Antifibrotic effects of the secretome of human adipose-derived mesenchymal stromal cells: in vitro studies

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ABSTRACT

Background: Fibrotic diseases represent an increasing cause of morbidity, mortality, and financial burden worldwide. Fibrosis is a pathological wound healing response characterized by aberrant accumulation of extracellular matrix (ECM) (e.g., collagen I). Transforming growth factor beta (TGF-β) is the main profibrotic factor. TGF-β activates fibroblasts into myofibroblasts (i.e., critical cellular effectors in fibrosis), promotes myofibroblast apoptosis resistance, increases ECM synthesis and deposition. Fibrosis can affect single or multiple organs. The prototypic multisystemic fibrotic disease is systemic sclerosis (SSc). Reversal of fibrosis requires eradicating the cause of injury, and/or eliminating myofibroblasts and remodeling the ECM. Currently, there are no treatments that result in fibrosis reversal. However, a recent proof-of-concept phase 1/2 open-label clinical trial provided preliminary evidence of efficacy for the use of human multipotent mesenchymal stromal cells (MSC) in severe diffuse SSc.

MSC are progenitor cells with proven anti-inflammatory and proangiogenic properties. These effects are mainly mediated by secreted soluble molecules and extracellular vesicles (i.e., MSC secretome). The contribution of the secretome to the MSC antifibrotic effects remains undefined. Reliable in vitro antifibrotic tests would enable the comparison of the antifibrotic effects of MSC from different donors. Those tests will also allow determining the pathogenic contribution of MSC to fibrotic diseases (e.g., SSc).

We hypothesize that in vitro the MSC secretome promotes fibrosis resolution and that SSc MSC have impaired antifibrotic effects.

This project aims are: (1) to establish reliable in vitro assays to assess the antifibrotic effects of the MSC secretome, and (2) to compare the antifibrotic potency of the MSC secretome from SSc and healthy (HC) individuals.

Methods: Adipose-derived MSC were isolated from 8 adult donors undergoing programmed surgery and characterized according to the criteria proposed by the International Society for Cellular Therapy. To evaluate the secretome, MSC conditioned medium (MSC-CM) was collected under standard conditions (resting) and following IFN-ɣ/TNF-α activation (primed). The MSC-CM antifibrotic effect was assessed in five in vitro assays: (1) inhibition of fibroblast migration; (2) inhibition of TGF-β induced fibroblast activation; (3) myofibroblast deactivation; (4) myofibroblast apoptosis sensitization, and (5) ECM remodeling. Readouts of these assays were: (1) Incucyte® Scratch Wound Analysis; (2, 3) Western blot analysis (α-SMA and procollagen I); (4) flow cytometry (Annexin V and DRAQ7); and (5) immunofluorescence (fibrillin I and fibronectin). For the comparison of SSc and age/sex-matched HC, MSC were obtained from subcutaneous adipose tissue of forearm skin biopsies. SSc MSC were isolated, characterized, and functionally evaluated.

Results: In vitro, adipose-derived MSC-CM inhibited fibroblast migration and TGF-β fibroblast activation without inducing fibroblast cell death. MSC-CM promoted myofibroblast deactivation and reduced the deposition of fibrillin-1 in the ECM. In contrast, MSC-CM did not restore myofibroblast apoptosis sensitivity. Compared to resting MSC-CM, primed MSC-CM were more potent at inhibiting TGF-β fibroblast activation and promoting myofibroblast deactivation. Resting MSC-CM from SSc and HC had similar clonogenicity, immunopotency, and in vitro antifibrotic effects.

Conclusions: In vitro assays allow the assessment of the MSC secretome antifibrotic effects. Priming enhances the anti-fibrotic effects of the MSC-secretome. Preliminary results suggest that in vitro, the antifibrotic properties of the SSc MSC secretome are preserved.

