Peterborough, UK), then chemiluminescent substrate (Amersham Pharmacia Biotech), and developed against photographic film (Hyperfilm ECL;Amersham Pharmacia Biotech) for between 30 sec and 5 mins.

For western blots for phosphorylated proteins PBS was replaced with Tris-Buffered Saline (TBS; 0.05M Tris Base, 0.9% NaCl, pH 7.6). Selected blots were washed and reprobed with a monoclonal antibody against β-actin (Sigma) or GAPDH (Abcam) to control for small variations in protein loading and transfer.

2.4.3 Protein Expression – Measurement of MCP-3 with ELISA

Serum, blister fluid and fibroblast cell culture supernatant samples were taken from patients with SSc, following consent, and were then stored at –20°C over a short period of time. Dermal interstitial fluid was derived by a suction blister technique from the lesional skin of SSc patients, and from the forearm skin of healthy controls(Dziadzio *et al.*, 2005). Fluid obtained using this technique has the properties of an interstitial fluid sample, and does not arise by exudation of fluid and protein from the vascular space.

Quantitative measurement of MCP-3 in these samples was undertaken using specific enzyme-linked immunosorbant assay (ELISA) kit (Biosource Europe S.A.). All samples were tested in triplicate, the minimum detectable dose of MCP-3 was less than 2 pg/ml. A standard curve was used as reference, this was obtained by increasing dilutions of a stock solution provided by manufacturer to obtain eight different concentrations, each of which

was also tested in duplicate. Briefly, 100 µl of each sample was added into each well of a 96-well microplate that was pre-coated with capture monoclonal antibody specific for MCP-3 and 50 ul of biotinylated monoclonal antibody for MCP-3. After 2 h incubation at room temperature, each well was thoroughly washed three times using an automated ELISA plate washer. 100 ul of diluted Streptavidin-peroxidase conjugate were then added to each well to bind to the biotinylated antibody and incubated for 30 min. After washing, 100 µl of chromogenic solution were added and incubated for 15 min. 100 µl of Stop solution were then added and the absorbance at 460nm was determined spectrophotometrically according to the manufacturer's instructions. Test sample MCP-3 concentrations (range 0-1000pg/ml) were determined from the standard calibration curve.

2.5 Immunohistochemical analysis of cryosections

2.5.1 Tissue processing and preparation

Murine and human dermal samples were obtained as described in Sections **2.1.3** and **2.2**. These tissue samples were snap frozen in liquid nitrogen and mounted onto cork with optima cutting compound (OCT, Miles, Cal), snap frozen in liquid nitrogen cooled isopentane (BDH, UK). Serial frozen sections (5µm) were cut on a Bright cryostat at -25^{o}C and air-dried for at least 1 hour.

2.5.2 Immunohistochemical staining

Prior to staining, sections were fixed in ice cold acetone for 10 minutes at 4^{o}C and then washed in PBS. Endogenous peroxidase was then quenched by incubation with 0.3% (v/v) hydrogen peroxide in methanol (VWR, UK) at room temperature for 15 minutes in the dark. Sections were then washed in PBS and non-specific binding of immunoglobulins was blocked by treatment with 2.5% (v/v) normal serum (Vector Laboratories, UK) for 30 minutes after which sections were incubated with primary antibodies for 1 hour at room temperature or overnight at 4^{o}C. The primary antibodies used were goat polyclonal anti-mouse MARC/MCP-3 and goat polyclonal anti-human MCP-3 IgG antibody. Other monoclonal antibodies used in this thesis were directed against cell-specific antigens including fibroblast, endothelial cell, α-smooth muscle actin and macrophage CD68 surface marker.

After washing in PBS, sections were incubated with a species-specific biotinylated secondary antibody (Vector Laboratories, UK) for 30 minutes, rinsed in PBS and incubated with Vectastain avidin biotin complex (ABC)-peroxidase conjugate,(Vector Laboratories, UK) for 30 minutes. After washing in PBS, sections were visualised using 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, UK). Sections were then washed in tap water, counterstained with Meyer's haematoxylin and mounted with aqueous media (Crystalmount, Biomeda, UK). Sections were viewed and photographed on an Olympus BH-2 photomicroscope. Controls included omission of the primary antibodies and primary antibodies substituted with goat isotype-matched IgG. All incubations were carried out at room temperature. For haematoxylin and eosin staining, sections were stained in haematoxylin for 1 minute and blued in 0.5 M sodium tetraborate for 15 secs. Sections were then stained in eosin for 1 minute, washed in water and dehydrated through the ethanol solutions and cleared in xylene before mounting.

2.6. Statistical Analysis

For quantitative variables, the mean ± SEM results from replicate samples, or from combined independent experiments where between-experiment variation allowed reliable combination of raw data, were compared. Statistical analysis was performed by the Student's unpaired *t* test. *p* values less than 0.05 were considered statistically significant.

CHAPTER 3: MOLECULAR ANALYSIS OF MOUSE MODELS OF FIBROSIS IDENTIFIES OVEREXPRESSION OF MCP-3

3.1 Introduction

Animal models are useful in providing clues for understanding biologically complex human diseases and for elucidating pathogenesis of diseases. They also provide the platform in which pharmacokinetics, toxicities and effectiveness of potentially novel therapies may be studied. Although there are currently no animal models that recapitulate the major features of SSc (vasculopathy, autoimmunity and fibrosis), several experimental models exist for studying the fibrotic process. Two important models that are particularly useful in elucidating the pathophysiology of fibrosis are Tsk1/+, the heritable mouse model of fibrosis and the transgenic mouse strain, a novel genetically engineered gain-of-function mouse model with spontaneous development of SSc-like fibrotic phenotype. First described more than half a century ago, the Tsk1 mouse model demonstrates the fibrotic phenotype with diffuse thickening and tethering of the skin as a result of a spontaneously occurring mutation. Although mice homozygous for the Tsk1 mutation die in utero at 8 to 10 days of gestation, heterozygous mice survive to develop thickened skin that is bound firmly to the underlying subcutaneous tissue.

The genetic basis for the phenotype has been localised to chromosome 2 as an inframe partial reduplication of the Fbn1 gene. The primary role of fibrillin is to form the scaffolding for elastic fibres. In Tsk1 mice, fibrillin appears to alter fibrillin matrix structure, with an increase in fibrillin in the superficial fascia of these animals. Tsk1 mice exhibit increased elastogenesis in the superficial fascia (gain-of-function) and a loss of the dense elastic fibre band normally found at the interface between the intradermal muscle and deep connective tissue (loss-of-function). In addition to the phenotypical similarities to SSc, Tsk mice also show pathological similarities to SSc in upregulated expression of microfibrillar proteins fibulin-2 and the expression of the mutated Fbn1 increases the incorporation of type I collagen and microfibril-associated glycoprotein 2 (MAGP-2) fibrillar structures within the ECM(Lemaire *et al.*, 2004). More recently, analysis of

lesional skin has demonstrated upregulation of the Wnt-γ catenin pathway and Wnt is shown to increase Fbn matrix formation *in vitro*, a key pathogenic feature in Tsk hypodermis(Bayle *et al.*, 2008).

Reported evidence further indicates that the immune system plays a role in modulating the fibrosis observed in the tight skin-1/+ mouse. It has been reported that Tsk/1+ mice are hyperresponsive to CD19 transmembrane signals leading to reduced IgM expression with increased serum IgG levels and spontaneous autoantibody production targeted against topoisomerase-1(Saito *et al.*, 2002). The reason for this aberrant immune response appeared to be the constitutive increase in tyrosine phosphorylation of CD19 in B cells from the Tsk1/+ mouse. In addition, CD19-mediated $[Ca^{2+}]_i$ responses, Vav phosphorylation and Lyn kinase activity were increased(Asano *et al.*, 2004a). In addition, adoptive transfer of both T and B cells from Tsk1/+ mice induces cutaneous collagen deposition in wild-type mice and deficiency in TGFβ, IL-4, IL-4 receptor, Stat-6 as well as the administration of anti-IL-4 antibody, IL-12 prevent or reduce skin fibrosis in Tsk1/+ mice(Ong *et al.*, 1999;McGaha *et al.*, 2001;Ong *et al.*, 1998;Tsuji-Yamada *et al.*, 2001;Kodera *et al.*, 2002). Taken together, these suggest immune dysregulation contribute to the fibrotic response in this murine model. In addition, there is evidence that Tsk fibrillin-1 alters TGFβ activity in the skin as well as altering the binding of fibrillin to latent TGFβ binding proteins. In addition, TGFβ deleted heterozygotes appear to demonstrate less fibrosis than do littermate Tsk mice. Furthermore, studies using hammerhead ribozymes specifically directed against the mutant fibrillin-1 transcript in skin fibroblasts derived from Tsk dermal biopsies revealed a striking decrease in the expression of TGFβ dependent genes, including CTGF and plasminogen activator inhibitor-1, in Tsk skin cells where the mutant fibrillin-1 had been deleted(Menon *et al.*, 2006). It is therefore logical that targeting TGFβ has been a key focus for the development of mouse models of SSc. Adenoviral gene transfer to transiently expose fibrosis-prone C57/BL6 mice to high levels of active TGFβ1 in the lung (Sime *et al.*, 1997;Kolb *et al.*, 2002) is associated with the development of profound parenchymal and pleural fibrosis. This transient transgene approach allows high level expression of active TGFβ which is largely targeted to the bronchial epithelium, although is not lineage specific. αvβ6 integrin knockout mice that cannot activate TGFβ1 develop an exaggerated

inflammatory response to bleomycin but are protected from fibrosis, suggesting that active TGFβ1 but not inflammation is essential for the fibrotic response(Munger *et al.*, 1999). Dissecting out the exact functional role of TGFβ pathways in SSc is hampered by its pleiotropic effects, its role in embryonic development, and the complexity of TGFβ regulatory mechanisms in vivo(Sonnylal *et al.*, 2007).

Fibroblasts are likely to be key effector cells in the late fibrotic stages of SSc, and considerable emphasis has been placed on delineating the role of TGFβ signalling in these cells. Targeting fibroblasts is challenging due to the paucity of lineage-specific marker genes especially cell-surface markers. Regulatory elements subcloned from type I collagen are expressed at an early stage in differentiating mesenchymal cells and have been used to generate promoter constructs that function as lineage-specific transgenes in fibroblasts(Rossert *et al.*, 1995).

In this thesis, lineage-specificity has been achieved by the use of a far-upstream transcriptional enhancer from the proα2(I)collagen (Col1a2) gene linked to a minimal promoter that directs expression to fibroblasts(Denton *et al.*, 2001;Bou-Gharios *et al.*, 1996). Delineation of DNase I-hypersensitive sites far upstream of the Col1a2 promoter sequence supports a key role in regulating expression of linked genes through direct binding of sequence specific transcription factors in collagen I producing cells(Bou-Gharios *et al.*, 1996). Not all type I collagen-producing cells express the transgene. Reporter mice show that the transgene is expressed specifically in cells of mesenchymal origin, mainly fibroblasts and some osteoblasts in areas of new bone growth suggesting recent differentiation from mesenchymal precursors. The promoter is important in controlling endogenous gene expression. The far upstream enhancer drives expression of Col1a2 in a spatio-temporal pattern similar to the endogenous gene; Col1a2 reporter mice show high levels of transgene expression in fibroblasts in the embryonic and neonatal period, whereas little transgene is expressed in adult mice from three weeks of age(Ponticos *et al.*, 2004).

In this thesis, a recently described novel transgenic mouse strain (TβRIIΔk) was used in which a potent fibroblast specific expression cassette subcloned from the proα2(I)collagen

gene drives the expression of a mutant kinase-deficient type II TGFβ receptor in fibroblasts but not other cell types. This experimental strategy with fibroblast-directed transgenesis allows selective disrupted TGFβ signalling while minimising effects in other cell types including endothelial cells or other immunological cells that are also important in the pathogenesis of SSc. The mutant TβRII construct encodes the extracellular and transmembrane portion of human TβRII; it can engage free TGFβ ligand at the cell surface, but cannot directly lead to the phosphorylation of TβRI or the initiation of downstream signalling (Weis-Garcia & Massague, 1996). This truncated receptor has previously been characterised as a competitive antagonist for TGFβ1 and has been shown to be a dominant negative inhibitor of TGFβ signalling in several experimental systems, albeit at high expression levels compared with wild type receptors (Brand & Schneider, 1995;Bottinger *et al.*, 1997;Brand *et al.*, 1993;Pannu *et al.*, 2004).

Modern methods of parallel examination of gene and protein expression have advanced the understanding of transgenic or spontaneously arising mutant mouse strains. In contrast to the limited numbers of mRNA transcripts that may be studied with Northern blot, the ability to simultaneously measure the transcription of large number of gene sequences has provided the opportunity to understand the pathogenesis of biologically complex diseases and also to potentially identify candidates for disease-related target molecules. In addition, it may identify biochemical pathways that can be used as targets for future therapeutic interventions. One of the most productive approaches has been to use gene expression profiling to investigate the biochemical properties of mutant mice. DNA arrays have been used for large-scale investigation of gene expression patterns and they are based on hybridisation of a labelled mixed probe onto large systematic, high intensity grids of cDNA fragments. In this thesis, the Clontech 1.2 Mouse Nylon Atlas Arrays were used which include over 1000 cDNAs spotted in a regular fashion onto a positively charged nylon membrane. This strategy has been used in several models for fibrotic diseases including the fibrotic plaques in Peyronie's disease and the mutagenic effect of nickel in murine fibroblasts(Qian *et al.*, 2004;Kowara *et al.*, 2005). A key advantage of this approach is that it seeks alterations in the mRNA profiles without predefined assumptions about disease progression. SSc, as a disease with progressive course, heterogeneous presentation with

incompletely understood pathogenesis presents itself as an ideal candidate disease for study through this approach. Of the three sample types (skin biopsies, peripheral blood and explanted cultured fibroblasts) that are available for microarray analysis, dermal fibroblasts are an attractive model system for study in SSc due to their accessibility, amenability to ex-vivo experimentation and they are known to retain some properties including positional memory and myofibroblast phenotype in cultures(Shi-Wen *et al.*, 2004;Rinn *et al.*, 2006).

3.2 Aims

Murine models including Tsk1 strain provide an important resource to study and delineate potential novel growth factors and cytokines in the pathogenesis of SSc. In addition, the significance of TGFβ in fibrosis led to the hypothesis that, targeted transgenesis using a lineage-specific promoter with sustained alteration in TGFβ signalling or responsiveness in fibroblasts may recapitulate the SSc phenotype. Analysis of both murine models may suggest an important interplay of TGFβ and other factors in driving the fibrotic response.

Experimental work described in this chapter aims to

1. Identify the most highly expressed key growth factor in Tsk1 fibroblasts,
2. Examine the potential expression of MCP-3 in TβRIIΔk model, and
3. Investigate the biochemical phenotype of TβRIIΔk murine model.

3.3 Methods

3.3.1 Fibroblast Culture

Fibroblast cultures were derived from skin biopsies from lower back of neonatal transgenic, Tsk1 or control littermate mice. Cells were cultured in the presence of antibiotics and passaged at confluence. Following 24 hours of culture in low serum (0.5% FCS) medium, 10^4 cells were seeded into each well of replicate 6 well tissue culture plates. At 24 hour intervals the cell layer was recovered and resuspended. Pharmacological inhibitor of the TβRI kinase (SD-208, Scios Inc., Freemont CA) was used to investigate the response of TGFβ regulated protein, CTGF expression to inhibition of TGFβ signalling pathway. For these experiments, fibroblasts were stimulated with recombinant TGFβ1 (4ng/ml) for 45 minutes in the presence or absence of SD-208.

The transgenic TβRIIΔk strain used in this thesis was generated by Professor Christopher Denton at University College London and University of Texas. In brief, a fibroblast-specific expression cassette was subcloned from the upstream region of the proα2(I)collagen gene. This incorporates a fragment between -19.5 and -13.5kb upstream of the transcription start site that, when linked to an endogenous minimal promoter drives gene expression at high levels in fibroblasts, but not in other type I collagen producing cells. Reporter genes linked to this promoter-enhancer show high level fibroblast-specific expression in embryonic development and postnatally. The mouse strain TβRIIΔk was generated by subcloning the cDNA encoding the extracellular and transmembrane portion of the human type II TGFβ receptor into the Sal1 site of the pCD3 expression vector. A bacterial β-galactosidase marker gene (LacZ) was co-expressed from a dicistronic transgene mRNA product via an encephalomyocarditis virus internal ribosome entry site sequence. The final transgene construct is shown in **Figure 3. 1**. Littermate wildtype C57/B6 mice were used as control animals.

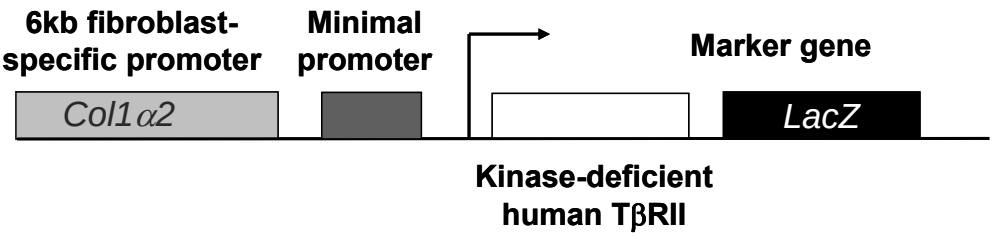


Figure 3.1 A fibroblast-specific expression cassette for transgenic mice that incorporates a 6kb fragment of the far upstream (-19.5 to -13.5 kb) region of the Col1a2 enhancer linked to a minimal Col1a2 promoter. The cDNA encoding for the kinase-deficient human TβRII is expressed at high levels in fibroblasts but not other type I collagen producing cells. A β-galactosidase marker gene was included that can be co-expressed to confirm the site and level of transgene expression.

3.3.2 Gene expression analysis

In order to compare differences in the gene expression between wildtype fibroblasts with Tsk1 and transgenic TβRIIΔk fibroblasts, low-density microarray analysis was undertaken. Entire cDNA populations were hybridised to BD Atlas™ cDNA mouse 1.2 expression arrays (Clontech, USA), which incorporate oligonucleotides specific for 1,176 mouse-gene transcripts; the cDNA-synthesis gene-specific primer mix, used for probe synthesis, is enriched for sequences corresponding to the cDNA for the genes on the array. In brief, mRNA was extracted, purified, and treated with DNase I (Qiagen, UK) to reduce genomic DNA contamination, as previously. Radio-labelled cDNA probe mixtures were synthesised by reverse transcription of each RNA population, using the cDNA synthesis primer mix, according to the manufacturer's instructions. Labelled cDNA probes were purified by column chromatography, using Atlas Nucleospin extraction spin columns. Label incorporation was assessed by scintillation counting with a Geiger counter, aiming for 2-10 x 10^6 counts per minute. Each BD Atlas™ cDNA Expression Array membrane was pre-hybridised (as described for northern blotting), then after denaturation to single strands, each radio-labelled probe mix was hybridised to separate BD Atlas arrays overnight with continuous agitation at 68°C. After a series of high-stringency washes, the membrane was exposed to a phosphorimager plate for 1-10 days at RT, depending on the signal intensity. Experiments were performed in parallel and so generally 4 membranes were probed at the same time. The hybridisation pattern and relative expression levels were analysed using AtlasImager software (Clontech, USA); normalisation of the expression data was achieved

by using the sum of the global intensities of the arrays, accounting for differences in the local background signal. Hybridisation signals are linearly related to the target cDNA concentration. A ratio of ≥2 between samples was taken to represent significant differential regulation and the difference must have been observed on at least two occasions in independent experiments to be considered significant. To minimise the effect of genetic background and gender, same sex (male) mice were used and littermate animals were examined. For each age of mouse pair compared, RNA was extracted independently from two wildtype or tight skin mice and analysed in parallel. For 6 week and neonatal samples each sample was analysed twice to confirm reproducibility.

3.3.3.Immunohistochemistry

For detection of MCP-3 protein on Tsk1 tissues, immunohistochemistry analysis with a biotin/streptavidin-based amplifying system was performed on mice skin sections. Serial frozen sections (5 μm) were cut on a cryostat at –30 ^{o}C and air-dried for an hour. Sections were fixed in ice-cold acetone and covered with 3% hydrogen peroxide for 10 minutes in the dark to block endogenous peroxidase activity. Slides were then blocked with 20% normal horse serum, and incubated with goat polyclonal anti-mouse MARC/MCP-3 IgG antibody (25 μg/ml in PBS, R & D Systems) for an hour at room temperature. After washing with PBS, sections were incubated with biotinylated horse anti-goat IgG diluted in PBS (7.5 μg/ml, BA-9500, Vector Lab, CA) for 30 minutes, rinsed and finally incubated with Vectastain Elite STR-ABC (Vector Lab, CA) reagent for 30 minutes. After washing, sections were visualised using 3-amino-9-ethylcarbazole(AEC) chromogen and H_2O_2 as substrate(SK-4200, Vector Lab, CA). Sections were then washed in tap water, counterstained with Carrazzis haematoxylin, and aqueously mounted with Gelmount (Biomeda, Foster City, CA). Sections were viewed and photographed on an Olympus BH-2 photomicroscope. Controls included omission of the primary antibodies and primary antibodies substituted with goat isotype-matched IgG. For detailed histological assessment of transgenic tissues, haematoxylin and eosin staining of these tissues were undertaken.

3.3.4.Western blot analysis of fibroblast protein expression

To extend the cDNA microarray data and to examine biochemical differences between Tsk1 and transgenic or wild type fibroblast cultures, a series of Western blot experiments were performed. In addition, responsiveness of cultured transgenic and wild type fibroblasts to recombinant TGFβ1 ligand were also undertaken. Fibroblast culture supernatants were concentrated by ammonium sulphate precipitation to selectively enrich samples for secreted matrix proteins. For supernatants, specific antibodies to collagen type I (Southern Biotechnology Inc., Birmingham, AL), fibronectin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), keratinocyte growth factor (FGF7) (R&D Systems) and goat anti-mouse MCP-3 antibody (R&D Systems) were used. Other studies examined CTGF (antibody supplied by FibroGen Inc.) and MMP13 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). All other antibodies were as described in Section **2.4.3**, and all protein extraction and western blotting methodologies performed as described in Section **2.4.3**.

3.4 Results

3.4.1 Gene expression analysis identifies overexpression of MCP-3 in neonatal Tsk1 fibroblasts

Parallel assessment of gene expression was performed using cDNA Microarray, comparing Tsk1 and wildtype littermate fibroblast (n=6) from skin biopsies at two time points: neonatal (1 week) and 12 weeks old. Neonatal fibroblasts expressed 244 (21%) of the 1176 genes represented on the array. These included genes for ECM turnover, growth factors, transcription factors and cell-related genes. At 12 weeks, 94 (8%) genes were expressed respectively. cDNA expression array templates for Tsk1 and wildtype neonatal fibroblasts are shown in **Figure 3.2**. At this time point, there was significant upregulation of tissue inhibitor of matrix metalloproteinase 3 (TIMP3), laminin β2 subunit (LAMC1), fibronectin 1 (FN1), thrombospondin 1 (TSP1), MCP-3, cytoplasmic dynein light chain 1 (dynein), Bcl-2 interacting protein (NIP3), inhibin βA subunit (inhibin), insulin-like growth factor binding protein 6 (IGFBP6), and cholecystokinin A receptor (CCKR). Conversely, there was down-regulation of cordon bleu protein (COBL), Cell Division Cycle 46 (CDC46), RAD23 ultraviolet excision repair protein (RAD23), laminin γ1 subunit (laminin), CD28 precursor, and interleukin-1 receptor (IL1R).

Differential expression was defined by 2-fold differences in hybridisation between control and Tsk1 RNA samples. Thus, 43 genes were differentially expressed neonatally but the number of differentially expressed transcript decreased to 24 at 12 weeks. For 13 genes, there was differential expression at either the neonatal or 12-week time point or at both of these time points. These genes, together with the normalised expression relative to the basal wild-type neonatal fibroblast levels, are listed in **Table 3.1**. More than half of the 43 genes that were differentially expressed neonatally
were not expressed consistently in adult samples, and these were not analyzed further. Conversely, 3 genes that were not differentially expressed in neonatal fibroblasts were found to be differentially expressed in adult cells, and these are included. Six genes including thrombospondin-1, osteopontin, hypoxia inducible factor-1α, tissue inhibitor of

metalloproteinase-3 and transforming growth factor β (TGFβ) showed sustained overexpression at all time-points. Several genes including biglycan and MCP-3 were overexpressed neonatally but suppressed in fibroblasts derived from established fibrotic skin. In neonatal cultures MCP-3 was the most overexpressed gene with more than 15-fold greater expression in neonatal Tsk1 compared with non-Tsk1 fibroblasts (**Figure 3.3**).

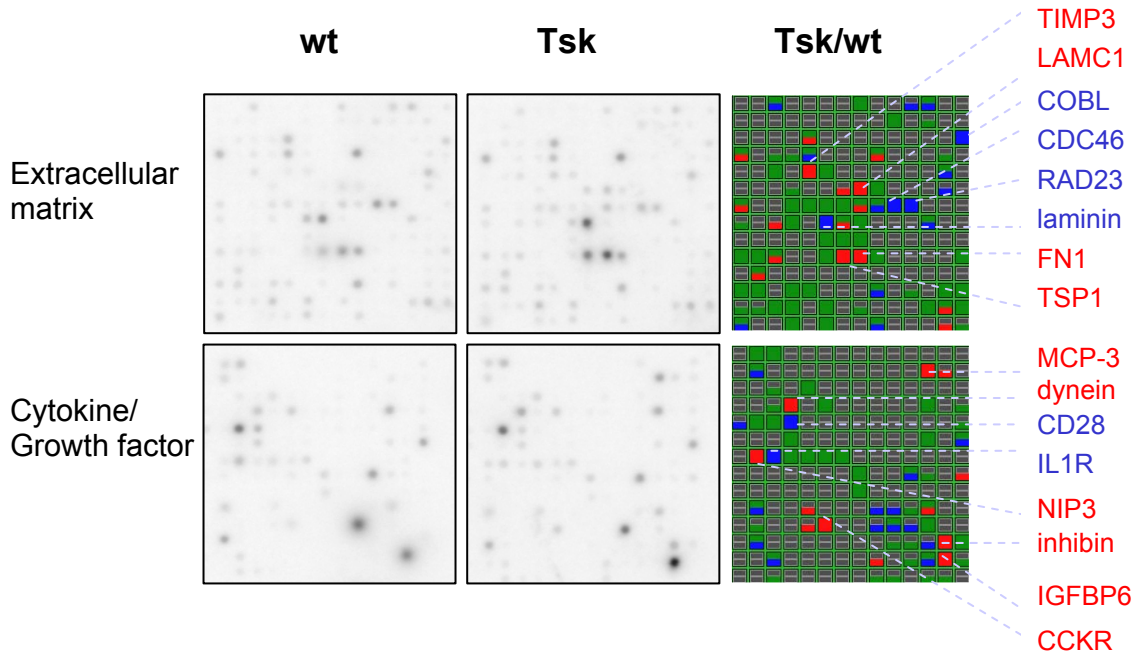


Figure 3.2 Overexpression of monocyte chemoattractant protein 3 (MCP-3) in type 1 tight-skin (Tsk1) neonatal mouse fibroblasts. Representative neonatal fibroblast gene-expression profiles are shown using portions of 2 mouse Atlas 1.2 cDNA arrays, corresponding to cytokine and growth factor or extracellular matrix transcripts. Tissue inhibitor of matrix metalloproteinase 3 (TIMP3), lamininβ2 subunit (LAMC1), fibronectin 1 (FN1), thrombospondin 1 (TSP1), MCP-3, cytoplasmic dynein light chain 1 (dynein), Bcl-2 interacting protein (NIP3), inhibinβA subunit (inhibin), insulin-like growth factor binding protein 6 (IGFBP6), cholecystokinin A receptor (CCKR), cordon bleu protein (COBL), Cell Division Cycle 46 (CDC46), RAD23 ultraviolet excision repair protein (RAD23), lamininγ1 subunit (laminin), CD28 precursor, and interleukin-1 receptor (IL1R).
Genes that are upregulated and downregulated are indicated in red and blue respectively. Genes that are similarly expressed in both samples are indicated in green. Half green/half red and half green/half blue indicate that although a difference (lower portion) was observed between the defined samples, the ratio (upper portion) is nonsignificant. The data are representative of triplicate experiments.

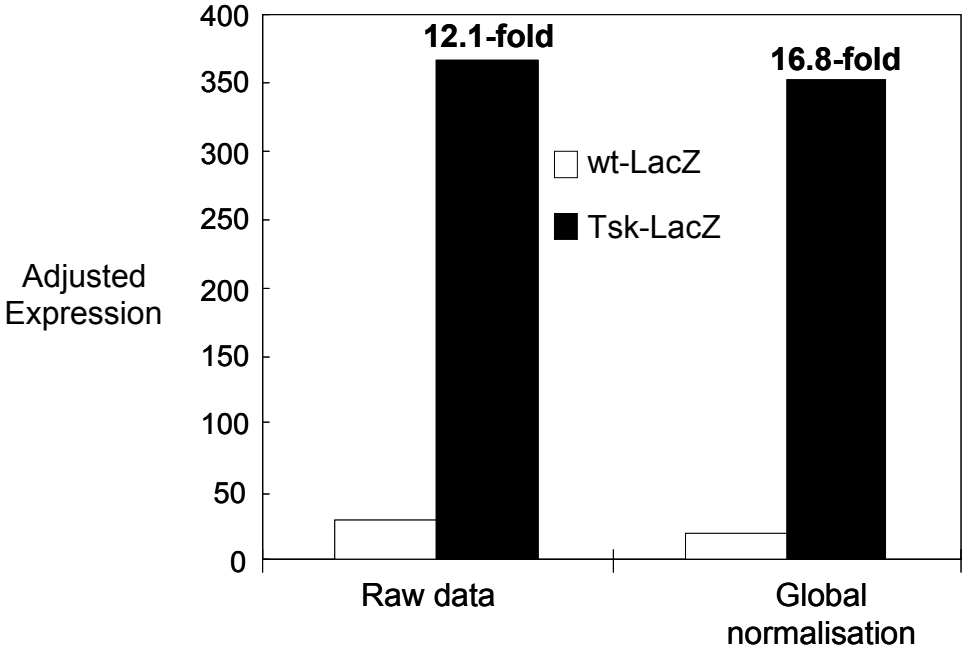


Figure 3.3 Overexpression of MCP-3 transcript on gene-expression profiling was quantified in a representative of triplicate experiments as shown with the histogram.

Gene Name	Prelesional (Neonatal)			Established (12 weeks)			GenBank accession no.
	Non-Tsk1	Tsk1	Ratio*	Non-Tsk1	Tsk1	Ratio*	
MCP-3(CCL7)	0.2	2.7	15.3	2.1	1.4	0.7	S71251
Monocyte colony-stimulating factor 1	0.3	3.4	10.9	0.4	0.8	2.2	X05010
Cluster differentiation antigen 44	0.1	0.7	4.9	0.6	1.1	1.8	M27129
TSP1	1.8	6.6	3.7	2.1	4.2	2.0	M87276
Hypoxia inducible factor 1α	0.2	0.7	3.0	0.4	0.2	2.0	U59496
TIMP3	0.2	0.6	3.0	0.4	0.7	2.0	L19622
Fibronectin 1	3.8	11.1	2.9	6.2	7.3	1.2	X82402
Osteopontin	1.8	4.3	2.4	6.8	8.6	1.3	J04806
Biglycan	5.1	10.1	2.0	8.4	4.2	0.5	L20276
TGFβ1	0.4	0.7	2.0	0.2	0.3	2.0	M13177
Integrin β1	0.4	0.6	1.6	1.0	2.1	2.2	Y00769
Keratinocyte growth factor (FGF7)	2.5	2.0	0.8	0.4	0.1	0.2	Z22703
Decorin	0.7	1.2	1.6	1.5	0.1	0.1	X53929

Table 3.1 Summary of consistently differentially expressed genes in neonatal or adult type 1 tight-skin (Tsk1) mouse fibroblasts. Genes listed are those with consistent differential expression either in early or late-stage Tsk1 in 3 experiments. Values for expression data are relative to the mean signal for the total signal intensity of expressed genes, after correction for local background signal.
*Differential gene expression in Tsk1 fibroblasts compared with non-Tsk1 littermate cells.

3.4.2 Overexpression of MCP-3 protein in Tsk1 fibroblast supernatants

Since many genes are also regulated post transcriptionally, western blot analysis was used to assess changes at the protein level. Upregulation of MCP-3 was observed in a series of 3 independent experiments with 2.4 fold overexpression in Tsk1 fibroblast culture supernatant compared to wildtype sample as shown in **Figure 3.4**.

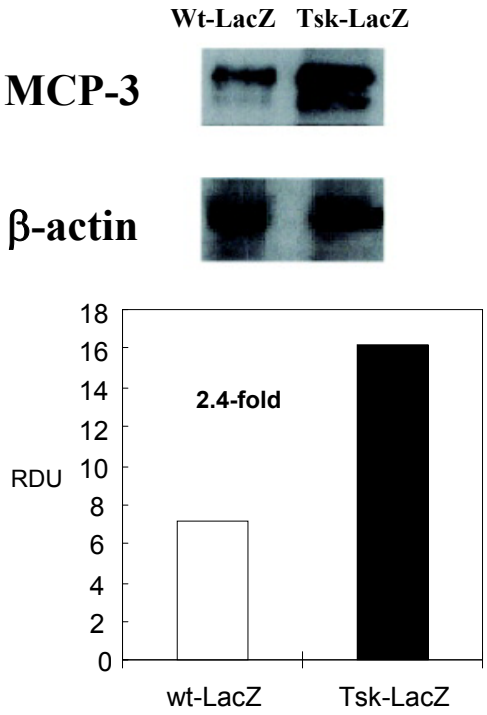


Figure 3.4 Overexpression of MCP-3 confirmed by Western blot analysis of Tsk1 and control (wild-type [Wt]) fibroblast culture supernatants. Cell-layer β-actin was used as a protein-loading control. Protein levels were quantified in a representative experiment as shown with the corresponding histogram in relative density units (RDU).

.

3.4.3 Dermal upregulation of MCP-3 in Tsk1

MCP-3 was detected abundantly in the dermal region in the skin biopsies from Tsk1 mice at 1 week and 3 weeks old. Non-specific perifollicular staining with antiMCP-3 antibody was observed in both control and Tsk1 skin sections. There was no inflammatory infiltrate detected in skin sections from both controls and Tsk1 mice (**Figure 3.5**), at all ages examined for 3 days post-natally to 12 weeks.

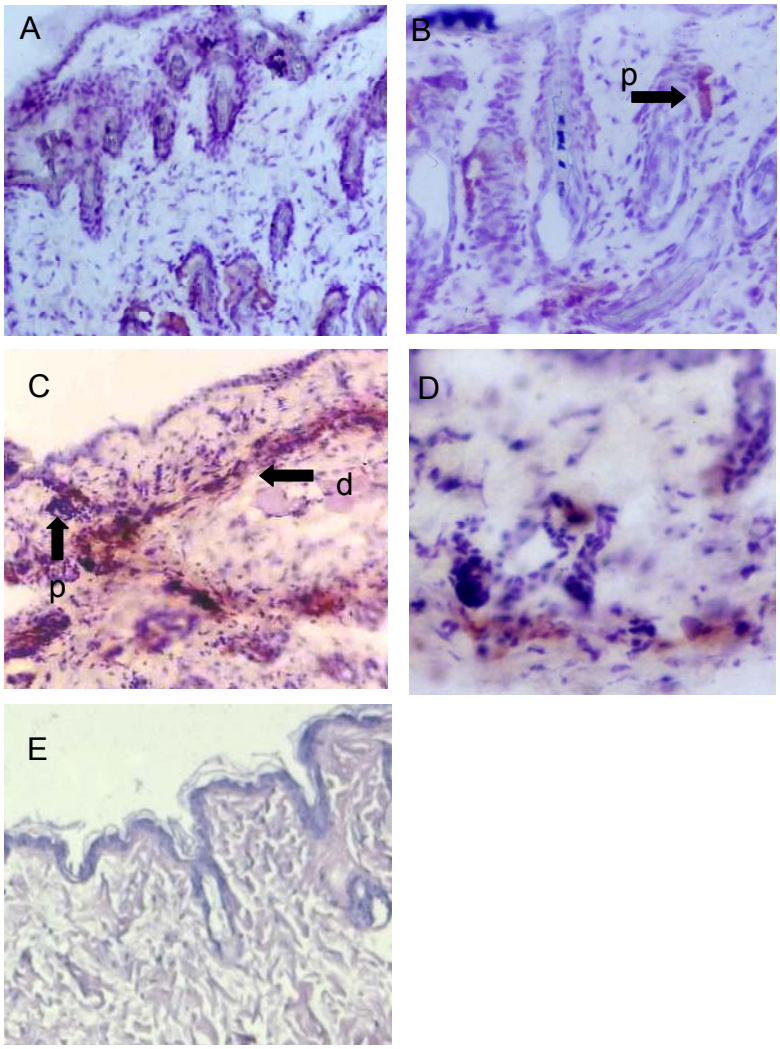


Figure 3.5 Upregulation of monocyte chemoattractant protein 3 expression in type 1 tight-skin (Tsk1) mouse skin. Frozen skin sections from 3-week-old healthy non-tight-skin (pallid) mice **(A** and **B)** and Tsk1 mice at 3 weeks **(C)** and 10 days **(D)** old were immunostained. Healthy adult mice show perifollicular (**p** with **arrow**) staining, whereas there is additional dermal expression (**d** with **arrow**) in Tsk1 samples. Tsk1 skin did not demonstrate any staining with isotype matched control IgG (**E**). (Original magnification × 240 in **A** and **C**; × 480 in **B** and **D**.)

3.4.4 MCP-3 is upregulated in transgenic model with activated TGFβ phenotype

Having identified MCP-3 as the most overexpressed transcript in the neonatal Tsk1 fibroblasts, the gene expression profile of transgenic fibroblasts was compared with wildtype fibroblasts with cDNA microarrays. Using the similar criteria as stipulated in Section **3.4.1**, 35 of the 1,176 genes represented on the array were differentially expressed by the neonatal transgenic fibroblasts. Representative portions of the cDNA expression arrays for neonatal fibroblasts are shown in **Figure 3.6 and Figure 3.7**.

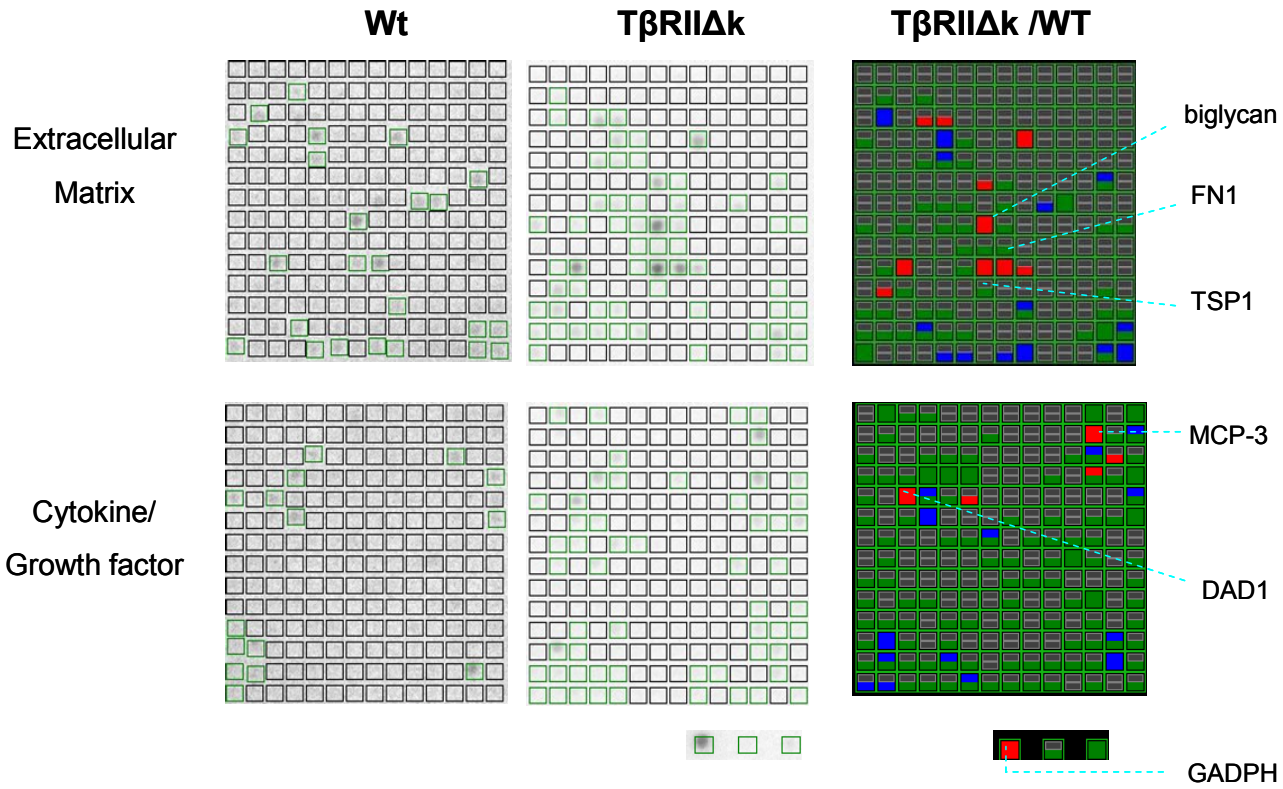


Figure 3.6 cDNA microarray hybridisation analysis of TβRIIΔk and wildtype neonatal fibroblast RNA. Representative neonatal fibroblast gene-expression profiles are shown using portions of 2 mouse Atlas 1.2 cDNA arrays, corresponding to cytokine and growth factor or extracellular matrix transcripts.
Fibronectin 1 (FN1), thrombospondin 1 (TSP1), MCP-3, Defender against Apoptotic Death-1 (DAD1), Glyceraldehyde-3-phosphate dehydrogenase (GADPH).
Genes that are similarly expressed in both samples are indicated in green. Half green/half red and half green/half blue indicate that although a difference (lower portion) was observed between the defined samples, the ratio (upper portion) is nonsignificant. The data are representative of triplicate experiments.

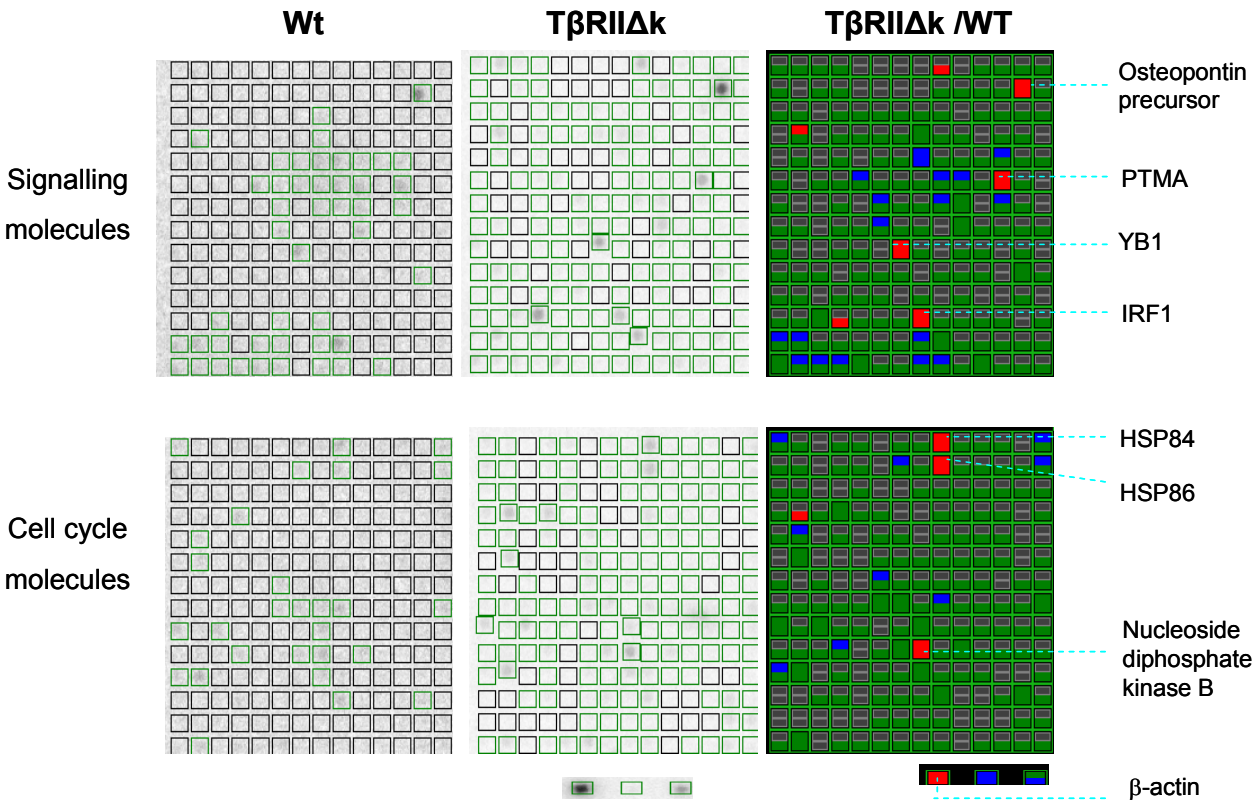


Figure 3.7 Representative neonatal fibroblast gene-expression profiles are shown using portions of 2 mouse Atlas 1.2 cDNA arrays, corresponding to signalling molecules-transcription factors and cell cycle molecules.
Prothymosin-alpha (PTMA), Y-Box DNA Binding protein (YB-1), Interferon regulatory factor-1 (IRF1), Heat shock protein84 (HSP84) and Heat shock protein86 (HSP86).
Genes that are similarly expressed in both samples are indicated in green. Half green/half red and half green/half blue indicate that although a difference (lower portion) was observed between the defined samples, the ratio (upper portion) is nonsignificant. The data are representative of triplicate experiments.

Some of the genes including those related to a range of matrix components, cytokines, growth factors and signalling intermediates in neonatal TβRIIΔk transgenic fibroblasts were significantly modulated are listed in **Table 3.2**. These are consistent with the complex profibrotic phenotype previously reported for these transgenic fibroblasts. There wee many similarities between the basal gene expression of transgenic fibroblasts with previously reported TGFβ1-treated wild type fibroblast gene expression. These include thrombospondin 1 precursor, osteopontin precursor, fibronectin, 84-kDa heat shock protein, prothymosin α, DAD1 and YB1 DNA-binding protein. Transcript levels of a number of growth factors were increased including several that promote fibrosis through activation of fibroblasts (fibronectin, osteopontin precursor and biglycan). Taken together, these results suggest that these transgenic fibroblasts demonstrate basal activation of TGFβ signalling pathways. MCP-3 was upregulated 7.3-fold compared with a 6-fold and 3.5-fold increase in fibronectin and thrombospondin 1 respectively. Western blot of transgenic fibroblast supernatants confirms the overexpression of MCP-3 protein (**Figure 3.8**).

Gene Name	Gene Code	Adjusted Expression		Ratio	Accession Number
		Controls	Transgenic		
MCP-3	D12b	3	22	7.3	Q03366
Fibronectin	F06j	14	93	6.6	X82402
Osteopontin precursor	B13b	23	115	5	P08721
Interferon regulatory factor 1	B08l	3	14	4.7	M21065
HSP86	C09b	3	13	4.3	M57673
GADPH	G27	14	58	4.1	M32599
Vimentin	F09d	10	37	3.7	X51438
DAD1	D03e	3	11	3.7	U83628
STAT-induced STAT inhibitor 1	E06k	3	11	3.7	AF180302
GPI	E01c	15	53	3.5	P06745
TSP1	F07j	6	21	3.5	M87276
ACTB	G43	64	211	3.3	M12481
Biglycan	F07h	23	67	2.9	L20276
NDK B	C08j	7	20	2.9	X68193
YB1 DNA binding protein	B07j	10	24	2.4	M60419
PTMA	B12f	11	23	2.1	X56135
Cathepsin D	F03j	13	27	2.1	X52886
HSP84	C09a	7	14	2	M18186
Guanine nucleotide binding protein	E13h	10	20	2	AF124384

Table 3.2 Selected upregulated genes in TβRIIΔk transgenic fibroblasts compared with wild type littermates (Clontech Atlas cDNA microarray).
MCP-3: Monocyte chemoattractant protein-3, HSP86: heat shock 86-kDa protein, GADPH: glyceraldehyde-3-phosphate dehydrogenase, DAD1: defender against cell death 1, GPI: glucose-6-phosphate isomerase, TSP1: thrombospondin 1 precursor, ACTB: cytoplasmic beta-actin, NDK B: nucleoside diphosphate kinase B, PTMA: prothymosin alpha, HSP84: 84-kDa heat shock protein

.

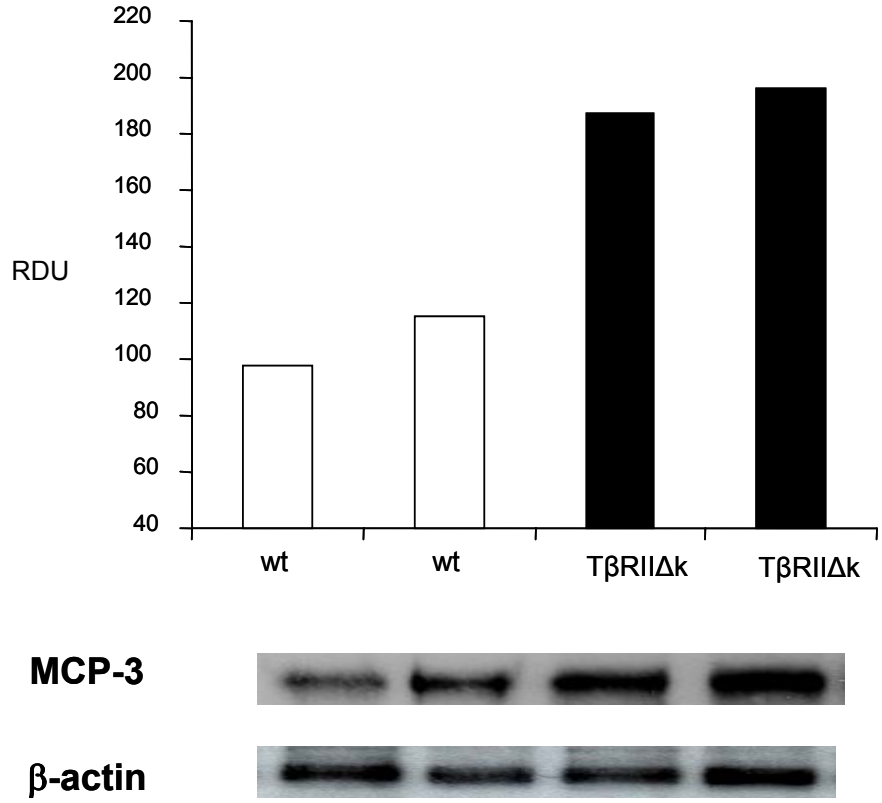


Figure 3.8 Upregulation of MCP-3 transcript was confirmed with Western blot with increased protein secretion by neonatal transgenic fibroblasts compared with wildtype fibroblasts. β-actin was used as protein-loading control. Protein levels were quantified in a representative experiment as shown with the corresponding histogram in relative density units (RDU).

3.4.5 Profibrotic phenotype of TβRIIΔk transgenic fibroblasts

All of the transgenic mice manifest increased thickness of the dermis by 12 weeks of age. This was especially apparent over the lower back with adherence of skin to underlying fascial layers. Histologically the dermis in a series of age 14 week old male mice was thickened with loss of the subcutaneous adipose layer (**Figure 3.9**).

