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The role of Microsomal prostaglandin synthase-1 (mPGES-1) and Ephrin B2 in Scleroderma

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The role of Microsomal prostaglandin synthase-1 (mPGES-1) and Ephrin B2 in Scleroderma

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RÉSUMÉ

La sclérodermie (sclérose systémique, ScS) est une maladie auto-immune du tissu conjonctif caractérisée par l'épaississement de la peau, l'apparition spontanée de lésions cicatricielles, des maladies des vaisseaux sanguins, divers degrés d'inflammation, en association avec un système immunitaire hyperactif. La pathogénèse exacte de cette maladie est inconnue et aucun traitement approprié n'est disponible. La fibrose est un élément distinctif de la maladie de ScS et est considérée résulter d'une incapacité à mettre fin de façon appropriée à la réponse normale de réparation des plaies. L'analyse histologique du stade initial de la ScS révèle une infiltration périvasculaire de cellules mononucléaires dans le derme, associée à une synthèse accrue de collagène dans les fibroblastes environnants. Ainsi, la compréhension des moyens de contrôler le stade inflammatoire de la ScS pourrait être bénéfique pour contrôler la progression de la maladie peu après son apparition. La mPGES-1 est une enzyme inductible qui agit en aval de la cyclo-oxygénase (COX) pour catalyser spécifiquement la conversion de la prostaglandine (PG) H2 en PGE2. La mPGES-1 joue un rôle clé dans l'inflammation, la douleur et l'arthrite; toutefois, le rôle de la mPGES-1 dans les mécanismes de fibrose, spécifiquement en rapport avec la ScS humaine, est inconnu. Mon laboratoire a précédemment montré que les souris à mPGES-1 nulle sont résistantes à la fibrose cutanée induite par la bléomycine, à l'inflammation, à l'épaississement cutané, à la production de collagène et à la formation de myofibroblastes. Sur la base de ces résultats, j'ai formulé l'hypothèse que l'inhibition pharmacologique de la mPGES-1 régulera à la baisse la production de médiateurs pro-inflammatoires et pro-fibreux au cours de la maladie de ScS. Afin d'explorer le rôle de la mPGES-1 dans l'inflammation et la fibrose associées à la maladie de ScS, j'ai d'abord examiné l'expression de la mPGES-1 dans la peau normale comparativement à des biopsies de peau extraites de patients atteints de ScS. Mes résultats ont montré que la mPGES-1 est nettement élevée dans la peau de patients atteints de ScS en comparaison avec la peau humaine normale. De plus, les niveaux de PGE2 dérivés de la mPGES-1 étaient également significativement plus élevés dans les fibroblastes cutanés isolés de patients atteints de ScS comparativement aux fibroblastes isolés de témoins sains. J'ai également étudié l'effet de l'inhibition pharmacologique de la mPGES-1 sur l'expression de marqueurs pro-fibreux. Mes études ont montré que l'expression de médiateurs pro-fibreux clés (α-SMA, endothéline-1, collagène de type 1 et facteur de croissance du tissu conjonctif (FCTC)) est élevée dans les fibroblastes cutanés ScS en comparaison avec les fibroblastes cutanés normaux. Un traitement avec un inhibiteur de la mPGES-1 a eu pour effet de réduire significativement l'expression de l'α-SMA, de l'endothéline-1, du collagène de type 1 mais pas du FCTC dans les fibroblastes ScS, sans effet significatif sur les fibroblastes normaux. J'ai en outre examiné l'effet de l'inhibition de la mPGES-1 sur des cytokines pro-inflammatoires clés impliquées dans la pathologie de la ScS, incluant IL-6, IL-8 et MCP-1. L'inhibition pharmacologique de la mPGES-1 a eu pour effet de réduire significativement les niveaux de production de cytokines pro-inflammatoires IL6, IL8 et MCP-1 dans les fibroblastes avec lésion ScS comparativement à des fibroblastes non traités. De plus, les patients atteints de ScS ont présenté des niveaux plus élevés de p-AKT, de p-FAK et de p-SMAD3 en comparaison avec les fibroblastes cutanés normaux. L'inhibiteur de la mPGES-1 a pu réguler à la baisse cette expression accrue de p-AKT et de p-FAK, mais pas de p-SMAD3, dans les fibroblastes ScS. Ces résultats ont suggéré que l'inhibition de la mPGES-1 pourrait être une méthode viable pour réduire le développement de sclérose cutanée et constituent une cible thérapeutique potentielle pour contrôler les mécanismes fibreux et inflammatoires associés à la pathophysiologie de la maladie de ScS.

L'un des autres processus critiques reliés à l'évolution de la réponse fibreuse associée à la maladie de ScS est la différenciation des fibroblastes en des cellules activées spécialisées

appelées myofibroblastes, responsables de déclencher une signalisation adhésive excessive et le dépôt excessif de matrice extracellulaire, conduisant à la destruction de l'architecture de l'organe. Ainsi, l'identification des facteurs endogènes qui initient/ favorisent la différenciation fibroblaste-myofibroblaste peut mener à des stratégies thérapeutiques prometteuses pour contrôler l'excès de signalisation adhésive et de fibrose associé à la maladie de ScS. Des études antérieures dans le domaine de la biologie du cancer ont suggéré que l'éphrine B2, une protéine transmembranaire appartenant à la famille des éphrines, est impliquée dans la signalisation adhésive et le remodelage extracellulaire. Cependant, son rôle dans la fibrose n'a jamais été exploré. Dans la deuxième partie de mon étude, j'ai donc étudié le rôle de l'éphrine B2 dans la fibrose. Mes études montrent que l'expression de l'éphrine B2 est significativement augmentée dans la peau humaine ScS comparativement à la peau normale. Plus important encore, le traitement *in vitro* de fibroblastes de la peau humaine normale avec de l'éphrine B2 recombinante est capable de transformer des fibroblastes en cellules myofibroblastiques manifestant toutes les caractéristiques myofibroblastiques typiques, incluant la formation accrue de fibres de tension, des adhérences focales, l'activation accrue de la FAK, un accroissement de l'expression et de la migration de fibroblastes et de leur adhérence à la fibronectine à la fois chez les fibroblastes cutanés normaux et ScS. En outre, j'ai traité des souris avec de l'éphrine B2 recombinante et montré que ces souris ont développé une fibrose cutanée significative associée à une épaisseur dermique et à une synthèse de collagène augmentées, une teneur en hydroxyproline (teneur en collagène) accrue et un nombre accru de myofibroblastes exprimant de l'α-SMA, une activation augmentée de la FAK et de marqueurs pro-fibreux incluant le collagène de type 1 et le FCTC.

Dans l'ensemble, mes études ont identifié deux médiateurs endogènes cruciaux impliqués dans la propagation de l'inflammation et de la fibrose associées à la maladie de ScS. L'inhibition de la mPGES-1 pourrait représenter une bonne stratégie alternative pour contrer l'inflammation et la fibrose au moins durant les stades précoces de la maladie de ScS. De plus, une signalisation excessive de l'éphrine B2 favorise la signalisation adhésive et fibreuse en déclenchant la différenciation de fibroblastes en myofibroblastes par l'activation de la voie de signalisation de la FAK. Ainsi, l'inhibition d'éphrine B2 bloquera la formation de fibroblastes-myofibroblastes et régulera à la baisse la fibrose associée à la maladie de ScS. En somme, la mPGES-1 et l'éphrine B2 semblent toutes deux des cibles attrayantes pour le traitement de la ScS et des troubles fibreux qui y sont reliés.

Mots-clés. Sclérose systémique, Microsomal prostaglandin synthase-1 (mPGES-1), Fibroblaste, Myofibroblaste, Éphrine B2, Éphrine B4.

SUMMARY

Scleroderma (Systemic sclerosis, SSc) is an autoimmune disease of the connective tissue featuring skin thickening, spontaneous scarring, and blood vessel disease, varying degrees of inflammation, associated with an overactive immune system. The exact pathogenesis of this disease is unknown and there is no appropriate treatment available. Fibrosis is a hallmark of SSc disease and is considered to arise due to an inability to appropriately terminate the normal wound repair response. Histological analysis of the initial stage of SSc reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts. Thus understanding how to control the inflammatory stage of SSc may be of benefit in controlling the progression of early onset disease. mPGES-1 is an inducible enzyme that acts downstream of cyclooxygenase (COX) to specifically catalyze the conversion of prostaglandin (PG) H2 to PGE2. mPGES-1 plays a key role in inflammation, pain and arthritis; however, the role of mPGES-1 in fibrotic mechanisms especially with respect to human SSc is unknown. My laboratory has previously shown that mPGES-1-null mice are resistant to bleomycin-induced skin fibrosis, inflammation, cutaneous thickening, collagen production and myofibroblast formation. Based on these results I hypothesized that pharmacological inhibition of mPGES-1 will downregulate the production of pro-inflammatory and pro-fibrotic mediators during SSc disease. To explore the role of mPGES-1 in inflammation and fibrosis associated with SSc disease, I first investigated the expression of mPGES-1 in normal skin compared to skin biopsies extracted from SSc patients. My results showed that mPGES-1 is markedly elevated in SSc skin compared to normal human skin. In addition, the levels of mPGES-1-derived PGE2 were also significantly higher in skin fibroblasts isolated from SSc patients compared to fibroblasts isolated from healthy controls. I further investigated the effect of pharmacological inhibition of mPGES-1 on the expression of pro-fibrotic markers. My studies showed the expression of key pro-fibrotic mediators (α-SMA, endothelin-1, collagen type 1 and connective tissue growth factor) are elevated in SSc skin fibroblasts compared to normal skin fibroblasts. Treatment with mPGES-1 inhibitor resulted in significant reduction in the expression of α-SMA, endothelin-1, collagen type 1 but not CTGF in SSc and normal fibroblasts. Further, I investigated the effect of mPGES-1 inhibition on key pro-inflammatory cytokines implicated in SSc pathology including IL-6, IL-8 and MCP-1. Pharmacological inhibition of mPGES-1 resulted in significant reduction in the production levels of pro-inflammatory cytokines, IL6, IL8 and MCP-1 in SSc-lesioned fibroblasts compared to untreated fibroblasts. In addition, SSc patients exhibited higher levels of p-AKT, p-FAK and p-SMAD3 compared to normal skin fibroblasts. mPGES-1 inhibitor was able to down regulate this increased expression of p-AKT, p-FAK but not p-SMAD3 in SSc fibroblasts. These results suggested that inhibition of mPGES-1 may be a viable method to alleviate the development of cutaneous sclerosis and is a potential therapeutic target to control fibrotic and inflammatory mechanisms associated with the pathophysiology of SSc disease.

One of the other critical processes associated with the evolution of fibrotic response associated with SSc disease is the differentiation of fibroblasts into specialized activated cells called myofibroblasts responsible for triggering excessive adhesive signaling and deposition of excessive extracellular matrix (ECM) leading to the destruction of organ architecture. Thus identifying endogenous factors which initiate/promote fibroblast-myofibroblast differentiation can lead to promising therapeutic strategies to control excessive adhesive signaling and fibrosis associated with SSc disease. Previous studies in cancer biology have suggested that ephrin B2, a transmembrane protein belonging to the family of ephrins, is involved in adhesive signaling and extracellular remodeling. However its role in fibrosis has never been explored. Therefore, in second part of my study, I investigated the role of ephrin B2 in fibrosis. My studies show ephrin

B2 expression is significantly enhanced in human SSc skin versus normal skin**.** Most importantly, *in vitro* treatment of normal human skin fibroblasts with recombinant ephrin B2 is able to transform fibroblasts into myofibroblastic cells exhibiting all typical myofibroblastic-characteristics including increased stress fibre formation, focal adhesions, increased activation of FAK, increased expression of and enhanced fibroblast migration and adhesion to fibronectin in both normal and SSc skin fibroblasts. Further, I treated mice with recombinant ephrin B2 and showed that these mice developed significant skin fibrosis associated with enhanced dermal thickness and collagen synthesis**,** increased hydroxyproline content (collagen content) and increased number of α-SMA-expressing myofibroblasts, enhanced activation of FAK and pro-fibrotic markers including type-I collagen and CTGF.

Overall, my studies have identified two crucial endogenous mediators involved in propagating inflammation and fibrosis associated with SSc disease. mPGES-1 inhibition may present a good alternative strategy to counteract inflammation and fibrosis at least during early stages of SSc disease. Further, excessive ephrin B2 signaling promotes adhesive and fibrotic signaling by triggering fibroblast to myofibroblast differentiation via activation of the FAK signaling pathway. Thus, inhibition of ephrin B2 will block fibroblast-myofibroblast formation and downregulate fibrosis associated with SSc disease. Overall, both mPGES-1 and ephrin B2 seems to be attractive targets for treatment of SSc and related fibrotic disorders.

Keywords. Systemic sclerosis, Microsomal prostaglandin synthase-1 (mPGES-1), Fibroblast, Myofibroblast, Ephrin B2, Ephrin B4.

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Manuscript #1 attached: Pharmacological Inhibition of mPGES-1 downregulates the expression of pro-fibrotic markers and the production of pro-inflammatory cytokines.

Ephrin B2 and Scleroderma

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LIST OF ABBREVIATIONS

bFGF: Basic fibroblast growth factor

BSA: Bovine serum albumin

CR- CHUM: University of Montreal Hospital Research Center

CTGF: Connective tissue growth factor

COX: Cyclooxygenase

DAB: Diaminobenzidine

dcSSc: Diffused cutaneous systemic sclerosis

DMEM: Dulbecco's Modified Eagle Medium

ECM: Extracellular Matrix

ET-1: Endothelin -1

Eph B2: Ephrin B2

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

IHC: Immunohistochemistry

IL: Interleukin**lcSSc**: Limited cutaneous systemic sclerosis

MCP: Monocyte chemotactic protein-1

MMP: Matrix Metalloproteinase

mPGES : Microsomal Prostaglandin synthase

PCR: Polymerase Chain Reaction

PGE2: Prostaglandin E2

PGH2: Prostaglandin H2

PDGF: Platelet-derived growth factor

SMA: Smooth muscle actin

SSc: Systemic sclerosis

TBS: Tris Buffered Saline

TGF: Transforming growth factor

TNF: Tumor Necrosis Factor

SDS: Sodium dodecyl sulfate

VEGF: Vascular Endothelial Growth Factor

WT: Wild Type

DEDICATION

This thesis is dedicated to my parents who have supported me all the way since the beginning of my studies.

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INTRODUCTION

SCLERODERMA (SYSTEMIC SCLEROSIS)

Scleroderma (Systemic sclerosis, SSc) is a fibroproliferative disorder associated with the production and accumulation of excessive fibrous connective tissue [1]. Primary features of SSc disease include autoimmunity, inflammation, obliterative vasculopathy and fibrosis. SSc does not just affect single organ and it spreads in large area of the skin and one or more internal organs such as kidneys, esophagus, heart, and lungs[2].

SSc has a global distribution. More women suffer from the disease than men[3] . The factors behind this gender disposition have not yet been elucidated and the overall incidence rate of SSc among adults in America is in the region of 20 for every 1 million persons annually. Statistics indicate that there was an increase in this rate between 1943 and 1973 but since then the rate has remained more or less constant. It is also reported that the prevalence rate of SSc among adults in America has remained more or less constant at 240 per million[4] . The frequency of occurrence of SSc in America is higher than that seen in UK, Asia, and continental Europe. There are between 75,000 and 100,000 people suffering from SSc in U.S. There is also a racial factor in the incidence and prevalence of SSc. The incidence of SSc is much higher in black women than in white women [4]

SSc is a leading cause of morbidity and case-specific mortality amongst all autoimmune rheumatic illnesses. Majority of the morbidity and mortality associated with SSc disease arises due to the development of complications that include gastrointestinal, renal, or cardiopulmonary diseases. Common organ involvement and manifestations in SSc disease include skin lesions,

gastrointestinal manifestations, cardiac involvement, pulmonary arterial hypertension (PAH), interstitial pulmonary fibrosis, and renal disease. Skin tightening and subcutaneous thickness may be the initial complaint that causes patients to seek for help. Generalized pruritus and cutaneous vasculitis are two more common cutaneous presentations where an underlying systemic disease may be present and will influence management[5]. Although skin manifestations are one of the most important components of clinical diagnosis and classification, studies have shown that life-threatening complications are independent from skin fibrosis [6]. Gastrointestinal manifestations represent the most common organ complications in patients suffering from SSc [7]. It is estimated that close to 90% of all patients with SSc manifest some form of gastrointestinal involvement with the most common of these being gastro-esophageal manifestations [8]. However, most of the gastrointestinal manifestations in patients with SSc disease are non-life threatening. Patients who have established SSc usually present with serious small intestine involvement causing dilation of small bowel loops leading to frequent bouts of intestinal pseudo-obstruction. Overgrowth of bacteria in the small intestine can then lead to reduced motility causing bloating, diarrhea, weight loss, cachexia, and malnutrition[7].

Involvement of the cardiac system is also one of the key determinants of mortality in patients with SSc which is largely seen in patients with the diffused cutaneous SSc form of systemic sclerosis[1] . It is however difficult to establish the precise percentage of cardiac involvement in patients with SSc due to diagnostic limitations[9]. Estimates suggest that the percentage of patients with SSc who have pericardial effusions is 35%. Involvement of the myocardium in patients with SSc has been attributed to fibrosis, ischemia, and myocarditis[1]. PAH is yet another organ complication that may occur in patients with SSc. PAH refers to elevated mean pulmonary artery pressure exceeding 25 mmHg when a person is resting, the right heart is catheterized and the pulmonary capillary wedge pressure (PCWP) is normal. This

complication occurs in both the limited systemic sclerosis (lcSSc) and the diffuse cutaneous sclerosis (dcSSc) forms of SSc and is a major cause of death in patients with SSc [10].

Interstitial pulmonary fibrosis has also been reported in patients with systemic fibrosis. The main types of interstitial lung disease observed in patients with SSc are the non-specific interstitial pneumonia (NSIP) and the usual interstitial pneumonia (UIP). Development of interstitial lung disease in people with SSc occurs insidiously and usually culminates in irreversible fibrosis of the lung. Reduced lung function is witnessed in only 15% of patients with SSc and this reduction usually occurs during the initial 4 years of disease [10] . The main types of renal manifestations that afflict patients with SSc are inflammatory renal pathology, chronic kidney disease, and scleroderma renal crisis (SRC). The most significant renal complication in SSc is SRC and it is seen in 10-15% of patients who have dcSSc. It is however very uncommon in patients with lcSSc as only 1-2% get to have SRC [11]. There is a high mortality of patients with SSc due to SRC [12]. Some evidence suggests that SRC may be triggered by intake of corticosteroids [13].

