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Assessing the activity of agonistic autoantibodies in systemic sclerosis and their effects on cultured vascular smooth muscle cells

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Ce mémoire intitulé:

Assessing the activity of agonistic autoantibodies in systemic sclerosis and their effects on cultured vascular smooth muscle cells

Présenté par:
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a été évalué par un jury composé des personnes suivantes:

Dr. Huy Ong, président-rapporteur
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Résumé

La sclérose systémique (ScS) est une maladie auto-immune dévastatrice d'étiologie inconnue. Le dysfonctionnement immunitaire, la fibrose et la vasculopathie sont les trois principales caractéristiques de cette maladie. Une récente étude a révélé un nouveau lien entre l'auto-immunité et la fibrose, par la présence d'auto-anticorps stimulant le récepteur du facteur de croissance dérivé des plaquettes (PDGFR) des fibroblastes. Ces auto-anticorps sont capables de stimuler les espèces réactives de l'oxygène et d'activer la kinase régulée par un signal extracellulaire (ERK1/2). L'hypothèse que nous formulons est que les cellules musculaires lisses vasculaires (VSMCs) exprimant conjointement les PDGFR, répondront elles aussi aux autoanticorps anti-PDGF-R. Le travail présenté ici vise à valider la présence d'auto-anticorps PDGFR dans les sérums de patients ScS, et à caractériser ensuite la réponse de VSMCs exposées à de l'immunoglobuline G (IgG) de ces sérums, en mesurant l'activation des cascades de signalisation spécifiques, ainsi que l'induction des gènes impliqués dans la réponse fibrotique.

Nos résultats démontrent la présence d'une fraction IgG stimulant une réponse phénotypique dans les cultures de VSMCs. Notamment, d'importantes régulations positive et négative des gènes pro-fibrotiques tgfb1 et tgfb2 respectivement, ont été observées dans les VSMCs exposées à des fractions de ScS-IgG. Les fractions de IgG positives pour l'activation de ERK étaient présentes dans la plupart, mais pas dans tous les échantillons de SSc (68%, 19/28), et moins présentes dans les contrôles 27% (11/3). Bien que, les fractions de SSc-IgG ont pu considérablement immunoprécipiter le PDGFR, l'utilisation d'un inhibiteur spécifique des récepteurs au PDGF (AG1296), n'a pas inhibé l'activation de ERK médiée par les fractions de SSc-IgG. Globalement, nos résultats indiquent la présence d'autoanticorps stimulants avec activité pro-fibrotique dans les sérums des patients ScS. Des travaux sont en cours pour identifier l'entité moléculaire responsable de la réponse d'IgG observée dans les cultures de VSMCs.

Mots-clés: Sclérose systémique, CMLV, PDGFR, auto-anticorps

Abstract

Systemic Sclerosis (SSc) is a devastating autoimmune disease of unknown etiology. Immune dysfunction, fibrosis and vasculopathy are the three major features of the disease; however, the interactions between these components are poorly understood. A novel link between autoimmunity and fibrosis has been proposed by the presence of stimulatory autoantibodies to the platelet-derived growth factor receptor (PDGFR) on fibroblasts. These autoantibodies were capable of stimulating reactive oxygen species and subsequent activation of ERK1/2. If the anti-PDGFR autoantibodies are present in the systemic circulation of SSc patients, they will most certainly encounter vascular smooth muscle cells (VSMCs). The latter are known to express the PDGFR and response to PDGF, which is a known phenotypic modulator of VSMCs. The work presented here seeks to readdress the presence of stimulatory anti-PDGFR autoantibodies in serum derived from SSc-patients and to characterize the effects of SSc-IgG on VSMCs by measuring the activation of specific signaling cascades and the induction of genes involved in fibrotic responses.

Our results demonstrate the presence of an IgG fraction stimulating a phenotypic response in cultured VSMCs. Notably, a significant up-regulation of the pro-fibrotic gene *tgfb1* and a significant down-regulation of the anti-fibrotic gene *tgfb2* were observed in VSMC exposed to SSc-IgG fractions. Positive IgG fractions for ERK activation were present in most, but not all, SSc samples (68%, 19/28), and they were less present in controls (27%) (3/11). Although, the SSc-IgG fractions were able to significantly immunoprecipitate the PDGFR, the use of a selective PDGFR inhibitor, AG1296, did not inhibit the activation of ERK mediated by SSc-IgG fractions. Altogether, our findings suggest the presence of stimulatory autoantibodies with profibrotic activity in serum derived form SSc patients. Work is in progress to identify the molecular entity responsible for the IgG response observed in cultured VSMCs.

Keywords: Systemic Sclerosis, vascular smooth muscle cells, PDGFR, autoantibodies

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LIST OF ABBREVIATIONS

Abs:	Antibodies
ACA:	Anticentromere Antibody
ACR:	American College of Rheumatology
ANA:	Antinuclear antibodies
APC:	Antigen-presenting cells
Anti-topo I:	Anti-topoisomerase I
AutoAbs:	Autoantibodies
BCR:	B cell receptor
BAL:	Bronchoalveolar lavage
CSRG:	Canadian scleroderma research group
CTGF:	Connective Tissue Growth Factor
DMEM:	Dulbecco's modified eagles medium
dSSc:	Diffuse systemic sclerosis
EC:	Endothelial cells
ECM:	Extracellular matrix
EGFR:	Epidermal growth factor receptor
ERK:	Extracellular regulated kinase
ET-1:	Endothelin-1
FAK:	Focal adhesion kinase
FVC:	Forced vital capacity
INF:	Interferon
IgG:	Immunoglobulin
IL:	Interleukin
IP:	Immunoprecipitation
JNK:	c-Jun N-terminal kinases
lSSc:	Limited systemic sclerosis
MAPK:	Mitogen-activated protein kinases

MCP:	Monocyte Chemo attractant protein
MIP:	Macrophage inflammatory protein
MMP:	Matrix metalloproteinase
NO:	Nitric Oxide
OA:	Osteoarthritis
PAH:	Pulmaonary arterial hypertension
PBS:	Phosphate buffered saline
PDGF:	Platelet derived growth factor
PDGFR:	Platelet derived growth factor receptor
PF:	Pulmonary fibrosis
ROS:	Reactive oxygen species
RTK:	Receptor Tyrosine Kinase
SBE:	SMAD-binding element
SSc:	Systemic sclerosis
Scl-GVHD:	sclerodermatous Graft-vs.-host disease
TCR:	T cells receptor
TGF-β:	Transforming growth factor-beta
TGFβR:	Transforming growth factor-beta receptor
Th2:	T-helper
TIMPs:	Tissue inhibitor of metallopreteinases
TKI:	Tyrosine Kinase Inhibitor
TSK1:	Tight skin 1
TSK2:	Tight skin 2
UCD-200:	University of California at Davis line of chicken.
VEGFR:	Vascular endothelial growth factor receptor
VSMCs:	Vascular smooth muscle cells

I dedicate this thesis to my family especially to my soul mate and life companion, Mohamad, to my lovely cute little daughter, Zara and to the greatest Mom on earth, Kamela. Her courage and success in fighting brain tumor taught me no matter what happens and no matter how bad it gets, there is always hope and there is always a light at the end of the tunnel

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Chapter 1: Introduction

1.1 Immune system and Tolerance

The immune system plays a major role in the protection of our body against infectious disease caused by bacteria, viruses or any other pathogen. This protection is achieved through two main immune responses: innate and acquired. The innate response provides the first line defence against many foreign substances. It consists of specialized cells and nonspecific molecules that respond quickly to the stimuli. However, the innate response is not effective against all pathogens and it lacks the ability to provide specific protective immunity against re-infection. The acquired response, in turns, provides a more diverse defence with much slower respond. It gains the advantage of targeting specific pathogen and generates a memory response protecting against re-infection with the same pathogen. The effectors cells involved in the adaptive immune response are acquired B and T cells. Each one of them bearing specific receptors: the B cell receptor (BCR) and those of T cells (TCR). These receptors can recognize a multitude of different antigens providing protection against most pathogens that we will encounter during life. The strong affinity interaction between a foreign molecule and its specific receptor induces the activation of these cells and causes their clonal expansion. These lymphocytes become specific and then migrate into the infected tissue causing inflammation and contributing to the elimination of the source of foreign antigen. Once this is done, the majority of specific lymphocytes disappear and the inflammation will subside (Janeway, 1997; Parham, 2009; Ranque, 2010).

Some developing B and T cells may carry receptor that bind to normal components of the human body, which is called self-antigen. To prevent this, several mechanisms have been evolved early in the foetal stage of life to induce a state of immunological tolerance towards self-antigen. During T cells development in the thymus, the cells are subjected to negative selection process where highly self-reactive cells are eliminated by apoptosis, macrophages and dendritic cells. Another mechanism is the absence of co-stimulatory

signals of antigen-presenting cells (APC); T cells become anergic and cannot induce immunity responses. There are also regulatory T cells that allow the suppression of auto-reactive T cells.

The same mechanisms of self tolerance are present in bone marrow during the maturation of B cells that are responsible for humeral immunity (antibodies production) (Janeway, 1997; Parham, 2009). These mechanisms of tolerance could be altered causing autoimmune diseases.

1.1.1 Autoimmune disease

Autoimmune diseases are caused by unwanted adaptive immune responses. They represent failures of the mechanisms that maintain self-tolerance. They can be caused by antibodies (Abs) that perturb a normal physiological function or by inflammatory T cells that damage healthy cells or tissue at a rate beyond the capacity of the body to repair. Some autoimmune disease are directed against antigens of one particular organ or tissue and are known as organ-specific or tissue-specific autoimmune disease, such as insulin-dependent diabetes. In contrast, others are directed against components common to all cells and are known as systemic autoimmune disease, such as systemic sclerosis (SSc) (Parham, 2009).

1.2 Systemic Sclerosis

SSc is a rare autoimmune connective tissue disease of unknown cause. Indeed, genetic and environmental factors could be the origin of the disease, but this still uncertain. SSc is highly heterogeneous and multisystemic in its clinical manifestations. The main organs affected are the skin, lungs, kidneys, gastrointestinal tract and heart.

The principle features of the disease are extensive fibrosis, vascular alteration and autoantibodies (autoAbs) against various cellular antigens (Gabrielli, 2009). The interaction between these features is complicated and not completely understood. However, researches and clinical symptoms suggest that vascular injury triggers the disease and leads to

inflammation and autoimmunity. Different Cytokines and autoAbs are involved in the disease. Cytokines production, caused by inflammatory cells, may stimulate fibroblast differentiation into myofibroblast. The latter will induce overproduction of collagen and other extracellular matrix (ECM) proteins leading to fibrosis (Varga, 2007).

1.2.1 Epidemiology and Demographics

In all epidemiology studies, SSc is reported to occur more frequently in women than in men with an overall ratio of approximately 3:1 (Chifflot, 2008). SSc onset occurs most likely in fifth decade and rarely in childhood and extreme elderly (Ansell, 1976; Czirjak, 1992; Ranque, 2010). Some races are more vulnerable to the disease than others are. For example, Choctaw Native Americans living in Oklahoma reported one of the highest SSc prevalence (Arnett, 1996). In addition, black Americans showed a higher age-specific incidence rate than white ones (Clements, 2004; Ranque, 2010).

SSc has a worldwide distribution (Silman, 1996). Its population have been characterized in patients from the United States, France, Japan, Australia and others (Scussel-Lonzetti, 2002). The reported incidence rates and prevalence show a wide variation among different countries. According to the epidemiological international studies of SSc, its incidence ranges from 2.7 to 23 new cases per million per year (Clements, 2004). While, the prevalence is estimated between 31 to 1470 cases per million and appears to be higher in North America and Australia as compared to Europe and Japan (Clements, 2004; Koopman, 2004; Ranque, 2010).

In Quebec, the prevalence rate of 443 cases per million was calculated and it differed greatly between sexes and ages. Prevalence was higher for older individuals and females, with a female to male ratio of 6:1 (Scussel-Lonzetti, 2002; Bernatsky, 2009).

Studies have also examined the patients' mortality and survival. Although deaths are more common in people diagnosed with SSc than in healthy individuals (Scussel-

Lonzetti, 2002), the 10-year cumulative survival after the physician diagnosis had improved from 54% in 1970 to 82% nowadays (Ranque, 2010).

1.2.2 Diagnosis and Classification

In 1980, the American College of Rheumatology (ACR) has defined diagnostic criteria for SSc that is 97 % sensitive and 98 % specific for the disease. The patient should fulfill the major criterion or two of the three minor criteria in order to be diagnosed with SSc. The major criterion is skin thickening proximal to the metacarpophalangeal joints. The minor criteria are having sclerodactyly (localized thickening and tightness in fingers or toes skin), digital pitting scars or bilateral basilar pulmonary fibrosis (Clements, 2004; Koopman, 2004).

SSc is a practically challenging problem for classification because it includes a very broad spectrum disease. The most widely accepted classification is the one based on the extent of skin involvement. Patients are classified into two main groups: limited SSc (lSSc) with skin involvement essentially limited to the hands and face; and diffuse SSc (dSSc) with skin involvement proximal to the elbows and knees. In patients with lSSc, visceral involvement is rare, with the exception of patients in whom pulmonary arterial hypertension (PAH), interstitial lung disease and/or bowel involvement eventually develop. Patients with dSSc experience visceral involvement including renal crisis, interstitial lung disease, PAH, heart and gastrointestinal tract involvement (Koopman, 2004; Ranque, 2010).

Classification based on serum autoantibodies is also characterized. Two major serum autoAbs in SSc patients have been recognized since 1980: anticentromere (ACA) and antitopoimerase I (anti-topo I) Abs. The latter is specific to SSc, and two-thirds of patients with this autoantibody have dSSc and pulmonary interstitial fibrosis. ACA is present primarily in patient with lSSc (Clements, 2004).

1.2.3 Etiology

The etiology of SSc is unknown but it seems to result from multifactorial processes such as alteration of immune system, genetics and environmental factors (Chifflot, 2008). Many substances have been implicated in the pathogenesis of this disease e.g. organic solvents, silica dust, vinyl chloride and silicone breast implants. Studies on these substances failed to establish a strong association between occurrence of SSc and exposure to any particular agents (Chifflot, 2008).

In addition, genetic factor had been investigated. Several studies found that a family history or a first degree relative has a significant risk factor for the disease. However, the absolute risk remains very low around 1% (Arnett, 2001; Zhou, 2001; Roberts-Thomson, 2006). Moreover, genetic, familial, and twin studies suggest that SSc occurs in genetically susceptible individuals (Feghali-Bostwick, 2005).

Overall, the pathogenesis of SSc appears to be influenced with both environmental and genetic factors (Ranque, 2010).

1.2.4 Clinical Manifestation

1.2.4.1 Fibrosis

Fibrosis is the pathological hallmark of SSc. Uncontrolled production of collagens and other ECM proteins by fibroblast residing in the skin, lungs and other vital organs leads to excess connective tissue accumulation. Over time, progressive build-up of connective tissue disrupts the normal tissue architecture of affected organs, causing their dysfunction and eventual failure. Thus, the fibrotic process contributes significantly to the morbidity and mortality of SSc (Varga, 2008; Ranque, 2010).

1.2.4.2 SSc Effect on the Skin

The skin is an organ severely affected in SSc. There are three evolving phases of skin thickening in individuals with SSc. First, the edematous phase, the patients complain of swollen fingers and edema in the dorsum of the hands, forearms, legs, feet and face. After that, the edema is gradually replaced by thickened tight skin which is called the indurative phase. During this phase, the dermis is markedly thickened and the epidermis is thinned. This leads to hair loss on the extremities, decreased sweating and mobility impairment of muscles, tendons and joints, which is called sclerodactyly. With time, the thickening of the skin freezes facial expression. Of note, this phase is rapid in dSSc, while it takes 15 to 20 years in lSSc form. Finally, in the atrophic phase, the thickened dermis softens and returns to a normal thickness. In addition, telangiectasias, which are red skin lesions caused by dilated blood vessels, increase in number and become the dominant visual feature for the patient.

In addition to fibrosis, SSc can cause ulceration of the skin fingers caused by ischemia. In some patients, it degenerates into necrosis. The fingers become painful and the patients have a poor quality of life. Calcium deposits (calcinosis) are also observed in patients with lSSc (Clements, 2004).

1.2.4.3 SSc Effect on the Lung

Pulmonary fibrosis (PF) is a common complication of SSc. 25 to 90% of patients suffering from SSc had PF. It is often severe and can cause death. PF can be found in both forms of the disease, but it is much more frequent in patients with the diffuse form. A significant loss of lung volume is seen in people with PF. Moreover, the macrophages and lymphocytes can be found in inter-alveolar space and inflammation of the lung is noticed.

Pulmonary arterial hypertension (PAH) is another severe complication observed in patients with SSc. It is a destructive vascular complication in the lungs with abrupt onset and often lethal outcome. PAH is caused by a thickening of pulmonary arteries. This

generates a decrease in diffusing capacity for carbon monoxide (DLCO) and forced vital capacity (FVC) that corresponds to the forced expiration (Clements, 2004).

The thickening of the pulmonary arteries is a result of several vascular changes that affect all three layers (i.e., intima, media and adventitia) of the pulmonary vessel. Each cell type (endothelial, smooth muscle and fibroblast) in the pulmonary vascular wall plays a specific role in the pathogenesis (Humbert, 2008). Endothelial cell (EC) injury is the critical early event in SSc-associated pulmonary vascular disease. It leads to alterations in endothelial function with increased production of vasoconstrictor mediators, such as endothelin-1 (ET-1), and decreased synthesis of prostacyclin and nitric oxide (NO) (Varga, 2002). In normal individuals, vasoactive molecules produced from EC adapt the pulmonary vascular smooth muscle cells (VSMCs) tone to the actual needs of the organism and keep the smooth muscle in a state of relaxation. In PAH, the imbalanced productions of vasoactive mediators lead to increased pulmonary vascular reactivity and abnormal vasoconstriction (Muller-Ladner, 2009).

Beside that, endothelial dysfunction triggers two cascades that contribute to the pulmonary artery thickening; vascular remodeling, and *in situ* thrombus formation (Varga, 2002). Besides its vasoconstrictive effects, ET-1 also promotes VSMCs proliferation. Hypertrophy of the intima and medial layers of pulmonary vessels due to VSMCs growth and proliferation causes significant luminal narrowing (Weissberg, 1990; Weber, 1994). Inflammation also plays a role in SSc-PAH, as patients have perivascular inflammatory cell infiltrates, such as T and B cells, and macrophages (Le Pavec, 2010). The immune response activation results in the production of autoAbs and fibrosis. This fibrotic response leads to adventitial fibrosis, furthering the vascular remodeling (Norton, 1970). This process can even progress to cause an almost complete occlusion of the pulmonary arterioles. Over all, vasoconstrion and luminal narrowing lead to elevated pulmonary vascular resistance and increase pulmonary artery pressure leading to SSc-PAH. (Figure 1, page 8, shows the pulmonary vascular remodeling in SSc-PAH).