RÉSUMÉ

Contexte: Les maladies fibrotiques représentent une cause croissante de morbidité, de mortalité et de charge financière. La fibrose est une réaction pathologique de cicatrisation caractérisée par une accumulation aberrante de la matrice extracellulaire (MEC). Le facteur de croissance transformant bêta (TGF-β) est le principal facteur profibrotique qui active les fibroblastes en myofibroblastes (les effecteurs cellulaires dans la fibrose), favorise la résistance des myofibroblastes à l'apoptose et augmente le dépôt de la MEC. La fibrose peut affecter un seul ou plusieurs organes. La maladie fibrotique multisystémique prototypique est la sclérose systémique (ScS). Pour inverser la fibrose, il faut éradiquer la cause de la lésion, et/ou éliminer les myofibroblastes et remodeler la MEC. Il n'existe aucun traitement qui permette d'inverser la fibrose. Cependant, un récent essai clinique ouvert de phase 1/2 de preuve de concept a suggéré l'efficacité de cellules stromales mésenchymateuses multipotentes humaines (CSM) dans la ScS diffuse sévère.

Les CSM sont des cellules progénitrices avec des propriétés anti-inflammatoires et proangiogéniques. Ces effets sont principalement médiés par des molécules solubles sécrétées et des vésicules extracellulaires [c'est-à-dire le sécrétome des CSM ou le milieu conditionné des CSM (CSM-MC)]. La contribution du sécrétome aux effets antifibrotiques des CSM reste indéfinie. Des tests antifibrotiques in vitro fiables permettraient de comparer les effets antifibrotiques des CSM provenant de différents donneurs. Ils permettront également de déterminer la contribution pathogénique des CSM aux maladies fibrotiques.

Nous émettons l'hypothèse qu'in vitro, le sécrétome des CSM favorise la résolution de la fibrose et que les CSM de la ScS ont des effets antifibrotiques altérés.

Les objectifs de ce projet sont: d'établir des tests in vitro fiables pour évaluer les effets antifibrotiques du CSM-MC, et de comparer la puissance antifibrotique du CSM-MC provenant de personnes atteintes de ScS et de témoins en santé (TS).

Méthodes: Des CSM dérivées de tissue adipeux ont été isolées de 8 donneurs adultes ayant subi une chirurgie programmée. Le CSM-MC a été collecté à partir de MSC cultivées dans des conditions standard (au repos) et après activation par IFN-ɣ/TNF-α (activé). L'effet antifibrotique du CSM-MC a été évalué dans cinq essais in vitro: l'inhibition de la migration des fibroblastes (‘Incucyte® Scratch Wound Analysis’); l'inhibition de l'activation des fibroblastes induite par le TGF-β et la désactivation des myofibroblastes (Western blot: α-SMA et procollagène I); la sensibilisation à l'apoptose des myofibroblastes (cytométrie de flux: Annexin V et DRAQ7) et le remodelage de la MEC (immunofluorescence: fibrilline I et fibronectine). Pour la comparaison entre la ScS et les TS, les CSM ont été obtenues à partir du tissu adipeux de biopsies de peau d'avant-bras. Les CSM-ScS et CSM-TS ont été caractérisées et évaluées fonctionnellement.

Résultats: In vitro, les CSM-MC dérivées de tissu adipeux ont inhibé la migration des fibroblastes et l'activation des fibroblastes par le TGF-β sans induire la mort cellulaire des fibroblastes. La CSM-MC a favorisé la désactivation des myofibroblastes et a réduit le dépôt de fibrilline-1 dans la MEC. En revanche, la CSM-MC n'a pas restauré la sensibilité des myofibroblastes à l'apoptose. Par rapport aux CSM-MC au repos, les CSM-MC activées étaient plus efficaces pour inhiber l'activation des fibroblastes par le TGF-β et favoriser la désactivation des myofibroblastes. Les CSM-MC au repos provenant de la

ScS et de TS avaient une clonogénicité, une immunopotence et des effets antifibrotiques in vitro similaires.

Conclusions: Les tests in vitro permettent d'évaluer les effets antifibrotiques du sécrétome des CSM. L'activation renforce les effets antifibrotiques du sécrétome de CSM. Les résultats préliminaires suggèrent qu'in vitro, les propriétés antifibrotiques du sécrétome de la CSM-ScS sont préservées.

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This thesis is dedicated to every patient

that comes to the consult with worries and uncertainties.

I hope science contributes to their relief.

CONTRIBUTIONS

All samples included in the experiments of this project were obtained through Ethics Review Board approved protocols (GEN-10-107: 'The effects of aging on human mesenchymal stem cells', and MEO-05-2022-8285, MP-05-2022-3080: 'Characterization of scleroderma multipotent mesenchymal stromal cells'). Adipose derived MSC were obtained from patients undergoing programmed cardiac surgery at the Royal Victoria Hospital (RVH) by Dr. Shum-Tim. Skin biopsy samples (Chapter 2, section 2.1.) were obtained by Dr Hudson from patients attending the Scleroderma Clinic at the Jewish General Hospital (JGH).