Subtypes of SSc disease

Systemic sclerosis is divided into specific mutually exclusive subsets because of their variable prognostic and diagnostic characteristics. The 2 main subsets of SSc are the limited systemic sclerosis (lcSSc) and the diffuse cutaneous sclerosis (dcSSc). The 2 subtypes are distinguished by the degree and scope of skin thickening and susceptibility to visceral involvement[14]. The autoantibody profile is also used to distinguish between the limited and diffuse disease forms[15] .

Limited Systemic Sclerosis (LcSSc)

In the lcSSc form of SSc disease, fibrosis is largely limited to the distal portions of the elbows or knees[16] . Skin involvement may also be witnessed in the face. Progress of fibrosis is usually

slow. Patients with this form of disease have a somewhat smaller risk of developing serious involvement of the interstitial lung [17].

Table 1: Differences between Limited cutaneous sclerosis (lcSSc) and Diffuse cutaneous sclerosis (dcSSc) [9]

Limited cutaneous sclerosis (lcSSc)	Diffuse cutaneous sclerosis (dcSSc)
Fibrosis limited to the distal portions of the elbows or knees	Fibrosis limited to the proximal portions of the trunk and extremities
Heart involvement is minimal	Heart involvement is severe in 10% of patients
Interstitial lung disease is severe in 15% of patients	Interstitial lung disease is severe in 15% of patients
PAH is seen in 10-15% of patients	PAH is seen in 5-10% of patients
Minimal kidney involvement	Kidney involvement is severe in 10-15% of patients
Concurrent primary biliary cirrhosis in 6-8% of patients	Large joint contractures
The overall survival rate is better than that of dcSSc	

Diffuse cutaneous sclerosis (dcSSc)

In the dcSSc form of SSc, fibrosis is largely limited to the proximal portions of the trunk and extremities [16]. Patients with dcSSc stand a higher risk of getting serious heart and kidney involvement than patients with the lcSSc form of disease [18] . Table 1 above depicts the primary distinguishing features between the 2 forms of SSc.

The main disadvantage of this subset classification method is that patients who have the early disease but show no visceral involvement and having or lacking minimal skin thickening fit nowhere in this scheme. To address this shortcoming, a new scheme for classifying subsets of SSc was proposed. In this scheme, it is possible to classify patients with early disease based on particular autoantibodies, changes in the nail fold capillary, and Reynaud's phenomenon. (An exaggeration of vasomotor responses to cold or emotional stress causes skin discoloration).

They can be grouped in the limited form of systemic sclerosis also termed as pre-scleroderma [16] . However, validation of the proposed scheme is yet to be done [15] .

Barnett et al [19] and Ferri et al [20] describe yet another scheme that can be used to subtype systemic sclerosis. In this scheme, 3 subtypes of systemic sclerosis have been described and these include limited cutaneous sclerosis, intermediate cutaneous sclerosis, and diffuse cutaneous sclerosis. In this scheme, the main feature of the limited cutaneous sclerosis subset is that the thickening of the skin is limited to digits and facial involvement may be present or absent. In intermediate cutaneous sclerosis, there is skin involvement in the limbs while in the diffuse cutaneous sclerosis; there is skin involvement in the trunk. This method of classifying subsets of SSc is better because its discrimination power is higher than that based on only 2 subsets [21]. Methods that only encompass skin changes and fail to factor in autoantibodies and imaging characteristics are inadequate and can hardly be used for the optimal classification of subsets of SSc. Classification of SSc can also be done based on the Preliminary Criteria for the Classification of SSc which was formulated by The American College of Rheumatology (ACR). This is a diagnostic criteria which has however been shown to be unsuitable for the diagnosis of SSc [10].

Symptoms

Reynaud's phenomenon is one of the earliest symptoms observed in SSc. Reynaud's phenomenon is a condition in which the toes and fingers undergo vasospasm due to cold. It occurs periodically, is reversible and manifests long before other signs of disease are observed [17]. In Reynaud's phenomenon, the toes, fingers and other extremities become discolored and may cause acral ulcer upon persistence [22] . Other common symptoms of SSc are telangiectases or obviously dilated blood vessels, sclerodactyly, vasculitis, and calcinosis in the hands, fingers and bony regions. Calcinosis refers to deposits of calcium in these areas[23].

Other symptoms include ulceration which may result in dry gangrene and fingertip loss, thickening of the skin, changes in the nail fold capillaries, SRC, malignant hypertension, PAH, and gastric antral vascular ectasia [3] . Symptoms are also dependent on the type and extent of organ involvement. Patients with renal involvement and specifically SRC may show non-specific symptoms such as fever, headache, dyspnea, and malaise[3]. Acute renal failure, pulmonary edema, hypertensive retinopathy and encephalopathy may be symptomatic in patients with end-organ damage. Coagulopathy is an uncommon symptom but thrombocytopenia and microangiopathic hemolytic anemia are common, occurring in 50% and 60% of patients respectively[23] .

Patients with SRC and SSc also manifest reduced significant systemic hypertension and reduced renal function [11] . Dry cough, dyspnea, and rare hemoptysis and chest pain are the main symptoms in patients with SSc who have interstitial pulmonary fibrosis. Pulmonary arterial hypertension (PAH) can be asymptomatic until it becomes very advanced. A common symptom is dyspnea while less common symptoms are syncope and chest pain. Symptoms of patients with gastrointestinal involvement include bloating, diarrhea, and weight loss, cachexia, malabsorption,

and malnutrition in severe cases. Classic symptoms are bowel pattern changes accompanied with abdominal distension and regular loose, floating and foul-smelling stools [7].

Etiology

SSc disease is highly heterogeneous [17] and the exact etiology of this debilitating disease is largely unknown [15] . Possible causative agents of SSc include viruses such as cytomegalovirus (CMV), exposure to silica, vinyl chloride, and organic solvents, and drugs [24] . In particular, Namboodiri et al [25] and Lunardi et al [26] have detected antibodies against CMV in patients with SSc. These antibodies do not only enhance endothelial cell apoptosis but also activate fibroblasts in cell culture assays, implying that they play a direct role in the damage of tissues in SSc. In addition, human CMV infection also causes an increase in the production of the connective tissue growth factor (CTGF) which is associated with the activation of fibroblasts and has been shown to play a role in pathological fibrosis[27].

Possible Genetic Association

There is a genetic association with SSc. SSc is inherited but not in a Mendelian fashion. There is a low disease concordance rate that does not exceed 5% among both dizygous and monozygous twins. The disease is seen more in families and less in the general population. Among families, the rate of the disease is 1.6% and this far exceeds the rate among the general population which is just 0.026% [17] . A positive family history of SSc is the biggest risk factor for the disease [28]. It has been observed that there are geographic clusters of the disease. Such clusters of SSc include the Chocktaw Native American Cluster and Italy and London clusters. The former cluster suggests that the disease is caused by yet to be identified genetic factors. Familial clustering has also been reported in Australia and the United States[4] .

Gender and the major histo-compatibilty complex (MHC) are also important genetic factors associated with SSc. The female to male ratio of SSc is 3:6.1 while people with systemic

sclerosis have a higher frequency of class I and class II MHC alleles. It has been shown that SSc is primarily associated with the linkage of DRw52 with DR5 and DR3 and that lung fibrosis is largely associated with B8-DR3-DRw52-DQB2. Presence of Antitopoisomerase (ATA) and DR52a can be used to predict pulmonary disease. Black women are more prone to the disease than white women [1].

Prognosis

The prognosis of SSc is variable and this is largely due to the variability of the disease spectrum [29]. Prognosis is improved by optimal and early treatment. There is no effective cure for SSc and people with dcSSc have a higher risk of mortality. The survival rate of patients with this form of disease is 55% at 10 years [30]. Varga et al [2] assert that better management of systemic sclerosis has led to an improvement in clinical outcomes. However, a cure for the disease has not yet been found and dcSSc has a higher fatality risk than the lcSSc form of disease [23].

The leading cause of death in SSc is pulmonary disease. Renal and cardiac diseases are also associated with poor prognoses. Whereas morbidity due to gastrointestinal disease has been noted, it is not easy to quantify the degree to which this occurs. There has been an improvement in the overall survival of people with SSc over the past few decades. The mean survival from diagnosis is estimated at 12 years. The prognosis also varies depending on the type and extent of organ involvement [30]. Patients with SSc and PAH have a median survival of between 1 and 3 years [31]. The mean survival for patients with SSc and severe pulmonary fibrosis is less than 3 years [31].

Pathogenesis

Even though the pathogenesis of SSc is complex and heterogeneous, the large number of studies carried out in the recent past has helped to shed more light on the pathophysiological events associated with this condition. It is characterized by sequence of events that are common among all the subsets of the disease. During pathogenesis of SSc, microvascular change is followed by inflammation and immune activation which eventually leads to fibrosis[32] .

Vascular injury caused by physical trauma, autoantibodies, viruses, and oxidative stress leads to activation of endothelial cells, leukocyte adhesion, vascular obliteration, and tissue hypoxia. These events trigger inflammation and autoimmunity resulting in production of growth factors and cytokines which cause activation of fibroblasts causing fibrosis [23] .

Microvascular Changes

The first events to occur during pathogenesis of SSc involve vascular injury. Vascular injury can be caused by autoantibodies that are cell-specific, physical trauma, granzymes, reactive oxygen species (ROS) that are generated as a result of ischemia and reperfusion, inflammatory cytokines, and vasculotropic viruses. Vascular injury is manifested by changes in the nail fold capillaries, cutaneous telangiectasia, malignant hypertension, PAH, and gastric antral vascular ectasia [3] . Vascular injury leads to the activation of endothelial cells and renders them dysfunctional as well [33] . It also results in changes in the permeability of capillaries, modified secretion of vasoactive mediators, and enhanced expression of the endothelial leukocyte adhesion molecule1 and VCAM-1[34] . Vascular injury also causes fibrinolytic and platelet pathways to become activated [35].

Once activated, the endothelial cells release endothelin-1 (ET-1). Endothelin-1 (ET-1) is a powerful vasoconstrictor, which also activates fibroblasts, enhances the proliferation of smooth

muscle cells, and induces the adhesion of leukocytes to the endothelium. Due to the vascular injury, vascular remodeling occurs and hypertrophy of the medial and intimal layers is seen together with adventitial fibrosis to result in the gradual narrowing of the lumen and its eventual elimination[36] . Apoptosis of the endothelial cells combines with the preceding processes to lead to cause the gradual loss of blood vessels and disruption of angiogenesis. Impaired formation of blood vessels has been attributed to the impaired differentiation and reduction of the CD34+ cells originating from the bone marrow [37, 38]. Consequently, hypoxia occurs and causes a significant increase in the expression of the vascular endothelial growth factor (VEGF) and its receptors [39], [34] .

Inflammation and Immune cell activation

The onset of inflammation in dcSSc is longer than that of lcSSc. In addition, there is an extensive spread of inflammation in the musculoskeletal and skin in the dcSSc form of SSc. This inflammation is accompanied by edema, which is indicative of changes in the permeability of the endothelium. As the disease progresses, widespread fibrotic changes occur and Reynaud's symptoms can manifest simultaneously with skin changes or can occur after the changes[4] .In contrast, the onset of lcSSc is much slower and there may be a pre-existence of Reynaud's phenomenon for a couple of years. In addition, the prominence of the vascular component is a characteristic feature of lcSSc and accounts for most of the manifestations of the disease including renal involvement, digital ulceration, and PAH. There is nevertheless a small amount of fibrosis and this is usually observed in the face, skin, gastrointestinal tract, and extremities[4].

Vascular injury leads to inflammation and autoimmunity. Following vascular injury, leukocytes are recruited. Chemokines such as monocyte chemoattractant protein 3 (MCP-3) and monocyte chemoattractant protein 1 (MCP-1) lead to the accumulation of mononuclear cells namely neutrophils and macrophages. Mononuclear cells produce cytokines such as interleukin 1

(IL-1) and TGFβ, which activate fibroblasts thereby initiating fibrogenesis. These cytokines also activate resident fibroblasts produced by pericytes and mesenchymal progenitor cells thereby further enhancing fibrinogenesis. Production of resident fibroblasts by pericytes is induced by the activity of the platelet-derived growth factor (PDGF), the basic fibroblast growth factor (bFGF), and the endothelin-1 (ET-1) on the pericytes. According to Varga et al [2] PDGF is a powerful mitogen and chemoattractant for fibroblasts and can stimulate them to produce TGFβ, IL-6, and MCP-1 and to generate collagen, proteoglycans, and fibronectin. Activated fibroblasts are also acted upon by TGFβ and connective tissue growth factor (CTGF) produced by the T helper cell 2 (TH2) leading to the formation of myofibroblasts which cause permanent scarring through tissue remodeling and fibrosis[4] .

There is a delicate balance between TH2 and TH1 cells and alteration of this balance is associated with increased fibrinogenesis. There is a shift towards TH2 predominance in SSc. Whereas TH1 cells predominantly secrete interferon gamma (IFNγ) and interleukin 2 (IL2), TH2 cells largely produce IL4, IL13, and IL5 [40]. The IFNγ inhibits the expression of collagen-encoding genes and abolishes the stimulatory actions of TGFβ. As such, IFNγ is a powerful inhibitor of the contraction of fibrogenesis since it inhibits the trans-differentiation of fibroblasts into myofibroblasts, the contraction of the extracellular matrix, and the proliferation of fibroblasts. The regulatory T cell also activates Myofibroblasts.

MAJOR PRO-INFLAMMATORY CYTOKINES IMPLICATED IN SSc DISEASE

Interleukin- 6 (IL- 6)

IL- 6 is a cytokine, which is produced by local tissues and later released in the circulation system. It is a polypeptide that comprises of 212 amino acids and is produced by various cells such as the T- cells and the monocytes. The molecular weight of IL- 6 ranges from 21- 29 kDa due to variable and extensive glycosylation and phosphorylation [41]. IL- 6 is vital in almost homeostatic perturbation situations such as trauma and acute infections [42]. It is also a multifunctional cytokine with a vital role in host defense due to its various ways of immune and hematopoietic activities [43]. IL-6 modulates various functions in the body such as apoptosis, cell differentiation and proliferation, and inflammation. Apart from its main function, IL- 6 also influences various body systems such as neural and endocrine systems, skeletal muscles and bone metabolism [44].

Studies by Yu et al showed that IL-6 contributes to the initiation and extension of the inflammatory process. During inflammation process, IL- 6 activates B and T lymphocytes and also stimulates hepatocytes to produce acute phase proteins [45]. Studies demonstrated that IL- 6 has anti-inflammatory and protective properties too. These properties include the ability to inhibit production of tumor necrosis factor (TNF), IL- 1 and macrophage inflammatory proteins [45].

IL- 6 is vital in activating fibroblasts to produce extracellular matrix whose excessive accumulation leads to SSc. Some Studies demonstrated that IL- 6 is highly expressed in patients with SSc especially during the early stages of the disease in inflammatory phase [46]. High IL- 6 expression is associated with more severe skin involvement at early stages. Fibroblasts isolated

and cultured from SSc lesional skin involvement produced higher level of IL- 6 compared with non lesional SSc samples [47].

Interleukin- 8 (IL- 8) and SSc

Interleukin- 8 (IL- 8) is a member of a family of structurally related proinflammatory factors that have a low molecular weight and are referred as the chemokine [48]. IL- 8 has a low molecular weight of approximately 8kDa. It is a non-glycosylated protein comprising of 72 amino acids. A number of studies have been carried out in the past to determine the possible changes in the serum levels of IL-8 in patients with SSc disease. According to a study by Reitamo and others [49], the levels of IL-8 and autoantibodies to IL-8 were significantly higher in patients with SSc disease [49]. In fact, the levels were undetectable in normal serum, but highly detectable in more than 12.5% of the patients. A study by Guang-bin Cui et al have also shown similar results [50] where the levels of the IL-8 were determined in mice induced with persistent inflammatory pain, such as the one experienced in SSc. In this case, it was found that in all the mice samples used, the level of serum IL-8 had raised significantly. The study indicates that the up-regulation of the IL-8 in mice is related to the activation of fibroblasts or mononuclear phagocytes and other immune cells [50]. In addition, such activation may be related to the production of the autoanitibodies targeting IL-8 molecules that are now detectable in the serum.

Monocyte chemo attractant protein- 1(MCP- 1) and SSc

MCP- 1 is an inflammatory chemokine that is produced predominantly by macrophages and endothelial cells. The expression of this chemokine increases in patients who have atherosclerotic lesions, thus, MCP-1 plays a vital role in artherogenesis [51]. MCP- 1 secretion is induced by the cytokine activation and also interaction of activated platelets with monocytes or endothelial cells. Research shows that MCP- 1 is a chemokine that links monocyte activation to vascular inflammation of patients with SSc [52] . Studies revealed that MCP- 1 has both proinflammatory

role and pro fibrotic role is SSc patients. In the early stages of SSc, MCP- 1 is released. It then attracts the T cells and mononuclear cells to the affected area. This leads to the production of pro-fibrotic cytokines such as IL- 4, which then activate the synthesis of ECM in dermal fibroblasts, and causes fibrosis in later stages of SSc [52]. Studies have shown that the inhibition of MCP-1 reduces the extent of SSc as well as atheroma in mice induced with hypercholesteroma [53]. Many other studies on targeting MCP-1 as a possible treatment for SSc patients revealed promising result in animal models [52]. Ongoing clinical trials are testing the MCP-1 antagonists on various disease as well as SSc patients.

Fibrosis

Fibrosis is the definitive feature of SSc [54]. It involves the gradual replacement of tissue architecture by the extracellular matrix (ECM), which is rich in collagen and other fibrotic components. Excessive ECM deposition in fibrotic organs leads to organ dysfunction [55]. Fibrosis commonly occurs in the lungs, skin, heart, gastrointestinal tract, endocrine glands, ligaments, and tendons, and it constitutes a large part of the mortality and morbidity that are associated with SSc[56].

Typically, the extracellular matrix is made up of 2 compartments, the cellular and connective tissue compartments [4]. According to Namboodiri et al. [25], the former compartment consists of inhabitant and infiltrating cells while the latter compartment consists of adhesion molecules, collagens, fibrillins, and proteoglycans. The extracellular matrix is also a reservoir for matricellular proteins as well as growth factors such as CTGF and TGFβ. These proteins regulate the differentiation, function continued existence of mesenchymal cells in concert with the connective tissue compartment.