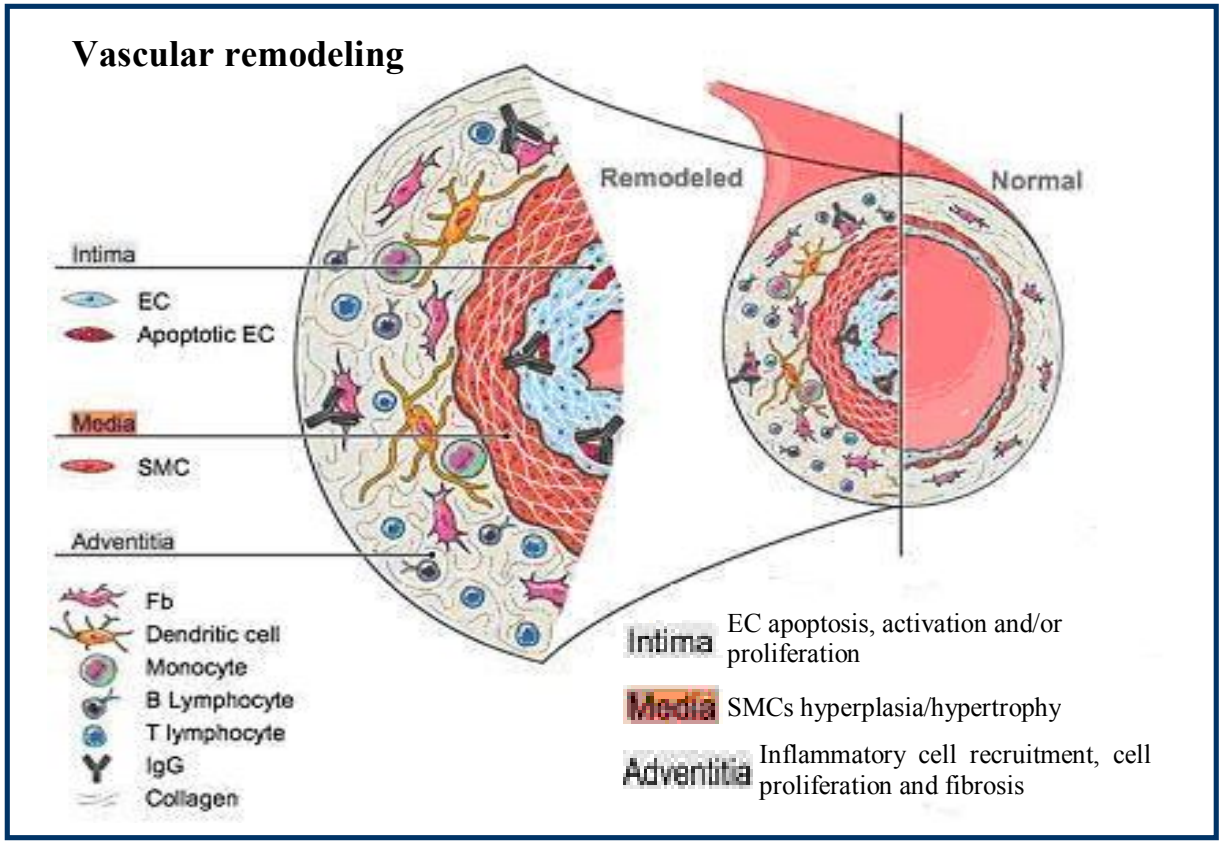


Figure 1: Pulmonary vascular remodeling in SSc-PAH. Modified from (Le Pavec, 2010).

1.2.4.4 SSc effect on other organs

In addition to skin and lungs, the heart, kidneys and gastrointestinal tract are subjected to fibrosis in SSc. Myocardial fibrosis is a hallmark of cardiac SSc. Cardiac involvement is found in up to 80% of patients who have SSc. In the SSc heart, significant interstitial and perivascular fibrosis are found, this will contribute to diastolic dysfunction as the disease progress. In addition, the heart vessels may be sclerotic and thus lead to dyspnea, palpitations and arrhythmia (Clements, 2004; Varga, 2008).

In the kidneys of patients with SSc, the glomeruli shrunk leading to chronic renal ischemia and other ischemic changes. Pathological changes can also occur in the

gastrointestinal tract of SSc patients at any level from the mouth to the rectum. The esophagus is virtually always affected with fibrosis. Replacement of the normal intestinal architecture results in disordered peristaltic activity, gastroesophageal reflux and small bowel dysmotility, pseudo-obstruction, and bacterial overgrowth (Clements, 2004; Varga, 2008).

Musculoskeletal involvement is evident in 1/3 to 1/2 of SSc patients and it is expressed as muscle weakness. In addition, Carpel tunnel syndrome is documented in SSc patients due to the fibrotic thickening of the tendon sheaths (Panayiotis, 2001).

Other clinical manifestations are Raynaud's phenomenon which affects almost all SSc patients (Panayiotis, 2001). It is caused by a vasoconstriction of arterioles after exposure to cold causing the skin to become pale, waxy-white or purple (Clements, 2004). Of note, PAH and Raynaud's syndrome share numerous similarities, not only on a clinical level with a substantial number of patients suffering both from Raynaud's syndrome and PAH, but also on a molecular level as both entities are characterized by endothelial dysfunction with reduced NO release and increased plasma levels of ET-1 (Muller-Ladner, 2009).

1.2.5 Animal models

The search for the ultimate etiology of SSc fibrosis, the problem of disease classification and the fact that the initial stage is generally not accessible in humans highlight the need for appropriate animal models in SSc. Animal models in SSc can be divided into two groups. In the first group, the pathologic phenotype is the result of a genetic mutation. These models are associated with the spontaneous development of SSc-like features. In the second group, the pathologic alterations are induced in normal animals by manipulation of their immune system or by administration of exogenous substances (Rogai, 2008; Yamamoto, 2009).

Examples of the first group are tight skin 1 (TSK1) and University of California at Davis line of chicken (UCD-200). First, in TSK1, the most striking features are: the presence of thickened fibrotic skin that is firmly bound to the subcutaneous and deep muscular tissue, and SSc-like activation of the TSK-1 fibroblasts. However, dermal sclerosis and vascular phenotype are not recognized, and additional non-SSc pathologies are noticed, including emphysema and kyphosis (Yamamoto, 2009; Beyer, 2010). Second, the UCD-200, this model of chickens spontaneously develops vascular damage, mononuclear cell infiltrates, fibrosis of the skin and internal organs, and polyarthritis. Thereby, it almost manifests the entire pathologic spectrum of SSc; it has a very early onset and shows rapid progression. Thus, UCD-200 provides a unique opportunity to study the earliest profibrotic events of SSc since the disease course closely resembles that of human SSc (Yamamoto, 2009; Beyer, 2010).

Examples of the second group are sclerodermatous Graft-versus-host disease (Scl-GVHD) and bleomycin-induced SSc. First, the Scl-GVHD mice exhibit skin thickening and pulmonary fibrosis after bone marrow transplantation. Thereby, systemic disease manifestations exist, but the sophisticated technical skills and difficulties in handling the immunocompromised mice are a major limitation (Yamamoto, 2009; Beyer, 2010). Second, the bleomycin-induced SSc is induced by the repeated injections of bleomycin into the back skins in mice. Histopathological examination revealed definite dermal sclerosis, which mimicked the histological features of human SSc; however, no major systemic manifestations are involved (Yamamoto, 2009; Beyer, 2010).

1.3 Pathogenesis of Systemic Sclerosis

Although the hallmark of SSc is widespread connective tissue fibrosis, the earliest and most frequent manifestations include blood vessel and immunological abnormalities. In recent years, research on SSc has evolved to provide a better understanding of the interdependence of the three major systems—namely, the vascular system, the immune system and the connective tissue involved in the disease (Hunzelmann, 2009). As studies

showed, vascular damage and leukocyte accumulation generate the molecular cues that control the profiles of soluble mediators. The latter regulate the aberrant behaviour of mesenchymal cells within connective tissues. Where, the dysregulated expression of these molecules and cells differentiation will contribute to the persistent fibrogenic response in SSc (Abraham, 2005). (Figure 2, page 12, shows a model of SSc pathogenesis). Herein we will discuss the role of each of these systems in SSc.

1.3.1 Vascular System

Vascular injury is the earliest, if not the primary, events in the pathogenesis of SSC. Histopathological evidence of vascular damage is present before fibrosis, and clinical manifestation such as Raynaud's phenomenon precede other disease manifestation (Kahaleh, 2004; Varga, 2007). Additional manifestations of SSc-associated vasculopathy include PAH, renal crisis and cutaneous telangiectasia (Clements, 2004). The most important cells involved in SSc vasculopathy are: endothelial cells (EC), vascular smooth muscle cells (VSMCs) and Pericytes.

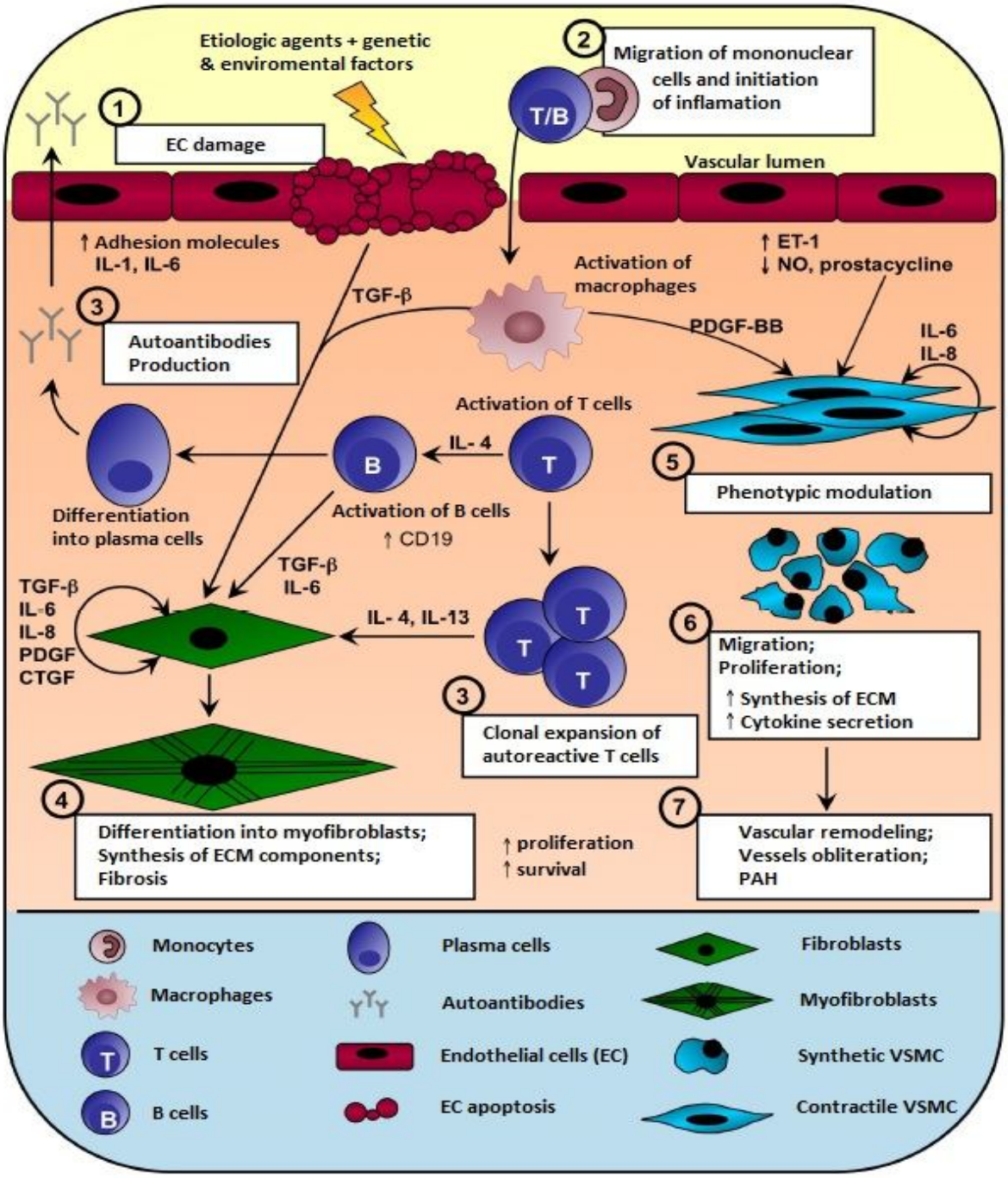


Figure 2: A model of SSc pathogenesis. Modified from (Robitaille, 2009).

It shows interaction between vascular system (EC & VSMCs), Immune system (B & T cells) and connective tissue (fibroblast & myofibroblasts) in SSc.

1.3.1.1 Endothelial Cells

The endothelium is a metabolically active tissue that under normal circumstances regulates regional blood flow, transportation of nutrients and migrations of blood cells while maintaining an antithrombotic lining in the vasculature (Cines, 1998). These important biologic functions are achieved through the production of a complex array of molecules including vasodilators (e.g. NO and prostacyclin), vasoconstrictors (e.g. ET-1), platelet-activating factor and cell adhesion molecules (e.g., selectins and integrins) (Schachna, 2002).

In SSc, the homeostatic function of endothelium is disrupted by EC activation and/or injury (Figure 2, page 12, step 1). The exact cause of EC activation or injury is unknown (Schachna, 2002). Moreover, endothelial regression in SSc suggests increase in EC apoptosis (Chen, 2003). The proposed mechanisms of endothelial apoptosis include damage by reactive oxygen specious (ROS) generated during ischemia/reperfusion, anti-endothelial Abs, inflammatory cytokines, abnormal ROS: NO ratios, vasculotropic viruses, altered basement membranes and decreased CD34 (Chen, 2003; Varga, 2007).

The loss of viable EC leads to loss of protective and vasodilating cytokines e.g. NO. The loss of vascular prostacycline favours platelet aggregation and *in situ* thrombosis (Clements, 2004). In addition, the vasoconstrictive mediator such as ET1 predominates and promotes leukocytes adhesion as well as intimal proliferation and fibrosblasts activation (Clements, 2004; Varga, 2007; Gabrielli, 2009). In addition, damage to the endothelium induces the release of proinflammatory mediators, chemokines and growth factors such as PDGF and TGFβ, which in turns, promote proliferation of smooth muscle cells and ECM deposition (Abraham, 2005).

Evidence for endothelial cell injury in SSc includes increased circulating levels of factor VIII/von Willebrand factor (endothelium's product produced in response to coagulation stimuli), increased level of circulating platelet aggregates, and increased levels of ET-1 (Clements, 2004).

1.3.1.2 Vascular Smooth Muscle Cells

VSMCs are located in the lining of blood vessels, mainly in level of the media, and play a key role in the development and regulation of vascular system. They provide first vasomotor and arterial tone necessary for maintenance of cardiovascular homeostasis. In addition, through their contraction or relaxation, they regulate blood flow and help maintain a constant blood pressure.

However, during a vascular injury, some VSMCs recruit at the injury to participate in the remodeling, repair and vascular growth. To accomplish these functions, the VSMCs have the ability to modulate their phenotype transiently and reversibly, a phenomenon known as phenotypic modulation (Chamley-Campbell, 1979). These cells develop from a mature differentiated phenotype called "contractile" phenotype to an immature, dedifferentiated one called "synthetic" (Chamley-Campbell, 1981).

In the media, the majority of normal adult blood vessels VSMCs are generally found in the contractile phenotype and are responsible for ensuring vascular tone through their elastic properties. These cells, in the contractile phenotype, are found in a quiescent state. Their synthetic activity is strongly reduced and the protein repertoire expression is almost exclusively limited to the proteins responsible for contraction. In contrast, VSMCs have a synthetic cellular machinery much more sophisticated, allowing them to proliferate, migrate and synthesize significantly more constituents of the ECM (Thyberg, 1990).

In vascular damage, VSMCs undergo contractile transient phenotypic modulation. First, they migrate, from the media toward the intima. Then, they proliferate and secrete glycoproteins, proteoglycans, cytokines and growth factors to participate actively in the process of wound repair. Once the injury is repaired, environmental factors within the same blood vessel return to their normal composition and physiological function allowing VSMCs to regain their phenotype and their properties contractile (Owens, 2004). However, under certain conditions, synthetic VSMCs persist the site of injury which, in the long term, can contribute to the development and/or progression of vascular disease (Owens, 2004).

VSMCs are capable, through their abilities of dedifferentiation and proliferation, to promote the development of a number of vascular diseases in SSc (Figure 2, page 12, steps 5, 6 &7). Evidence suggests that activation of VSMCs leads to migration of these cells into the intimal layer of the vessel, where they differentiate into a myofibroblast (Kirk, 1995). This transformation of the smooth muscle cell may mediate the progressive fibrosis or proliferation of the intima that is typical of SSc vascular disease. The exact mediator of the smooth muscle cell activation is unknown, but speculation includes the release of mediators from the activated endothelium (e.g., ET-1) and platelets (e.g., platelet derived growth factor (PDGF), a very well known phenotypic modulator of VSMCs) (Schachna, 2002).

In addition, VSMCs in SSc are responsible for abnormal vasoconstrictive response, best exemplified by Raynaud's phenomenon. Some studies suggest that VSMCs in SSc is hyper-responsive to α-adrenergic stimuli, thus leading to abnormal cold-induced vasoconstrictive responses (Kahaleh, 2008).

1.3.1.3 Pericytes

Pericytes are mesenchymal cells that normally reside in the walls of microvessels in intimate contact with the underlying endothelium. They play a role in maintaining vascular homeostasis. In SSc, Pericytes in the lesion overexpress several cytokine receptors, including PDGFR (Rajkumar, 1999). These cells proliferate and contribute to increased wall thickness, since activated pericytes can transdifferentiate into collagen-producing fibroblasts and myofibroblasts (Helmbold, 2004).