Every experiment of this project was performed by Marianela Brizio under the supervision of Dr. Colmegna except the:

- Isolation and phenotype characterization of some MSC that was performed by previous members of Dr. Colmegna's laboratory (Dr. Lora, Anastasia Cheng, Peter Jeon and others).
- Fibroblast migration assay (section 2.4.1. and 3.3.2.) that was performed by Alice Freton and Dr. Lora.
- Dose-dependent experiment shown in Figure 12 C that was done by Ingrid Anaya.

The concept and design of this thesis was directed by Dr. Colmegna and reviewed by Dr Dominique Farge and Dr. Joyce Rauch.

ABBREVIATIONS

ADAM12	Disintegrin and metalloproteinase domain-containing protein 12
AT	Adipose tissue
BCL-2	B-cell lymphoma 2
BM	Bone marrow
CKD	Chronic kidney disease
CLD	Chronic liver disease
CM	Conditioned medium
CTGF	Connective tissue growth factor
DMEM	Dulbecco's modified Eagle's medium
DPP4	Dipeptidyl peptidase 4
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
EGFR	Epidermal growth factor receptor
ESRD	End-stage renal disease
FABP4	Fatty acid binding protein 4
FGF	Fibroblast growth factor
FNDC4	Fibronectin type III domain containing 4
GAG	Glycosaminoglycan
GvHD	Graft versus host disease
HGF	Hepatocyte growth factor
HLA	Human leucocyte antigen
IDO	Indoleamine 2,3-dioxygenase 1 enzyme
IFN-ɣ	Interferon-gamma
IGF1R	Insulin-like growth factor 1 receptor
IL-10	Interleukin-10
IPF	Idiopathic pulmonary fibrosis
LAP	Latency associated peptide
LLC	Large latent complex
lncRNA	Long noncoding RNA

LOX	Lysyl oxidases
LRP1	Low-density lipoprotein-receptor related protein 1
LTBP	Latent TGF-β binding protein
MFGE8	Milk fat globule-EGF factor 8 protein
MMF	Mycophenolate mofetil
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal cells
NASH	Non-alcoholic steatohepatitis
PAI-1	Plasminogen activator inhibitor-1
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2
PPARɣ	Peroxisome proliferator- activated receptor gamma
RAAS	Renin–angiotensin–aldosterone system
RWD	Relative wound density
SFRP2	Secreted frizzled-related protein 2
SLRPs	Small leucine-rich proteoglycans
Smad	Small mothers against decapentaplegic
SSc	Systemic sclerosis
TGF-β	Transforming growth factor- beta
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor- alpha
tPA	Tissue plasminogen activator
TβRI	TGF- β receptor type I
TβRII	TGF- β receptor type II
UC	Umbilical cord
VEGF	Vascular endothelial growth factor
WB	Western blot
α-SMA	Alpha-smooth muscle actin

CHAPTER 1. BACKGROUND

Section 1. Wound healing: a fundamental repair process for human survival

1.1.1. Definition and phases

Wound healing is the replacement and regeneration of destroyed or damaged tissue by newly produced tissue [1]. This is a fundamental evolutionarily preserved process for human survival [2]. Although there are multiple causes of tissue damage (e.g., trauma, chemicals, toxins, drugs, immune-mediated, and ischemic), they all trigger the same immediate repair response, which consists of four overlapping and regulated steps: hemostasis, inflammation, cell proliferation, and tissue regeneration [2, 3].