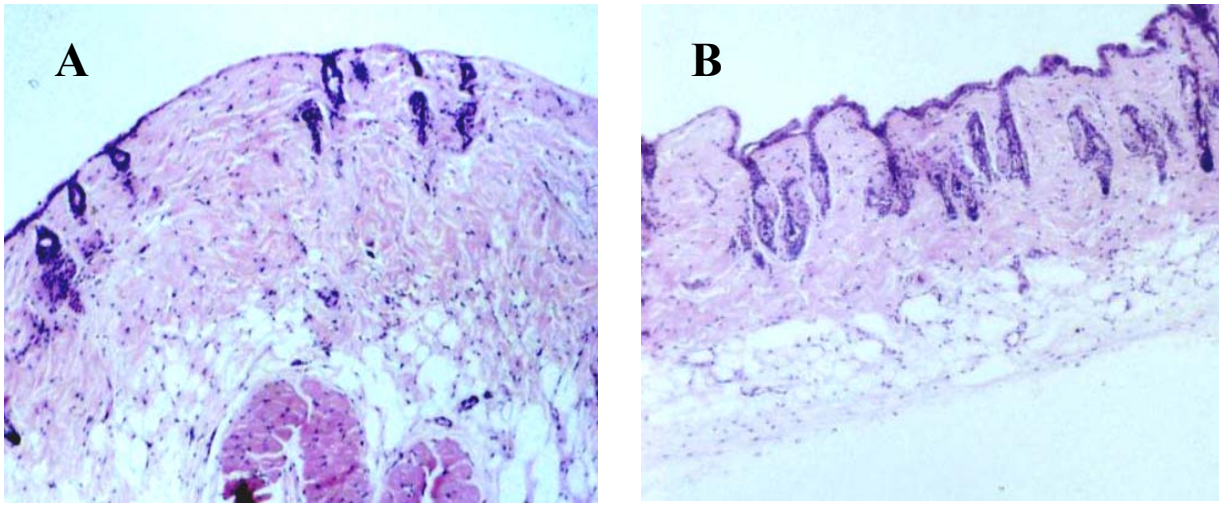


Figure 3.9 Dermal fibrosis in adult transgenic mice. Compared to wildtype mice(B), the skin of adult mice (A) was thickened and representative sections (haematoxylin and eosin) for littermate male mice aged 14 weeks show increased dermal thickness and loss of subcutaneous fat.

Western blotting confirms the histological changes observed for the transgenic mice with high levels of type I collagen expression in fibroblast supernatants and the levels were comparable to those seen in wildtype cells after treatment with TGFβ1. However, this was not further influenced by exogenous TGFβ1 (**Figure 3.10**). Western blot analysis was undertaken to extend the changes observed from the expression profiling for the neonatal transgenic fibroblasts in **Table 3.2**. Fibronectin expression by wildtype cells was strongly induced by TGFβ1 whilst transgenic cells had elevated basal levels. Induction was seen in transgenic cells treated with TGFβ1, but the relative response was less than for wildtype fibroblasts. For keratinocyte growth factor (FGF7) there was substantial down-regulation of the protein level in culture media from wildtype cells after treatment with TGFβ1, and transgenic fibroblasts had a significantly lower level of FGF7 than control cells, with a proportionately smaller suppressive effect of TGFβ1 (**Figure 3.11**). Overall, western blot data confirm that expression of a truncated kinase-deficient type II TGFβ receptor in explanted dermal fibroblasts is associated with a profibrotic phenotype and this finding is consistent with the increased dermal thickness as shown in **Figure 3.9**. Secondly, the transgenic fibroblasts demonstrate a diminished albeit varied responses to exogenous TGFβ1, with fibronectin retaining significant responsiveness.

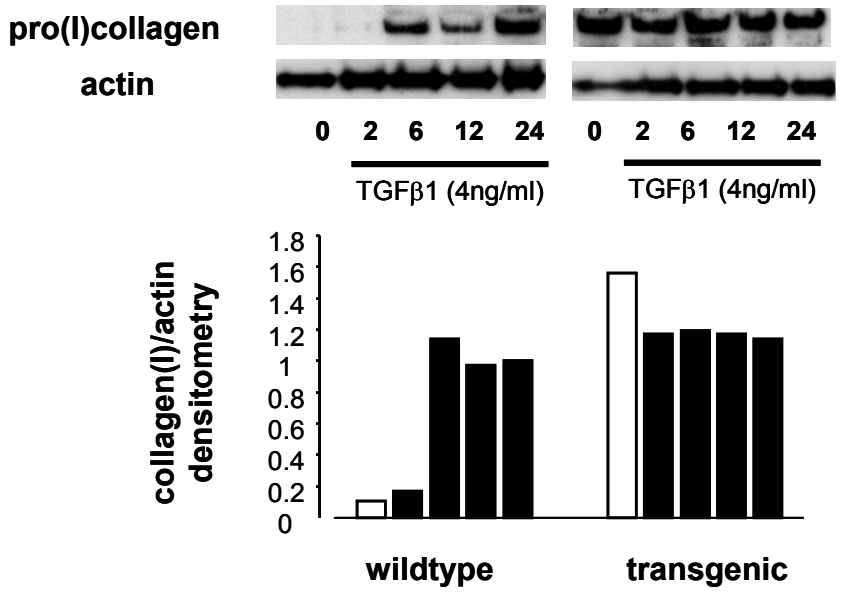


Figure 3.10 Biochemical phenotype of TβRIIΔk dermal fibroblasts. Pro(I) collagen overexpression and refractoriness to TGFβ1 demonstrated by Western blot analysis of dermal fibroblast supernatants after stimulation by recombinant murine TGFβ1 (4ng/ml).

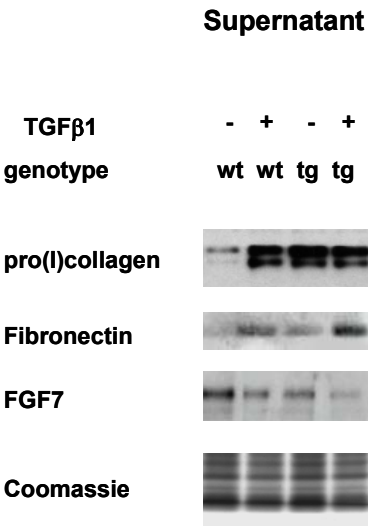


Figure 3.11 Transgenic fibroblasts demonstrate altered protein expression and blunted response to recombinant TGFβ1. Transgenic (tg) or wildtype (wt) neonatal fibroblasts were derived from littermates treated overnight with TGFβ1. Cultured fibroblasts supernatants demonstrated induction of Type I collagen and fibronectin by TGFβ1 in wildtype cells and increased expression at basal level by transgenic fibroblasts. FGF7 is suppressed by TGFβ1 and transgenic cells produce lower levels constitutively. FGF7 and fibronectin secretion by transgenic fibroblasts is modulated by TGFβ1 but the proportionate change is less than in wild type cells. Coomassie staining of a representative gel shows total protein loading. Data are representative of three independent experiments.

3.4.6 Transgenic fibroblasts have a TGFβ1 activated phenotype

Plasminogen activator inhibitor-1 (PAI-1) and CCN2/CTGF represents prototypic markers of TGFβ activation and to explore the expression of these TGFβ regulated proteins in transgenic fibroblasts, a series of independent experiments confirmed that compared to wildtype cells, there was upregulation of PAI-1 protein by transgenic fibroblasts, and this expression was relatively refractory to recombinant TGFβ1 stimulation. This refractoriness was sustained up to examination at later time points up to 72 hours after TGFβ1 stimulation. The TGFβ regulated profibrotic cytokine connective tissue growth factor (CTGF) was examined in parallel experiments. There was extremely low level of CTGF expression by wildtype cells but it was strongly induced by TGFβ1 at 6 hours. Expression then returned to basal over 72 hours. Transgenic fibroblasts demonstrated substantial constitutive CTGF production, consistent with their in vivo fibrotic phenotype, and expression was further induced to a peak levels similar to TGFβ1 treated wildtype cells, representing an increase of around 50% from basal transgenic level, although maximum induction occurred at 24 rather than 6 hours (**Figure 3.12**). This result suggests that these transgenic fibroblasts have sustained refractoriness to recombinant TGFβ1.

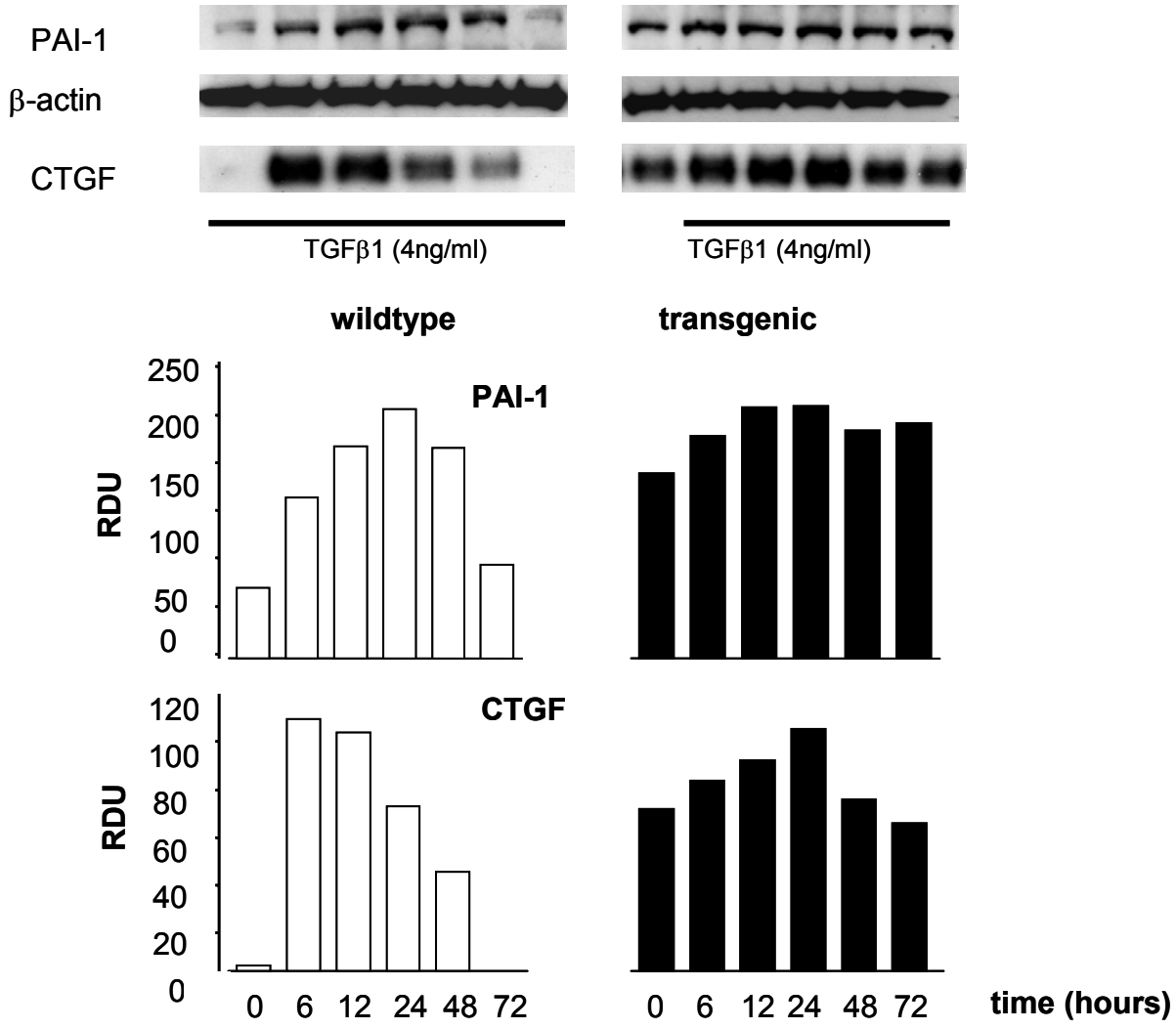


Figure 3.12 Altered basal and TGFβ-induced PAI-1 and CTGF expression by transgenic fibroblasts. Increased expression of PAI-1 protein in fibroblast lysates on Western blot returned to basal level at 72 hours for wild type cells but sustained overexpression was demonstrated in transgenic fibroblasts. Similar pattern of overexpression was observed for CTGF protein in transgenic fibroblasts but maximum expression occurred at 24 hours compared to 6 hours for wild type cells. These data are representative of duplicate samples from two independent experiments.

To confirm that activation of TGFβ signalling pathway is responsible for the constitutive overexpression of pro(I)collagen or CTGF, SD-208, a novel TβRI-kinase (ALK5) inhibitor was used. Inhibition of ALK5 reduced pro(I)collagen and CTGF expression in transgenic fibroblasts to levels seen in littermate wildtype cells (**Figure 3.13**).

We also examined expression of the major type I collagen degrading enzyme MMP13 in mice. Levels of both precursor and active enzyme were substantially increased in transgenic fibroblast compared with littermate wildtype cells, suggesting that protein overproduction rather than reduced degradation is more likely the cause of increased matrix collagen in the TβRIIΔk transgenic mice (**Figure 3.14**).

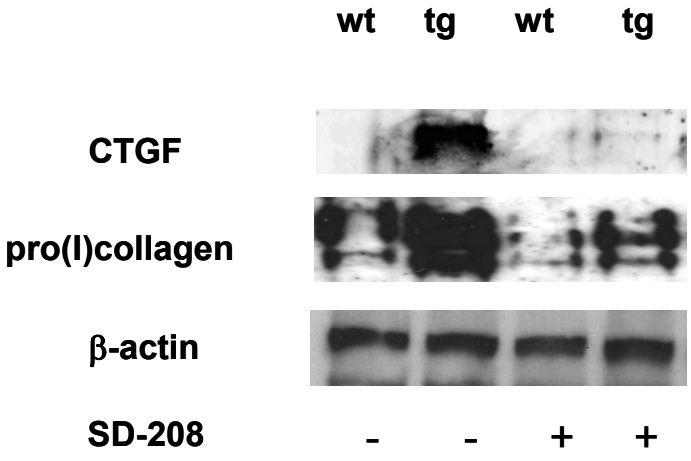


Figure 3.13 Transgenic fibroblasts demonstrate TGFβ-dependent phenotype. Collagen and CTGF protein overexpression is abrogated by TβRI kinase (ALK5) inhibition (SD-508). These data are representative of four independent experiments.

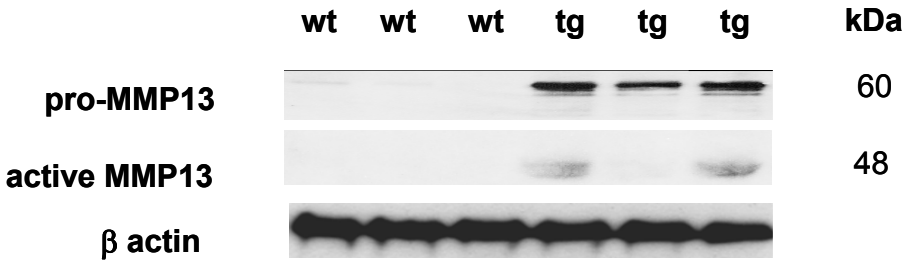


Figure 3.14 Transgenic fibroblasts overexpress the major fibrillar collagen-degrading protease MMP-13. These data are representative of three independent experiments.

3.5 Discussion

There are a number of approaches for determination broad differences in gene expression in different tissue or cell samples. Other studies have used a variety of technologies including differential-display reverse transcription-polymerase chain reaction, ribonuclease protection assay and Northern blotting to examine gene-expression profiles in connective tissue diseases, including SSc. Candidate genes suggested by these approaches have included fibronectin, protease nexin 1, interleukin-1α and CTGF. However, MCP-3 was not identified in these earlier studies, this may reflect intrinsic differences in the SSc skin biopsy samples examined or in the fundamental properties of each of the methods used for examining the differential gene expression, and emphasises the value of using multiple approaches to address the same question.

Overexpression of chemokines and related receptor genes including MCP-3 has been described in gene-cluster analysis of data from high-density gene-chip experiments studying whole-lung samples in the bleomycin-induced model of murine pulmonary fibrosis. Also, in a recent analysis of Luzina et al of gene-expression profiles with lung inflammation, there was increased expression of chemokines and chemokine receptor genes associated with a greater risk for lung fibrosis(Luzina *et al.*, 2002). These studies suggest that cDNA microarray approaches are valuable as a screening approach to identify potential targets for further study. However, these results must be interpreted with caution, and repetition of studies is important, including validation with independently prepared samples.

Homogeneous populations of dermal fibroblasts from Tsk1, transgenic and wild-type mice were used to reduce the biological variability, which may otherwise limit data analysis. However, even with this starting material, there was substantial variation when independently prepared samples were compared, presumably due to differences in cell cycle and other biological parameters, thus, many of the differentially expressed genes initially seen in neonatal fibroblasts were observed in only some littermate pairs. Replication of data is essential to reduce background differences in gene expression that are

independent of fundamental differences in the cell lines, even in genetically homogeneous samples such as sex-matched littermates of inbred mouse lines. Only a few genes were consistently differentially expressed, and low-density microarrays such as those used for screening in this study have relatively few transcripts. More complete assessment using high-density gene chips may be more amenable to formal statistical and bioinformatic analyses.

It has been proposed that the myriad outcomes in SSc may represent different diseases that might ultimately be influenced by expression profiling. Many studies have reported characteristic phenotypic features of explanted dermal SSc fibroblasts with upregulation of TGFβ pathway including members of CCN family, thrombospondin 1 and PAI-1(Gardner *et al.*, 2004). Several authors have also demonstrated extensive evidence of the TGFβ and Wnt pathways in vivo(Gardner *et al.*, 2006;Whitfield *et al.*, 2003) with upregulation of many TGFβ targets including extracellular matrix proteins with fibrillar collagens, small leucine-rich family of proteoglycans (SLRPs) and members of the CCN family. However, explanted fibroblasts did not display the same consistent gene expression profile in particular the collagen transcripts suggesting that cultured fibroblasts appear not to completely mirror the in vivo SSc fibrotic phenotype. The reasons for this are unclear but possible explanations include presence of nonfibroblast cell types that drive the disease process and the loss of the cytokine milieu and matrix with the process of explantation and growth in monolayer. On the other hand, explanted dermal fibroblasts remain an attractive model system to study in SSc largely due to its amenability to ex vivo experimentation and accessibility. In addition, some of its key features including positional memory and myofibroblast phenotype remain intact(Shi-Wen *et al.*, 2004;Rinn *et al.*, 2006).

Despite recent controlled clinical trials(Hoyles *et al.*, 2006;Nihtyanova & Denton, 2008), the assessment and treatment of SSc remains challenging, in part due to an incomplete understanding of pathogenesis. Using murine models that recapitulate SSc phenotypes, modern methods of parallel examination of gene and protein expression have contributed significantly in the understanding of SSc pathogenesis. Gene expression profiling of these

animal models of SSc will permit testing of hypotheses for pathogenesis in vivo and to investigate the biochemical properties of mutant mice.

The identification of MCP-3 as a key soluble factor, primarily known for its key role in leucocyte migration in two major fibrotic murine models is intriguing. This provides the first evidence for upregulation of MCP-3 in a murine model with TGFβ-activated phenotype, suggesting that MCP-3 may be dependent on TGFβ in fibrosis. In addition, there is now increasing evidence that the related chemokine, MCP-1 acts in concert with TGFβ in driving fibrosis. There is however, few studies that has directly implicate involvement of MCP-3 in fibrosis. This will be explored further in this thesis. It is also plausible that early mediators such as MCP-3 might induce other factors such as TGFβ1 and perhaps other downstream candidates such as CTGF or platelet-derived growth factor are induced later. The ability of fibroblasts to secrete these factors supports a model of autocrine or paracrine local regulatory pathways in pathogenesis. Potential consecutive induction of fibroblast-derived mediators also emphasises the importance of disease stage-specific approaches to targeted molecular therapies in SSc.

Several authors have reported upregulation of MCP-3 in autoimmune diseases including primary biliary cirrhosis and inflammatory bowel disease. Whilst autoimmunity has been described in Tsk1 mice, this aspect has not been well studied in the transgenic fibroblasts. However, autoimmunity has been demonstrated in pancreatic acinar cells harbouring kinase-deficient type II TGFβ receptor construct(Hahm *et al.*, 2000). It is therefore possible that whilst this effect may be tissue-specific, autoimmunity may be highly relevant in this fibroblast-directed transgenic murine model. Apart from these studies, other evidence that may suggest possible involvement of MCP-3 in fibrotic process includes, its upregulation in usual interstitial pneumonia and SSc-related lung fibrosis, and also, in an experimental model of radiation-induced lung fibrosis (Choi *et. al*., 2004; Yanaba *et. al*., 2006; Johnston *et. al*., 2002).

In addition to prompting the present study of MCP-3, the gene expression analysis of Tsk1 skin provides some additional information regarding the pathogenesis of the tight-skin

mouse phenotype. The lack of inflammatory infiltrate on immunohistochemistry is entirely consistent with the absence of vasculopathy in this murine model.

While recognizing that ~fold-change in mRNA may not necessarily correlate with biological importance, only the most dysregulated genes were considered. The expression profiling of Tsk1 and transgenic fibroblasts replicates the findings from previous studies that have reported characteristics phenotypic features of explanted dermal SSc fibroblasts with upregulation of TGFβ pathway with approximately half of the constitutively differentially expressed genes in transgenic fibroblasts showing a similar pattern in wild type cells after TGFβ1 stimulation(Denton *et al.*, 2003). Moreover, the gene expression analysis for neonatal Tsk1 fibroblasts revealed striking differences neonatally with upregulation of MCP-3 and monocyte colony-stimulating factor 1, although some of these differences were not sustained at later time points. A smaller number of genes, mostly related to ECM turnover, were consistently upregulated including TIMP3 and TGFβ at all time points. There is however, some disparity between protein and mRNA expression for MCP-3. The mRNA expression level was about ~15-fold greater in the Tsk1 than wild type fibroblasts; whereas the protein level was 2.4-fold greater by Western blot analysis. Similar discrepancy was observed for transgenic fibroblasts, albeit at lower fold difference compared to wild type fibroblasts. Whilst these results support overexpression of MCP-3 secretion by Tsk1 and transgenic fibroblasts, it suggests that these may not solely dependent upon increased mRNA, and that posttranslational modification and even mRNA stability may be relevant in determining protein expression.

Recent compelling evidence has demonstrated that in SSc and other fibrotic diseases, reduced ECM turnover is a contributory factor for collagen accumulation and the development of dermal fibrosis. The turnover of the ECM depends upon the balance between MMPs and TIMPs. Gene expression profilings for both models suggest altered expression of a subset of MMPs and TIMPs, and this mirrors the cohort of MMP genes upregulated by TGFβ in other studies. In addition to upregulation of MMP-13 in chronic dermal wound, several authors have documented the critical role of MMP-13 in model of liver fibrosis in MMP-13-/-mice, and this was associated with downregulation of

TGFβ(Uchinami *et al.*, 2006). Moreover, MMPs including MMP-13 may modulate chemokine activity by cleaving chemokines including MCP-3 at their NH2-terminals, and therefore converting them into antagonistic derivatives. In addition, MMPs may also establish chemokine gradient in vivo and therefore regulate the migration of inflammatory infiltrate. For example, MMP9 was recently shown to be required for formation of transepithelial gradients of MCP-3 in a murine model for asthma(Corry *et al.*, 2004). While inflammation is not a feature in Tsk1, it remains unclear if chemokine-regulated inflammation is important in TβRIIΔk model.

Apart from these two models that demonstrate overexpression of MCP-3, other murine models of pulmonary fibrosis induced by irradiation has also reveal elevation of several key chemokines including MCP-3 and its receptors, CCR1 and CCR2. It is likely that these chemokines are responsible for the chronic inflammatory recruitment and activation observed in this model of irradiation-induced fibrosis(Johnston *et al.*, 2002). However, unlike Tsk1 and TβRIIΔk mice, the fibrotic response was not observed in all murine strains. Myofibroblastic expression of MCP-3 was also recently described in an experimental murine model of bleomycin-induced lung fibrosis with resistance to Fas-induced apoptosis as a way of evading immune surveillance and thus allowing for their uninterrupted accumulation with resulting collagen deposition in the ECM(Wallach-Dayan *et al.*, 2007).

The pathogenic mechanisms for Tsk1/+ model however is not well-defined and it has been argued that it does not completely reproduce the SSc phenotype(Baxter *et al.*, 2005). In contrast to human SSc, these mice have subcutaneous hyperplasia with relative sparing of the dermis. In addition, these mice demonstrate tethering and thickening of the hypodermal tissue in contrast to the dermal fibrosis seen in SSc. Other major phenotypical differences between the Tsk model and human SSc included the emphysematous lung changes in the murine model rather than fibrosis and the absence of vasculopathy.

Transgenic models on the other hand provide an in vivo system to explore molecular mechanisms that account for the temporal, spatial and stimulus-responsive regulation of an individual transgene. Accordingly, transgenic models such as TβRIIΔk have demonstrated

the pathophysiological fibrotic consequences of increased mouse transgene with thickened dermis with increased collagen deposition. The expression of a non-signalling type II TGFβ receptor in fibroblasts results in paradoxical activation of downstream signalling pathways with basally increased expression of PAI-1 and CTGF. Moreover, experiments using SD-208 strongly suggest that activation of the type I TGFβ receptor, ALK5 is a key determinant of the profibrotic phenotype in this transgenic murine strain.

To explain the sustained refractoriness of the transgenic fibroblasts to exogenous TGFβ1, it has now been demonstrated that the level of transgenic receptor expression and that of endogenous wildtype receptor appears to be critical; low level expression of TβRIIΔk appears to lead to increased wildtype receptor levels. This may be due to heterodimerisation of wildtype and mutant receptors that results in a signalling receptor complex that is less susceptible to degradation(Denton *et al.*, 2005). Higher levels of mutant receptor act as a dominant negative inhibitor of signalling. At higher concentrations, homodimers of mutant receptor are likely to form and these may inhibit responsiveness by direct competition for ligand with wild type receptors (**Figure 3.15**). Therefore, for some proteins such as fibronectin, there is regulation and for these, inhibitory pathways may be preferentially activated in transgenic fibroblasts in response to exogenous TGFβ1.

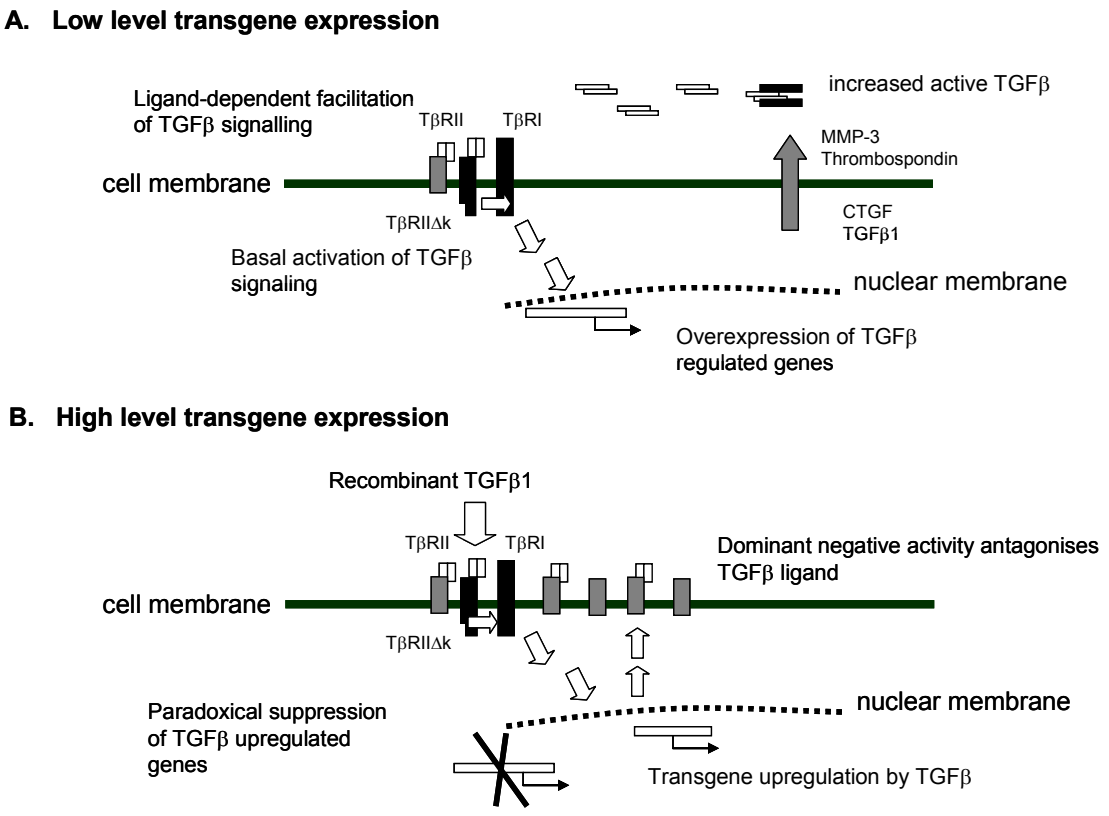


Figure 3.15 Diagram summarizing activation of the TGFβ ligand-receptor axis in TβRIIΔk transgenic mice. **A**. Expression of the mutant receptor at low levels enhances activation of the endogenous type I TGFβ receptor. Downstream consequences include upregulation of TGFβ1 and other gene products that promote TGFβ activity or activate the latent TGFβ complex. This leads to net activation of TGFβ signalling. **B**. In contrast, in response to recombinant TGFβ1, there is significant elevation of transgene expression. Higher level of transgene expression is inhibitory and abrogates signalling (Denton *et al.*, 2005).

These results should be considered in the context of previous studies of other transgenic mice harbouring kinase-deficient type II TGFβ receptor constructs. Although this transgene operates as a dominant negative inhibitor of TGFβ activity in vitro, it appears to induce much more complex effects in vivo in transgenic mice. Most reported phenotypes are consistent with perturbed TGFβ signalling, seen most clearly when cell type-specific or tissue-specific promoters have been used. TβRIIΔk expression in mouse epidermal cells resulted in a hypertrophic epidermal phenotype with refractoriness to TGFβ1-induced growth inhibition associated with enhanced sensitivity to chemical carcinogenesis(Wang *et al.*, 1997). When expressed in chondrocytes, TβRIIΔk transgene regulated by a

metallothionein promoter was associated with degenerative skeletal changes suggestive of defective TGFβ response(Serra *et al.*, 1997). Increased trabecular bone was demonstrated with TβRIIΔk expression in osteoblasts(Filvaroff *et al.*, 1999), whereas in mammary stromal cells, impaired mammary differentiation and development was demonstrated (Joseph *et al.*, 1999). By contrast, expression in epithelial cells in the mammary gland (Gorska *et al.*, 1998), prostate (Kundu *et al.*, 2000), and intestine (Hahm *et al.*, 2002) has been associated with epithelial hyperplasia, consistent with the role of TGFβ in negatively regulating epithelial cell proliferation. In addition, recent publications have used TβRIIΔk to unravel the complex role of TGFβ in autoimmunity. Expression in T lymphocytes resulted in a lymphoproliferative disease, similar to the TGFβ1 null mouse phenotype, and also increased susceptibility to autoimmune hepatitis. Under the influence of a *pS2* mouse trefoil peptide promoter in pancreatic acinar cells, TβRIIΔk is associated with increased susceptibility to cerulein-induced pancreatitis characterised by severe inflammatory cell infiltration, increased cytokines IL-4 and IL-10, T- and B-cell hyperactivation, IgG-type autoantibodies against pancreatic acinar cells, and IgM-type autoantibodies against pancreatic ductal epithelial cells(Hahm *et al.*, 2000). In addition, there was marked increase in MMP-3 and MMP-9 expression in pancreatic acinar cells. Interestingly, when the transgene is regulated by a mouse metallothionein promoter, there was increased cell proliferation in pancreatic acinar cells with perturbed acinar differentiation resulting in fibrosis, neoangiogenesis and mononuclear infiltration, associated with a paradoxical up-regulation of TGFβ expression in transgenic acinar cells(Bottinger *et al.*, 1997). Overall, previous transgenic models with tissue-directed expression of TβRIIΔk all show blunted ligand responsiveness. Disparate results using the same TβRIIΔk construct are likely to reflect tissue-specific TGFβ responses.

Independent confirmation of the paradoxical activation of downstream signalling and increased extracellular TGFβ bioactivity through mechanisms comes from the recently described human Loeys-Dietz syndrome (Loeys *et al.*, 2005). This is an autosomal dominant aortic aneurysm syndrome with affected patients having widely spaced eyes, cleft palate and widespread vascular dilatation with increased risk of aortic dissection or rupture.

Based on current knowledge of altered TGFβ signalling pathway in aortic dissections associated with Marfan syndrome, sequencing of TGFβR1 and TGFβR2 in 52 families with a history of Loeys-Dietz syndrome identified disease-causing somatic mutations in these two receptors: a majority of which occur either adjacent to or within the serine/threonine kinase domains of TβRII and TβRI. There were however no phenotypic differences between those who harbour TGFβR1 versus TGFβR2 mutations. These receptor mutations identified were specific to this syndrome. The paradoxical increased TGFβ signalling observed experimentally in the aortic wall would suggest that these mutations are either gain of function or compensatory mechanism to overcome these mutations. Recently, these receptor mutations have been described in Marfan Syndrome but these are predicted to result in loss of function. However, it remains unclear whether the abnormal phenotype in Marfan syndrome is a consequence of increased TGFβ signalling. Further support for potential alterations in TGFβ signalling arising in the context of this type of kinase domain mutation is provided by the identification of kinase domain mutations in another member of the TGFβ superfamily of receptors, the bone morphogenetic protein receptor type II (BMPRII) in familial pulmonary arterial hypertension (PAH) (Morrell *et al.*, 2001;Lane *et al.*, 2000;Thomson *et al.*, 2000). The BMPRII receptor has an analogous role in BMP signalling to TβRII for TGF-β ligand; absent kinase activity leads to activation of downstream pathways, and smooth muscle cells from pulmonary arteries of patients heterozygous for mutations in the signalling domain of BMPRII show altered responses to TGFβ1 as well as BMPs (Morrell *et al.*, 2001). However, this mutation has not been found in SSc (Tew *et al.*, 2002).

3.6 Summary

In conclusion, the studies presented in this chapter established the overexpression of MCP-3 in two major murine models for fibrosis: Tsk1/+ and the novel transgenic TβRIIΔk strains. These mice provide a valuable model for studies in human fibrosis, including SSc, probably best reflecting the established fibrotic phase of the disease. Taken together these results suggest that in addition to its major role as a chemoattractant for mononuclear cells, MCP-3 may be potentially profibrotic. In addition, its overexpression in the transgenic model with genetically determined perturbation of TGFβ signalling in fibroblasts suggests that both these factors may share a biological and functional significance in driving fibrosis.

.

CHAPTER 4: MCP-3 AS A PROFIBROTIC MEDIATOR IN MOUSE MODELS OF SCLERODERMA

4.1 Introduction

Since the first description in 1977, chemokines are primarily known for their major and eponymous function in induction of directional cell migration, thus coordinating leucocyte recruitment both in physiological and pathological conditions. There has been an increasing amount of information correlating the chemokine system with the pathogenesis of different human diseases. Most of these are thought to be attributable to its major role in chemotaxis.

MCP-3, as a key member of CC chemokine family, binds cell-surface glycosaminoglycans and forms stable gradients in order to institute an inflammatory focus. Like MCP-1, it has largely been studied for its main role in recruitment of a variety of mononuclear inflammatory cells. Increased fibroblastic expression of MCP-3 has been demonstrated following stimulation by cytokines including IL-13 and IFNγ(Menten *et al.*, 1999;Wills-Karp, 2004;Wynn, 2004). Overexpression of MCP-3 has been associated with several major autoimmune and inflammatory diseases including asthma, atherosclerosis and multiple sclerosis. Interestingly, more recently, increased expression of MCP-3 has been demonstrated in sera from cohort of patients with SSc, and lung fibrosis(Yanaba *et al.*, 2006). The mechanisms underlying these observations are unclear but it is postulated that MCP-3 mediates the leucocyte chemotaxis and migration through endothelial into the affected organ tissues leading to the interaction between leucocytes and fibroblasts. The related chemokine, MCP-1 has been studied extensively in SSc and although it has been demonstrated that targeting MCP-1 may have a therapeutic effect in mouse models of SSc or fibrotic disorders, it is unknown whether this strategy is effective in human diseases.

In these fibrotic diseases of which SSc is the prototypic fibrotic disorder, excessive ECM production notably type I collagen is a major feature. The regulation of ECM accumulation and type I collagen gene expression in SSc is tightly controlled at multiple coordinated steps, among them transcriptional regulation is a critical step. The cis acting elements that

control the activity of the proximal promoter are also involved in mediating the transcriptional response to cytokines which are central in tissue remodelling and fibrotic diseases. In SSc increased transcriptional activation of type I collagen has been shown in lesional tissues(Herrmann *et al.*, 1991). Explanted cells from affected areas maintain this phenotype for several passages in vitro. The regulation of the expression of the two collagen I components (the α1 and α2 chains) genes has been subject of intense research over the last decade and several control elements have been described for both collagen I α chain genes. The understanding of collagen gene regulation has been greatly advanced with the increasing use of transgenic mice. Expression patterns of different proximal promoter constructs of the murine collagen gene linked to a reporter transgene revealed a modular arrangement of separate cell-specific cis-acting elements that can activate the mouse pro-α(I) collagen gene in different type I collagen-producing cells, with certain critical sequences directing expression of collagen genes to specific mesenchymal cell types at the appropriate time in embryonic development(Rossert *et al.*, 1995). A potent fibroblast-specific enhancer element has been described in the 6-kb sequence of the murine proα2(I) collagen gene (Col1a2) between -19.5 kb and -13.5 kb upstream of the transcription start site. This region contains some DNase-hypersensitivity sites that have been demonstrated only in the collagen type 1 producing cells, and this element has been referred to as the far-upstream proα2(I) enhancer element. This enhancer is capable of directing high-level fibroblast-specific expression to reporter genes linked to endogenous and heterologous minimal promoters in transgenic mouse embryos. Further experiments demonstrated that distinct sequences within the hypersensitive sites are essential for both the functional integrity of the enhancer and tissue specific elements that direct expression in mesenchymal cells of internal tissues(De *et al.*, 2002). Comparison of murine and human sequences has led to the identification of regions of high genomic homology between both species. This homology encompasses the locations of the five DNase I hypersensitive sites as well as the composition of their sequences. These segments termed as MH-I, MH-2 and MH-3 were shown to have 85%, 90% and 88% homology(Antoniv *et al.*, 2001). This positional and compositional conservation of intergenic sequences represent powerful evidence of critical ancestral role in gene regulation. In addition to the far-upstream fibroblast-specific enhancer in the murine Col1a2 gene, it has also been demonstrated that the human far-

upstream region of COL1A2 acts as a strong tissue-specific enhancer in concert with the proximal minimal promoter, and is required for full gene expression(Ramirez *et al.*, 2006). More recently, transgenic mice experiments have revealed that the enhancer sequence is important in determination of the fibrotic phenotype of Tsk/1+ mice(Denton *et al.*, 2001). Using different transgene constructs of the enhancer elements linked to β-galactosidase reporter gene, activation of LacZ in Tsk mice was demonstrated via the 2-kb and 6-kb sequences suggesting that these regions contain sites that respond to activating factors in the Tsk/1+ mice. These transgene constructs for the mouse and human proα2(I) collagen genes are shown in **Figure 2.1**. Taken together, these data demonstrate that the far upstream enhancer element exert significant influence on collagen production via the both Col1a2 and COL1A2 minimal promoters and this thesis will draw upon these elements to study the effect of recombinant MCP-3 on the modulation of collagen gene expression in the fibrotic process.

4.2 Aims

Overexpression of MCP-3 has been established in two major murine models of fibrosis: Tsk/1+ and the transgenic TβRIIΔk murine strains in which there is activated TGFβ activated phenotype with increased dermal collagen expression. Using a novel physiological bioassay with cultured dermal fibroblasts from 2kb-LacZ transgenic mice and also, transient transfection of the proα2(I) collagen gene promoter-constructs, the possible direct or indirect profibrotic effect of MCP-3 on ECM gene expression was examined.

4.3 Methods

4.3.1 Cell culture and transfection of reporter constructs

Fibroblasts from neonatal transgenic 2kb-LacZ line, Tsk1 and non-Tsk1 were used for these experiments. The 2kb-LacZ cell lines provide a physiological assay for transactivation of a genomic β-galactosidase reporter for which endogenous expression in vivo recapitulates that of endogenous type I collagen genes. All cell lines were maintained in DMEM (Gibco) as described in Section **2.2**.

4.3.2 Transfection of reporter constructs and assessment of MCP-3 responsiveness

Transactivation of collagen(I) gene by different concentrations of recombinant MCP-3 was compared with recombinant MCP-1 using transgenic mice which harbours a fibroblast-specific 2kb-LacZ reporter transgene in fibroblasts culture. In addition, transient transfection was used to assess MCP-3 and TGFβ1 responsiveness in control dermal fibroblasts with the 2kb-LacZ collagen promoter construct. Analyses were performed in 24-well plates using Lipofectamine-plus, as described in Section **2.2.3**. For each experiment, transfection was performed when the cell layer was 60-70% confluent in a 24-well tissue-culture plate. Reporter gene expression was determined using the Galactolight assay kit (Tropix, Bedford, MA).

4.3.3 Protein kinase inhibitors and neutralising antibody to TGFβ1.

The inhibitors used in this study included SD-208, an inhibitor of the TβRI kinase (1 μM, Scios Inc., Fremont CA) which is a selective 2,4-disubstituted pteridine-derived TGFβ RI kinase that demonstrates in vitro specificity for TβRI kinase of over 100-fold compared with TGFβRII kinase and at least 20-fold over members of a panel of related protein kinases. SD-208 was kept as 20 mM stock solution at -20^0C until use. Other protein kinase inhibitors include SB203580 (20 μm, Calbiochem, La Jolla, CA), a highly specific and cell-permeable inhibitor of p38 MAP kinase (IC_{50} = 34 nM *in vitro*) with no significant inhibition of JNK and p42 MAP kinase at 100 μM; LY294002 (50 μM, Calbiochem, La Jolla, CA) is a cell-permeable and specific phosphatidylinositol 3-kinase inhibitor (IC_{50} =

1.4 μM) that acts on the ATP-binding site of the enzyme and does not affect the activities of MAP kinase and PKC even at 50 μM and 1D11, a neutralising murine TGFβ monoclonal antibody which recognises both mouse and human TGF-β1 (5 μg/ul, R&D Systems, Oxford UK). All inhibitors were dissolved to a desired stock concentration and further dilutions were prepared in sterile DMEM unless otherwise stated and carrier alone added as appropriate. In the experiments in which the effect of these inhibitors diluted in DMEM was studied, DMSO-diluted treated cells were used as controls.

4.3.4 Protein Expression

Cultured fibroblast supernatants were collected and concentrated by ammonium sulphate precipitation to selectively enrich samples for secreted matrix proteins. Specific antibodies to collagen type I, a rabbit polyIgG (1:2000 dilution, Novotec) was used. All protein extraction and Western blotting methodologies performed as described in Section **2.4.3**, unless stated otherwise. Monoclonal antibody against β-actin (Sigma, St. Louis, MO) was used to control for variation in cell number between cultures.

4.4 Results

4.4.1 Transactivation of proα2(I) collagen gene expression by MCP-3 in 2kb-LacZ transgenic murine fibroblasts

Initially, transgenic fibroblasts from the 2kb-LacZ line were used, since these cells provide a physiological assay for transactivation of a genomic β-galactosidase reporter for which endogenous expression in vivo recapitulates that of endogenous type I collagen genes. Concentrations between 10ng/ml and 250ng/ml of recombinant chemokine were evaluated in early experiments. Preliminary time-course analysis from a series of duplicate experiments revealed that initial induction occurred at 6 hours with up-regulation of 34 ± 15% above baseline, which subsequently rose and then declined to 25 ± 22% above baseline at 48 hours. There was maximal induction of the transgene expression at 24 hours with (Mean±Sem %) 133±28%, p=0.02 at 10 ng/ml, 186±38%, p=0.01 at 100 ng/ml and 230±45%, p=0.05 at 250ng/ml compared to basal (**Figure 4.1**). This showed very similar dose-response characteristics to that previously observed for the related chemokine MCP-1 (Gharaee-Kermani *et al.*, 1996) (**Figure 4.2**) with maximal induction 90.0±20% and 115±16% for MCP-3 and MCP-1 respectively at 24 hours. Concentrations between 100-500 ng/ml gave consistent effect and these were used in the majority of later studies. A series of independent experiments (n = 5) showed that MCP-3 200ng/ml and 400 ng/ml increased the transactivation of the reporter transgene, with a maximal mean (±SEM) change above baseline levels of 80 ± 31% (p = 0.01) at 24 hours (**Figure 4.3**).

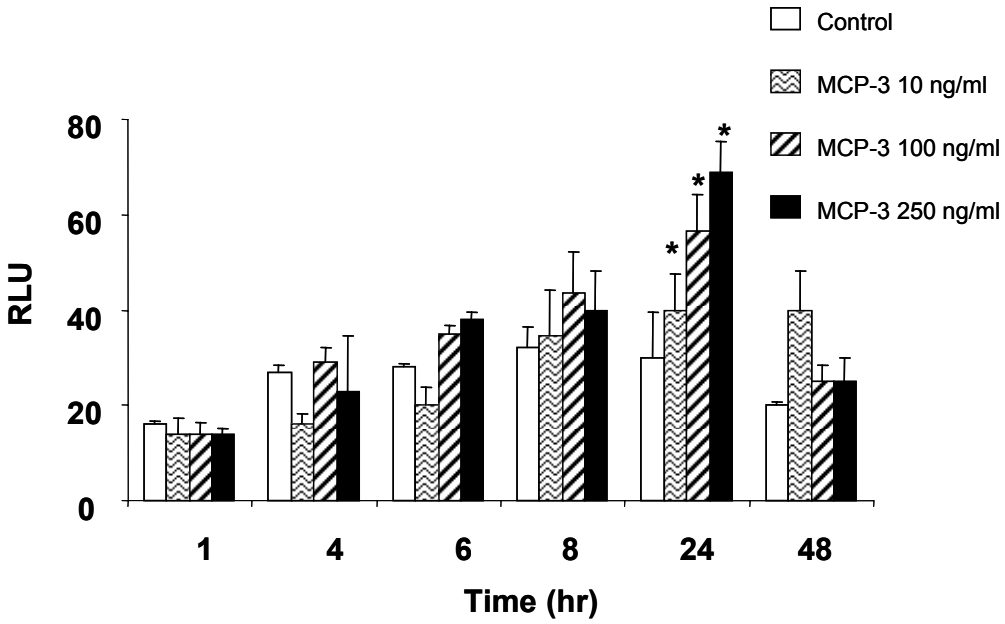


Figure 4.1 Time and dose-dependent transactivation of the 2kb-LacZ transgene expression by variable dose of MCP-3 (10-250ng/ml). Fibroblasts were grown to 80% confluence and serum starved in DMEM with 0.2%FCS for 24 h. Cells were then incubated for 1, 4, 6, 8, 24 and 48 hours in the presence of recombinant MCP-3. Values are the mean and SEM of triplicate samples and are representative of 3 independent experiments. * = $p < 0.05$ by Student's paired t-test.

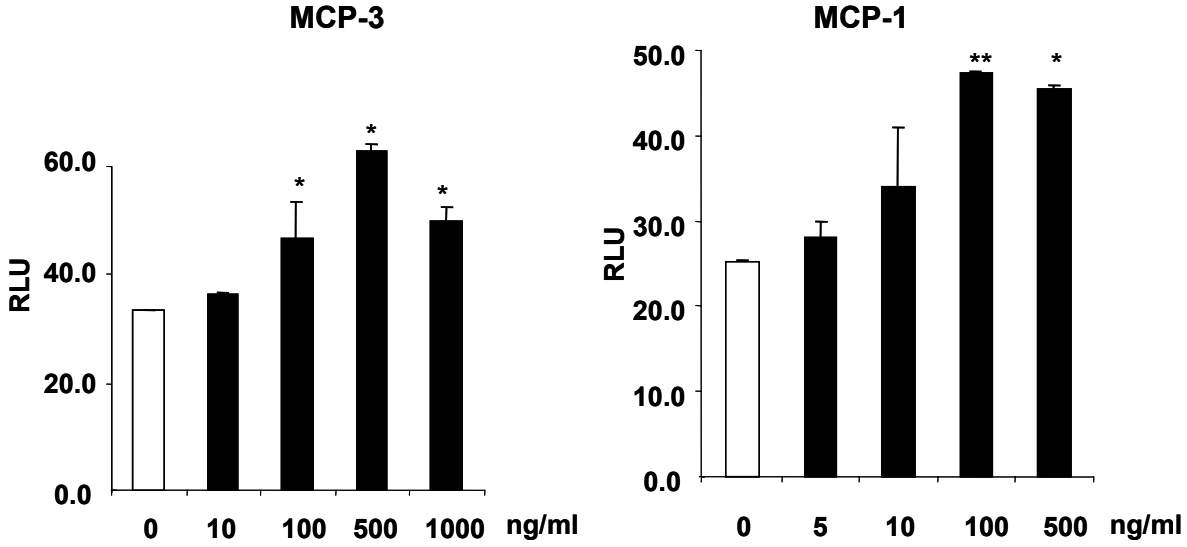


Figure 4.2 Dose-response in activation of 2kb-LacZ transgenic fibroblasts by MCP-3 and MCP-1 at 24 hours. Fibroblasts cultured from the skin of transgenic mice with high-level fibroblast-specific expression of β-galactosidase (2kb-LacZ) were grown to 80% confluence and serum starved for 24 hours before incubated with recombinant MCP-3 and MCP-1 to their effect on extracellular matrix gene expression. Values are the mean and SEM of triplicate samples and are representative of 5 independent experiments. *=P<0.05; **=P<0.01 as determined by Student's unpaired t-test. RLU=relative luminescent units.

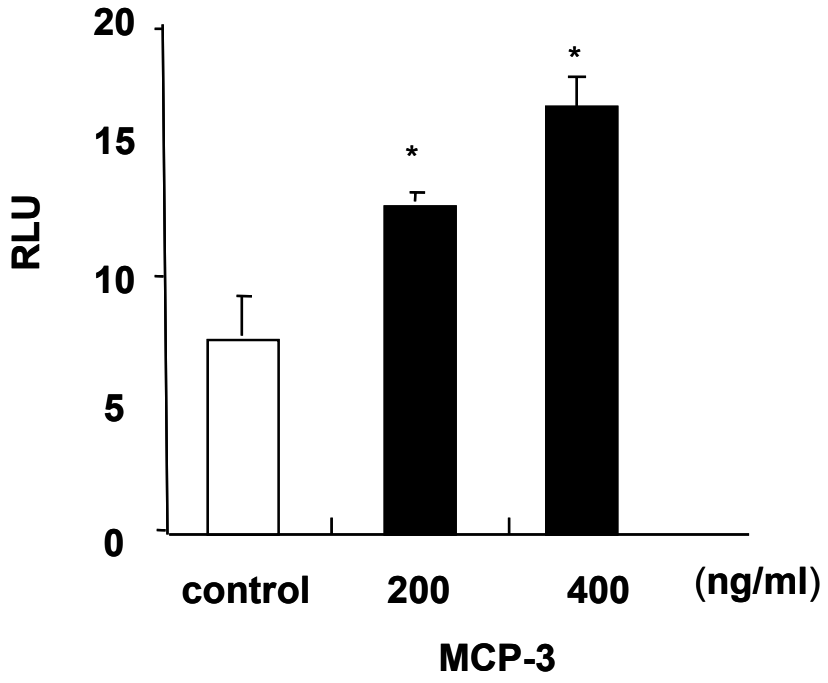


Figure 4.3 Activation of murine proα2(I) collagen (Col1a2) gene expression by MCP-3 in transgenic 2kb-LacZ fibroblasts. Fibroblasts cultured from the skin of transgenic mice with high-level fibroblast-specific expression of β-galactosidase (2kb-LacZ) were used to examine MCP-3 activation of ECM gene expression. Values are the mean and SEM of triplicate samples and are representative of 5 independent experiments. *=$P<0.05$ as determined by Student's unpaired t-test. RLU=relative luminescent units.