1.3.2 Connective tissue

1.3.2.1 Extracellular Matrix (ECM)

The ECM is a highly organised and dynamic arrangement of macromolecules that provides structural support for tissues and cells, and serves as a repository of information. The ECM consists of a cellular compartment of resident and infiltrating cells, and of a

connective tissue compartment composed of collagens, proteoglycans, elastins, fibrillins and adhesion molecules. The ECM also serves as the major reservoir for secreted growth factors such as TGFβ, and matricellular proteins such as connective tissue growth factor (CTGF), which, together with the connective tissue compartment, provide the cues that control differentiation, proliferation, function and survival of resident cells (Varga, 2008).

Fibrosis is characterized pathologically by replacement of normal tissue architecture with collagen-rich ECM, thus results in functional impairment of affected organ. It is the result of alteration in the dynamic balance between the accumulation and degradation of the ECM in tissue. Excessive connective tissue accumulation mainly collagen types I, III and VI, is due to overproduction by fibroblasts and related mesenchymal cells activated by soluble factors such as growth factors (TGFβ, PDGF); in an autocrine and/or paracrine manner, or by cell-cell or cell-ECM interactions. Impaired ECM degradation and expansion of the pool of mesenchymal cells in lesional tissues further contribute to ECM accumulation. Evidence suggests that both of these pathways are implicated in SSc, and resident fibroblasts are the pivotal effectors cells in the process (Clements, 2004; Varga, 2008).

1.3.2.2 Fibroblasts and myofibroblasts

Fibroblasts are the most common cells of connective tissue and are responsible of the ECM and collagen synthesis. Thus, they play critical role in maintaining the structural integrity of connective tissues and in wound healing. Under the influence of appropriate extracellular signals, fibroblasts are induced to synthesize collagens and other ECM macromolecules, to adhere to and contract connective tissue, to secrete growth factors cytokines and chemokines, to express surface receptors for these ligands, and to undergo transdifferentiation into myofibroblasts. Together, these properties (biosynthetic, proinflammatory, contractile, and adhesive) enable fibroblasts to mediate effective wound healing.

In contrast to physiologic conditions, where the fibroblast repair program is controlled tightly and self-limited, pathological fibrosis is characterized by sustained and amplified fibroblast activation. This results in exaggerated ECM accumulation and remodeling. Inappropriate fibroblast activation is the fundamental pathogenetic alteration underlying fibrosis in SSc (Varga, 2008).

The mechanism of fibroblast hyperactivation in SSc is presently unknown. However, many studies have recently highlighted some soluble factors synthesized by the fibroblasts themselves, the EC and mononuclear cells, which are responsible for this activation. Among these factors, TGFβ plays such an important role in activation and survival of fibroblasts. It induces cell proliferation and decreases the susceptibility of fibroblasts to apoptosis (Blobe, 2000; Jelaska, 2000). TGFβ may also induce the differentiation of fibroblasts into myofibroblasts, which are present in large quantities in SSc lesions (Figure 2, page 12, step 4). These cells usually persist longer and are responsible for the overproduction of collagen in injured tissues (Jelaska, 1996; Desmouliere, 2005).

Some intrinsic defects, such as genetic and phenotypic alterations have also been observed in fibroblasts from SSc patients. Among these, several alterations in signaling pathways those regulate the expression of genes encoding for ECM components., In particular, alterations at certain family members of SMADs, which are involved in the production of collagen type I, are reported (Dong, 2002; Varga, 2002; Mori, 2003). SSc fibroblasts also exhibit other features that could encourage the accumulation of collagen in patients with the disease such as decreased production of collagenase (Takeda, 1994), increased production of collagenase inhibitors (Kirk, 1995) and increased susceptibility to certain growth factors (Denton, 1997). Overall, many of the characteristics of SSc fibroblasts resemble those of healthy fibroblasts stimulated by TGFβ (Ihn 2008), suggesting that TGFβ is a key mediator of tissue fibrosis in SSc.

Myofibroblasts express the cytoskeletal protein alpha smooth muscle actin, and synthesize collagens and other ECM components. Their primary physiologic role is contraction of early granulation tissue during normal wound healing, where myofibroblasts are detected transiently and then disappeared. Removal of myofibroblasts from the lesion by means of apoptosis is a crucial step in wound resolution.

In contrast, in pathological fibrogenesis, myofibroblasts persist in lesional tissue, resulting in excessively contracted ECM characteristic of chronic scar. In addition, they are a major source of TGFβ during the fibrotic response. The presence of alpha smooth muscle actin-positive myofibroblasts is associated strongly with fibrotic disorders and SSc, but is absent from normal skin (Varga, 2008).

1.3.2.3 Collagen

Collagen belongs to a large family that comprises at least 19 distinct proteins encoded by at least 31 separate genes. All collagens consist of three polypeptides assembled into a unique triple-helical structure. The fibrillar collagens types, which are collagen I, II and III, account for more than 70% of total collagens content of tissues. Although type I collagen is ubiquitous throughout the body, the distribution of type II collagen is restricted to the cartilaginous tissue and vitreous humor. Type III collagen is usually in close association with type I and present in appreciable amounts in the skin, aorta, gut and lungs (Clements, 2004).

Patients with SSc have an increase in collagen types 1 and 3, with type 1 being the most abundant. Type 1 collagen is encoded by the *col1a1* and *col1a2* genes, which are at least partly controlled by the transcription factor SP1 (Philips, 1995). Increased SP1 binding activity has been recorded in sclerodermic fibroblasts and its activity has shown to be associated with increased gene expression of type 1 collagen in patients with SSc (Hitraya, 1998). Gene expression of type 1 collagen is also affected by TGFβ, which indicates a possible synergistic profibrotic interaction between SP1 and the TGFβ pathway

via the SMAD3/4 complexes (Zhang, 2000). Reduced amounts of SMAD7, which is an inhibitor of collagen gene expression, have also been reported in SSc, which suggests that the loss of this inhibitory effect allows TGFβ to stimulate unfettered, excessive accumulation of ECM (Charles, 2006).

1.3.2.4 Profibrotic Cytokines

As illustrated above, profibrotic cytokines are a major driving force in the activation of fibroblasts and fibroblast-like cells, resulting in increased release of ECM protein. They are often upregulated throughout the course of the disease. Potent pro-fibrotic cytokines, produced by various cells, include interleukin (IL-4 and IL-6), chemokines (monocyte chemoattractant protein-1 (MCP-1)), connective tissue growth factor (CTGF), transforming growth factor β (TGFβ) and platelet-derived growth factor (PDGF) (Sakkas, 2004) (Figure 3, page 20). Both IL-4 and IL-6 stimulate ECM synthesis (White, 1996; Bruns, 1997). IL-4 also stimulates fibroblast proliferation and promote T helper (Th2) type immune response (Haustein, 1998). This response suppresses IFNγ, which is an inhibitor of collagen synthesis. MCP-1, produced by endothelial cells and other cells, also has profibrotic actions via activating collagen and TGFβ1 gene expression in fibroblasts (Gharaee-Kermani, 1996). CTGF produced by fibroblasts, acts with TGFβ to stimulate collagen synthesis (Igarashi, 1995). However, the best-characterized profibrotic cytokines in SSc are TGFβ and PDGF. (Table I, page 26, summarizes their role in SSc). Hereafter, we will discuss in details these two cytokines and their role is SSc.

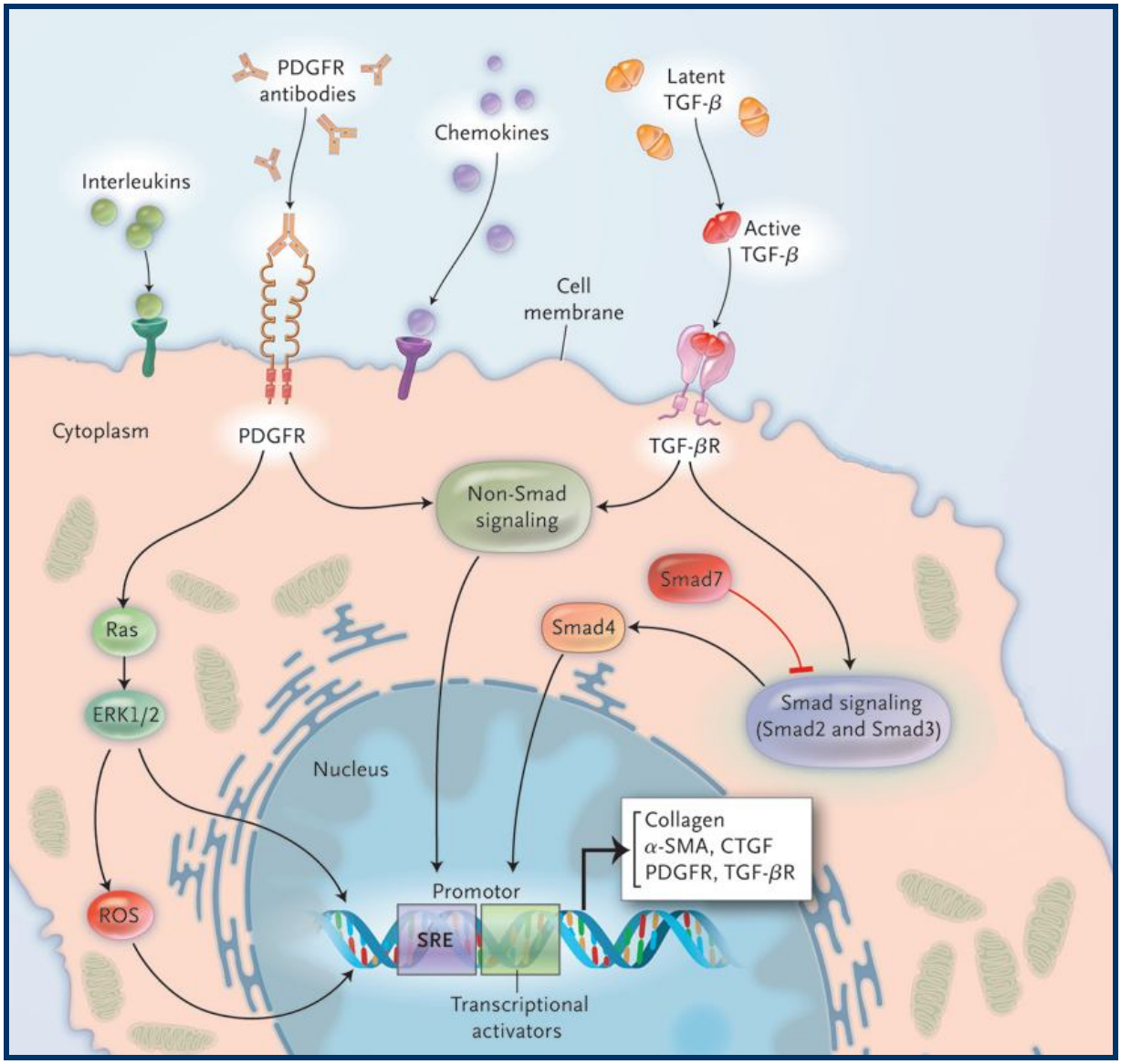


Figure 3: Activation of Fibroblasts in SSc. Adapted from (Gabrielli, 2009).

Profibrotic cytokins such as interleukins, chemokines, PDGF and TGFβ trigger signaling cascades in fibroblasts. For example, the phosphorylation of SMAD2 triggers a signaling cascade from SMAD3 to SMAD1, which interacts with SMAD4 and regulates gene transcription in the nucleus. Activation of TGFβ and TGFβR also results in the activation of pathways not involving SMAD proteins, modulating transcription factors. These pathways intersect with pathways induced by activation of PDGFR, leading to a complex intracellular signaling network. Production of ECM, cytokines and cytokine receptors is thereby stimulated; these participate in regulatory loops to sustained fibroblast activation.

1.3.2.4.1 Transforming Growth Factor-beta

TGFβ is considered the master regulator of physiologic fibrogenesis (wound healing) and pathological fibrogenesis. In addition, it has essential roles in normal tissue repair, angiogenesis, immune regulation, cell proliferation and cell differentiation. It is secreted by platelets, monocytes/macrophages, T cells and fibroblasts. Furthermore, it binds to most cell types since they express its specific cell surface receptors (Gu, 2008).

There are three TGFβ isoforms: TGFβ1, TGFβ2 and TGFβ3. They are structurally almost identical but exert different biological functions (Gu, 2008). Each isoform is encoded by a distinct gene and synthesized as part of large precursor molecule containing a propeptide region. The TGFβ isoform is cleaved from the propeptide before the precursor is secreted by the cell, but remains attached to the propeptide by noncovalent bonds. After it has been secreted, most TGFβ is stored in the ECM as a large latent complex composed of TGFβ, its propeptide, and a protein called latent TGFβ-binding protein (Sinha, 1998); (Blobe, 2000). Activation of latent TGFβ to its biologically active form that is capable of inducing cellular responses can be mediated by integrins, thrombospondins or matrix metalloproteinases (MMPs) (Gu, 2008; Varga, 2008).

Cell surface receptors for TGFβ family ligands are distinguished from those of other growth factors and cytokines by their specificity for phosphorylation of serine or threonine, rather than tyrosine residues. Receptor complexes are heterotetrameric, consisting of two 'type II' receptors that bind ligand, and two signal transducing 'type I' receptors which, in most instances, cannot bind ligand directly and thus are considered to act downstream of the type II receptor. TGFβ may also be presented to the type II receptor by the accessory proteins betaglycan and endoglin (Leask, 2004).

Upon ligand binding, an assembly of a heteromeric complex is initiated, and stabilized by interactions between the cytoplasmic domains of the type II and type I receptors (Roberts, 1999). Thus, once activated, TGFβ binds to the heteromeric receptor complex consisting of one TGFβ type I and one TGFβ type II receptor, and triggers an

intracellular signal transduction cascade that leads to the induction of target genes (Massague, 2006).

TGFβ signal transduction pathway involves phosphorylation of TGFβRI, which is a transmembrane serine-threonine kinase that in turn phosphorylates SMAD2 and SMAD3. The latter transduce the signals to the nucleus where they activate downstream gene transcription. Ligand-induced signal transduction through the SMAD proteins is tightly controlled by the endogenous inhibitors SMAD7 (Varga, 1995; Pannu, 2004). Although the SMAD pathway is considered to be the central conduit for signals from the TGFβ receptors, emerging evidence highlights the importance of non-SMAD pathways (Moustakas, 2005). In fact, recent studies indicate novel roles for some non-SMAD pathways, such as the tyrosine kinase c-ABL (Daniels, 2004) and early growth response 1 (Chen, 2006), in mediating the stimulation of profibrotic responses induced by TGFβ in fibroblasts. These novel non-SMAD pathways interact with each other and with SMAD proteins in complex, cell lineage–specific signaling networks (Varga, 2007; Gu, 2008).

In SSc, SMAD-dependent or SMAD-independent signaling downstream of TGFβ has been extensively characterized in sclerodermic cells (Figure 3, page 20). Evidences for the involvement of SMAD dependent pathway are reduced fibrosis in an animal model of SSc via SMAD3 disruption (Lakos, 2004), and impaired inhibitory function of SMAD7 in SSc fibroblasts (Asano, 2004). Overall, dysregulated expression or function of activating and inhibitory SMAD proteins and their cofactors has been documented in SSc fibroblasts. It may also contribute to the initiation or propagation of the abnormal fibrogenic response (Varga, 1995; Pannu, 2004). However, evidence for the involvement of SMAD-independent pathway is the elimination of collagen I and III expression in scleroderma cells via inhibiting protein kinase C delta, geranyl transferase1 or stress-activated protein kinase p38 (Rosenbloom, 2000; Hayashida, 2007).

Evidences for the involvement of TGFβ in SSc are the DNA microarray analysis, which indicates that a group of TGFβ–dependent genes are overexpressed in biopsy

specimens from skin lesions in patients. Furthermore, in a mouse model recapitulating the clinical and histological features of SSc, enhanced TGFβ signaling in fibroblasts causes skin fibrosis. In addition, enhanced TGFβ expression has been extensively detected in sclerodermic lesions. Noteworthy, beside the TGFβ role in SSc as the strongest myofibroblast inducer, it also modulates the expression of various cytokine receptors, including receptors for TGFβ and PDGF (Gabrielli, 2009).

More specifically, most studies available in literature about the TGFβ role in SSc and all evidences mentioned above are related to TGFβ1. In fact, it is undisputed that TGFβ1 is a potent profibrotic cytokine, since it stimulates fibroblasts chemotaxis, proliferation, expression of α-SMA, and synthesis of collagen as well as other ECM proteins. Furthermore, it inhibits ECM degradation by decreasing the synthesis of MMP and increasing the synthesis of tissue inhibitor of MMP (Massague, 1990; Nabel, 1993; Gu, 2008). However, the specific function of the other two TGFβ isoforms in the pathogenesis of SSc remains unclear. Although much effort has been made to elucidate their pathologic role in SSc, their specific functions remain elusive since results from various studies are contradictory (Prelog, 2005; Sgonc, 2008).

1.3.2.4.2 Platelet Derived Growth Factor (PDGF)

The PDGF family is composed of four different polypeptide chains, the traditional PDGF-A and PDGF-B, and more recently discovered PDGF-C and PDGF-D. The biologically active PDGF protein forms disulphide-bonded dimmers including the four homodimers PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD, and one heterodimer PDGF-AB. PDGF-A and PDGF-B are processed intracellularly and secreted in their active form, while PDGF-C and PDGF-D are secreted as latent factors requiring proteolytic activation (Betsholtz, 2003).

PDGFs exert their biological activities by activating two structurally related tyrosine kinase receptors, PDGFRα and PDGFRβ. Ligand-induced receptor homo or

heterodimerization leads to autophosphorylation of specific tyrosine residues within the cytoplasmic domain. Dimers of PDGF-A (PDGF-AA) activates PDGFRα homodimers, exclusively, while dimers of PDGF-B (PDGF-BB) is capable of activating PDGFRα, PDGFRβ homodimers and PDGFRαβ heterodimer (Betsholtz, 2003). Each of PDGFR is characterized by an extracellular binding part and a large intracellular part with a tyrosine kinase domain (Malarkey, 1995). The extracellular part is composed of five immunoglobulin-like domains (Ig-like), however the ligand-binding function resides in the first three domains of the receptors. e.g., Ig-like domain 3 of the PDGFα receptor contains epitopes of particular importance for PDGF-AA binding, while the PDGF-BB binding epitopes reside in Ig-like domains 1 and 2 (Miyazawa, 1998; Wiesmann, 2000).