Hemostasis is the first phase following an injury. Tissue damage triggers the coagulation cascade leading to platelet influx and the formation of a fibrin and fibronectin clot. Platelet degranulation releases multiple cytokines and growth factors, including transforming growth factor-beta (TGF-β) [3]. This is followed by an acute inflammatory reaction with neutrophil recruitment due to complement activation, platelet activation, and the presence of microorganisms [2]. This is the start of the inflammatory phase. Following 48 h, monocytes are attracted to the site of injury and differentiate into macrophages which contributes to limiting infection, clearing debris, and attracting fibroblasts [4]. The third wound-healing phase occurs two to ten days after injury [5]. The proliferation phase is characterized by the migration and proliferation of fibroblasts, endothelial cells and epithelial cells. These cellular components, together with immune cells, generate a loose connective tissue with new capillaries (i.e., granulation tissue) and an immature extracellular matrix (ECM) that replaces the initial clot [6]. The granulation tissue typically grows from the base of a wound. The ECM, initially composed of a network of type III

collagen, mechanically stabilizes the damage and immobilizes growth factors required to promote angiogenesis and stromal regeneration, including TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) [7]. These factors also attract fibroblasts from the edge of the wound or the bone marrow and activate them into myofibroblasts. Activated myofibroblasts have a contractile capacity that promotes closing the wound edges [8]. In addition, fibroblasts and myofibroblasts interact and produce stronger, long-stranded type I collagen transforming the ECM into a mature scar. The last stage of wound repair, remodeling and regeneration, occurs two to three weeks after injury. This phase, which can last for more than twelve months, requires the cessation of the inflammatory response and a switch in myofibroblast function. Myofibroblasts can deactivate and return to the low activity state characteristic of fibroblasts in healthy tissues. As an alternative, they can assume roles that were not characteristic of their precursor cells, such as becoming scar-resolving or senescent cells. Myofibroblasts can also be eliminated by apoptosis (programmed cell death) [9]. Apoptosis of immune cells and myofibroblasts, together with the reversion of the myofibroblast phenotype, are fundamental to limit ECM production, achieving a proper wound-healing response [10]. Myofibroblasts that do not undergo apoptosis adopt a new role cleaving ECM fibers and secreting proteases [i.e., matrix metalloproteinases (MMPs)]. This promotes ECM degradation and remodeling through crosslinking fibrillar collagen I, thereby strengthening the repaired tissue [9, 11]. Failure in myofibroblast apoptosis or ECM remodeling, is associated with abnormal wound healing (i.e., fibrosis) [9].

Wound healing is key to preserving the structural characteristics of an organ and stimulates the migration, activation, and differentiation of functional cell types [12]. The ultimate restoration of the functioning of an organ depends on its regenerative potential, which varies from organ to organ. For example, the liver has a high regenerative capacity, and 70% of its function can be re-established after injury [13]. In most other organs, an injury would result in scar tissue that preserves organ structure but not function. The wound-healing process is summarized in Figure 1.

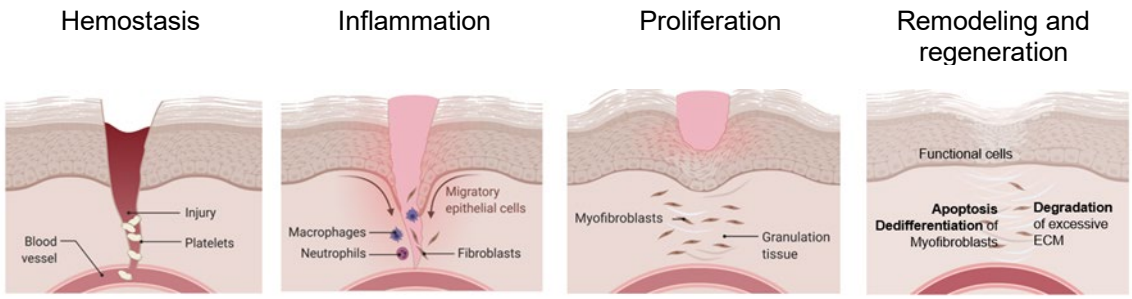


Figure 1. Wound healing process

Section 2. Fibrosis: a pathological mechanism of repair

1.2.1. Definition

Fibrosis is a pathological tissue repair process characterized by aberrant accumulation of ECM components, mainly fibrillar collagen type I, that results in persistent and non-resolving scarring [14]. The accumulation of ECM alters the structure and function of the affected organ, resulting in distorted architecture, impaired regeneration, and, ultimately, organ failure [15]. The hallmark of tissue fibrosis is the persistence of activated myofibroblasts and the absence of ECM degradation and remodeling. TGF-β is the main profibrotic factor given its critical role in myofibroblast activation (Section

1.2.4.1.). The culprit of fibrosis is still unknown, but repetitive or severe tissue damage and chronic inflammation contribute to abnormal tissue repair [10].