4.4.2 Transactivation of proα2(I) collagen gene expression by MCP-3 in transient transfection of wildtype murine fibroblasts with murine and human collagen promoter constructs

Data from transgenic mice were confirmed using transient transfection of wild-type fibroblasts using the same 2kb-Col1a2-LacZ construct with a mean (SEM) activation above baseline of 63.1 ± 23% with MCP-3 and 158 ± 54% with TGFβ1, p=0.01 respectively (**Figure 4.4**). To further delineate responsive elements in the upstream fragment of the 5' region of the mouse proα2(I)collagen gene, transient transfection of cultured wildtype fibroblasts with a 350bp minimal promoter of Col1a2 was undertaken. As with the larger 2kb-LacZ construct, there was a dose-dependent response in upregulation of Col1a2 minimal promoter with (% basal expression ± sem) mean activation of 172 % ± 51 and 185% ± 31, p = 0.02 at 200 ng/ml and 400 ng/ml respectively from a series of 3 independent experiments as shown in **Figure 4.5**, suggesting that the upstream elements previously implicated in selective activation are not essential for the effect of MCP-3.

Although sequence conservation of the proximal and distal regulatory elements of Col1a2 has been confirmed, previous studies suggest that differences in gene regulation can occur between species. Data for mouse constructs was confirmed using human promoter-reporter constructs. **Figure 4.6** summarises the results from 3 independent experiments using this construct with maximal induction of collagen reporter gene expression (% basal expression ± sem) of 152 ± 14 % at 200ng/ml. The time course for stimulation of collagen gene expression suggested that a second mediator may be elaborated by MCP-3 activated fibroblasts. Since TGFβ is found abundantly and released from activated fibroblasts and is known to increase the gene expression of collagen I and III in vitro, the effect of anti-TGFβ antibody on this stimulation of collagen reporter gene expression was examined. In addition, recent reports have explored the role of TGFβ in mediating the effect of other chemokines, MCP-1 and CCL18 on collagen expression using 1D11(R&D Systems)(Gharaee-Kermani *et al.*, 1996;Luzina *et al.*, 2006). The results show that MCP-3 stimulation of fibroblast collagen synthesis was partially inhibited with percentage change (mean ± sem, % change) 31.0 ± 6.7%, p = 0.05 in the presence of anti-TGFβ antibody. In

addition, there was a dose-dependent response with the antibody with maximal neutralisation effect at 50μg/ml as shown in **Figure 4.7**. This is consistent with previous findings that MCP-1 stimulation of fibroblast collagen expression was partially inhibited in the presence of anti-TGFβ antibody at concentrations found to completely inhibit TGFβ activity in the conditioned medium(Gharaee-Kermani *et al.*, 1996).

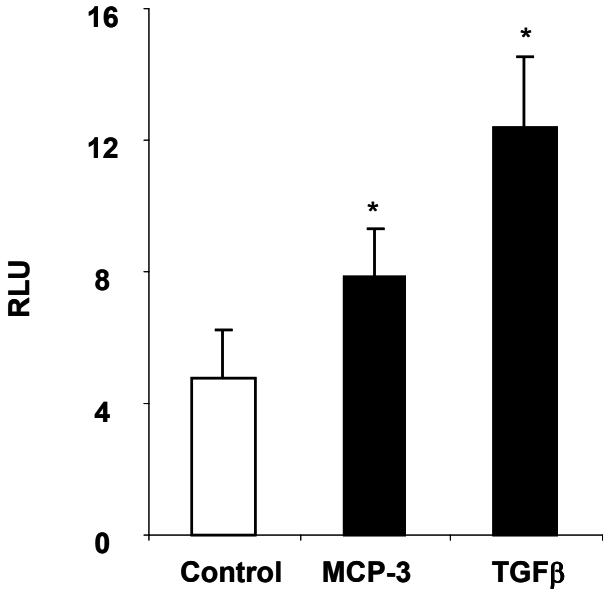


Figure 4.4 Activation of 2kb-Col1a2-LacZ reporter gene by recombinant MCP-3 in transient transfection is shown as reporter gene expression in a series of independent experiments. Activation of 2kb-LacZ promoter construct by MCP-3 (200ng/ml) approaches to that of recombinant TGFβ1 (4ng/ml) at 24 hours. * = $P<0.05$ by Student's unpaired t-test. RLU=relative luminescence units.

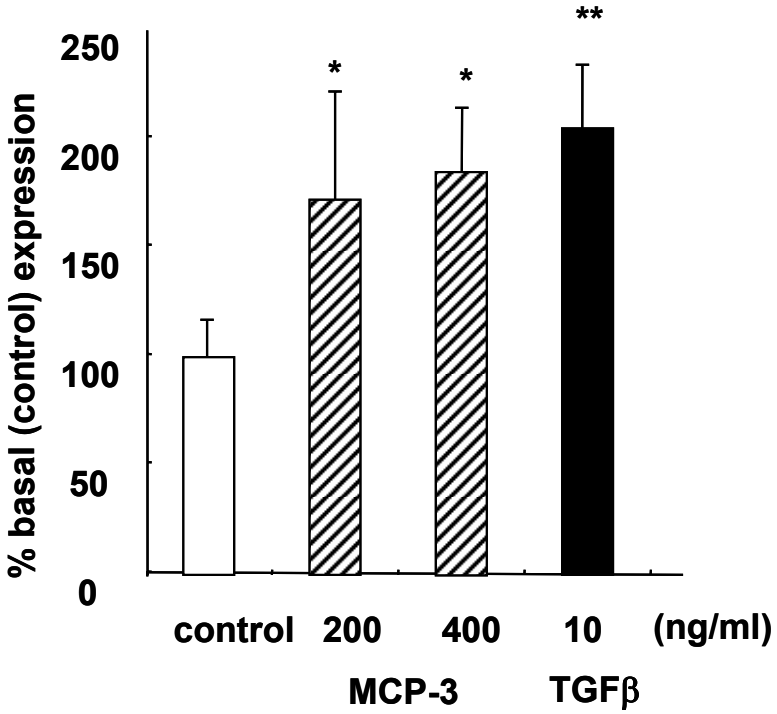


Figure 4.5 Activation of murine proα2(I) collagen (Col1a2) gene expression by MCP-3. Transactivation of the LacZ reporter gene was examined in wildtype fibroblasts harbouring -350bp endogenous Col1a2 promoter with MCP-3 200 ng/ml and 400 ng/ml in transient transfection in 3 independent experiments for the minimal promoter construct.

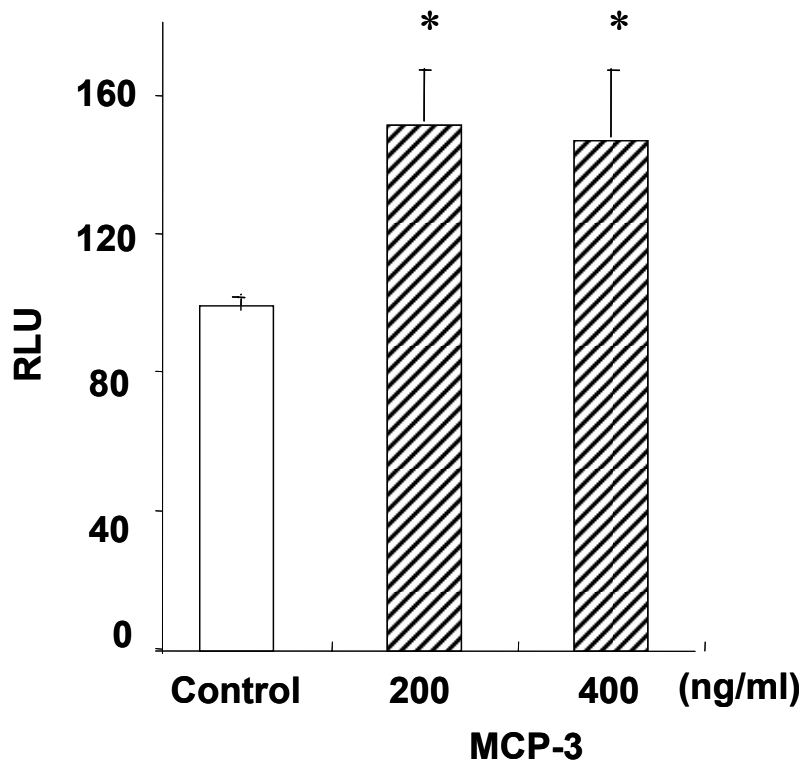


Figure 4.6 Activation of a human proα2(I) collagen (COL1A2) gene in transient transfection. Activation of this minimal promoter is expressed as the mean and SEM percentage of basal expression from 3 independent experiments and corrected for transfection efficiency.

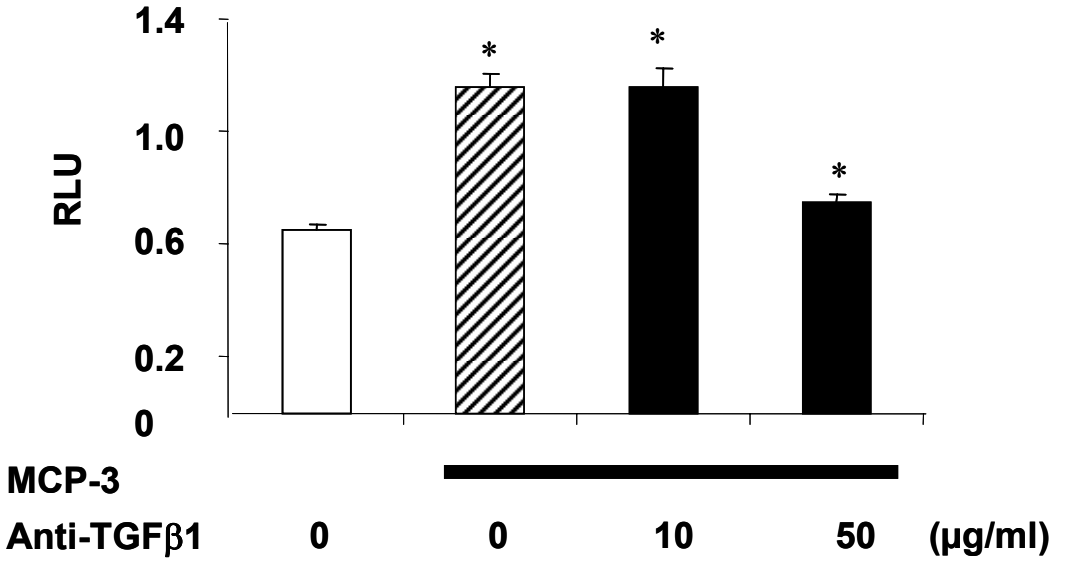


Figure 4.7 Activation of a human proα2(I) collagen (COL1A2) gene reporter by MCP-3 via a TGFβ-dependent mechanism. Anti-TGFβ antibody significantly reduces MCP-3-induced activation of human COL1A2 promoter constructs at 24 hours. Data are representative of 3 independent experiments and expressed as the mean and SEM of triplicate samples. *=$p<0.05$ by Student's paired t-test.

4.4.3 Upregulation of collagen protein expression by MCP-3 is mediated by MAPK, PI3K and TGFβ signalling pathways

To extend and confirm data obtained from COL1A2 promoter activation assays, the effect of the specific pharmacological pathways of potential signalling pathways activated, directly or indirectly, in response to MCP-3 was investigated in dermal fibroblasts. Confluent dermal fibroblasts were stimulated with recombinant MCP-3 for 24 hours in the presence of inhibitors of p38 MAPK, TGFβ and its associated receptor and PI3K pathways. Normalised quantitation of protein expression by scanning densitometry with Western blot analysis demonstrated that there is an increase in type I collagen by recombinant MCP-3. There was consistent reduction of the collagen type I protein expression with SB203580 and LY294002. Anti-TGFβ antibody (1D11) and receptor blockade (SD-208) also substantially reduced expression of collagen protein (**Figure 4.8**). The effect of ID11 is greater than that with SD-208 suggesting that complete inhibition with the former.

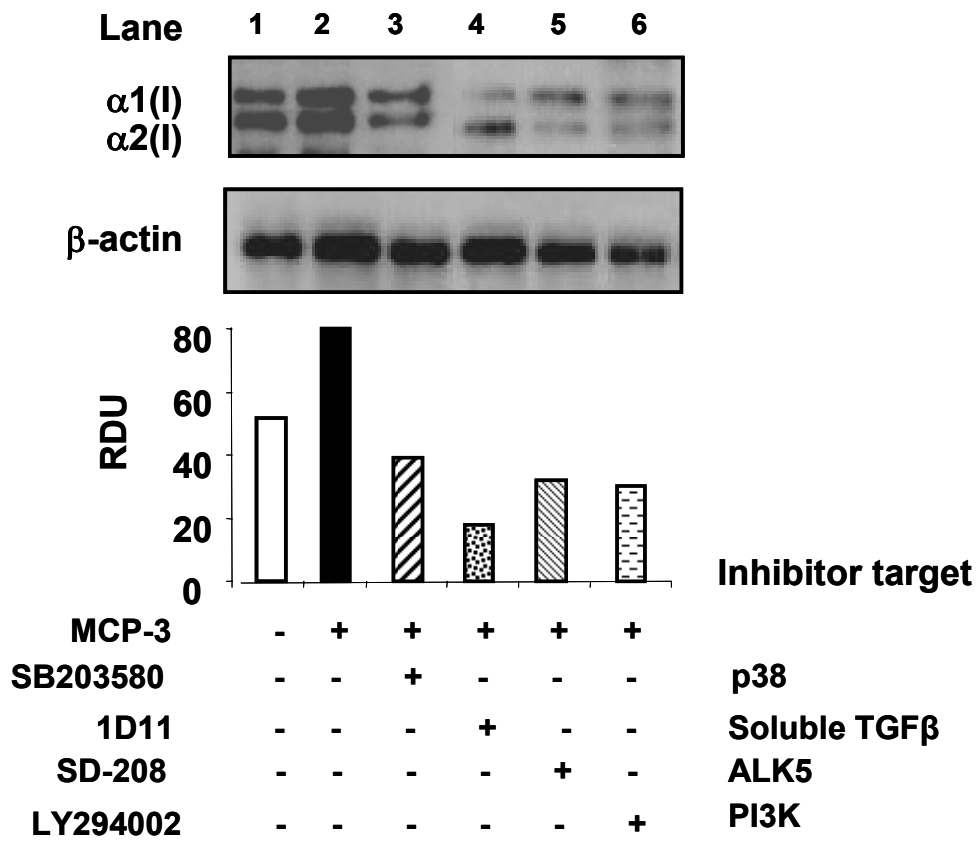


Figure 4.8 Effect of key pharmacological inhibitors of TGFβ, PI3K and p38 pathways on the upregulated expression of type I collagen in wild type fibroblasts. Expression level of type I collagen in wild type fibroblasts was determined by immunoblotting of tissue culture media using antibodies to type I collagen. The addition of the selective p38 MAPK inhibitor (SB203580), PI3K inhibitor (LY294002) and TGFβ inhibitors (1D11 and ALK5 inhibitor, SD-208) abolished the upregulated expression of type I collagen. Antibodies against β-actin were also used as loading control. Type I procollagen protein levels were defined as the mean band density of the α1(I) and α2(I) procollagen proteins, which was quantitated by scanning densitometry (bottom). Representative result of three independent experiments was shown in relative density units (RDU).

4.5 Discussion

There is growing evidence that chemokines may affect the homeostasis of ECM, and excessive synthesis of type I collagen by fibroblasts in the dermis is a pathological hallmark of SSc. There is emerging interest to implicate the contribution of inflammation to fibrotic response and there are studies to support the pivotal role of chemokines in linking the association between inflammation and fibrogenesis. For example, in murine model of unilateral ureteral obstruction, marked interstitial macrophage infiltration was observed with increase induction of tubular MCP-1 in the obstructed kidney(Morrissey & Klahr, 1998). Similarly, in a model of bleomycin-induced lung fibrosis, CXCR-2 mediated neutrophil recruitment appears to be essential for mediating the lung fibrosis in this model(Russo *et al.*, 2008). Moreover, treatment with CXCR2 antagonists led to reduction in neutrophil airway transmigration and collagen deposition. Other indirect evidence to suggest the central role of CC chemokines in fibrosis is derived from in vitro data that blockade of CCR1, an important receptor for both MCP-3 and MCP-1 suppresses the renal fibrosis induced by unilateral ureter obstruction and the pulmonary fibrosis induced by bleomycin(Tokuda *et al.*, 2000).

However it is also conceivable that lung and renal fibrosis may occur as a result of other mechanisms in the absence of inflammation via disrupted cross-talk between epithelial cells and fibroblasts(Lange-Sperandio *et al.*, 2007). Therefore, the lack of inflammatory infiltrate in Tsk1 as demonstrated in Chapter 3 is intriguing. For this reason, the effect of MCP-3 on ECM synthesis was examined in this chapter. The transcriptional regulation of the murine pro-α2(I) collagen gene represents a widely used experimental model to study the molecular mechanisms responsible for collagen I biosynthesis in particular its responsiveness to cytokines and growth factors that are central in the aetiopathogenesis of fibrotic conditions. The data showed increased proα2(I) collagen promoter activity in response to MCP-3 stimulation with similar dose-response characteristics to those previously reported for MCP-1(Gharaee-Kermani *et al.*, 1996). Although relatively high concentrations of recombinant cytokine were needed for maximal effect, the threshold for activation was often an order of magnitude lower, and likely to reflect expression levels in

the pericellular space in vivo. For the promoter assay, initial studies with transgenic murine fibroblasts containing a 2-kb upstream fragment driving high-level fibroblast-specific expression to reporter genes was used. Such use of transgenic cells represents a physiological approach to studies of collagen-gene activation in vitro, since the transgene is stably integrated in chromatin and is known to reflect endogenous collagen-gene expression in vivo.

Reporter genes regulated by different murine and human promoter sequences were also examined in transient transfection studies with early-passage fibroblast cultures. Transactivation of Col1a2 in Tsk1 skin has previously been shown to depend on far-upstream fibroblast-specific elements as well as sequences within the proximal promoter, with both proximal and distal regulatory sequences appearing to be responsive to TGFβ. In contrast, the data presented in this chapter suggests that MCP-3-induced Col1a2 activation may be independent of the upstream enhancer. Whilst it has been reported that the far upstream elements may directly respond to TGFβ1 and the presence of several Smad-binding elements are consistent with this, this do not completely exclude the possibility that the upstream enhancer element is acting by amplification of responses mediated via the proximal promoter of the Col1a2 which also include a Smad3-binding consensus sequence in addition to putative Sp1 and CBF binding sites. One of the five DNase I-hypersensitive sites in chromatin identified in the murine proα2(I) collagen gene lies within the 350bp promoter which is associated with high levels of expression, or that it provides position-independent expression in transgenic mice and it may therefore regulate the expression of linked genes through the binding of sequence specific transcription factors(Liau *et al.*, 1986). Moreover, it has been demonstrated that the 350bp proximal proα2(I) collagen promoter alone can direct, albeit a low level of reporter expression in transgenic mice. Therefore, this raises the possibility that the profibrotic effects of MCP-3 as shown in this study are only partly mediated via TGFβ1. Consistent with this, the data on collagen protein expression supports the role of MAPK p38 kinase and PI3K in the upregulation of collagen by recombinant MCP-3. Moreover, collagen gene upregulation in the fibrotic Tsk1 skin is likely to occur via a number of pathways that may depend both on TGFβ1 and on other factors. In the Tsk1 murine model, targeted mutations in either the signalling chain of

the IL-4 receptor or signal transducer and activator of transcription 6 (STAT6) prevents the cutaneous hyperplasia in Tsk mice, suggesting the importance of IL-4 and it has been shown that IL-4 may induce significant levels of MCP-1 production in stromal cells(McGaha *et al.*, 2001;Ong *et al.*, 1999;Lee *et al.*, 2003). Conversely, MCP-1 upregulates IL-4 mRNA expression and protein production(Lukacs *et al.*, 1997). Thus, it is possible that mutual induction of IL-4 and CC chemokines may be important in the fibrotic response in Tsk1 mice.

Consistent with this observation is, in this study, the time course of maximal induction, which was between 16 and 24 hours, with little effect before 6 hours. It nevertheless remains possible that TGFβ may be a co-factor as well as a potential downstream mediator of collagen-gene activation especially since collagen-gene activation was not completely abrogated by a high concentration of anti-TGFβ antibody. Conversely, there are recent reports suggesting overexpression of CC chemokine, CCL-18 may directly activate ECM gene expression in cultured human and dermal fibroblasts. The authors reported that in response to CCL18, recruitment of Sp1 and constitutive basal activity of Smad3 which is independent of TGFβ bioactivity are necessary for collagen expression in lung fibroblasts(Luzina *et al.*, 2006). It is interesting that other studies have suggested that the collagen expression regulated by basal Smad3 activity may be mediated via p38 MAPK and PI3K pathways(Furukawa *et al.*, 2003;Peron *et al.*, 2001). Using pharmacological inhibitors targeted against these two key pathways, the findings presented in this chapter is consistent with these reported studies.

At the cell surface TGFβ ligands initially engage the type II high affinity receptor TβRII, inducing a conformational change in this receptor to allow dimerisation with type I receptor (also known as activin like kinase, ALK) and its phosphorylation. The active complex predominantly transmits the signals via Smads, which translocate into the nucleus acting as transcription factors. Seven ALKs have been described of which ALK5 activates Smad2/3. These two Smads are specific mediators of TGFβ/activin signalling and Smad3 appears to be a key element in the signal transduction pathways involved in the in vivo fibrotic process. Smad3, but not Smad2, has been demonstrated to be involved in transducing the signal from TGFβ receptors to the COL1A2 promoter. The data presented here is entirely

consistent with this as there is down regulation of collagen protein induction with ALK5 inhibitor (SD208) and a pan-specific anti TGFβ antibody (1D11). TGFβ may activate other signalling cascades including MAPK pathways including p38, Erk and JNK kinase pathways, some of which regulate Smad activation but others may induce responses unrelated to transcription. It is noteworthy that other chemokines including CCL18 have been demonstrated to transcriptionally upregulates collagen expression via similar MAPK signalling pathways. There have been several reports to support an Akt-dependent pathway in basal type I procollagen expression in human dermal fibroblasts and in TGFβ1-induced type I procollagen expression(Kim *et al.*, 2008). Consistent with this, a recent report demonstrated that mast cell-induced type I collagen expression is upregulated via a PI3K/Akt pathway in keloid fibroblasts(Zhang *et al.*, 2006).

4.6 Summary

Data presented in this chapter suggest that MCP-3 may have a profibrotic effect by induction of Type I collagen gene in both transgenic 2kb-LacZ fibroblasts and wildtype fibroblasts transiently transfected with murine and human minimal promoter for collagen gene. The data also supports that the effect on collagen gene and protein appears to be partly mediated via TGFβ1 bioactivity. Additionally, a TGFβ-independent effect on collagen protein expression appears to be mediated via MAPK p38 and PI3K signalling pathways.

CHAPTER 5: EXPRESSION OF MCP-3 IN SCLERODERMA

5.1 Introduction

Animal models provide valuable insight into potential mechanisms and key mediators that drive the disease in particular in relation to the fibrotic response in the mouse strains described in Chapter 3. However, it is important to evaluate the significance of these results from animal models and to relate these findings to the human fibrotic diseases. A number of studies have demonstrated the crucial role of fibrogenic cytokines and chemokines in initiating and/or leading to the sequential events of fibrosis. Chemokines are chemotactic cytokines, and have been shown to be involved in various diseases. Moreover, chemokines are in an exclusive position to integrate inflammatory events and reparative processes and are therefore, important modulators of skin wound healing. For example, unlike normal skin, fibroblasts expressing CXCL1 and its corresponding receptor CXCR2 have been observed recently in keloids that occur during dermal wound healing in genetically predisposed individuals(Nirodi *et al.*, 2000). Similarly, high concentrations of CXCL4 within and in close vicinity to the platelet plug as a result of platelet degranulation immediately after wounding may lead to the arrest and accumulation of migrating/infiltrating fibroblasts, and therefore limiting excessive scarring(Watson *et al.*, 1994). In addition, chemokines have been shown to act directly or indirectly on resident cells, thereby regulating reepithelialisation, angiogenesis, and also myofibroblast differentiation(Werner & Grose, 2003). Chemokines might also be involved in the regulation of skin homeostasis as suggested for CXCL12. This chemokine is constitutively expressed in dermal fibroblasts and blood vessels of human skin but downregulated after injury, suggesting that it may function as a homeostatic regulator of tissue remodelling(Fedyk *et al.*, 2001).

CC chemokines have been demonstrated to be upregulated in a number of different pathological processes, including synovial pannus of rheumatoid joints, autoimmune lesions of multiple sclerosis, affected mucosal surfaces in ulcerative colitis and Crohn's disease, lung inflammation in chronic bronchitis, sarcoidosis, and asthma, and the vascular inflammation that characterises atherosclerosis (**Table 5.1**). For example, in the rheumatoid synovium, macrophages not only are activated by MCP-1 but also produce this

chemokine(Koch *et al.*, 1992). More recently, elevated circulating levels of several chemokines have been demonstrated in SSc patients (**Table 5.2**).

Pathogenic Disease state	Diseases	Chemokines and receptors involved
Inflammatory	Arthritis; colitis	Inflammatory CC and CXC
Infectious	Acute and chronic bacterial and viral infections; sepsis	CXCR4 and CCR5; Inflammatory CC and CXC
Autoimmune	SLE; Multiple sclerosis Type I diabetes	Inflammatory CC CXCL9,-10 and CXCR3 and CCR4
Allergic	Asthma	CCL11 and CCR3, CCL22 and CCR4 CCL1 and CCR8
Neoplasia	Metastasis; Angiogenesis	CXCL12 and CXCR4; CCL19 and CCR7
Graft rejection	Heart allograft Renal allograft Lung allograft	CCL5, CCL2, CCL3 and CCR5 CXCL8 and CXCR1 CXCL9, CXCL10, CXCL11 and CXCR3
Vascular	Atherosclerosis	CCL2 and CCR2, CX3CL1 and CX3CR1

Table 5.1 Chemokines and their receptors associated with major human diseases. The principal human diseases associated with different chemokines and their respective receptors are listed. As an integral part of the leucocytic recruitment to sites of infection and inflammatory diseases, chemokines and their receptors are implicated in the pathophysiology of many important diseases.

Chemokines	References
CC Chemokine	
CCL2/MCP-1 CCL3/MIP-1α CCL4/MIP-1β CCL17/TARC CCL18/PARC CCL22/MDC CCL27/CTACK	(Hasegawa *et al.*, 1999;Scala *et al.*, 2004) (Atamas *et al.*, 2003) (Fujii *et al.*, 2004) (Hayakawa *et al.*, 2005)
CXC chemokine	
CXCL1/GROα CXCL8/IL-8	(Reitamo *et al.*, 1993;Furuse *et al.*, 2003)
CX3C chemokine	
CX3CL1/fractalkine	(Hasegawa *et al.*, 2005)

Table 5.2 Elevated serum levels of chemokines in SSc.
MCP=Monocyte chemoattractant protein; MIP=Macrophage inflammatory protein; TARC=thymus and activation-regulated chemokine; MDC=macrophage-derived chemokine; CTACK=cutaneous T-cell-activating chemokine; GROα-growth-related oncogene α.

Among the CC chemokines, there is accumulating evidence that MCP-1 is essential in the development of various fibrotic conditions including SSc(Distler *et al.*, 2008). MCP-1 is expressed by fibroblasts, keratinocytes, inflammatory cells and endothelial cells whereas normal skin shows no MCP-1 expression(Distler *et al.*, 2001). One of the earliest studies to suggest this identified that SSc fibroblasts, but not normal fibroblasts, promote leucocyte migration across endothelial cell monolayers through an MCP-1-dependent mechanism(Denton *et al.*, 1998), and other studies have subsequently confirmed overexpression of MCP-1 in SSc tissues and by lesional fibroblasts in culture(Hasegawa *et al.*, 1999;Galindo *et al.*, 2001). Up-regulation of MCP-1 has also been described in several chronic inflammatory disorders(Tucci *et al.*, 2006;Van *et al.*, 1999). Whilst there are numerous studies to implicate MCP-3 in recruitment of inflammatory infiltrate in major inflammatory diseases including asthma and multiple sclerosis, the role of this chemokine in fibrotic diseases remains largely unexplored. Serum levels and spontaneous production

levels of peripheral blood mononuclear cells of MIP-1α and MIP-1β were raised in SSc patients compared with normal controls(Hasegawa *et al.*, 1999). In addition, elevated levels of MIP-1α correlated with pulmonary fibrosis and other reports have also demonstrated increased MIP-1α levels in BAL fluid in SSc patients with alveolitis. Similarly, CCL18 is increased in pulmonary fibrosis and its levels reflect the disease activity than did other serum markers of pulmonary fibrosis in SSc(Atamas *et al.*, 2003). Serum cutaneous T-cell attracting chemokine (CTACK/CCL27) levels were increased in SSc and it is also overexpressed in the sclerotic skin of SSc patients(Hayakawa *et al.*, 2005).

5.2 Aims

Identification of MCP-3 as a highly overexpressed gene in fibroblasts from neonatal Tsk1 mice raised the possibility that this chemokine might have a role in the pathogenesis of fibrosis in these mice. The previous chapter has shown that MCP-3 has a novel in vitro profibrotic activity and given that the Tsk1 strain is regarded as a model for human SSc, this led me to examine the expression of this chemokine in the skin biopsy specimens and fibroblasts cultured from a series of SSc patients. Using dermal fibroblast cell lines established from SSc patients and healthy age matched controls the following questions were addressed:

1. Is MCP-3 overexpressed in SSc?
2. If this is increased, are there any there are patterns of expression of this chemokine in relation to the subset and stage of disease?

5.3 Methods

5.3.1 Cell culture

5.3.1.1 Cell lines

All dermal fibroblast cells from patients with dcSSc were maintained as described in Section **2.2.1.** For experiments, cells were used between passage 2 and 5.

5.3.1.2 Fibroblast conditioned media

Control and SSc dermal fibroblast cell lines were maintained in DMEM until fully confluent. Media was replaced by DMEM (Invitrogen, UK) containing 0.2%FCS (Sigma, UK) supplemented with L-glutamine, penicillin G (100 U/ml) and streptomycin sulphate (100 µg/ml) (Invitrogen, UK) and the conditioned media collected 24 h later.

5.3.2 Protein expression

5.3.2.1 Collection of blister fluid

Dermal interstitial fluid was sampled from the fibrotic forearm skin of patients and from the forearm of healthy controls using a suction blister device (Dermovac, Ventipress), with applied 290–310 mmHg suction for 3–4 h, after which dermal fluid was sampled using a 21-gauge needle and stored at –70°C prior to analysis. This is a standard technique used in dermatological research for the collection of dermal interstitial fluid. Previous studies show that the sieve function of the capillary basement membrane is preserved during suction blister formation and that the fluid collected is representative of dermal interstitial fluid(Vermeer *et al.*, 1979).

5.3.2.2 Western blot analysis

To evaluate the differential expression of MCP-3 and MCP-1 in dermal fibroblasts from SSc and healthy controls, cultured fibroblast supernatants were collected after overnight incubation of skin samples in conditioned medium (200µl per well). All protein extraction and Western blotting methodologies performed as described in Section **2.4.3**, unless stated otherwise.

5.3.2.3 Antibodies

All antibodies unless stated otherwise were used as described in Section **2.4.3.3**. These include goat anti-human MCP-1 antibody, goat anti-human MCP-3 antibody (100 μg/ml) (all from R&D Systems, Oxford, UK). Parallel blots of cell lysates and supernatants from each culture were probed with a monoclonal antibody against β-actin (Sigma, St. Louis, MO) to control for variation in cell number between cultures.

5.3.2.4 Immunohistochemical staining

Dermal punch biopsy samples were obtained from 22 patients with dcSSc (14 with early disease and 8 with established dcSSc), 6 with lcSSc, and 11 healthy controls. For detection of MCP-3 protein, immunohistochemistry analysis with a biotin/streptavidin-based amplifying system was performed on these dermal sections. Serial sections of frozen dermal tissues were stained for MCP-3 protein with goat polyclonal anti-human MCP-3 IgG antibody (25 μg/ml in PBS; R&D Systems). The major patterns of distribution for MCP-3 staining were determined for each subset of SSc and healthy controls. Additional mouse monoclonal antibodies were used to identify microvascular pericytes (αSMA, clone 1A4 1:400, Sigma-Aldrich, Poole), endothelial cells (PAL-E 1:100, Monosan, Uden, The Netherlands), macrophages (CD68 1:50, Dako) and fibroblasts (AS02 1:500, Oncogene). Controls included an exchange of primary antibodies with isotype-matched corresponding IgG antibodies.

5.3.2.5 Assay for MCP-3 in SSc

MCP-3 levels in the sera, cultured fibroblasts media and blister fluid were assayed by commercially available sandwich ELISA (R&D Systems Inc., Oxford, UK). Standard curve was derived from the stock solution of 10,000pg/ml with a mean minimum detectable dose of 1pg/ml.

5.4 Results

5.4.1 Upregulation of MCP-3 protein in SSc dermal fibroblast cultures

Having established fibroblastic MCP-3 protein upregulation in Tsk1 and TβRIIΔk transgenic murine models, parallel analyses of dermal fibroblast culture media from early stage dcSSc were compared with matched controls. The level of MCP-3 immunoreactivity in SSc fibroblast supernatants (mean ± SEM relative density units [RDU] 4.92 ± 1.12 in controls versus 8.27 ± 0.5 in SSc; p = 0.02) was greater than that observed for MCP-1 (RDU 3.84 ± 0.73 versus 7.87 ± 0.14, respectively; p = 0.01), as shown in **Figure 5.1**.

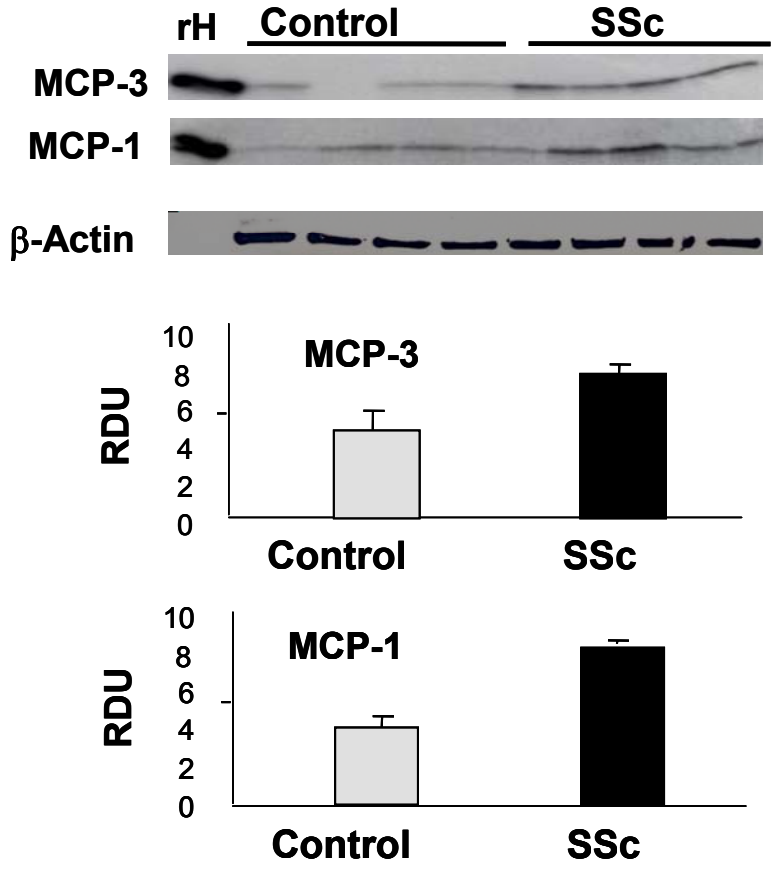


Figure 5.1 Overexpression of MCP-3 and MCP-1 by lesional SSc human skin fibroblasts. Fibroblasts culture media from representative normal or SSc dermal fibroblast cultures were analysed using antibodies to MCP-3 and MCP-1. Cell layer β-actin staining was used as a loading control, and recombinant human MCP-3 and MCP-1(rH) were used to confirm primary antibody specificity (Top panel). Densitometry analysis summarising 3 independent experiments shows the mean and SEM protein levels for MCP-3 and MCP-1 in relative density units (RDU) (Bottom panel).

5.4.2 Dermal overexpression of MCP-3 in subsets of cutaneous SSc

There was variable amount of specific immunostaining for MCP-3 in the lower epidermal layer, in both healthy control and SSc biopsy samples. However, as discussed in Chapter 3.4.3 with Tsk1 skin, SSc lesional skin sections showed dermal expression of MCP-3 as well as additional strong MCP-3 expression in and around the blood vessels at sites of mononuclear cell infiltrates. The majority of the inflammatory mononuclear cells in SSc skin were positive for CD68 (**Figure 5.2**). Consistent with the data on mRNA and protein expression, additional immunostaining studies using anti-AS02 which recognises Thy-1 antigen on fibroblasts suggest that MCP-3 is localised away from vascular or epithelial structures. Additional immunostaining with monoclonal antibody against PAL-E, an endothelial cell surface antigen and α-SMA antibodies suggests that MCP-3 may localise with endothelial and pericytes (**Figure 5.3**).

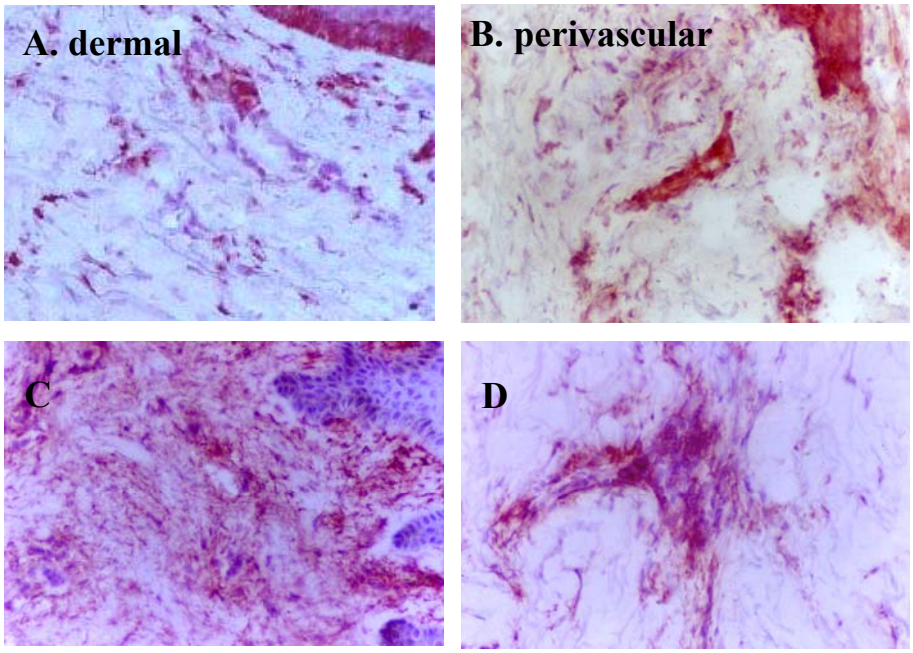


Figure 5.2 Increased MCP-3 expression in early-stage diffuse cutaneous systemic sclerosis (dcSSc) skin biopsy samples. Dermal MCP-3 localisation with vascular immunostaining is observed in A and B respectively. Most of these samples were positive for the fibroblast marker AS02 and monocyte/macrophage marker CD68 in juxtaposed sections (shown in C and D respectively in bottom two panels). (Original magnification x240)

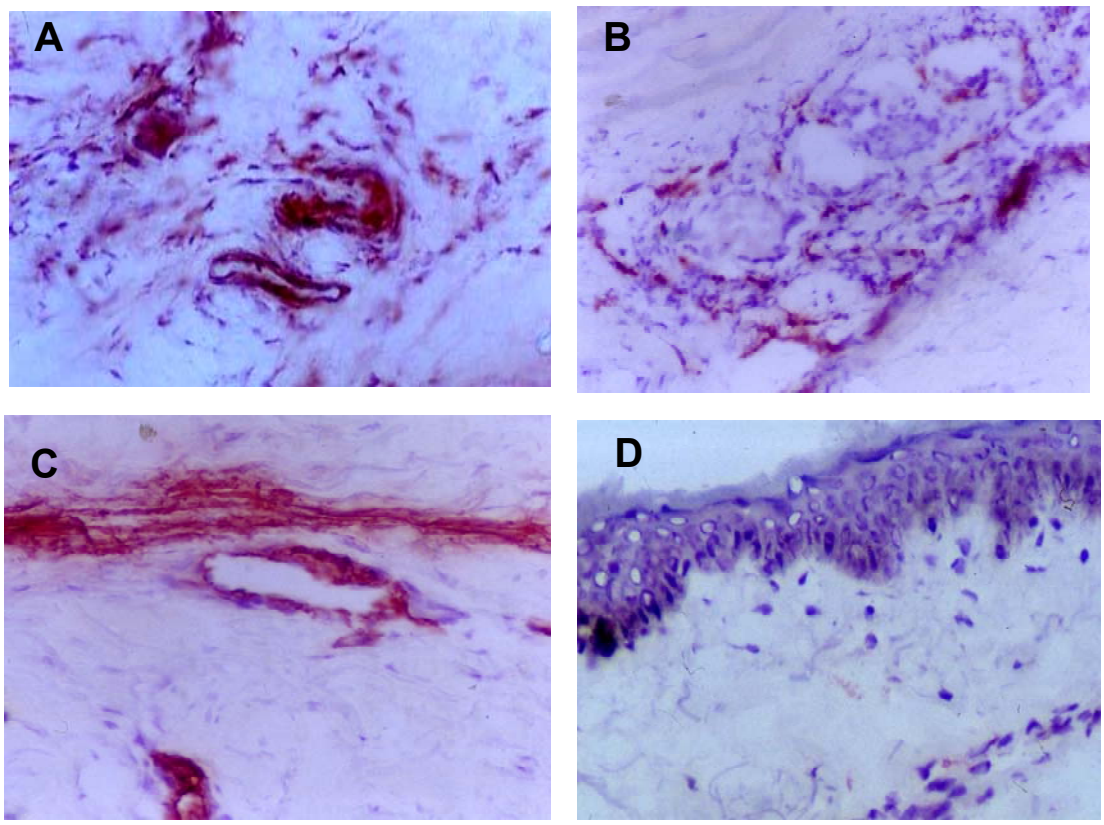


Figure 5.3 Increased perivascular MCP-3 expression in dcSSc. Shown in panel A, in these skin biopsies from patients with dcSSc, the pattern of immunostaining for perivascular MCP-3 infiltration appears to localise with endothelial cells and pericytes, demonstrated in panels B and C respectively. In contrast, control skin samples did not demonstrate any significant immunostaining with IgG with normal goat IgG as shown in panel D. (Original magnification x240)

There was significantly more MCP-3 expression in skin biopsies from patients with early-onset diffuse disease compared to established diffuse or limited cutaneous SSc indicating that it is associated with inflammatory or progressive skin disease **(Figure 5.4)**. Negative controls with IgG isotype-matched antibodies showed no staining.

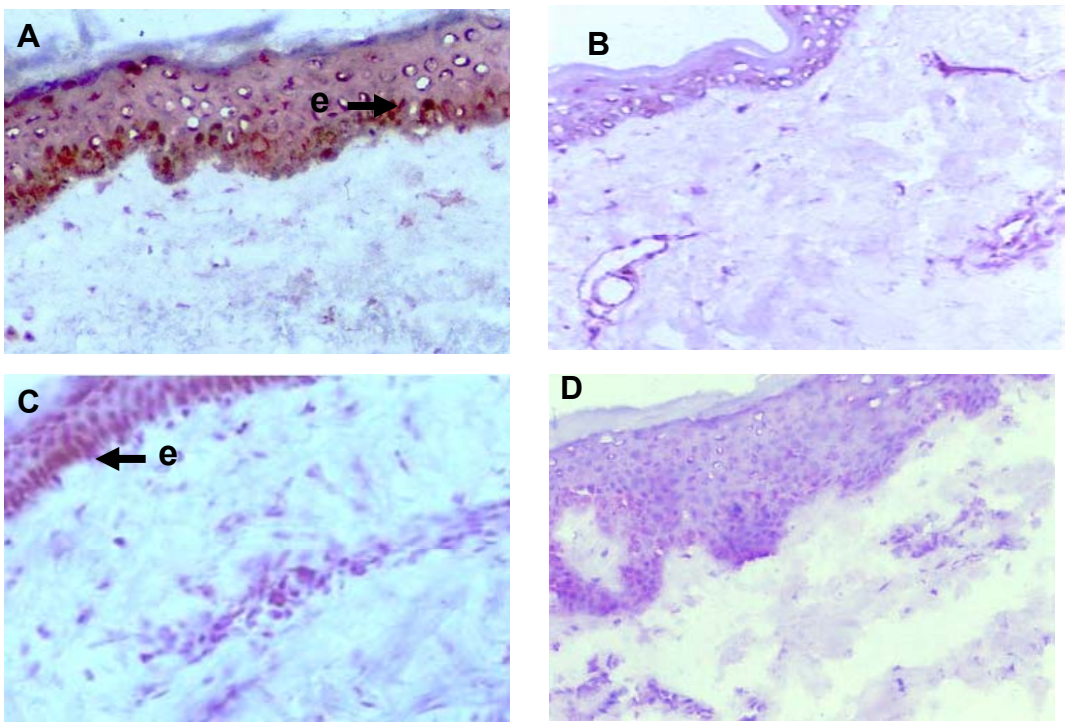


Figure 5.4 Expression of MCP-3 in different subsets of SSc and healthy controls. There is a variable amount of epidermal staining (e with arrow) in skin sections from patients with established dcSSc (A) and in clinically uninvolved early dcSSc skin (B) or limited cutaneous SSc (lcSSc) skin (C) and in skin from healthy controls (D). Minimal vascular MCP-3 immunostaining is detected in uninvolved skin from patients with established dcSSc (A) and lcSSc (C). (Original magnification × 240.)

Table 5.3 summarises the staining patterns observed in SSc skin biopsy specimens. Immunostaining for MCP-3 was associated with an inflammatory infiltrate with vascular localisation in the majority of skin sections from patients with early dcSSc (n=14) as well as more diffuse dermal staining. Diffuse dermal staining was absent in the skin sections from patients with established dcSSc and lcSSc.

Pattern	Control n = 11	Early dcSSc n = 14	Established dcSSc n = 8
Inflammatory infiltrate	0	12	2
Perivascular	0	10	2
Diffuse dermal	0	10	0
Epidermal	8	14	6

Table 5.3 Histological characteristics of dermal expression of MCP-3 in SSc and healthy controls. The values represent the number of samples displaying the respective staining patterns for MCP-3. Early dcSSc is defined by <2 years' duration.

5.4.3 Overexpression of MCP-3 in biological fluids in SSc

Having established overexpression of MCP-3 in the dermis of early active diffuse SSc on Western blot analysis and immunohistochemistry, I examined whether similar upregulation of MCP-3 is observed in the sera, cultured fibroblast media and blister fluid samples assayed by ELISA in 15 controls and patients with dcSSc. There was elevated sera expression of MCP-3 in dcSSc, 25.7 ± 4.1 (mean ± SEM) compared to controls, 13.2 ± 2.7 pg/ml, p=0.02. Interestingly, there was heterogeneous expression of MCP-3 among the patients with dcSSc with a subset of patients demonstrating higher mean level, 35.8±3.9 (Mean± SEM) (n=6) compared to the remaining patients with a mean 14.8±3.4 (**Figure 5.5**). This is in contrast to the generally increased MCP-3 immunoreactivity in the dermis of early dcSSc as discussed in Section **5.4.2.** This may be related to MCP-3 sequestration in the early stage disease.

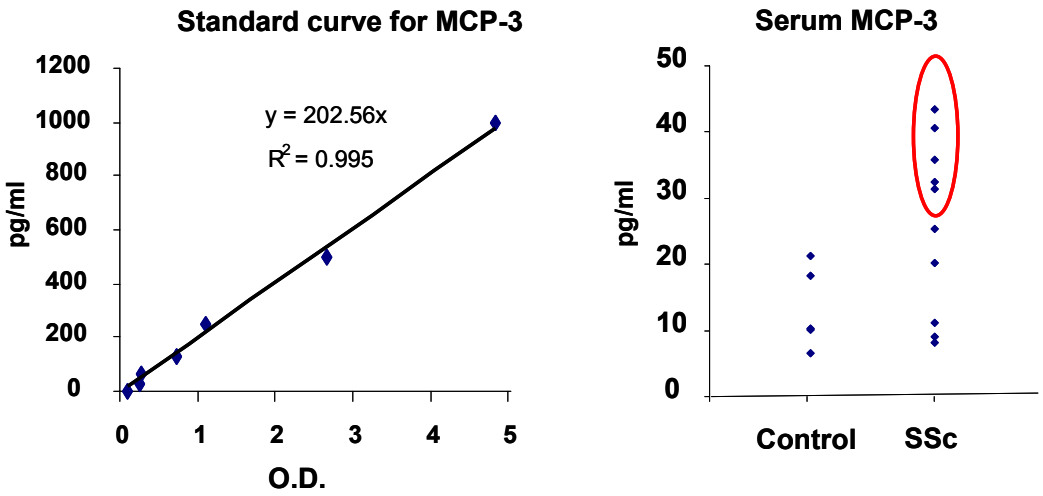


Figure 5.5 Serum level of MCP-3 in early-stage dcSSc. Shown on the left is the standard curve for MCP-3 levels. On the right, there was increased level of MCP-3 in the sera from a subset of patients with early-stage dcSSc compared to matched healthy controls. O.D.: Optical density.

There was overexpression of protein in the explanted fibroblasts supernatant (Mean±SEM) in SSc, 796±323 pg/ml (n=4) compared to controls 617±282 pg/ml (n=4), as shown in **Table 5.4**. This is consistent with results on Western blot experiment on cultured fibroblasts supernatant in Section **5.4.1**. However, there was no difference in MCP-3 expression in blister fluid with 181±16.4 in controls (n=2) and 193.4±4.9 in SSc samples (n=2).

	Normal	SSc
n =	4	4
mean	617	796
sem	282	323
median	433	606

Table 5.4 Expression levels of MCP-3 in fibroblast culture supernatants were determined in pg/ml using specific ELISA kit. (p=0.04 as determined by Student's unpaired t-test).

5.5 Discussion

There is increasing evidence to implicate CC chemokines in various fibrotic conditions. Previous chapters have indicated that MCP-3 is overexpressed in murine models for fibrosis and the data in this chapter suggests that this chemokine is also overexpressed in human SSc. The array data presented in Chapter 3 suggest that MCP-3 is upregulated neonatally in Tsk1 skin and not overexpressed at 12 weeks. These findings are consistent with the more frequently observed MCP-3 overexpression in early dcSSc skin as compared with biopsy samples of skin with established disease. This would suggest that MCP-3 may be an important initiator in the cascade of mediators leading to dermal fibrosis. This is similar to the characteristics of other profibrotic mediators including TGFβ1 in skin samples which often show little expression in established lesional skin of SSc (Querfeld *et al.*, 1999).

The significance of the differences in staining patterns for MCP-3 in SSc is unclear. Diffuse dermal expression was observed in dcSSc skin samples. The lack of uniform staining for MCP-3 protein may suggest metabolic heterogeneity in fibroblasts activity in collagen synthesis. This was also observed with dermal expression of MCP-1 in SSc, with predilection for areas close to blood vessels (Galindo *et al.*, 2001). More recently, our group also demonstrated similar heterogeneity in fibroblastic expression for CCR2, the key receptor for MCP-1 and MCP-3 in early stage dcSSc(Carulli *et al.*, 2005).
Furthermore, fibroblast cultures from various levels of involved SSc skin revealed that the highest collagen-producing populations were found at the lower levels of the dermis and that there was a synchronised increase in type I and type III collagens and fibronectin. These suggest that fibroblasts at the lower dermis and around the blood vessels were the most active, suggesting fibroblast clonal selection that expresses a phenotype for high matrix protein synthesis.