For PDGF receptors, the key event following growth factor stimulation is autophosphorylation within the intracellular domain accompanied by dimerization of the receptor. Autophosphorylation of the PDGF receptor occurs within a number of regions including the kinase insert domain. This phosphorylation promotes interaction of the receptor with a number of target proteins or enzymes and induces certain conformational change. Thus, leads to induction of several signaling pathways including phosphstidylinositol 3 kinase (PI3), Ras-MAPK, Src family kinases and phospholipase Cγ (PLCγ) (Malarkey, 1995). Such induction results in cellular proliferation, chemotaxis and actin reorganization (Betsholtz, 2003). Noteworthy, PDGFRα and β are structurally similar and activate overlapping signal transduction pathways resulting in overlapping biological properties in vitro (Trojanowska, 2008).

In SSc pathogenesis, PDGF plays an important role. It is almost undetectable in healthy skin or lung, whereas immunohistochemical studies have revealed increased presence of PDGF and PDGF receptors in SSc skin biopsies. In addition, expression of PDGF-B was detected in endothelial cell lining of small capillaries and in the infiltrating cells (Gay, 1989; Klareskog, 1990). PDGF-A was also prominently expressed in small capillaries, around the hair follicles and in selective stromal cells (Yamakage, 1992).

Likewise, elevated levels of PDGF-A and PDGF-B were found in bronchoalveolar lavage (BAL) fluid obtained from SSc patients (Ludwicka, 1995).

An interesting observation was made regarding TGFβ regulation of PDGFRα in sclerodemic fibroblasts. Indeed, unlike normal fibroblasts, which are unaffected by TGFβ treatment or show decreased PDGFRα expression in response to TGFβ, SSc fibroblasts respond to TGFβ with up-regulation of PDGFRα (Yamakage, 1992). As a result, TGFβ treatment renders SSc fibroblasts more responsive to the subsequent mitogenic stimulation with PDGF. This unique characteristic is present in both skin fibroblasts and fibroblasts obtained from SSc BAL fluid (Ludwicka, 1995). Relevant to this finding, another study have shown that sclerodermic fibroblasts express elevated levels of endogenous IL-1α, which in turn stimulates production of PDGF-A (Kawaguchi, 1999). These findings imply the existence of an autocrine PDGF-A/PDGFRα loop operating in SSc fibroblasts.

In addition, beneficial effects of selective inhibitors of PDGF signaling on dermal fibrosis had been reported (Akhmetshina, 2008). The functional significance of the activation of PDGF signaling in SSc fibroblasts has not been fully evaluated, but it may contribute to the enhanced proliferation and migratory and contractile potential of cultured SSc fibroblasts. Noteworthy, recent study has also suggested that sera from patients with SSc contain pathological autoAbs directed against PDGF receptors (Figure 3, page 20) (Baroni, 2006) (this study will be discussed later in details, page 32). These autoAbs were capable of stimulating reactive oxygen species and subsequent activation of extracellular regulated kinase 1/2 (ERK1/2). This intriguing study, if confirmed, may provide additional support for the persistent activation of PDGF signaling and its contribution to SSc fibrosis. This new concept, however, awaits additional independent confirmation.

Table I: Pro-fibrotic cytokines involved in the pathogenesis of SSc.

	TGFβ	PDGF
Main cell source	Macrophages, fibroblasts, T cells, B cells, platelets, EC	Platelets, macrophages, EC, fibroblasts
Pathogenic relevance	- Induces proliferation of fibroblasts and production of CTGF and ET-1; - Stimulates synthesis of collagens, fibronectin, proteoglycans; - Inhibits ECM degradation by reduced synthesis of MMP and induction of TIMP-1;	- Serves as mitogen and chemoattractant for fibroblasts and VSMCs; - Induces synthesis of collagen, fibronectin, proteoglycans; - Stimulates secretion of TGFβ1, MCP-1 and IL-6;
Effect in scleroderma	- Stimulates expression of TGFβ and PDGF receptors; - Increased levels of TGFβ in skin in some studies; - Elevated expression and phosphorylation levels of SMAD2 or SMAD3 effectors of TGFβ signaling pathway; - Elevated levels of TGFβRI in vivo;	- Elevated expression of PDGF and PDGFR in skin; - Increased levels in BAL biologic fluids;

1.3.3 Immune System

The innate and adaptive immune systems both have a role in the pathogenesis of SSc (Abraham, 2005). The activation of the immune system is another early and predominating event in SSc. Monocytes are among the first cells to migrate into tissues, but they seem to play an important role throughout the disease, since they are found predominantly in skin biopsies taken at both the early and later phases (Ishikawa, 1992; Kraling, 1995). Once activated and migrated to the damaged tissues, monocytes/macrophages secrete several soluble factors that can participate not only in (as mentioned earlier):

1. EC activation, increasing synthesis and expression of integrins, thereby accentuating the fixing circulating leukocytes on vascular endothelium;
2. The fibrotic process, influencing growth, differentiation and chemotaxis of fibroblasts;
3. The activation of VSMCs;

But, also in:

4. Activation of other immune cells, such as T and B cells , perpetuating the inflammatory cycle (Jimenez, 2004) (Figure 2, page 12, step 2).

1.3.3.1 T cells

T cells in skin lesions of SSc patients are predominantly CD4+. They display markers of activation, exhibit oligoclonal expansion and predominantly Th2 cells. Th2 predominant profile is defined by increased levels of anti-inflammatory and profibrotic cytokines such as IL-4 and TGFβ, and reduced production of proinflammatory cytokines such as IFNγ (Clements, 2004; Abraham, 2005). These characteristics parallel the increased serum levels of cytokines derived from Th2 cells in SSc (Gabrielli, 2009). Thus, T cells appear to play a role in fibrosis through activation of monocytes and direct release of profibrotic cytokines (Clements, 2004).

1.3.3.2 B cells

B cells and plasma cells are occasionally seen among cells infiltrating the skin and lung of SSc patients (Lafyatis, 2007; Gu, 2008). An activated B cell signature has been demonstrated by microarray analysis in SSc skin (Varga, 2007). Naive B cells and memory B cells from SSc patients are both activated and they express increasable levels of CD19, which is a cell surface signaling receptor that regulates B cell responses. Transgenic mice overexpressing CD19 spontaneously develop high titers of anti-topo I specific Abs associated with SSc (Varga, 2007). In addition, depletion of B cells resulted in amelioration of skin fibrosis in the TSK1/+ mouse (Hasegawa, 2006). This suggests that altered B cells function in SSc might account not only for autoAbs production but also for fibrosis, since activated B cells secrete IL-6, which directly stimulates fibroblast (Gu, 2008; Gabrielli, 2009).

1.3.3.2.1 Autoantibodies (AutoAbs)

Each systemic autoimmune disease is characterized by the elaboration of a distinct group of autoAbs that target a limited number of ubiquitously expressed antigens (Harris, 2003). These autoAbs may arise as secondary events either to molecular mimicry or consequent to repeated cell damage and release of nuclear antigens (Clements, 2004). Virtually, all patients with SSc have detectable serum autoAbs that are either associated with the disease phenotype such as antinuclear antibodies (ANA) or with the disease pathogenesis like anti-endothelial cell antibodies, anti-matrix metalloproteinase antibodies, antifibrillin-1 antibodies and antifibroblast antibodies (Chung, 2004). (The latter pathological roles in SSc are illustrated in Table II, page 29).

Table II: Autoantibodies with direct pathogenicity in SSc. Adapted from (Chung, 2004).

Autoantibodies	Role of pathogenesis	Clinical associations
Anti-endothelial cells	Incite vascular injury by inducing endothelial cell apoptosis	Ischemic digital infracts; PAH
Antifibroblasts	Induce fibroblast production ICAM-I and IL-6 leading to vascular damage and ECM production	Limited SSc
Anti-metalloproteinase	Inhibit MMP-1 collagenase activity	Diffuse SSc
Antifibrillin-1	Induce instability in microfibrils resulting in ECM accumulation	Choctaws American Indian and Japanese ethnic background

More than 90% of SSc patients have ANA (e.g. ACA, anti-topo I and antinucleolar Abs). The major autoAbs in SSc are directed against CENP-A through F, DNA-topo-I and less frequently against antinucleolar antigens. SSc-specific autoAbs are associated with the major subtypes of the disease and with specific disease manifestation. ACA are found in 20 to 30 % of SSc patients and are associated with lSSc, significantly reduced frequency of pulmonary fibrosis and lower mortality. However, anti-topo I Abs are detected in 9 to 20% of SSc patients and are associated with dSSc, pulmonary fibrosis and increased mortality. In Addition, heterogeneous group of mutually exclusive autoAbs, which are called antinucleolar Abs, are seen in 15 to 40% of SSc patients. Of these, anti-RNA-polymerase I and III Abs that are highly specific for SSc and they are associated with diffuse cutaneous involvement, SSc-related renal crisis and greater mortality (Gu, 2008). (Table III, page 30, is a list of the known ANA in SSc).

Table III: Autoantibodies and their clinical associations in patients with SSc. Adapted from (Chung, 2004).

Autoantibodies	Cutaneous involvement	Clinical associations
Anti-topo I	Diffuse	PF, peripheral vascular disease, cardiac involvement, and malignancies
Anticentromere (ACA)	Limited	CREST syndrome, ischemic digital loss, isolated pulmonary hypertension and renal disease
Antihistone	Limited> Diffuse	Severe PF, cardiac and renal disease, poor prognosis
Antipolymyositis	Diffuse or Limited	Severe Raynaud's phenomenon, arthritis, pulmonary disease, calcinosis, myositis and benign course
Anti-Th/To	Limited	Buffy fingers, small bowl movements, hypothyrodism, PF and renal crisis
Anti-U3-snoRNP (fibrillarin, Mpp10, h-U355k)	Diffuse or Limited	African-American ethnicity, cardiac and renal disease, and gastrointestinal involvement
Anti-UI-SnRNP proteins Anti-UI-snRNA	Diffuse or Limited	Severe Raynaud's phenomenon, MCTD, PF and renal crisis
Anti-RNA polymerase	Diffuse	renal crisis
Anti-B23	Limited> Diffuse	PAH and malignancies

1.4 Treatment

Treatment of SSc is largely empirical and rarely evidence based (Maddison, 2002). It has been somewhat haphazard and treatment has often been benefiting from the experience gained from treating other connective tissue diseases. The choice of therapy is complex and depends on the type of SSc (diffuse versus limited); the stage of illness (early versus late); and the specific organ-related problems faced by the individual patient (Charles, 2006).

At present, there are no known treatments to change the overall course of SSc. However, survival has improved dramatically in recent years due to the effectiveness of treatments directed at specific internal organ features. These include drugs for PAH such as epoprostenol, treprostinil and iloprost, which can supply prostacyclin that the pulmonary vascular endothelium itself no longer supplies in adequate quantities. Moreover, bosentan, which is ET receptor antagonist, is also used because, as mentioned earlier, ET (vasoconstrictor mediator) is frequently increased in the serum of patients with SSc-PAH. In addition, other drugs are used for other SSc manifestations. Angiotensin-converting enzyme inhibitors (ACEI) are the preferred choice in the management of renal crisis, the fatal complication of SSc. Moreover, immunosuppressive, such as, cyclophosphamide, d-penicillamine, methotrexate, and corticosteroid are used for pulmonary inflammation (Charles, 2006). Furthermore, antifibrotic therapy e.g. INFα and β, and recombinant human relaxin have been tested in controlled trials but did not show any benefits (Panayiotis, 2001).

Tyrosine kinases (TK) are promising targets for antifibrotic treatment approaches in SSc due to their central role in production and release of ECM. Investigation for tyrosine kinase inhibitors (TKIs) is currently available. The TKI imatinib, a dual inhibitor of Abelson kinase (c-Abl) and PDGF receptor, is currently investigated in clinical proof-of-concept trials for the treatment of patients with SSc (Akhmetshina, 2009). In addition, further TKIs such as nilotinib (Bcr-Abl tyrosine kinase inhibitor) and dasatinib (SRC-

family protein-tyrosine kinase inhibitor) with potent antifibrotic effects are emerging from preclinical studies. In contrast to TKIs of c-Abl and PDGF receptor, the role of vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) TKIs in the treatment of SSc remains unclear (Akhmetshina, 2008; Beyer, 2010).

UVA phototherapy could be implicated in treatment of SSc because of its major immunosuppressive effects via the induction of apoptosis of T cells, impairment of cell proliferation and the induction of inhibitory cytokines like IL-10. However, results of various studies are conflicting and controlled studies are currently in progress (Hunzelmann, 2010).

Clinical research on therapies for SSc is currently very active. The future and present trials should focus on the cellular mediators in SSc as potential therapeutic targets (Charles, 2006).

1.5 Rational, Hypothesis and Objectives

1.5.1 Anti-PDGFR Stimulatory AutoAbs in SSc Link between Autoimmunity and Fibrosis

A common feature of all the Abs found in SSc is their inability to activate selective signaling pathways. Their production is probably secondary to cell breakdown induced by inflammation. Recently, a novel class of Abs has been discovered in SSc patients. They appear to be the first identified link between the activation of immune system and tissue fibrosis. The novel link is between B-cell-mediated autoantibody production and upregulated PDGFR signaling in SSc that was provided by Baroni et al. study (Baroni, 2006). This Italian group showed that there are autoAbs from sera of patients of SSc that bind and stimulate PDGFR in fibroblasts, which in turn stabilizes RAS and induce ERK1/2. Induction of ERK1/2 increases levels of reactive oxygen species (ROS). The long-term persistence of ROS and ERK1/2 ultimately results in the stimulation of collagen gene

expression (Baroni, 2006). This interesting study provides additional support for the persistent activation of PDGFR signaling and its contribution to SSc fibrosis. However, other studies contradict the presence of agonistic Abs to PDGFR (Classen, 2009; Loizos, 2009). Noteworthy, the PDGFR stimulatory autoAbs found not to be specific for SSc. They have also been detected in graft versus host patients (Svegliati, 2007) and in systemic lupus erythematosus (SLE) (Kurasawa, 2010). Thus, the presence of stimulatory autoAbs to PDGFR in SSc must be verified in larger number of patients affected by SSc and different laboratories.

Vascular complications are another hallmark of SSc. In SSc, the homeostatic function of endothelium is disrupted by EC activation and/or injury. Endothelial cell (EC) injury is the critical early event in SSc-associated pulmonary vascular disease such as PAH. In PAH, EC injury leads to alterations in endothelial function with increased production of vasoconstrictor mediators and decreased synthesis of vasodilator ones (Varga, 2002). In addition, damage to the endothelium in SSc induces the release of proinflammatory mediators, chemokines and growth factors such as PDGF and TGFβ, which in turns, promote proliferation of smooth muscle cells and ECM deposition (Abraham, 2005).

The investigation of the effects of anti-PDGFR on EC and VSMCs would bring the vascular components of SSc into the picture and provide better understanding to the interacting systems in this disease. Therefore, if anti-PDGFR autoAbs were present in the circulation of SSc patients, they would most certainly encounter VSMCs. The latter are known to express the PDGFR and response to PDGF, which is a known phenotypic modulator of VSMCs (Rensen, 2007). Thus, we hypothesized that VSMCs will response to autoAbs against the PDGFR leading to the activation of ERK and the induction of genes involved in fibrotic responses.

In order to prove this hypothesis the following specific objectives will be investigated in this study (Figure 4, page 35).

1.5.2 Objective 1: Involvement of SSc-IgG in activating downstream pathway of PDGFR in VSMCs

There are several signaling cascades induced by PDGFRs. One of these downstream pathway is MAPK. The latter has been proposed to play an important role in cell growth and differentiation stimulated by PDGF (Malarkey, 1995). It is possible that autoAbs to PDGFR could act as mitogen just as the endogenous cytokine and induce ERK1/2. Thus ERK phosphorylation will be measured by immunoblot analysis to assess the response of VSMCs to autoAbs from SSc patients.

1.5.3 Objective 2: Involvement of SSc-IgG in affecting fibrotic genes in VSMCs

According to Baroni et al study, upregulated PDGFR signaling by SSc autoAbs results in the stimulation of collagen gene expression. Since patients with SSc have an increase in collagen types 1 and 3 (Philips, 1995), we will test the effect of SSc-IgG on collagen 1 (*col1a1)* and collagen *3 (col III)* genes expression. In addition, we will also assess the effect of these autoAbs on *tgfb* genes since TGFβ1 is a key mediator of tissue fibrosis (Ihn, 2008). Indeed, in SSc, the role of TGFβ1 as a fibroblasts' activator and a potent stimulator of collagen production is well defined, but the specific function of the other two isoforms in the pathogenesis of SSc remains unclear (Ihn, 2008). Thus, we will study the effect of SSc-IgG on the mRNA expression of the three different isoform (*tgfb1, b2* and *b3*). The study of the 5 different genes will be accomplished by the RT-qPCR method.

1.5.4 Objective 3: Involvement of PDGFR in the molecular events noticed in VSMCs in response to SSc-IgGs

The third objective is to verify the involvement of PDGFR in the molecular events noticed in VSMCs in response to SSc-IgGs. In order to achieve this, two assays will be

performed. First, we will immunoprecitpitate the PDGFR by SSc-IgG and controls. Second, we will use the highly characterized PDGFR tyrosine kinase inhibitor, the tyrphostin AG1296.