Fibrosis can be organ-specific or multisystemic and can be a primary or secondary pathogenic mechanism in highly prevalent diseases. Organ-specific fibrotic diseases include myelofibrosis, kidney, pancreas or heart fibrosis, cirrhosis, and pulmonary fibrosis [10]. In contrast, the prototypic profibrotic systemic disease is systemic sclerosis (SSc, also known as scleroderma) with other examples including graft versus host disease and nephrogenic systemic fibrosis [16].

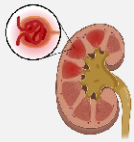
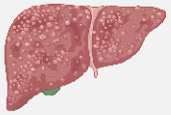
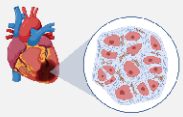
1.2.2. Burden

An uncontrolled fibrotic response is implicated in multiple prevalent diseases, in particular in most chronic inflammatory diseases. Fibrosis development usually takes a similar course independent of the underlying organ or disease and can be considered a final pathological process of a maladaptive repair [17]. Fibrotic diseases represent an increasing cause of morbidity, mortality, and financial burden worldwide [18]. The annual combined incidence of major fibrosis-related diseases is approximately 5,000 per 100,000 person-years [19] and are reported to contribute to 30-45% of all-cause mortality in developed countries [10, 20]. However, several groups have called for accurate estimates of specific fibrotic diseases [21, 22]. The burden of fibrosis is not only explained by the large number of affected individuals, but also by the incomplete understanding of the pathogenesis of the fibrotic process, the marked etiological and clinical heterogeneity, the absence of validated biomarkers, and, most importantly, the lack of a ‘cure for fibrosis’ [16]. The economic impact of fibrosis, although difficult to assess precisely, is estimated to be in the tens of billions of dollars resulting in a huge burden to public health [2].

Three specific examples help illustrate the increasing and global burden of fibrotic diseases. Chronic kidney disease (CKD) has a major impact in global health as it affects 10% of the world's population [23] and half of adults above age 70 [24]. Renal fibrosis (i.e., glomerulosclerosis, tubular atrophy and dilation, tubulointerstitial fibrosis, and capillary rarefaction) is the common endpoint of almost all progressive kidney diseases [25]. Fibrosis in CKD is a progressive process that not only deteriorates the kidney, but also the heart function [17]. Between 1990 and 2017, the prevalence of CKD has increased by 29.3% (26.4 to 32.6) and the global increase in mortality from CKD by 41.5% (35.2 to 46.5) [26]. Mortality from CKD and cardiovascular disease deaths attributable to impaired kidney function caused 4.6% (4.3 to 5.0) of global deaths in 2017. This made CKD the 12th leading cause of death globally in 2017 compared to being the 17th cause in 1990 [26]. Similar trends are observed in other fibrotic diseases, including chronic liver disease (CLD). Cirrhosis ranked 11th as a leading cause of death and 15th as a leading cause of morbidity, accounting for 2.2% of deaths and 1.5% of disability-adjusted life years worldwide in 2016. The absolute number of CLD cases (inclusive of any stage of disease severity) in 2017 was estimated at 1.5 billion worldwide [27]. Cirrhosis accounted for an estimated 1.32 million deaths in the same year [21]. As a final example to highlight the burden of fibrosis, myocardial fibrosis is a significant global health problem associated with nearly all forms of heart disease. Myocardial fibrosis leading to heart failure is characterized by interstitial fibrosis, chamber remodeling, and reduced ventricular compliance [28]. Heart failure is a predominant cause of mortality in the United States, that accounts for nearly 800,000 annual deaths. Its direct and indirect costs in 2011 were ~$320 billion and predictions suggest that they will rise to >$900 billion by 2030 [29].

Given the burden of fibrosis, understanding its pathogenesis particularly by deciphering the specific checkpoints that regulate the fibrotic process and promote fibrosis resolution is fundamental. A better understanding of fibrosis may enable the development of novel approaches to fibrosis modulation and the reduction of personal, societal, and global suffering. *My project will contribute new evidence to this purpose.*

Table 1. Organ fibrosis: associated etiologies and prevalence [30-35].