Fibroblasts and Myofibroblasts

Fibroblasts are cells found in the connective tissue throughout the body which produce collagen and other proteins found in the extracellular spaces of cell [57]. They have a vital role in matrix deposition, matrix degradation and also in growth factor secretion as well as inflammatory response and control [58]. Fibroblasts migrate within tissues through a process known as cell migration; Cell migration is a cellular process that has a role in disease and health such as wound healing, immune response and tissue development.[59] Fibroblasts take part on wound healing since they have the ability to move to the site of the wound to repair damaged tissue and eventually heal the wound [60]. Tissue injury and microenvironmental changes are important stimuli for phenotype transition of fibroblasts to myofibroblasts. In response to tissue injury and as a change in normal intracellular environment, fibroblasts acquire actin fibers, the stress fibers which are the hallmark of stable protomyofibroblasts. The final step of fibroblast differentiation to myofibroblasts is the expression of α-SMA in protomyofibroblasts. The generation of α-SMA needs TGF-β 1and β2, ED-A fibronectin (an isoform de novo expressed during wound healing and fibrotic changes) and the high extracellular matrix stress.

Fibrosis is largely executed by the differentiation of fibroblasts to myofibroblasts [61]. Myofibroblasts express the stress fibers that lead to ECM contraction. Myofibroblasts also produce α-SMA (α-smooth muscle actin), which is an important contractile factor [62]. Moreover, fibroblasts produce collagen in response to stimuli from inflammatory cells, platelets, and epithelial and endothelial cells. Stimuli also cause fibroblasts to secrete other molecules of the ECM that attach, contract, organize, and remodel connective tissue. In fibrotic disorders, myofibroblasts exhibit defective apoptosis process resulting in the maintenance of the fibrosis [63]. Cytokines and growth factors are also produced by fibroblasts, which can also undergo trans-differentiation to form contractile myofibroblasts [64]. Several growth factors and signaling

pathways have been shown to be implicated in the pathogenesis of fibrosis. Pro-fibrotic proteins, including TGF-β, Endothelin-1, and connective tissue growth factor, are believed to play an important role in the pathogenesis of fibrosis [65],[33].

Transforming growth factor-β (TGF-β)

TGF-β is an important regulatory cytokine that has diverse effects on cell differentiation, proliferation, survival, and remodeling [66, 67]. At least three isoforms of TGF-β have been identified in mammals, but only TGF-β1 has been shown to play a pivotal role in wound healing and fibrosis. TGF-β is stored in a latent form in ECM, and it binds to the latent TGF-β binding factor (LTF). When the proteolysis of the carboxyterminal in LTF occurs, TGF-β is converted to its active form and signaling starts through the TGF-β specific receptors. TGF-β has 2 types of receptors with several subtypes. There are five Activin receptor–like kinases (ALKs) for type II. There are seven type I receptors [68]. When TGF-β binds to the receptors, the aggregation of both receptors occurs consequently, and TβRII (TGF-β Receptor type II) activates TβRI (TGF-β Receptor type I). The signaling cascade occurs through the phosphorylation of the SMAD proteins. Moreover, TGF-β also acts through other signaling pathways. The mitogen-activated protein kinase (MAPK), P38, and Jun-kinase (JNK) cascade are other pathways [69], [70]. Upon the activation of TGF-β, the expression of collagens and fibronectin increases, which causes the matrix deposition. Furthermore, TGFβ inhibits the activation of matrix metalloproteinases (MMPS) that degrade the ECM [71], [72]. Studies by Desmouliere et al show that TGF plays an important role in the differentiation of myofibroblasts through α-smooth muscle actin (α-SMA) activation [73]. In their study, the administration of TGF-β in rats induced the formation of granulation tissue with high expressions of α-SMA in myofibroblasts, which is specific for TGF-β. Choi et al demonstrated that knocking down TGF-β expression using anti-sense RNA

decreases the fibrotic tissue after injury [74]. Studies by Bonniaud et al show that an overexpression of TGF-β in lungs leads to lung fibrosis in mice [75].

Signaling Pathways

SMAD signaling pathway

The SMAD pathway is the major pathway involved in transmitting signals from the TGFβ receptors. As already indicated before, the extracellular matrix is a reservoir of inactive or latent TGFβ. The dormancy of the latent TGFβ is maintained by the latent TGFβ binding proteins (LTBPs). The TGFβ is activated by plasmin, integrins, THY-1, and thrombospondins and attaches to the cell surface receptors namely TGFβRII and TGFβRI. An intracellular signal transduction cascade is triggered due to the binding of the TGFβ to the receptors and this leads to activation of the target genes. The receptors are serine-threonine kinases and they cause phosphorylation of SMAD proteins [76].

When SMAD2 and SMAD3 are phosphorylated, they create hetero-complexes with SMAD4 and move into the nucleus from the cytoplasm. In the nucleus, the hetero-complexes attach to the cis-acting DNA sequence (CAGAC), which characterizes the consensus SMAD-binding element (SBE). The SBE is present in the promoters of a large number of genes that can be induced by TGFβ. After attachment to the SBE, recruitment of transcriptional factors to the DNA by the activated SMAD proteins occurs and this induces the transcription of the collagen-encoding genes. Inhibition of SMAD-dependent signal transduction is mediated by SMAD7. Studies have shown that SSc is associated with changes in the activation and inhibition of specific cofactors and proteins involved in the SMAD signaling pathway [2], [23] ,[77].

The Non-SMAD pathways

The non-SMAD pathways also play a critical role in the pathogenesis of SSc. The non-SMAD pathway involves the activation of the focal adhesion kinase (FAK), MAPK, Jun kinase (JNK), calcineurin, TGFβ activated kinase 1 and lipid kinases such as AKT and PI3K. . Bujor et al studied the role of AKT in deposition of collagen by normal dermal fibroblasts. They discovered that the basal production of collagen was hindered when AKT was inhibited. Inhibition of AKT also led to elevated production of matrix metalloproteinase 1 (MMP1) and elimination of the inhibitory effect of TGFβ on MMP1. The findings demonstrate that AKT is profibrotic as it increases the synthesis and reduces the degradation of collagen. It was thus concluded that AKT plays a role in fibrosis in SSc [78].

Focal adhesion kinase (FAK)

Focal Adhesion kinase (FAK) is a 125 kD protein which plays an important role in the focal adhesion dynamics between cells, as well as in motility and cell survival. FAK is phosphorylated in response to growth factors, integrin, and other stimulation [79]. Studies demonstrate that the phosphorylation of the FAK (p-FAK) is involved in myofibroblast differentiation and plays a role in the pathogenesis of SSc[80]. In SSc patients, myofibroblasts have the ability to produce α-SMA with the stimulation of TGF-β. For this induction, TGF-β needs focal adhesion kinase phosphorylation on the Tyr-397 site [81]. Moreover, studies by Mimura et al [80] demonstrate that P-FAK on the Tyr-397 site is also higher in the myofibroblasts of SSc patients as compared to that in normal fibroblasts. These results also confirm the possible role of p-FAK in the pathogenesis of SSc by TGF-β signaling and α-SMA expression in myofibroblasts.

Endothelin-1

Endothelin-1 (ET-1), which is a potent vasoconstrictor secreted from endothelial cells, plays a critical role in the pathogenesis of SSc[82]. Studies by Abraham el al [83] show that ET-1 is overexpressed by SSc fibroblasts, thus confirming the possible role of ET-1 in SSc and other

fibrotic disorders. Studies by Mutsaers et al [84] demonstrate that ET-1 levels are elevated in animal models of lung fibrosis. ET-1 binds to ETA and ETB receptors on fibroblasts directly to induce the differentiation of myofibroblasts [85]. Moreover, studies of lung fibrosis also show that ET-1 induces elevated levels of α-SMA through Akt and the ras/MEK/ERK signaling pathway [86, 87].

Connective Tissue Growth Factor

Connective tissue growth factor (CTGF) is a cysteine-rich protein and a member of the CCN superfamily, plays a direct role in fibrosis as well as an indirect role through the creation of a favorable environment [88] for other factors that induce the fibrosis in fibrotic disorders. CTGF is a promoting factor for the adhesion of fibroblasts to fibronectin [84, 89]; it also helps TGF-β to induce cell adhesion to fibronectin and other ECM components [90]. Moreover, CTGF increases the effect of the ET-1 and TGF-β signaling pathway and indirectly increases the fibrosis effect [91]. Studies by Sato et al [92] show that serum CTGF is higher in SSc patients as compared to control samples. Furthermore, CTGF has a positive correlation with skin fibrosis and pulmonary fibrosis in SSc patients. CTGF seems to be involved in maintaining the fibrotic phase of SSc [92].

Table 2: Pathways and signaling molecules dysregulated in SSc [15]

Molecule	Changes in SSc fibroblasts
Cofactors and transcription **factors**	
SMAD2/3	Accumulates in the nucleus and becomes phosphorylated constitutively
SMAD7	Expression is reduced

PPARγ	Expression is reduced
SP1	Becomes phosphorylated constitutively
P300/CBP	Expression is increased, binds constitutively to SMAD2/3
FLI-1	Expression is reduced
Kinases	
FAK1	Activated in a constitutive manner
PKC-δ	Expression is increased
ERK	Activated in a constitutive manner
Surface receptors	
TGFβ receptors	Expression of TGFβR1 and TGFβR11 is elevated
Integrin $\alpha_\gamma\beta_3$	Expression is increased
Integrin $\alpha_\gamma\beta_5$	Expression is increased
PDGFRα	Expression is increased
PDGFRβ	Expression is increased

Current treatment, drugs in market and drugs in clinical trials for SSc disease

Treatment options for SSc remain a challenge because of the unclear pathogenesis of this autoimmune disease. However, those immune-modulators which target blood vessels and aid in recognition, management of end-organ damage, adjunctive therapies like light, physical and psychotherapy are considered most effective to treat this multi-factorial disease. The search for new drugs that work as anti-fibrotic agents is probably one of the most active areas of research in this field. Open label studies with Revimmune drug therapy have shown a desirable effect on the immune system of patients suffering from SSc but the trials are ongoing for more data to confirm efficacy of the drug[93]. Controlled clinical trials with Imatinib mesylate (Gleevec) have been carried out to determine the safety and tolerability in patients. Proven effectiveness (anti-fibrotic effect) and low incidence of side effects was observed as a result [94]. A platelet gel for treating digital ulcers is currently in clinical trials along with others (anti-fibrotic agents like interferon gamma, D-penicillamine, kolchichicine, calcitriol) which reduce excessive production of collagens and other connective tissue proteins to prevent and control symptoms like skin fibrosis[95]. Clinical trials are ongoing with drugs like Orencia, and MQX-503 whereas efficacy of D-penicillamine is supported by retrospective, prospective and double-blind controlled trial

even though these studies could not differentiate more efficacious form of drug in terms of dose[96]. Drugs like methotrexate, cyclosporine, nifedine, iloprost have all been studied in controlled trials with variable outcomes and a considerable number of trials have proved nifedipine, a calcium channel blocker as a gold standard. Randomized trials on the drug - cyclophosphamide confirm the moderate clinical benefits seen in patients with early, symptomatic disease[97]. Many studies are ongoing on finding an appropriate treatment for SSc; however there is not any approved treatment, which can stop this disorder completely. **I anticipate that targeting inflammation during early phases of SSc disease could be a better therapeutic option. Therefore it's essential to identify mediators, which are responsible for initiating inflammatory response during early phases of SSc disease. Another option is to identify endogenous mediators, which initiate the differentiation of fibroblasts to myofibroblasts and promote adhesive and fibrotic signaling.** For instance, a variety of in vitro and in vivo studies using murine models of fibrotic diseases suggest that FAK inhibitors exhibit potent antifibrotic effects, thus making them attractive drugs for fibrotic disorders seen in the clinic. In recent years, several orally bioavailable ATP-competitive FAK inhibitors have been developed by pharmaceutical companies and have entered early into human clinical trials [98]. One of the first clinically available specific FAK inhibitors was PF-562, 271, which inhibited FAK phosphorylation in vivo in a dose-dependent fashion in several human s.c. xenograft models [99]. Recently the present authors showed that PF-562, 271 also prevented bleomycin-induced lung fibrosis in a mouse model. The Phase I study using PF-562, 271 was performed in patients with head and neck, prostatic and pancreatic cancer (clinical trial #NCT00666926, http://clinicaltrials.gov/). Clinically, PF-562, 271 prolonged disease stabilization in a subgroup of patients. Due to the low toxicity of this drug, combination therapies with blocking antibodies or antagonists/inhibitors of profibrotic factor receptors seem possible. However, to our knowledge, there are no clinical studies that have reported the effects of FAK inhibitors in any fibrotic diseases.

Arachidonic Acid Pathway

Arachidonic acid plays an important role in many physiological processes. The pathway has an important role on generation of pain and inflammation as well as for maintenance of homeostasis. Arachidonic acid is formed by the activity of phospholipase A2 on cell membrane phospholipids.

There are 2 main pathways for the metabolism of arachidonic acid and these are the 5-lipoxygenase (5-LO) and the cyclooxygenase (COX) pathways. In the 5-LO pathway, 5-HPETE is formed by the activity of 5-lipooxygenase enzymes on arachidonic acid and this is the precursor for several leukotrienes such as LTB4, LTC4, LTD4, and LTE4. In the COX pathway, the cyclo-endoperoxide PGG2 is formed in reactions catalyzed by the cyclooxygenase enzymes [100].

There are many different types of cyclooxygenases including COX-1, COX-2, and COX-3. The PGG2 is then catalyzed to PGH2, which is then converted into prostanoids such as thrombooxanes (TXA2), prostaglandins such as PGD2, PGE2 and PGF2α, and prostacyclins such as PGI2. Prostaglandins catalyze the modulation of immune function; leukotrienes add molecular oxygen to particular double bonds in polyunsaturated fatty acids and thrombooxanes are potent vasoconstrictors and enhance the aggregation of platelets[100] . PGE2 is the commonest prostanoid as it is produced by many cell and tissue types and its spectrum of activity is wide. It acts on the G-coupled EP1, EP2, EP3, and EP4 receptors and together with PGI2, it is the main prostanoid involved in inflammation and pain [101], [102] . Formation of PGE2 from PGH2 is catalyzed by the microsomal prostaglandin E synthase-1 (mPGES-1) and this is depicted in the diagram below [103].

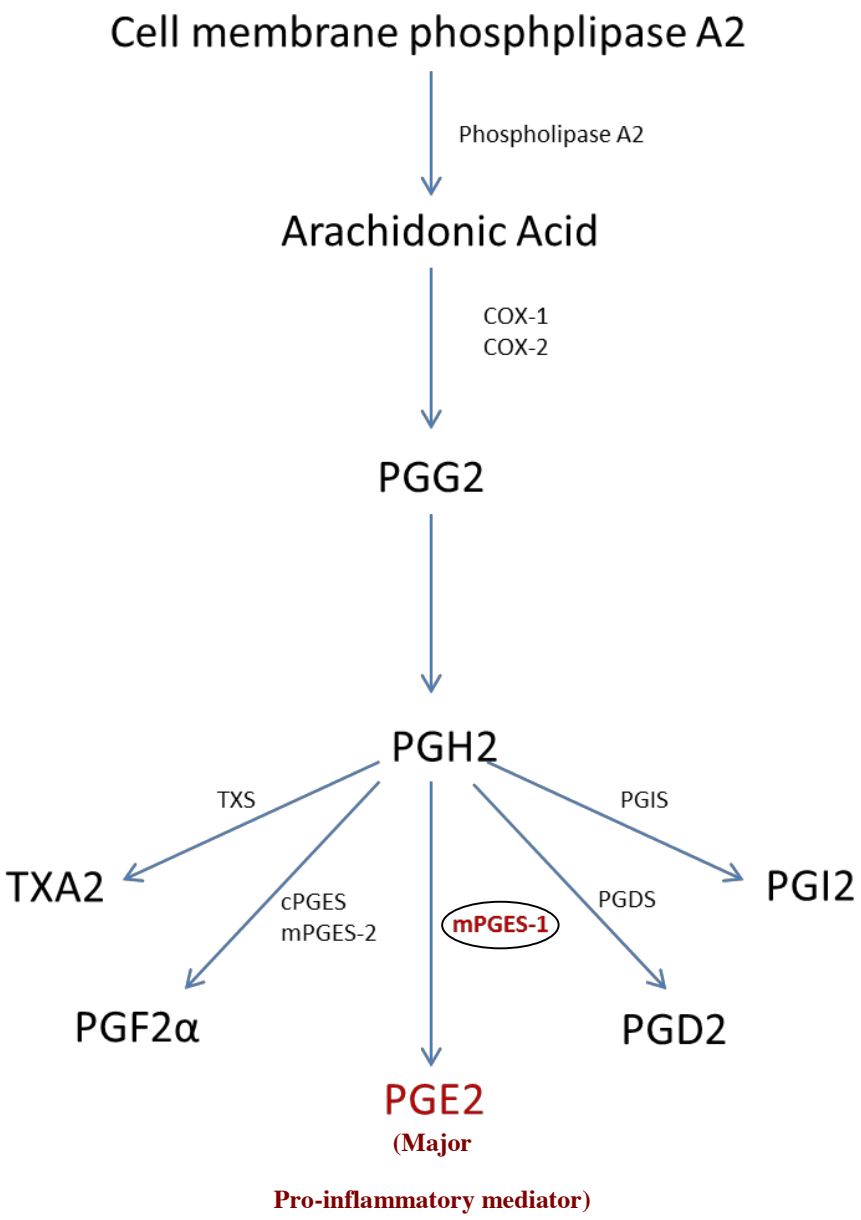

Diagram 1: Illustration of the pathway involved in biosynthesis of prostaglandin E2 (PGE2).

In the diagram above, mPGES=microsomal prostaglandin E synthase, PG=prostaglandin, TPX=thromooxane A2 receptor, TXA2=thrombooxane A2. As shown, conversion of arachidonic acid to PGH2 is mediated by the cycloxgenases COX-1 and COX-2. TXA2, PGE2, PGD2, and PGI2 are synthesized in reactions mediated by TXA2 synthases and PGI2 synthases respectively[103]

Microsomal Prostaglandin E Synthase-1 (mPGES-1)

Microsomal prostaglandin E_2 synthases (mPGES) are enzymes that catalyze the conversion of PGH_2 to PGE_2 [104]. Thus far, three PGE synthases, namely cytosolic PGE synthase (cPGES), mPGES-1 and mPGES-2, have been characterized [104-106]. cPGES is localized in the cytosolic region of cells and tissues under basal conditions and is most likely to be involved in the homeostatic production of PGE_2 [106]. mPGES-2 is also constitutively expressed in wide variety of tissues and cell types and is synthesized as a Golgi membrane associated protein [107]. In contrast, mPGES-1 is induced in response to inflammation, and acts downstream of cyclooxygenases (COX) [108, 109]. PGE2, the final metabolite of cyclooxygenase pathway, has a variety of endogenous functional effects [110]. Besides its role in the initiation and perpetuation of inflammatory processes, PGE-2 helps the blood clot formation, protect the gastrointestinal tract by increasing the mucus formation and also takes part in labor by constricting the uterine[111].

mPGES-1 and its derived PGE2 in inflammation and fibrosis

mPGES-1 has been shown to be a critical mediator of inflammation, pain, angiogenesis, fever, bone metabolism and tumorgenesis [102, 112-114]. Previous studies have shown that mPGES-1 expression is elevated in tissues and cells of various inflammatory diseases including rheumatoid arthritis (RA) and osteoarthritis (OA) [108, 109, 115, 116]. mPGES-1 null mice are resistant to chronic inflammation of joints in the models of collagen induced arthritis (CIA) and collagen antibody induced arthritis (CAIA) [102, 112]. We have also shown that mPGES-1 is induced during skin wound healing process in mice [117].