Epidermal staining for MCP-3 was observed in the skin samples from SSc: however, this finding was also detected in the healthy controls. Interestingly, it has also been recently

reported that there is a similar lack of specificity of epidermal expression for MCP-1 expression in early stage dcSSc(Carulli *et al.*, 2005). However other authors have documented strong MCP-1 expression in keratinocytes particularly at the lower epidermal layers(Galindo *et al.*, 2001). Similar perifollicular staining was also observed in both control and lesional samples, and these patterns are unlikely to be of pathogenic significance, although it is apparently specific and therefore may reflect a physiological function for MCP-3. Although this present study did not examine receptor dermal expression, strong immunoreactivity for CCR2 and CCR3, the key receptors for MCP-3 has been described in SSc and atopic dermatitis respectively(Carulli *et al.*, 2005;Wakugawa *et al.*, 2001).

By immunostaining, MCP-3 was more frequently expressed in vascular and perivascular inflammatory mononuclear cells and scattered fibroblasts in the dermis of SSc patients especially those with early diffuse SSc. Other chemokines are also expressed in similar distribution (**Table 5.5**). The vascular and perivascular staining observed in early-stage dcSSc may reflect a role for fibroblast-derived MCP-3 in mononuclear cell extravasation, and the colocalisation with CD68-positive cells supports this. These findings are also consistent with recent in vivo evidence that has established the important parallel contributions of MCP-3 and MCP-1 in CCR2-mediated inflammatory monocyte recruitment from bone marrow to inflamed tissues in murine model of thioglycollate-induced peritonitis and *L .monocytogenes* infection(Jia *et al.*, 2008;Tsou *et al.*, 2007). It is possible that MCP-3 overexpression recruits macrophages into the SSc skin, leading to the initiation of skin fibrosis and production of other inflammatory or fibrotic cytokines.

Chemokines	Epidermis	Dermis			References
		Cellular Infiltrate	Fibroblasts	Endothelial cells	
CCL2/MCP1	++	+	+	+	(Distler *et al.*, 2001;Hasegawa *et al.*, 1999;Galindo *et al.*, 2001)
CCL5	++	-	-	-	(Anderegg *et al.*, 2000)
CXCL8	+	+		++	(Koch *et al.*, 1993)
CX3CL1				++	(Hasegawa *et al.*, 2005)

Table 5.5 Upregulated chemokine expression in SSc skin.

The localisation of MCP-3 in α-SMA pericytes is consistent with recent findings that MCP-1 colocalised with α-SMA pericytes(Carulli *et al.*, 2005). Pericytes are believed to be critical for vascular homeostasis in wound healing and fibrosis. Thus, in early dcSSc, microvascular pericytes become activated and expressed PDGFβ receptor and may be a source of tissue myofibroblasts. Myofibroblasts are important in the pathogenesis of fibrotic diseases. Fibroblasts when activated primarily by growth factors including TGFβ acquire smooth muscle cell-like features including stress fibre formation as well as de novo expression of α-SMA and specific ECM proteins. Persistence of myofibroblasts has been postulated to be the common feature of fibrotic disease.

Whilst the data suggests that there was consistency in the overexpression of MCP-3 in the cultured fibroblasts supernatants from dcSSc compared to healthy controls, quantified by both ELISA and Western blot analysis, the measured expression of this chemokine in the sera indicates heterogeneity with a subset of patients expressing high levels of MCP-3. This mirrors the recently reported findings that there is considerable heterogeneity in expression of MCP-1 and the cognate receptor for MCP-1 and MCP-3, CCR2 between cultured fibroblasts from different patients with early dcSSc(Carulli *et al.*, 2005). Interestingly, among these populations of early dcSSc fibroblasts, those that express CCR2 receptor

appear to have a myofibroblast phenotype. It is also possible that heterogeneity in the expression of chemokines and their receptors by fibroblasts may be partly genetically determined.

Expression of MCP-3 in different biological fluids was studied to examine the localisation of this chemokine in SSc. For example, suction blister fluid measures locally produced growth factors and cytokines in the extracellular fluid. Although these factors may be localised in these body compartments, the functional significance of this is unclear. It has been demonstrated that N and C-terminal CTGF cleavage products are found in biological fluids such as uterine flushings(Ball *et al.*, 1998), but their biological relevance is not known. In SSc, it has been reported that although, whole CTGF and C-terminal CTGF were present only at low levels and showed no difference between the different disease subgroups, only N-terminal CTGF levels were elevated in SSc blister fluids from dcSSc patients when compared to healthy controls(Dziadzio *et al.*, 2005). Other studies have demonstrated elevated soluble forms of ICAM-1 and IL-2R in suction blister fluid samples from early SSc suggestive of activation of endothelial cells and T cells in SSc although it was noted there was significant overlap in the measured values for these factors in both SSc and control samples(Sondergaard *et al.*, 1998). From the data presented in this chapter, there was no difference in MCP-3 expression in dcSSc and controls and this may reflect the small numbers of samples studied. However, the absence of any differential MCP-3 expression may also suggest that MCP-3 is internalised once it is bound to GPCRs or degraded.

It is interesting that in inflammatory bowel diseases, expression of MCP-3 was strictly correlated with disease severity (Uguccioni *et al.*, 1999). It is possible that MCP-3 may serve as a severity marker in SSc. Similar correlation of chemokine expression and disease activity has been demonstrated in other autoimmune diseases including rheumatoid arthritis and SLE(Ellingsen *et al.*, 2001;Narumi *et al.*, 2000).

5.6 Summary

This chapter extends the observations made from previous chapters that MCP-3, in addition to be overexpressed in the murine model for fibrosis, is also upregulated in SSc in particular in the active stage of diffuse disease with significant expression in the dermis and perivascular distribution albeit some heterogeneity in its expression. This may be a key determinant of the clinical disease phenotype and that expression of MCP-3 in early stage dcSSc may be a useful marker for disease severity or predictor of outcome.

CHAPTER 6: REGULATION OF MCP-3 EXPRESSION

6.1 Introduction

MCP-3 is one of the pluripotent CC-chemokines and may be induced in various cell types in response to endogeneous or exogeneous stimuli including inflammatory cytokines TNFα. For example, induction of MCP-3 was expressed by cultured fibroblasts treated with a combination of IL-1β and IFNγ and its expression in airway smooth muscle cells was suppressed with anti-inflammatory steroids(Menten *et al.*, 1999;Pype *et al.*, 1999). In contrast to homeostatic chemokines (e.g. CXCL13) which are constitutively expressed, inflammatory or induced chemokines including MCP-3 are transcriptionally regulated during inflammation and mediate the recruitment of inflammatory cells to target tissues.

The intracellular signalling regulating MCP-3 secretion is largely unknown. A recent study suggests that PI3 kinase-Akt/PKB pathway mediates MCP-3 secretion in neuronal cells, and therefore confers a survival advantage(Bandyopadhyay *et al.*, 2007). Similarly, PI3K, and not protein kinase C mediates MCP-3 secretion by mononuclear cells stimulated by combination of IL-1β and LL-37, a host defence peptide which modulates the innate immune responses. In addition, this study supported previous reports that MCP-3 expression, like its related chemokine, MCP-1, is regulated in part by the transcription factor NFκB (Yu *et al.*, 2007). In contrast, other authors have reported the role of MAPK p38, ERK and JNK kinase in induction of MCP-3 in human airway smooth muscle cells(Wuyts *et al.*, 2003). It is therefore likely that the expression of MCP-3 is regulated in a cell-specific and context-specific manner.

The increasing number of reports that demonstrated upregulation of MCP-3 in various inflammatory and autoimmune diseases suggests that regulation of this chemokine expression may contribute to the aetiopathogenesis of these conditions. For example, recent studies have demonstrated distinct regional expression regulation for MCP-3 in correlation with chronic inflammation in multiple sclerosis brains(Banisor *et al.*, 2005). These observations are consistent with previous studies, and therefore, support the

involvement of MCP-3 in the development of inflammatory demyelination. Previously, Van Damme analysed the transcription regulation of MCP-3 promoter and identified an active AP-1-like element at position -37 and an Ets-like element in the region between -190 and –172 upstream to the transcription start site that inhibited the promoter activity in MG-63 cells(Murakami *et al.*, 1997). Further studies have demonstrated several positive and negative regulatory elements in the promoter in response to various cytokines including IL-1β in different cells lines including MG-63 cells, monocytes, osteosarcoma tumour cells and endothelial cells. In contrast, there have been few studies on regulation of fibroblastic expression of MCP-3. Upregulation of MCP-3 expression in dermal fibroblasts by IL-4 may be mediated by the AP-2 cis element within 1.2 kb proximal to the transcription start site. In addition, the presence of two putative NF-κB transcription sites within the promoter may underlie the induction of MCP-3 mRNA expression by TNFα in dermal fibroblasts(Hein *et al.*, 1999).

6.2 Aims

The previous chapters have demonstrated that MCP-3 is overexpressed in murine models of fibrosis and SSc, raising the possibility of a novel role as a profibrotic mediator. Moreover, its upregulation in TβRIIΔk model with activated TGFβ-dependent pathways suggests MCP-3 may be regulated by TGFβ.

To explore the potential synergism between these mediators, I examine the effect of TGFβ on MCP-3 bioactivity.

This chapter aims to:

1. Determine the regulation of MCP-3 gene and protein expression by TGFβ
2. Elucidate signalling pathways governing the activation of MCP-3 expression by TGFβ

6.3 Methods

6.3.1 Generation of MCP-3 1kb promoter reporter gene construct

To explore the molecular mechanisms underlying the activation of MCP-3 induction by TGFβ in dermal fibroblasts, a 1kb upstream fragment of the murine MCP-3 promoter containing the TATAA box and transcription start site, flanked by BglII and HindIII was generated from the MCP-3-pUC19 construct (**Figure 6.1**) and cloned into the luciferase reporter vector pGL3 (Promega) as described below.

6.3.1.1 Culture and purification of MCP-3-pUC19

A pUC19 plasmid cloning vector containing a 4.7 kb Pst1 fragment of the MCP-3 proximal and upstream promoter inserted at the Pst1 site was kindly donated by Professor Van Damme, Rega Institute, Belgium, and this was used to generate a 1kb upstream promoter sequence(Murakami *et al.*, 1997).

The MCP-3 promoter-containing pUC19 DNA was transformed into the E.coli strain DH5α as described in Section **2.3.3**. DNA was isolated using the Qiagen Plasmid Maxi Prep kit (Qiagen) as per the manufacturer's instructions. Briefly, an overnight culture of the *E.coli* strain DH5α containing the pUC19 plasmid was inoculated into 500 ml of LB and agitated overnight. DNA was prepared as described in Section **2.3.4.2** taking care to avoid vortexing to prevent DNA shearing. Upon binding of the DNA to the Qiagen-tip 500, ATP-dependent exonuclease was applied to digest any contaminating genomic DNA, leaving only supercoiled pUC19 DNA remaining. The plasmid DNA was eluted, precipitated with 0.7 volumes of isopropanol and collected by centrifugation. The DNA pellet was resuspended in an appropriate volume of TE buffer and the concentration was determined by UV spectrophotometer (Hitachi U-2001).

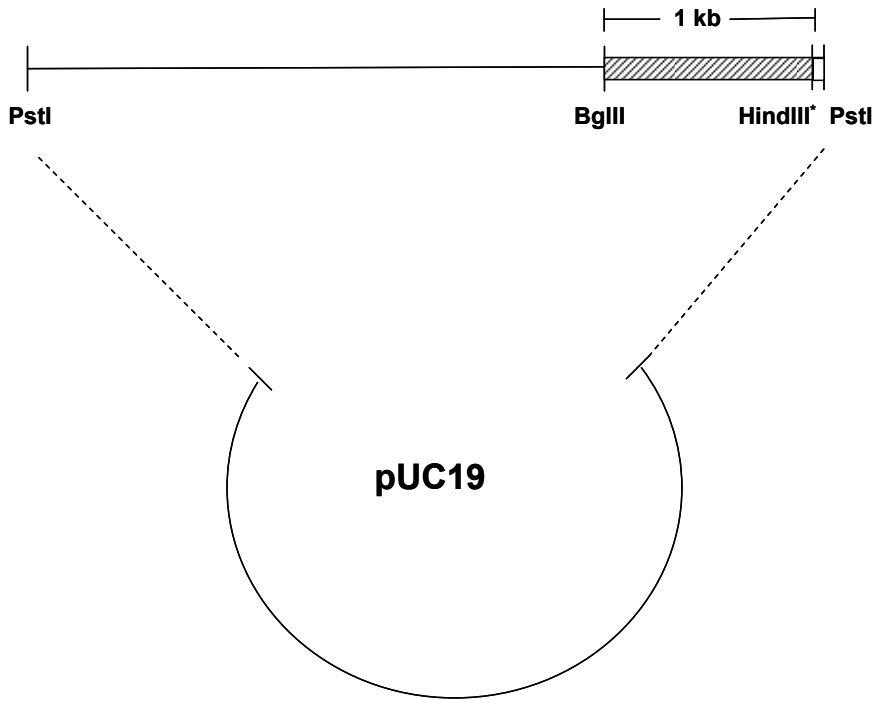


Figure 6.1 pUC19 cloning vector incorporating the 4.4kb MCP-3 promoter inserted at the Pst1 restriction site. The shaded area on the promoter was amplified to generate the 1kb fragment upstream to the transcription start site with BglII and HindIII restriction sites(Murakami *et al.*, 1997). *The HindIII restriction enzyme site was generated.

6.3.1.2 Generation of MCP-3 1kb promoter-construct

A 1kb promoter fragment was amplified using polymerase chain reaction (PCR) and oligonucleotides that were designed to amplify the specific region of the MCP-3 promoter adjacent to the BglII site at position -1025, upstream of the transcription start site. The oligonucleotide primers annealed to the MCP-3 promoter are forward primer 5'-GC**AGATCT**TGATATGCTGTGCAG-3' at position -1025 and reverse primer 5'-GC**AAGCTT**GAGAATTGGAGGTTTCTGGTT-3' at position +52 upstream of the transcription start site respectively. The forward primer contains a BglII (endogenous) restriction enzyme site and the reverse primer contains a HindIII (exogenous) restriction enzyme site **(Figure 6.2).** A PCR reaction mixture containing 115ng of the MCP-3 promoter-pUC19 (57.7ng/ul) as template; 50pmol of each oligonucleotide primer; and 38ul of master mix (5ul 1x *Pfu* DNA polymerase (Promega, UK) with 10x Reaction Buffer with MgSO4 and 10mM dNTPs) was over laid with oil to prevent evaporation and placed in an

automated thermal cycler PTC-100 (MJ research, Inc). *Pfu* DNA polymerase which has proofreading properties was added after the addition of dNTPS to ensure high fidelity amplification.

MCP3F1
5'-GC**A GAT CT**T GAT ATG CTG TGC AG-3'

Amount	Concentration (Volume 1 ml)	33 pmol/μl	Length 23-mer
8.4 OD	Volume for 100 pmol/μl	332 μl	GC Content 47.8%
235 μg	Molecular weight	7095 g/mol	Scale 0.01 μmol
33.2 nmol	T_m	60.6 ^{0}C	Purification HPSF

MCP3R1
5'-GC**A AGC TT**G AGA ATT GGA GGT TTC TGG TT-3'

Amount	Concentration (Volume 1 ml)	67 pmol/μl	Length 29-mer
21.8 OD	Volume for 100 pmol/μl	673 μl	GC Content 44.8%
607 μg	Molecular weight	9019 g/mol	Scale 0.01 μmol
67.3 nmol	T_m	65.3 ^{0}C	Purification HPSF

Figure 6.2 Oligonucleotide sequences for the two primers used to amplify the 1kb-upstream region of the murine MCP-3 promoter. The MCP3F1 contains the BglII restriction enzyme site and the reverse primer, MCP3R1 contains a HindIII restriction enzyme site.

After being initially denatured for 2 minutes at 95 C with a single cycle, the MCP-3 promoter was amplified for 25 cycles (95^0C for 30 secs, 50^0C for 30 secs, 72^0C for 2 minutes). This was followed by final extension at 72^0C for 5 minutes to ensure all the PCR products are completely blunted and the reaction mixture was cooled overnight at 6^0C for 30 hours. Using the agarose gel electrophoresis (0.8%), the quality of the amplified blunt PCR product was verified to demonstrate a discrete band at 1kb (**Figure 6.3**). The appropriate fragment was isolated and purified using QIAquick gel extraction kit (Qiagen) and QIAquick PCR Purification kit (Qiagen).

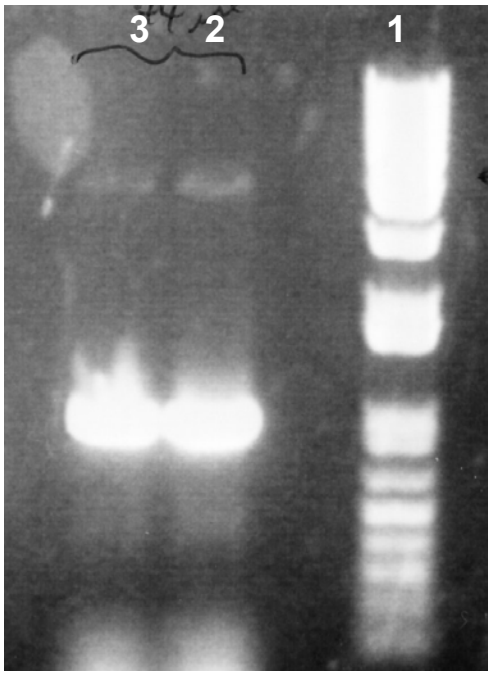


Figure 6.3 Generation of MCP-3 1kb promoter fragment by PCR. The 1kb upstream fragment of the murine MCP-3 promoter, flanked by BglII and HindIII, was amplified using PCR with primers containing a BglII restriction enzyme site and HindIII restriction enzyme site. ***Lane 1***: 1 kb Plus DNA ladder (Invitrogen); ***Lane 2 and 3***:1 kb fragment of MCP-3 promoter.

The blunt-end PCR product was directly inserted into pCR-BluntII-TOPO cloning plasmid vector (3.5kb) as per manufacturer's instruction (Invitrogen, Paisley, UK). This cloning strategy was preferred to direct digestion of the promoter fragment with ligation into pGL3 Basic Vector because the number of bases in the primers used in the PCR (**Figure 6.2**) was too few to ensure effective digestion for direct ligation into the reporter vector. The cloned plasmid was analysed with restriction digest with BglII and HindIII to confirm the presence of the 1kb insert (**Figure 6.4**). The construct is then transformed into competent *E. coli* cells, isolated and resuspended in an appropriate volume of TE buffer. Its concentration was determined by UV spectrophotometer at 250ng/ul.

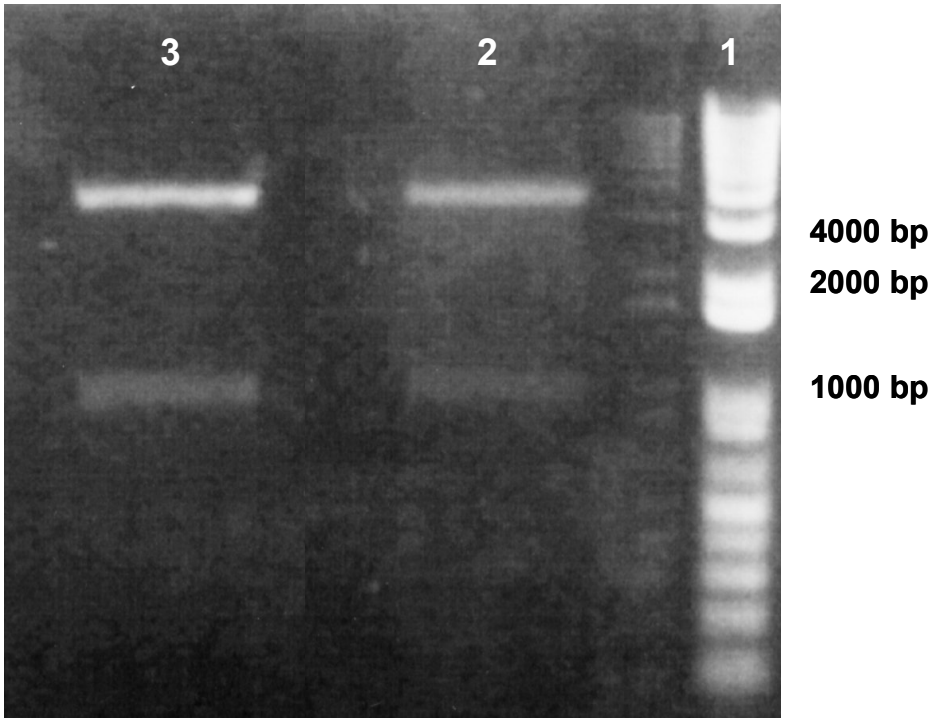


Figure 6.4 Generation of 1 kb promoter-construct with pCR-BluntII-TOPO cloning plasmid vector. Following insertion of the PCR product with the 1kb promoter-construct into pCR-BluntII-TOPO cloning plasmid vector, the cloned plasmid was restriction digested with BglII and HindIII and the 1 kb fragment was isolated (*Lanes 2 and 3*). *Lane 1*: 1 kb Plus DNA ladder. All restriction enzymes were used at 10 U/μg.

6.3.1.3 DNA Ligation for 1kb MCP-3 promoter fragment into pGL3 reporter vector

To clone the 1kb promoter construct into basic pGL3-Luciferase reporter vector (Promega), both TOPO cloning plasmid vector containing the 1kb-MCP-3 promoter construct(250ng/ul) and basic pGL3-plasmid vectors(2.44μg/ul) were digested with *Bgl*II and *Hind*III. The digested fragments were resolved by agarose gel electrophoresis as described in Section **2.3.6** (**Figure 6.5**).

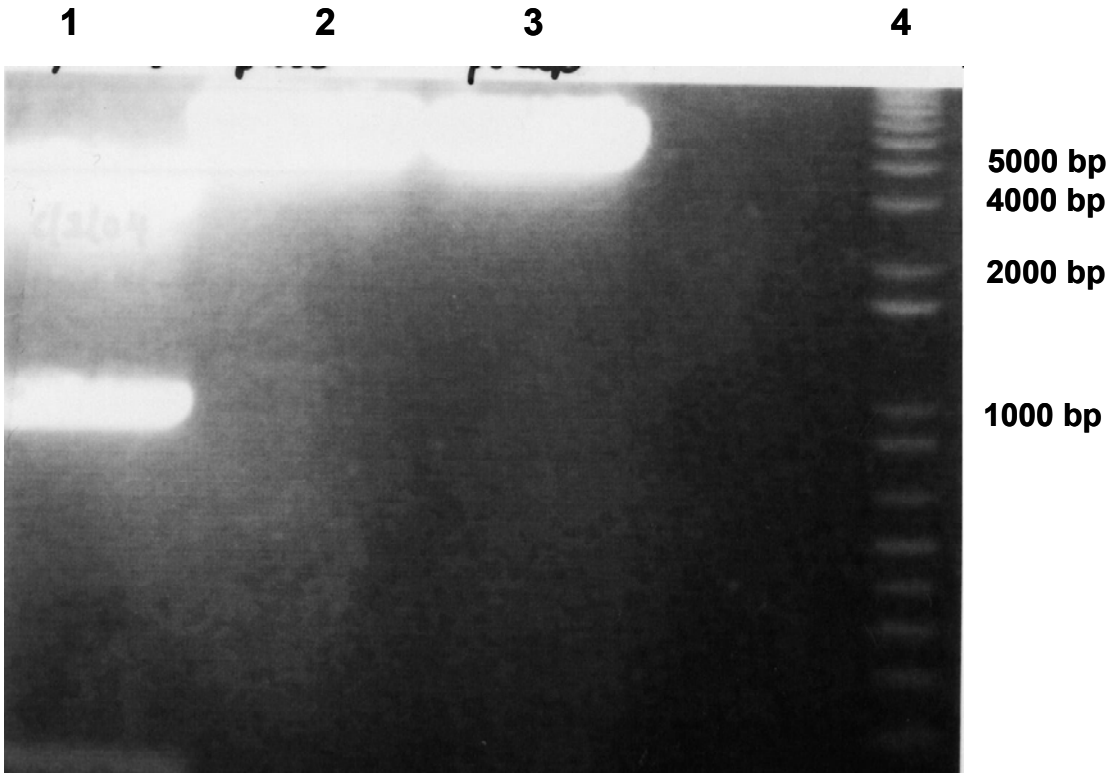


Figure 6.5 Restriction digestion of cloned plasmid with 1kb MCP-3 promoter-construct and pGL3-Basic plasmid vector. *Lane 1*: 1kb MCP-3 promoter with TOPO cloning plasmid vector digested with with BglII and HindIII to yield the 1 kb fragment of MCP-3 and the 3.5kb linearised TOPO cloning plasmid vector, *Lane 2 and 3*: pGL3-Basic plasmid vector linearised with similar restriction enzymes to yield the full-length 4.8 kb fragment. *Lane 4*: 1 kb Plus DNA ladder. All restriction enzymes were used at 10 U/μg.

The appropriate fragment was gel isolated and concentration determined by UV spectrophotometer. The concentration of 1kb MCP-3 promoter was 15ng/μl and for linearised pGL3-Luciferase vector is 50ng/μl. The linearised pGL3 vector and purified 1kb MCP-3 fragment were ligated using T4 DNA ligase (Promega) with equal molar ratios of DNA fragments from pGL3 basic (BglII/HindIII digested) and MCP-3 promoter (BglII/HindIII digested) in 20 μl 10 mM Tris-Cl ph 7.4, 10 mM Mg^{2+} , 1 mM ATP, 1 mM DTT using 1 U T4 DNA ligase (Promega) and 60ng of 1kb MCP-3-promoter fragment and 75ng of linearised pGL3-Luciferase vector, at 16^0C for 16 hours.

Ligation reactions were transformed into DH5α *E.coli* (Invitrogen) and plasmid DNA amplified using DNA Miniprep method (Qiagen). The ligated product was validated by diagnostic restriction digestion with BglII and HindIII, and resolved with agarose gel electrophoresis (**Figure 6.6**). Two other restriction digestion sites, NheI and BamHI were selected to validate the ligated product as shown in **Figure 6.7**.

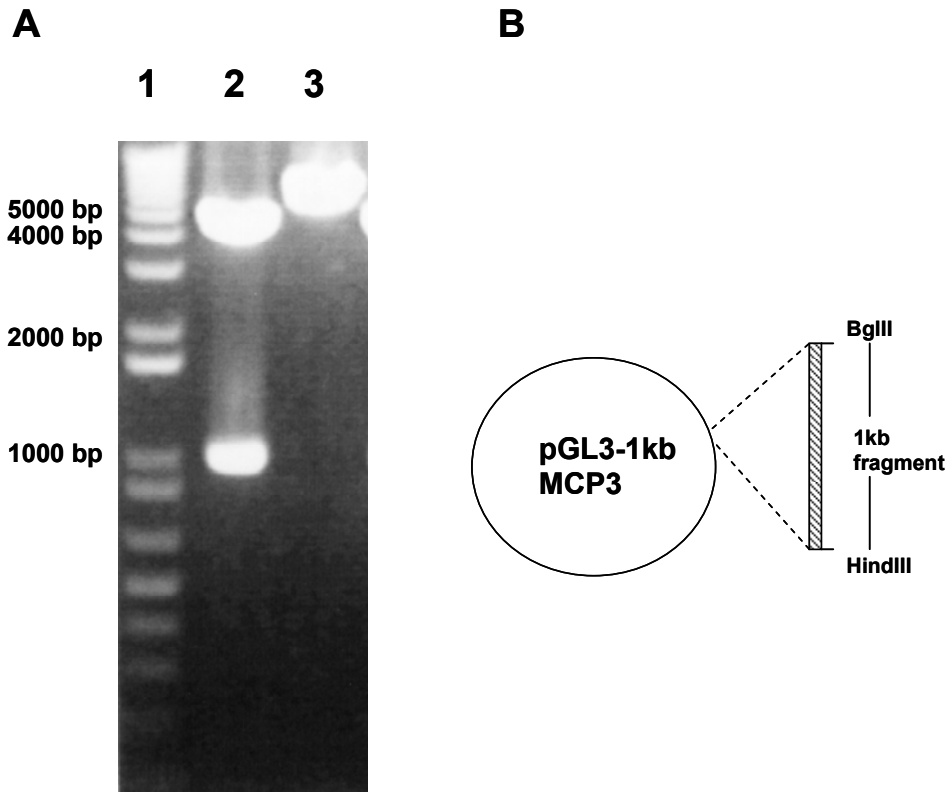
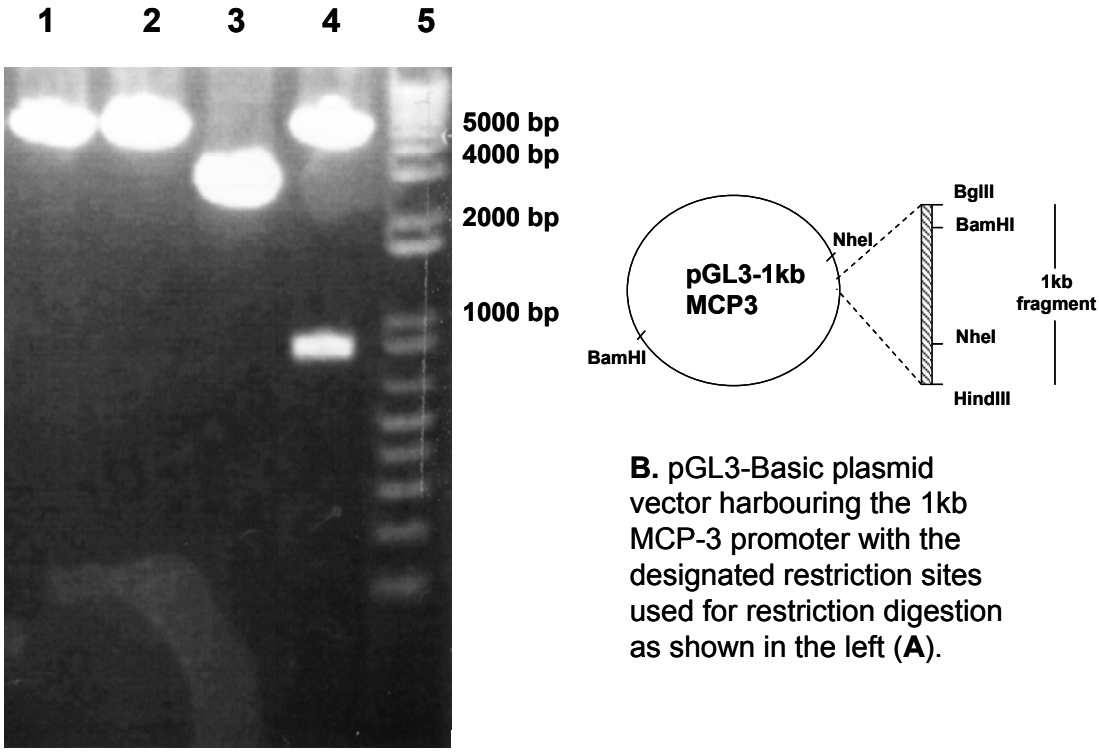


Figure 6.6 A. Diagnostic restriction digestion of ligated pGL3-Basic vector containing the 1kb-promoter fragment. **A.** *Lane 1*: 1 kb Plus DNA ladder. *Lane 2*: Ligated product restriction digested with BglII and HindIII to yield the 1kb MCP-3 promoter fragment and the linearised 4.8kb pGL3-Basic vector. *Lane 3*: Ligated product linearised with HindIII to yield a 5.8kb fragment.
B. pGL3-Basic plasmid vector harbouring the 1kb-MCP-3 promoter construct with designated restriction sites for BglII and HindIII.



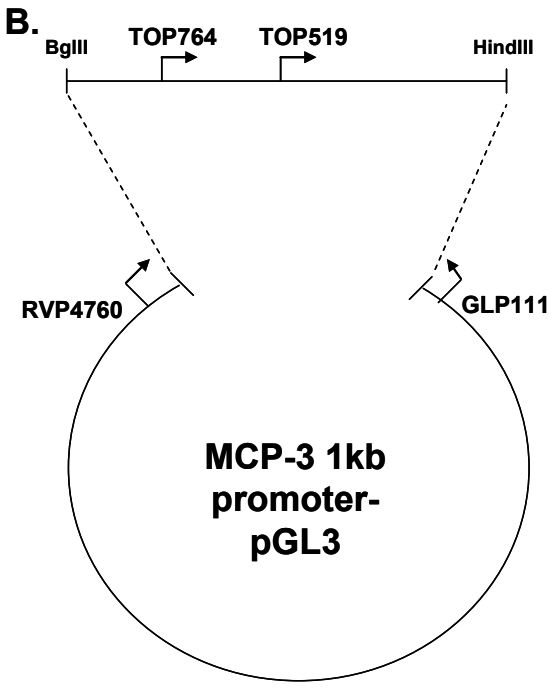
B. pGL3-Basic plasmid vector harbouring the 1kb MCP-3 promoter with the designated restriction sites used for restriction digestion as shown in the left (**A**).

Figure 6.7 A. Diagnostic restriction digestion of ligated pGL3-Basic vector containing the 1kb-promoter fragment. **A.** *Lane 1*: pGL3-Basic vector linearised with BamHI to yield its full-length 4.8kb fragment *Lane 2*: pGL3-Basic vector linearised with NheI to yield its full length 4.8kb fragment. *Lane 3*: Ligated product restriction digest with BamHI to yield two fragments, 2.8kb and 2.9 kb fragments *Lane 4*: Ligated product restriction digested with Nhe1 to yield 2 fragments at 809bp and 4.9kb bands. *Lane 5*: 1 kb Plus DNA ladder.
B. pGL3-Basic plasmid vector containing the 1kb-MCP-3 promoter construct with designated restriction sites for BamHI and NheI.

To verify that the correct promoter fragment has been generated and to confirm the direction of insertion, sequencing analysis with MWG-Biotech using the primers shown in **Table 6.1** were undertaken. Two internal oligonucleotide primers within the 1kb promoter sequence and two external oligonucleotide primers adjacent to the multiple cloning sites of pGL3 reporter vector were used for sequencing analysis. The concentration of pGL3-Basic plasmid vector containing the 1kb MCP-3 promoter was determined with UV spectrophotometer at 3.47μg/ul.

A. Primers	Oligonucleotide	No of bases of good quality sequence
TOP764:	5'-CTG CTG GCT GTT GAA GTC –3'	1035
TOP519:	5'-GCC ACC AGG ATT TAA GAC AGT G-3'	697
GLP111:	5'-CTT TAT GTT TTT GGC GTC TTC CA-3'	986
RVP4760:	5'-CTA GCA AAA TAG GCT GTC CC-3'	886

Figure 6.8 Sequencing of 1kb MCP-3 promoter fragment in pGL3 reporter vector.



A. Oligonucleotide primers used to sequence the inserted promoter fragment in pGL3-Luciferase reporter vector. GLP111: This is used for sequencing counterclockwise upstream of luciferase gene (111bp on pGL3 Basic Vector), RVP4760: This is used for sequencing clockwise across the upstream multiple cloning sites (4760bp on pGL3 Basic Vector). With these two primers, there was an overlap of 695 bp of good quality sequence within the 1kb MCP-3 promoter fragment. For the remaining two oligonucleotide primers: TOP764 and TOP519 at –764bp and –519bp respectively, annealed to the MCP-3 promoter as indicated in **B**, 5' to the transcriptional start site and both primers are used for sequencing clockwise.

6.3.2 Cell culture

Control and transgenic TβRIIΔk fibroblast cell lines were used in the course of the studies presented within this chapter. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, UK) supplemented with 10% fetal calf serum (FCS; Gibco, UK), 100 units/ml penicillin, and 100mg/ml streptomycin, and cultured in a humidified atmosphere of 5% CO_2 in air. The cells were then serum starved with 0.2% FCS for 24 hours, and then stimulated with appropriate recombinant cytokines. Where appropriate, pharmacological inhibitors were added to cultured fibroblasts 45 minutes prior to stimulation with the recombinant cytokines: ERK inhibitor U0126 (20μM; Promega, Southampton, United Kingdom), SB203580 (20μM) and Calphostin (500nM) (all from Calbiochem, La Jolla, CA), Curcumin (20μm; Sigma, St. Louis, MO) and SD-208, inhibitor of the TβRI kinase (1μM, Scios Inc., Fremont CA) and cultured for an additional 24 hours.

6.3.3. Smad3 siRNA transfection studies

To study the effect of TGFβ signalling pathway on TGFβ-mediated expression of MCP-3 protein in fibroblast supernatants, Smad3 siRNA (20 nM and 80 nM, Santa Cruz Biotechnology, Santa Cruz, CA), transfected into cultured fibroblasts stimulated with recombinant TGFβ1, was used to knock down expression of Smad3 protein. As a negative control, non-silencing or control siRNA (80 nM) with non-targetting 20-25 nucleotide siRNA is used, and total Smad3 and GAPDH expression in culture media and cell lysates were used to confirm downregulation of Smad3 and as loading control respectively. Rabbit polycolonal anti-total-Smad3 was obtained from Cell Signalling Technology (Beverly, MA). Experimental procedures were performed as outlined in **2.2.3.4.**

6.4 Results

6.4.1 Upregulation of MCP-3 protein by TGFβ in wildtype murine fibroblast cultures

To investigate the effect of TGFβ on the expression of MCP-3 in murine dermal fibroblasts, confluent dermal fibroblasts were incubated with recombinant TGFβ1. MCP-3 protein was induced in the fibroblast supernatants treated with recombinant TGFβ1 with different concentrations at 4-20ng/ml (**Figure 6.9**). Much interest has focused on the role of the Smad family of proteins in TGFβ signalling in fibroblasts, that the activated TGFβ receptor complex phosphorylates Smad3, which in turn associates with Smad4 to form complexes that determine many of the major effects of TGFβ on transcriptional activation. Therefore, to explore the effect of this canonical Smad signalling pathway on MCP-3 induction by TGFβ, cultured dermal fibroblasts transfected with Smad3 siRNA were incubated with recombinant TGFβ1. As shown in **Figure 6.10**, the increase in MCP-3 protein in the fibroblast supernatants was blunted in the presence of Smad3 siRNA.

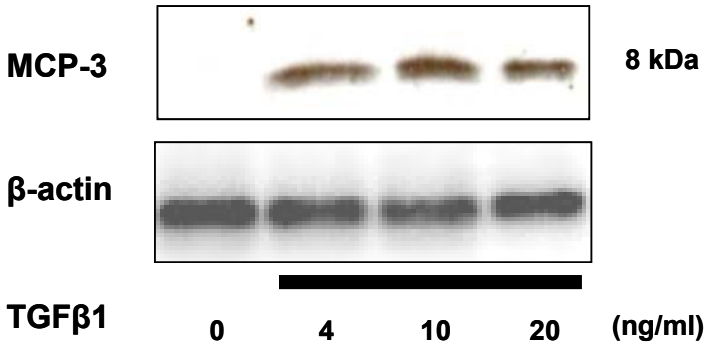


Figure 6.9 Upregulation of MCP-3 protein expression by recombinant TGFβ1 in wildtype murine fibroblast supernatants.

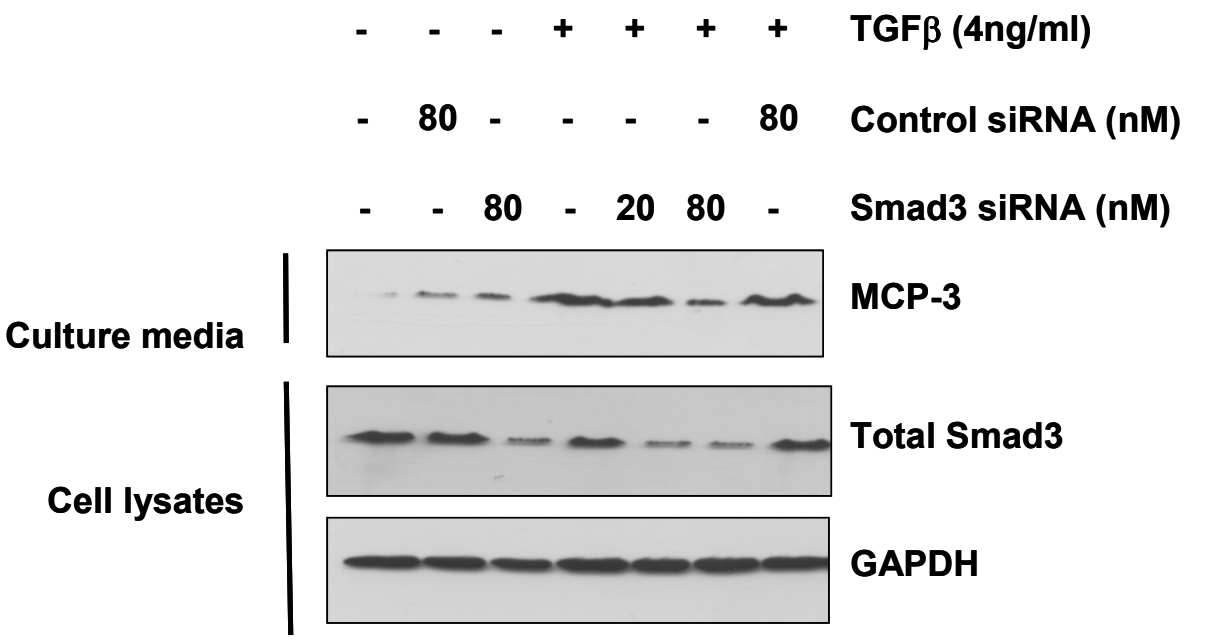


Figure 6.10 Upregulation of MCP-3 protein expression by recombinant TGFβ1 is mediated by TGFβ signalling pathway. Smad3-specific siRNA dose-dependently suppresses TGFβ-induced MCP-3 protein expression towards basal levels in murine fibroblasts without affecting constitutive GAPDH levels. The panels represent MCP-3 (upper), Total Smad3 (middle) and GAPDH (bottom) Western blots. Cell lysate GAPDH was used as a protein-loading control.

6.4.2 Transcriptional activation of MCP-3 promoter by TGFβ in wildtype and transgenic TβRIIΔk fibroblasts

As discussed in Chapter 3, upregulation of MCP-3 transcript and protein in transgenic TβRIIΔk mice provides the first evidence that MCP-3 is regulated by TGFβ. Therefore, to investigate if this induction may be ascribed to transcriptional activation mediated by TGFβ, the response of MCP-3 promoter to recombinant TGFβ1 was examined in wildtype fibroblasts. Recombinant TGFβ1 stimulated the promoter activity of MCP-3 and time-course experiment demonstrated that there is little upregulation at 6-18 hrs with significant upregulation at later time point. Compared to basal level, there is time-dependent activation of MCP-3 promoter by recombinant TGFβ1 with (Mean±SEM, p = 0.01) 95.9±4.5% and 109±6.4% at 24 and 48-hour time-points respectively (**Figure 6.11**).

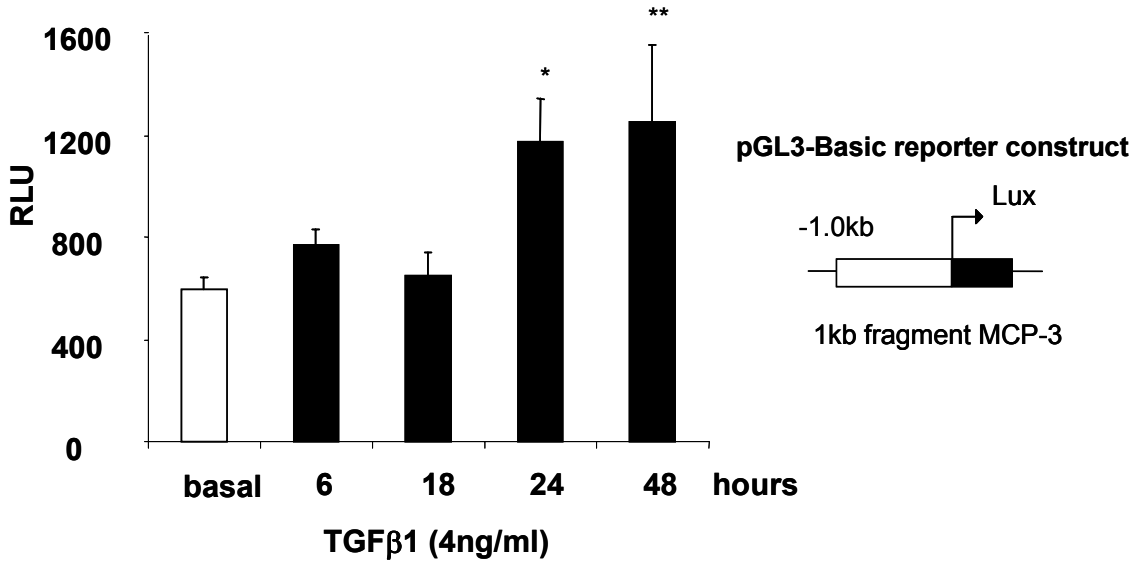


Figure 6.11 Time course for transactivation of the murine MCP-3 gene in transient transfection of neonatal murine fibroblasts is shown as reporter gene expression in 3 independent experiments for the upstream 1kb MCP-3 promoter construct linked to luciferase reporter gene. Assays were performed in triplicate, and the average is shown with the standard error of the mean, (* $p<0.05$, ** $p<0.01$, by Student's unpaired t-test. RLU=Relative Luminescent Units).

Expression studies of TGFβ-regulated PAI-1 and CCN2 (Chapter 3) demonstrated that transgenic TβRIIΔk fibroblasts were partially refractory to recombinant TGFβ1 and that

there is upregulation of MCP-3 in the transgenic fibroblasts. Therefore, to explore whether similar altered TGFβ responsiveness may be observed with MCP-3, the activation of MCP-3 promoter was compared in wildtype with that of transgenic TβRIIΔk murine fibroblasts in the presence or absence of recombinant TGFβ1. Compared to the TGFβ1-dependent activation in wild type cells, there was basal promoter activation by transgenic fibroblasts (157 ± 57 %) but there was no further upregulation by recombinant TGFβ1(**Figure 6.12**).

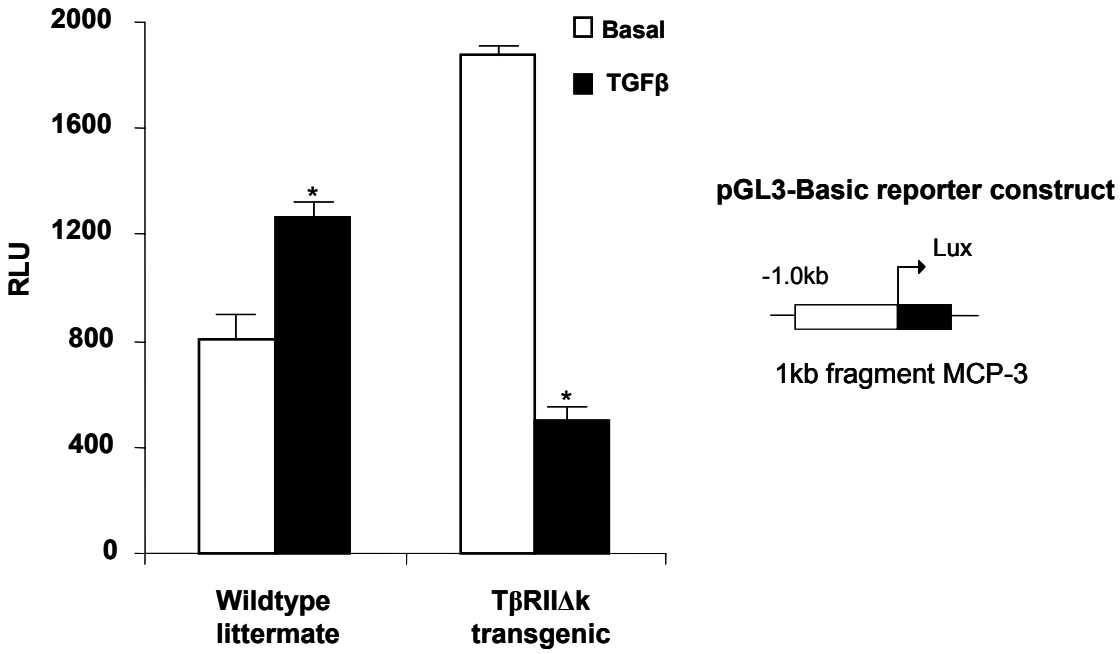


Figure 6.12 Altered TGFβ1-dependent MCP-3 promoter activation in transgenic TβRIIΔk fibroblasts. Neonatal fibroblasts transiently transfected with 1kb-MCP-3 promoter construct were stimulated with TGFβ1 (4ng/ml). Compared to the marked induction of MCP-3 promoter by TGFβ1 in wildtype cells, the transgenic fibroblasts are much less responsive to recombinant TGFβ1 although there is basal activation of the promoter in the transgenic fibroblasts.

6.4.3 Activation of MCP-3 promoter expression by TGFβ via distinct MAPK pathways

To assess the signalling pathways mediating TGFβ1 upregulation of the MCP-3 promoter, a series of pharmacological inhibitors were used. There was significant activation of MCP-3 promoter by recombinant TGFβ1 with 184±15.5% (Mean±SEM % basal expression), p=0.02. SD208 and U0126 reduced this activation by 70.7±9.6%, p=0.03 and 73.2±9.4%, p=0.01 respectively (**Figure 6.13**). There was partial inhibition of the promoter activation with SB203580 and curcumin by 51.5±13.8%, p=0.05 and 38.0±14.8%, p=0.02 respectively. Calphostin also partially abrogated the promoter response by 45.1±14.4%, p=0.01. These results suggest that apart from TGFβ signalling pathways, several key members of the MAPK family, AP-1 and PKC are also necessary for the TGFβ-mediated activation of MCP-3 gene expression in fibroblasts.

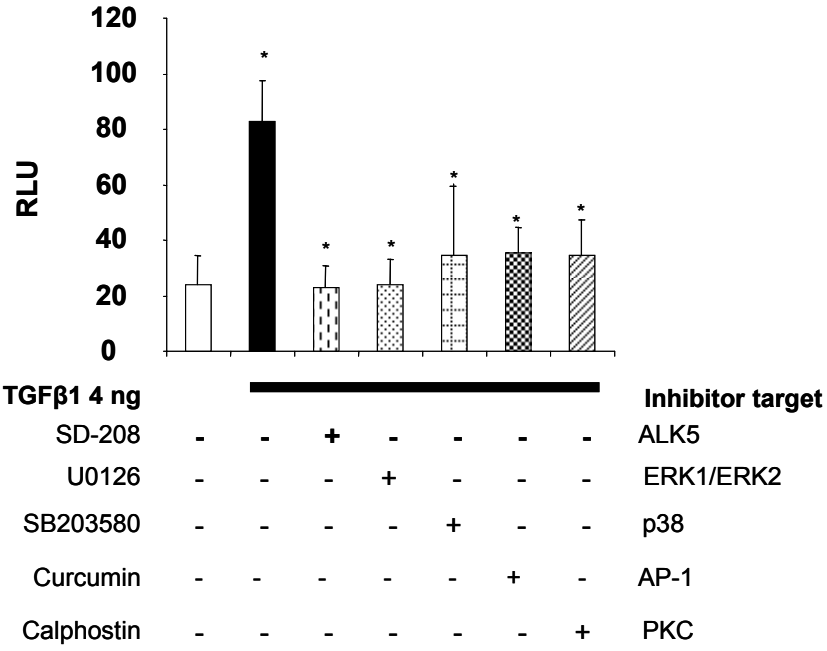


Figure 6.13 Regulation of activation of MCP-3 promoter by TGFβ is demonstrated in 3 independent experiments. Transient transfections of the murine fibroblasts were carried out using the indicated pharmacological inhibitors as described in Methods Section **6.3.2.** (*P<0.05 as determined by Student's unpaired t-test, RLU = relative luminescence units.