Organ Fibrosis					
Top 3 etiologies	Diabetes mellitus Hypertension Glomerulonephritis	Viral hepatitis Alcohol induced Non-alcoholic steatohepatitis (NASH)	Hypertension Coronary artery disease Aortic stenosis	Idiopathic pulmonary fibrosis Occupational diseases Sarcoidosis	Physical injury Systemic sclerosis Graft versus host disease
Prevalence	CKD ~ 10% ESRD ~ 0.2%	Cirrhosis (autopsy studies) ~ 5-10%	Diastolic heart Failure ~1%	IPF: 0.33-4.51 per 10,000 persons	Systemic sclerosis: 7.2–33.9 and 13.5–44.3 per 100,000 individuals in Europe and North America

1.2.3. Clinical prototype: Systemic sclerosis

SSc is the prototypical systemic inflammatory fibrotic disease [36]. It affects approximately 40,000 Canadians and represents a model for most other fibrotic processes. Two main subtypes of SSc are defined according to the extent of skin involvement: diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc. dcSSc (referred as SSc throughout this thesis) is the subtype of greater concern because of its rapid progression and high prevalence of early lung, heart, and kidney involvement. SSc is a chronic multisystem, autoimmune disease characterized by the pathogenic triad of early inflammatory features, vascular hyper-reactivity, and progressive skin and internal organs

fibrosis. SSc severely impacts patients' quality of life (e.g., finger ulcers, joint contractures, gastroesophageal reflux disease, malabsorption, fecal incontinence, and exertional dyspnea) and accounts for high mortality (i.e., mortality rates in SSc are 3.5 times higher than in the general population) [37-41]. There is no cure for SSc [42]. Immunosuppressive drugs (e.g., cyclophosphamide and mycophenolate mofetil) have at best modest effects in SSc stabilizing disease without improving survival [42, 43]. A minority of highly selected SSc patients with early rapidly progressive skin disease are candidates for autologous hematopoietic stem cell therapy (HSCT) [40, 44-47]. In those patients, HSCT improves survival, skin thickness, and lung function compared to intravenous cyclophosphamide (moderate-certainty evidence) and enhances their health-related quality of life [44, 48-51]. HSCT also normalizes the SSc molecular signatures by 'correcting' the autoreactive immune response [52]. However, HSCT has a high risk of early treatment-related mortality and serious short- and long-term adverse events, such that only a minority of patients (<5%) are eligible [53, 54]. SSc has an unmet therapeutic need since safe and effective disease-modifying therapies are lacking, and both long-term morbidity and mortality remain unacceptably high. *In subsequent sections of this thesis, I will refer to SSc and focus on the function of mesenchymal stromal cells (MSC, section 1.4.6.) and their potential contribution to fibrosis.*

1.2.4. Effectors

Multiple factors are implicated in fibrogenesis. They include mechanical traction, innate and adaptive cellular components of the inflammatory response, multiple growth factors and cytokines, fibroblasts and myofibroblasts, endothelial cells, and ECM. *Here, we will limit the discussion to myofibroblasts, TGF-β and ECM, the three effectors*

in the in vitro assays we established to assess the antifibrotic effects of the conditioned medium of mesenchymal stromal cells (MSC-CM).

1.2.4.1. Myofibroblasts

Myofibroblasts are heterogeneous contractile non-muscle cells, activated in response to tissue injury with the primary task of repairing lost or damaged ECM. As described in Section 1, collagen production and myofibroblast contraction are part of the normal wound healing response and crucial to replace the damaged tissue [8]. However, the persistence of myofibroblasts in tissues leads to a distorted ECM and impairs tissue regeneration. For this reason, myofibroblasts are attractive cell targets for therapeutics [55].

Myofibroblast activation involves the up-regulation of profibrotic genes that results in increased synthesis of ECM (e.g., different types of collagens, fibronectin, and glycosaminoglycans) and the acquisition of contractile properties due to the neo-expression of the alpha-smooth muscle isoform of actin (α-SMA) and polymerization of granular actin into contractile actin bundles called stress fibers [56]. The modification of the cytoskeleton into organized stress fibers is a unique characteristic of myofibroblasts that distinguishes them from other non-muscle cells [56]*. In this project, myofibroblasts were required to fulfill three minimal requirements: the expression of α-SMA, the formation of stress fibers in vitro (contractile capacity) and collagen I synthesis [7].*

The 'myofibroblast phenotype', is acquired by several cells following activation due to a biochemical stimuli, such as TGF-β, and due to mechanical traction generated by an injury [8]. Most myofibroblasts derive from local fibroblasts. Less frequently, myofibroblasts originate from epithelial and endothelial cells through a process known as

‘epithelial-mesenchymal transition’. The latter involves the loss of apical-basal polarity and tight intercellular junctions from epithelial and endothelial cells, that acquire a mesenchymal expression profile [57, 58].