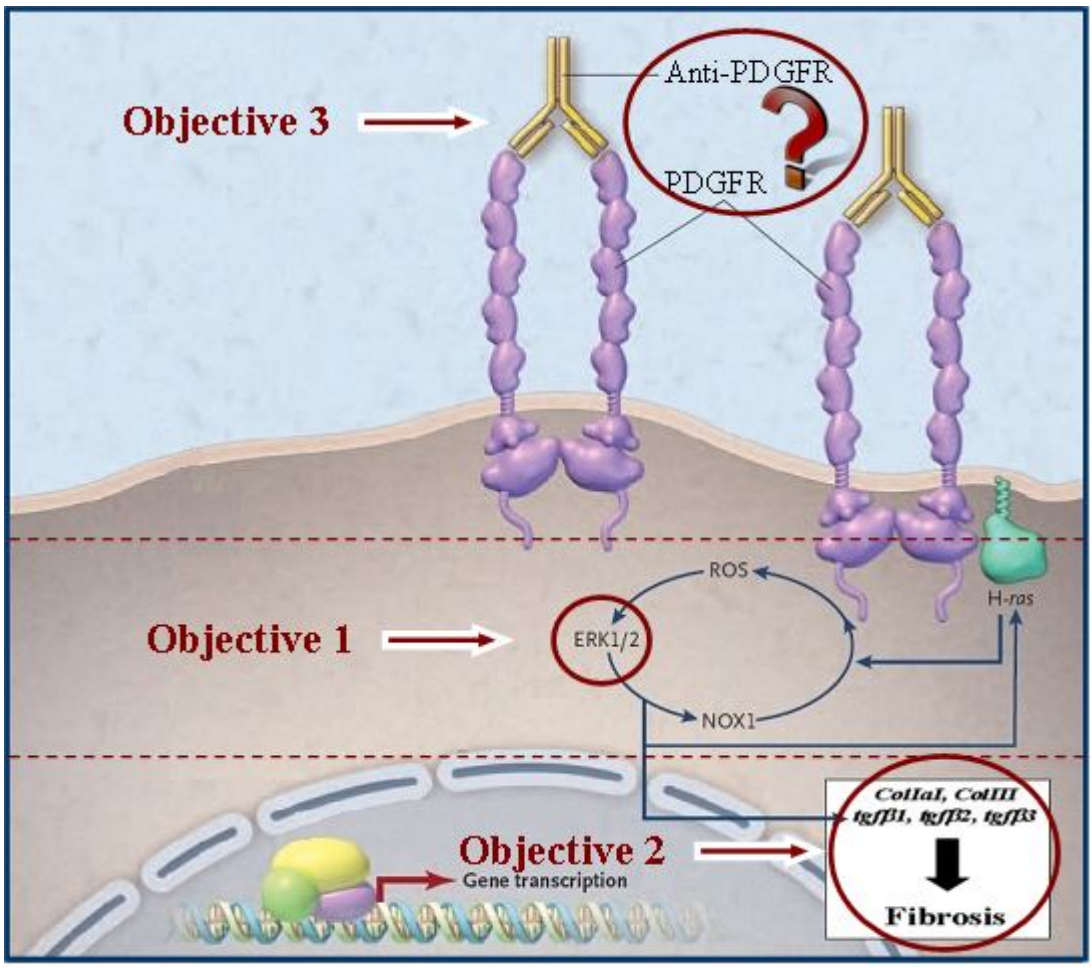


Figure 4: Schematic diagram represents the objectives of the research. Modified from (Baroni, 2006).

Chapter 2: Material and method

2.1 Patients' characterization

The Canadian scleroderma research group (CSRG) provided us with sera samples of controls and SSc patients as well. Nine controls were used in our study. The controls were either normal people or patients with osteoarthritis (OA), which is a degenerative disease of degradation of the joint with no inflammation or systemic autoimmunity features (Cooke, 1987). The controls were age and sex matched. The SSc patients' sera were randomly selected from CSRG bank. We studied 19 patients with SSc (2 men and 17 women) with a median age of 51 years (range 23 to 75). All patients have disease duration less than 5 years with a diffuse type of the disease. These patients had not received any immunosuppressive treatment. Of 19 patients, seven had anti-RNA polymerase antibodies and four had anti Scl-70 antibodies. (Demographic characteristics of patient outlined in Table IV, page 36).

Table IV: Demographic characteristics of SSc patients involved in this study

Characteristics	Sex		Race		Age	
	Male	Female	White	Others	Median	Range
Systemic Sclerosis (N=19)	2	17	17	2	51	23-75
Duration of disease	< 5 years					

2.2 Reagents (antibodies, agonists)

PDGF-BB and TGFβ1 was purchased from R&D system (Minneapolis). Phospho-ERK1/2 (Thr202-Tyr204) antibody (#9101) and anti-ERK1/2 (#9102) are from Cell Signaling Technology (Beverly, MA). Anti-PDGFRβ (# 06-495) was purchased from Up State Millipore, while the (PDGF) α and β receptors inhibitor AG1296 (# 658551) was purchased from Calbiochem (Gibbstown, NJ).

2.3 IgG purification

The IgG fractions where purified from sera of controls and SSc patients by affinity chromatography using Protein A/G agarose 15ml settled resin (Pierce). Sera samples (300µl) were diluted 1:1 with a binding buffer, phosphate buffered saline (PBS), and then applied to a gravity-flow column (Pierce) packed with 300µl of protein A/G allowing the IgG binding. Then, the protein A/G column is washed from the non-bound serum components by several washing steps (4 washes with PBS 5ml and three washes with 1M NaCl 200µl). Finally, IgG elution buffer 200µl (Pierce) is used to elute the IgGs and the eluted IgG fractions are neutralized using 20µl of Tris- HCL PH 8.5.

The mature native PDGF isoforms have a molecular weight of ~30,000 Dalton (Heldin, 1999). Thus, a size-exclusion chromatography was added to the purification procedure to remove trace amounts of contaminating cytokines. **A**micon Ultra-4 device (Millipore) 100K is used for this purpose. It concentrates the sample to be collected from the filter and allow the flow of any molecule with a molecular weight less than 100,000 Dalton.

2.4 Cell Culture

Rat aortic VSMCs were obtained from Dr. Darren Richard (Hôtel-Dieu de Québec, QC, Canada) and grown in high glucose Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cultured cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Quiescent VSMCs were obtained by incubation of 80% confluent cell cultures in serum-free high glucose DMEM, Ham's F-12 (1:1) supplemented with 15mM Hepes (pH 7.4), 0.1% low endotoxin bovine serum albumin (Sigma, Saint Louis, MO), and 5 g/ml transferrin (Sigma, Saint Louis, MO) for 48 hours.

For experiments with pharmacological inhibitors, the cells were treated with vehicle alone or with the indicated concentrations of the inhibitor for 30 min before addition of PDGF-BB or IgG fractions.

2.5 Immunoblot analysis

After the different treatments, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and whole cell extracts were prepared using Triton X-100 lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 50mM sodium fluoride, 5mM EDTA, 40mM β-glycerophosphate, 1mM sodium orthovanadate, 10^{-4}M phenylmethylsulfonyl fluoride, 10^{-6}M leupeptin, 10^{-6}M pepstatin A, 1% Triton X-100, 10% glycerol) for 30 min at 4°C. All cellular extract is subjected to centrifugation at 13,000g for 10 min, and were quantified by the BCA method. Equal amounts of lysate proteins (20-50 g) were subjected to electrophoresis on 7.5, or 10% acrylamide gels. Proteins were electrophoretically transferred onto a nitrocellulose membrane for two hours at 4ºC in a transfer buffer with methanol (14.4g/L glycine, 3g/L TRIS and 20% methanol). Then the membrane is blocked for an hour in a solution of TBS 0.1% Tween 20 containing 5% milk and then incubated in primary antibody with specifications of each. The membrane is then washed 5 times 5 minutes in TBS 1% Tween 20 then incubated for 2 hours in secondary antibody coupled to peroxidase. The membrane was washed again, 5 times 5 minutes in TBS 1% Tween 20 and then the presence of proteins of interest is revealed by using ECL+ (enhanced chemiluminescence).

2.6 RT real time-PCR

Total RNA was isolated using RNeasyMini kit (QIAGEN) according to manufacturer's instructions. Spectrophotometric quantification of RNA samples was performed. Accepted ratio of (A_{260}/A_{280}) for pure RNA was 1.9-2.1 in 10mM Tris-Cl PH 7.5. RNA samples were further analysed on a Bioanalyzer system at Genomic platform,

Institute for Research in Immunology and Cancer (IRIC), to verify RNA integrity before proceeding to QPCR assay. Total RNA (2µg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random primers (Applied Biosystems) as described by the manufacturer. Real-time PCRs were subsequently performed using the Fast SYBR Green Master Mix (Applied Biosystems) with the following rat forward and reverse primers (Table V, page 39):

Table V: Sequences of primers used to study genes expressions.

Gene	Primers
colIII	FWD 5`AGATGCTGGTGCTGAGAAG3`
	REV 5`TGGAAAGAAGTCTGAGGAAGG3`
tgfβ1	FWD 5`CCTGGAAAGGGCTCAACAC3`
	REV 5`CAGTTCTTCTCTGTGGAGCTGA 3`
tgfβ2	FWD 5`AGTGGGCAGCTTTTGCTC3`
	REV 5`GTAGAAAGTGGGCGGGATG3`
tgfβ3	FWD 5`AGTGGCTGTTGCGGAGAG 3`
	REV 5` GCTGAAAGGTATGACATGGACA3`

Quanitect primer assay (QIAGEN) was used for *col1a1*. β-*actin* endogenous control was used for sample standardization. The qPCRs were performed using the following schedule: initial step of 3 min at 95˚C, followed by 40 cycles of: 5 sec at 95˚C and 30 sec at 60˚C. All reactions were run in triplicate and the average values of Cts were used for quantification. The relative quantification of target genes was determined using the ΔΔCT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an

endogenous control gene (β-*actin*) (ΔCT = Ct target – Ct CTRL) and compared with a calibrator: ΔΔCT = ΔCt Sample - ΔCt Calibrator. Relative expression (RQ) was calculated with the formula $RQ = 2^{-\Delta\Delta CT}$.

2.7 Co-immunoprecipitaion

500µg of whole cell extracts were incubated for 4 hours at 4°C with 2µg of anti-PDGFRβ (Up State, Millipore), 200µg of SSC or controls-IgGs. The latter were preabsorbed to 50µl protein-A-sepharose beads. The immune complexes were washed four times with Triton X-100 lysis buffer and 2X Laemmli's sample buffer was added. The immunoprecipitated protein analysed by immunoblot analysis.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 for Mac (GraphPad Software, San Diego, CA). Comparison of two groups was carried out using a two-tailed unpaired *t*-test, and comparison of more than two groups was carried out with one-way ANOVA and a Bonferroni post-test. Statistical significance was accepted at a *P*-value below 0.05.

Chapter 3: Results

3.1 SSc-IgG has growth properties on VSMCs

In general, PDGF-stimulated cell proliferation is mediated via the activation of Ras and the downstream MAPK signaling pathway (Bonner, 2004). We hypothesized that SSc-IgG will act as the endogenous cytokine PDGF and will induce ERK in VSMCs. To prove this hypothesis, first a kinetic assay in rat VSMCs is performed to demonstrate that VSMCs respond to PDGF and to determine the optimal time for ERK phosphorylation through PDGFR in these cells. A specific concentration of PDGF-BB**,** which is capable of activating PDGFRα, PDGFRαβ and PDGFRβ (Betsholtz, 2003), is used for different time course. The maximal phosphorylation of ERK was obtained after 5 minutes of PDGF-BB stimulation and reduced with longer exposition time (Figure 5, page 41)**.** Thus, 5 minutes is the optimal time used to expose VSMCs to SSc-IgG.

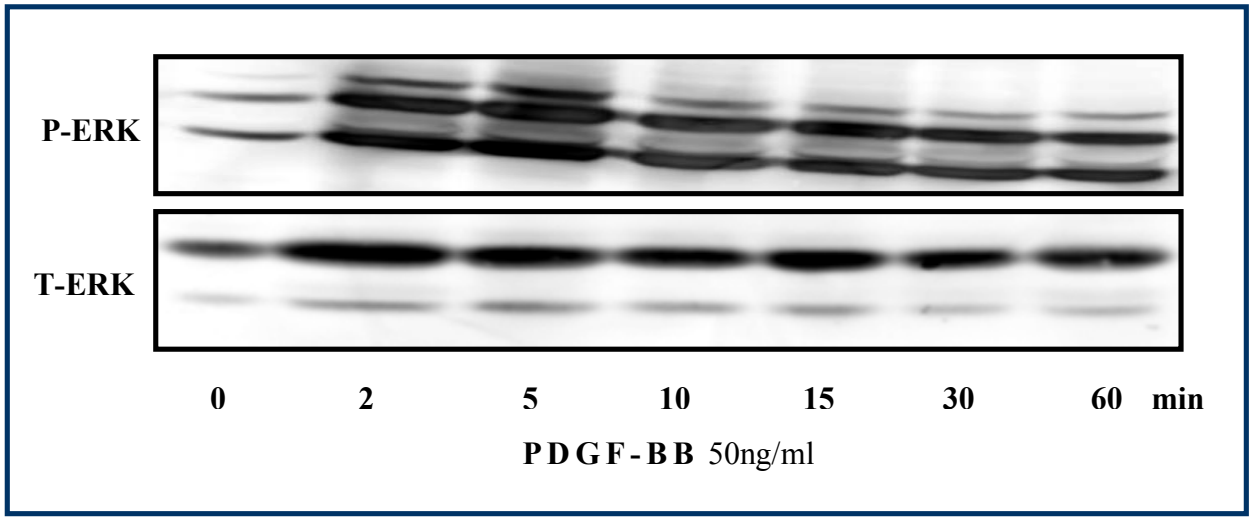


Figure 5: Time course effect of PDGF on ERK activation in VSMCs

Quiescent VSMCs left untreated or exposed to PDGF-BB 50ng/ml for time indicated. Then cells lysates were separated on 10% SDS gel and subjected to immunoblotting analysis using indicated antibodies. This result is representative of three independent experiments.

Total IgG is purified from patients with SSc and controls to eliminate the confounding effect of PDGF or other cytokines present in the sera. Then, cells were exposed to different SSc-IgGs as well as IgGs from controls. PDGF-BB is used to control efficiency of the experimental procedure. The use of an anti-phospho ERK1/2 (Thr202-Tyr204) antibody in Western blot analysis revealed that SSc-IgG were able to phosphorylate ERK more than controls-IgG (Figure 6, page 42). The phosphor ERK signals in response to most (68%, 19/28) SSc-IgG samples was markedly stronger than in controls, of which only 27% (3/11) gave an above-basal signal. Of which gives evidence that VSMCs respond to SSc-IgG and activate MAPK cascade, a downstream pathway of growth factors receptors.

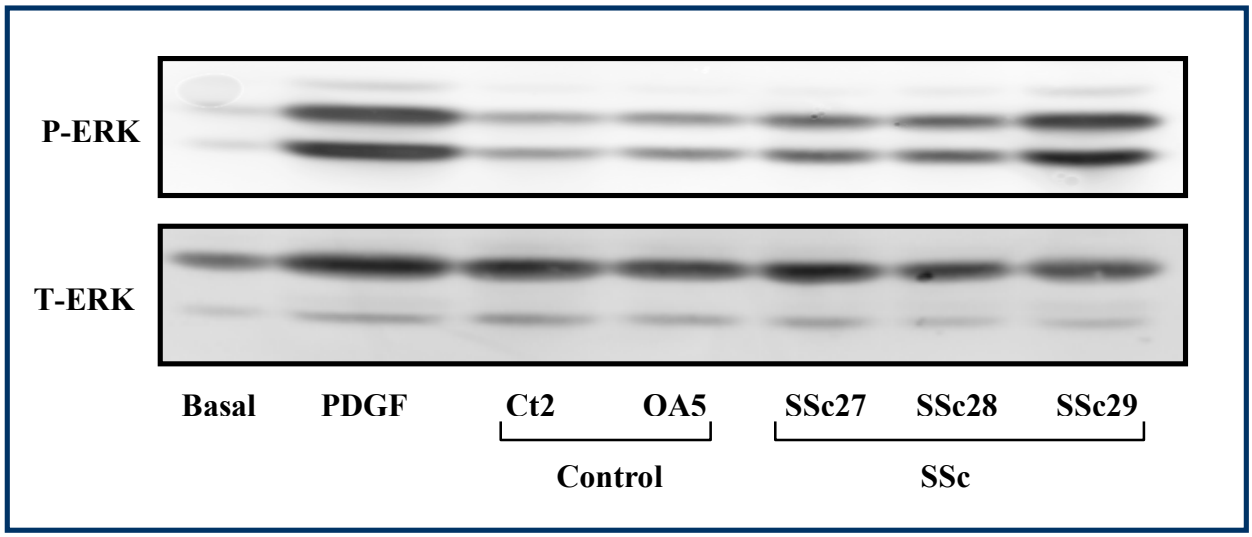


Figure 6: SSc-IgGs phosphorylate ERK more than controls-IgG in VSMCs

Quiescent cells were left untreated or exposed to PDGF-BB 50ng/ml, 200µg of purified IgG of controls or SSc patients for 5 minutes. Then ERK phosphorylation was measured by immunoblot analysis. Normalization of ERK phosphorylation is accomplished by stripping the nitrocellulose membrane and reprobing with an antibody against total ERK. This result is representative of five independent experiments.

3.2 SSc- IgG has a profibrotic role in VSMCs

It has been reported that upregulated PDGFR signaling by SSc autoAbs results in the stimulation of collagen gene expression in fibroblast (Gabrielli, 2006). To test the capacity of SSc-IgG to affect collagen expression in VSMCs, we verified first PDGFR activation aptitude to induce *col1a1* and *colIII* expression. Thus, time course assay with PDGF is performed to validate the optimal time for collagen induction (Figure 7, page 44). TGFβ1, a very well known inducer of collagen expression in VSMCs, is used to control efficiency of the assay (Massague, 1990; Miyazawa, 1995). As a result, we found that PDGF neither induced *col1a1* (Figure 7A) nor *colIII* (Figure 7B) for the time-course indicated, even though TGFβ1 was able to induce *col1a1* and *colIII* gene expression by almost 4.5 folds at 72 hours (Figure 7 A&B).