6.5 Discussion

Data presented in Chapter 3 demonstrate that MCP-3 is upregulated in a murine model with activated TGFβ-phenotype. This suggests that MCP-3 expression may be dependent on TGFβ and the results presented in this chapter showed that the upregulation of MCP-3 protein and gene in cultured fibroblasts is transcriptionally regulated by TGFβ. This appears to be mediated by recruitment of ALK5 receptor and activation of both the canonical Smad and MAPK signalling pathways

Few studies have been performed on regulation of MCP-3 expression. Several studies have demonstrated a significant association between polymorphisms within MCP-3 promoter and inflammatory diseases including multiple sclerosis(Fiten *et al.*, 1999;Nelissen *et al.*, 2002). However, a more recent study on Korean patients with asthma failed to detect an association between four SNPs including one in the 1kb promoter region and the risk of developing asthma(Park *et al.*, 2005). Other work has highlighted a novel transcriptional regulatory role for β-catenin, a major protein in the Wnt signalling transduction pathway, on MCP-3 promoter expression in dermal fibroblasts. In this study, regulation of MCP-3 expression by activated form of β-catenin appears to involve direct association of the β-catenin complex with a putative T-cell factor/lymphocyte enhancer factor (Tcf/LEF)-binding motif, ATCAAAG in the MCP-3 promoter(Fujita *et al.*, 2000). Because the Tcf/LEF complex also recruits various coactivators or corepressors to modulate transcription, it is conceivable that these associated molecules in combination may determine the function of the complex, and this may be relevant in regulation of MCP-3 expression by TGFβ, another key target gene for Wnt signalling in fibroblasts.

It has been demonstrated that cytokine activation of the chemokines are largely transcriptionally regulated. For example, the induction of MCP-1 expression is mediated in part through two tandem AP-1 sites and an NF-κB site in the proximal promoter of this gene depending on the cell type and stimuli applied(Martin *et al.*, 1997). In contrast, the role of Smad signalling pathway in regulation of chemokine expression has been explored in a small number of studies. Interestingly, a recent study has identified MCP-1 as a target gene in genome-wide analysis of TGFβ-modulated genes in endothelial cells(Wu *et al.*,

2006). The same group also demonstrated that TGFβ-mediated induction of MCP-1 is Smad-dependent and that this involves direct binding of Smad3 and Smad4 to the Smad-binding element (SBE) with CAGAC sequence on the MCP-1 promoter in human vascular endothelial and hepatoma HepG2 cells(Ma *et al.*, 2007). Putative transcription factor binding sites, AP-1 (-37 bp), SBE, CAGACA (-732 bp) and Sp1 (-745 bp) on the 1kb MCP-3 promoter have been identified using TFSEARCH (Searching Transcription Factor Binding Sites V1.3, putative score>0.85(Murakami *et al.*, 1997) and these may contribute to the transcriptional activation of MCP-3 by TGFβ. The moderate responsiveness of the 1kb MCP-3 promoter to recombinant TGFβ1 in murine fibroblasts suggests that additional TGFβ responsive regions may be responsible, and a possible explanation for this is the presence of an additional putative SBE identified at -2.2kb upstream to the 1kb promoter. It is also well-known that Smad proteins regulate transcription in collaboration with other factors(Massague *et al.*, 2005), but it is unclear what factors may work in concert with the Smad proteins to modulate MCP-3 expression. This may potentially involve direct DNA binding or through interaction with coactivators (eg. p300/CBP) or corepressors (eg. TG-interacting factor, SnoN and c-Ski) or sequence-specific transcription factors (eg. AP-1, TFE3, Sp1, Evi-1 and GATA-3). The role of Smad-dependent signalling pathways and TGFβ in chemokine expression was also recently explored in human astrocytic lines in regulation of MCP-1 transcription. In this study, whilst Smad-3 stimulates transcription of MCP-1, Smad-4, on the other hand, had no effect on the basal activity of the MCP-1 promoter, but it showed the ability to decrease both Smad-3-mediated transcription of the MCP-1 promoter(Abraham *et al.*, 2003). Therefore, the intracellular concentrations of Smad3 and Smad4 can modulate expression of MCP-1 in these cells, and this interaction may also be relevant in the transcriptional activation of MCP-3 by recombinant TGFβ1. Interestingly, TGFβ was reported to inhibit MCP-1 expression in macrophages via an antagonistic effect of Smad3 on AP-1 activity and this discrepancy highlights the cell type-specific regulation of CC chemokines by TGFβ(Feinberg *et al.*, 2004).

It has recently been demonstrated that histone acetylation at the upstream regulatory regions for MCP-1 is associated with the assembly of Sp1 is necessary for PDGF and TNFα induced expression of MCP-1(Ping *et al.*, 1999;Ping *et al.*, 2000). This will in turn

facilitate stabilisation of the assembly of transcription factors at both proximal and distal regulatory regions to drive chemokine expression. It is plausible that similar mechanism may underlie the induction of MCP-3 by TGFβ.

The basal upregulation of MCP-3-promoter in TβRIIΔk fibroblasts is consistent with the basal induction of other TGFβ-regulated proteins including PAI-1 and CTGF as discussed in Chapter 3. However, the suppressive effect of recombinant TGFβ1 on the upregulation of MCP-3 promoter parallels the observation reported with altered response of TβRIIΔk fibroblasts to other TGFβ-regulated reporter constructs including Col1a2 and fibronectin(Denton *et al.*, 2005). For some of these genes, differential effects were observed with modest induction of PAI-1 receptor activity but the basal activity for other promoter constructs (3TP, Col1a2 and fibronectin) was suppressed in transgenic fibroblasts. The apparent paradoxical downregulation of these TGFβ responsive genes including MCP-3 in transgenic fibroblasts at these later time points to TGFβ1 is explained by the partial regulation of the fibroblast-specific expression cassette in the transgene by exogenous TGFβ. In addition, the transgene in this transgenic model functions as a dominant negative inhibitor of TGFβ signalling at high expression levels in the presence of recombinant TGFβ1.

The data in this chapter suggest that induction of ALK5 by TGFβ is important in transcriptional activation of MCP-3 expression. Whilst ALK5 is well-established to be closely associated with TGFβ signalling pathway, there is growing evidence that some of the downstream effects mediated by ALK5 may be independent of Smad2/3(Pannu *et al.*, 2007). For example, constitutive overexpression of CTGF in SSc fibroblasts is Smad2/3 independent and blockade of Smad signalling targeting ALK5 kinase did not normalise CTGF levels in SSc fibroblasts(Holmes *et al.*, 2001;Chen *et al.*, 2006). In addition, in vitro experimental model on angiogenesis demonstrated that TGFβ and ALK5 pathways have a distinct gene expression profiling on endothelial cells(Wu *et al.*, 2006).

Overexpression of MCP-3 in inflammatory diseases such as asthma has been demonstrated to be mediated by MAPK signalling pathways including p38, ERK and JNK in human airway smooth muscle cells(Wuyts *et al.*, 2003). In addition, AP-1 has been demonstrated to regulate MCP-3 mRNA and protein secretion from cultured cervical fibroblasts, suggesting the involvement of AP-1 in the cyclic stretch-associated signal transduction(Abraham *et al.*, 2003;Takemura *et al.*, 2004). The signalling pathways that regulate chemokine activation appear to be largely cytokine-specific and tissue-specific. Recent studies using cultured fibroblasts infected with retroviruses encoding TAK1K63W, an inactive mutant of the protein kinase, TGFβ activated kinase (TAK1) demonstrated that constitutive and inducible expression of MCP-3 is strikingly dependent on MAPK Jun and p38, but not PI3K signalling pathways(Thiefes *et al.*, 2005). The data in this chapter showed that similar signalling pathways are important in MCP-3 gene activation by TGFβ in fibroblasts.

6.6 Summary

Overall the experimental data presented confirms that TGFβ induces MCP-3 expression via elements within the proximal 1kb promoter fragment upstream to the transcription start site in which several putative transcriptional factor binding sites including SBE have been identified. The activation of MCP-3 promoter construct by TGFβ appears to be mediated by recruitment of ALK5 receptor and is dependent on activation of both the canonical Smad3 and MAPK signalling pathways. In addition, the lack of responsiveness of the MCP-3 promoter construct to exogenous TGFβ in transgenic TβRIIΔk strain is entirely consistent with the previously reported refractoriness of similar TGFβ-regulated genes in this profibrotic murine model.

CHAPTER 7: INTERPLAY BETWEEN TGFβ AND MCP-3 IN FIBROSIS

7.1 Introduction

In the pathogenesis of fibrosis it is likely that a number of cytokines or growth factors act in concert to promote a fibrotic microenvironment and lead to the development of a profibrotic population of fibroblasts. There is an increasing body of evidence to suggest a potentially important regulatory loop between chemokines and TGFβ in inflammatory and fibrotic diseases. TGFβ is a multifunctional cytokine that regulates the growth, differentiation and function of immune and non-immune cells. A major effect of TGFβ on mesenchymal cells is its activation of ECM deposition via induction of collagen types I, III, IV, VII and X, fibronectin and proteoglycans. This role is further enhanced by its inhibitory effect of matrix degradation, decreasing synthesis of proteases and increasing levels of protease inhibitors. Data from previous chapters have demonstrated MCP-3 as a potential profibrotic mediator. In addition, overexpression of MCP-3 was demonstrated in a murine model of fibrosis with a TGFβ-dependent phenotype, strongly suggesting that there is an important crosstalk between these two important mediators. Since intracellular signalling pathways activated by these ligands may be similar and there may be regulation of the level of activity of each ligand by the other it is plausible that interplay between them is highly relevant.

The central role of a canonical Smad-dependent signalling pathway for TGFβ is firmly well-established but it is also recognised that non-Smad pathways may drive fibrosis, including members of the MAPK family. The identification of non-Smad signalling pathways preceded the discovery of Smads (Libby *et al.*, 1986). MAPK constitute a family of serine/threonine kinases that are central in the signalling cascades regulating a wide array of intracellular processes such as cell growth, differentiation, apoptosis and cellular responses to external stress signals. In response to these signals, they activate nuclear transcription factors such as c-jun, Elk-1 and c-Fos. Categorised into five subfamilies; $MAPK^{ERK1/2}$, the $MAPK^{p38}$, $MAPK^{ERK3/4}$, $MAPK^{ERK5}$ and the $MAPK^{jnk}$, the specific MAPK gives its name to the pathways that utilise them i.e. $MAPK^{ERK1/2}$ is referred to as the ERK1/2 pathway (Moustakas & Heldin, 2005). These protein kinases share 60-70%

identity but differ in the sequence and size of their activation loop, and are activated by different stimuli, and each MAPK subclass consists of several isoforms and members, which often have distinct functions.

Activation of the MAPK signalling cascade involves a series of three protein kinases consisting of a MAPK that is activated by the dual phosphorylation of Thr and Tyr residues in a TXY motif by specific upstream MAPK kinases (MKKs), which in turn are phosphorylated by MAPK kinase kinases. The TXY motif is unique to each of the MAPK subclasses, where X is Glu, Pro and Gly in ERK, JNK and p38 MAPK respectively. In general, ERK1 and ERK2 also known as p44 and p42 MAPKs are prototypically activated by mitogenic stimuli and growth factors, whereas JNK and p38 MAPK are activated predominantly by environmental stresses such as osmotic changes, ultraviolet light, heat shock and inflammation. In many cell types TGF-β has been shown to activate ERK1/2, p38 and JNK, although the link between activated receptor and cytoplasmic effector in some cases remain to be determined (Derynck & Zhang, 2003;Javelaud & Mauviel, 2005). In addition MAPK activation by TGF-β is cell-type dependent (Abecassis *et al.*, 2004;Engel *et al.*, 1999;Leask *et al.*, 2003).

Physiologically non-Smad TGF-β activated pathways elicit downstream intracellular effects in at least three ways. Firstly, direct modification of Smad function in which phosphorylation of the linker region in Smad2 and Smad3 by ERK has both positive and negative effects on Smad activity (Hayashida *et al.*, 2003;Kretzschmar *et al.*, 1999). For example, ERK, a MAPK family member, phosphorylates serine residues in the linker regions of Smad1-3 and ERK inhibition reduces TGFβ-stimulated Smad phosphorylation as well as collagen biosynthesis in mesangial cells, suggesting that ERK activation is necessary for an optimal response to TGFβ(Hayashida *et al.*, 2003); whereas the p38 substrate MSK1 kinase promotes the association of Smad3 with the co-activator p300 (Abecassis *et al.*, 2004). In contrast, it has been reported that ERK1/2 activation has a potent suppressive effect on type I and III collagen expression by normal human skin fibroblasts(Reunanen *et al.*, 2000). A second mechanism involves functional modulation by Smads. For example, JunB is expressed at low levels in fibroblasts but is transcriptionally

induced by TGF-β via Smads and in turn promotes TGF-β activation of COL1A2 and PAI-1 (Chang & Goldberg, 1995) Thirdly, activation may be independent of Smads with activation by TGF-β receptors directly. For example several TGF-β responsive extracellular matrix genes and matrix regulatory enzymes such as Urokinase-type plasminogen activator receptor and fibronectin genes are regulated by MAPK pathways independent of the Smad signalling pathway (Yue *et al.*, 2004;Hocevar *et al.*, 1999). A schematic overview of the canonical and non-canonical TGFβ signalling is shown in **Figure 7.1**.

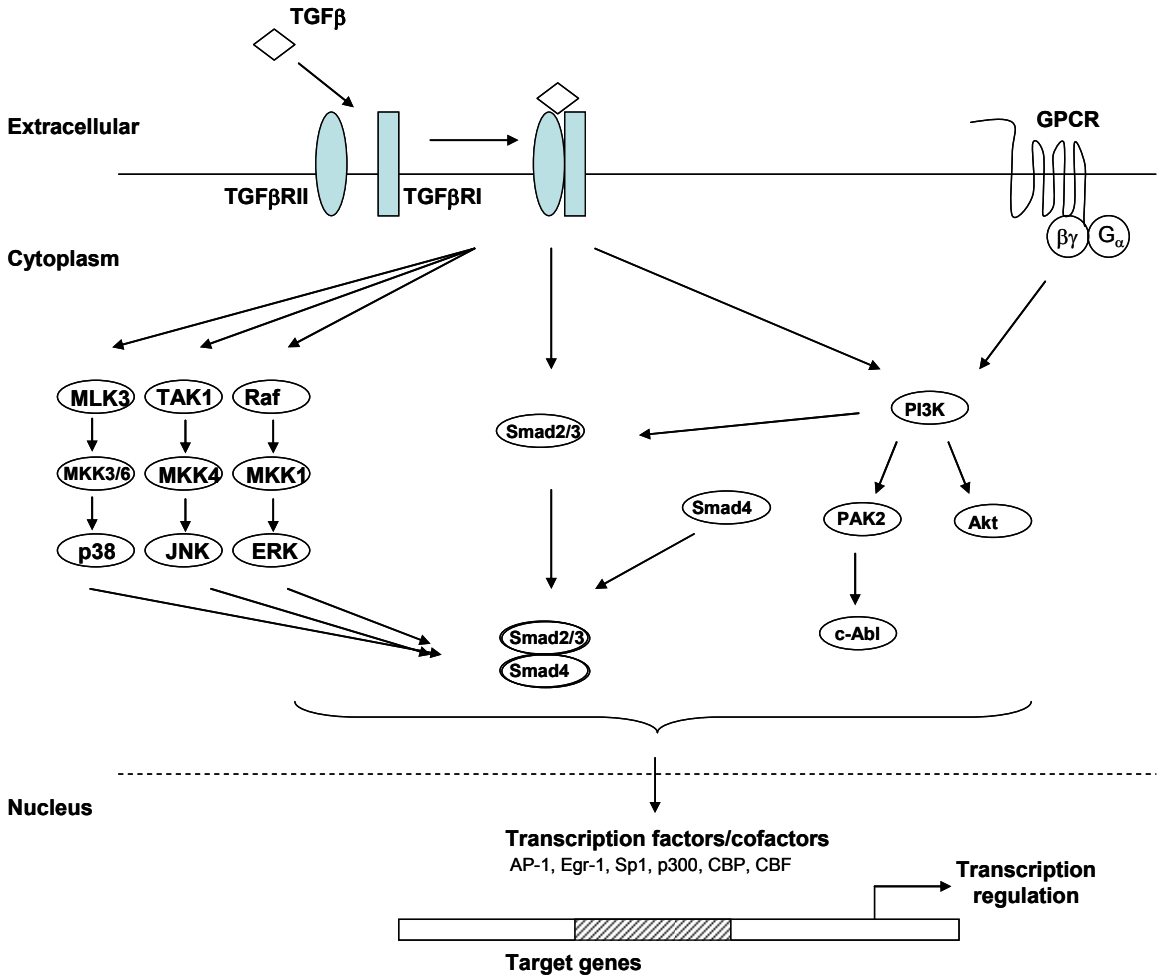


Figure 7.1 Schematic representation of the TGFβ signalling cascade. Modulation of the Smad canonical signalling through TGFβRI by additional MAPK and PI3K pathways is essential for most cellular responses to TGFβ. Initiation of signalling requires the binding of TGFβ to TGFβ receptor type II, resulting in the recruitment and phosphorylation of TGFβ receptor type I to produce a heteromeric complex that activates downstream signalling pathways. Activated TGFβRI then recruits and phosphorylates R-Smad such as Smad2 and Smad3, leading to the association with Smad4 and translocation of the formed heterocomplex into the nucleus. TGFβ also interacts with MAPK (p38, JNK and ERK) whereby TGFβ is able to activate MAPK signalling but also be modulated by it through phosphorylation of the Smad linker domain. PI3K which is activated by GPCR may also mediate TGFβ signalling via Smad2 phosphorylation. The translocated heterocomplex binds to the target gene with other transcription factors including Sp1 and cooperates with p300/CBP to regulate gene expression.

More recently, other signalling pathways besides the MAPK have also been shown to mediate TGFβ signalling such as the PI3K pathways. PI3Ks phosphorylate inositol-containing lipids at the D-3 position of the inositol ring. They are divided into three classes in mammalian cells. Class III PI3Ks produce phosphatidylinositol (PtdIns)-3-P, which is constitutively present in all cells. Class I and class II PI3Ks can utilise PtdIns, PtdIns-4-P and PtdIns-4,5-P_2. Of all the PI3K members, class I is the best characterised and they exist as heterodimers of a 110-kDa catalytic subunit (p110α, p110β, p110δ, and p110γ) and an adaptor/regulator subunit (p85α, p85β, p55, and p101)(Cantley, 2002). Unlike other PI3K members, PI3Kγ is activated by GPCRs via binding of the βγ subunits of G proteins. Following PI3K activation, PIP_3 recruits the phosphoinositide-dependent kinase (PDK)-1 and Akt/PKB, bringing these proteins into proximity at the plasma membrane where Akt is phosphorylated on Thr^{308} by PDK-1(Cantley, 2002). This is followed by phosphorylation at Ser^{473}. Once activated, Akt leaves the plasma membrane to phosphorylate intracellular substrates. Akt has also been shown to translocate the nucleus where it can phosphorylate transcription factors(Toker, 2000).

Recently Bakin et al. have shown that the inhibition of PI3K blocks TGFβ1–induced Smad2 phosphorylation, suggesting that Smad proteins are potential targets of the PI3K pathway(Bakin *et al.*, 2000). In fact, the inhibition of PI3K was shown to result in a redistribution of Smad anchor for receptor activation (SARA), which directly interacts with Smad2/3 and functions to recruit Smad2 for phosphorylation to the activated TGFβ receptor complex(Tsukazaki *et al.*, 1998), and the attenuation of both TGFβ-induced Smad2 phosphorylation and transcriptional activation(Itoh *et al.*, 2002). Furthermore, the basal activity of PI3K was shown to be necessary for COL1A2 mRNA stabilization and to be indispensable for the establishment of the constitutive activation of TGFβ/Smad3 signalling in SSc fibroblasts(Asano *et al.*, 2004b). In addition, PI3Kγ when expressed in leucocytes and vascular endothelium is important in chemotaxis. Therefore, in response to GPCR agonists, including chemokines, PI3Kγ coordinates the leucocyte transmigration across the endothelium and the absence of PI3Kγ has been shown to confer resistance to the development of inflammatory pathologies including lung injury and airway inflammation(Hirsch, 2006;Medina-Tato *et al.*, 2007). In addition, these non-Smad

signalling signalling pathways may be activated by other growth factors including epidermal growth factor, hepatocyte growth factor, oncogenic Ras, and Angiotensin II. These will therefore act in concert to modulate the TGFβ/Smad signalling response.

7.2 Aims

Overexpression of MCP-3 in both human and animal models of fibrosis, and the identification of MCP-3 expression in TβRIIΔk model led to the hypothesis that interaction between MCP-3 and TGFβ may be important in regulation of fibrotic response. Thus to investigate this hypothesis that the interplay between these two mediators may have in modulation of the signalling response in the fibrotic microenvironment, I proposed to investigate

1. To examine the effect of MCP-3 on TGFβ signalling pathways,
2. To examine the effect of MCP-3 and TGFβ on collagen expression and its regulatory pathways,
3. To investigate the fibroblastic gene response to MCP-3 and TGFβ.

7.3 Methods

7.3.1 Cell culture

7.3.1.1 Cell lines

All primary dermal fibroblast cells were maintained as described in Section **2.2.1**. For experiments, cells were used between passage 2 and 5.

7.3.1.2 Fibroblast conditioned media

Dermal fibroblast cell lines were maintained in DMEM (Invitrogen, UK) supplemented with 10% fetal calf serum (Gibco BRL) until fully confluent. Media was replaced by DMEM (Invitrogen, UK) containing 0.2% FCS (Sigma, UK) supplemented with L-glutamine, penicillin G (100 U/ml) and streptomycin sulphate (100 μg ml^{-1}) (Invitrogen, UK) and the conditioned media (CM) collected 24 h later.

7.3.2 Preparation of RNA and Northern blot Analysis for Collagen expression

Total RNA was isolated from wildtype murine fibroblasts treated with recombinant MCP-3 and TGFβ using TRIzol (Invitrogen) according to the manufacturer's instructions. Levels of specific transcripts were determined by Northern blotting, following separation of RNA on 1% agarose/formaldehyde gels and capillary transfer to Hybond N+ membrane (Amersham Biosciences). Filters were hybridised and probed with cDNA fragment specific for conserved region of the pro-α2(I) collagen mRNA as previously described(Shi-Wen *et al.*, 2000). 18 S rRNA band intensity was used to confirm equal gel loading. Probes were labelled with [α-^{32}P]dCTP to a specific activity of 10^9 dpm/mg, using the Megaprime™ random priming method (Amersham Biosciences). Levels of transcripts were determined from signal intensity measured by autoradiography.

7.3.3 Analysis of gene expression by microarray

To determine the differential fibroblastic response to treatment with MCP-3 and TGFβ1, expression profiling was conducted as described in Section **2.3.9**. Dermal fibroblasts were cultured to confluence. Media were changed and exposed to 200ng/ml of recombinant MCP-3, 4ng/ml of recombinant TGFβ1, both factors together and serum-free culture medium for four hours. At the end of the treatment period, total RNA was harvested as

described in Section **7.3.2**. Each pooled RNA sample was applied to the mouse Atlas 1.2 array as outlined in Chapter 2 and experiments were performed in parallel. Differential gene expression was assessed using AtlasImage software (ClonTech) according to manufacturer's protocol.

7.3.4 Smad3 siRNA transfection of cultured fibroblasts

To examine whether Smad signalling pathways may regulate the crosstalk between MCP-3 and TGFβ in fibroblastic collagen expression, a series of protein expression analyses were undertaken using cultured fibroblast supernatants pre-treated with recombinant MCP-3. For these experiments, wildtype dermal fibroblasts were transfected with Smad3 and control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) following manufacturer's instructions as discussed in Section **2.2.3.4**. A nonsilencing siRNA with a scrambled sequence that will not lead to degradation of Smad3 was used as a negative control.

7.3.5 Protein expression

All antibodies were as described in Section **2.4.3**, and all protein extraction and western blotting methodologies performed as described in Section 2.4.3, unless stated otherwise. For indicated experiments on activation of Smad-independent signalling pathways by recombinant MCP-3 and TGFβ1 at various time point intervals, antibodies against phospho-p38 and phospho-Akt (Cell Signalling Technology, Beverly, MA) were used and membranes were incubated with anti-phospho-p38 and anti-phospho-Akt (Ser473) monoclonal antibody (1:1,000) respectively overnight at 4°C. As a loading control, immunoblotting was also performed using antibodies against total p38 and total Akt (1:1,000) respectively.

7.3.6 Protein Kinase inhibitors

The following inhibitors were used for this study: ERK inhibitor U0126 (20μM; Promega, Southampton, United Kingdom), SB203580 (20μM), LY294002 (50μM), Calphostin (500nM) (all from Calbiochem, La Jolla, CA) and cultured for an additional 24 hours in the presence or absence of recombinant chemokine, MCP-3 (200ng/ml) and recombinant TGFβ1 (4ng/ml). Reporter gene activity was measured by luminometry (Turner Designs,

Sunnyvale, CA) using luciferase and β-galactosidase assays (Tropix Inc. Bedford, MA) according to the manufacturers' instructions. Values given are means ± standard errors of triplicate assays from three individual experiments.

7.4 Results

7.4.1 Activation of TGFβ-regulated promoter constructs, 3TP-Luciferase and COL1A2-LacZ in wildtype murine fibroblasts

To determine whether MCP-3 could activate TGFβ-regulated promoter-reporter construct, reasoning that this could arise as a result of direct activation of signalling intermediates that are also regulated by TGFβ or alternatively by secondary induction or activation of TGFβ ligand by MCP-3. **Figure 7.2** demonstrates consistent dose-dependent activation of TGFβ-responsive promoter 3TP-Luciferase by recombinant MCP-3 (128 ± 21.3%) 200 ng and (148 ± 20.5 %) 400 ng compared to (174 ± 39.6) in the presence of TGFβ 4ng/ml (mean ± SEM, % Basal).

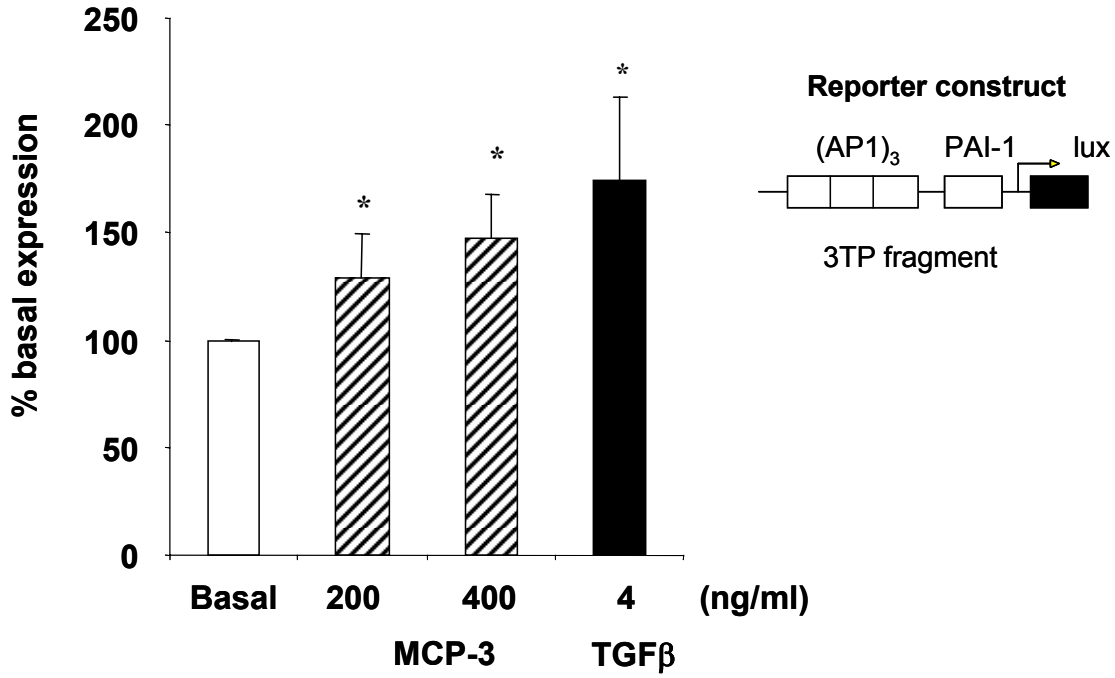


Figure 7.2 Activation of TGFβ-regulated pathways by MCP-3
Upregulation of transcriptional activity of 3TP-Luciferase reporter gene by MCP-3 in transient transfection. Values are the mean and SEM of triplicate samples and are representative of 3 independent experiments. *p<0.05 vs. basal cells.

Similarly, as discussed in Chapter 4, recombinant MCP-3 upregulated pro-α2(I) promoter-reporter construct (COL1A2-LacZ) in dermal fibroblasts (227 ± 91%, p=0.04). Activity of this construct has been shown to reflect transcription of the endogenous COL1A2 gene and is also upregulated by TGFβ. Since TGFβ is a potent stimulator of ECM gene expression *in vitro*, the effect of anti-TGFβ antibody and TGFβ receptor blockade in modulating this activation of collagen reporter gene expression was examined to assess whether upregulation might be occurring via TGFβ. As shown in **Figure 7.3**, the collagen gene activation, in the presence of ID11 and SD-208, by recombinant MCP-3 was significantly reduced by 39.5± 11.3% (p=0.04) and 61.5± 16.5% (p=0.02) respectively. Together these results suggest that MCP-3 has an important role in mediating TGFβ-regulated pathways and its effect on ECM gene expression is also regulated by TGFβ.

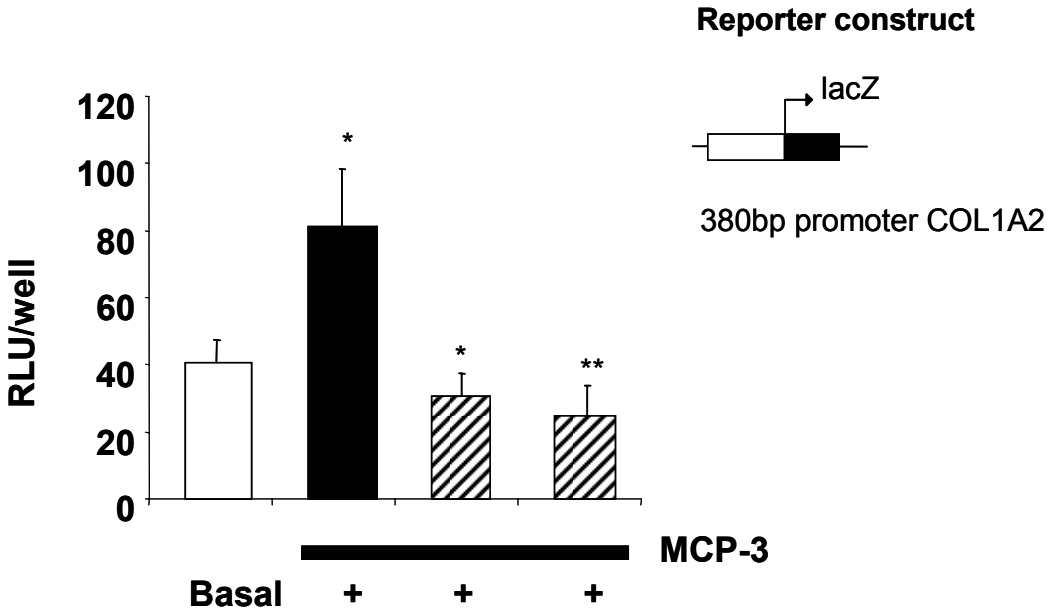


Figure 7.3 TGFβ-dependent activation of COL1A2 gene expression by MCP-3 in wildtype murine fibroblasts
Transactivation of the human proα2(I) collagen (COL1A2) gene reporter by MCP-3 via a TGFβ-dependent mechanism is shown. Inhibitors of TGFβ and its cognate receptor, ALK5 were used in transient transfection in 3 independent experiments for the minimal promoter. * $p<0.05$, ** $p<0.01$ as determined by Student's unpaired *t*-test. RLU=relative luminescent units.

7.4.2 Upregulation of Collagen Type 1 transcript and protein by MCP-3 and TGFβ

In parallel experiments, a time-course analysis of Col1a2 mRNA and protein levels from wildtype murine fibroblasts stimulated with recombinant MCP-3 and TGFβ1 were determined using Northern blot and Western blotting gel electrophoresis.

As demonstrated in **Figure 7.4**, in response to recombinant MCP-3, there was upregulation of collagen transcript peaking at 8 hours followed by maximal protein expression at 48 hours. Similar pattern was observed with recombinant TGFβ1 with maximal collagen mRNA expression at 8 hours sustained up to 24 hours with maximal protein expression at 48 hours.

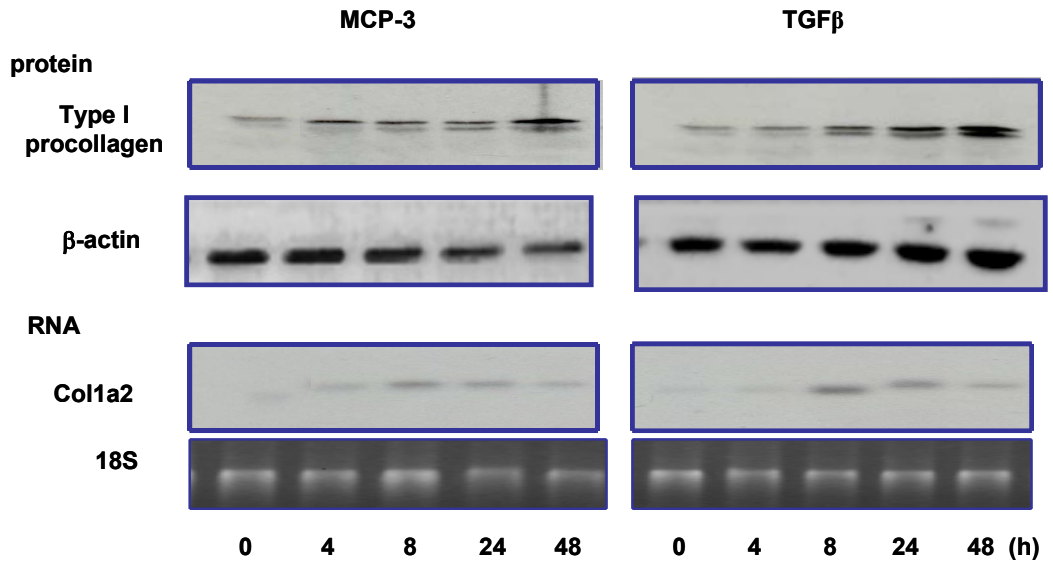


Figure 7.4 Time course induction of type I procollagen by MCP-3 and TGFβ in wildtype murine fibroblasts.
Western blot analysis of dermal fibroblast supernatants after stimulation by recombinant murine MCP-3 (200ng/ml) and TGFβ1 (4 ng/ml) demonstrates induction of pro(I) collagen (Top panel). Blots were also probed with an anti-actin antibody to establish that lanes were equally loaded. Bottom panel demonstrates Northern blot analysis with early induction of Col1a2 mRNA in response to MCP-3 and TGFβ1 in wildtype fibroblasts.

7.4.3 Regulation of COL1A2 expression by MCP-3 and TGFβ

Having established that TGFβ signalling pathway may mediate the induction of collagen expression by MCP-3, this was further explored using siRNA targeting one of the key mediators in the canonical TGFβ signalling pathway, Smad3 in murine fibroblasts. Consistent with the earlier observation in **Figure 7.3**, type I procollagen expression was increased with recombinant MCP-3 (200ng/ml) and in contrast to the fibroblasts transfected with the non-silencing siRNA, there is dose-dependent attenuation in collagen expression with Smad3 siRNA (20-80nM) transfection. This suggests that MCP-3-induced collagen secretion is partially mediated by Smad3-dependent signalling (**Figure 7.5**).

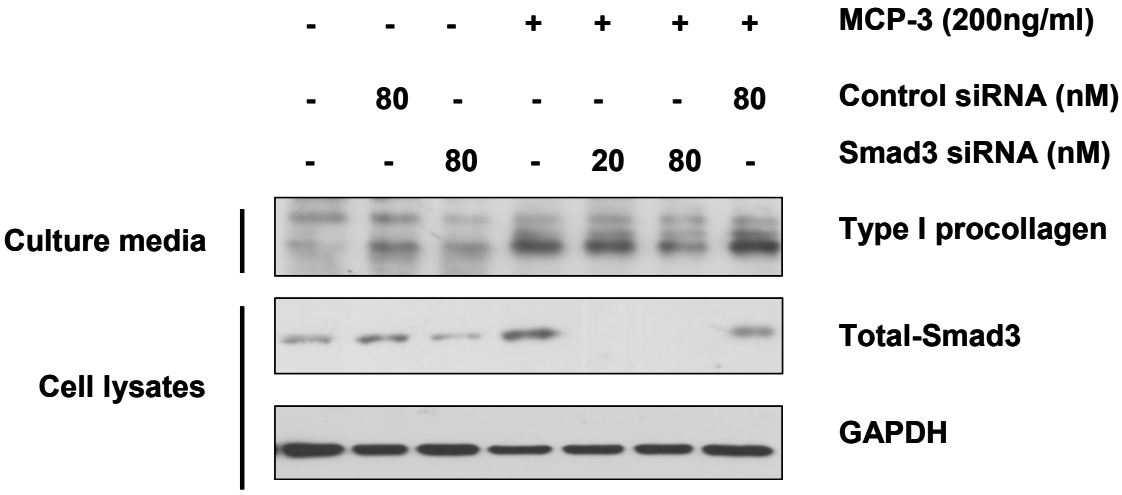


Figure 7.5 Activation of type I procollagen by MCP-3 via canonical Smad pathway
MCP-3 induced type I procollagen expression is dose-dependently reduced by Smad3 siRNA in murine dermal fibroblasts. Cells were transfected with control (80 nM) or Smad3 siRNA (20 and 80 nM) for 24 hours and then incubated with 200 ng/ml MCP-3 in fresh serum-free media for 24 hours. Total Smad3 and GAPDH (in whole cell lysates) and type I procollagen (in culture media) expression were analysed by Western blotting. The results are representative of three independent experiments.

7.4.4 Activation of COL1A2 promoter expression by MCP-3 and TGFβ is mediated by distinct MAPK and PI3K signalling pathways

To determine if there are distinct patterns of activation of collagen gene expression for MCP-3 and TGFβ, a series of pharmacological inhibitors were used following treatment of dermal fibroblasts for 24 hours. A series of independent experiments demonstrated that recombinant MCP-3 and TGFβ1 increased the transactivation of COL1A2 promoter with a mean (±SEM) change above baseline levels of 53.5 ± 6.1%, = 0.005 and 43.4±14.6%, p=0.04 respectively (**Figure 7.6**). This upregulation was inhibited by SB203580 by 64.0±7.7% and 50.7±6.5% (Mean±SEM%, p=0.05) with recombinant MCP-3 and TGFβ1 respectively. Similar inhibition was shown with LY294002 by 63.8±3.7%, p=0.005 with recombinant MCP-3, but a smaller degree of inhibition with recombinant TGFβ1 by 27.4±15.4%, p=0.02. Transactivation was partially inhibited by calphostin, by 32.3±9.2%, p=0.04 and 32.9±15.5%, p=0.04 with recombinant MCP-3 and TGFβ1 respectively. There was little change in the COL1A2 activation by U0126 for both MCP-3 and TGFβ. Together, these results suggest a role for p38 MAPK in the activation of collagen gene expression by both factors.

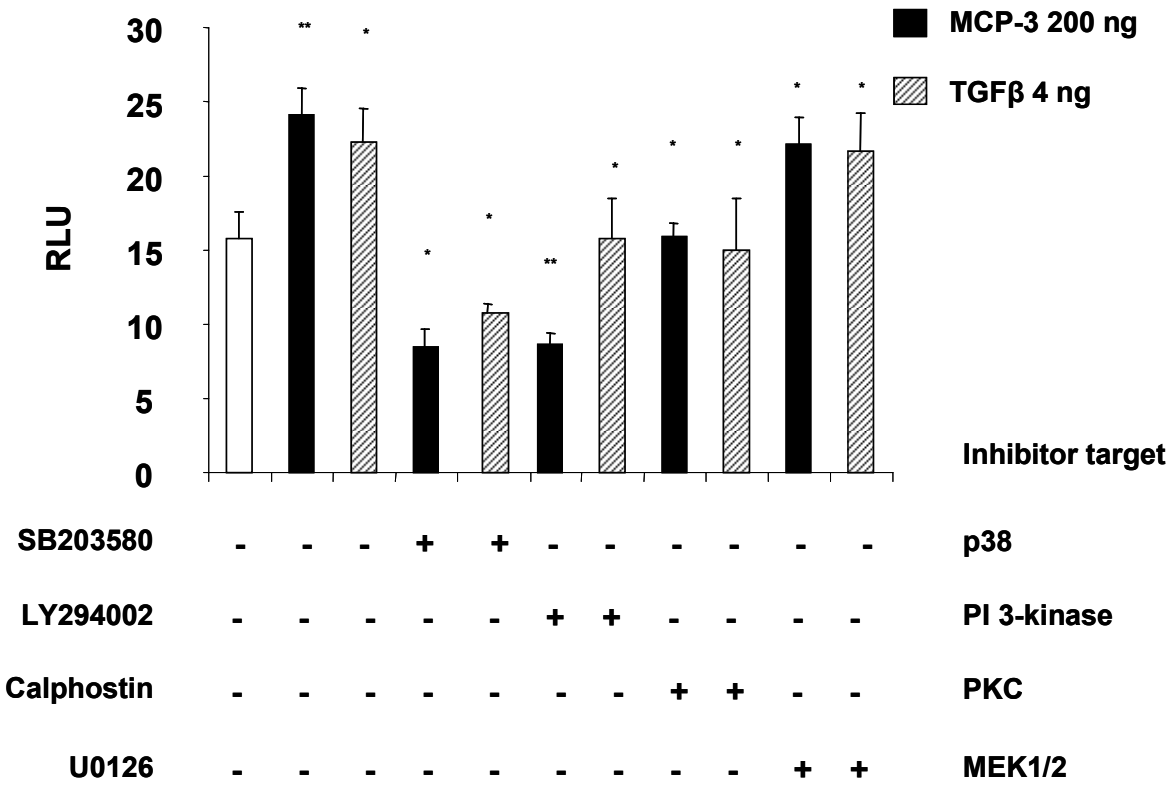


Figure 7.6 Inhibition of MCP-3 and TGFβ-activation of COL1A2 promoter
Murine dermal fibroblasts were serum-starved for 24 h and pretreated with the indicated amounts of various inhibitors for 1 h prior to the addition of MCP-3 (200 ng/ml) and TGFβ1 (4ng/ml) for 24 h. SB203580, LY294002, Calphostin and U0126 were dissolved in Me2SO. The controls were incubated with an equal concentration of Me2SO. p38 MAPK and PI3K signalling pathways differentially mediates the upregulation of collagen promoter in transient transfection by MCP-3 and TGFβ1. The means ± SEM% of four independent experiments are shown. *= $p < 0.05$, ** = $p < 0.01$, by Student's unpaired t-test. RLU=relative luminescent units.

7.4.5 Differential activation of p38 MAPK and Akt phosphorylation by MCP-3 and TGFβ in wildtype fibroblasts

Since the inhibition of p38 MAPK and PI3K pathways differentially decreased the induction of type I collagen expression in wildtype dermal fibroblasts by both TGFβ and MCP-3, activation of both signalling pathways by these factors were studied with immunoblotting using antibodies specific for activated forms of p38 MAPK and Akt. Thus, p38 MAPK Thr /Tyr phosphorylation occurred as early as 20 minutes after treatment with recombinant MCP-3 and the response was sustained up to 1 hour. In contrast, phosphorylation was observed with recombinant TGFβ1 at later time points with a maximum at 8 hours. These results suggest that although, p38 MAPK phosphorylation appears to be central in regulation of collagen gene expression, the kinetics underlying this expression revealed differential time profile for both these factors. Similar pattern was demonstrated with Akt phosphorylation with recombinant MCP-3 peaking early at 20 minutes. In contrast, Akt phosphorylation was observed to occur later at 2-4 hours with recombinant TGFβ1. These results are summarized in **Figure 7.7**.

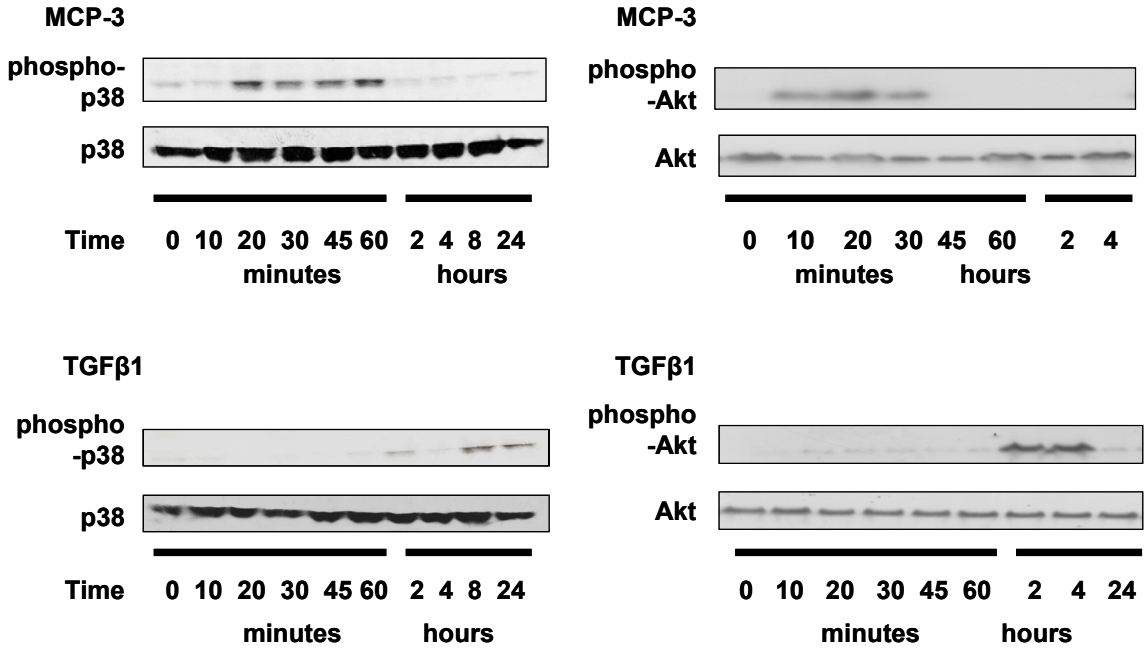


Figure 7.7 Activation of p38 and PI3K by TGFβ and MCP-3 in dermal fibroblasts. Phosphorylation of p38 MAPK and PI3K in normal fibroblasts was determined by immunoblotting using antibodies specific for phosphorylated, activated forms of p38 MAPK (Thr180/Tyr182) and Akt respectively. Dermal fibroblasts were serum-starved for 24 hours and treated with either 200ng/ml MCP-3 or 4ng/ml of TGFβ1 for the indicated times. Antibodies against p38 MAPK and PI3K were also used to confirm that the protein concentrations of p38 MAPK and Akt were maintained with TGFβ and MCP-3. This is a representative of three independent experiments

7.4.6 Differential gene expression in response to MCP-3 and TGFβ

Insofar the data suggests that there may be interplay between TGFβ and MCP-3 in regulation of collagen activation. To test whether there are direct synergistic or additive effects on fibroblast gene expression, a series of gene array studies was performed focussing on early time point 4 hours during which previous studies have shown that there is significant induction of selected known direct TGFβ target genes with 4 ng/ml of recombinant TGFβ1(Renzoni *et al.*, 2004). Normalisation of the expression data was achieved by using the sum of the global intensities of the arrays. Adjusted expression level was set to be at least 10 to allow meaningful comparative interpretation of the data. Relative changes of 2.0 or greater were defined as upregulation and downregulation respectively.

In a series of experiments, MCP-3 alone resulted in upregulation of 8 genes more than 2-fold. These were osteopontin precursor, rac alpha serine/threonine kinase, Osteoblast-specific factor2 (OSF2), cytoplasmid dynein light chain 1, ubiquitin, PAI-1, vimentin and rab2 ras-related protein. 3 genes including MCP-3, G2/mitotic-specific cyclin A1 and biglycan were downregulated by MCP-3 by at least 2-fold. Incubation of dermal fibroblasts with recombinant TGFβ1 was associated with upregulation of 8 genes: all of which, excluding vimentin and PAI-1, were also upregulated by MCP-3. The remaining two genes were Fas1 receptor and interleukin-7 receptor alpha. Biglycan and G2/mitotic-specific A1 were downregulated by TGFβ1.

27 genes were expressed with combination with recombinant MCP-3 and TGFβ1, of which 10 were upregulated by at least two-fold. These were brain factor1 (BF1), PAI-1, osteopontin precursor, rac alpha serine threonine kinase, OSF2, thrombospondin 2 precursor (TSP2), Insulin-like growth factor binding protein-6 (IGFBP6), cytoplasmid dynein light chain 1, glucose-6-phosphate isomerase (Gpi1) and vimentin.
Some of these genes including those related to a range of matrix components, cytokines, growth factors and signalling intermediates that are upregulated and downregulated in wildtype fibroblasts treated with a combination of recombinant MCP-3 and TGFβ1 are shown in **Tables 7.1** and **7.2** respectively.

Gene name	Accession Number	Gene code	Adjusted Expression				Ratio		
			Basal	TGFβ	MCP3	TGFβ +MCP3	TGFβ	MCP3	TGFβ +MCP3
BF1	U36760	B02a	1	8	3	19	8	3	19
PAI	M33960	F04m	2	4	11	28	2	5.5	14
OP	P08721	B13b	1	37	25	10	37	25	10
RAC-PK-alpha	P31749	D02l	1	16	14	10	16	14	10
OSF2	D13664	A01n	2	11	10	17	5.5	5	8.5
TSP2	L07803	F07f	4	1	5	14	0.25	1.25	3.5
IGFBP6	X81584	D13m	4	6	9	13	1.5	2.25	3.25
CDLC1	M25825	D04d	6	24	16	18	4	2.67	3
G6PI	U89408	E01c	11	14	7	29	1.27	0.64	2.64
Vimentin	X51438	F09d	11	8	22	24	0.727	2	2.18
NFTFp65	M61909	B09g	50	57	75	94	1.14	1.5	1.88
TSP1	M87276	F07j	42	22	37	63	0.52	0.88	1.5
FN1	X82402	F08j	16	9	17	22	0.56	1.06	1.37
ubiquitin	X51703	G11	105	238	213	139	2.26	2.03	1.32
Decorin	X53929	F08g	13	4	12	16	0.31	0.92	1.23
MCP-3	S71251	D12b	24	55	10	29	2.29	0.42	1.2
IL7r	M29697	D06g	7	21	5	7	3	0.71	1
DAD1	U83628	D03e	15	16	28	15	1.06	1.87	1
Tb4	X16053	D12d	14	24	8	14	1.71	0.57	1

Table 7.1 Genes upregulated in wildtype murine fibroblasts in response to MCP-3, TGFβ and MCP-3 and TGFβ.

BF1: Brain factor 1, PAI: Plasminogen activator inhibitor, OP: Osteopontin precursor, Rac-PKα: rac alpha serine/threonine kinase, OSF2: Osteoblast-specific factor 2 precursor, TSP2: thrombospondin 2 precursor, IGFBP6: Insulin-like growth factor binding protein-6, CDLC1: Cytoplasmic dynein light chain 1, G6PI: glucose-6-phosphate isomerase, NFTFp65: NF-kappa-B transcription factor p65 subunit, TSP1: thrombospondin 1 precursor, FN1: fibronectin 1 precursor, MCP-3: Monocyte chemoattractant protein-3, IL7rα: Interleukin-7 receptor alpha, DAD1: Defender against cell death 1, Tβ4: Thymosin beta-4.