In fibrosis (e.g., SSc), myofibroblasts are resistant to apoptosis [59]. This resistance is mediated by TGF-β–dependent and -independent pro-survival mechanisms. The balance between multiple pro-apoptotic and anti-apoptotic BCL-2 family proteins instructs myofibroblasts to survive despite the normal proapoptotic signals accumulated during the wound healing process [9]. Targeting the BCL-2 pathway that mediates resistance to apoptosis induces cell death and reverses dermal fibrosis in mouse models of SSc [60]. *In this project, we confirmed that TGF-β treatment confers apoptosis resistance to myofibroblasts.*

Myofibroblasts are found in granulation and fibrotic tissues as well as in the stroma of tumors but are not present in healthy tissues [61]. The increased number of myofibroblasts in fibrotic organs (i.e. skin biopsies from SSc patients) correlates with clinical measures of fibrosis (i.e. modified Rodnan skin score) [62]. Of interest, recent studies allowed tracking specific fibroblast subsets that derive into myofibroblasts in SSc [63-65]. Single-cell RNA sequencing of SSc skin biopsy samples showed a population of dermal fibroblasts expressing secreted frizzled-related protein 2/Dipeptidyl Peptidase 4 (SFRP2/DPP4) that differentiated into myofibroblasts characterized by an upregulation of SFRP4, ADAM12 and CTGF [63]. Knockdown of ADAM12 in mesenchymal cells was sufficient to limit generation of myofibroblasts and collagen accumulation [65]. Moreover, perivascular cells in diffuse cutaneous SSc overexpress activated ADAM12 and are

related to fibrosis [64]. *We assessed the expression of ADAM12 and CTGF as part of the myofibroblast characterization.*

TGF-β activation induces a metabolic reprogramming of myofibroblasts to facilitate energy-consuming cellular functions (i.e., protein synthesis), these altered metabolic pathways contribute to fibrosis in several ways [66]. Lactate, generated by glycolysis to provide a rapid energy source, reduces extracellular pH, activating TGF-β and perpetuating the myofibroblast phenotype. Glycolysis also increases the amounts of pyruvate that are converted into acetyl-CoA in the mitochondrial matrix before entering the citric acid cycle. Intermediate metabolites, such as succinate, are generated from acetyl-CoA which promotes fibrosis. Dysregulated glycolysis is implicated in experimental models of fibrosis, and inhibition of glycolysis reduces ECM accumulation [67]. Activated fibroblasts also undergo changes in amino acid metabolism upregulating glutaminase-1 (*GLS-1*) [68]. In lung myofibroblasts, inhibition of *GLS-1* decreases the expression of collagens but does not modify fibronectin, elastin, or α-SMA. In vivo, the inhibition of *GLS-1* ameliorates bleomycin- and TGF-β1-induced pulmonary fibrosis [10]. *We measured GLS-1 expression to assess for the metabolic reprograming of myofibroblasts following TGF-β induction*.

1.2.4.2. TGF-β

The TGF-β family of growth factors is the most extensively studied mediator of fibroblast activation, of which TGF-β is likely to play the greatest role in pathological fibrosis. TGF-β is a pleiotropic dimeric growth factor involved in embryonic development, tissue homeostasis in adulthood, and in disease states such as fibrosis and cancer [69]. There are three isoforms identified in mammals: TGF-β1, TGF-β2 and TGF-β3 [70]. TGF-

β1 plays a fundamental role in wound healing and is the most potent profibrotic growth factor modulating proliferation and differentiation, cell adhesion, immune responses, and extracellular matrix deposition [9]. TGF-β1 increases matrix protein synthesis and modulates the balance between MMPs and their antagonists, tissue inhibitors of metalloproteinases (TIMPs) [71]. These multiple functions are modulated by TGF-β1 synthesis, the extracellular activation from its latent form and subsequent stimulation of several molecular pathways.