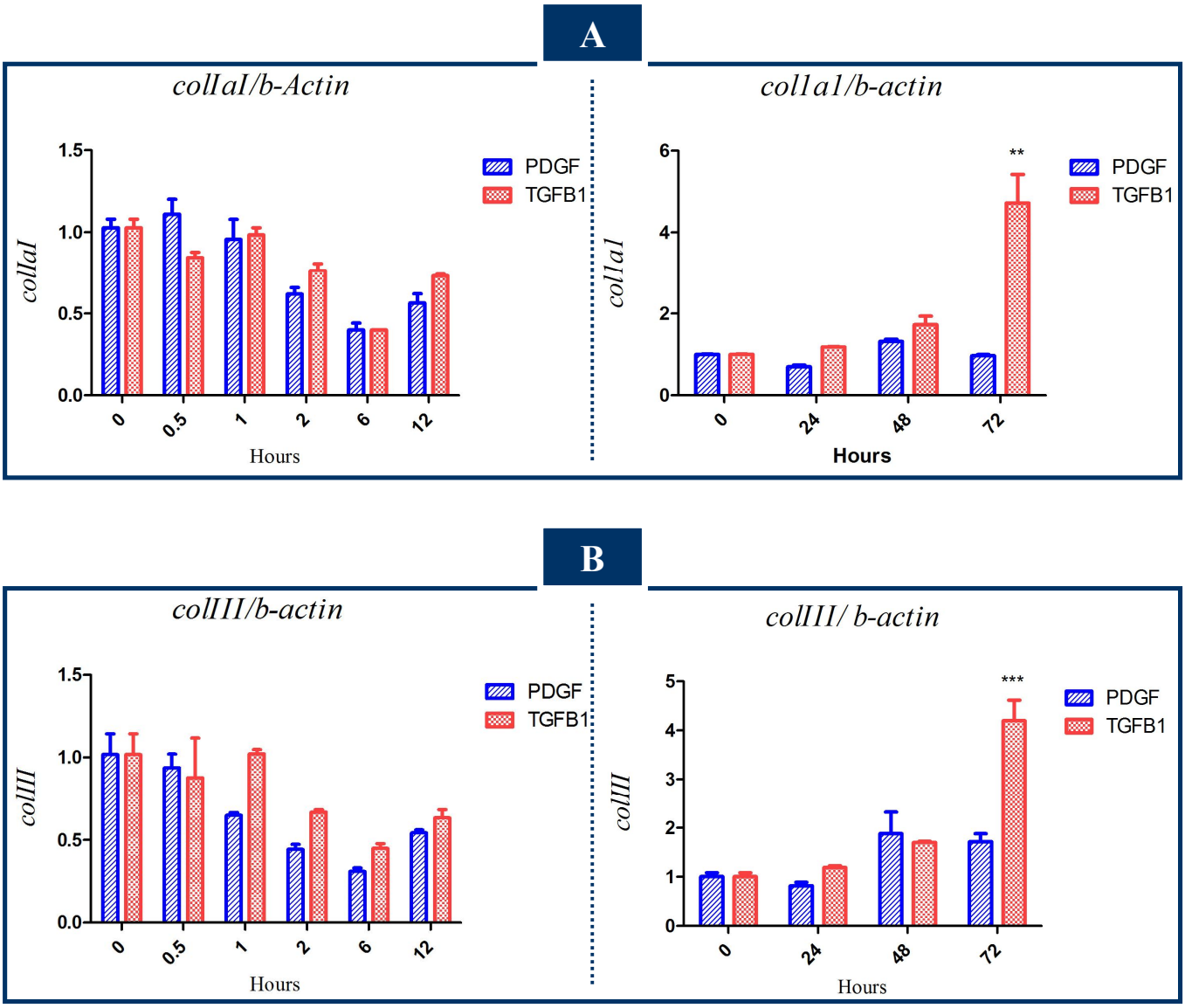


Figure 7: Time course of PDGF effects on *col1a1* and *colIII* genes expression in VSMCs

Quiescent VSMCs were treated with PDGF-BB (50ng/ml) or TGFβ1 (10ng/ml) for the time indicated. RT-QPCR was used to detect messenger RNA encoding *col1a1* (A) and *colIII* (B). Fold changes were calculated relative to the average of triplicate untreated samples by the comparative CT formula $2^{-\Delta\Delta CT}$. Representative result of two independent experiments is shown.

Previous data obtained in vitro indicated that PDGF mediates procollagen synthesis only indirectly through enhancing the expression of *tgf*b genes in macrophage and in fibroblast in vitro (Pierce, 1988; Van Obberghen-Schilling, 1988). Therefore, in accordance with a lack of a significant effect of PDGF in inducing collagen genes following 72 hours of exposition, our data could correlate with this proposition. Thus, we further investigate PDGF effect on *tgf*b genes by time-course assay to validate the best time to expose cells to SSc-IgG.

It is well documented that the three TGFβ isoforms are structurally almost identical but exert different biological function and each isoform is encoded by a distinct gene (Blobe, 2000). Thereby, we studied the effect of PDGF on the expression of three different isoforms (*tgfb1, tgfb2* and *tgfb3)*. *Tgfb* genes showed variable responses to PDGF (Figure 8, page 46). The *tgfb1* and *tgfb3* expression were significantly upregulated ~3 folds. An increase in *tgfb1* can be detected within 1h following PDGF addition; it reaches a peak by 2h and carries on to 6h (Figure 8A). *Tgfb3* increase can be detected as early as 30 minute to reach the maximum induction at 1 to 2h (Figure 8C). In contrast to *tgfb1* and *tgfb3, tgfb2* expression is negatively regulated by PDGF. Its downregulation noticed at 2h, reached a maximal 0.15 fold reduction at 6h and remained low by 12h (Figure 8B). Thereby, the PDGF common influence on *tgfb* genes expression was observed at 2h. Thus, it is the target time chosen to study SSc-IgG effect on these genes.

Of note, it has been previously reported that TGFβ1 positively regulates its own expression in fibroblast cells (Van Obberghen-Schilling, 1988). Noteworthy, our data replicate the autoinduction of *tgfb1* expression at 2h in VSMCs as well (Figure 8A). Thus, it is used as additional positive control in the following RT-qPCR assays.

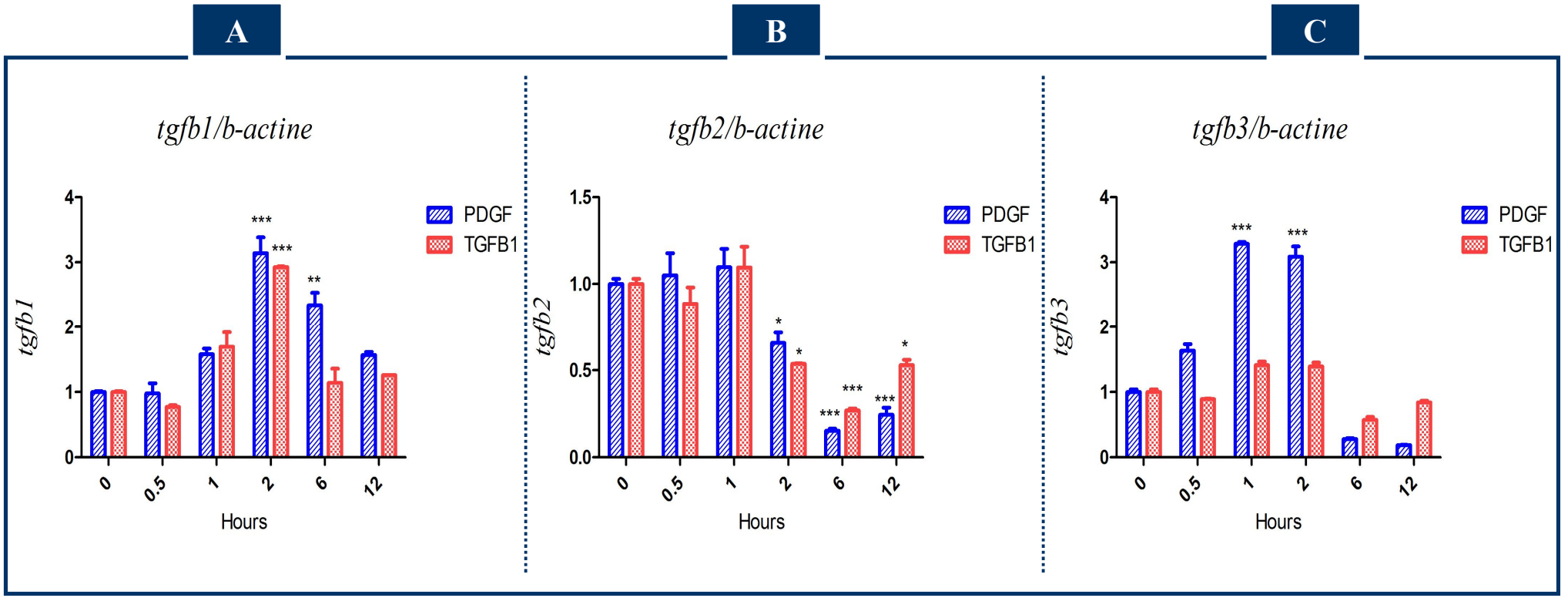


Figure 8: Time course of PDGF effects on *tgfb* genes expression in VSMCs

Quiescent VSMCs were treated with PDGF-BB (50ng/ml) or TGFβ1 (10ng/ml) for the time indicated. RT-QPCR was used to detect messenger RNA encoding *tgfb1*, *tgfb2* and *tgfb3*. Fold changes were calculated relative to the average of triplicate untreated samples by the comparative CT formula $2^{-\Delta\Delta CT}$. Representative results of one of two experiments are shown. *** $p<0.001$, ** $p<0.01$, * $p<0.05$

3.2.1 SSc-IgG induces *tgfb1*and *tgfb3* gene expression in VSMCs

SSc-IgGs effect on *tgfb* genes expression is investigated in two cohorts of patients. In the first one, RT-QPCR analysis showed that *tgfb1* mRNA levels are ~2 folds high by SSc-IgG but not by control IgG (Figure 9 A1, page 48). The difference between SSc-IgG effect and control-IgG on *tgfb*1 were highly significant (Figure 9 A2). Similarly, in the second cohort, *tgfb*1 expression was upregulated by SSc-IgG (Figure 9 B1). Even though some controls rise up *tgfb*1 level, the induction by SSc-IgG was significantly higher than controls with a p value <0.001 (Figure 9 B2).

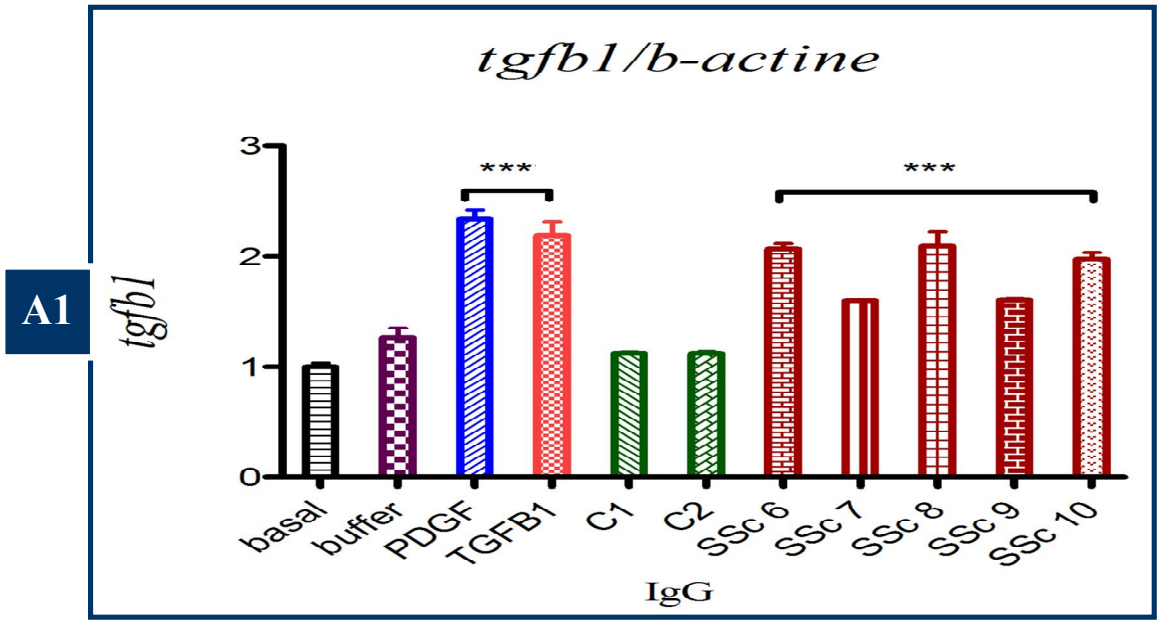
*Tgfb*3 expression revealed a similar pattern as *tgfb*1 in the first cohort with 2.5-4.8 folds increase in response to SSc-IgG (Figure 10 A1&A2, page 50). However, its expression in the second cohort was less, since results showed that the difference between SSc-IgG effect and control-IgG were not significant (Figure 10 B1&B2).

Although the role of TGFβ3 in the pathogenesis of SSc is difficult to describe, various studies suggest its fibrotic role (Shinozaki, 1997; Querfeld, 1999). And, as mentioned before, TGFβ1 is a potent fibrogenic cytokine. Thus, our data suggest a role of SSc-IgG in inducing profibrotic genes.

3.2.2 SSc-IgGs reduced *tgfb2* expression in VSMCs

Unexpected finding is that SSc-IgG caused a ~0.32-0.58 fold decrease in *tgfb*2 mRNA level in the experiments of two groups presented in (Figure 11 A1&B1, page 52). Moreover, the reduction of *tgfb*2 expression was significantly higher in response to SSc-IgG than controls in both groups (Figure 11 A2&B2).

Noteworthy, upon investigating SSc-IgGs effect on the expression of collagen type I and III, RT-qPCR analysis showed that SSc-IgGs neither induce mRNA level of *col1a1* nor *colIII* in VSMCs after 2h exposition (data not shown).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tgfb1
3
2
1
0
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buffer
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TGFB1
C1
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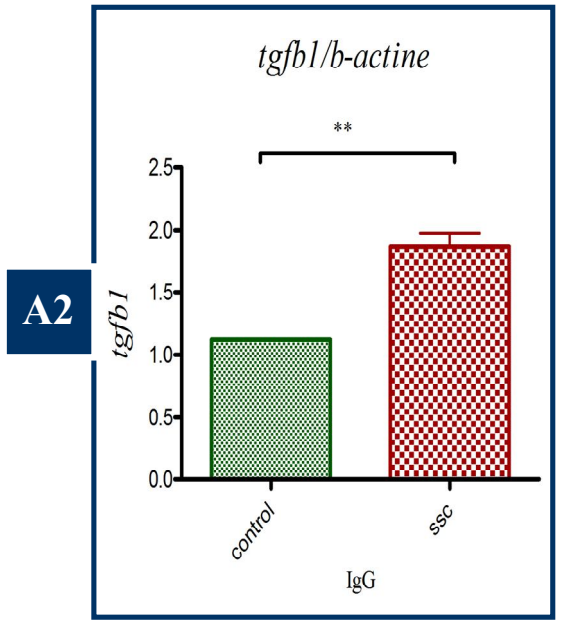
SSc 6
SSc 7
SSc 8
SSc 9
SSc 10
IgG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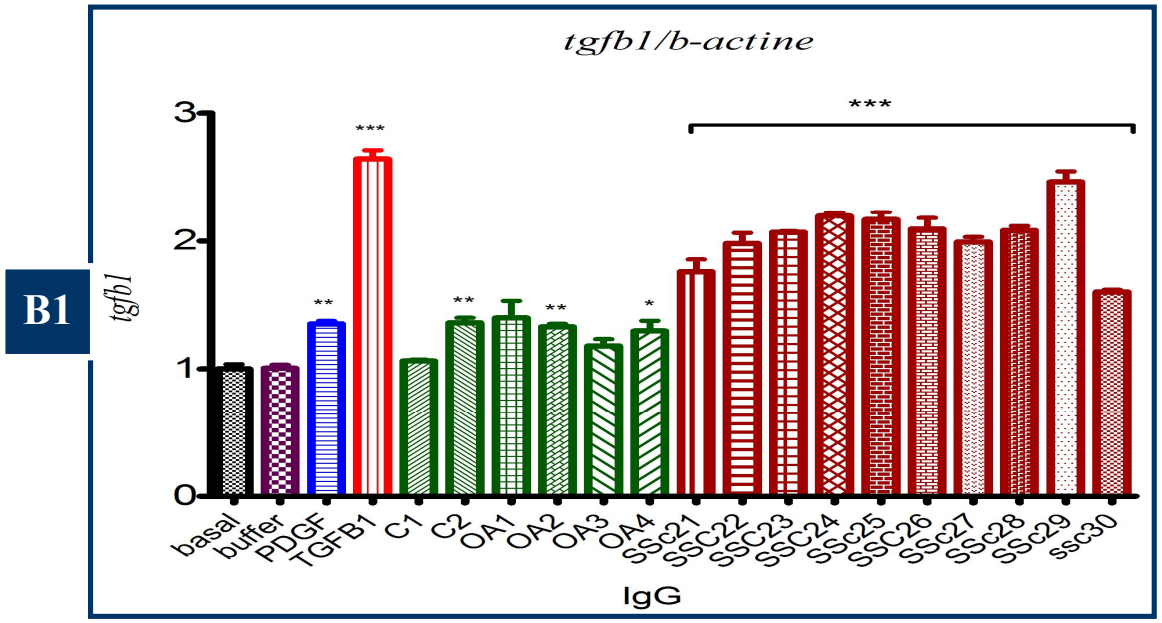
0.0
control
ssc
IgG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tgfb1
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0
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*
basal
buffer
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C2
OA1
OA2
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SSc28
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ssc30
IgG