Gene name	Accession Number	Gene code	Adjusted Expression				Ratio		
			Basal	TGFβ	MCP3	TGFβ +MCP3	TGFβ	MCP3	TGFβ +MCP3
Cyclin A1	X84311	B09m	561	173	280	173	3.24	2.0	3.24
Granzyme C	X12822	F03e	10	4	9	5	2.5	1.1	2
KGF	Z22703	D14f	25	9	8	14	2.78	3.1	1.78
NDKb	X68193	C08j	46	50	42	26	0.92	1.09	1.76
Biglycan	L20276	F07h	52	11	26	30	4.72	2	1.73
BRCA2	U89652	C08i	9	14	10	7	0.64	0.9	1.28
GNBP	AF124384	E13h	19	11	25	15	1.72	0.76	1.26
NMLC3	U04443	F09j	12	8	14	10	1.5	0.85	1.2

Table 7.2 Genes downregulated in wildtype murine fibroblasts in response to MCP-3, TGFβ and MCP-3 and TGFβ.
Cyclin A1: G2/mitotic-specific cyclin A1, KGF: Keratinocyte growth factor, NDKb: Nucleoside diphosphate kinase B, BRCA2: Breast cancer type 2 susceptibility protein, GNBP: Guanine nucleotide binding protein, NMLC3: non-muscle myosin light chain 3.

Overall, three distinct patterns observed with the combination treatment: synergistic effect for BF1, PAI, OSF2, TSP2, IGFBP6, Gpi1 and vimentin; an apparent reduction in synergism with osteopontin precursor and breast cancer type 2 susceptibility protein (BRCA2), and downregulation of biglycan and keratinocyte growth factor (FGF-7). The differential response for representative genes is shown in **Figure 7.8.**

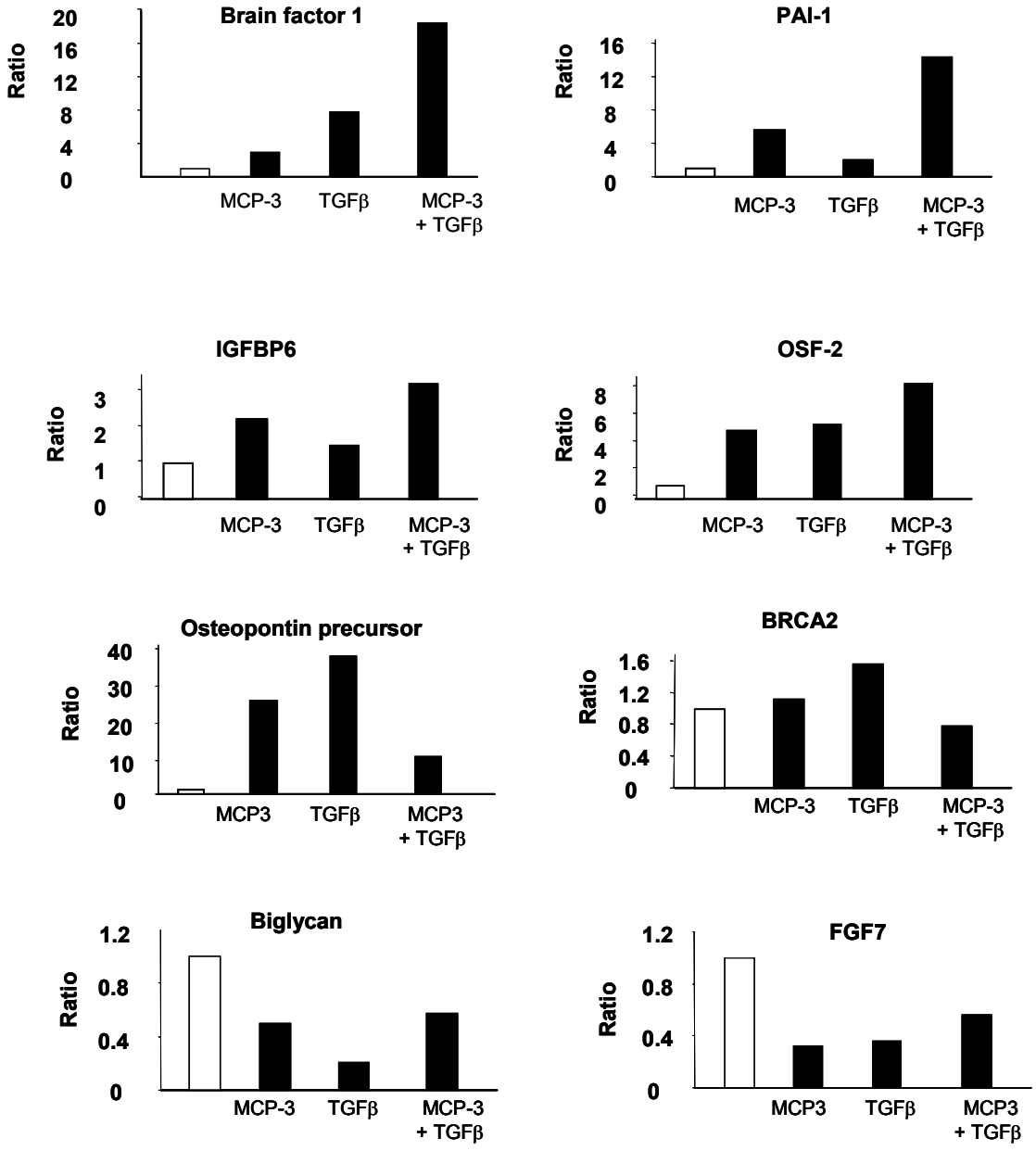


Figure 7.8 Comparative differential gene expression in dermal fibroblasts treated with combination of MCP-3 and TGFβ1

Expression pattern for representative genes are is shown with synergistic effect for plasminogen activator inhibitor (PAI-1), brain factor 1 (BF1), osteoblast-specific factor 2 precursor (OSF2) and Insulin-like growth factor binding protein-6 (IGFBP6). This synergy is less marked with osteopontin and BRCA2 with downregulation of Biglycan and FGF7.

7.5 Discussion

Persistent fibrosis is likely to be determined by interplay of key mediators with an appropriate microenvironment. Previous chapters have highlighted the potential interplay between TGFβ and MCP-3 in particular induction of MCP-3 by TGFβ may set the stage whereby the initial response to injury may regulate inflammation and the profibrotic effects of MCP-3 might contribute to progressive accumulation of ECM. Whether this interaction is bidirectional (that is, MCP-3 induces TGFβ and TGFβ in turn induces MCP-3) is an issue that is complex and unresolved, and has not been examined to date in fibroblasts as the key effector cells in skin fibrosis. Given the involvement of pathobiological events in dermal fibroblasts in fibrotic diseases and the fact that they produce both TGFβ and MCP-3 and respond to these cytokines, this chapter explored whether this interaction between the two factors may be of particular relevance in progressive dermal fibrosis because it provides a positive feedback relationship between TGFβ and MCP-3 and thereby confers a self-perpetuating cycle of inflammation and fibrogenesis, with the latter representing a signature characteristic of fibrotic diseases. Specifically, the interplay between TGFβ and MCP-3 in induction of ECM gene and protein expression and activation of downstream signalling pathways was investigated.

In Chapter 4, it was demonstrated that, using pharmacological inhibitors of TGFβ receptor, the profibrotic effect of MCP-3 appears to be mediated via TGFβ. Consistent with this, the data presented in this Chapter demonstrates down regulation of collagen protein expression with ALK5 inhibitor and Smad3 siRNA, supporting the role of canonical Smad-dependent pathways in MCP-3-mediated collagen expression. In addition, collagen expression is also influenced by the net effect of the interaction between these Smad molecules and transcription factors including Sp1 which is closely regulated by modifications to the chromatin structure. For example, the balance of the two key regulators, histone acetyltransferase and histone deacetylase plays a critical role in regulation of collagen transcription by altering the chromatin structure. Ghosh et. al. recently demonstrated that inhibition of histone deacetylation with trichostatin A in fibroblasts resulted in suppression of TGFβ-induced Sp1 level and consequently, its interaction with Smad complex and COL1A2 promoter activity(Ghosh *et al.*, 2007). Apart from TGFβ, other factors may

activate the Smad signalling pathway. TGFBP-3 has been demonstrated to stimulate Smad2/3 phosphorylation and may potentiate TGFβ-stimulated Smad phosphorylation in vitro. This may be a novel mechanism in which other chemokines such as MCP-3 may interact with TGFβ in regulating fibrotic response. Experimental evidence to support this comes from CCR2 deficient mice model in which there is reduced TGFβ-mediated myofibroblast differentiation with abrogation of Smad3 and Smad4 expression suggesting an important crosstalk between CCR2-regulated chemokines, MCP-1 and MCP-3 with TGFβ, and presumably their respective receptors and signalling pathways(Gharaee-Kermani *et al.*, 2003). Other studies also highlighted the role of Smad3 and Smad4 in mediating the effect of the related chemokine, MCP-1 on epithelial-myofibroblast transition, vascular inflammation and angiogenesis by TGFβ(Feinberg *et al.*, 2004;Ma *et al.*, 2007;Tan *et al.*, 2005). Given that these two chemokines are closely related, it is not inconceivable that MCP-3 may share some of these important biological functions that are increasingly recognised to be important in regulating fibrotic response.

In contrast to TGFβ1, the preferential mediation of collagen gene activation via PI3K by MCP-3 is consistent with the well-established GPCR activation upon binding with MCP-3 resulting in dissociation of G-protein complex into Gα and Gβγ subunits. Gα induces the activation of the PI3K pathway, while the Gβγ subunits activate phospholipase C and induce Ca^{2+} influx and protein kinase C activation(Barberis & Hirsch, 2008). The role of the latter pathway is supported by the partial inhibition of MCP-3-mediated Collagen gene activation by Calphostin, a specific inhibitor targeted against protein kinase C. The early activation of Akt by MCP-3 in murine fibroblasts is consistent with previously reported in monocytic cell lines, and in some cases a biphasic response in phosphorylation of Akt has been reported to occur within the first hour of treatment with MCP-3(O'Boyle *et al.*, 2007).

The kinetics of p38 and PI3K pathway stimulation by both TGFβ and MCP-3 correlates well with the kinetics of the stimulation of collagen gene expression by both factors. The slow kinetics of p38 and PI3K by TGFβ compared to MCP3 is consistent with modulation of transcriptional activation of collagen by anti-TGFβ antibody at 24 hours. It is possible that at the earlier time points, other TGFβ-target genes such as PAI are upregulated by

MCP-3 as shown on our microarray data. Results from Western blot analysis supports this observation with delayed response of TGFβ on MCP-3-mediated collagen protein biosynthesis at 48 hours. This is consistent with other studies in which induction of collagen mRNA occurs after 3 hours post-TGFβ addition and remains elevated for up to 72 h(Ishikawa *et al.*, 1990;Varga *et al.*, 1994). It is possible that p38 and PI3K contributes to maintaining the specific transcription complexes mediating collagen gene regulation by both these factors.

Interestingly, TGFβ1 may modulate inflammatory response by altering chemokine receptor expression. A recent study has demonstrated that TGFβ may upregulate CCR1 in fetal astrocytes, and thereby facilitate the binding of its cognate receptors including MCP-3. It is plausible that similar mechanism may occur in fibroblasts(Han *et al.*, 2000).

Although ERKs, p38 and PI3K have been shown to interact with the TGFβ pathway in the regulation of ECM deposition and turnover, the mechanisms through which TGFβ mediates these MAPK pathways and its biological consequences are not well-characterised. The involvement of p38 in TGFβ stimulation of collagen and fibronectin was recently demonstrated in dermal fibroblasts and the TGFβ response was greatly enhanced by the presence of p38α(Ihn *et al.*, 2005). PI3K is activated in response to a variety of stimuli, including cytokines and growth factors. TGFβ can also activate PI3K via phosphorylation of its effector Akt. Inhibition of this pathway can abrogate TGFβ-induced Smad2 phosphorylation and transcription activation of the collagen 1 gene in mesangial cells(Runyan *et al.*, 2004). In addition, expression of other key profibrotic mediators such as tenascin-C has been shown to regulated by PI3K in dermal fibroblasts in SSc(Jinnin *et al.*, 2006).

TGFβ may activate other signalling cascades including MAPK pathways including p38, Erk and JNK kinase pathways, some of which regulate Smad activation but others may induce responses unrelated to transcription. Significantly, TAK1 has been shown to be an important upstream activator of MKK6, an upstream dual specificity kinase which in turn

activates p38. Activation of this pathway resulted in phosphorylation of activating factor 2 (ATF-2) and enhancement of the complex formed between Smad4 and ATF-2.This in turn leads to activation of the transcription partners for Smads, potentiating the TGFβ response. In contrast, an interesting feature of non-Smad signalling proteins such as MAPK and PI3K is their ability to modulate Smad activity, most frequently negatively. This may explain the apparent reduction of some candidate fibroblast gene expression such as osteopontin precursor and BRCA2 in the array data with combination of MCP-3 and TGFβ compared to treatment of the fibroblasts with each factor alone.

For some gene products including ECM and growth factors OSF2, TSP2, IGFBP6 and BF1, there is synergy between MCP-3 and TGFβ whilst there is downregulation of Biglycan and FGF7. OSF2 or periostin can interact with other ECM proteins such as fibronectin and collagen I and may affect fibroblastic migration via its interaction with integrins(Kudo *et al.*, 2007). Brain Factor 1 which has been shown to antagonise the TGFβ-signalling pathway either by association with the transcriptional coactivator, FAST1(Dou *et al.*, 2000) or Smad proteins(Rodriguez *et al.*, 2001) and therefore, has an inhibitory effect on its transcriptional activation. IGFBP6 is important in regulating IGF bioactivity and this has been shown to be overexpressed in hepatic, renal and dermal fibrosis(Gentilini *et al.*, 1998;Seseke *et al.*, 2004). Some of the effects by both MCP-3 and TGFβ are antagonistic, such as a facilitatory effect by TGFβ and an inhibitory effect by MCP-3 on prothymosin beta-4, important for cellular migration and angiogenesis with possible effect on reduction of collagenase activity(Cavasin, 2006). For genes that are downregulated, there is a synergistic effect for several genes including Cyclin A1, which is important in G1 phase of cell cycle, and FGF7 and Nucleoside diphosphate kinase B which are important for cellular proliferation and differentiation for skin homeostasis and wound repair(Braun *et al.*, 2007).

Previous studies have demonstrated that excessive matrix protein deposition during the fibrotic process is largely related to an increase in the rate of transcription of type 1 collagen genes, COL1A1 and COL1A2. The TGFβ responsive sequences have been localised to the 330bp of the transcription start site of the COL1A2 promoter.

In addition to TGFβ, an expanding repertoire of cytokines and chemokines that regulate mesenchymal cell function has been demonstrated to be overexpressed or dysregulated in SSc. These soluble factors including IL-13 and IL-4, act in concert with TGFβ in driving the fibrotic response. IL-13 induces the production of latent TGFβ1 in macrophages and can also serve as an indirect activator of TGFβ by upregulating MMPs that cleave the LAP. Moreover, IL-13 is a potent inducer of MCP-3 and it is likely these mediators may be directly involved in the crosstalk between MCP-3 and TGFβ. Moreover, there is now accumulating evidence to suggest that there is an important regulatory loop between the related chemokine, MCP-1 and TGFβ in experimental models of inflammatory diseases such as Thy-1 nephritis(Schneider *et al.*, 1999). Similar findings were detected with colocalisation immunohistochemical studies with CCR2 and TGFβ in renal model of fibrosis. Recent reports have demonstrated that in lung fibroblasts and glomerular cells, MCP-1 can stimulate TGFβ via transcription mechanisms(Gharaee-Kermani *et al.*, 1996). In contrast, there are studies to suggest that growth factors and cytokines may act independently of TGFβ to stimulate collagen deposition(Kaviratne *et al.*, 2004). Stimulation of COL1A2 promoter activity by CCL18 in lung fibroblasts is another example of Smad activation independent of TGFβ and requiring Sp1 participation(Luzina *et al.*, 2006). This is also supported by recent evidence that fibrocytes may be directly recruited to the lung by CXC chemokine receptor 4 (CXCR4), CC chemokine receptor 7 (CCR7) and CCR2(Moore *et al.*, 2005;Phillips *et al.*, 2004). In addition, the chemotactic and angiogenic response of chemokines may contribute to the vascular remodelling, an important aspect of the fibrotic response in SSc and diabetic retinopathy(Shireman, 2007).

7.6 Summary

This results support the role of TGFβ in regulating of collagen expression by MCP-3 in murine fibroblasts. Although there appears to be a central role for TGFβ receptor (ALK5) and Smad-3 dependent pathways in driving the production of collagen by MCP-3, there is a significant contribution by the non-Smad signalling via p38 and PI3K pathways. An additive effect of these two agonists was demonstrated by comparative microarray analysis for key TGFβ regulated transcripts including PAI-1 and OSF2. Together, these results confirm cross-talk between MCP-3 and TGFβ that may be critical in the development of fibrosis.

CHAPTER 8: FINAL DISCUSSION AND FUTURE DIRECTIONS

Activation of the immune system and replacement of specialised smooth muscle or epithelial structures with collagen-rich ECM by fibroblasts are hallmarks in the pathogenesis of SSc. The molecular mechanisms underlying the infiltration of inflammatory cells into the skin and the subsequent activation of fibroblasts are areas of major research interest. The main cell types responsible for fibrosis are the fibroblast and its contractile counterpart, the myofibroblast, which has a biochemical phenotype intermediate between a smooth muscle cell and a typical interstitial fibroblast. Most studies support an interaction of different cell types orchestrated by pro-inflammatory and profibrotic cytokines. Chemokines are leucocyte chemoattractants that cooperate with these profibrotic cytokines in the development of fibrosis by recruiting myofibroblasts, macrophages and other key effector cells to sites of tissue injury. The general aims of the studies presented in this thesis were to investigate the expression of MCP-3 in two major murine models of fibrosis and SSc, and to explore the regulation of MCP-3 expression with its interplay with the profibrotic TGFβ in fibroblasts. In this chapter, I will summarise and discuss the results presented in Chapters 3, 4, 5, 6 and 7 in particular to consider the extent to which results presented in this thesis support the hypotheses introduced in Chapter 1.

Although a number of distinct mouse strains have been studied as putative animal models of SSc, most do not completely recapitulate the major attributes of the disease such as the overlapping vascular, immunological and fibrotic pathological states; female predominance; temporal and spatial evolution and progressive multiorgan damage. Nevertheless, they provide essential insight into specific quantifiable features in particular fibrosis. The two mouse models examined in this thesis offer complementary insight into the pathogenesis of fibrosis and permit key common pathways and mediators to be identified that may potentially be important in disease manifestations in SSc. In Chapter 3, gene expression profiling has demonstrated that MCP-3 is upregulated in both these fibrotic murine models for SSc. A key consideration into these gene expression studies is the tissue used for analysis, and these may include dermal biopsies, peripheral blood cells and as

presented in this thesis, cells grown in culture from explant murine dermal biopsies. It is known that explanted fibroblasts from lesional skin of patients with SSc display an abnormal activated phenotype that persists during their serial passage in vitro, indicating autonomous alteration in cell function. In addition, the explanted fibroblasts may also reflect the in vivo metabolic heterogeneity in that subpopulations of fibroblasts are selected to express a phenotype for high matrix synthesis. However, it has been observed that normal fibroblasts transfected with the TGFβ receptor demonstrate a more SSc-like transcript profile than fibroblasts cultured from SSc patients(Pannu *et al.*, 2006), suggesting the importance of the dermal microenvironment in influencing the SSc fibroblast phenotype. Whilst this may imply that experiments performed on cultured fibroblasts alone may not completely mirror the characteristics of the fibroblasts in vivo, a potential problem with samples such as whole blood or dermal biopsies lies with the difficulty in reliably differentiating signals from the different components of these tissues. It would therefore potentially be difficult to evaluate this data or to dissect the specific contributions from each cellular component of the affected tissues. Using gene expression profiling of explanted cultured fibroblasts from murine models for fibrosis, the results from Chapter 3 are supported by a similar study on murine model for chronic colitis with fibrosis. In this study, there was sustained upregulation of inflammation-related genes including MCP-3 and its receptor, CCR2 at a stage that coincides with active and chronic inflammation(Wu & Chakravarti, 2007). Moreover, both MCP-3 and CCR2 remained overexpressed after the inflammation had subsided suggesting that MCP-3 may have additional role in establishment of fibrosis. This is particularly relevant in Tsk1/+ as there is absence of inflammatory response in this murine model and would likewise suggest that MCP-3 may have a profibrotic effect. Moreover, upregulation of MCP-3 and its receptors, CCR1 and CCR2 was also reported in a global gene expression analysis of murine graft versus host disease in which irradiated BALB/c (H-2d) mice transplanted across minor histocompatibility loci with B10.D2 (H-2d) bone marrow and spleen cells develop skin thickening and lung fibrosis, along with cutaneous immune cell infiltrates, TGFβ1 overproduction, and upregulation of collagen synthesis. The authors reported that overexpression of these chemokines was associated with the early influx of immune cells, particularly macrophages and T cells into the dermis(Zhou *et al.*, 2007), and this is

consistent with the primary role of chemokines in leucocyte recruitment to inflammatory sites.

The significance of TGFβ in fibrosis led to the hypothesis that, targeted transgenesis using a lineage-specific promoter with sustained alteration in TGFβ signalling or responsiveness in fibroblasts may recapitulate the SSc phenotype. In this transgenic model described in this thesis, fibroblast-directed expression of a mutant kinase TβRIIΔk resulted in fibrosis. Because TβRIIΔk behaves as a dominant negative inhibitor of TGFβ signalling in cultured fibroblasts, this transgene was predicted to confer protection from fibrosis. However, these TβRIIΔk mice develop early skin fibrosis, whereas only a proportion develop spontaneous lung fibrosis at later time points, mimicking the variable lung involvement in SSc. Cultured skin fibroblasts show sustained transgene expression and a TGFβ1 activated phenotype with basally increased expression of PAI-1, CTGF, Collagen I and other markers of TGFβ signalling, similar to wildtype cells treated with TGFβ1. In addition, diminished responses to exogenous TGFβ1 were seen. Thus, the TβRIIΔk strain provides a more complete model for the late stage fibrotic phase of SSc than in other models. Like the Tsk/1+ model, fibroblastic MCP-3 was also upregulated in TβRIIΔk model and this suggests that MCP-3 expression is upregulated by TGFβ.

Apart from the increased markers of TGFβ signalling, there was upregulation of MMPs including MMP-13 in the transgenic fibroblasts. In addition, there was increased TIMP-2 transcript in Tsk1/+ fibroblasts. MMP-13 is a major collagenolytic enzyme leading to rapid turnover of fibrillar collagens, tenascin, fibronectin and fibrillin-1 in addition to its key role in the MMP activation cascade. In the transgenic fibroblasts, it is likely that some of the overexpressed MMPs including MMP-13 are critical in activation of latent TGFβ latent complex which in turn contribute to fibrogenesis. In support of this, it has been demonstrated that the increased TGFβ bioactivity in the transgenic fibroblasts was partly MMP-dependent with reduction in active TGFβ activity in the supernatants from cultured transgenic fibroblasts treated with a broad-spectrum MMP inhibitor(Denton *et al.*, 2005). It is also well-recognised that chemokines undergo posttranslational processing both by

glycosylation and proteolytic processing with MMPs in which minor modifications of the NH2-terminus of chemokines may alter the biological activity or target cell specificity. MCP-3 may be cleaved by MMP-1,-2,-3 and MMP-13. Interestingly, truncated MCP-3 has been detected in synovial fluid of inflammatory arthritis. As a result, truncated MCP-3 is devoid of chemotactic activity and calcium signalling activity but retains receptor-binding properties. Moreover, MMP bioactivity may be regulated by MCP chemokines. Since MMP activity would increase proteolysis and reduce ECM accumulation, these data seems somewhat contradictory to the outcome of increased ECM in these two models. It is likely that the altered balance between MMPs and TIMPs in these murine models represent a regulatory feedback in the fibrotic microenvironment leading to exaggerated accumulation of ECM(Takeda *et al.*, 1994;Bou-Gharios *et al.*, 1994). Apart from activation of latent TGFβ by MMPs, one other possible explanation may be that a substantial increased in the biosynthesis or activity of TIMP overcomes the effect of the increased MMP-13 levels, and therefore net TIMP levels remain greater in skin lesions from these two fibrotic murine strains mice than in wild-type mice. Additional experiments to evaluate the effect of MMP on MCP-3 will clarify the extent to which proteolytic processing may have on its profibrotic effect. As most current immunological methods that rely on antibodies do not discriminate between authentic and posttranslationally modified chemokine isoforms, this would necessitate more detailed analysis such as mass spectrometry to identify these modifications. However, in a broader context, discrimination between intact and differently processed chemokines in body fluids is a difficult task and a challenge for future clinical research.

In addition to the increasing evidence to support overexpression of chemokines in fibrosis, several studies have reported a direct effect of chemokines on fibroblasts. Yamamoto et al (Yamamoto *et al.*, 2001)demonstrated a significant increase of α1(I) collagen mRNA after incubation of cultured dermal fibroblasts with recombinant MCP-1 by Northern blot analysis. The non-inflammatory role for MCP-1 was also recently explored in the bleomycin model of fibrosis which demonstrated increased abnormal collagen fibrillogenesis compared to MCP-1(-/-) murine strain(Ferreira *et al.*, 2006). Consistent with this, the upregulation of MCP-3 in TβRIIΔk and Tsk1/+ models suggests that MCP-3, apart

from its role as pro-inflammatory mediator, may modulate ECM synthesis by fibroblasts. This was confirmed with upregulation of collagen gene in a series of transfection experiments with transgenic fibroblasts harbouring a far-upstream enhancer of Type I collagen gene and fibroblasts transiently transfected with murine and human minimal promoter sequences. Additionally, the data also revealed that the induction of Col1a2 activation might be independent of the upstream enhancer, suggesting that the profibrotic effect may be only partially mediated by TGFβ. Consistent with this, there was incomplete inhibition of collagen gene activation with the neutralising antibody against TGFβ1 by MCP-3. Moreover, MCP-3 mediated-induction of collagen protein appears to be mediated by MAPK p38 and PI3K signalling pathways. Interestingly, our group has demonstrated that in addition to the in vitro activation of far-upstream fibroblast-specific elements by TGFβ, additional fibroblast-specific pathways may be involved in the collagen gene upregulation in the Tsk1/+ murine model, suggesting that other factors other than TGFβ may be important in the fibrotic Tsk1/+ mice. Whilst it is well-established that the Col1a2 and COL1A2 promoter is activated by TGFβ, there are instances in which the collagen gene is upregulated through mechanisms that are either independent of or parallel to TGFβ. IL-4 and IL-13, which stimulate collagen gene promoter activity are two such examples(Jinnin *et al.*, 2004;McGaha *et al.*, 2003). In these studies, the loss of IL-4 in Tsk mice reduces TGFβ production and abrogates cutaneous hyperplasia whereas loss of IL-13 attenuates infection-induced liver fibrosis in mice independently of TGFβ.

Although murine models provide an invaluable insight into the potential pathogenic disease mechanisms, it is critical to consider pathogenesis in the context of human fibrotic diseases. SSc represents a prototypic fibrotic disease and access to lesional skin biopsies makes them a useful investigative tool to test candidate mediators identified from the murine models. The results presented in this thesis that MCP-3 may be an important initiator in the cascade of mediators leading to dermal fibrosis. Interestingly, it has been reported that MCP-3 is upregulated in experimental models of inflammation although its physiological function, for example in wound healing has not been fully elucidated. It is noteworthy that recent studies with murine strains deficient for MCP family of CC chemokines and CCR2 genes have demonstrated that MCP-3 and CCR2 may play a critical role in maintenance of

normal blood monocytes as well as recruitment of inflammatory circulatory monocytes to sites of inflammation. Compared to MCP-1(-/-) murine strains, there was significantly greater retention of marrow monocytes in the MCP-3(-/-) and CCR2(-/-) mice(Tsou *et al.*, 2007). Interestingly, this effect appears to be specific to these two members of the MCP chemokine family. Whilst this result supports a non-redundant activity for these chemokines, it is unclear whether activation of CCR1 or CCR3, the other receptors for MCP-3, contributes to monocyte recruitment. These results are replicated in another inflammatory murine model with infection to Listeria monocytogenes in which the defect in monocyte emigration from the marrow was again more pronounced in the MCP-3(-/-) mice than in MCP-1(-/-) mice strain. From these studies, it was suggested that both MCP-1 and MCP-3 might work in parallel to monocytic recruitment in response to inflammatory stimuli. Taken together, these observations may be particularly relevant in the early inflammatory phase of SSc with increased colocalisation of MCP-3 in the perivascular dermis in early stage dcSSc strongly suggesting that MCP-3 may be important in its chemotactic function for mononuclear infiltrate in particular monocytes and T cells in early active disease.

The extent to which each chemokine MCP-1 and MCP-3 contributes to this dermal inflammatory infiltrate in SSc is difficult to determine as a double knockout MCP-1 and MCP-3 mouse strain is not currently available. However, studies on CCR2 knockout mice suggest impaired monocytic recruitment to sites of injury. In contrast, studies on lung fibrosis with mice deficient for CCR2 demonstrated that these mice were protected from fibrosis and this protective effect was independent of the inflammatory infiltrate suggesting that CCR2 and its ligands may have a direct profibrotic effect(Moore *et al.*, 2001). It may be that MCP-3 has a dual pathogenic role in both early and late stage of the SSc. Recruitment of inflammatory mononuclear cells by MCP-3 to the lesional dermal area would occur early followed by the scattered fibroblastic expression of MCP-3 in the established stage of the disease with secretion of ECM proteins. More recently, our group demonstrated that a subset of dermal fibroblasts from patients with early dcSSc expresses CCR2, the receptor for both MCP-1 and MCP-3, and double-labelling experiments revealed that CCR-2 positive fibroblasts expressed α-smooth muscle actin, suggesting that ligands

for CCR2 may stimulate the differentiation of a distinct subset of resting fibroblasts into metabolically active myofibroblasts in early stage of the disease(Carulli *et al.*, 2005). Although this study did not directly examine MCP-3 expression, MCP-3 is a major ligand for CCR2 and these results may account for the heterogeneity in the fibroblastic expression of MCP-3 in the lesional skin. Interestingly, it has recently been reported that CCR2 may be critical for the recruitment of fibrocytes and this may account for the protective effect against fibrosis in mice deficient for CCR2(Moore *et al.*, 2005). Whilst there is in vitro evidence that fibrocytes migrate to MCP-1, the role of MCP-3 in recruitment of fibrocytes remains unexplored.

My findings also suggest that there is heterogeneity in MCP-3 expression levels in the sera in particular there was differential increase in a subset of patients with dcSSc. This observation is in keeping with the heterogeneity of the disease presentation and course. In support of this, recent studies have demonstrated that upregulation of MCP-3 is correlated with diffuse subset, skin score and presence and severity of lung fibrosis(Yanaba *et al.*, 2006;Choi *et al.*, 2004). Whilst the number of patients studied for this experiment was small, its potential role of MCP-3 as a biomarker to identify prognostic and therapeutic response that may allow early and accurate identification of patients merits further assessment.

The regulatory mechanisms governing the physiological expression and upregulation of MCP-3 expression in fibrosis have not been well studied. Whilst MCP-3 transcript is expressed in many human tissues including lung in low abundance, its regulatory control mechanisms remain unclear(Murakami *et al.*, 1997). Like other MCPs, its gene is mapped to chromosome 17q11 and its transcriptional regulation has been examined using deletion fragments of the upstream 1kb promoter region in several cells lines including human osteosarcoma cell lines. Moreover, transcriptional regulation of MCP-3 expression has been studied in human dermal fibroblasts in response to cytokines including IL-4 and TNFα, suggesting that interplay between MCP-3 and other growth factors may be pivotal in its expression(Hein *et al.*, 1999). Similar findings were also recently reported on the critical role of β-catenin on MCP-3 promoter expression in dermal fibroblasts(Fujita *et al.*, 2000).

It has been proposed that this effect is mediated via putative transcriptional factor binding sites including AP-1 and NF-κB sites within the promoter.

The initial finding with upregulation of MCP-3 transcript and protein in the TβRIIΔk murine model proposes that there is an important crosstalk between MCP-3 and TGFβ. This was further confirmed by results presented in Chapter 6 that MCP-3 gene and protein are transcriptionally regulated by TGFβ in murine fibroblasts. Furthermore, I demonstrated that this activation of MCP-3 by TGFβ is mediated via ALK5 receptor and required recruitment of both the canonical Smad and MAPK signalling pathways. It is likely that the transcriptional activation of MCP-3 expression depends on the cell type and stimuli applied. To further examine the transcriptional regulation of MCP-3 by TGFβ1, promoter reporter constructs with deletion fragments and point mutation constructs with the pGL3 plasmid vector may be used to map the response elements within the 1kb promoter fragment used in this thesis. Further promoter deletion constructs may be extended to include the putative Smad-binding element binding site(-2.2kb) identified on TFSEARCH. Results from these promoter deletion mapping studies may be extended with DNase I footprinting and EMSA using a region of DNA spanning the transcriptional factor binding sites within the MCP-3 promoter as a target. This may be confirmed with Chromatin immunoprecipitation (ChIP) analysis. This methodology provides a mechanistic insight into the in vivo binding activity between transcription factors and MCP-3 promoter and also to dissect out the histone modifications and the assembly of transcription factors at these binding sites. For example, trichostatin A, a histone deacetylase inhibitor is able to trigger MCP-1, suggesting that histone acetylation alone can induce MCP-1 expression(Ping *et al.*, 1996).

To explore further the crosstalk between MCP-3 and TGFβ, I established that MCP-3 may be important in modulating TGFβ-regulated pathways and that MCP-3 mediated activation of COL1A2 is also dependent on TGFβ. Further confirmation of this results may be undertaken with determination of the biological activity of both active and latent TGFβ in MCP-3 treated conditioned media from fibroblasts, using the functionally relevant PAI-1 promoter linked to the luciferase reporter gene stably integrated into mink lung epithelial

cells(Abe *et al.*, 1994). Results presented in Chapter 7 supported the pivotal role of canonical TGFβ signalling pathways in regulation of collagen expression by MCP-3. In addition, non-Smad signalling pathways via p38 and PI3K appear to contribute to the activation of collagen by MCP-3. The role of PI3K/AKT has been explored in several animal models and has been implicated in several fibrotic mechanisms including EMT. EMT-like changes were observed in rats exposed to chronic hyperglycaemia and TGFβ is thought to be the primary cytokine driver of EMT in kidney with elevated PKB/Akt phosphorylation in the affected kidneys compared to controls(Kattla *et al.*, 2008). Treatment of animals with rapamycin has been shown to attenuate diabetes-induced kidney disease in rats and also reduced elevated activated mTOR (mammalian target of rapamycin) and PKB/Akt in the glomeruli of these animals(Lloberas *et al.*, 2006). Consistent with this, a recent report showed that TGFβ-induced collagen overexpression in human dermal fibroblasts was completely inhibited by rapamycin(Kim *et al.*, 2008). These findings suggest that therapeutic strategies targeting PKB/Akt signalling may be appropriate in fibrotic diseases although there may be other signalling pathways that may be activated as consequence to overcome the targeted inhibition.

The interplay between these two factors is strongly supported by a recent report in an experimental model of renal allograft. In this study, both factors enhance intratubular cytotoxicity during acute rejection and T cell-mediated EMT leading to graft fibrosis during chronic allograft failure(Al-Hamidi *et al.*, 2008). Moreover, the abrogation of fibrotic response in the knockout mice for CCR1 and CCR2, the major receptors for MCP-3 provide indirect evidence of the potential role of MCP-3 in the development of fibrosis. Interestingly, the reduction of fibrosis in these knockout mice was associated with decreased IL-13 production. Previous studies have shown that the profibrotic activities of IL-13 involve both direct fibroblast activation and indirect mechanisms via stimulation of TGFβ. It is therefore likely that these factors may act in synergy to mediate the profibrotic effect and IL-13 would be a potential candidate for further study in its role in the crosstalk between TGFβ and MCP-3. Comparative gene expression analysis with these two factors suggests that a cohort of TGFβ-regulated transcripts including OSF2 is upregulated by MCP-3 and TGFβ. In support of this, fibroblastic OSF2 has also been recently

demonstrated to be upregulated by TGFβ and IL-13, suggesting that these factors act in synergy in promoting ECM accumulation(Blanchard *et al.*, 2008). Similar interplay among these factors and CC chemokines has been reported to be critical in both human and murine model of lung fibrosis(Murray *et al.*, 2008).

There are several approaches by which the studies described in this thesis may be expanded and developed towards a better understanding of the expression and regulation of MCP-3 in fibrosis and SSc. In vivo evidence for the role of MCP-3 in fibrosis may be established with administration of a neutralising antibody against MCP-3 in both the Tsk1/+ and TβRIIΔk models. Similar technique has been used to determine the effect of anti-MCP-1 antibody on the induction of dermal sclerosis in the bleomycin-induced fibrosis model with reduction in inflammatory mononuclear infiltrate and collagen expression(Yamamoto & Nishioka, 2003). Studies from chemokine and chemokine receptor knockout targetting CCR2 and MCP-1 have established that CCR2 signalling is important in the fibrotic response by regulating chemokine expression and fibroblast responsiveness to TGFβ. MCP-1(-/-) has been used in models of dermal wound repair and dermal fibrosis. However, the caveat is that these knockout mouse models may provide unexpected results. For example, in the MIP-1α(-/-) murine model for dermal wound, the results suggest that MIP-1α although important in recruitment of inflammatory infiltrate appear to be inconsequential in normal wounds. It is likely that the MIP-1α(-/-) mice may have developed compensatory developmental mechanisms that overcame the loss of this single chemokine(Low *et al.*, 2001). Moreover, the redundancy property of chemokines and their corresponding receptors may alter the phenotype otherwise expected for the knockout mouse strain. Interestingly, in the MCP-1(-/-) wound model, there was no effect in inflammatory cell infiltrate compared to the wildtype, and the effect on epithelialisation and vasculogenesis was far more significant than collagen synthesis, suggesting that compensatory mechanisms may be operational or that there is differential requirement for MCP-1 in different inflammatory disease environment(Low *et al.*, 2001).

Similar studies targetting MCP-3 would be helpful to clarify and confirm the results in this thesis in particular with reference to its role in regulation of inflammation and fibrotic

response. Mice genetically deficient for MCP-3 (the 129/Sv strain) has recently been described and they have no obvious developmental abnormalities and were born at the expected Mendelian ratios(Tsou *et al.*, 2007). The effect of subcutaneous injection of bleomycin into the skin of MCP-3(-/-) mice may be compared to the dermal fibrosis induced in wild-type mice. As discussed in Chapter 1, the bleomycin mouse model represents a well-established in vivo model for fibrosis that produces dermal lesions similar to SSc. A similar study has recently been reported for MCP-1(-/-) in bleomycin mouse model which demonstrated a markedly diminished response to fibrotic stimulus. Alternatively, MCP-3(-/-) mice may be crossed with Tsk1/+ mice to generate MCP-3$^{-/-}$ Tsk/+ mice. Our group has previously reported the regulation of collagen gene by mating transgenic mice harbouring a far-upstream fibroblast-specific Col1a2 enhancer with Tsk/1+ mice(Denton *et al.*, 2001). In these experiments, assessment of histological (collagen and inflammatory infiltrate) and biochemical response, and measurement of TGFβ levels may extend our understanding of the profibrotic role of MCP-3 in fibrotic process. In addition, a conditional knockout of the gene postnatally using the site-specific Cre/LoxP recombination strategy will allow controlled spatial and temporal deactivation of the gene(Sonnylal *et al.*, 2007), and therefore eliminate the possibility of compensatory mechanisms and avoid the developmental consequences in a genetically deficient mice targeting MCP-3 gene.

Overall, the work described in this thesis has provided evidence to support a potential role for MCP-3 in the pathogenesis of fibrosis and suggests that there may be important links between MCP-3 and the profibrotic TGFβ in ECM production. Whilst some of the activity of MCP-3 may be related to its recruitment of mononuclear infiltrate to affected skin, there is evidence to suggest that, with TGFβ, it may have an additional profibrotic effect. This study represents a further step towards unraveling the molecular mechanisms that underlie the inflammatory and fibrotic responses in SSc and has raised additional issues for further experimental investigations. Furthermore, demonstration that MCP-3 may at least under experimental conditions and in murine models lead to ECM accumulation suggests that therapeutic strategies aiming to modulate chemokine and chemokine receptor activity in vivo may eventually be beneficial in systemic sclerosis or other fibrotic diseases.

6. Reference List

Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, & Rifkin DB (1994). An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* **216**, 276-284.

Abecassis L, Rogier E, Vazquez A, Atfi A, & Bourgeade MF (2004). Evidence for a role of MSK1 in transforming growth factor-beta-mediated responses through p38alpha and Smad signaling pathways. *J Biol Chem* **279**, 30474-30479.

Abraham S, Sawaya BE, Safak M, Batuman O, Khalili K, & Amini S (2003). Regulation of MCP-1 gene transcription by Smads and HIV-1 Tat in human glial cells. *Virology* **309**, 196-202.

Agarwal SK, Tan FK, & Arnett FC (2008). Genetics and genomic studies in scleroderma (systemic sclerosis). *Rheum Dis Clin North Am* **34**, 17-40.

Aiba S, Tabata N, Ohtani H, & Tagami H (1994). CD34+ spindle-shaped cells selectively disappear from the skin lesion of scleroderma. *Arch Dermatol* **130**, 593-597.

Al-Dhaher FF, Pope JE, & Ouimet JM (2008). Determinants of Morbidity and Mortality of Systemic Sclerosis in Canada. *Semin Arthritis Rheum*.

Al-Hamidi A, Pekalski M, Robertson H, Ali S, & Kirby JA (2008). Renal allograft rejection: The contribution of chemokines to the adhesion and retention of alphaE(CD103)beta7 integrin-expressing intratubular T cells. *Mol Immunol*.

Allen SJ, Crown SE, & Handel TM (2007). Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* **25**, 787-820.

Amara A, Gall SL, Schwartz O, Salamero J, Montes M, Loetscher P, Baggiolini M, Virelizier JL, & Arenzana-Seisdedos F (1997). HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. *J Exp Med* **186**, 139-146.

Anderegg U, Saalbach A, & Haustein UF (2000). Chemokine release from activated human dermal microvascular endothelial cells--implications for the pathophysiology of scleroderma? *Arch Dermatol Res* **292**, 341-347.

Anders HJ, Belemezova E, Eis V, Segerer S, Vielhauer V, Perez dL, Kretzler M, Cohen CD, Frink M, Horuk R, Hudkins KL, Alpers CE, Mampaso F, & Schlondorff D (2004). Late onset of treatment with a chemokine receptor CCR1 antagonist prevents progression of lupus nephritis in MRL-Fas(lpr) mice. *J Am Soc Nephrol* **15**, 1504-1513.

Anders HJ, Vielhauer V, Frink M, Linde Y, Cohen CD, Blattner SM, Kretzler M, Strutz F, Mack M, Grone HJ, Onuffer J, Horuk R, Nelson PJ, & Schlondorff D (2002). A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. *J Clin Invest* **109**, 251-259.

Andersson-Sjoland A, de Alba CG, Nihlberg K, Becerril C, Ramirez R, Pardo A, Westergren-Thorsson G, & Selman M (2008). Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis. *Int J Biochem Cell Biol* **40**, 2129-2140.

Andrikopoulos K, Liu X, Keene DR, Jaenisch R, & Ramirez F (1995). Targeted mutation in the col5a2 gene reveals a regulatory role for type V collagen during matrix assembly. *Nat Genet* **9**, 31-36.

Antoniv TT, De VS, Wells D, Denton CP, Rabe C, de CB, Ramirez F, & Bou-Gharios G (2001). Characterization of an evolutionarily conserved far-upstream enhancer in the human alpha 2(I) collagen (COL1A2) gene. *J Biol Chem* **276**, 21754-21764.

Aragay AM, Mellado M, Frade JM, Martin AM, Jimenez-Sainz MC, Martinez A, & Mayor F, Jr. (1998). Monocyte chemoattractant protein-1-induced CCR2B receptor desensitization mediated by the G protein-coupled receptor kinase 2. *Proc Natl Acad Sci U S A* **95**, 2985-2990.

Arnett FC, Cho M, Chatterjee S, Aguilar MB, Reveille JD, & Mayes MD (2001). Familial occurrence frequencies and relative risks for systemic sclerosis (scleroderma) in three United States cohorts. *Arthritis Rheum* **44**, 1359-1362.

Asano N, Fujimoto M, Yazawa N, Shirasawa S, Hasegawa M, Okochi H, Tamaki K, Tedder TF, & Sato S (2004a). B Lymphocyte signaling established by the CD19/CD22 loop regulates autoimmunity in the tight-skin mouse. *Am J Pathol* **165**, 641-650.

Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, & Tamaki K (2004b). Phosphatidylinositol 3-kinase is involved in alpha2(I) collagen gene expression in normal and scleroderma fibroblasts. *J Immunol* **172**, 7123-7135.

Atamas SP, Luzina IG, Choi J, Tsymbalyuk N, Carbonetti NH, Singh IS, Trojanowska M, Jimenez SA, & White B (2003). Pulmonary and activation-regulated chemokine stimulates collagen production in lung fibroblasts. *Am J Respir Cell Mol Biol* **29**, 743-749.

Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, & Arteaga CL (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**, 36803-36810.

Ball DK, Surveyor GA, Diehl JR, Steffen CL, Uzumcu M, Mirando MA, & Brigstock DR (1998). Characterization of 16- to 20-kilodalton (kDa) connective tissue growth factors (CTGFs) and demonstration of proteolytic activity for 38-kDa CTGF in pig uterine luminal flushings. *Biol Reprod* **59**, 828-835.

Bandyopadhyay S, Jeong KH, Hansen JT, Vassilev PM, Brown EM, & Chattopadhyay N (2007). Calcium-sensing receptor stimulates secretion of an interferon-gamma-induced monokine (CXCL10) and monocyte chemoattractant protein-3 in immortalized GnRH neurons. *J Neurosci Res* **85**, 882-895.

Banisor I, Leist TP, & Kalman B (2005). Involvement of beta-chemokines in the development of inflammatory demyelination. *J Neuroinflammation* **2**, 7.

Barberis L & Hirsch E (2008). Targeting phosphoinositide 3-kinase gamma to fight inflammation and more. *Thromb Haemost* **99**, 279-285.

Barisic-Dujmovic T, Boban I, & Clark SH (2008). Regulation of collagen gene expression in the Tsk2 mouse. *J Cell Physiol* **215**, 464-471.

Barnett AJ & Coventry DA (1969). Scleroderma. 1. Clinical features, course of illness and response to treatment in 61 cases. *Med J Aust* **1**, 992-1001.

Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A, Avvedimento EV, & Gabrielli A (2006). Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med* **354**, 2667-2676.

Bataille F, Rohrmeier C, Bates R, Weber A, Rieder F, Brenmoehl J, Strauch U, Farkas S, Furst A, Hofstadter F, Scholmerich J, Herfarth H, & Rogler G (2008). Evidence for a role of epithelial mesenchymal transition during pathogenesis of fistulae in Crohn's disease. *Inflamm Bowel Dis*.

Baum B, Settleman J, & Quinlan MP (2008). Transitions between epithelial and mesenchymal states in development and disease. *Semin Cell Dev Biol* **19**, 294-308.

Baxter RM, Crowell TP, McCrann ME, Frew EM, & Gardner H (2005). Analysis of the tight skin (Tsk1/+) mouse as a model for testing antifibrotic agents. *Lab Invest* **85**, 1199-1209.

Bayle J, Fitch J, Jacobsen K, Kumar R, Lafyatis R, & Lemaire R (2008). Increased expression of Wnt2 and SFRP4 in Tsk mouse skin: role of Wnt signaling in altered dermal fibrillin deposition and systemic sclerosis. *J Invest Dermatol* **128**, 871-881.

Becvar R, Stork J, Pesakova V, Stanova A, Hulejova H, Rysova L, Zatloukalova A, Zatloukal P, Jachymova M, & Pourova L (2005). Clinical correlations of potential activity markers in systemic sclerosis. *Ann N Y Acad Sci* **1051**, 404-412.

Belch JJ, Veale DJ, & Colleridge T (1993). Large vessel disease in CREST. *Ann Rheum Dis* **52**, 837-838.

Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, & Strieter RM (2000). CXC chemokines in angiogenesis. *J Leukoc Biol* **68**, 1-8.

Bennett RM (1990). Scleroderma overlap syndromes. *Rheum Dis Clin North Am* **16**, 185-198.

Berahovich RD, Miao Z, Wang Y, Premack B, Howard MC, & Schall TJ (2005). Proteolytic Activation of Alternative CCR1 Ligands in Inflammation. *J Immunol* **174**, 7341-7351.

Birnboim HC & Doly J (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513-1523.

Blanchard C, Mingler MK, McBride M, Putnam PE, Collins MH, Chang G, Stringer K, Abonia JP, Molkentin JD, & Rothenberg ME (2008). Periostin facilitates eosinophil tissue infiltration in allergic lung and esophageal responses. *Mucosal Immunol* **1**, 289-296.

Boiardi L, Macchioni P, Meliconi R, Pulsatelli L, Facchini A, & Salvarani C (1999). Relationship between serum RANTES levels and radiological progression in rheumatoid arthritis patients treated with methotrexate. *Clin Exp Rheumatol* **17**, 419-425.

Bolad I & Delafontaine P (2005). Endothelial dysfunction: its role in hypertensive coronary disease. *Curr Opin Cardiol* **20**, 270-274.

Bolster MB, Ludwicka A, Sutherland SE, Strange C, & Silver RM (1997). Cytokine concentrations in bronchoalveolar lavage fluid of patients with systemic sclerosis. *Arthritis Rheum* **40**, 743-751.

Bona CA, Murai C, Casares S, Kasturi K, Nishimura H, Honjo T, & Matsuda F (1997). Structure of the mutant fibrillin-1 gene in the tight skin (TSK) mouse. *DNA Res* **4**, 267-271.

Bordron A, Dueymes M, Levy Y, Jamin C, Leroy JP, Piette JC, Shoenfeld Y, & Youinou PY (1998). The binding of some human antiendothelial cell antibodies induces endothelial cell apoptosis. *J Clin Invest* **101**, 2029-2035.

Borroni EM, Buracchi C, de la Torre YM, Galliera E, Vecchi A, Bonecchi R, Mantovani A, & Locati M (2006). The chemoattractant decoy receptor D6 as a negative regulator of inflammatory responses. *Biochem Soc Trans* **34**, 1014-1017.

Bottinger EP, Jakubczak JL, Roberts IS, Mumy M, Hemmati P, Bagnall K, Merlino G, & Wakefield LM (1997). Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas. *EMBO J* **16**, 2621-2633.

Bou-Gharios G, Garrett LA, Rossert J, Niederreither K, Eberspaecher H, Smith C, Black C, & Crombrugghe B (1996). A potent far-upstream enhancer in the mouse pro alpha 2(I) collagen gene regulates expression of reporter genes in transgenic mice. *J Cell Biol* **134**, 1333-1344.

Bou-Gharios G, Osman J, Black C, & Olsen I (1994). Excess matrix accumulation in scleroderma is caused partly by differential regulation of stromelysin and TIMP-1 synthesis. *Clin Chim Acta* **231**, 69-78.

Bradham DM, Igarashi A, Potter RL, & Grotendorst GR (1991). Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* **114**, 1285-1294.

Brand T, MacLellan WR, & Schneider MD (1993). A dominant-negative receptor for type beta transforming growth factors created by deletion of the kinase domain. *J Biol Chem* **268**, 11500-11503.

Brand T & Schneider MD (1995). Inactive type II and type I receptors for TGF beta are dominant inhibitors of TGF beta-dependent transcription. *J Biol Chem* **270**, 8274-8284.