My laboratory is the first to investigate the role of mPGES-1 in fibrosis using animal models. In our recent study, [118] we investigated the effect of mPGES-1 genetic deletion in mice model of bleomycin-induced skin fibrosis. Our study revealed that mPGES-1-null mice were resistant to

bleomycin-induced skin fibrosis associated with reduced inflammation, myofibroblast formation, cutaneous thickening, and collagen production in the mouse dermis [118]. Bleomycin-induced fibrosis is an inflammation-driven mice model and inflammation is involved with the onset of fibrosis including SSc disease [23, 119, 120]. It is well established that PGE_2 the product of mPGES-1, is one of the major pro-inflammatory mediator upregulated during inflammation. Given the known role of mPGES-1 in driving inflammatory responses, this study strongly suggested that mPGES-1 may play a key role in the initial, inflammatory stages of SSc disease.

Studies on PGE2 further demonstrate its role in fibrosis. Studies by Harding et al showed that treatment of neonatal rat ventricular with PGE2 increases the phosphorylation of AKT and fibroblast proliferation that can initially lead to cardiac fibrosis [121]. Another study by khozani et al showed a considerable increase in the thickness of the vessels of full skin draft treated with PGE2 compared to control skin draft. This study also confirms the possible effect of PGE2 on fibroblast proliferation and fibrosis [122]. Although it is presumed to be a pro-inflammatory mediator in inflammation process, and pro-fibrotic mediator in skin fibroblasts and some other fibrotic disorder, PGE_2 has an anti-inflammatory and antifibrotic activity too [123]. As we know, uncontrolled activity of fibroblasts contributes significantly in the development of fibrotic lung diseases. For instance, collagen synthesis by fibroblasts results in scarring and fibrosis [124]. During the development of lung fibrosis, the production, and signaling of PGE_2 is often diminished [125]. cAMP-activated protein kinase A (PKA) underpins the inhibition of fibroblast activation. In this view, PGE_2 exerts its anti-fibrotic activity through similar cAMP signaling pathway, thus impaired production of PGE_2 in fibroblasts has been associated with fibrosis of the lower airway [124], [126]. Therefore, in lung cells PGE_2 is observed to inhibit multiple fibroblast functions especially fibroblast proliferation, migration, collagen synthesis, and differentiation of myofibroblast. PGE_2 also reduces the cross-linking of collagen by enhancing the degradation of

fibroblast collagen and reducing production of lysyl oxidase. Therefore, understanding the mechanism of these PGE_2 inhibition activities may provide useful insights into the pathogenesis of lung fibrosis [123]. PGE_2 has a direct influence in the survival of lung fibroblasts. Lung cells treated with PGE_2 exhibited a dose-dependent increase in fibroblast apoptosis (fibroblast death) therefore, inhibiting fibrogenesis. In this case, PGE_2 induces apoptotic fibroblast death through E prostanoid-2 and 4 (EP2-EP4) receptor signaling pathway [127]. An animal model study, [128] established that PGE_2 protects the lungs of rodents from fibrotic lung diseases including bleomycin-induced lung dysfunction as described by Oury et al.[129]. However, following bleomycin-induced lung dysfunction administration of PGE_2 does not provide therapeutic benefit against lung fibrosis and dysfunction [128]. Similarly, fibrotic lung fibroblasts extracted from mice with bleomycin-induced fibrosis or patients with idiopathic pulmonary fibrosis are resistant to the collagen inhibitory action of PGE_2. In individuals with pulmonary fibrosis, plasminogen activation system is observed to be dysregulated. On the other hand, PGE_2 inhibits the expression of plasminogen activator inhibitor-1 (PAI-1), which is a key profibrotic molecule [123].

However, reactivation of plasminogen to plasmin restores the antifribrotic activity of PGE_2 in bleomycin-induced and idiopathic pulmonary fibrosis [124]. Down-regulation of PGE_2 synthesis in interstitial lung fibrosis is the underpinning mechanism of the pathogenesis of lung fibrosis. According to Bauman et al [130], activation of plasminogen induces the synthesis and release of PGE_2 in fibroblasts. For instance, induction of plasminogen in lungs extracted from mice with bleomycin-induced fibrosis results in upregulation of PGE_2 synthesis in the epithelial cells of alveolar, lung fibroblasts, and fibrocytes. This results in enhanced anti-fibrotic activity.

Given these considerations, it is likely that mPGES-1 and its derived PGE2 may play a complex dual role during SSc disease. It may contribute to the initiation of fibrogenesis through its ability to promote inflammation, mPGES-1 may actually act to control the overexpression of profibrotic

genes in established lesions [131]. Further, mPGES-1 derived PGE2 could exhibit differential effects i.e. anti- or pro-fibrotic depending on the tissue such as skin (pro-fibrotic) and lungs (anti-fibrotic). Therefore, it is critical to understand the exact role of mPGES-1 and its derived PGE2 in the pathophysiology of SSc disease.

Ephrins

The ephrins and ephrin (Eph) receptors belong to the subfamily of protein tyrosine kinases. Eph receptors have glycosylated extracellular domains with ligand-biding sites similar to immunoglobulin. The ligand-binding sites are adjacent to a cysteine-rich region and two repeats of fibronectin type III [132]. Their glycosylated extracellular domains interact with appropriate ephrin ligands in the neighboring cells. Such interactions generate bi-directional signaling pathways. Therefore, ephrins and Eph receptors play significant roles in various key biological processes such as cell morphology, intercellular interactions (communication), cell boundaries formation, cell migration, insulin regulation, immune function, angiogenesis including various aspects of cancer[133]. Expression patterns of Eph receptors and their corresponding ephrin ligands have been observed in cancerous cells and tumorous blood vessels. These strongly suggest that Eph receptors play substantial role in tumorigeneses and cancer development. Therefore, the use of Eph receptors as new therapeutic targets is promising approach to cancer treatment [134] . In the current human genomics, 8 ephrin ligands and 14 Eph receptors have been identified and characterized [135]. There are two subgroups; A or B, of both Eph receptors and their corresponding ligands. The subgroups of Eph receptors is based on the nature of their interaction with their corresponding ephrin ligands. On the contary the subgroups of ephrin ligands are based on their structure [136].

The Ephrin signaling pathway starts with the binding of EphB receptor tyrosine kinases (RTKs) to transmembrane ephrinB ligands followed by the activation of both Ephrin receptors [137]. The critical role of Ephrins has been shown on central nervous system development as Ephrins act as a mediator of adjacent cell migration on the axonal part of neurons to their specific destination [138, 139]. Furthermore, Ephrin receptors have the ability to conduct the reverse signaling pathway [140]. The exact mechanism of how the reverse signaling pathway occurs is not well recognized. However, studies show that these responses are distinguishable from the intracellular signal activated in Ephrin receptor-expressing cells [141].

Ephrin B2 in extra-cellular matrix remodelling and adhesive signalling

Role of ephrin B2 in fibroblast biology and SSc disease is unknown. Majority of the studies performed in cancer and endothelial cells suggest a critical role of ephrin B2 and its receptor ephB4 in cell migration, adhesion and ECM remodelling. In mouse malignant melanoma cells it has been shown that overexpression of ephrin-B2 leads to the formation of multiple lamellipodia, enhanced polymerisation of actin fibers, and induction of focal adhesion complexes with activation of FAK[142]. Furthermore, ephrin-B2-overexpressing B16 cells display a significant increase of β1-integrin-mediated attachment to matrix components such as laminin and fibronectin and enhanced cell migration in both Boyden chamber invasion experiments as well as in *in vitro* scratch-wound assays[142]. Ephrin-B2 and its receptor EphB4 have also been shown to mediate cell adhesion and migration functions between arterial and venous endothelial cells [143, 144]. Ephrin-B2 deficient smooth muscle cells display impaired cell adhesion, spreading, polarized migration, and the induction of FAK. Furthermore, another study shows that mice cells overexpressing ephrin B2 exhibit increased adhesion and antibody against ephrin B2 results in loss of adhesion[145]. Further, antibody against ephB4 receptor also results in loss of cell adhesion [145]. In an isolated report, it was shown that expression of ephrin B2 and its receptor

eph B4 was enhanced in skin of patients with early diffuse SSc[146]. However, this study did not explore the role of ephrin B2 and ephB4 in fibroblast biology and fibroblast functions including fibroblast-myofibroblast differentiation, migration, adhesion and fibrosis associated with SSc disease.

PURPOSE OF THE STUDY

Scleroderma (Systemic sclerosis, SSc) is a prototypic multisystem fibrotic disease, and is considered to be initiated by a combination of microvascular injury, inflammation and autoimmunity culminating in fibroblast activation and fibrosis. Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts. Thus understanding how to control the inflammatory stage of SSc may be of benefit in controlling the progression of early onset disease. mPGES-1 is an inducible enzyme that acts downstream of cyclooxygenase (COX) to specifically catalyze the conversion of prostaglandin (PG) H2 to PGE2. mPGES-1 plays a key role in inflammation, pain and arthritis; however, the role of mPGES-1 in fibrotic mechanisms especially with respect to human SSc is unknown. Our recent study using mPGES-1 KO mice showed that compared to WT mice, mPGES-1-null mice were resistant to bleomycin-induced fibrosis, inflammation, cutaneous thickening, collagen production and myofibroblast formation as mentioned [118]. These results suggested that inhibition of mPGES-1 may be a viable therapeutic strategy to alleviate the development of inflammation and fibrosis associated with the pathophysiology of SSc disease.

Another key step in the development of fibrosis is the differentiation of fibroblasts to myofibroblasts responsible for production of excessive amount of ECM and fibrosis. Previous studies in cancer biology have suggested that ephrin B2, a transmembrane protein belonging to

ephrin family, is a mediator of adhesive signaling and extracellular remodeling. However its role in fibrosis has never been explored. Till date only one report has demonstrated that the expression of ephrin B2 is enhanced in SSc skin fibroblasts of early diffused SSc [146]. Our purpose of study is to define role of ephrin B2 in fibroblast to myofibroblast differentiation and its role in fibrosis associated with SSc disease.

HYPOTHESIS, AIMS AND OBJECTIVES

Part 1:

mPGES-1 expression is significantly elevated in human SSc skin compared to normal human skin. Also, mPGES-1 deficient mice are resistant to bleomycin induced fibrosis. Therefore, I **hypothesize** that pharmacological inhibition of mPGES-1 will result in downregulating the production of pro-inflammatory and pro-fibrotic mediators during SSc disease. To test this hypothesis, I isolated fibroblasts from skin biopsies obtained from patients with SSc disease and normal subjects and determine:

Aim#1:

A) To determine the expression of pro-fibrotic markers in the presence/absence of mPGES-1 inhibitor in SSc versus control skin fibroblasts.

(B) To determine the production of Pro-inflammatory cytokines in the presence/absence of mPGES-1inhibitor.

(C) To study the Expression of p-FAK and p-AKT in the presence/absence of mPGES-1inhibitor in SSc versus normal skin fibroblasts.

See manuscript #1 for results.

Part 2:

Previous studies in cancer biology have suggested that ephrin B2 is involved in adhesive signaling and extracellular remodeling. However its role in fibrosis has never been explored. Therefore, in second part of my study, I investigated the role of ephrin B2 in fibrosis using fibroblasts from skin biopsies obtained from patients with SSc disease and normal subjects. Further, I assessed the role of ephrin B2 in fibrosis by treating mice with recombinant ephrin B2.

I hypothesize that ephrin B2 is involved in promoting the fibroblast to myofibroblast differentiation and fibrosis associated with SSc disease. To test this hypothesis, I determined:

Aim#2:

(A) The expression of ephrin B2 in SSc versus normal skin fibroblasts.

(B) Role of ephrin B2 in the differentiation of fibroblast to myofibrolasts.

(C) Role of ephrin B2 in fibroblast migration and adhesion.

(D) The effect of recombinant Ephrin B2/Fc treatment on mouse skin.

See manuscript #2 for results.

Manuscript #1

Pharmacological Inhibition of mPGES-1 downregulates the expression of pro-fibrotic markers and the production of pro-inflammatory cytokines.

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(Completed and to be submitted)

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Authors Contribution: P.G., M.K.

Experimental procedure: P.G., M.B, G.P, F.M., D.H., M.K.

Abstract

Objective. To determine the specific role of microsomal prostaglandin E synthase-1 (mPGES-1) in scleroderma (SSc) disease using skin fibroblasts isolated from normal and SSc patients.

Methods: Skin fibroblasts were isolated by punch biopsies from the forearm of healthy individuals and those with diffuse cutaneous scleroderma in DMEM containing 10% fetal bovine serum. Donors were age-, site- and sex-matched. Experimental protocols were approved by the Ethics Committee. Cells were cultured in the presence/absence of mPGES-1 inhibitor (provided by Merck Frosst Canada) for 18 hours and then the expression of pro-fibrotic and pro-inflammatory cytokines were determined.

Results: The immunohistochemical and western blotting findings showed that the expression levels of mPGES-1 were higher in SSc patients versus Normal Patients (NP). Therefore, we further determined if by pharmacological inhibition of mPGES-1, we could alter the gene expression profile of pro-fibrotic mediators and pro-inflammatory cytokines implicated in SSc disease. Our studies showed that the expression of both pro-fibrotic mediators (α-SMA, endothelin-1, collagen type 1 and connective tissue growth factor) and pro-inflammatory cytokines (IL-6, IL-8 and MCP-1) were significantly higher in SSc skin fibroblasts compared to (NP) fibroblasts. Treatment with mPGES-1 inhibitor significantly decreased the expression of pro-fibrotic mediators with only a numeric reduction in CTGF. Moreover, treatment with mPGES-1 inhibitor reduced the expression of pro-inflammatory cytokines in both normal as well as SSc fibroblasts with significant decrease in the latter. In addition, SSc patients exhibited higher levels of p-AKT, p-FAK and p-SMAD3. mPGES-1 inhibitor was able to down regulate this increased expression of p-AKT, p-FAK but not p-SMAD3 in SSc fibroblasts.

Conclusions:

These results indicated that elevation of mPGES-1 during SSc could be a contributing factor in the pathology of SSc and blocking mPGES-1 could be beneficial.

Introduction

Scleroderma (Systemic sclerosis, SSc) is an autoimmune disease of the connective tissue featuring skin thickening, spontaneous scarring, blood vessel disease, varying degrees of inflammation, associated with an overactive immune system. There is currently no approved treatment for this fibrotic disorder and all the treatments are symptomatic. [147]. Even though the pathogenesis of SSc is complex and heterogeneous, the large number of studies carried out in the recent past has helped to shed more light on the pathogenesis of the condition. Analysis of the initial stage of SSc reveals perivascular infiltrates of mononuclear cells in the dermis, leukocyte adhesion, vascular obliteration, and tissue hypoxia which triggers inflammation and autoimmunity resulting in production of growth factors, cytokine and chemokine synthesis in the surrounding fibroblasts [148] ,[149]. MCP-1(Monocyte chemotactic protein-1), also called CCL2, is a human protein encoded in the CCL2 gene [150] . It belongs to the Cysteine-Cysteine motif chemokine family and exists as a small cytokine. It largely recruits memory T cells, monocytes and dendritic cells to area with inflamed or injured tissues [151]. Its mode of action is through chemotactic activity and mostly for basophils and monocytes [152] . It also contributes to pro-inflammatory effect by catalyzing synthesis of proteins in SSc fibroblasts. During inflammation, the principal chemokine (MCP-1) recruits monocytes, macrophages and activated lymphocytes. This is aided by a potent inducer of CXCR1 and CXCR2, a chemical signal, which

attracts neutrophils to the site of inflammation. The overall event is eased by the pro-inflammatory activity of IL-6 by inhibiting TNF-α [47]. IL-8 acts through its chemoattractant activity where it induces chemostasis on neutrophils [153]. Similarly, as a pro-inflammatory cytokine IL-6 signals for cell recruitment through cell surface receptor type 1 complex that is composed of ligand binding IL-6Rα and signal transducers gp130[154].

TGFβ is also an important molecular determinant of fibrosis in SSc[155]. It is not only the major regulator of both pathological and physiological fibrogenesis but also plays critical roles in the repair of tissues, cell differentiation and proliferation, regulation of immune function, and angiogenesis[156] . When TGF-β binds to the receptors, the aggregation of both receptors occurs consequently and TβRII activates TβRI and the signaling cascade occurs through the phosphorylation of the SMAD proteins [157]. However, the SMAD pathway is the main pathway used to transmit signals from the TGFβ receptors. The dormancy of the latent TGFβ is maintained by the latent TGFβ binding proteins (LTBPs) [158]. An intracellular signal transduction cascade is triggered due to the binding of the TGFβ to the receptors and this leads to activation of the target genes [159]. When TGF-β is stimulated, it utilizes p-FAK (Phosphorylated Focal adhesion kinase) on Tyr-397 site, which is abnormally elevated in SSc [80].

Through the promotion of adhesion of fibroblasts to fibronectin, connective tissue growth factor (CTGF) provides favorable environment for other factors to induce fibrosis in SSc[160]. TGF-β also helps in inducing adhesion to fibronectin and ECM components. Besides all factors mentioned above, increased Endothelin-1 (ET-1) in the plasma and biopsies of forearm of SSc patients has been identified. Some studies have shown that patients with Idiopathic lung fibrosis have higher level of ET-1 in their Bronchoalveolar lavage [33].