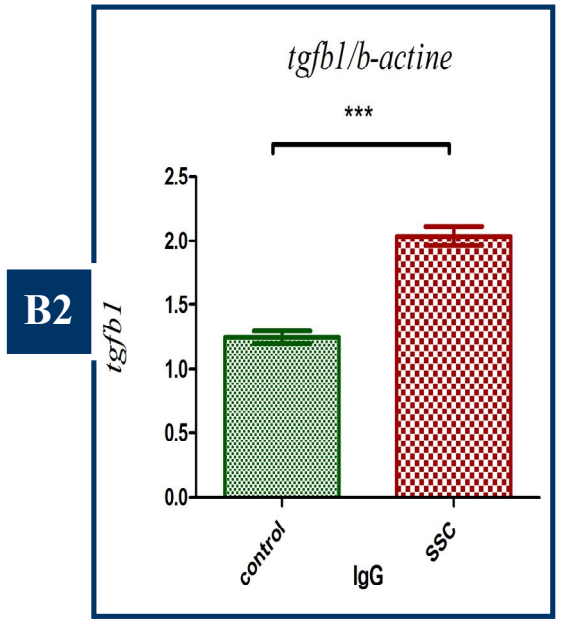
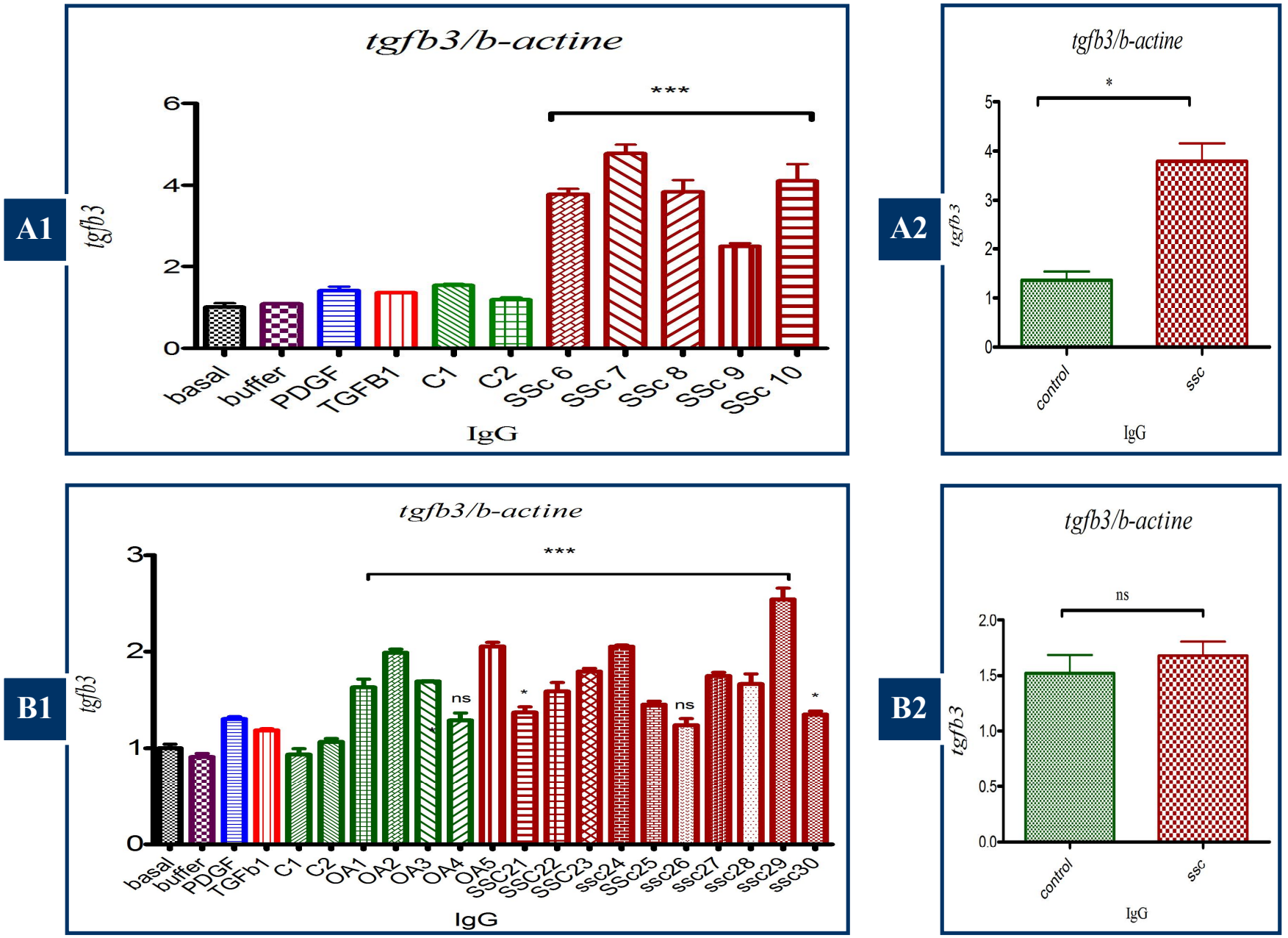

B2
tgfb1/b-actine
***
tgfb1
2.5
2.0
1.5
1.0
0.5
0.0
control
SSC
IgG

Figure 9: Upregulation of *tgfb*1 expression by SSc antibodies in VSMCs

(A1, B1) Quiescent VSMCs were left untreated or exposed to buffer (used in IgG purification), PDGF-BB (50ng/ml), TGFβ1 (10ng/ml), control-IgGs (C, OA) or SSC-IgGs for two hours. RT-QPCR was used to detect messenger RNA encoding *tgfb*1. Fold changes were calculated relative to the average of triplicate untreated samples by the comparative CT formula $2^{-\Delta\Delta CT}$.
(A2, B2) unpaired t-test comparing two groups.
*** $p<0.001$, ** $p<0.01$, * $p<0.05$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SSc 10
IgG
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OA1
OA2
OA3
OA4
OA5
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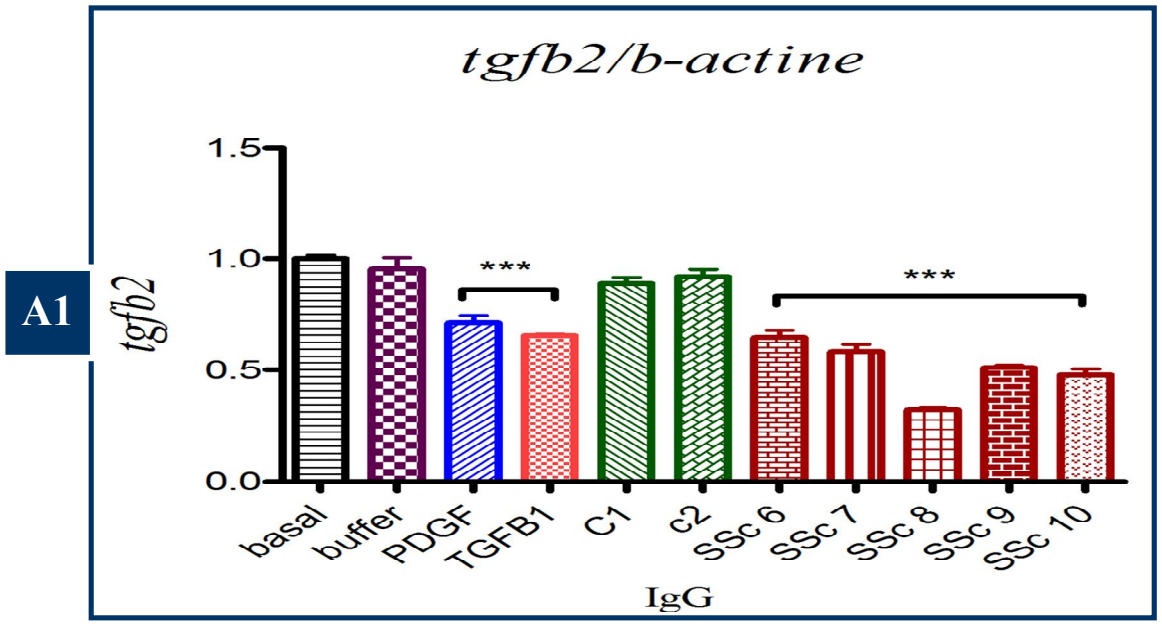
SSC22
SSC23
ssc24
SSc25
ssc26
ssc27
ssc28
ssc29
ssc30
IgG
B2
tgfb3/b-actine
tgfb3
ns
control
ssc
IgG

Figure 10: Upregulation of *tgfb3* expression by SSc antibodies in VSMCs

(A1, B1) Quiescent VSMCs were left untreated or exposed to buffer (used in IgG purification), PDGF-BB (50ng/ml), TGFβ1 (10ng/ml), control-IgGs (C, OA) or SSC-IgGs for two hours. RT-QPCR was used to detect messenger RNA encoding *tgfb*3. Fold changes were calculated relative to the average of triplicate untreated samples by the comparative CT formula $2^{-\Delta\Delta CT}$.
(A2, B2) unpaired t-test comparing two groups.
*** $p<0.0001$, *** $p<0.001$, * $p<0.05$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tgfb2
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1.0
0.5
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***
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PDGF
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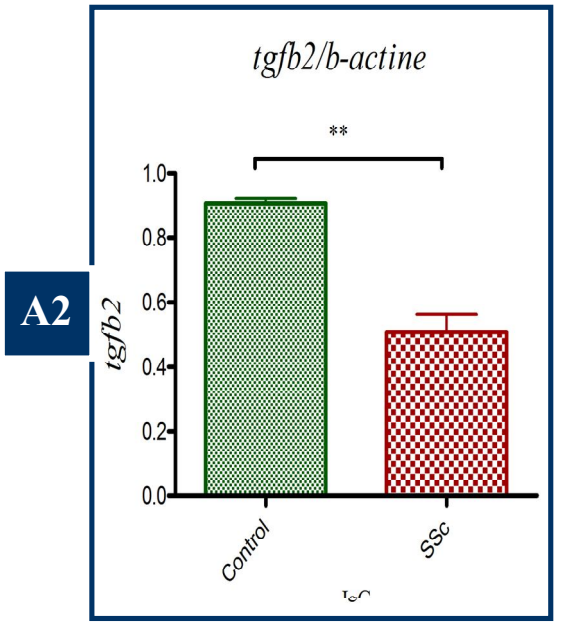
SSc 6
SSc 7
SSc 8
SSc 9
SSc 10
IgG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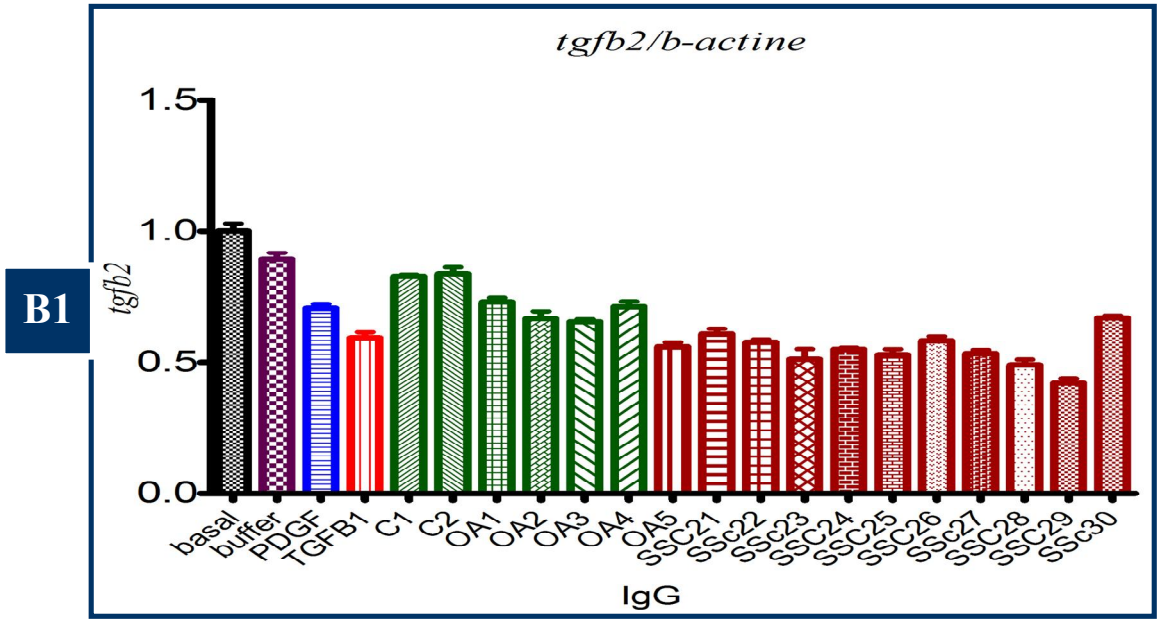
Control
SSc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0.0
basal
buffer
PDGF
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C2
OA1
OA2
OA3
OA4
OA5
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SSc22
SSc23
SSC24
SSC25
SSC26
SSc27
SSC28
SSC29
SSc30
IgG

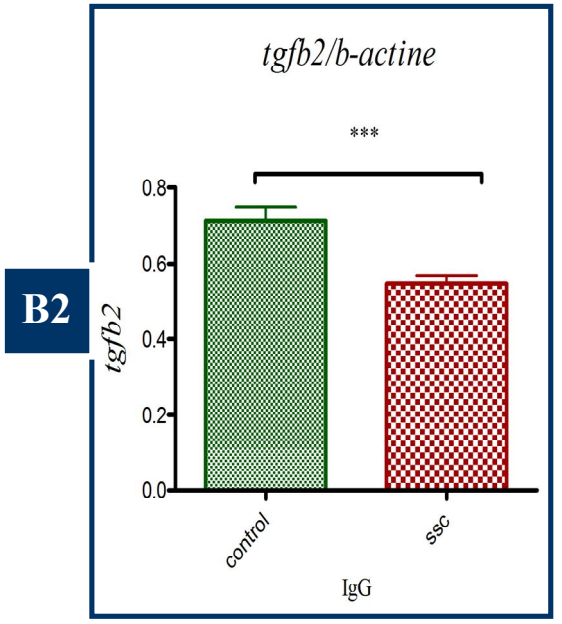
B2
tgfb2/b-actine
***
tgfb2
0.8
0.6
0.4
0.2
0.0
control
ssc
IgG

Figure 11: Downregulation of *tgfb*2 expression by SSc antibodies in VSMCs

(A1, B1) Quiescent VSMCs were left untreated or exposed to buffer (used in IgG purification), PDGF-BB (50ng/ml), TGFβ1 (10ng/ml), control-IgGs (C, OA) or SSC-IgGs for two hours. RT-QPCR was used to detect messenger RNA encoding *tgfb*2. Fold changes were calculated relative to the average of triplicate untreated samples by the comparative CT formula $2^{-\Delta\Delta CT}$.
(A2, B2) unpaired t-test comparing two groups.
*** $p<0.001$, ** $p<0.01$

3.3 Involvement of PDGF receptor in the molecular events noticed in VSMCs in response to SSc-IgGs

To prove that ERK-inducing activity was mediated by the activation of PDGFR, we performed two additional experiments. First, we inquired whether the SSc-IgG could immunoprecipitate PDGF receptor from a whole cellular extract of VSMCs (Figure 12A, page 55). Both control and SSc patients immunoprecipitated the PDGFR; however the SSc significantly immunoprecipitate more PDGFR than control (Figure 12 B2). Only faint PDGFR bands were detected in control treatments, while 75% of the SSc-IgG samples tested immunoprecipitated the PDGFR much more strongly. Second, to further substantiate the role of PDGFR, we used the highly characterized PDGFR tyrosine kinase inhibitor, the tyrphostin AG1296. Dose response assay with AG1296 was performed on VSMCs (Figure 13A, page 57). 5µM is the optimal concentration for AG1296 to inhibit ERK phosphorylation triggered by PDGF since higher concentration may cause unselective inhibition. Surprisingly, the use of AG1296 did not block these SSc-IgG-induced ERK phosphorylations, despite effectively blocking these signals in PDGF-stimulated cells (Figure 13B). Together, the results of the last two experiments suggest that either these properties of SSc-IgGs are not mediated through PDGFR or their effects interfere with PDGFR independently of its kinase activity.

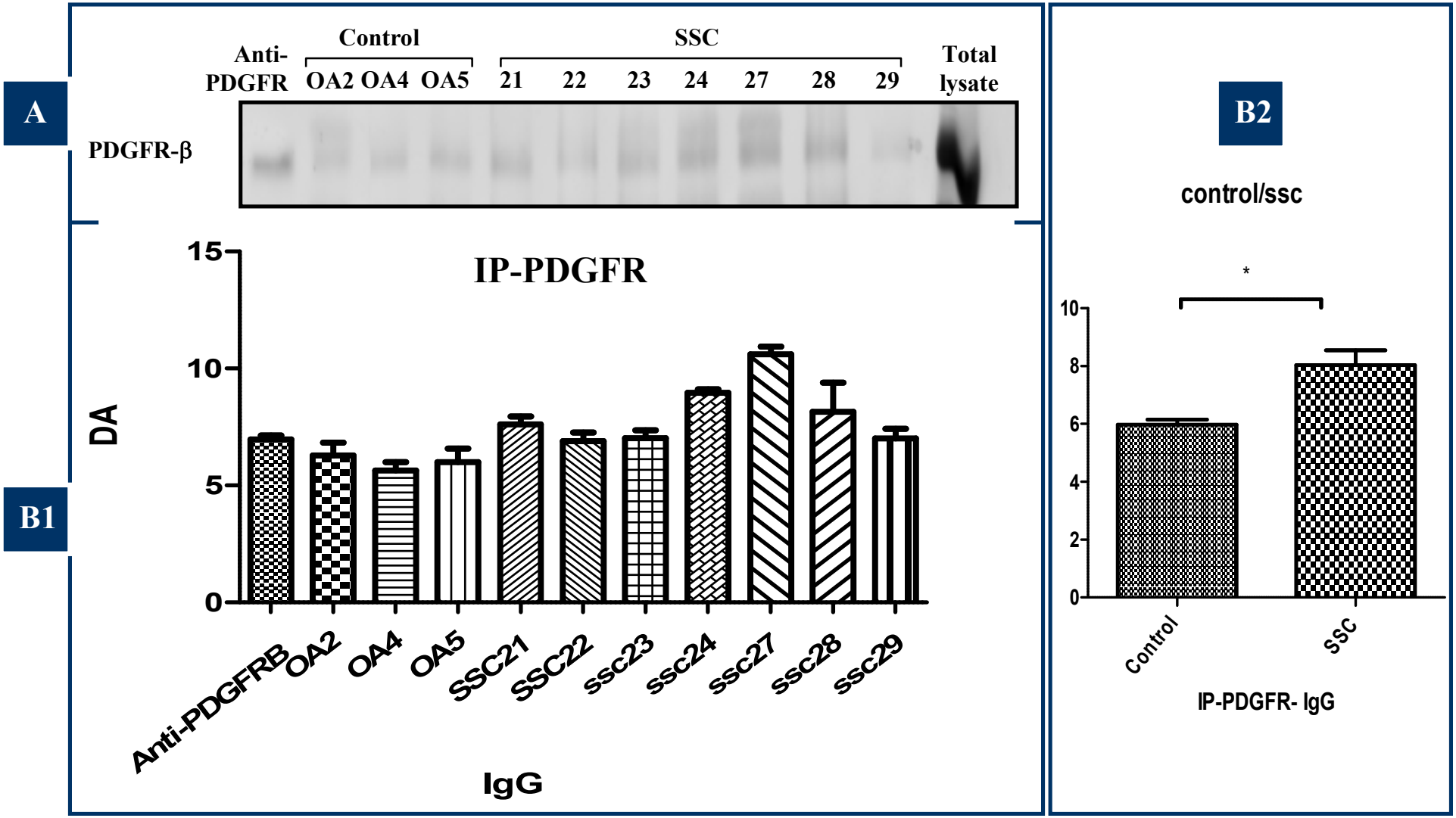
A
Anti-PDGFR
Control
OA2 OA4 OA5
SSC
21 22 23 24 27 28 29
Total lysate
PDGFR-β
B1
IP-PDGFR
DA
15
10
5
0
Anti-PDGFRB
OA2
OA4
OA5
SSC21
SSC22
ssc23
ssc24
ssc27
ssc28
ssc29
IgG
B2
control/ssc
*
10
8
6
4
2
0
Control
SSC
IP-PDGFR- IgG