Braun S, Mauch C, Boukamp P, & Werner S (2007). Novel roles of NM23 proteins in skin homeostasis, repair and disease. *Oncogene* **26**, 532-542.

Brodmerkel CM, Huber R, Covington M, Diamond S, Hall L, Collins R, Leffet L, Gallagher K, Feldman P, Collier P, Stow M, Gu X, Baribaud F, Shin N, Thomas B, Burn T, Hollis G, Yeleswaram S, Solomon K, Friedman S, Wang A, Xue CB, Newton RC, Scherle P, & Vaddi K (2005). Discovery and pharmacological characterization of a novel rodent-active CCR2 antagonist, INCB3344. *J Immunol* **175**, 5370-5378.

Bruns M, Herrmann K, & Haustein UF (1994). Immunologic parameters in systemic sclerosis. *Int J Dermatol* **33**, 25-32.

Bryan C, Howard Y, Brennan P, Black C, & Silman A (1996). Survival following the onset of scleroderma: results from a retrospective inception cohort study of the UK patient population. *Br J Rheumatol* **35**, 1122-1126.

Bujor AM, Pannu J, Bu S, Smith EA, Muise-Helmericks RC, & Trojanowska M (2008). Akt Blockade Downregulates Collagen and Upregulates MMP1 in Human Dermal Fibroblasts. *J Invest Dermatol*.

Burdick MD, Murray LA, Keane MP, Xue YY, Zisman DA, Belperio JA, & Strieter RM (2005). CXCL11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. *Am J Respir Crit Care Med* **171**, 261-268.

Bustos ML, Frias S, Ramos S, Estrada A, Arreola JL, Mendoza F, Gaxiola M, Salcedo M, Pardo A, & Selman M (2007). Local and circulating microchimerism is associated with hypersensitivity pneumonitis. *Am J Respir Crit Care Med* **176**, 90-95.

Cantley LC (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.

Carter PH (2002). Chemokine receptor antagonism as an approach to anti-inflammatory therapy: 'just right' or plain wrong? *Curr Opin Chem Biol* **6**, 510-525.

Carulli MT, Ong VH, Ponticos M, Shiwen X, Abraham DJ, Black CM, & Denton CP (2005). Chemokine receptor CCR2 expression by systemic sclerosis fibroblasts: evidence for autocrine regulation of myofibroblast differentiation. *Arthritis Rheum* **52**, 3772-3782.

Carulli MT, Spagnolo P, Fonseca C, Welsh KI, duBois RM, Black CM, & Denton CP (2008). Single-nucleotide polymorphisms in CCL2 gene are not associated with susceptibility to systemic sclerosis. *J Rheumatol* **35**, 839-844.

Cavasin MA (2006). Therapeutic potential of thymosin-beta4 and its derivative N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after infarction. *Am J Cardiovasc Drugs* **6**, 305-311.

Cepeda EJ & Reveille JD (2004). Autoantibodies in systemic sclerosis and fibrosing syndromes: clinical indications and relevance. *Curr Opin Rheumatol* **16**, 723-732.

Cerinic MM, Valentini G, Sorano GG, D'Angelo S, Cuomo G, Fenu L, Generini S, Cinotti S, Morfini M, Pignone A, Guiducci S, Del RA, Kalfin R, Das D, & Marongiu F (2003). Blood coagulation, fibrinolysis, and markers of endothelial dysfunction in systemic sclerosis. *Semin Arthritis Rheum* **32**, 285-295.

Chang E & Goldberg H (1995). Requirements for transforming growth factor-beta regulation of the pro-alpha 2(I) collagen and plasminogen activator inhibitor-1 promoters. *J Biol Chem* **270**, 4473-4477.

Chang HY, Chi JT, Dudoit S, Bondre C, van de RM, Botstein D, & Brown PO (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci U S A* **99**, 12877-12882.

Chen K, Wei Y, Alter A, Sharp GC, & Braley-Mullen H (2005). Chemokine expression during development of fibrosis versus resolution in a murine model of granulomatous experimental autoimmune thyroiditis. *J Leukoc Biol* **78**, 716-724.

Chen Y, Shi-Wen X, Eastwood M, Black CM, Denton CP, Leask A, & Abraham DJ (2006). Contribution of activin receptor-like kinase 5 (transforming growth factor beta receptor type I) signaling to the fibrotic phenotype of scleroderma fibroblasts. *Arthritis Rheum* **54**, 1309-1316.

Chizzolini C (2008). T cells, B cells, and polarized immune response in the pathogenesis of fibrosis and systemic sclerosis. *Curr Opin Rheumatol* **20**, 707-712.

Choi ES, Jakubzick C, Carpenter KJ, Kunkel SL, Evanoff H, Martinez FJ, Flaherty KR, Toews GB, Colby TV, Kazerooni EA, Gross BH, Travis WD, & Hogaboam CM (2004). Enhanced monocyte chemoattractant protein-3/CC chemokine ligand-7 in usual interstitial pneumonia. *Am J Respir Crit Care Med* **170**, 508-515.

Christner PJ, Artlett CM, Conway RF, & Jimenez SA (2000). Increased numbers of microchimeric cells of fetal origin are associated with dermal fibrosis in mice following injection of vinyl chloride. *Arthritis Rheum* **43**, 2598-2605.

Christner PJ, Peters J, Hawkins D, Siracusa LD, & Jimenez SA (1995). The tight skin 2 mouse. An animal model of scleroderma displaying cutaneous fibrosis and mononuclear cell infiltration. *Arthritis Rheum* **38**, 1791-1798.

Chvatchko Y, Proudfoot AE, Buser R, Juillard P, Alouani S, Kosco-Vilbois M, Coyle AJ, Nibbs RJ, Graham G, Offord RE, & Wells TN (2003). Inhibition of airway inflammation by amino-terminally modified RANTES/CC chemokine ligand 5 analogues is not mediated through CCR3. *J Immunol* **171**, 5498-5506.

Cipriani P, Guiducci S, Miniati I, Cinelli M, Urbani S, Marrelli A, Dolo V, Pavan A, Saccardi R, Tyndall A, Giacomelli R, & Cerinic MM (2007). Impairment of endothelial cell differentiation from bone marrow-derived mesenchymal stem cells: new insight into the pathogenesis of systemic sclerosis. *Arthritis Rheum* **56**, 1994-2004.

Claman HN, Jaffee BD, Huff JC, & Clark RA (1985). Chronic graft-versus-host disease as a model for scleroderma. II. Mast cell depletion with deposition of immunoglobulins in the skin and fibrosis. *Cell Immunol* **94**, 73-84.

Clements P, Lachenbruch P, Siebold J, White B, Weiner S, Martin R, Weinstein A, Weisman M, Mayes M, Collier D, & . (1995). Inter and intraobserver variability of total skin thickness score (modified Rodnan TSS) in systemic sclerosis. *J Rheumatol* **22**, 1281-1285.

Clements PJ, Hurwitz EL, Wong WK, Seibold JR, Mayes M, White B, Wigley F, Weisman M, Barr W, Moreland L, Medsger TA, Jr., Steen VD, Martin RW, Collier D, Weinstein A, Lally E, Varga J, Weiner SR, Andrews B, Abeles M, & Furst DE (2000). Skin thickness score as a predictor and correlate of outcome in systemic sclerosis: high-dose versus low-dose penicillamine trial. *Arthritis Rheum* **43**, 2445-2454.

Cohen SN, Chang AC, & Hsu L (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. *Proc Natl Acad Sci U S A* **69**, 2110-2114.

Comerford I, Litchfield W, Harata-Lee Y, Nibbs RJ, & McColl SR (2007). Regulation of chemotactic networks by 'atypical' receptors. *Bioessays* **29**, 237-247.

Corry DB & Kheradmand F (2002). Biology and therapeutic potential of the interleukin-4/interleukin-13 signaling pathway in asthma. *Am J Respir Med* **1**, 185-193.

Corry DB, Kiss A, Song LZ, Song L, Xu J, Lee SH, Werb Z, & Kheradmand F (2004). Overlapping and independent contributions of MMP2 and MMP9 to lung allergic inflammatory cell egression through decreased CC chemokines. *FASEB J* **18**, 995-997.

Cowie RL (1987). Silica-dust-exposed mine workers with scleroderma (systemic sclerosis). *Chest* **92**, 260-262.

Cowper SE, Kuo PH, & Bucala R (2007). Nephrogenic systemic fibrosis and gadolinium exposure: association and lessons for idiopathic fibrosing disorders. *Arthritis Rheum* **56**, 3173-3175.

De VS, Ponticos M, Antoniv TT, Wells DJ, Abraham D, Partridge T, & Bou-Gharios G (2002). Identification of the key regions within the mouse pro-alpha 2(I) collagen gene far-upstream enhancer. *J Biol Chem* **277**, 9286-9292.

Del PN, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, Maglione W, Comina DP, Vitali C, Fraticelli P, Gabrielli A, Cortelezzi A, & Lambertenghi-Deliliers G (2006). Bone marrow endothelial progenitors are defective in systemic sclerosis. *Arthritis Rheum* **54**, 2605-2615.

DeMarco PJ, Weisman MH, Seibold JR, Furst DE, Wong WK, Hurwitz EL, Mayes M, White B, Wigley F, Barr W, Moreland L, Medsger TA, Jr., Steen V, Martin RW, Collier D, Weinstein A, Lally E, Varga J, Weiner SR, Andrews B, Abeles M, & Clements PJ (2002). Predictors and outcomes of scleroderma renal crisis: the high-dose versus low-dose D-penicillamine in early diffuse systemic sclerosis trial. *Arthritis Rheum* **46**, 2983-2989.

Denton CP, Lindahl GE, Khan K, Shiwen X, Ong VH, Gaspar NJ, Lazaridis K, Edwards DR, Leask A, Eastwood M, Leoni P, Renzoni EA, Bou GG, Abraham DJ, & Black CM (2005). Activation of key profibrotic mechanisms in transgenic fibroblasts expressing kinase-deficient type II Transforming growth factor-{beta} receptor (T{beta}RII{delta}k). *J Biol Chem* **280**, 16053-16065.

Denton CP, Shi-Wen X, Sutton A, Abraham DJ, Black CM, & Pearson JD (1998). Scleroderma fibroblasts promote migration of mononuclear leucocytes across endothelial cell monolayers. *Clin Exp Immunol* **114**, 293-300.

Denton CP, Zheng B, Evans LA, Shi-Wen X, Ong VH, Fisher I, Lazaridis K, Abraham DJ, Black CM, & de CB (2003). Fibroblast-specific expression of a kinase-deficient type II transforming growth factor beta (TGFbeta) receptor leads to paradoxical activation of TGFbeta signaling pathways with fibrosis in transgenic mice. *J Biol Chem* **278**, 25109-25119.

Denton CP, Zheng B, Shiwen X, Zhang Z, Bou-Gharios G, Eberspaecher H, Black CM, & de CB (2001). Activation of a fibroblast-specific enhancer of the proalpha2(I) collagen gene in tight-skin mice. *Arthritis Rheum* **44**, 712-722.

Derynck R & Zhang YE (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584.

Devalaraja MN & Richmond A (1999). Multiple chemotactic factors: fine control or redundancy? *Trends Pharmacol Sci* **20**, 151-156.

Dick EA, Aviv R, Francis I, Hamilton G, Baker D, Black C, Platts A, & Watkinson A (2001). Catheter angiography and angioplasty in patients with scleroderma. *Br J Radiol* **74**, 1091-1096.

Distler JH, Akhmetshina A, Schett G, & Distler O (2008). Monocyte chemoattractant proteins in the pathogenesis of systemic sclerosis. *Rheumatology (Oxford)*.

Distler O, Pap T, Kowal-Bielecka O, Meyringer R, Guiducci S, Landthaler M, Scholmerich J, Michel BA, Gay RE, Matucci-Cerinic M, Gay S, & Muller-Ladner U (2001). Overexpression of monocyte chemoattractant protein 1 in systemic sclerosis: role of platelet-derived growth factor and effects on monocyte chemotaxis and collagen synthesis. *Arthritis Rheum* **44**, 2665-2678.

Dong C, Zhu S, Wang T, Yoon W, Li Z, Alvarez RJ, ten DP, White B, Wigley FM, & Goldschmidt-Clermont PJ (2002). Deficient Smad7 expression: a putative molecular defect in scleroderma. *Proc Natl Acad Sci U S A* **99**, 3908-3913.

Dooley A, Gao B, Bradley N, Abraham DJ, Black CM, Jacobs M, & Bruckdorfer KR (2006). Abnormal nitric oxide metabolism in systemic sclerosis: increased levels of nitrated proteins and asymmetric dimethylarginine. *Rheumatology (Oxford)* **45**, 676-684.

Dooley S, Hamzavi J, Breitkopf K, Wiercinska E, Said HM, Lorenzen J, ten Dijke P, & Gressner AM (2003). Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats. *Gastroenterology* **125**, 178-191.

Dou C, Lee J, Liu B, Liu F, Massague J, Xuan S, & Lai E (2000). BF-1 interferes with transforming growth factor beta signaling by associating with Smad partners. *Mol Cell Biol* **20**, 6201-6211.

Duncan MR & Berman B (1991). Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. *J Invest Dermatol* **97**, 686-692.

Dziadzio M, Usinger W, Leask A, Abraham D, Black CM, Denton C, & Stratton R (2005). N-terminal connective tissue growth factor is a marker of the fibrotic phenotype in scleroderma. *QJM* **98**, 485-492.

Eis V, Luckow B, Vielhauer V, Siveke JT, Linde Y, Segerer S, De Lema GP, Cohen CD, Kretzler M, Mack M, Horuk R, Murphy PM, Gao JL, Hudkins KL, Alpers CE, Grone HJ, Schlondorff D, & Anders HJ (2004). Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction. *J Am Soc Nephrol* **15**, 337-347.

Elias JA, Lee CG, Zheng T, Ma B, Homer RJ, & Zhu Z (2003). New insights into the pathogenesis of asthma. *J Clin Invest* **111**, 291-297.

Ellingsen T, Buus A, & Stengaard-Pedersen K (2001). Plasma monocyte chemoattractant protein 1 is a marker for joint inflammation in rheumatoid arthritis. *J Rheumatol* **28**, 41-46.

Elsner J, Petering H, Hochstetter R, Kimmig D, Wells TN, Kapp A, & Proudfoot AE (1997). The CC chemokine antagonist Met-RANTES inhibits eosinophil effector functions through the chemokine receptors CCR1 and CCR3. *Eur J Immunol* **27**, 2892-2898.

Elsner J, Petering H, Kimmig D, Wells TN, Proudfoot AE, & Kapp A (1999). The CC chemokine receptor antagonist met-RANTES inhibits eosinophil effector functions. *Int Arch Allergy Immunol* **118**, 462-465.

Engel ME, McDonnell MA, Law BK, & Moses HL (1999). Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* **274**, 37413-37420.

Fanning GC, Welsh KI, Bunn C, Du BR, & Black CM (1998). HLA associations in three mutually exclusive autoantibody subgroups in UK systemic sclerosis patients. *Br J Rheumatol* **37**, 201-207.

Fedyk ER, Jones D, Critchley HO, Phipps RP, Blieden TM, & Springer TA (2001). Expression of stromal-derived factor-1 is decreased by IL-1 and TNF and in dermal wound healing. *J Immunol* **166**, 5749-5754.

Feinberg MW, Shimizu K, Lebedeva M, Haspel R, Takayama K, Chen Z, Frederick JP, Wang XF, Simon DI, Libby P, Mitchell RN, & Jain MK (2004). Essential role for Smad3 in regulating MCP-1 expression and vascular inflammation. *Circ Res* **94**, 601-608.

Ferreira AM, Takagawa S, Fresco R, Zhu X, Varga J, & DiPietro LA (2006). Diminished induction of skin fibrosis in mice with MCP-1 deficiency. *J Invest Dermatol* **126**, 1900-1908.

Ferri C, Azzi A, & Magro CM (2005). Parvovirus B19 and systemic sclerosis. *Br J Dermatol* **152**, 819-820.

Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, & Kitani A (2006). IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat Med* **12**, 99-106.

Filvaroff E, Erlebacher A, Ye J, Gitelman SE, Lotz J, Heillman M, & Derynck R (1999). Inhibition of TGF-beta receptor signaling in osteoblasts leads to decreased bone remodeling and increased trabecular bone mass. *Development* **126**, 4267-4279.

Fioretti F, Fradelizi D, Stoppacciaro A, Ramponi S, Ruco L, Minty A, Sozzani S, Garlanda C, Vecchi A, & Mantovani A (1998). Reduced tumorigenicity and augmented leukocyte infiltration after monocyte chemotactic protein-3 (MCP-3) gene transfer: perivascular accumulation of dendritic cells in peritumoral tissue and neutrophil recruitment within the tumor. *J Immunol* **161**, 342-346.

Fiten P, Vandenbroeck K, Dubois B, Van Coillie E, Nelissen I, Van Damme J, Ligers A, Hillert J, Andersson M, Olsson T, & Opdenakker G (1999). Microsatellite polymorphisms in the gene promoter of monocyte chemotactic protein-3 and analysis of the association between monocyte chemotactic protein-3 alleles and multiple sclerosis development. *J Neuroimmunol* **95**, 195-201.

Fleischmajer R, Gay S, Meigel WN, & Perlish JS (1978). Collagen in the cellular and fibrotic stages of scleroderma. *Arthritis Rheum* **21**, 418-428.

Fonseca C & Denton CP (2007). Genetic association studies in systemic sclerosis: more evidence of a complex disease. *J Rheumatol* **34**, 903-905.

Fonseca C, Renzoni E, Sestini P, Pantelidis P, Lagan A, Bunn C, McHugh N, Welsh KI, du Bois RM, Denton CP, Black C, & Abraham D (2006). Endothelin axis polymorphisms in patients with scleroderma. *Arthritis Rheum* **54**, 3034-3042.

Frazier K, Williams S, Kothapalli D, Klapper H, & Grotendorst GR (1996). Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. *J Invest Dermatol* **107**, 404-411.

Fujii H, Shimada Y, Hasegawa M, Takehara K, & Sato S (2004). Serum levels of a Th1 chemoattractant IP-10 and Th2 chemoattractants, TARC and MDC, are elevated in patients with systemic sclerosis. *J Dermatol Sci* **35**, 43-51.

Fujita M, Furukawa Y, Nagasawa Y, Ogawa M, & Nakamura Y (2000). Down-regulation of monocyte chemotactic protein-3 by activated beta-catenin. *Cancer Res* **60**, 6683-6687.

Fukakusa M, Bergeron C, Tulic MK, Fiset PO, Al Dewachi O, Laviolette M, Hamid Q, & Chakir J (2005). Oral corticosteroids decrease eosinophil and CC chemokine expression but increase neutrophil, IL-8, and IFN-gamma-inducible protein 10 expression in asthmatic airway mucosa. *J Allergy Clin Immunol* **115**, 280-286.

Furst DE, Clements PJ, Steen VD, Medsger TA, Jr., Masi AT, D'Angelo WA, Lachenbruch PA, Grau RG, & Seibold JR (1998). The modified Rodnan skin score is an accurate reflection of skin biopsy thickness in systemic sclerosis. *J Rheumatol* **25**, 84-88.

Furukawa F, Matsuzaki K, Mori S, Tahashi Y, Yoshida K, Sugano Y, Yamagata H, Matsushita M, Seki T, Inagaki Y, Nishizawa M, Fujisawa J, & Inoue K (2003). p38 MAPK mediates fibrogenic signal through Smad3 phosphorylation in rat myofibroblasts. *Hepatology* **38**, 879-889.

Furuse S, Fujii H, Kaburagi Y, Fujimoto M, Hasegawa M, Takehara K, & Sato S (2003). Serum concentrations of the CXC chemokines interleukin 8 and growth-regulated oncogene-alpha are elevated in patients with systemic sclerosis. *J Rheumatol* **30**, 1524-1528.

Gabrielli A, Svegliati S, Moroncini G, Pomponio G, Santillo M, & Avvedimento EV (2008). Oxidative stress and the pathogenesis of scleroderma: the Murrell's hypothesis revisited. *Semin Immunopathol* **30**, 329-337.

Galdo FD, Sotgia F, de Almeida CJ, Jasmin JF, Musick M, Lisanti MP, & Jimenez SA (2008). Decreased expression of caveolin 1 in patients with systemic sclerosis: Crucial role in the pathogenesis of tissue fibrosis. *Arthritis Rheum* **58**, 2854-2865.

Galindo M, Santiago B, Rivero M, Rullas J, Alcami J, & Pablos JL (2001). Chemokine expression by systemic sclerosis fibroblasts: abnormal regulation of monocyte chemoattractant protein 1 expression. *Arthritis Rheum* **44**, 1382-1386.

Gardner H, Shearstone JR, Bandaru R, Crowell T, Lynes M, Trojanowska M, Pannu J, Smith E, Jablonska S, Blaszczyk M, Tan FK, & Mayes MD (2006). Gene profiling of scleroderma skin reveals robust signatures of disease that are imperfectly reflected in the transcript profiles of explanted fibroblasts. *Arthritis Rheum* **54**, 1961-1973.

Gardner H, Strehlow D, Bradley L, Widom R, Farina A, de FA, Peyman J, Koteliansky V, & Korn JH (2004). Global expression analysis of the fibroblast transcriptional response to TGFbeta. *Clin Exp Rheumatol* **22**, S47-S57.

Gentilini A, Feliers D, Pinzani M, Woodruff K, & Abboud S (1998). Characterization and regulation of insulin-like growth factor binding proteins in human hepatic stellate cells. *J Cell Physiol* **174**, 240-250.

Gharaee-Kermani M, Denholm EM, & Phan SH (1996). Costimulation of fibroblast collagen and transforming growth factor beta1 gene expression by monocyte chemoattractant protein-1 via specific receptors. *J Biol Chem* **271**, 17779-17784.

Gharaee-Kermani M, McCullumsmith RE, Charo IF, Kunkel SL, & Phan SH (2003). CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. *Cytokine* **24**, 266-276.

Ghosh AK (2002). Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. *Exp Biol Med (Maywood)* **227**, 301-314.

Ghosh AK, Mori Y, Dowling E, & Varga J (2007). Trichostatin A blocks TGF-beta-induced collagen gene expression in skin fibroblasts: involvement of Sp1. *Biochem Biophys Res Commun* **354**, 420-426.

Giannini E & Boulay F (1995). Phosphorylation, dephosphorylation, and recycling of the C5a receptor in differentiated HL60 cells. *J Immunol* **154**, 4055-4064.

Gifford RW, Jr. & Hines EAJr (1960). Prognostic significance of Raynaud's phenomenon and other clinical characteristics of systemic scleroderma. A study of 271 cases. *Circulation* **21**, 1088-1095.

Gillitzer R & Goebeler M (2001). Chemokines in cutaneous wound healing. *J Leukoc Biol* **69**, 513-521.

Gilman AG (1987). G proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56**, 615-649.

Gomer RH (2008). Circulating progenitor cells and scleroderma. *Curr Rheumatol Rep* **10**, 183-188.

Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, Martinez A, Dorf M, Bjerke T, Coyle AJ, & Gutierrez-Ramos JC (1998). The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* **188**, 157-167.

Gordon KJ & Blobe GC (2008). Role of transforming growth factor-beta superfamily signaling pathways in human disease. *Biochim Biophys Acta* **1782**, 197-228.

Gorska AE, Joseph H, Derynck R, Moses HL, & Serra R (1998). Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Differ* **9**, 229-238.

Greco G, Mackewicz C, & Levy JA (1999). Sensitivity of human immunodeficiency virus infection to various alpha, beta and gamma chemokines. *J Gen Virol* **80 (Pt 9)**, 2369-2373.

Green MC, Sweet HO, & Bunker LE (1976). Tight-skin, a new mutation of the mouse causing excessive growth of connective tissue and skeleton. *Am J Pathol* **82**, 493-512.

Grone HJ, Weber C, Weber KS, Grone EF, Rabelink T, Klier CM, Wells TN, Proudfood AE, Schlondorff D, & Nelson PJ (1999). Met-RANTES reduces vascular and tubular damage during acute renal transplant rejection: blocking monocyte arrest and recruitment. *FASEB J* **13**, 1371-1383.

Gruschwitz MS, Moormann S, Kromer G, Sgonc R, Dietrich H, Boeck G, Gershwin ME, Boyd R, & Wick G (1991). Phenotypic analysis of skin infiltrates in comparison with peripheral blood lymphocytes, spleen cells and thymocytes in early avian scleroderma. *J Autoimmun* **4**, 577-593.

Habibipour S, Oswald TM, Zhang F, Joshi P, Zhou XC, Dorsett-Martin W, & Lineaweaver WC (2003). Effect of sodium diphenylhydantoin on skin wound healing in rats. *Plast Reconstr Surg* **112**, 1620-1627.

Hadida F, Vieillard V, Autran B, Clark-Lewis I, Baggiolini M, & Debre P (1998). HIV-specific T cell cytotoxicity mediated by RANTES via the chemokine receptor CCR3. *J Exp Med* **188**, 609-614.

Hagood JS, Prabhakaran P, Kumbla P, Salazar L, MacEwen MW, Barker TH, Ortiz LA, Schoeb T, Siegal GP, Alexander CB, Pardo A, & Selman M (2005). Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am J Pathol* **167**, 365-379.

Hahm KB, Im YH, Lee C, Parks WT, Bang YJ, Green JE, & Kim SJ (2000). Loss of TGF-beta signaling contributes to autoimmune pancreatitis. *J Clin Invest* **105**, 1057-1065.

Hahm KB, Lee KM, Kim YB, Hong WS, Lee WH, Han SU, Kim MW, Ahn BO, Oh TY, Lee MH, Green J, & Kim SJ (2002). Conditional loss of TGF-beta signalling leads to increased susceptibility to gastrointestinal carcinogenesis in mice. *Aliment Pharmacol Ther* **16 Suppl 2**, 115-127.

Hamamdzic D, Harley RA, Hazen-Martin D, & LeRoy EC (2001). MCMV induces neointima in IFN-gammaR-/- mice: intimal cell apoptosis and persistent proliferation of myofibroblasts. *BMC Musculoskelet Disord* **2**, 3.

Han Y, Wang J, Zhou Z, & Ransohoff RM (2000). TGFbeta1 selectively up-regulates CCR1 expression in primary murine astrocytes. *Glia* **30**, 1-10.

Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, & Matsushima K (1994). Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* **56**, 559-564.

Haribabu B, Richardson RM, Fisher I, Sozzani S, Peiper SC, Horuk R, Ali H, & Snyderman R (1997). Regulation of human chemokine receptors CXCR4. Role of phosphorylation in desensitization and internalization. *J Biol Chem* **272**, 28726-28731.

Hasegawa M, Fujimoto M, Kikuchi K, & Takehara K (1997). Elevated serum levels of interleukin 4 (IL-4), IL-10, and IL-13 in patients with systemic sclerosis. *J Rheumatol* **24**, 328-332.

Hasegawa M, Sato S, Echigo T, Hamaguchi Y, Yasui M, & Takehara K (2005). Up regulated expression of fractalkine/CX3CL1 and CX3CR1 in patients with systemic sclerosis. *Ann Rheum Dis* **64**, 21-28.

Hasegawa M, Sato S, Fujimoto M, Ihn H, Kikuchi K, & Takehara K (1998). Serum levels of interleukin 6 (IL-6), oncostatin M, soluble IL-6 receptor, and soluble gp130 in patients with systemic sclerosis. *J Rheumatol* **25**, 308-313.

Hasegawa M, Sato S, & Takehara K (1999). Augmented production of chemokines (monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1alpha (MIP-1alpha) and MIP-1beta) in patients with systemic sclerosis: MCP-1 and MIP-1alpha may be involved in the development of pulmonary fibrosis. *Clin Exp Immunol* **117**, 159-165.

Haugh JM, Codazzi F, Teruel M, & Meyer T (2000). Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J Cell Biol* **151**, 1269-1280.

Haustein UF, Ziegler V, Herrmann K, Mehlhorn J, & Schmidt C (1990). Silica-induced scleroderma. *J Am Acad Dermatol* **22**, 444-448.

Hayakawa I, Hasegawa M, Matsushita T, Yanaba K, Kodera M, Komura K, Takehara K, & Sato S (2005). Increased cutaneous T-cell-attracting chemokine levels in sera from patients with systemic sclerosis. *Rheumatology (Oxford)* **44**, 873-878.

Hayashida T, Decaestecker M, & Schnaper HW (2003). Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. *FASEB J* **17**, 1576-1578.

Hein H, Schluter C, Kulke R, Christophers E, Schroder JM, & Bartels J (1999). Genomic organization, sequence analysis and transcriptional regulation of the human MCP-4 chemokine gene (SCYA13) in dermal fibroblasts: a comparison to other eosinophilic beta-chemokines. *Biochem Biophys Res Commun* **255**, 470-476.

Heldin CH & Westermark B (1999). Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol Rev* **79**, 1283-1316.

Helwig U, Lammers KM, Gionchetti P, Rizzello F, Campieri M, & Uguccioni M (2000). MCP-3 in inflammatory bowel disease. *Gut* **47**, 155.

Herrmann K, Heckmann M, Kulozik M, Haustein UF, & Krieg T (1991). Steady-state mRNA levels of collagens I, III, fibronectin, and collagenase in skin biopsies of systemic sclerosis patients. *J Invest Dermatol* **97**, 219-222.

Hesselstrand R, Scheja A, & Akesson A (1998). Mortality and causes of death in a Swedish series of systemic sclerosis patients. *Ann Rheum Dis* **57**, 682-686.

High WA, Ayers RA, Chandler J, Zito G, & Cowper SE (2007). Gadolinium is detectable within the tissue of patients with nephrogenic systemic fibrosis. *J Am Acad Dermatol* **56**, 21-26.

Hirsch E (2006). Signal transduction in inflammation. Perspective clues from the leukocyte-endothelium interface. *Thromb Haemost* **95**, 3-4.

Hocevar BA, Brown TL, & Howe PH (1999). TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. *EMBO J* **18**, 1345-1356.

Hodge G, Hodge S, Reynolds PN, & Holmes M (2005). Up-regulation of Interleukin-8, Interleukin-10, Monocyte Chemotactic Protein-1, and Monocyte Chemotactic Protein-3 in Peripheral Blood Monocytes in Stable Lung Transplant Recipients: Are Immunosuppression Regimens Working? *Transplantation* **79**, 387-391.

Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, & Leask A (2001). CTGF and SMADs, maintenance of scleroderma phenotype is independent of SMAD signaling. *J Biol Chem* **276**, 10594-10601.

Horuk R, Shurey S, Ng HP, May K, Bauman JG, Islam I, Ghannam A, Buckman B, Wei GP, Xu W, Liang M, Rosser M, Dunning L, Hesselgesser J, Snider RM, Morrissey MM, Perez HD, & Green C (2001). CCR1-specific non-peptide antagonist: efficacy in a rabbit allograft rejection model. *Immunol Lett* **76**, 193-201.

Hoyles RK, Ellis RW, Wellsbury J, Lees B, Newlands P, Goh NS, Roberts C, Desai S, Herrick AL, McHugh NJ, Foley NM, Pearson SB, Emery P, Veale DJ, Denton CP, Wells AU, Black CM, & du Bois RM (2006). A multicenter, prospective, randomized, double-blind, placebo-controlled trial of corticosteroids and intravenous cyclophosphamide

followed by oral azathioprine for the treatment of pulmonary fibrosis in scleroderma. *Arthritis Rheum* **54**, 3962-3970.

Hoyles RK, Khan K, Shiwen X, Howat SL, Lindahl GE, Leoni P, du Bois RM, Wells AU, Black CM, Abraham DJ, & Denton CP (2008). Fibroblast-specific perturbation of transforming growth factor beta signaling provides insight into potential pathogenic mechanisms of scleroderma-associated lung fibrosis: exaggerated response to alveolar epithelial injury in a novel mouse model. *Arthritis Rheum* **58**, 1175-1188.

Hu B, Wu Z, & Phan SH (2003). Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am J Respir Cell Mol Biol* **29**, 397-404.

Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, & Rabinovitch M (2004). Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* **43**, 13S-24S.

Humbert M, Ying S, Corrigan C, Menz G, Barkans J, Pfister R, Meng Q, Van DJ, Opdenakker G, Durham SR, & Kay AB (1997). Bronchial mucosal expression of the genes encoding chemokines RANTES and MCP-3 in symptomatic atopic and nonatopic asthmatics: relationship to the eosinophil-active cytokines interleukin (IL)-5, granulocyte macrophage-colony-stimulating factor, and IL-3. *Am J Respir Cell Mol Biol* **16**, 1-8.

Ihn H (2008). Autocrine TGF-beta signaling in the pathogenesis of systemic sclerosis. *J Dermatol Sci* **49**, 103-113.

Ihn H, Sato S, Fujimoto M, Kikuchi K, Kadono T, Tamaki K, & Takehara K (1997). Circulating intercellular adhesion molecule-1 in the sera of patients with systemic sclerosis: enhancement by inflammatory cytokines. *Br J Rheumatol* **36**, 1270-1275.

Ihn H, Yamane K, Asano Y, Kubo M, & Tamaki K (2002). IL-4 up-regulates the expression of tissue inhibitor of metalloproteinase-2 in dermal fibroblasts via the p38 mitogen-activated protein kinase dependent pathway. *J Immunol* **168**, 1895-1902.

Ihn H, Yamane K, & Tamaki K (2005). Increased phosphorylation and activation of mitogen-activated protein kinase p38 in scleroderma fibroblasts. *J Invest Dermatol* **125**, 247-255.

Irvine KM, Andrews MR, Fernandez-Rojo MA, Schroder K, Burns CJ, Su S, Wilks AF, Parton RG, Hume DA, & Sweet MJ (2008). Colony-stimulating factor-1 (CSF-1) delivers a proatherogenic signal to human macrophages. *J Leukoc Biol*.

Ishikawa O, Yamakage A, LeRoy EC, & Trojanowska M (1990). Persistent effect of TGF-beta 1 on extracellular matrix gene expression in human dermal fibroblasts. *Biochem Biophys Res Commun* **169**, 232-238.

Isogai Z, Ono RN, Ushiro S, Keene DR, Chen Y, Mazzieri R, Charbonneau NL, Reinhardt DP, Rifkin DB, & Sakai LY (2003). Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. *J Biol Chem* **278**, 2750-2757.

Isono M, Chen S, Hong SW, Iglesias-de la Cruz MC, & Ziyadeh FN (2002). Smad pathway is activated in the diabetic mouse kidney and Smad3 mediates TGF-beta-induced fibronectin in mesangial cells. *Biochem Biophys Res Commun* **296**, 1356-1365.

Itoh F, Divecha N, Brocks L, Oomen L, Janssen H, Calafat J, Itoh S, & Dijke PP (2002). The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-beta/Smad signalling. *Genes Cells* **7**, 321-331.

Jacobsen S, Halberg P, & Ullman S (1998). Mortality and causes of death of 344 Danish patients with systemic sclerosis (scleroderma). *Br J Rheumatol* **37**, 750-755.

Jaffee BD & Claman HN (1983). Chronic graft-versus-host disease (GVHD) as a model for scleroderma. I. Description of model systems. *Cell Immunol* **77**, 1-12.

Jain R, Shaul PW, Borok Z, & Willis BC (2007). Endothelin-1 induces alveolar epithelial-mesenchymal transition through endothelin type A receptor-mediated production of TGF-beta1. *Am J Respir Cell Mol Biol* **37**, 38-47.

Jang MK, Kim JY, Jeoung NH, Kang MA, Choi MS, Oh GT, Nam KT, Lee WH, & Park YB (2004). Oxidized low-density lipoproteins may induce expression of monocyte chemotactic protein-3 in atherosclerotic plaques. *Biochem Biophys Res Commun* **323**, 898-905.

Javelaud D & Mauviel A (2005). Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene* **24**, 5742-5750.

Jia T, Serbina NV, Brandl K, Zhong MX, Leiner IM, Charo IF, & Pamer EG (2008). Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection. *J Immunol* **180**, 6846-6853.

Jimenez SA & Artlett CM (2005). Microchimerism and systemic sclerosis. *Curr Opin Rheumatol* **17**, 86-90.

Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, & Tamaki K (2006). Upregulation of tenascin-C expression by IL-13 in human dermal fibroblasts via the phosphoinositide 3-kinase/Akt and the protein kinase C signaling pathways. *J Invest Dermatol* **126**, 551-560.

Jinnin M, Ihn H, Mimura Y, Asano Y, Yamane K, & Tamaki K (2005). Effects of hepatocyte growth factor on the expression of type I collagen and matrix metalloproteinase-1 in normal and scleroderma dermal fibroblasts. *J Invest Dermatol* **124**, 324-330.

Jinnin M, Ihn H, Yamane K, & Tamaki K (2004). Interleukin-13 stimulates the transcription of the human alpha2(I) collagen gene in human dermal fibroblasts. *J Biol Chem* **279**, 41783-41791.

Johnston CJ, Williams JP, Okunieff P, & Finkelstein JN (2002). Radiation-induced pulmonary fibrosis: examination of chemokine and chemokine receptor families. *Radiat Res* **157**, 256-265.

Jones SA, Moser B, & Thelen M (1995). A comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2. Chemokine mediated activation of p42/p44 MAP-kinase (ERK-2). *FEBS Lett* **364**, 211-214.

Joseph H, Gorska AE, Sohn P, Moses HL, & Serra R (1999). Overexpression of a kinase-deficient transforming growth factor-beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol Biol Cell* **10**, 1221-1234.

Jun JB, Kuechle M, Min J, Shim SC, Kim G, Montenegro V, Korn JH, & Elkon KB (2005). Scleroderma fibroblasts demonstrate enhanced activation of Akt (protein kinase B) in situ. *J Invest Dermatol* **124**, 298-303.

Kadono T, Kikuchi K, Ihn H, Takehara K, & Tamaki K (1998). Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts. *J Rheumatol* **25**, 296-301.

Kadono T, Kikuchi K, Sato S, Soma Y, Tamaki K, & Takehara K (1995). Elevated plasma endothelin levels in systemic sclerosis. *Arch Dermatol Res* **287**, 439-442.

Kahaleh B (2008). Vascular disease in scleroderma: mechanisms of vascular injury. *Rheum Dis Clin North Am* **34**, 57-71.

Kahaleh MB (1991). Endothelin, an endothelial-dependent vasoconstrictor in scleroderma. Enhanced production and profibrotic action. *Arthritis Rheum* **34**, 978-983.

Kahaleh MB, Sherer GK, & LeRoy EC (1979). Endothelial injury in scleroderma. *J Exp Med* **149**, 1326-1335.

Kahlmann D, valos-Misslitz AC, Ohl L, Stanke F, Witte T, & Forster R (2007). Genetic variants of chemokine receptor CCR7 in patients with systemic lupus erythematosus, Sjogren's syndrome and systemic sclerosis. *BMC Genet* **8**, 33.

Karrer S, Bosserhoff AK, Weiderer P, Distler O, Landthaler M, Szeimies RM, Muller-Ladner U, Scholmerich J, & Hellerbrand C (2005). The -2518 promotor polymorphism in the MCP-1 gene is associated with systemic sclerosis. *J Invest Dermatol* **124**, 92-98.

Kasama T, Strieter RM, Lukacs NW, Lincoln PM, Burdick MD, & Kunkel SL (1995). Interleukin-10 expression and chemokine regulation during the evolution of murine type II collagen-induced arthritis. *J Clin Invest* **95**, 2868-2876.

Kattla JJ, Carew RM, Heljic M, Godson C, & Brazil DP (2008). Protein kinase B/Akt activity is involved in renal TGF-{beta}1-driven epithelial-mesenchymal transition in vitro and in vivo. *Am J Physiol Renal Physiol* **295**, F215-F225.

Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, Wakefield LM, Letterio JJ, & Wynn TA (2004). IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. *J Immunol* **173**, 4020-4029.

Kennedy KJ, Strieter RM, Kunkel SL, Lukacs NW, & Karpus WJ (1998). Acute and relapsing experimental autoimmune encephalomyelitis are regulated by differential expression of the CC chemokines macrophage inflammatory protein-1alpha and monocyte chemotactic protein-1. *J Neuroimmunol* **92**, 98-108.

Kim DG, Kang HM, Jang SK, & Shin HS (1992). Construction of a bifunctional mRNA in the mouse by using the internal ribosomal entry site of the encephalomyocarditis virus. *Mol Cell Biol* **12**, 3636-3643.

Kim S, Lee Y, Seo JE, Cho KH, & Chung JH (2008). Caveolin-1 increases basal and TGF-beta1-induced expression of type I procollagen through PI-3 kinase/Akt/mTOR pathway in human dermal fibroblasts. *Cell Signal* **20**, 1313-1319.

Kishimoto T (1989). The biology of interleukin-6. *Blood* **74**, 1-10.

Kitagawa K, Wada T, Furuichi K, Hashimoto H, Ishiwata Y, Asano M, Takeya M, Kuziel WA, Matsushima K, Mukaida N, & Yokoyama H (2004). Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* **165**, 237-246.

Kobayashi H, Nishimaki T, Kaise S, Suzuki T, Watanabe K, Kasukawa R, & Suzuki T (1999). Immunohistological study endothelin-1 and endothelin-A and B receptors in two patients with scleroderma renal crisis. *Clin Rheumatol* **18**, 425-427.

Koch AE (2005). Chemokines and their receptors in rheumatoid arthritis: future targets? *Arthritis Rheum* **52**, 710-721.

Koch AE, Kronfeld-Harrington LB, Szekanecz Z, Cho MM, Haines GK, Harlow LA, Strieter RM, Kunkel SL, Massa MC, Barr WG, & . (1993). In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis. Their role in early and late disease. *Pathobiology* **61**, 239-246.

Koch AE, Kunkel SL, Harlow LA, Johnson B, Evanoff HL, Haines GK, Burdick MD, Pope RM, & Strieter RM (1992). Enhanced production of monocyte chemoattractant protein-1 in rheumatoid arthritis. *J Clin Invest* **90**, 772-779.

Kodera T, McGaha TL, Phelps R, Paul WE, & Bona CA (2002). Disrupting the IL-4 gene rescues mice homozygous for the tight-skin mutation from embryonic death and diminishes TGF-beta production by fibroblasts. *Proc Natl Acad Sci U S A* **99**, 3800-3805.

Kolb M, Bonniaud P, Galt T, Sime PJ, Kelly MM, Margetts PJ, & Gauldie J (2002). Differences in the fibrogenic response after transfer of active transforming growth factor-beta1 gene to lungs of "fibrosis-prone" and "fibrosis-resistant" mouse strains. *Am J Respir Cell Mol Biol* **27**, 141-150.

Kolek V, Petrek M, Szotkowska J, & du Bois RM (2002). CC and C chemokine expression in pulmonary sarcoidosis. *Eur Respir J* **20**, 1206-1212.

Kolodsick JE, Toews GB, Jakubzick C, Hogaboam C, Moore TA, McKenzie A, Wilke CA, Chrisman CJ, & Moore BB (2004). Protection from fluorescein isothiocyanate-induced fibrosis in IL-13-deficient, but not IL-4-deficient, mice results from impaired collagen synthesis by fibroblasts. *J Immunol* **172**, 4068-4076.

Kondo K, Okada T, Matsui T, Kato S, Date K, Yoshihara M, Nagata Y, Takagi H, Yoneda M, & Sugie I (2001). Establishment and characterization of a human B cell line from the lung tissue of a patient with scleroderma; extraordinary high level of IL-6 secretion by stimulated fibroblasts. *Cytokine* **13**, 220-226.

Kowara R, Karaczyn A, Cheng RY, Salnikow K, & Kasprzak KS (2005). Microarray analysis of altered gene expression in murine fibroblasts transformed by nickel(II) to nickel(II)-resistant malignant phenotype. *Toxicol Appl Pharmacol* **205**, 1-10.

Kraling BM, Maul GG, & Jimenez SA (1995). Mononuclear cellular infiltrates in clinically involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. *Pathobiology* **63**, 48-56.

Kretzschmar M, Doody J, Timokhina I, & Massague J (1999). A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* **13**, 804-816.

Kubo M, Czuwara-Ladykowska J, Moussa O, Markiewicz M, Smith E, Silver RM, Jablonska S, Blaszczyk M, Watson DK, & Trojanowska M (2003). Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. *Am J Pathol* **163**, 571-581.

Kudo Y, Siriwardena BS, Hatano H, Ogawa I, & Takata T (2007). Periostin: novel diagnostic and therapeutic target for cancer. *Histol Histopathol* **22**, 1167-1174.

Kulkarni AB, Ward JM, Yaswen L, Mackall CL, Bauer SR, Huh CG, Gress RE, & Karlsson S (1995). Transforming growth factor-beta 1 null mice. An animal model for inflammatory disorders. *Am J Pathol* **146**, 264-275.

Kundu SD, Kim IY, Yang T, Doglio L, Lang S, Zhang X, Buttyan R, Kim SJ, Chang J, Cai X, Wang Z, & Lee C (2000). Absence of proximal duct apoptosis in the ventral prostate of transgenic mice carrying the C3(1)-TGF-beta type II dominant negative receptor. *Prostate* **43**, 118-124.

Kurose H, Katada T, Amano T, & Ui M (1983). Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via alpha-adrenergic, cholinergic, and opiate receptors in neuroblastoma x glioma hybrid cells. *J Biol Chem* **258**, 4870-4875.

Kuwana M, Kaburaki J, Arnett FC, Howard RF, Medsger TA, Jr., & Wright TM (1999). Influence of ethnic background on clinical and serologic features in patients with systemic sclerosis and anti-DNA topoisomerase I antibody. *Arthritis Rheum* **42**, 465-474.

Kuziel WA, Morgan SJ, Dawson TC, Griffin S, Smithies O, Ley K, & Maeda N (1997). Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. *Proc Natl Acad Sci U S A* **94**, 12053-12058.

Lane KB, Machado RD, Pauciulo MW, Thomson JR, Phillips JA, III, Loyd JE, Nichols WC, & Trembath RC (2000). Heterozygous germline mutations in BMPR2, encoding a TGF-beta receptor, cause familial primary pulmonary hypertension. The International PPH Consortium. *Nat Genet* **26**, 81-84.

Lange-Sperandio B, Trautmann A, Eickelberg O, Jayachandran A, Oberle S, Schmidutz F, Rodenbeck B, Homme M, Horuk R, & Schaefer F (2007). Leukocytes induce epithelial to mesenchymal transition after unilateral ureteral obstruction in neonatal mice. *Am J Pathol* **171**, 861-871.

Le HM, Martin M, & Haas C (1999). A syndrome resembling human systemic sclerosis (scleroderma) in MRL/lpr mice lacking interferon-gamma (IFN-gamma) receptor (MRL/lprgammaR-/-). *Clin Exp Immunol* **115**, 281-287.

Leask A, Holmes A, Black CM, & Abraham DJ (2003). Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. *J Biol Chem* **278**, 13008-13015.

Lee CG, Kang HR, Homer RJ, Chupp G, & Elias JA (2006). Transgenic modeling of transforming growth factor-beta(1): role of apoptosis in fibrosis and alveolar remodeling. *Proc Am Thorac Soc* **3**, 418-423.

Lee EB, Zhao J, Kim JY, Xiong M, & Song YW (2007). Evidence of potential interaction of chemokine genes in susceptibility to systemic sclerosis. *Arthritis Rheum* **56**, 2443-2448.

Lee YW, Hennig B, & Toborek M (2003). Redox-regulated mechanisms of IL-4-induced MCP-1 expression in human vascular endothelial cells. *Am J Physiol Heart Circ Physiol* **284**, H185-H192.

Lemaire R, Farina G, Kissin E, Shipley JM, Bona C, Korn JH, & Lafyatis R (2004). Mutant fibrillin 1 from tight skin mice increases extracellular matrix incorporation of microfibril-associated glycoprotein 2 and type I collagen. *Arthritis Rheum* **50**, 915-926.

LeRoy EC (1974). Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. *J Clin Invest* **54**, 880-889.

LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA, Jr., Rowell N, & Wollheim F (1988). Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* **15**, 202-205.

LeRoy EC & Medsger TA, Jr. (2001). Criteria for the classification of early systemic sclerosis. *J Rheumatol* **28**, 1573-1576.

Letterio JJ, Geiser AG, Kulkarni AB, Dang H, Kong L, Nakabayashi T, Mackall CL, Gress RE, & Roberts AB (1996). Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. *J Clin Invest* **98**, 2109-2119.

Li Q, Park PW, Wilson CL, & Parks WC (2002). Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. *Cell* **111**, 635-646.

Liau G, Szapary D, Setoyama C, & de CB (1986). Restriction enzyme digestions identify discrete domains in the chromatin around the promoter of the mouse alpha 2(I) collagen gene. *J Biol Chem* **261**, 11362-11368.

Libby J, Martinez R, & Weber MJ (1986). Tyrosine phosphorylation in cells treated with transforming growth factor-beta. *J Cell Physiol* **129**, 159-166.

Lin CL, Suri RM, Rahdon RA, Austyn JM, & Roake JA (1998). Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. *Eur J Immunol* **28**, 4114-4122.

Lloberas N, Cruzado JM, Franquesa M, Herrero-Fresneda I, Torras J, Alperovich G, Rama I, Vidal A, & Grinyo JM (2006). Mammalian target of rapamycin pathway blockade slows progression of diabetic kidney disease in rats. *J Am Soc Nephrol* **17**, 1395-1404.

Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, Meyers J, Leitch CC, Katsanis N, Sharifi N, Xu FL, Myers LA, Spevak PJ, Cameron DE, De Backer J, Hellemans J, Chen Y, Davis EC, Webb CL, Kress W, Coucke P, Rifkin DB, De Paepe AM, & Dietz HC (2005). A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet* **37**, 275-281.

Low QE, Drugea IA, Duffner LA, Quinn DG, Cook DN, Rollins BJ, Kovacs EJ, & DiPietro LA (2001). Wound healing in MIP-1alpha(-/-) and MCP-1(-/-) mice. *Am J Pathol* **159**, 457-463.

Ludwicka-Bradley A, Tourkina E, Suzuki S, Tyson E, Bonner M, Fenton JW, Hoffman S, & Silver RM (2000). Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation. *Am J Respir Cell Mol Biol* **22**, 235-243.

Lukacs NW, Chensue SW, Karpus WJ, Lincoln P, Keefer C, Strieter RM, & Kunkel SL (1997). C-C chemokines differentially alter interleukin-4 production from lymphocytes. *Am J Pathol* **150**, 1861-1868.

Luther SA & Cyster JG (2001). Chemokines as regulators of T cell differentiation. *Nat Immunol* **2**, 102-107.

Luzina IG, Atamas SP, Wise R, Wigley FM, Xiao HQ, & White B (2002). Gene expression in bronchoalveolar lavage cells from scleroderma patients. *Am J Respir Cell Mol Biol* **26**, 549-557.

Luzina IG, Tsymbalyuk N, Choi J, Hasday JD, & Atamas SP (2006). CCL18-stimulated upregulation of collagen production in lung fibroblasts requires Sp1 signaling and basal Smad3 activity. *J Cell Physiol* **206**, 221-228.

Ma J, Wang Q, Fei T, Han JD, & Chen YG (2007). MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration. *Blood* **109**, 987-994.

Mackiewicz Z, Sukura A, Povilenaite D, Ceponis A, Virtanen I, Hukkanen M, & Konttinen YT (2002). Increased but imbalanced expression of VEGF and its receptors has no positive effect on angiogenesis in systemic sclerosis skin. *Clin Exp Rheumatol* **20**, 641-646.

Madani G, Katz RD, Haddock JA, Denton CP, & Bell JR (2008). The role of radiology in the management of systemic sclerosis. *Clin Radiol* **63**, 959-967.

Makhluf HA, Stepniakowska J, Hoffman S, Smith E, LeRoy EC, & Trojanowska M (1996). IL-4 upregulates tenascin synthesis in scleroderma and healthy skin fibroblasts. *J Invest Dermatol* **107**, 856-859.

Mantovani A (1999). The chemokine system: redundancy for robust outputs. *Immunol Today* **20**, 254-257.

Mantovani A, Bonecchi R, & Locati M (2006). Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* **6**, 907-918.