Our focus is on Microsomal **prostaglandin synthase-1(mPGES-1)** an inducible enzyme that acts downstream of cyclooxygenase (COX) to specifically catalyze the conversion of prostaglandin (PG) H2 to PGE2 [104],[105]. mPGES-1 plays a key role in inflammation, pain and arthritis [112]; However, the role of mPGES-1 in fibrotic mechanisms especially with respect to human SSc is unknown. Our recent study using mPGES-1 knockout mice showed that compared to WT mice, mPGES-1-null mice were resistant to bleomycin-induced fibrosis, inflammation, cutaneous thickening, collagen production and myofibroblast formation [161]. These results suggested that inhibition of mPGES-1 may be a viable approach to alleviate the development of cutaneous sclerosis and is a potential therapeutic target to control fibrotic and inflammatory mechanisms associated with the pathophysiology of SSc disease. Therefore, in our studies, we used the **mPGES-1 Inhibitor,** provided by Merk Frosst Canada to investigate the power of this inhibitor on decreasing the inflammatory stage of the SSc patients.

Materials and methods

Materials

Human Fibroblast Culture: Dermal fibroblasts were isolated from explant culture of 4 mm punch biopsies from the forearm of healthy individuals and those with diffuse cutaneous scleroderma and cultured in DMEM containing 10% fetal bovine serum (Invitrogen). Donors were age-, site- and sex-matched. The Ethics Committee approved experimental protocols. All participants were recruited, under informed written consent. Cells were cultured and after reaching confluence were starved overnight and for 18 hours in the presence or absence of mPGES-1 inhibitor (1 mmol) provided by Merck Frosst Canada.

Western blotting

Protein from Normal as well as SSc fibroblasts was extracted. Cells were lysed in Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS), and the protein content of the lysates was determined using bicinchoninic acid protein assay reagent (Pierce Rockford) with bovine serum albumin (BSA) as the standard. Cell lysates were adjusted to equal amounts of protein and then were applied to SDS-polyacrylamide gels (10–20%) for electrophoresis. Next, the proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10 mM TBS containing 0.1% tween- 20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with the respective antibodies in TBS-T. After washing the membranes with TBS-T, the membranes were incubated overnight with horseradish peroxidase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:10,000 dilution in TBS-T containing 5% skim milk) at 4°C. After further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad Chemidoc Apparatus.

Histological and IHC studies

4 mm punch biopsies from the forearm of healthy individuals and those with diffuse cutaneous scleroderma were isolated and embedded in paraffin wax. Sections (0.5 μm) were cut using a microtome (Leica) and collected on Superfrost Plus slides (Fisher Scientific). Sections were then de-waxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. Immunolabeling of mPGES-1 was performed using the DakoCytomation LSAB+ System-HRP kit (Carpinteria, CA). Immunohistochemical procedures

were performed according to the manufacturer's recommendations. Briefly, endogenous peroxide was blocked using 0.5% H2O2 in methanol for 5 minutes. Non-specific IgG binding was blocked by incubating sections with bovine serum albumin (0.1%) in PBS for 1 hour and then incubated with primary antibody for mPGES-1 (1:1000) in a humidified chamber and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes followed by incubation with streptavidin for 30 minutes. The chromogen diaminobenzidine tetrahydrochloride (DAB), was added till sufficient color development and sections counterstained with Harris's hematoxylin.

RNA isolation and Real-Time PCR

Skin fibroblasts from SSc and Normal patients were cultured as above. Total RNA was isolated from control Fibroblasts, and SSc Fibroblasts using TRIzol (Invitrogen) (RNeasy; QIAGEN), reverse transcribed and amplified using TaqMan Assays-on-Demand (Applied Biosystems) in a reaction solution containing two unlabeled primers and 6-carboxyfluorescein-labelled TaqMan MGB probe Samples were combined with One-Step MasterMix (Eurogentec). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems). The expression values were standardized to values obtained with control Polymerase RNA primers using the ΔCt method. All primers for each target gene are available from Applied Biosystems Assay on demand. Data was normalized to Polymerase mRNA levels and represent averages and standard error of the mean (SEM) from direct comparison of SSc and control Skin fibroblasts. Statistical significance of Real-Time PCR results was determined by one-way analysis of variance.

ELISA

The Fluorokine® MAP Multiplex Assay System with Luminex 200 detection equipment (R&D Systems Minneapolis, MN, USA) were used for the determination of IL6 (sensitivity of 0.36 pg/ml), IL8 (sensitivity of 0.39 pg/ml), IL17 (sensitivity of 0.39 pg/ml), IL4 (sensitivity of 1.75 pg/ml), MCP-1 (sensitivity of 0.16 pg/ml), TNF-α (sensitivity of 0. 60 pg/ml). Diluted microparticles were prepared. The microparticles were equipped with analyte-specific antibodies and were added to a sample of interest where the antibodies bind to their respective substrates. Biotinylated antibodies were subsequently added to the sample and bind the microparticle-affiliated analytes. Finally, a streptavidin–phycoerythrin conjugate was added to the sample, which binds the biotinylated antibodies. The Data from Fluorokine® MAP was analyzed with QIAGEN LiquiChip System Software Version 2.3.

Statistical analysis

Statistical significance of qPCR results was determined by two-way analysis of variance with the Bonferroni post-test using GraphPad Prism 3.00 for Windows. For other assays, statistical analysis was evaluated by the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Increased expression of mPGES-1 and PGE2 production in SSc patient fibroblasts versus normal patient fibroblasts

Immunohistochemical findings using the antibody recognised mPGES-1 protein showed higher amount of mPGES-1 protein in SSc fibroblasts compared to Normal fibroblasts (Figure 1A). In addition, the level of prostaglandin E2 (specific metabolic of mPGES-1) are significantly elevated in SSc fibroblasts compared to normal human skin fibroblasts (Figure 1B). These results

indicated that elevation of mPGES-1 could be a contributing factor in pathology of SSc and blocking mPGES-1 could be beneficial in counteracting fibrosis relate to SSc.

Effect of mPGES-1 pharmacological inhibition on gene expression of pro- fibrotic markers in SSc fibroblasts versus normal human fibroblasts

The expression of pro-fibrotic markers (α- SMA, Collagen type 1, EN -1 and CTGF has been determined. The expression of α- SMA increased in SSc patients versus normal patients. Treatment with mPGES-1 inhibitor significantly decreased the expression of α- SMA in SSc fibroblasts (Figure 2A). Moreover, the expression of Collagen type 1 also increased in SSc patients versus normal patients. Treatment with mPGES-1 inhibitor significantly decreased the expression of Collagen type 1 in SSc fibroblasts (Figure 2B). As described in (Figure 2C), EN-1 which is our third profibrotic markers detected in the experiment was higher in SSc patients versus normal patients and again out treatment with mPGES-1 inhibitor significantly declined the expression of SSc skin fibroblasts. Furthermore, the expression of CTGF was higher in SSc patients versus normal patients; However, There was only a partial reduction in the expression of SSc fibroblasts in the presence of mPGES-1 inhibitor treatment (Figure 2D).

Effect of mPGES-1 pharmacological inhibition on gene expression of pro-inflammatory markers in SSc fibroblasts versus normal human fibroblasts

The production of pro-inflammatory cytokines (IL6, IL8, and MCP-1) has been determined. The production of MCP-1 was higher in SSc fibroblasts compared to normal fibroblasts and treatment with mPGES-1 inhibitor significantly decreased the level of MCP-1 in normal and SSc fibroblasts (Figure 3A). Also, the level of IL6 was higher in SSc fibroblasts as expected and treatment with mPGES-1 inhibitor significantly declined the expression of IL8 in SSc fibroblasts.

However in normal fibroblasts we had a numeric reduction in expression of IL6 (Figure 3B). IL8 production was higher in SSc fibroblasts and treatment with mPGES-1 inhibitor reduced the expression of IL8 significantly (Figure 3C). SSc patients exhibited higher levels of p-AKT and p-FAK. mPGES-1 inhibitor was able to down regulate this increased expression of p-AKT, p-FAK (Figure 3D).

Discussion

In recent years, a significant effort has been made toward an appropriate treatment for fibrotic disorders. mPGES-1 in cyclooxygenase pathway and its key role in inflammation responses made it an attractive target for anti- inflammatory therapies. Although its role in fibrosis has not been well recognized, many studies on human cells and mice are ongoing. In one study on human lung fibrosis, the role of prostaglandin E2 (PGE2) which is the metabolite of mPGES-1 has been monitored and implicated that PGE2 has an important role in interstitial fibrosis due to the ability of PGE2 to prevent from fibroblast proliferation, migration, and collagen secretion [162] . Therefore down regulation of the PGE2 in interstitial lung fibrosis is an important factor in the pathogenesis of this disorder.

Our studies have shown that mPGES-1 is over expressed in human dermal SSc fibroblasts and in bleomycin-induced skin sclerosis in mice. Moreover, mPGES-1 null mice were resistant to bleomycin-induced inflammation, cutaneous thickening, collagen production and myofibroblast formation compared to WT mice. In addition our studies demonstrated that, the level of PGE2 which is the metabolite of mPGES-1 is higher in SSc skin fibroblast[118]. Considering the pivotal role of mPGES-1 as a major pro-inflammatory enzyme upregulated in inflammatory cascade and as study on interstitial lung fibrosis implicated lower level of PGE2, it is

understandable that inflammation plays a biphasic role in fibrosis. In fact, prostacyclins restrict the activation of fibroblasts after tissue injury but, in response to the original injury, may promote recruitment of inflammatory cells and lead to secondary activation of fibroblasts [163]. Therefore, in our studies on human skin cell fibroblasts, we investigated the role of mPGES-1 on human SSc fibroblasts by using mPGES-1 inhibitor, a synthetic inhibitor, provided by Merck Frosst Canada) to study the expression of pro-fibrotic markers and the production of pro – inflammatory cytokines in the presence/absence of mentioned treatment. Our analysis on pro-inflammatory cytokines (IL-6, IL-8, MCP-1) demonstrated that mPGES-1 inhibitor is not only able to downregulate the production of pro-inflammatory cytokines significantly in SSc skin fibroblasts but also may cause a numeric reduction in normal skin fibroblasts too; furthermore, the reduction in expression of pro-fibrotic markers has been determined in SSc skin fibroblasts .In one study on human lung fibroblasts researchers found that α-SMA, the pro- fibrotic marker which is high in SSc, is induced by transforming growth factor-beta (TGF-β), which requires focal adhesion kinase (FAK) phosphorylation on its Tyr-397 site . Therefore, FAK phosphorylation is high in SSc and treatment with TGF-β antisense can decrease the phosphorylation of FAK [80]. Moreover, acutely transforming retrovirus (AKT) is a serine/threonine kinase that plays important roles in survival, cell regulation and collagen deposition. Studies demonstrated that blocking AKT using pharmacological inhibitors, small interfering RNA (siRNA), and a dominant-negative AKT mutant led to inhibition of the basal type I collagen production. Furthermore, inhibition of AKT increased the basal matrix metalloproteinase 1 (MMP1) production and reversed the inhibitory effect of TGF- β on MMP1 gene expression. SSc fibroblasts were more sensitive to AKT inhibition, with respect to collagen and MMP1 production. These findings suggest that in SSc skin fibroblasts, AKT can directly contribute to elevated collagen and it may represent an attractive target for therapy of SSc fibrosis

[78]. These results are of considerable interest, give a clue to find an appropriate treatment for SSc patients; Moreover, the observation that mPGES-1 inhibitor reduces the pro-inflammatory and pro-fibrotic cytokines in SSc has important implications in understanding of pathophysiology of SSc. Indeed, our research and the study on mPGES-1 null mice certainly may lead to the development of new therapeutic strategies in the treatment of SSc and possibly other fibrotic disorders.

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Ephrin B2 and Scleroderma

Ephrin B2 is overexpressed in human Scleroderma skin and mediates fibroblast migration, spreading and adhesion, and induces fibrosis in mice

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Abstract

Objectives

The role of ephrin B2, a member of ephrin family belonging to the largest sub-family of membranous receptor protein-tyrosine kinases, in the pathophysiology of scleroderma (SSc) disease is unknown. In the present study we explored the potential of ephrin B2 in mediating fibrotic and adhesive signalling associated with the pathophysiology of SSc disease.

Methods

Skin sections were obtained by punch biopsies from the forearm of healthy individuals and those with cutaneous SSc. Extracted biopsies were then used immunohistochemistry, western blotting,

Real-Time PCR and isolation of dermal fibroblast for cell culture based assays. Cultured fibroblasts were treated with recombinant human ephrin B2/Fc and subjected to fibroblast function assays such as migration, adhesion and stress fibre formation. Mice were daily injected subcutaneously with recombinant human ephrin B2/Fc (100µg/Kg/mouse) for two weeks and degree of fibrosis was determined.

Results

Our IHC, Real-Time PCR and western blot analysis confirmed that Ephrin B2 expression was elevated in SSc skin compared to normal skin. Treatment of normal and SSc fibroblasts with recombinant human ephrin B2/Fc resulted in enhanced cell migration, adhesion to fibronectin, cell spreading, cell-cell contact, stress fiber formation and increased expression of p-FAK and α-SMA (myofibroblast formation) compared to untreated fibroblasts. Furthermore, mice treated with recombinant mouse ephrin B2/Fc induced significant skin fibrosis in mice associated with enhanced collagen deposition, dermal thickness, hydroxyproline content, α-SMA-expressing fibroblasts and increased expression of p-FAK, type I collagen and CTGF.

Conclusion

We for the first time show that ephrin B2 is a key mediator of fibrosis and targeting ephrin B2 could be a new therapeutic option to counteract SSc disease manifestations.

Introduction

Scleroderma (Systemic sclerosis, SSc) is an autoimmune disease for which there is currently no appropriate treatment. While the eitology of this debilitating disease is unknown, SSc disease is associated with production and accumulation of excessive fibrous connective tissue [2]. It is believed that elevated and activated adhesive signaling is involved in promoting fibrogenesis and is a key phenotypic hallmark of fibrotic cells [164].

The Eph receptors and their ephrin ligands belong to the largest sub-family of membranous receptor protein-tyrosine kinases. Based on their structures and sequence relationships, ephrins are divided into the ephrin-A class (anchored to the membrane by a glycosyl-phosphatidylinositol linkage) and ephrin-B class (transmembrane proteins) [136]. Binding of EphB receptor tyrosine kinases (RTKs) to transmembrane ephrin B ligands at the surface of adjacent cells initiates a cascade of signaling events in both the receptor- and the ligand-expressing cells [165]. Ephrins, first identified as axon guidance molecules, have since been shown to regulate several biological functions including animal development, control of extracellular matrix remodelling, adhesive signalling and cell migration [165-169]. They have been shown to mediate cell migration and cell positioning during tissue modelling programs including gastrulation and patterning of vascular, skeletal and nervous systems during the development of invertebrate and vertebrate embryos [137, 170-172]. In mouse malignant melanomas cells it has been shown that overexpression of ephrin-B2 leads to the formation of multiple lamellipodia, enhanced polymerisation of actin fibers, and induction of focal adhesion complexes with constitutive activation of focal adhesion kinase (FAK) [142]. Furthermore, ephrin-B2-overexpressing B16 cells display a significant increase of β1-integrin-mediated attachment to matrix components such as laminin and fibronectin and enhanced cell migration in both Boyden chamber invasion experiments as well as

in in vitro scratch-wound assays [142]. Ephrin-B2 and its receptor EphB4 have also been shown to mediate cell adhesion and migration functions between arterial and venous endothelial cells [143, 144].

In a recent report, it was shown that expression of ephrin B2 and its receptor eph B4 was enhanced in skin of patients with early diffuse systemic sclerosis [146]. However, the role of ephrins in fibroblast function in SSc disease is still unknown. In the present study we first determined the expression of ephrin B2 in SSc skin versus normal human skin and further determined the role of ephrin B2 in fibroblast functions including migration, adhesion, spreading and adhesive signalling using fibroblasts isolated from normal donors and SSc patients with cutaneous involvement. In addition, we treated mice (intradermal injections) with recombinant mouse ephrin B2/Fc and determined its potential to cause fibrosis in mice.

Materials and Methods

Normal Human and SSc skin biopsies

6 mm punch biopsies from the forearm of healthy individuals and SSc patients with clinical cutaneous involvement were performed. The biopsies were cut into 2 sections, one was used for isolation and culture of skin fibroblasts and the other for the whole tissue studies. The biopsies were extracted from 9 normal female donors (Age range between 49-65 years old) and 7 female SSc patients with clinical cutaneous involvement of the biopsied site (Patient ages ranged between 45-63 years old). All experimental protocols were approved by the Institutional Ethics Committee. All SSc subjects were part of the registry of the Canadian Scleroderma Research Group and provided informed written consent. Skin biopsies were then: (1) Processed for immunohistochemistry; (2) Homogenized and processed for western blotting or Real-Time PCR;

or (3) Used for isolation of dermal fibroblast which were cultured in DMEM containing 10% fetal bovine serum (Invitrogen) and cell culture based assays were performed.

Immunohistochemistry

Biopsies from the forearm of healthy individuals and those with SSc were embedded in paraffin wax. Sections (0.5 μm) were cut using a microtome (Leica) and collected on Superfrost Plus slides (Fisher Scientific). Sections were then de-waxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. Immunolabeling of ephrin B2 was performed using the DakoCytomation LSAB+ System-HRP kit (Carpinteria, CA). Immunohistochemical procedures were performed according to the manufacturer's recommendations. Briefly, endogenous peroxide was blocked using 0.5% H2O2 in methanol for 5 minutes. Non-specific IgG binding was blocked by incubating sections with bovine serum albumin (0.1%) in PBS for 1 hour and then incubated with primary antibody for ephrin B2 (Sigma-Aldrich; 1:1000 dilution) in a humidified chamber and left overnight at 4°C. Next, sections were incubated with the biotinylated link for 30 minutes followed by incubation with streptavidin for 30 minutes. The chromogen diaminobenzidine tetrahydrochloride (DAB) was then added till sufficient color development.

Western blotting

Normal and SSc skin explants were homogenized in 50 mM Tris-buffered saline (TBS) containing 0.1% sodium dodecyl sulfate (SDS) and protease inhibitors (leupeptin, pepstatin A) and the protein content was determined using bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard. Homogenates were adjusted to equal equivalents of protein and then were applied to SDS–polyacrylamide gels (10%) for

electrophoresis as described before [173]. The proteins were electroblotted onto polyvinylidene fluoride membranes. After the membranes were blocked in 10 mM TBS containing 0.1% tween-20 (TBS-T) and 5% skim milk, the membranes were probed for 1.5 hours with primary antibody for ephrin B2 (Sigma-Aldrich, USA; 1:1000 dilution) or β-actin (Sigma-Aldrich, USA; 1:1000 dilution) in TBS-T. After washing the membranes with TBS-T, the membranes were incubated overnight with horseradish peroxidase-conjugated anti-rabbit or horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) (1:10,000 dilution in TBS-T containing 5% skim milk) at 4°C. After further washing with TBS-T, protein bands were visualized with an enhanced chemiluminescence system using a Bio-Rad Chemidoc Apparatus.