Figure 12: SSc-IgG pulled down PDGFR more than controls

(A) 500μg of VSMC whole cell extract is subjected to immunoprecipitation using 200μg of Purified IgG from control and SSC patients. The immunoprecipitated protein is then analysed by immunoblot analysis using anti-PDGFRβ antibody. "Total lysate" indicates immunoblots of total protein. Representative results of one of two experiments are shown.
(B1) is the results of densitometric analysis of two independent experiments.
(B2) is the difference between SSc and controls in these experiments.
* $p<0.05$

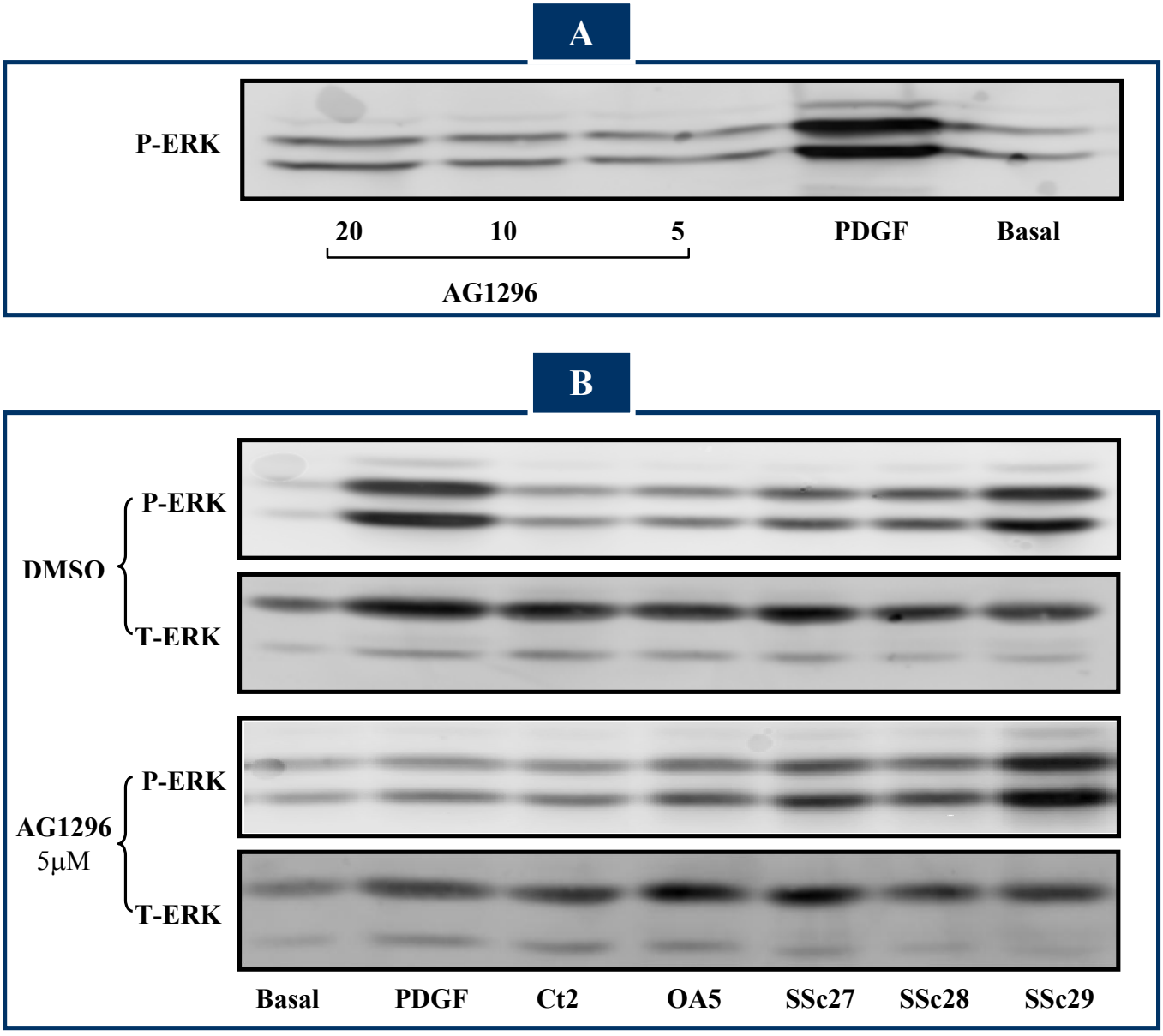

Figure 13: ERK phosphorylation in response to SSc-IgG was independent of the tyrosine kinase phosphorylation of the PDGFR

(A) Cells were pre-exposed to the concentrations indicated of AG1296 30 min before stimulation with PDGF. The ERK phosphorylation was measured by immunoblot analysis. (B) Cells were pre-exposed to 5µM AG 1296 or DMSO 30 min before stimulation with PDGF 50ng/ml or purified IgG from control and SSc patients. Cell extracts were prepared and subjected to immunoblotting analysis using indicated antibodies. One of two independent experiments with similar results is shown.

Chapter 4: Discussion

Our results, in term of ERK activation, indicate that autoAbs from SSc patients were able to phosphorylate ERK in VSMCs more than controls (Figure 6, page 42). However, the SSc-IgG effect was not noticed in all SSc patients. Although the Italian group (Baroni et al) claimed that the stimulatory Abs to the PDGFR were found in all patients with SSc, serological studies in CSRG laboratories showed that anti-PDGFR autoAbs exist in almost 5% of patients tested not all (unpublished observation). In addition, our findings correlate with the fact that SSc is very heterogeneous and that the frequency of SSc autoAbs varies depending on the ethnic and racial groups (Okano, 1996; Harvey, 1999). I.e. in Quebec, frequency of anti-topo I in French Canadian with diffuse SSc is the lowest reported ~13.8% (Scussel-Lonzetti, 2002). Nevertheless, in Italy, the frequency of anti-topo I was over 50% (Giordano, 1986; Ferri, 1991). Thus, whether the discovered autoAbs to VSMCs in this study are the anti-PDGFR autoAbs or not, it is predicted not to find these autoAbs in all SSc patients and they are not a specific hallmark to SSc.

We further investigate the effect of SSc-IgG on genes involved in fibrogenic response (Ihn, 2008). Even though, the TGFβ superfamily has a shared structure and similar signaling pathways, they have overlapped biological effects (Ihn, 2008). It is well known that the most potent profibrotic stimulus to fibroblasts is TGFβ1. In SSc, it is well documented that it activates fibroblasts and stimulates collagen production, but the specific function of the other two isoforms remains unclear because results from various studies are contradictory (Cotton, 1998; Denton, 2001). Among these, immunohistochemical studies concluded that TGFβ 2 and 3 play important roles in the induction of collagen synthesis in SSc, since collagen mRNA was found to be induced in the same areas where their expression was noted (Kulozik, 1990; Querfeld, 1999). Whereas, other studies showed no significant difference in levels of these isoforms' expression in patients with SSc and control (Coker, 2001; Whitfield, 2003). Lately, microarray study on gene expression patterns in SSc skin showed reduced TGFβ2 expression in SSc skin biopsy samples as compared with healthy control skin (Whitfield, 2003). Moreover, studies in the BAL fluid of patients with SSc found that concentrations of TGFβ2 levels are reduced, as compared

with controls (Meloni, 2004). Interestingly, a study on UCD-200 line of chickens, the only spontaneous animal model showing the 3 main hallmarks of SSc, i.e., vascular alterations, mononuclear cell infiltration and fibrosis, demonstrated that TGFβ2 significantly inhibited the expression of procollagen leading to the same levels as seen in controls (Prelog, 2005). In addition, in the same study, the constitutive overproduction of the procollagen mRNA and the diminished synthesis of TGFβ2 in untreated UCD-200 also suggest that TGFβ2 is an antifibrotic cytokine. These results shed light on the contradictory observations regarding the role of TGFβ2 in human SSc and support its role as antifibrotic cytokine. Although TGFβ3 expression and its role in SSc are still less defined, various studies suggest its fibrotic role (Shinozaki, 1997; Querfeld, 1999; Coker, 2001; Sgonc, 2008).

In our study, we analyzed the influence of SSc-IgG on the three TGFβ isoforms' expression. We found autoAbs to VSMCs that are able to induce mRNA level of the *tgfb1* (Figure 9, page 48) and *tgfb3* (Figure 10, page 50), whereas *tgfb2* is significantly reduced (Figure 11, page 52). *Tgfb1* was upregulated by 2-fold ($p<0.001$) in VSMC treated with SSc-IgG, as compared to those treated with control IgG. *Tgfb2* mRNA was downregulated by 20 to 40% ($p<0.001$) in SSc-IgG treated VSMCs. These data give insight, for the first time, to the existence of pathological autoAbs to VSMCs in SSc patients that are able to create imbalance between pro and antifibrotic cytokines in favour of fibrosis. We reported upregulation of *tgfb3* expression in SSc-IgG treated VSMCs more than control-IgG treated ones; however, the difference between these two groups was not significant. Further experiment is suggested to conclusively rule out the SSc-IgG effect on *tgfb3* expression.

Interestingly, some control-IgG induced *tgfb1* and reduced *tgfb2* expressions as well (Figure 9, page 48; Figure 11, page 52). As mentioned earlier, also some controls-IgG induced ERK phosphorylation. Similar observations have been reported in studies on stimulatory PDGFR autoAbs (Balada, 2008; Classen, 2009; Loizos, 2009). The existence of such autoAbs in normal people that possess lower effect on the molecular events noticed with SSc-IgG raised two possibilities.

First, Abs in controls may not recognise the same specific epitope that trigger the receptor in SSc or have a lower affinity to that epitope. In other words, it is not only the presence or absence of these autoAbs but also the location of the epitope recognised by them in their specific receptor. Thus, possibly these autoAbs exist in normal people however they have lower affinity to their receptor to trigger a pathological response. Such phenomenon had been discussed in Graves' disease since thyroid-stimulating hormone receptor (TSHR) has been detected not only in Graves' disease, but also in 55% of euthyroid control subjects (Atger, 1999). In the case of the TSHR, autoAbs directed against most of the regions on the surface of the protein will have no pathologic consequences. Whereas if Abs occur against some discrete domain(s) of the receptor, they will provoke hyperthyroidism or hypothyroidism. Thus, the localization of the epitopes recognized by anti-TSHR Abs distinguishes patients with Graves' disease from euthyroid subjects (Atger, 1999).

The Second possibility is that these autoAbs may even be directed against the same conformational epitope, but their molecular characteristics may differ. This indeed was found by Latrofa et al in Graves' disease as well (Latrofa, 2004). Thus, these autoAbs in healthy subjects may be the precursors of the more potent SSc-associated autoAbs and that an antigen driven process in genetically susceptible individuals leads to affinity maturation and IgG class switching of these naturally occurring autoAbs, which in turn, would become pathogenic and disease-specific when determined by functional assay (Latrofa, 2004). Thereby, the occurrence of pathogenic rather than non-pathogenic Abs in different subjects may be due either to their genetic background or to an encounter with a cross-reactive antigen (Guilbert, 1982; Tron, 1989; Coutinho, 1995).

Noteworthy, AutoAbs from SSc sera induced neither *col1a1* nor *col III* in our study (data not shown). It is possible that SSc-IgG could indirectly induce collagen expression via the imbalance between pro and antifibrotic TGFβ isoforms or via TGFβ1 itself. In addition, it is known that fibroblast activation in SSc may be a result of stimulation by

autocrine TGFβ signaling (Ihn, 2008). Likewise, *tgfβ*1 induced by SSc-IgG could activate autocrine TGFβ signaling in VSMCs and induce collagen deposition.

We next immunoprecipitate PDGFR by IgG from SSc and controls to verify if the autoAbs that we found are the stimulatory anti-PDGFR Abs previously described. Both control and SSc patients immunoprecipitated the PDGFR, but the SSc-IgG significantly immunoprecipitate more PDGFR than control ones (Figure 12, page 55). However, the use of AG1296, a specific inhibitor of the PDGFR kinases did not block these IgG-induced ERK phosphorylations, despite effectively blocking these signals in PDGF-stimulated cells (Figure 13, page 57). Taken together, these findings indicate two possibilities. The first possibilty is that SSc-IgG activity on VSMCs is not mediated through PDGFR. This open the door for lots of other alternative explanations, for example, the SSc-IgG effect in our report could be mediated through the TGFβ receptor itself. It is well documented that in addition to the effects on Smad phosphorylation, TGFβ can signal via Ras and Rac proteins and activate certain MAP kinases including ERK 1 and 2 (Engel, 1999; Park, 2002). One study reported that in this pathway Ras/MEK/ERK potentiates CTGF gene expression in fibroblasts. Thus, the TGFβ induction of Ras/MEK/ERK seems to be profibrotic (Leask, 3003). Although, SSc-IgG effect in our study could be mediated through this signaling pathway, there are lots of other receptor in literature could explain the SSc-IgG effects noticed in our study. Thus the use of modern and more comprehensive mass sperctrometry analysis (ms/ms) to identify the interactions between SSc-IgG and their specific receptor will be a more convenient and time saving method than approaching pharmacologically each of the possible receptors that may be involved.

The second possibility is that SSc-IgG effects are mediated through PDGFR; however, this effect is independent of the tyrosine kinase phosphorylation of the PDGFR. Similar apparently tyrosine kinase-independent activities by PDGF have been reported. A study demonstrated that erg-1 by PDGF occurs in the absence of tyrosine kinase activity. While another found that, the inhibition of tyrosine kinase activity does not affect the increase in GaG chain elongation that is induced by PDGF. Such results suggest the

existence of PDGF signaling pathways that operate independently from PDGFR tyrosine kinase (Mundschau, 1994; Schonherr, 1997). Thus, in our study, PDGFR knockdown experiment is eagerly awaited in order to conclusively verify the involvement of the PDGFR in the effects of SSc antibodies.

4.1 PDGFR stimulatory autoAbs in SSc: lessons learned and outcomes

As mentioned earlier, autoAbs against PDGFRs were first reported by Baroni et al (Baroni, 2006). They demonstrated that sera from patients with SSc stimulates PDGFR-positive fibroblasts, but not PDGFR-negative cells, to produce ROS and to activate Ha-Ras ERK1/2 signaling in normal fibroblasts, resulting in the expression of collagen and α-smooth muscle actin. They also reported that sera IgG from patients with SLE or healthy control individuals did not stimulate fibroblasts to produce ROS. This study has not been replicated and many recent reports could not show the existence of these stimulatory anti-PDGFR Abs. Among these, the group of Classen et al, they purified immunoglobulins from 37 SSc patients and tested PDGFR activation by these Abs using four different bioassays, i.e., cell proliferation, ROS production, signal transduction, and receptor phosphorylation. They showed that IgGs from patients with SSc had little effect on PDGFR signaling, including the proliferation of fibroblasts and PDGFRα phosphorylation (Classen, 2009). Whereas, another group reported that purified IgG from patients with SSc exhibited no agonist activity in a cell-based PDGFRα phosphorylation assay, and did not stimulate a mitogenic response or MAPK activation in a PDGFRα-expressing cell line (Loizos, 2009). Moreover, the same study using electrochemiluminescence binding assay showed that sera samples from normal individuals and SSc patients could bind to PDGFRs (Loizos, 2009). Similarly, using non-bioactive assays in Balada et al study anti-PDGFRα Abs were detected in sera from normal controls and SSc patients as well (Balada, 2008). Furthermore, two recent studies reported that these anti-PDGFR Abs are not specific to SSc since they have been found in extensive chronic graft-versus-host disease (ecGVHD), a disease with SSc-

like clinical manifestation (Svegliati, 2007) and in patients with SLE (Kurasawa, 2010). The findings arising from these studies raise the question as whether these agonistic autoAbs to PDGFR in SSc exist or whether experimental variations play a role in these variant results.

There are possible scenarios highlighted by Baroni et al group to clarify variations between their study and the above ones. First, they explained that the IgG purification process should involve size-exclusion chromatography, which removes small molecules. This step is essential to remove trace amounts of contaminating cytokines such as PDGF or TGFβ. Absence of this step may lead to misleading results (Classen, 2009; Gabrielli, 2009; Loizos, 2009). Second, the use of inconvenient cell lines that have low levels of cell surface PDGFR. Such cells are perfectly suitable for the detection of PDGF biological activity, but they may be not able to respond to low-affinity IgG due to competition by the Fc receptor (Classen, 2009; Gabrielli, 2009; Loizos, 2009). Third, the epitope recognized by these autoAbs is a conformational structure, recognized only in its native configuration. Difference in the PDGFR conformation in experiments could account for the differences in the results of studies.

However, these explanations are unlikely in our study because the IgG purification steps involved a concentrating additional step to exclude contaminants. In addition, IgG purification process was done according to protocol provided from Baroni et al group to overcome the IgG sensitivity and instability described by them (unpublished observations). Although, we used VSMCs, not fibroblast, these cells are responsive to PDGF and express enormous amount of PDGFR that will reduce the chance for competition by Fc receptor (Lindqvist, 2001). Moreover, VSMCs were stayed in serum free media for two days to ensure quiescence. RTKs maintain in an inactive configuration in quiescent cells, which is suitable for these Abs binding (Schlessinger, 2000; Blume-Jensen, 2001; Schlessinger, 2003).

In spite of that, so far we have been unable to prove that effects noticed in VSMCs in this study are mediated by PDGFR. Indeed, we also found autoAbs in some healthy control as well, and these autoAbs seem to be present in most, but not all, SSc patients. The autoAbs in this study are not a specific hallmark for SSc; however, they have a critical role in the fibrotic process via affecting TGFβ gene expressions.

4.2 Conclusion and Perspectives

In conclusion, we have identified, for the first time, autoAbs to VSMCs in patient with SSc, which provide a novel link between B cell mediated antibody production, vascular alteration and fibrosis in SSc. They appear to upregulate the profibrotic *tgfb1* gene, and downregulate the antifibrotic *tgfb2* gene, creating imbalance between fibrotic genes in favour of fibrosis. Although they do bind to the PDGFR of VSMCs, and although ERK phosphorylation is upregulated by the SSc-IgG, we could not prove that these effects are mediated by the PDGFR. Thus, the next step will be to clarify the involvement of the PDGFR by PDGFR knockdown experiments and/or pharmacological approaches to identify the SSc-Abs bound receptor. This step will explain part of the cause of vasculopathy in SSc and open the door for new therapeutic targets in treating the disease. Moreover, the existence of these antibodies should be replicated in larger number of patients and from other population.

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