Mantovani A, Garlanda C, Locati M, Rodriguez TV, Feo SG, Savino B, & Vecchi A (2007). Regulatory pathways in inflammation. *Autoimmun Rev* **7**, 8-11.

Marasini B, Cossutta R, Selmi C, Pozzi MR, Gardinali M, Massarotti M, Erario M, Battaglioli L, & Biondi ML (2005). Polymorphism of the fractalkine receptor CX3CR1 and systemic sclerosis-associated pulmonary arterial hypertension. *Clin Dev Immunol* **12**, 275-279.

Martin T, Cardarelli PM, Parry GC, Felts KA, & Cobb RR (1997). Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1. *Eur J Immunol* **27**, 1091-1097.

Massague J, Seoane J, & Wotton D (2005). Smad transcription factors. *Genes Dev* **19**, 2783-2810.

Matucci CM & Kahaleh MB (2002). Beauty and the beast. The nitric oxide paradox in systemic sclerosis. *Rheumatology (Oxford)* **41**, 843-847.

Mayes MD, Lacey JV, Jr., Beebe-Dimmer J, Gillespie BW, Cooper B, Laing TJ, & Schottenfeld D (2003). Prevalence, incidence, survival, and disease characteristics of systemic sclerosis in a large US population. *Arthritis Rheum* **48**, 2246-2255.

McCormick LL, Zhang Y, Tootell E, & Gilliam AC (1999). Anti-TGF-beta treatment prevents skin and lung fibrosis in murine sclerodermatous graft-versus-host disease: a model for human scleroderma. *J Immunol* **163**, 5693-5699.

McGaha T, Saito S, Phelps RG, Gordon R, Noben-Trauth N, Paul WE, & Bona C (2001). Lack of skin fibrosis in tight skin (TSK) mice with targeted mutation in the interleukin-4R alpha and transforming growth factor-beta genes. *J Invest Dermatol* **116**, 136-143.

McGaha TL, Le M, Kodera T, Stoica C, Zhu J, Paul WE, & Bona CA (2003). Molecular mechanisms of interleukin-4-induced up-regulation of type I collagen gene expression in murine fibroblasts. *Arthritis Rheum* **48**, 2275-2284.

McHugh NJ, Whyte J, Harvey G, & Haustein UF (1994). Anti-topoisomerase I antibodies in silica-associated systemic sclerosis. A model for autoimmunity. *Arthritis Rheum* **37**, 1198-1205.

McMillan TR, Moore BB, Weinberg JB, Vannella KM, Fields WB, Christensen PJ, van Dyk LF, & Toews GB (2008). Exacerbation of established pulmonary fibrosis in a murine model by gammaherpesvirus. *Am J Respir Crit Care Med* **177**, 771-780.

McQuibban GA, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, & Overall CM (2002). Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* **100**, 1160-1167.

Medina-Tato DA, Ward SG, & Watson ML (2007). Phosphoinositide 3-kinase signalling in lung disease: leucocytes and beyond. *Immunology* **121**, 448-461.

Medsger TA, Jr., Silman AJ, Steen VD, Black CM, Akesson A, Bacon PA, Harris CA, Jablonska S, Jayson MI, Jimenez SA, Krieg T, LeRoy EC, Maddison PJ, Russell ML, Schachter RK, Wollheim FA, & Zacharaie H (1999). A disease severity scale for systemic sclerosis: development and testing. *J Rheumatol* **26**, 2159-2167.

Meloni F, Caporali R, Marone BA, Paschetto E, Morosini M, Fietta AM, Patrizio V, Bobbio-Pallavicini F, Pozzi E, & Montecucco C (2004). BAL cytokine profile in different interstitial lung diseases: a focus on systemic sclerosis. *Sarcoidosis Vasc Diffuse Lung Dis* **21**, 111-118.

Mendoza FA, Artlett CM, Sandorfi N, Latinis K, Piera-Velazquez S, & Jimenez SA (2006). Description of 12 cases of nephrogenic fibrosing dermopathy and review of the literature. *Semin Arthritis Rheum* **35**, 238-249.

Menon RP, Menon MR, Shi-Wen X, Renzoni E, Bou-Gharios G, Black CM, & Abraham DJ (2006). Hammerhead ribozyme-mediated silencing of the mutant fibrillin-1 of tight skin mouse: insight into the functional role of mutant fibrillin-1. *Exp Cell Res* **312**, 1463-1474.

Menten P, Proost P, Struyf S, Van CE, Put W, Lenaerts JP, Conings R, Jaspar JM, De GD, Billiau A, Opdenakker G, & Van DJ (1999). Differential induction of monocyte chemotactic protein-3 in mononuclear leukocytes and fibroblasts by interferon-alpha/beta and interferon-gamma reveals MCP-3 heterogeneity. *Eur J Immunol* **29**, 678-685.

Minty A, Chalon P, Guillemot JC, Kaghad M, Liauzun P, Magazin M, Miloux B, Minty C, Ramond P, Vita N, & . (1993). Molecular cloning of the MCP-3 chemokine gene and regulation of its expression. *Eur Cytokine Netw* **4**, 99-110.

Mirolo M, Fabbri M, Sironi M, Vecchi A, Guglielmotti A, Mangano G, Biondi G, Locati M, & Mantovani A (2008). Impact of the anti-inflammatory agent bindarit on the chemokinome: selective inhibition of the monocyte chemotactic proteins. *Eur Cytokine Netw* **19**, 119-122.

Mirzadegan T, Diehl F, Ebi B, Bhakta S, Polsky I, McCarley D, Mulkins M, Weatherhead GS, Lapierre JM, Dankwardt J, Morgans D, Jr., Wilhelm R, & Jarnagin K (2000). Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. *J Biol Chem* **275**, 25562-25571.

Miyazawa K, Shinozaki M, Hara T, Furuya T, & Miyazono K (2002). Two major Smad pathways in TGF-beta superfamily signalling. *Genes Cells* **7**, 1191-1204.

Miyazono K, Olofsson A, Colosetti P, & Heldin CH (1991). A role of the latent TGF-beta 1-binding protein in the assembly and secretion of TGF-beta 1. *EMBO J* **10**, 1091-1101.

Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, & Toews GB (2005). CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol* **166**, 675-684.

Moore BB, Paine R, III, Christensen PJ, Moore TA, Sitterding S, Ngan R, Wilke CA, Kuziel WA, & Toews GB (2001). Protection from pulmonary fibrosis in the absence of CCR2 signaling. *J Immunol* **167**, 4368-4377.

Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, & Trembath RC (2001). Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta(1) and bone morphogenetic proteins. *Circulation* **104**, 790-795.

Morrissey JJ & Klahr S (1998). Differential effects of ACE and AT1 receptor inhibition on chemoattractant and adhesion molecule synthesis. *Am J Physiol* **274**, F580-F586.

Morse J, Barst R, Horn E, Cuervo N, Deng Z, & Knowles J (2002). Pulmonary hypertension in scleroderma spectrum of disease: lack of bone morphogenetic protein receptor 2 mutations. *J Rheumatol* **29**, 2379-2381.

Motomura Y, Khan WI, El-Sharkawy RT, Verma-Gandhu M, Verdu EF, Gauldie J, & Collins SM (2006). Induction of a fibrogenic response in mouse colon by overexpression of monocyte chemoattractant protein 1. *Gut* **55**, 662-670.

Moustakas A & Heldin CH (2005). Non-Smad TGF-{beta} signals. *J Cell Sci* **118**, 3573-3584.

Moustakas A, Souchelnytskyi S, & Heldin CH (2001). Smad regulation in TGF-beta signal transduction. *J Cell Sci* **114**, 4359-4369.

Mueller A, Mahmoud NG, Goedecke MC, McKeating JA, & Strange PG (2002). Pharmacological characterization of the chemokine receptor, CCR5. *Br J Pharmacol* **135**, 1033-1043.

Mukerjee D, St GD, Coleiro B, Knight C, Denton CP, Davar J, Black CM, & Coghlan JG (2003). Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: application of a registry approach. *Ann Rheum Dis* **62**, 1088-1093.

Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, & Sheppard D (1999). The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**, 319-328.

Murakami K, Nomiyama H, Miura R, Follens A, Fiten P, Van CE, Van DJ, & Opdenakker G (1997). Structural and functional analysis of the promoter region of the human MCP-3 gene: transactivation of expression by novel recognition sequences adjacent to the transcription initiation site. *DNA Cell Biol* **16**, 173-183.

Murphy PM (2002). International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev* **54**, 227-229.

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, & Power CA (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**, 145-176.

Murray LA, Argentieri RL, Farrell FX, Bracht M, Sheng H, Whitaker B, Beck H, Tsui P, Cochlin K, Evanoff HL, Hogaboam CM, & Das AM (2008). Hyper-responsiveness of IPF/UIP fibroblasts: Interplay between TGFbeta1, IL-13 and CCL2. *Int J Biochem Cell Biol*.

Nakao A, Fujii M, Matsumura R, Kumano K, Saito Y, Miyazono K, & Iwamoto I (1999). Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* **104**, 5-11.

Narumi S, Takeuchi T, Kobayashi Y, & Konishi K (2000). Serum levels of ifn-inducible PROTEIN-10 relating to the activity of systemic lupus erythematosus. *Cytokine* **12**, 1561-1565.

Navratilova Z, Lukac J, Mrazek F, Kriegova E, Bucova M, Bosak V, & Petrek M (2008). MCP-1 -2518 A/G single nucleotide polymorphism in slovak patients with systemic sclerosis. *Mediators Inflamm* **2008**, 204063.

Needleman BW, Wigley FM, & Stair RW (1992). Interleukin-1, interleukin-2, interleukin-4, interleukin-6, tumor necrosis factor alpha, and interferon-gamma levels in sera from patients with scleroderma. *Arthritis Rheum* **35**, 67-72.

Nelissen I, Dubois B, Goris A, Ronsse I, Carton H, & Opdenakker G (2002). Gelatinase B, PECAM-1 and MCP-3 gene polymorphisms in Belgian multiple sclerosis. *J Neurol Sci* **200**, 43-48.

Newman JH, Wheeler L, Lane KB, Loyd E, Gaddipati R, Phillips JA, III, & Loyd JE (2001). Mutation in the gene for bone morphogenetic protein receptor II as a cause of primary pulmonary hypertension in a large kindred. *N Engl J Med* **345**, 319-324.

Nguyen VA, Sgonc R, Dietrich H, & Wick G (2000). Endothelial injury in internal organs of University of California at Davis line 200 (UCD 200) chickens, an animal model for systemic sclerosis (Scleroderma). *J Autoimmun* **14**, 143-149.

Nibbs R, Graham G, & Rot A (2003). Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6. *Semin Immunol* **15**, 287-294.

Nickoloff BJ, Xin H, Nestle FO, & Qin JZ (2007). The cytokine and chemokine network in psoriasis. *Clin Dermatol* **25**, 568-573.

Nietert PJ & Silver RM (2000). Systemic sclerosis: environmental and occupational risk factors. *Curr Opin Rheumatol* **12**, 520-526.

Nihtyanova SI & Denton CP (2008). Current approaches to the management of early active diffuse scleroderma skin disease. *Rheum Dis Clin North Am* **34**, 161-179.

Nirodi CS, Devalaraja R, Nanney LB, Arrindell S, Russell S, Trupin J, & Richmond A (2000). Chemokine and chemokine receptor expression in keloid and normal fibroblasts. *Wound Repair Regen* **8**, 371-382.

Nishimaki T, Aotsuka S, Kondo H, Yamamoto K, Takasaki Y, Sumiya M, & Yokohari R (1999). Immunological analysis of pulmonary hypertension in connective tissue diseases. *J Rheumatol* **26**, 2357-2362.

Noble PW (2003). Idiopathic pulmonary fibrosis. New insights into classification and pathogenesis usher in a new era therapeutic approaches. *Am J Respir Cell Mol Biol* **29**, S27-S31.

O'Boyle G, Brain JG, Kirby JA, & Ali S (2007). Chemokine-mediated inflammation: Identification of a possible regulatory role for CCR2. *Mol Immunol* **44**, 1944-1953.

O'Hayre M, Salanga CL, Handel TM, & Allen SJ (2008). Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. *Biochem J* **409**, 635-649.

Oh SP, Seki T, Goss KA, Imamura T, Yi Y, Donahoe PK, Li L, Miyazono K, ten Dijke P, Kim S, & Li E (2000). Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A* **97**, 2626-2631.

Ong C, Wong C, Roberts CR, Teh HS, & Jirik FR (1998). Anti-IL-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. *Eur J Immunol* **28**, 2619-2629.

Ong CJ, Ip S, Teh SJ, Wong C, Jirik FR, Grusby MJ, & Teh HS (1999). A role for T helper 2 cells in mediating skin fibrosis in tight-skin mice. *Cell Immunol* **196**, 60-68.

Oshima M, Maeda A, Ishioka S, Hiyama K, & Yamakido M (1999). Expression of C-C chemokines in bronchoalveolar lavage cells from patients with granulomatous lung diseases. *Lung* **177**, 229-240.

Pannu J, Gardner H, Shearstone JR, Smith E, & Trojanowska M (2006). Increased levels of transforming growth factor beta receptor type I and up-regulation of matrix gene program: A model of scleroderma. *Arthritis Rheum* **54**, 3011-3021.

Pannu J, Gore-Hyer E, Yamanaka M, Smith EA, Rubinchik S, Dong JY, Jablonska S, Blaszczyk M, & Trojanowska M (2004). An increased transforming growth factor beta receptor type I:type II ratio contributes to elevated collagen protein synthesis that is resistant to inhibition via a kinase-deficient transforming growth factor beta receptor type II in scleroderma. *Arthritis Rheum* **50**, 1566-1577.

Pannu J, Nakerakanti S, Smith E, ten DP, & Trojanowska M (2007). Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. *J Biol Chem* **282**, 10405-10413.

Pantelidis P, Southcott AM, Black CM, & du Bois RM (1997). Up-regulation of IL-8 secretion by alveolar macrophages from patients with fibrosing alveolitis: a subpopulation analysis. *Clin Exp Immunol* **108**, 95-104.

Park BL, Kim LH, Choi YH, Cheong HS, Park HS, Hong SJ, Choi BW, Lee JH, Uh ST, Park CS, & Shin HD (2005). Association analysis of monocyte chemotactic protein-3 (MCP3) polymorphisms with asthmatic phenotypes. *J Biochem Mol Biol* **38**, 77-81.

Peron P, Rahmani M, Zagar Y, Durand-Schneider AM, Lardeux B, & Bernuau D (2001). Potentiation of Smad transactivation by Jun proteins during a combined treatment with epidermal growth factor and transforming growth factor-beta in rat hepatocytes. role of phosphatidylinositol 3-kinase-induced AP-1 activation. *J Biol Chem* **276**, 10524-10531.

Petrek M, Cermakova Z, Hutyrova B, Micekova D, Drabek J, Rovensky J, & Bosak V (2002). CC chemokine receptor 5 and interleukin-1 receptor antagonist gene polymorphisms in patients with primary Sjogren's syndrome. *Clin Exp Rheumatol* **20**, 701-703.

Phelps RG, Murai C, Saito S, Hatakeyama A, Andrikopoulos K, Kasturi KN, & Bona CA (1998). Effect of targeted mutation in collagen V alpha 2 gene on development of cutaneous hyperplasia in tight skin mice. *Mol Med* **4**, 356-360.

Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, & Strieter RM (2004). Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* **114**, 438-446.

Ping D, Boekhoudt G, & Boss JM (1999). trans-Retinoic acid blocks platelet-derived growth factor-BB-induced expression of the murine monocyte chemoattractant-1 gene by blocking the assembly of a promoter proximal Sp1 binding site. *J Biol Chem* **274**, 31909-31916.

Ping D, Boekhoudt G, Zhang F, Morris A, Philipsen S, Warren ST, & Boss JM (2000). Sp1 binding is critical for promoter assembly and activation of the MCP-1 gene by tumor necrosis factor. *J Biol Chem* **275**, 1708-1714.

Ping D, Jones PL, & Boss JM (1996). TNF regulates the in vivo occupancy of both distal and proximal regulatory regions of the MCP-1/JE gene. *Immunity* **4**, 455-469.

Polentarutti N, Introna M, Sozzani S, Mancinelli R, Mantovani G, & Mantovani A (1997). Expression of monocyte chemotactic protein-3 in human monocytes and endothelial cells. *Eur Cytokine Netw* **8**, 271-274.

Ponath PD, Qin S, Ringler DJ, Clark-Lewis I, Wang J, Kassam N, Smith H, Shi X, Gonzalo JA, Newman W, Gutierrez-Ramos JC, & Mackay CR (1996). Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J Clin Invest* **97**, 604-612.

Ponticos M, Abraham D, Alexakis C, Lu QL, Black C, Partridge T, & Bou-Gharios G (2004). Col1a2 enhancer regulates collagen activity during development and in adult tissue repair. *Matrix Biol* **22**, 619-628.

Poormoghim H, Lucas M, Fertig N, & Medsger TA, Jr. Systemic sclerosis sine scleroderma: demographic, clinical, and serologic features and survival in forty-eight patients. Arthritis Rheum. 43[2], 444-451. 2000.
Ref Type: Abstract

Postlethwaite AE, Holness MA, Katai H, & Raghow R (1992). Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* **90**, 1479-1485.

Postlethwaite AE, Shigemitsu H, & Kanangat S (2004). Cellular origins of fibroblasts: possible implications for organ fibrosis in systemic sclerosis. *Curr Opin Rheumatol* **16**, 733-738.

Powell N, Humbert M, Durham SR, Assoufi B, Kay AB, & Corrigan CJ (1996). Increased expression of mRNA encoding RANTES and MCP-3 in the bronchial mucosa in atopic asthma. *Eur Respir J* **9**, 2454-2460.

Power CA, Clemetson JM, Clemetson KJ, & Wells TN (1995). Chemokine and chemokine receptor mRNA expression in human platelets. *Cytokine* **7**, 479-482.

Prescott RJ, Freemont AJ, Jones CJ, Hoyland J, & Fielding P (1992). Sequential dermal microvascular and perivascular changes in the development of scleroderma. *J Pathol* **166**, 255-263.

Prockop DJ & Kivirikko KI (1995). Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* **64**, 403-434.

Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, Borlat F, Wells TN, & Kosco-Vilbois MH (2003). Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc Natl Acad Sci U S A* **100**, 1885-1890.

Proudfoot AE, Power CA, Hoogewerf AJ, Montjovent MO, Borlat F, Offord RE, & Wells TN (1996). Extension of recombinant human RANTES by the retention of the initiating methionine produces a potent antagonist. *J Biol Chem* **271**, 2599-2603.

Pype JL, Dupont LJ, Menten P, Van CE, Opdenakker G, Van DJ, Chung KF, Demedts MG, & Verleden GM (1999). Expression of monocyte chemotactic protein (MCP)-1, MCP-2, and MCP-3 by human airway smooth-muscle cells. Modulation by corticosteroids and T-helper 2 cytokines. *Am J Respir Cell Mol Biol* **21**, 528-536.

Qian A, Meals RA, Rajfer J, & Gonzalez-Cadavid NF (2004). Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture. *Urology* **64**, 399-404.

Quan TE, Cowper SE, & Bucala R (2006). The role of circulating fibrocytes in fibrosis. *Curr Rheumatol Rep* **8**, 145-150.

Querfeld C, Eckes B, Huerkamp C, Krieg T, & Sollberg S (1999). Expression of TGF-beta 1, -beta 2 and -beta 3 in localized and systemic scleroderma. *J Dermatol Sci* **21**, 13-22.

Rajkumar VS, Sundberg C, Abraham DJ, Rubin K, & Black CM (1999). Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. *Arthritis Rheum* **42**, 930-941.

Ramirez F, Tanaka S, & Bou-Gharios G (2006). Transcriptional regulation of the human alpha2(I) collagen gene (COL1A2), an informative model system to study fibrotic diseases. *Matrix Biol* **25**, 365-372.

Randone SB, Guiducci S, & Cerinic MM (2008). Systemic sclerosis and infections. *Autoimmun Rev*.

Reitamo S, Remitz A, Varga J, Ceska M, Effenberger F, Jimenez S, & Uitto J (1993). Demonstration of interleukin 8 and autoantibodies to interleukin 8 in the serum of patients with systemic sclerosis and related disorders. *Arch Dermatol* **129**, 189-193.

Renzoni E, Lympany P, Sestini P, Pantelidis P, Wells A, Black C, Welsh K, Bunn C, Knight C, Foley P, & du Bois RM (2000). Distribution of novel polymorphisms of the interleukin-8 and CXC receptor 1 and 2 genes in systemic sclerosis and cryptogenic fibrosing alveolitis. *Arthritis Rheum* **43**, 1633-1640.

Renzoni EA, Abraham DJ, Howat S, Shi-Wen X, Sestini P, Bou-Gharios G, Wells AU, Veeraraghavan S, Nicholson AG, Denton CP, Leask A, Pearson JD, Black CM, Welsh KI, & du Bois RM (2004). Gene expression profiling reveals novel TGFbeta targets in adult lung fibroblasts. *Respir Res* **5**, 24.

Reunanen N, Foschi M, Han J, & Kahari VM (2000). Activation of extracellular signal-regulated kinase 1/2 inhibits type I collagen expression by human skin fibroblasts. *J Biol Chem* **275**, 34634-34639.

Ribeiro S & Horuk R (2005). The clinical potential of chemokine receptor antagonists. *Pharmacol Ther* **107**, 44-58.

Rinn JL, Bondre C, Gladstone HB, Brown PO, & Chang HY (2006). Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Genet* **2**, e119.

Rippe RA, Schrum LW, Stefanovic B, Solis-Herruzo JA, & Brenner DA (1999). NF-kappaB inhibits expression of the alpha1(I) collagen gene. *DNA Cell Biol* **18**, 751-761.

Roberts AB, McCune BK, & Sporn MB (1992). TGF-beta: regulation of extracellular matrix. *Kidney Int* **41**, 557-559.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, & . (1986). Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc Natl Acad Sci U S A* **83**, 4167-4171.

Rodriguez C, Huang LJ, Son JK, McKee A, Xiao Z, & Lodish HF (2001). Functional cloning of the proto-oncogene brain factor-1 (BF-1) as a Smad-binding antagonist of transforming growth factor-beta signaling. *J Biol Chem* **276**, 30224-30230.

Rojas-Ramos E, Avalos AF, Perez-Fernandez L, Cuevas-Schacht F, Valencia-Maqueda E, & Teran LM (2003). Role of the chemokines RANTES, monocyte chemotactic proteins-3 and -4, and eotaxins-1 and -2 in childhood asthma. *Eur Respir J* **22**, 310-316.

Rossert J, Eberspaecher H, & de Crombrugghe B (1995). Separate cis-acting DNA elements of the mouse pro-alpha 1(I) collagen promoter direct expression of reporter genes to different type I collagen-producing cells in transgenic mice. *J Cell Biol* **129**, 1421-1432.

Runyan CE, Schnaper HW, & Poncelet AC (2004). The phosphatidylinositol 3-kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen I expression in response to transforming growth factor-beta1. *J Biol Chem* **279**, 2632-2639.

Russo RC, Guabiraba R, Garcia CC, Barcelos LS, Roffe E, Souza AL, Amaral FA, Cisalpino D, Cassali GD, Doni A, Bertini R, & Teixeira MM (2008). Role of the Chemokine Receptor CXCR2 in Bleomycin-induced Pulmonary Inflammation and Fibrosis. *Am J Respir Cell Mol Biol*.

Ruzek MC, Jha S, Ledbetter S, Richards SM, & Garman RD (2004). A modified model of graft-versus-host-induced systemic sclerosis (scleroderma) exhibits all major aspects of the human disease. *Arthritis Rheum* **50**, 1319-1331.

Saharinen J, Taipale J, & Keski-Oja J (1996). Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. *EMBO J* **15**, 245-253.

Saito E, Fujimoto M, Hasegawa M, Komura K, Hamaguchi Y, Kaburagi Y, Nagaoka T, Takehara K, Tedder TF, & Sato S (2002). CD19-dependent B lymphocyte signaling thresholds influence skin fibrosis and autoimmunity in the tight-skin mouse. *J Clin Invest* **109**, 1453-1462.

Saito S, Nishimura H, Phelps RG, Wolf I, Suzuki M, Honjo T, & Bona C (2000). Induction of skin fibrosis in mice expressing a mutated fibrillin-1 gene. *Mol Med* **6**, 825-836.

Sakkas LI, Chikanza IC, & Platsoucas CD (2006). Mechanisms of Disease: the role of immune cells in the pathogenesis of systemic sclerosis. *Nat Clin Pract Rheumatol* **2**, 679-685.

Samuel CS, Zhao C, Yang Q, Wang H, Tian H, Tregear GW, & Amento EP (2005). The relaxin gene knockout mouse: a model of progressive scleroderma. *J Invest Dermatol* **125**, 692-699.

Sato S, Fujimoto M, Hasegawa M, & Takehara K (2004). Altered blood B lymphocyte homeostasis in systemic sclerosis: expanded naive B cells and diminished but activated memory B cells. *Arthritis Rheum* **50**, 1918-1927.

Sato S, Hasegawa M, Fujimoto M, Tedder TF, & Takehara K (2000). Quantitative genetic variation in CD19 expression correlates with autoimmunity. *J Immunol* **165**, 6635-6643.

Sato S, Hasegawa M, & Takehara K (2001). Serum levels of interleukin-6 and interleukin-10 correlate with total skin thickness score in patients with systemic sclerosis. *J Dermatol Sci* **27**, 140-146.

Sawaya HH, Jimenez SA, & Artlett CM (2004). Quantification of fetal microchimeric cells in clinically affected and unaffected skin of patients with systemic sclerosis. *Rheumatology (Oxford)* **43**, 965-968.

Scala E, Pallotta S, Frezzolini A, Abeni D, Barbieri C, Sampogna F, De Pita O, Puddu P, Paganelli R, & Russo G (2004). Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. *Clin Exp Immunol* **138**, 540-546.

Schenk S, Mal N, Finan A, Zhang M, Kiedrowski M, Popovic Z, McCarthy PM, & Penn MS (2007). Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* **25**, 245-251.

Schneider A, Panzer U, Zahner G, Wenzel U, Wolf G, Thaiss F, Helmchen U, & Stahl RA (1999). Monocyte chemoattractant protein-1 mediates collagen deposition in experimental glomerulonephritis by transforming growth factor-beta. *Kidney Int* **56**, 135-144.

Seder RA, Marth T, Sieve MC, Strober W, Letterio JJ, Roberts AB, & Kelsall B (1998). Factors involved in the differentiation of TGF-beta-producing cells from naive CD4+ T cells: IL-4 and IFN-gamma have opposing effects, while TGF-beta positively regulates its own production. *J Immunol* **160**, 5719-5728.

Seemayer CA, Viazov S, Neidhart M, Bruhlmann P, Michel BA, Gay RE, Roggendorf M, & Gay S (2001). Prevalence of TTV DNA and GBV-C RNA in patients with systemic sclerosis, rheumatoid arthritis, and osteoarthritis does not differ from that in healthy blood donors. *Ann Rheum Dis* **60**, 806-809.

Serpier H, Gillery P, Salmon-Ehr V, Garnotel R, Georges N, Kalis B, & Maquart FX (1997). Antagonistic effects of interferon-gamma and interleukin-4 on fibroblast cultures. *J Invest Dermatol* **109**, 158-162.

Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, & Moses HL (1997). Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. *J Cell Biol* **139**, 541-552.

Seseke F, Thelen P, & Ringert RH (2004). Characterization of an animal model of spontaneous congenital unilateral obstructive uropathy by cDNA microarray analysis. *Eur Urol* **45**, 374-381.

Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, & Wick G (2000). Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95. *Arthritis Rheum* **43**, 2550-2562.

Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, & Wick G (1996). Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *J Clin Invest* **98**, 785-792.

Shah M, Foreman DM, & Ferguson MW (1995). Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. *J Cell Sci* **108 (Pt 3)**, 985-1002.

Shand L, Lunt M, Nihtyanova S, Hoseini M, Silman A, Black CM, & Denton CP (2007). Relationship between change in skin score and disease outcome in diffuse cutaneous systemic sclerosis: application of a latent linear trajectory model. *Arthritis Rheum* **56**, 2422-2431.

Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, Leask A, & Abraham DJ (2004). Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. *Mol Biol Cell* **15**, 2707-2719.

Shi-Wen X, Pennington D, Holmes A, Leask A, Bradham D, Beauchamp JR, Fonseca C, du Bois RM, Martin GR, Black CM, & Abraham DJ (2000). Autocrine overexpression of CTGF maintains fibrosis: RDA analysis of fibrosis genes in systemic sclerosis. *Exp Cell Res* **259**, 213-224.

Shireman PK (2007). The chemokine system in arteriogenesis and hind limb ischemia. *J Vasc Surg* **45 Suppl A**, A48-A56.

Shuman S (1991). Recombination mediated by vaccinia virus DNA topoisomerase I in Escherichia coli is sequence specific. *Proc Natl Acad Sci U S A* **88**, 10104-10108.

Shuman S (1994). Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. *J Biol Chem* **269**, 32678-32684.

Silman AJ & Black C (1988). Increased incidence of spontaneous abortion and infertility in women with scleroderma before disease onset: a controlled study. *Ann Rheum Dis* **47**, 441-444.

Sime PJ, Xing Z, Graham FL, Csaky KG, & Gauldie J (1997). Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* **100**, 768-776.

Somanath PR, Chen J, & Byzova TV (2008). Akt1 is necessary for the vascular maturation and angiogenesis during cutaneous wound healing. *Angiogenesis*.

Sondergaard K, Stengaard-Pedersen K, Zachariae H, Heickendorff L, Deleuran M, & Deleuran B (1998). Soluble intercellular adhesion molecule-1 (sICAM-1) and soluble interleukin-2 receptors (sIL-2R) in scleroderma skin. *Br J Rheumatol* **37**, 304-310.

Sonnylal S, Denton CP, Zheng B, Keene DR, He R, Adams HP, Vanpelt CS, Geng YJ, Deng JM, Behringer RR, & de Crombrugghe B (2007). Postnatal induction of transforming growth factor beta signaling in fibroblasts of mice recapitulates clinical, histologic, and biochemical features of scleroderma. *Arthritis Rheum* **56**, 334-344.

Spencer-Green G (1998). Outcomes in primary Raynaud phenomenon: a meta-analysis of the frequency, rates, and predictors of transition to secondary diseases. *Arch Intern Med* **158**, 595-600.

Stafford S, Li H, Forsythe PA, Ryan M, Bravo R, & Alam R (1997). Monocyte chemotactic protein-3 (MCP-3)/fibroblast-induced cytokine (FIC) in eosinophilic inflammation of the airways and the inhibitory effects of an anti-MCP-3/FIC antibody. *J Immunol* **158**, 4953-4960.

Steen VD & Medsger TA, Jr. (2001). Improvement in skin thickening in systemic sclerosis associated with improved survival. *Arthritis Rheum* **44**, 2828-2835.

Steen VD & Medsger TA, Jr. (2000). Severe organ involvement in systemic sclerosis with diffuse scleroderma. *Arthritis Rheum* **43**, 2437-2444.

Steen VD, Medsger TA, Jr., Osial TA, Jr., Ziegler GL, Shapiro AP, & Rodnan GP (1984). Factors predicting development of renal involvement in progressive systemic sclerosis. *Am J Med* **76**, 779-786.

Storb U, O'Brien RL, McMullen MD, Gollahon KA, & Brinster RL (1984). High expression of cloned immunoglobulin kappa gene in transgenic mice is restricted to B lymphocytes. *Nature* **310**, 238-241.

Stratton RJ, Coghlan JG, Pearson JD, Burns A, Sweny P, Abraham DJ, & Black CM (1998). Different patterns of endothelial cell activation in renal and pulmonary vascular disease in scleroderma. *QJM* **91**, 561-566.

Stratton RJ, Pompon L, Coghlan JG, Pearson JD, & Black CM (2000). Soluble thrombomodulin concentration is raised in scleroderma associated pulmonary hypertension. *Ann Rheum Dis* **59**, 132-134.

Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980). Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* **23**, 581-590.

Svegliati S, Olivieri A, Campelli N, Luchetti M, Poloni A, Trappolini S, Moroncini G, Bacigalupo A, Leoni P, Avvedimento EV, & Gabrielli A (2007). Stimulatory autoantibodies to PDGF receptor in patients with extensive chronic graft-versus-host disease. *Blood* **110**, 237-241.

Swaminathan S, Ahmed I, McCarthy JT, Albright RC, Pittelkow MR, Caplice NM, Griffin MD, & Leung N (2006). Nephrogenic fibrosing dermopathy and high-dose erythropoietin therapy. *Ann Intern Med* **145**, 234-235.

Tager RE & Tikly M (1999). Clinical and laboratory manifestations of systemic sclerosis (scleroderma) in Black South Africans. *Rheumatology (Oxford)* **38**, 397-400.

Takagawa S, Lakos G, Mori Y, Yamamoto T, Nishioka K, & Varga J (2003). Sustained activation of fibroblast transforming growth factor-beta/Smad signaling in a murine model of scleroderma. *J Invest Dermatol* **121**, 41-50.

Takeda K, Hatamochi A, Ueki H, Nakata M, & Oishi Y (1994). Decreased collagenase expression in cultured systemic sclerosis fibroblasts. *J Invest Dermatol* **103**, 359-363.

Takemura H, Suzuki H, Fujisawa H, Yuhara T, Akama T, Yamane K, & Kashiwagi H (1998). Enhanced interleukin 6 production by cultured fibroblasts from patients with systemic sclerosis in response to platelet derived growth factor. *J Rheumatol* **25**, 1534-1539.

Takemura M, Itoh H, Sagawa N, Yura S, Korita D, Kakui K, Hirota N, & Fujii S (2004). Cyclic mechanical stretch augments both interleukin-8 and monocyte chemotactic protein-3 production in the cultured human uterine cervical fibroblast cells. *Mol Hum Reprod* **10**, 573-580.

Takeuchi A, Usui Y, Takeuchi M, Hattori T, Kezuka T, Suzuki J, Okunuki Y, Iwasaki T, Haino M, Matsushima K, & Usui M (2005). CCR5-deficient mice develop experimental autoimmune uveoretinitis in the context of a deviant effector response. *Invest Ophthalmol Vis Sci* **46**, 3753-3760.

Tan XY, Zheng FL, Zhou QG, Duan L, & Li Y (2005). [Effect of bone morphogenetic protein-7 on monocyte chemoattractant protein-1 induced epithelial-myofibroblast transition and TGF-beta1-Smad 3 signaling pathway of HKC cells]. *Zhonghua Yi Xue Za Zhi* **85**, 2607-2612.

Tanuma N, Sakuma H, Sasaki A, & Matsumoto Y (2006). Chemokine expression by astrocytes plays a role in microglia/macrophage activation and subsequent neurodegeneration in secondary progressive multiple sclerosis. *Acta Neuropathol* **112**, 195-204.

Tashkin DP, Elashoff R, Clements PJ, Goldin J, Roth MD, Furst DE, Arriola E, Silver R, Strange C, Bolster M, Seibold JR, Riley DJ, Hsu VM, Varga J, Schraufnagel DE, Theodore A, Simms R, Wise R, Wigley F, White B, Steen V, Read C, Mayes M, Parsley E, Mubarak K, Connolly MK, Golden J, Olman M, Fessler B, Rothfield N, & Metersky M (2006). Cyclophosphamide versus placebo in scleroderma lung disease. *N Engl J Med* **354**, 2655-2666.

Tedder TF, Inaoki M, & Sato S (1997). The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. *Immunity* **6**, 107-118.

Terada Y, Hanada S, Nakao A, Kuwahara M, Sasaki S, & Marumo F (2002). Gene transfer of Smad7 using electroporation of adenovirus prevents renal fibrosis in post-obstructed kidney. *Kidney Int* **61**, 94-98.

Tew MB, Arnett FC, Reveille JD, & Tan FK (2002). Mutations of bone morphogenetic protein receptor type II are not found in patients with pulmonary hypertension and underlying connective tissue diseases. *Arthritis Rheum* **46**, 2829-2830.

Thelen M (2001). Dancing to the tune of chemokines. *Nat Immunol* **2**, 129-134.

Thiefes A, Wolter S, Mushinski JF, Hoffmann E, ttrich-Breiholz O, Graue N, Dorrie A, Schneider H, Wirth D, Luckow B, Resch K, & Kracht M (2005). Simultaneous blockade of NFkappaB, JNK, and p38 MAPK by a kinase-inactive mutant of the protein kinase TAK1 sensitizes cells to apoptosis and affects a distinct spectrum of tumor necrosis factor [corrected] target genes. *J Biol Chem* **280**, 27728-27741.

Thomson JR, Machado RD, Pauciulo MW, Morgan NV, Humbert M, Elliott GC, Ward K, Yacoub M, Mikhail G, Rogers P, Newman J, Wheeler L, Higenbottam T, Gibbs JS, Egan J, Crozier A, Peacock A, Allcock R, Corris P, Loyd JE, Trembath RC, & Nichols WC (2000). Sporadic primary pulmonary hypertension is associated with germline mutations of the gene encoding BMPR-II, a receptor member of the TGF-beta family. *J Med Genet* **37**, 741-745.

Thornton S, Duwel LE, Boivin GP, Ma Y, & Hirsch R (1999). Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. *Arthritis Rheum* **42**, 1109-1118.

Tilton B, Ho L, Oberlin E, Loetscher P, Baleux F, Clark-Lewis I, & Thelen M (2000). Signal transduction by CXC chemokine receptor 4. Stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* **192**, 313-324.

Ting WW, Stone MS, Madison KC, & Kurtz K (2003). Nephrogenic fibrosing dermopathy with systemic involvement. *Arch Dermatol* **139**, 903-906.

Toker A (2000). Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol Pharmacol* **57**, 652-658.

Tokuda A, Itakura M, Onai N, Kimura H, Kuriyama T, & Matsushima K (2000). Pivotal role of CCR1-positive leukocytes in bleomycin-induced lung fibrosis in mice. *J Immunol* **164**, 2745-2751.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, & Brown RA (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* **3**, 349-363.

Topham PS, Csizmadia V, Soler D, Hines D, Gerard CJ, Salant DJ, & Hancock WW (1999). Lack of chemokine receptor CCR1 enhances Th1 responses and glomerular injury during nephrotoxic nephritis. *J Clin Invest* **104**, 1549-1557.

Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, Mack M, & Charo IF (2007). Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. *J Clin Invest* **117**, 902-909.

Tsuji-Yamada J, Nakazawa M, Takahashi K, Iijima K, Hattori S, Okuda K, Minami M, Ikezawa Z, & Sasaki T (2001). Effect of IL-12 encoding plasmid administration on tight-skin mouse. *Biochem Biophys Res Commun* **280**, 707-712.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, & Wrana JL (1998). SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. *Cell* **95**, 779-791.

Tsuneyama K, Harada K, Yasoshima M, Hiramatsu K, Mackay CR, Mackay IR, Gershwin ME, & Nakanuma Y (2001). Monocyte chemotactic protein-1, -2, and -3 are distinctively expressed in portal tracts and granulomata in primary biliary cirrhosis: implications for pathogenesis. *J Pathol* **193**, 102-109.

Tucci M, Quatraro C, Frassanito MA, & Silvestris F (2006). Deregulated expression of monocyte chemoattractant protein-1 (MCP-1) in arterial hypertension: role in endothelial inflammation and atheromasia. *J Hypertens* **24**, 1307-1318.

Uchinami H, Seki E, Brenner DA, & D'Armiento J (2006). Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. *Hepatology* **44**, 420-429.

Uguccioni M, Gionchetti P, Robbiani DF, Rizzello F, Peruzzo S, Campieri M, & Baggiolini M (1999). Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. *Am J Pathol* **155**, 331-336.

Valentini G, D'Angelo S, Della RA, Bencivelli W, & Bombardieri S (2003). European Scleroderma Study Group to define disease activity criteria for systemic sclerosis. IV. Assessment of skin thickening by modified Rodnan skin score. *Ann Rheum Dis* **62**, 904-905.

Van Damme J, Proost P, Lenaerts JP, & Opdenakker G (1992). Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J Exp Med* **176**, 59-65.

van de WJ & Gershwin ME (1985). Animal model of human disease. Avian scleroderma. An inherited fibrotic disease of white Leghorn chickens resembling progressive systemic sclerosis. *Am J Pathol* **120**, 478-482.

Van Den Steen PE, Wuyts A, Husson SJ, Proost P, Van DJ, & Opdenakker G (2003). Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *Eur J Biochem* **270**, 3739-3749.

Van Dop C, Yamanaka G, Steinberg F, Sekura RD, Manclark CR, Stryer L, & Bourne HR (1984). ADP-ribosylation of transducin by pertussis toxin blocks the light-stimulated hydrolysis of GTP and cGMP in retinal photoreceptors. *J Biol Chem* **259**, 23-26.

Van Obberghen-Schilling E, Roche NS, Flanders KC, Sporn MB, & Roberts AB (1988). Transforming growth factor beta 1 positively regulates its own expression in normal and transformed cells. *J Biol Chem* **263**, 7741-7746.

Van CE, Van DJ, & Opdenakker G (1999). The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev* **10**, 61-86.

Van DJ, Proost P, Lenaerts JP, & Opdenakker G (1992). Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J Exp Med* **176**, 59-65.

Vancheeswaran R, Magoulas T, Efrat G, Wheeler-Jones C, Olsen I, Penny R, & Black CM (1994). Circulating endothelin-1 levels in systemic sclerosis subsets--a marker of fibrosis or vascular dysfunction? *J Rheumatol* **21**, 1838-1844.

Varga J & Abraham D (2007). Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* **117**, 557-567.

Varga J, Rudnicka L, & Uitto J (1994). Connective tissue alterations in systemic sclerosis. *Clin Dermatol* **12**, 387-396.

Varga JA & Trojanowska M (2008). Fibrosis in systemic sclerosis. *Rheum Dis Clin North Am* **34**, 115-143.

Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den BF, Dinant HJ, Lee Y, Wyant T, Jacobson EW, Baeten D, & Tak PP (2008). Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial. *Arthritis Rheum* **58**, 1931-1939.

Vermeer BJ, Reman FC, & van Gent CM (1979). The determination of lipids and proteins in suction blister fluid. *J Invest Dermatol* **73**, 303-305.

Verrecchia F, Laboureau J, Verola O, Roos N, Porcher R, Bruneval P, Ertault M, Tiev K, Michel L, Mauviel A, & Farge D (2007). Skin involvement in scleroderma--where histological and clinical scores meet. *Rheumatology (Oxford)* **46**, 833-841.

Verrecchia F, Mauviel A, & Farge D (2006). Transforming growth factor-beta signaling through the Smad proteins: role in systemic sclerosis. *Autoimmun Rev* **5**, 563-569.

Vestergaard C, Just H, Baumgartner NJ, Thestrup-Pedersen K, & Deleuran M (2004). Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. *Acta Derm Venereol* **84**, 353-358.

Vicenzi E, Alfano M, Ghezzi S, Gatti A, Veglia F, Lazzarin A, Sozzani S, Mantovani A, & Poli G (2000). Divergent regulation of HIV-1 replication in PBMC of infected individuals by CC chemokines: suppression by RANTES, MIP-1alpha, and MCP-3, and enhancement by MCP-1. *J Leukoc Biol* **68**, 405-412.

Vittal R, Horowitz JC, Moore BB, Zhang H, Martinez FJ, Toews GB, Standiford TJ, & Thannickal VJ (2005). Modulation of prosurvival signaling in fibroblasts by a protein kinase inhibitor protects against fibrotic tissue injury. *Am J Pathol* **166**, 367-375.

Wakefield LM & Roberts AB (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr Opin Genet Dev* **12**, 22-29.

Wakugawa M, Nakamura K, Akatsuka M, Kim SS, Yamada Y, Kawasaki H, Tamaki K, & Furue M (2001). Expression of CC chemokine receptor 3 on human keratinocytes in vivo and in vitro--upregulation by RANTES. *J Dermatol Sci* **25**, 229-235.

Wallach-Dayan SB, Golan-Gerstl R, & Breuer R (2007). Evasion of myofibroblasts from immune surveillance: a mechanism for tissue fibrosis. *Proc Natl Acad Sci U S A* **104**, 20460-20465.

Wang F, Herzmark P, Weiner OD, Srinivasan S, Servant G, & Bourne HR (2002). Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat Cell Biol* **4**, 513-518.

Wang XJ, Greenhalgh DA, Bickenbach JR, Jiang A, Bundman DS, Krieg T, Derynck R, & Roop DR (1997). Expression of a dominant-negative type II transforming growth factor beta (TGF-beta) receptor in the epidermis of transgenic mice blocks TGF-beta-mediated growth inhibition. *Proc Natl Acad Sci U S A* **94**, 2386-2391.

Watson JB, Getzler SB, & Mosher DF (1994). Platelet factor 4 modulates the mitogenic activity of basic fibroblast growth factor. *J Clin Invest* **94**, 261-268.

Weis-Garcia F & Massague J (1996). Complementation between kinase-defective and activation-defective TGF-beta receptors reveals a novel form of receptor cooperativity essential for signaling. *EMBO J* **15**, 276-289.

Wells TN, Power CA, Shaw JP, & Proudfoot AE (2006). Chemokine blockers--therapeutics in the making? *Trends Pharmacol Sci* **27**, 41-47.

Wenzel J, Henze S, Worenkamper E, Basner-Tschakarjan E, Sokolowska-Wojdylo M, Steitz J, Bieber T, & Tuting T (2005). Role of the chemokine receptor CCR4 and its ligand thymus- and activation-regulated chemokine/CCL17 for lymphocyte recruitment in cutaneous lupus erythematosus. *J Invest Dermatol* **124**, 1241-1248.

Wenzel SE, Trudeau JB, Barnes S, Zhou X, Cundall M, Westcott JY, McCord K, & Chu HW (2002). TGF-beta and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. *J Immunol* **169**, 4613-4619.

Werner S & Grose R (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev* **83**, 835-870.

Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, McCalmont TH, Brown PO, Botstein D, & Connolly MK (2003). Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A* **100**, 12319-12324.

Wigley FM (2009). Vascular disease in scleroderma. *Clin Rev Allergy Immunol* **36**, 150-175.

Willis BC, duBois RM, & Borok Z (2006). Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc* **3**, 377-382.

Wills-Karp M (2004). Interleukin-13 in asthma pathogenesis. *Curr Allergy Asthma Rep* **4**, 123-131.

Wolf G, Jocks T, Zahner G, Panzer U, & Stahl RA (2002). Existence of a regulatory loop between MCP-1 and TGF-beta in glomerular immune injury. *Am J Physiol Renal Physiol* **283**, F1075-F1084.

Worda M, Sgonc R, Dietrich H, Niederegger H, Sundick RS, Gershwin ME, & Wick G (2003). In vivo analysis of the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay. *Arthritis Rheum* **48**, 2605-2614.

Wu D, Peng F, Zhang B, Ingram AJ, Gao B, & Krepinsky JC (2007). Collagen I induction by high glucose levels is mediated by epidermal growth factor receptor and phosphoinositide 3-kinase/Akt signalling in mesangial cells. *Diabetologia* **50**, 2008-2018.

Wu F & Chakravarti S (2007). Differential expression of inflammatory and fibrogenic genes and their regulation by NF-kappaB inhibition in a mouse model of chronic colitis. *J Immunol* **179**, 6988-7000.

Wu L, Fan J, Matsumoto S, & Watanabe T (2000). Induction and regulation of matrix metalloproteinase-12 by cytokines and CD40 signaling in monocyte/macrophages. *Biochem Biophys Res Commun* **269**, 808-815.

Wu X, Ma J, Han JD, Wang N, & Chen YG (2006). Distinct regulation of gene expression in human endothelial cells by TGF-beta and its receptors. *Microvasc Res* **71**, 12-19.

Wuyts WA, Vanaudenaerde BM, Dupont LJ, Demedts MG, & Verleden GM (2003). Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-kappaB in IL-1beta-induced chemokine release in human airway smooth muscle cells. *Respir Med* **97**, 811-817.

Wynn J, Fineberg N, Matzer L, Cortada X, Armstrong W, Dillon JC, & Kinney EL (1985). Prediction of survival in progressive systemic sclerosis by multivariate analysis of clinical features. *Am Heart J* **110**, 123-127.

Wynn TA (2004). Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nat Rev Immunol* **4**, 583-594.

Yamakage A, Kikuchi K, Smith EA, LeRoy EC, & Trojanowska M (1992). Selective upregulation of platelet-derived growth factor alpha receptors by transforming growth factor beta in scleroderma fibroblasts. *J Exp Med* **175**, 1227-1234.

Yamamoto T, Eckes B, & Krieg T (2001). Effect of interleukin-10 on the gene expression of type I collagen, fibronectin, and decorin in human skin fibroblasts: differential regulation by transforming growth factor-beta and monocyte chemoattractant protein-1. *Biochem Biophys Res Commun* **281**, 200-205.

Yamamoto T, Eckes B, Mauch C, Hartmann K, & Krieg T (2000). Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1 alpha loop. *J Immunol* **164**, 6174-6179.

Yamamoto T & Nishioka K (2003). Role of monocyte chemoattractant protein-1 and its receptor,CCR-2, in the pathogenesis of bleomycin-induced scleroderma. *J Invest Dermatol* **121**, 510-516.

Yamamoto T & Nishioka K (2005). Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: current update and future perspective. *Exp Dermatol* **14**, 81-95.

Yamamoto T, Takagawa S, Katayama I, & Nishioka K (1999). Anti-sclerotic effect of transforming growth factor-beta antibody in a mouse model of bleomycin-induced scleroderma. *Clin Immunol* **92**, 6-13.

Yanaba K, Komura K, Kodera M, Matsushita T, Hasegawa M, Takehara K, & Sato S (2006). Serum levels of monocyte chemotactic protein-3/CCL7 are raised in patients with

systemic sclerosis: association with extent of skin sclerosis and severity of pulmonary fibrosis. *Ann Rheum Dis* **65**, 124-126.

Yang YC, Piek E, Zavadil J, Liang D, Xie D, Heyer J, Pavlidis P, Kucherlapati R, Roberts AB, & Bottinger EP (2003). Hierarchical model of gene regulation by transforming growth factor beta. *Proc Natl Acad Sci U S A* **100**, 10269-10274.

Youn BS, Mantel C, & Broxmeyer HE (2000). Chemokines, chemokine receptors and hematopoiesis. *Immunol Rev* **177**, 150-174.

Yu J, Mookherjee N, Wee K, Bowdish DM, Pistolic J, Li Y, Rehaume L, & Hancock RE (2007). Host defense peptide LL-37, in synergy with inflammatory mediator IL-1beta, augments immune responses by multiple pathways. *J Immunol* **179**, 7684-7691.

Yuan W, Yufit T, Li L, Mori Y, Chen SJ, & Varga J (1999). Negative modulation of alpha1(I) procollagen gene expression in human skin fibroblasts: transcriptional inhibition by interferon-gamma. *J Cell Physiol* **179**, 97-108.

Yue J, Sun B, Liu G, & Mulder KM (2004). Requirement of TGF-beta receptor-dependent activation of c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (Sapks) for TGF-beta up-regulation of the urokinase-type plasminogen activator receptor. *J Cell Physiol* **199**, 284-292.

Zhang Q, Kelly AP, Wang L, French SW, Tang X, Duong HS, Messadi DV, & Le AD (2006). Green tea extract and (-)-epigallocatechin-3-gallate inhibit mast cell-stimulated type I collagen expression in keloid fibroblasts via blocking PI-3K/AkT signaling pathways. *J Invest Dermatol* **126**, 2607-2613.

Zhang Y, McCormick LL, Desai SR, Wu C, & Gilliam AC (2002). Murine sclerodermatous graft-versus-host disease, a model for human scleroderma: cutaneous cytokines, chemokines, and immune cell activation. *J Immunol* **168**, 3088-3098.

Zhou L, Askew D, Wu C, & Gilliam AC (2007). Cutaneous gene expression by DNA microarray in murine sclerodermatous graft-versus-host disease, a model for human scleroderma. *J Invest Dermatol* **127**, 281-292.

Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, & Elias JA (2002). IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* **168**, 2953-2962.

Zlotnik A & Yoshie O (2000). Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127.