Additionally, normal patient fibroblasts were cultured to confluence and treated with/without ephrin B2/Fc (Recombinant human ephrin B2/Fc; Creative BioMart, USA, 4μg/ml; concentration of 4μg/ml was chosen for actual experiments based on our pilot experiments in which 4μg/ml was the most effective concentration in inducing α-SMA expression without affecting cell viability) for 24 hours and protein was extracted and western blotting for p-FAK (Cell Signalling, USA; 1:1000 dilution), Total-FAK (Cell Signalling, USA; 1:1000 dilution), α-SMA (Sigma-Aldrich, USA; 1:1000 dilution) and β-actin (Sigma-Aldrich; 1:1000 dilution) was performed as described above.

RNA isolation and Real-Time PCR

Total RNA was isolated from normal human and SSc skin explants using TRIzol (Invitrogen) (RNeasy; QIAGEN), reverse transcribed and amplified using TaqMan Assays-on-Demand (Applied Biosystems) in a reaction solution containing two unlabeled primers and 6-carboxyfluoroscein-labelled TaqMan MGB probe. Samples were combined with One-Step

MasterMix (Eurogentec). Amplified sequences were detected using the ABI Prism 7900HT sequence detector (Applied Biosystems). The expression values were standardized to values obtained with GAPDH primers using the ΔCt method and presented as averages and standard error of the mean (SEM) from direct comparison of SSc and normal human skin.

Adhesion, migration and cell spreading assay

Normal and SSc skin fibroblasts were isolated and cultured as described above. Following confluence, cells were pre-treated for 24 hours in the absence/presence of recombinant human ephrin B2/Fc (4μg/ml). Cells were then lifted using 2 mmol/L EDTA in PBS and seeded at $1x10^5$/ml for 30 min at 37°C in Dulbecco's Modified Eagle's Medium containing 2% bovine serum albumin on glass Lab-Tek chamber slides in 24-well plates (Nunc) coated with 10 μg/ml fibronectin (Sigma). Background adhesion was measured using BSA-coated plates. After washing, adhered cells were trypsinized and counted to account for adhesive ability of the cells pre-treated with/without recombinant human ephrin B2/Fc.

For *in vitro* migration assay, cultured normal human skin fibroblasts were grown in 12-well plates. Medium was removed and cells were once rinsed with serum-free medium + 0.1% BSA and were cultured for 24 hours in serum-free medium + 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a blue pipette tip (approximately 1.3-mm width). The wells were washed two times to remove detached cells or cell debris. The cells were then cultured in serum-free medium in the presence/absence of recombinant human ephrin B2/Fc (4μg/ml). Mitomycin C (10 μg/ml, Sigma) was always included in the media to prevent cell proliferation. Images of the scratched areas under each condition were photographed at 0 and 18 hours post-injury.

To account for stress fibre formation, cell spreading and expression of α-SMA and F-FAK, cells were allowed to adhere in the presence/absence of recombinant human ephrin B2/Fc for 12 hours on fibronectin-coated plates and immunofluorescence using anti-vinculin antibody, rhodamine-phalloidin staining, and α-SMA antibody was performed.

Subcutaneous treatment of mice with ephrin B2

Subcutaneous injections using mice recombinant ephrin B2/Fc were performed using the methodology previously reported for bleomycin-induced model of fibrosis [174, 175]. 6 weeks old C57/BL6 mice received 100µl subcutaneous injections of ephrin B2/Fc (100µg/Kg/mouse) into a single location on the shaved back of mice once daily for 2 weeks. Control mice received sunbcutaneous injections of PBS for 2 weeks. Following two-week treatment with either PBS or ephrin B2/Fc, mice were further housed for 2 weeks and killed by CO_2 euthanasia and skin samples were collected for histology, immunohistochemistry, hydroxyproline assay and western blotting. Institutional animal ethics committee approved all experimental protocols.

Histological Assessment of Collagen content

Sections (0.5 µm) were cut using a microtome (Leica) and collected on Superfrost Plus slides (Fisher Scientific). Sections were then de-waxed in xylene and rehydrated by successive immersion in descending concentrations of alcohol. To assess the effects of ephrin B2 treatment on collagen synthesis, trichrome collagen stain was employed as previously described [174, 175]. Briefly, collagen content in each section was assessed by three blinded observers using the following assessment criteria: 0 signifies: No collagen fibres; 1 signifies: Few collagen fibres; 2 signifies: Moderate amount of collagen fibres; 3 signifies: Excessive amount of collagen fibres.

Assessment of Inflammation

To assess inflammation, the sections were stained with hematoxylin and Eosin (H&E; Fisher Scientific). H&E stain was performed according to the manufacturer's recommendation. The effect of ephrin B2 treatment on inflammation (degree of mononuclear cell influx) was graded on a scale of 0-3 by three separate blinded observers. 0 signifies: No mononuclear cells; 1 signifies: Few mononuclear cells; 2 signifies: Moderate mononuclear cells; 3 signifies: Extensive mononuclear cells.

Hydroxyproline assay

Hydroxyproline assay was performed as a marker of collagen content in PBS-treated and ephrin B2/Fc-treated skin using the method previously described [176]. Skin tissues were homogenized in saline, hydrolyzed with 2N NaOH for 30 min at 120 °C, followed by the determination of hydroxyproline by modification of the Neumann and Logan's reaction using Chloramine T and Ehrlich's reagent using a hydroxyproline standard curve and measuring at 550 nm. Values were expressed as μg of hydroxyproline per mg of protein.

α-SMA Immunohistochemistry

Sections were cut and processed as described above. Immunolabeling of α-SMA was performed using the DakoCytomation LSAB+ System-HRP kit (Carpinteria, CA). Immunohistochemical procedures were performed according to the manufacturer's recommendations. Briefly,

endogenous peroxide was blocked using 0.5% H_2O_2 in methanol for 5 minutes. Non-specific IgG binding was blocked by incubating sections with bovine serum albumin (0.1%) in PBS for 1 hour and then incubated with primary antibody for α-SMA (1:1000) in a humidified chamber and left overnight at 4°C. Next, sections were incubated with biotinylated link for 30 minutes followed by incubation with streptavidin for 30 minutes. The chromogen diaminobenzidine tetrahydrochloride (DAB), was then added till sufficient color development and sections counterstained with Harris's hematoxylin.

Statistical analysis

Statistical significance of qPCR results was determined by two-way analysis of variance with the Bonferroni post-test using GraphPad Prism 3.00 for Windows. For other assays, statistical analysis was evaluated by the two-tailed Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Ephrin B2 is overexpressed in human SSc skin

We first determined the expression of ephrin B2 in normal human skin versus SSc skin by immunohistochemistry. Low expression of Ephrin B2 was observed in both epidermis and dermal regions of the normal skin. However, Ephrin B2 was strongly expressed in both epidermis and dermis of SSc skin (Figure 1a). Next, we determined the expression of ephrin B2 in normal human skin versus SSc skin via western blotting and real-time PCR. Our data further confirmed

that ephrin B2 protein expression (Figure 1b) and mRNA expression (Figure 1c) was significantly elevated in SSc skin compared to normal human skin.

Ephrin B2 treated fibroblasts exhibit enhanced actin stress fibre formation, cell spreading, and increased phosphorylation of FAK and α-SMA expression

We next determined if treatment of normal and SSc skin fibroblasts with recombinant human ephrin B2/Fc can affect stress fibre formation and cell spreading. As expected, our results first showed that untreated SSc fibroblasts exhibited greater actin stress fibre formation and cell spreading on fibronectin compared to untreated normal skin fibroblasts, as revealed with rhodamine-phalloidin (red) staining and anti-vinculin antibody (green) (Figure 2A and B). Treatment of normal and SSc skin fibroblasts with ephrin B2/Fc resulted in enhanced stress fibre formation and cell spreading on fibronectin compared to untreated normal and untreated SSc fibroblasts respectively. It should be noted that Ephrin B2/Fc-treated normal fibroblasts exhibited phenotypic characteristics of SSc-like myofibroblasts with increased stress fibres and cell spreading. Further, we also consistently noticed that treatment of both normal as well as SSc fibroblasts with ephrin B2/Fc resulted in increased cell-cell contact with a mesh-like appearance.

Since recombinant human ephrin B2/Fc treatment produced enhanced cell migration, adhesion and spreading, we next determined if treatment of normal and SSc skin fibroblasts with recombinant human ephrin B2/Fc can affect the phopsphorylation of FAK, a key player in adhesive signalling. As expected, our results first showed that untreated SSc fibroblasts exhibited greater phosphorylation of FAK compared to untreated normal skin fibroblasts (Figure 2C). Treatment of normal and SSc fibroblasts with ephrin B2/Fc resulted in increased phosphorylation of FAK compared to untreated normal and untreated SSc fibroblasts respectively.

We further determined if recombinant human ephrin B2/Fc treatment showed any effect on myofibroblast formation, we used indirect immunofluorescence analysis with an anti–α-SMA antibody. Our results first showed that untreated SSc fibroblasts exhibited greater expression of α-SMA–containing stress fibers compared to untreated normal skin fibroblasts (Figure 2D). Treatment of normal and SSc skin fibroblasts with ephrin B2/Fc resulted in increased expression of α-SMA–containing stress fibers compared to untreated normal and untreated SSc fibroblasts respectively. α-SMA staining further confirmed that ephrin B2/Fc-treated normal fibroblasts exhibited phenotypic characteristics of SSc-like myofibroblasts.

Ephrin B2-treated normal fibroblasts exhibit enhanced protein expression of p-FAK and α-SMA Since our immunofluorescence study showed that ephrin B2/Fc treatment resulted in increased phosphorylation of FAK and α-SMA expression in skin fibroblasts, we further confirmed this observation in normal human fibroblasts by western blotting. Our results confirmed that normal human skin fibroblasts treated with ephrin B2/Fc exhibited enhanced phosphorylation of FAK and increased expression of α-SMA compared to untreated fibroblasts (Figure 2E)

Ephrin B2 treated fibroblasts exhibit enhanced migration and adhesion to fibronectin

We next determined if treatment of normal and SSc skin fibroblasts with recombinant human ephrin B2/Fc can affect fibroblast migration and adhesion to fibronectin. Using an in vitro scratch assay, we first observed that untreated SSc skin fibroblasts exhibited greater migration rate compared to untreated normal human skin fibroblasts. Treatment with ephrin B2/Fc significantly ($P<0.05$) enhanced the rate of fibroblast migration in both normal as well as SSc fibroblasts compared to untreated normal and untreated SSc fibroblasts respectively (Figure 3A, B). Next,

our results showed that untreated SSc skin fibroblasts exhibited significantly ($P<0.05$) increased number of fibroblasts adhering to fibronectin compared to untreated normal human skin fibroblasts. Treatment with ephrin B2/Fc significantly ($P<0.05$) enhanced the number of cells adhering to fibronectin in both normal as well as SSc fibroblasts compared to untreated normal and untreated SSc fibroblasts respectively (Figure 3C).

Mice treated with mice recombinant Ephrin B2/ Fc exhibit dermal fibrosis

Mice recombinant ephrin B2/Fc (or PBS as a control) was injected subcutaneously once daily for two weeks followed by 2 weeks of further housing of mice without any treatment. At 4 weeks, tissue biopsies from the back of the mouse were extracted and subjected to histological and biochemical analyses. Trichrome staining showed that treatment with ephrin B2 resulted in significant development of dermal fibrosis in mice associated with increased dermal thickness and collagen score (Figure 4A-C) compared to PBS-treated mice. It was interesting to observe that fibrosis associated with 2-week treatment with ephrin B2 was comparable to what we usually observe with established model of bleomycin-induced skin fibrosis.

We further assessed the effect of ephrin B2 treatment on any degree of inflammation (mononuclear cell influx) induced by ephrin B2 treatment. Blinded histological analysis using H&E staining showed presence of very few mononuclear cells within the dermis of both PBS-treated and ephrin B2-treated mice with no significant differences between both treatments (4D), suggesting a minimal contribution of inflammation towards development of fibrosis in ephrin-B2-treated mice.

Mice treated with mice recombinant Ephrin B2/Fc exhibit enhanced collagen content and α-SMA-expressing myofibroblasts

Hydroxyproline analysis was performed to determine the collagen content. Our results showed a significant increase in collagen content in ephrin B2-treated skin versus PBS-treated skin. (Figures 5A), confirming the results obtained from histological analysis of collagen staining.

As α-SMA-expressing myofibroblasts are a hallmark of SSc disease [174, 175], we further assessed the effect of ephrin B2 treatment on the induction of α-SMA-expressing myofibroblasts. We first subjected skin sections of ephrin B2- or PBS-treated mice to immunohistochemical analysis with an anti-α-SMA antibody. Compared to PBS-treated skin, a markedly elevated numbers of myofibroblasts were detected in ephrin B2-treated skin (Figure 5C). These data were further confirmed by western blot analysis, which confirmed increased protein expression of α-SMA in ephrin B2-treated skin compared to PBS-treated skin (Figure 5B).

Mice treated with mouse recombinant Ephrin B2/Fc exhibit enhanced expression of p-FAK, type-I collagen and CTGF

We further assessed if treatment with ephrin B2 had any effect on the phosphorylation of FAK in vivo. Skin sections isolated from ephrin B2-treated mice exhibited significant phosphorylation of FAK compared to low expression observed in PBS-treated sections in western blot. (Figure 6A). In addition, skin biopsies isolated from ephrin B2-treated mice exhibited significant increase in the mRNA expression of type I collagen and CTGF compared to PBS-treated sections (Figure 6B and C).

Discussion

Fibrosis associated with SSc disease is characterized by excess deposition of extracellular matrix components. Myofibroblast are the activated fibroblasts that produce large amount of collagen and are primarily the major cell types among others contributing towards the pathology of SSc disease [177]. It is now widely accepted that in fibrotic disorders, excessive adhesive signalling plays an active role. Excessive adhesive signalling results in execrated cell-cell and cell-ECM interactions and contributes to activation and promotion of fibrotic signalling mechanisms. Thus, targeting factors which control adhesive signalling such as cell-cell and cell-ECM interactions could present us with new and promising therapeutic targets to counteract fibrotic mechanisms associated with SSc and related disorders.

In cancer studies, members of ephrins family, especially ephrin B2 has been shown to control extracellular matrix remodelling and adhesive signalling [142]. In early diffuse systemic sclerosis skin, a recently published study reported that ephrin B2 and its receptor eph B4 were elevated compared to normal human skin [146]. However, the role of ephrin B2 associated with SSc disease remains unknown.

In the present study we first show that ephrin B2 is elevated in SSc skin from patients with cutaneous involvement compared to normal human skin. We next show that treatment of normal and SSc skin fibroblasts with recombinant human ephrin B2/Fc enhanced several key fibroblast functions including migration, adhesion to fibronectin, stress fibre formation, α-SMA expression (myofibroblast formation) and enhanced adhesive signalling implicated in the pathophysiology of SSc disease. Our results further showed that ephrin B2/Fc treatment resulted in the increased phosphorylation of FAK in normal human fibroblasts. It is now well established that FAK is a

key regulator of cell adhesion, proliferation, survival, migration and myofibroblast differentiation of scleroderma fibroblasts [80, 178, 179]. Indeed, the basis of the myofibroblast phenotype is an increased ability to adhere to and contract ECM. FAK-deficient fibroblasts show significantly decreased cell migration, and re-expression of FAK in FAK-deficient cells restores their migratory ability [180]. Further, pharmacologic inhibition of FAK inhibits TGF-β1-induced expression of α-SMA (a myofibroblast marker) [80, 181]. It has also been reported that α-SMA expression was increased through the interaction between integrins and ECM, especially fibronectin, via phosphorylation of FAK [179]. Thus the ability of ephrin B2 to induce phosphorylation of FAK may in part be driving the fibroblast functions such as increased migration, adhesion to fibronectin, stress fibre formation, α-SMA expression (myofibroblast formation) (Figure 6C).

Since ephrin B2 treatment of normal human and SSc fibroblasts promoted several fibrotic functions, we next determined if treatment of mice with subcutaneous injections of recombinant ephrin B2/Fc can induce fibrosis in mice. Out of 5 mice treated with ephrin B2/Fc, all mice consistently developed significant dermal fibrosis associated with increased dermal thickness, collagen deposition, and hydroxyproline content and α-SMA-expressing fibroblasts. Interestingly, we did not observe any differences in the degree of mononuclear cell influx (inflammation) with or without ephrin-B2 treatment, suggesting that inflammation may play a minimal role in the development of fibrosis upon treatment with ephrin B2.

We further observed that ephrin B2/Fc-treatment was able to significantly induce the phosphorylation of FAK in vivo, consistent to what we observed in vitro with normal human and SSc fibroblasts treated with ephrin B2. In addition to FAK phosphorylation, treatment of mice with ephrin B2/Fc also upregulated the expression of collagen type I, a major component of ECM

as well as CTGF, a key profibrotic marker associated with fibrosis. Thus induction of phosphorylation of FAK and subsequent increase in adhesive signalling could be contributing factor development of fibrosis in ephrin B2-treated mice.

Currently there is no perfect mouse model that mimics each aspect of SSc pathology. Most often used model of skin fibrosis, bleomycin-induced model of skin scleroderma, utilizes repeated application of bleomycin, an anti-tumor antibiotic originally isolated from the fungus *Streptomyces verticillus* [182], to induce inflammation and subsequent fibrosis in skin [118, 183]. The results presented in this study show that ephrin B2-induced dermal fibrosis could be used as another suitable model of skin fibrosis with minimal involvement of inflammation.

Overall, these data suggest that targeting ephrin B2 could be beneficial in counteracting the profibrotic and abnormal adhesive signalling in SSc and related diseases. Although beyond the scope of the present study, future studies should be directed towards understanding more in depth role of ephrin B2 in SSc disease using pharmacological as well as genetic approach. It would be interesting to see if pharmacological inhibition of ephrin B2 or/and fibroblast-specific ephrin B2 knockout mice resist excessive adhesive signalling and are protected from fibrosis in animals models of skin fibrosis.

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Figure Legends:

Figure 1: Expression of ephrin B2 is elevated in SSc skin versus normal human skin: (A) Immunohistochemistry for ephrin B2 showed increased expression of ephrin B2 in SSc skin versus normal human skin. Representative data from n=4/group is shown. **(B and C)** Western blot and real-time PCR results showed increased protein and mRNA expression of ephrin B2 in SSc skin versus normal skin. Data from n=6 per group is shown.

Figure 2. Ephrin B2 treated normal and SSc fibroblasts exhibit enhanced actin stress fibre formation, cell spreading, increased phosphorylation of FAK and α-SMA expression (myofibroblast formation). (A and B) Untreated SSc fibroblasts exhibited greater actin stress fibre formation and cell spreading on fibronectin compared to untreated normal skin fibroblasts, as revealed with rhodamine-phalloidin (red) staining (A) and anti-vinculin antibody (green) (B). Treatment of normal and SSc skin fibroblasts with ephrin B2/Fc resulted in enhanced stress fibre formation and cell spreading on fibronectin compared to untreated normal and untreated SSc fibroblasts respectively. **(C)** Untreated SSc fibroblasts exhibited greater phosphorylation of FAK compared to untreated normal human skin fibroblasts. Treatment of normal and SSc fibroblasts with ephrin B2/Fc resulted in increased phosphorylation of FAK compared to untreated normal and untreated SSc fibroblasts respectively. **(D)** Untreated SSc fibroblasts exhibited greater expression of α-SMA–containing stress fibers compared to untreated normal skin fibroblasts. Treatment of normal and SSc skin fibroblasts with ephrin B2/Fc resulted in increased expression of α-SMA–containing stress fibers compared to untreated normal and untreated SSc fibroblasts respectively. Representative photo from n=5 separate observations/group is shown. **(E)** Western

blot results show increased phosphorylation of FAK and increased expression of α-SMA in ephrin B2/Fc treated normal human fibroblasts compared to untreated normal human fibroblasts. Data from at least n=4 per group is shown.

Figure 3. Ephrin B2 treated normal and SSc fibroblasts exhibit enhanced migration and adhesion to fibronectin: (A and B). Normal and SSc skin fibroblasts were subjected to in vitro migration (scratch assay) in the presence/absence of recombinant human ephrin B2/Fc and rate of migration of fibroblasts was calculated at 0 and 18 hours post scratch. Untreated SSc skin fibroblasts exhibited significantly greater ($P<0.05$) migration rate compared to untreated normal human skin fibroblasts. Treatment with ephrin B2/Fc significantly ($P<0.05$) enhanced the rate of fibroblast migration in both normal as well as SSc fibroblasts compared to untreated normal and untreated SSc fibroblasts respectively. Data from n=4 per group is shown. **(C)** Normal and SSc skin fibroblasts were subjected to adhesion assay as detailed in methods section. Untreated SSc skin fibroblasts exhibited significantly ($P<0.05$) increased number of fibroblasts adhering to fibronectin compared to untreated normal human skin fibroblasts. Treatment with ephrin B2/Fc significantly ($P<0.05$) enhanced the number of cells adhering to fibronectin in both normal as well as SSc fibroblasts compared to untreated normal and untreated SSc fibroblasts respectively. Data from n=6 per group is shown. ($^{+}$) Refers to statistical significance of $P<0.05$ between untreated normal human fibroblasts versus untreated SSc fibroblasts. (*) Refers to statistical significance of $P<0.05$ between ephrin B2/Fc-treated normal human fibroblasts or ephrin B2/Fc-treated SSc fibroblasts versus untreated normal human fibroblasts or untreated SSc fibroblasts respectively.

Figure 4. Mice treated with mouse recombinant Ephrin B2 Fc exhibit dermal fibrosis

(A) Trichrome staining was performed to account for collagen content (degree of fibrosis) and dermal thickness in response to treatment with mouse recombinant Ephrin B2 Fc (2 week treatment; dose 100ug/Kg/mouse once daily subcutaneously). **(B)** Blind histological analysis in trichrome stained sections showed that ephrin B2-tretated mice exhibited enhanced collagen score compared to PBS-treated mice. **(C)**Ephrin B2-treated mice exhibited significantly increased dermal thickness compared to PBS-treated mice **(D)** Blind histological analysis in H&E stained sections showed that ephrin B2-treated mice did not exhibit any differences in the degree of monocluear cell influx (inflammation score) compared to PBS-treated mice. *, $p<0.05$; ephrin B2-treated mice compared to PBS-treated mice. Representative data from n=5 separate animals/treatment group is shown.

Figure 5. Mice treated with mouse recombinant Ephrin B2 Fc exhibit increased collagen content and myofibroblast formation *in vivo*.

(A) Hydroxyproline analysis showed increased collagen content in ephrin-B2-treated mice compared to PBS-treated mice. **(B).** Western blot analysis with an anti-α-SMA antibody showed enhanced α-SMA expression ephrin B2-treated skin versus PBS-treated skin. *, $p<0.05$; ephrin B2-treated mice compared to PBS-treated mice. Representative data from n=5 separate animals/treatment group is shown. **(C)** Immunohistochemistry using anti-α-SMA antibody showed increased number of α-SMA expressing myofibroblasts in ephrin B2-treated skin versus PBS-treated skin.

Figure 6. Mice treated with mouse recombinant Ephrin B2/Fc exhibit increased phosphorylation of FAK and increased expression of type-I collagen and CTGF *in vivo*.

(A) Western blot analysis show increased phosphorylation of FAK in ephrin B2-treated skin versus PBS-treated skin. **(B)** qPCR data show increased mRNA expression of Collagen type CTGF and I in ephrin B2-treated skin versus PBS-treated skin. *, $p<0.05$; ephrin B2-treated mice compared to PBS-treated mice. Representative data from n=5 separate animals/treatment group is shown. **(C)** Schematic representation of the role of ephrin B2 in fibroblast differentiation, migration, adhesion, extracellular matrix production and fibrosis.

General Discussion

My research so far has demonstrated the role of two key endogenous mediators involved in the pathophysiology of SSc disease. Firstly, I explored the role of pro-inflammatory enzyme

(mPGES-1) in SSc disease by determining the effect of pharmacological inhibition of mPGES-1 on the expression of pro-fibrotic and pro-inflammatory markers using skin fibroblasts isolated from normal and SSc patients. Secondly, I explored the role of adhesive factor (ephrin B2) in fibroblast to myofibroblast differentiation and subsequently its role in fibrosis using skin fibroblasts isolated from normal and SSc patients as well as treating mice with recombinant ephrin B2.

Targetting mPGES-1 in SSc disease

Previous studies in my laboratory investigated the role of mPGES-1 in skin fibrosis using bleomycin-induced mice model. In this study, mPGES-1 null mice were used and results showed that mPGES-1-null mice were resistant to bleomycin-induced skin fibrosis associated with reduced inflammation, myofibroblast formation, cutaneous thickening, and collagen production in the mouse dermis [118]. Bleomycin-induced fibrosis is an inflammation-driven mice model and inflammation is involved with the onset of fibrosis including SSc disease [23, 119, 120]. Given the known role of mPGES-1 in driving inflammatory responses, this study strongly suggested that mPGES-1 might play a key role in the initial, inflammatory stages of SSc disease.

To further explore the role of mPGES-1 in inflammation and fibrosis associated with SSc disease, I first investigated the expression of mPGES-1 in normal skin compared to skin biopsies extracted from SSc patients. My results showed that mPGES-1 is markedly elevated in SSc skin compared to normal human skin. In addition, the levels of mPGES-1-derived PGE2 were also significantly higher in skin fibroblasts isolated from SSc patients compared to fibroblasts isolated from healthy controls. I further investigated the effect of pharmacological inhibition of mPGES-1 on the expression of pro-fibrotic markers. My studies showed the expression of key pro-fibrotic mediators (α-SMA, endothelin-1, collagen type 1 and connective tissue growth factor) are elevated in SSc skin fibroblasts compared to normal skin fibroblasts, in line with previous reports

[83, 92, 184]. Treatment with mPGES-1 inhibitor resulted in significant reduction in the expression of α-SMA, endothelin-1, collagen type 1 but not CTGF in SSc fibroblasts, with no significant effect on normal fibroblasts. Further I investigated the effect of mPGES-1 inhibition on key pro-inflammatory cytokines implicated in SSc pathology including IL-6, IL-8 and MCP-1 [46], [185], [186]. Pharmacological inhibition of mPGES-1 resulted in significant reduction in the production levels of pro-inflammatory cytokines, IL6, IL8 and MCP-1 in SSc-lesioned fibroblasts compared to untreated fibroblasts. In addition, SSc patients exhibited higher levels of p-AKT, p-FAK and p-SMAD3 compared to normal skin fibroblasts. mPGES-1 inhibitor was able to down regulate this increased expression of p-AKT, p-FAK but not p-SMAD3 in SSc fibroblasts.

Overall, the first part of my thesis shows that pharmacological inhibition of mPGES-1 could be beneficial in counteracting both pro-fibrotic and pro-inflammatory components of SSc disease. Further pre-clinical studies are required to test the efficacy and safety of mPGES-1 pharmacological inhibition in vivo in mice before these inhibitors can be deemed safe for clinical trials.

Targeting ephrin B2 in SSc disease

One of the critical processes associated with the evolution of fibrotic response associated with SSc disease is considered to arise from a dysregulated wound healing response where fibroblasts differentiate into specialized activated cells called myofibroblasts. Accumulation of large amount of myofibroblasts is responsible for triggering excessive adhesive signaling and deposition of excessive extracellular matrix (ECM) leading to the destruction of organ architecture[187, 188]. Thus identifying endogenous factors which initiate/promote fibroblast-myofibroblast

differentiation can lead to promising therapeutic strategies to control excessive adhesive signaling and fibrosis associated with SSc disease. Prior to my study, the role of ephrin B2 in fibrosis associated with SSc disease was unknown. My studies show that that ephrin B2 expression is significantly enhanced in human SSc skin versus normal skin**.** Most importantly, *in vitro* treatment of normal human skin fibroblasts with recombinant ephrin B2 is able to transform fibroblasts into myofibroblastic cells exhibiting all typical myofibroblastic-characteristics including increased stress fibre formation, focal adhesions, increased activation of FAK and enhanced fibroblast migration and adhesion to fibronectin in both normal and SSc skin fibroblasts. Further, I treated mice with recombinant ephrin B2 and showed that these mice developed significant skin fibrosis associated with enhanced dermal thickness and collagen synthesis**,** increased hydroxyproline content (collagen content) and increased number of α-SMA-expressing myofibroblasts, enhanced activation of FAK and pro-fibrotic markers including type-I collagen and CTGF. These results provide compelling evidence that ephrin B2 is a key mediator of fibroblast-myofibroblast differentiation and promotes fibrotic and adhesive signaling associated with SSc disease.

Conclusion

SSc disease is multifactorial and multistage disease. My studies have identified two crucial endogenous mediators involved in propagating inflammation and fibrosis associated with SSc disease. mPGES-1 inhibition may present a good alternative strategy to counteract

inflammation and fibrosis at least during early stages of SSc disease. Further, excessive ephrin B2 signaling promotes adhesive and fibrotic signaling by triggering fibroblast to myofibroblast differentiation via activation of the FAK signaling pathway. Thus, inhibition of ephrin B2 will block fibroblast-myofibroblast formation and downregulate fibrosis associated with SSc disease. Overall, both mPGES-1 and ephrin B2 seems to be attractive targets for treatment of SSc and related fibrotic disorders.

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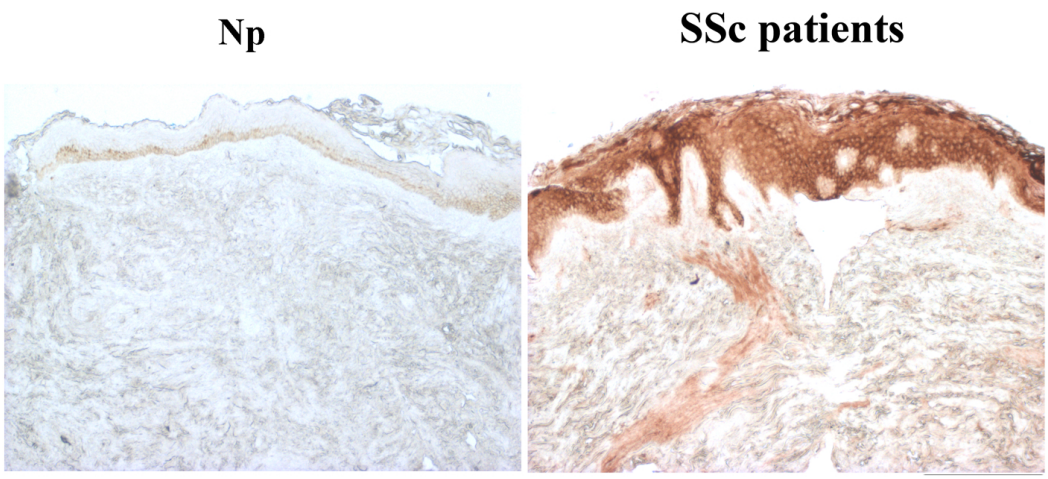
A



B

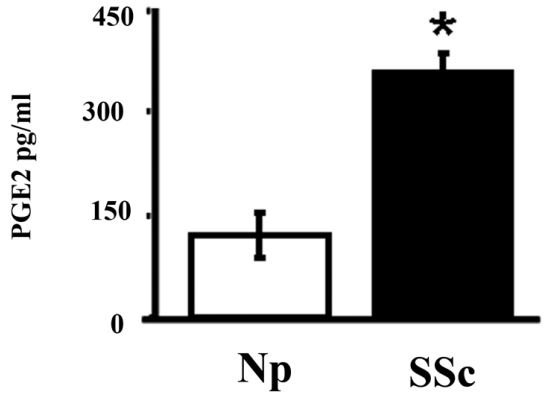


Figure 1. (A) Increased expression of mPGES -1 in SSc patient versus Normal patient (Np) by immunohistochemistry. Representative figure from n=6 different specimen/group. (B) Increased level of PGE2 in supernatent of fibroblasts isolated from SSc patients versus Np . n= 6/group * = Pvalue <0.05.

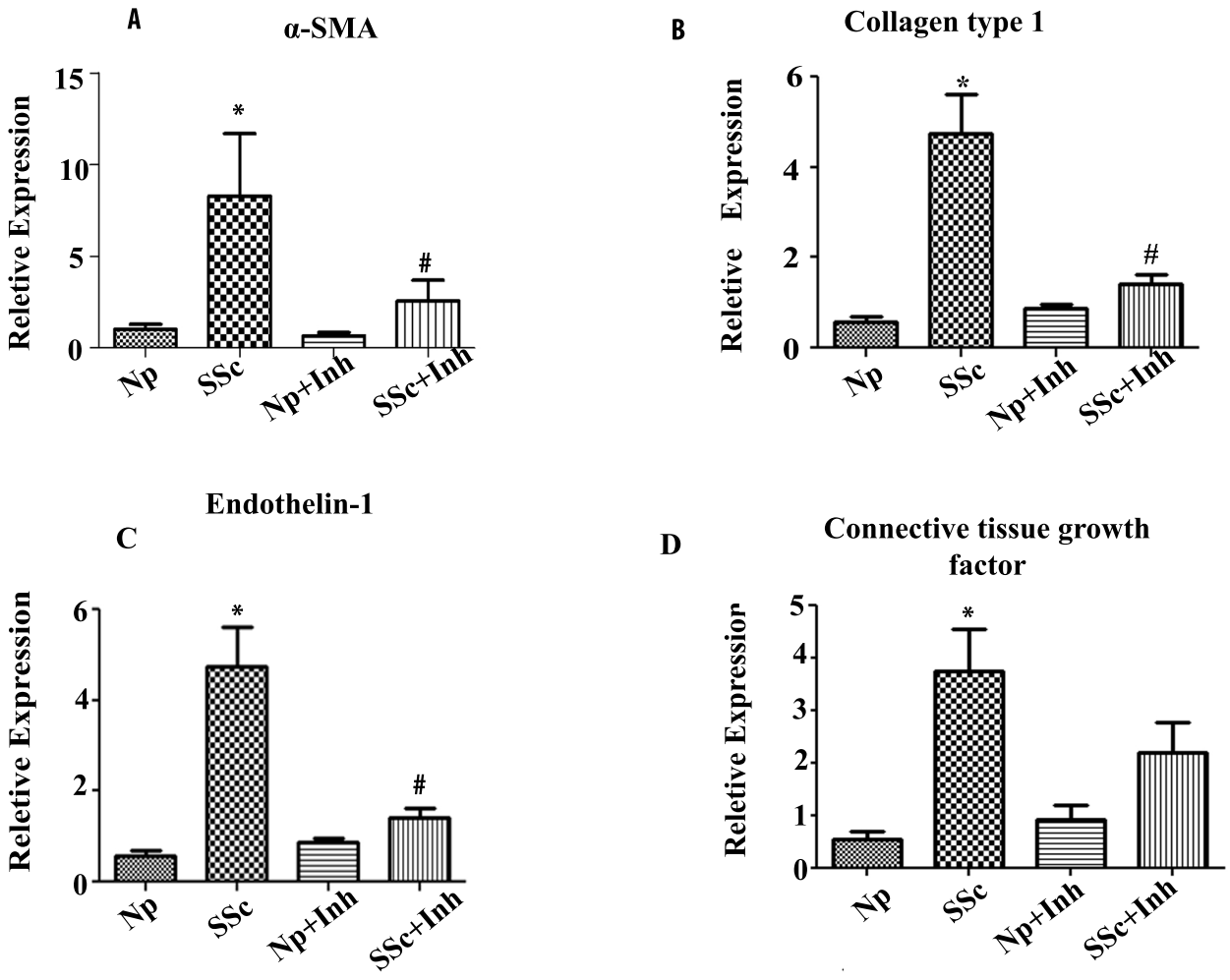


Figure 2. (A) Increased mRNA expression of α-SMA in SSc patient fibroblasts versus Normal patient (Np) fibroblasts . Treatment with mPGES-1 inhibitor significantely decreased the expression of α-SMA in SSc fibroblasts . (B) Increased mRNA expression of Collagen type 1 in SSc patient fibroblasts versus Np fibroblasts . Treatement with mPGES-1 inhibitor significantely decreased the expression of α-SMA in SSc fibroblasts . (C) Increased mRNA expression of Endothelin-1 (ET-1) in SSc patient fibroblasts versus Np . Treatment with mPGES-1 inhibitor significantely increased the expression of ET-1 in SSc fibroblasts . (D) Increased mRNA expression of Connective tissue growth factor (CTGF) in SSc patient fibroblasts versus Np . Treatment with mPGES-1 inhibitor decreased the expression of CTGF in SSc fibroblasts . * = pvalue <0.05 . n=7 forearm skin fibroblasts from each group.

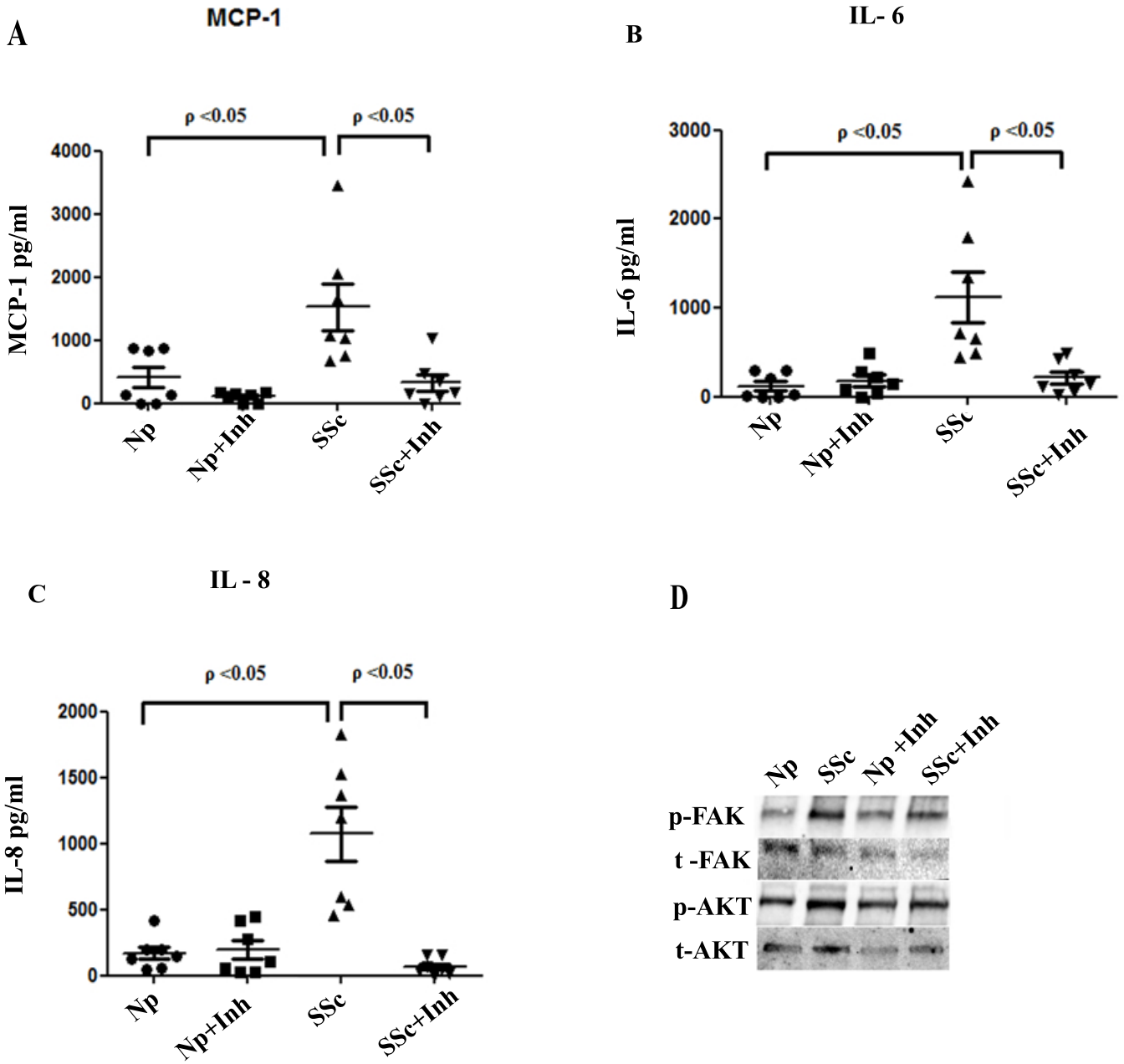


Figure (3). Monocyte chemotactic protein -1 (MCP-1) secretion of dermal fibroblasts isolated from forarm of SSc donors and normal donors in the presence/absence of mPGES-1 inhibitor. MCP-1 was analysed by Elisa in the supernatent of fibroblasts after 18 hours of starvation. (B) Interleukin-6 (IL-6) secretion of dermal fibroblasts isolated from forearm of SSc and normal donors in the presence/absence of mPGES-1 inhibitor. IL-6 was analysed by Elisa in the supernatent of fibroblasts after 18 hours of starvation. (C) Interleukin-8 (IL-8) secretion of dermal fibroblasts isolatedfrom forearm of SSc and normal donors in the presence/absence of mPGES-1 inhibitor. IL-8 was analysed by Elisa in the supernatant of fibroblasts after 18 hours of starvation. (D) SSc patients exhibited higher level of p-AKT and p-FAK. mPGES-1 inhibitor was able to downregulate p-AKT and p-FAK in SSc patients.

Figure 1

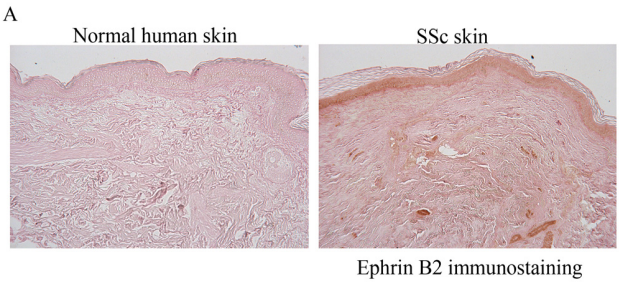

A
Normal human skin
SSc skin
Ephrin B2 immunostaining
B
Normal human skin
SSc skin
Ephrin B2
β-actin
56 KDa
42 KDa
C
Ephrin B2
Relative expression
5
2.5
0
*
Normal human skin
SSc skin

Figure 2

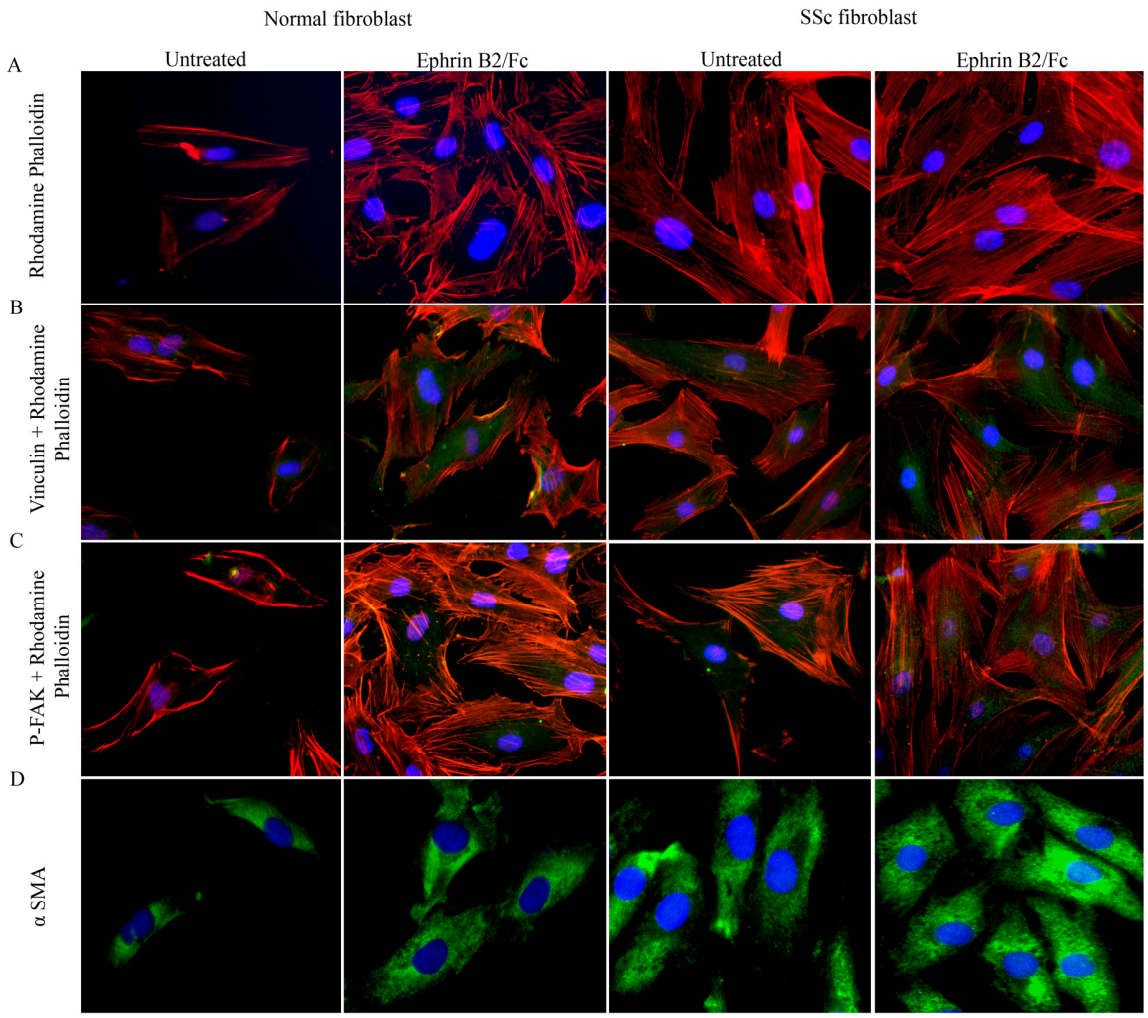

Normal fibroblast
SSc fibroblast
A
Untreated
Ephrin B2/Fc
Untreated
Ephrin B2/Fc
Rhodamine Phalloidin
B
Vinculin + Rhodamine Phalloidin
C
P-FAK + Rhodamine Phalloidin
D
α SMA
E
Untreated
Ephrin B2/Fc
P-FAK
125 KDa
T-FAK
125 KDa
α SMA
42 KDa
β-actin
42 KDa

Figure 3

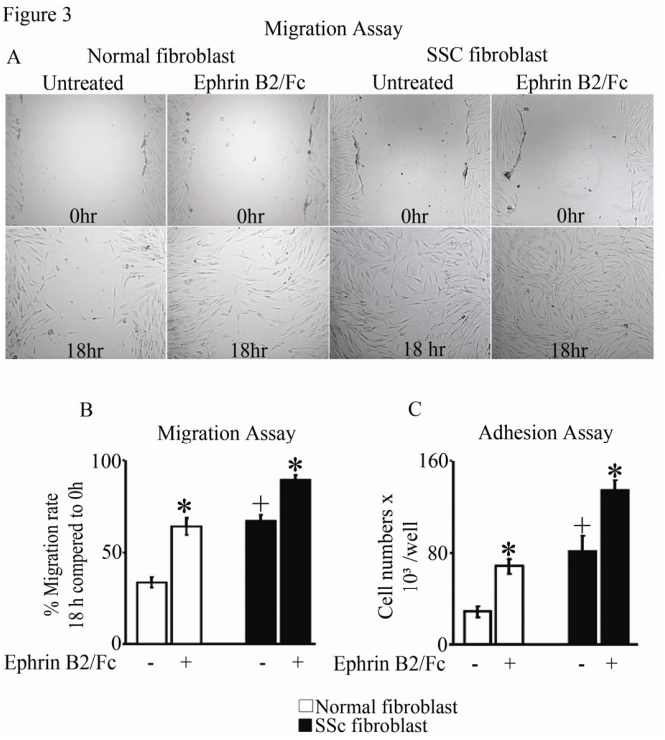

Migration Assay
A
Normal fibroblast
SSC fibroblast
Untreated
Ephrin B2/Fc
Untreated
Ephrin B2/Fc
0hr
0hr
0hr
0hr
18hr
18hr
18 hr
18hr
B
Migration Assay
% Migration rate
18 h compered to 0h
100
50
0
Ephrin B2/Fc - + - +
C
Adhesion Assay
Cell numbers x
10³ /well
160
80
0
Ephrin B2/Fc - + - +
Normal fibroblast
SSc fibroblast

Figure 4

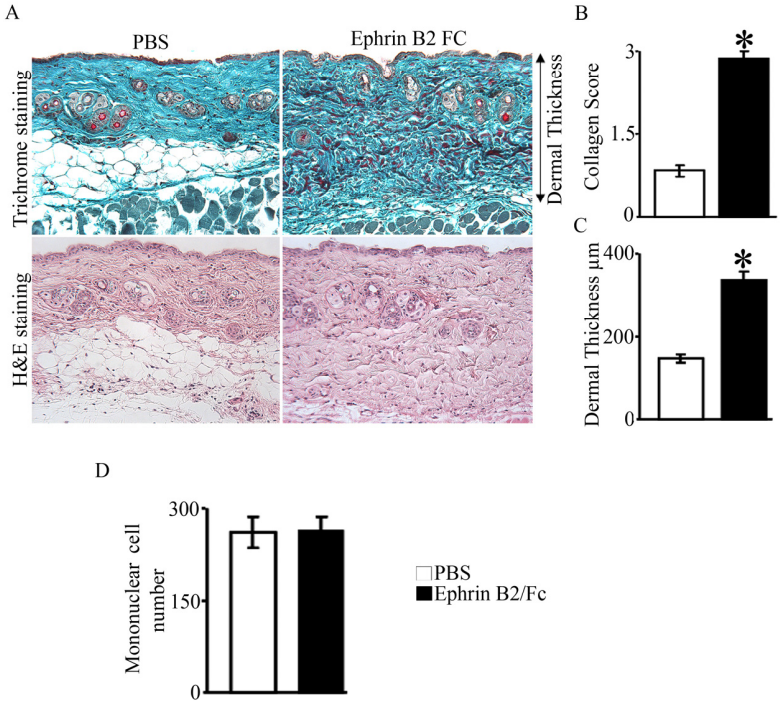

A
PBS
Ephrin B2 FC
Trichrome staining
H&E staining
Dermal Thickness
B
Collagen Score
3
1.5
0
*
C
Dermal Thickness µm
400
200
0
*
D
Mononuclear cell number
300
150
0
PBS
Ephrin B2/Fc

Figure 5

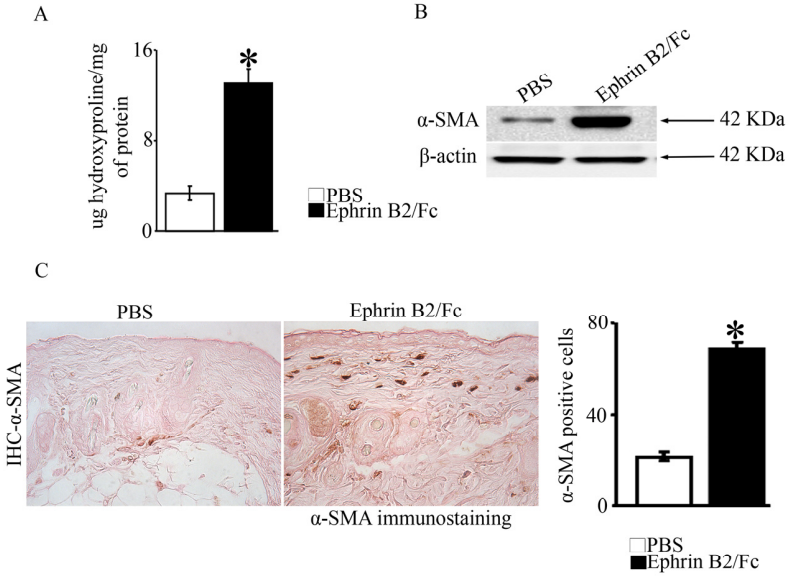
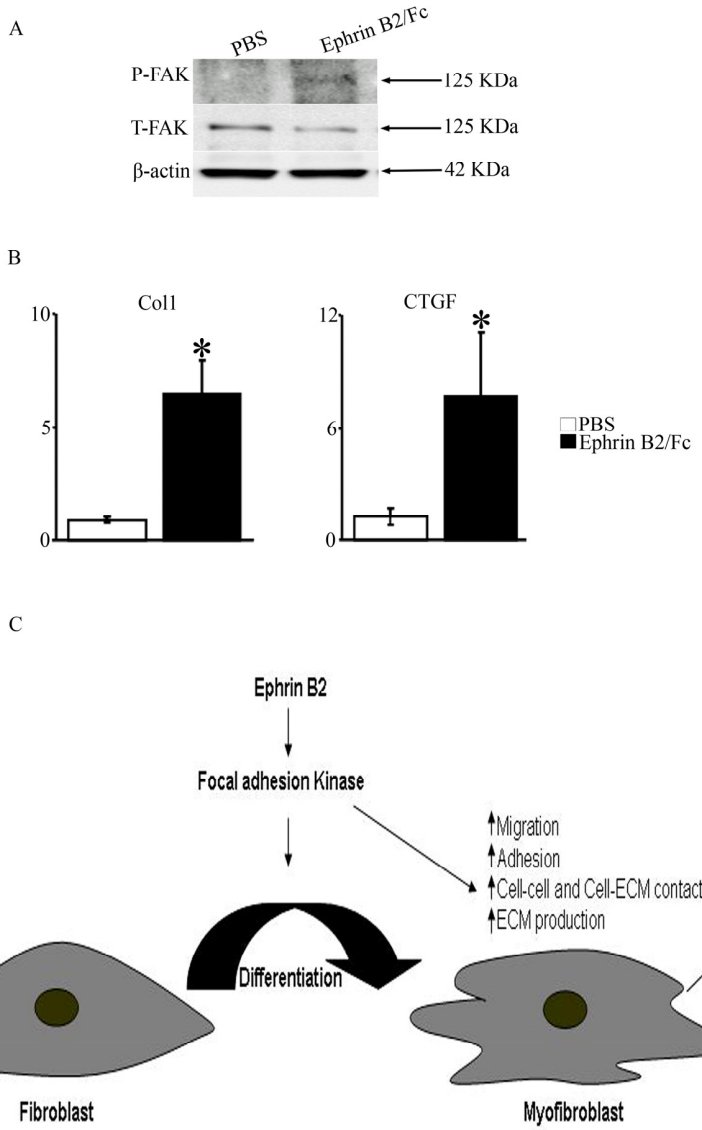

A
16
8
0
ug hydroxyproline/mg of protein
*
PBS
Ephrin B2/Fc
B
PBS
Ephrin B2/Fc
α-SMA
β-actin
42 KDa
42 KDa
C
PBS
Ephrin B2/Fc
IHC-α-SMA
α-SMA immunostaining
80
40
0
α-SMA positive cells
*
PBS
Ephrin B2/Fc

Figure 6


A
PBS
Ephrin B2/Fc
P-FAK
T-FAK
β-actin
125 KDa
125 KDa
42 KDa
B
Col1
CTGF
10
5
0
12
6
0
*
*
PBS
Ephrin B2/Fc
C
Ephrin B2
Focal adhesion Kinase
Migration
Adhesion
Cell-cell and Cell-ECM contact
ECM production
Fibrosis
Differentiation
Fibroblast
Myofibroblast