TGF-β1 is produced by multiple cells: fibroblasts, macrophages [72], platelets [73], T cells [74] and mast cells [75]. TGF-β1 is synthesized as a homodimer containing a mature 25 kDa portion and a latency-associated peptide (LAP). In the endoplasmic reticulum, the homodimer is linked to a latent TGF-β binding protein (LTBP) [76]. This complex is transported to the Golgi where the LAP is cleaved and rebounds non-covalently, conferring latency to TGF-β restricting its activity [76]. The latent TGF-β is secreted in a large latent complex (LLC: TGF-β, LAP and LTBP) or as the TGF-β/LAP complex, known as the small latent complex. These complexes are proteolytically cleaved to be activated in an integrin-mediated process [28]. LTBP allows the ECM to sequester latent TGF-β by proteoglycans, fibrillin, collagens, or fibronectin, and can be released when damage occurs. Multiple biochemical and biomechanical factors release TGF-β1 from the latency complex [76]. The best characterized activators are cell-surface integrins in association with ECM traction [76]. Thrombospondin-1, MMPs, reactive oxygen species, low pH and lactic acid are also involved in releasing latent TGF-β1 [76]. To ensure that the TGF-β signal does not persist after activation, mature TGF-β is rapidly cleared from the extracellular space. This is partially attributed to α_2-macroglobulin that

mediates TGF-β endocytosis [77]. There are other factors that have been reported to play an inhibitory role sequestering TGF-β, such as decorin, a small proteoglycan that binds and blocks its activity [78].

TGF-β molecular pathways are triggered by its binding to a membrane receptor serine/threonine kinase family of type I (TβRI) and type II (TβRII) receptors [79]. The TβRI, also known as activin receptor-like kinase (ALK), is thought to be predominantly responsible for the fibrotic activities of TGF-β1. The TGF-β1 canonical pathway involves the phosphorylation of Smad2/3, which subsequently binds Smad4 and translocates to the nucleus [80]. The complex acts as a transcription factor, inducing the activation of numerous pro-fibrotic genes. Fibroblasts isolated from Smad3-deficient mice are resistant to TGF-β1-induced expression of ECM proteins [81, 82]. The persistence of activated fibroblasts is considered the result of perpetual TGF-β signaling [83].

In addition to the Smad2/3-mediated pathways, TGF-β can also induce non-canonical signaling that involves several mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK) and p38 [84, 85]. Persistent activation of these non-Smad pathways, promoting survival of myofibroblasts, seems to be predominant in fibrotic diseases [85, 86]. TGF-β1 induces the expression of ADAM12 by activating the PI3K and MAPK signaling pathways [87]. In adult tissues, the expression of ADAM12 is extremely low, but increases under pathological conditions, including carcinogenesis [88] and SSc [64]. A role for p38 in mediating TGF-β-induced collagen I expression has been associated with an increased phosphorylation and activation of p38 in SSc fibroblasts [53]. Moreover, in pre-clinical studies p38 blockage had therapeutic benefit in lung and renal fibrosis [89]. The TGF-β/ALK1/Smad1/5 pathway also plays a role in fibrosis [85].

In a subset of SSc fibroblasts, up regulation of collagen I and connective tissue growth factor (CTGF/CCN2) does not involve Smad2/3 activation but is mediated by ALK1/Smad1 and ERK1/2 pathways. CTGF/CCN2 is also a primary effector of this pathway, thus establishing an autocrine loop that amplifies TGF-β signaling [90]. In vitro, Smad-mediated signaling and TGF-β–induced non-Smad pathways are often interconnected.

The TGF-β pathway is regulated by several mechanisms in order to protect the injured tissue from an overactive fibrotic response. A subclass of Smads, called the inhibitory Smads (Smad6 and Smad7), antagonizes TGF-β signaling [12]. On one hand, Smad7 competes with Smad2/3 for binding to the activated TβRI, thereby preventing the phosphorylation of Smad2/3. Also, Smad7 acts as an adaptor protein that recruits Smurf1/2, resulting in the ubiquitination of TβRI, followed by receptor degradation, and termination of signaling. In SSc, Smad7 inhibitory function was reported to be altered [91]. TβRI is increased in SSc fibroblasts compared with normal fibroblasts, and the overexpression of Smurf1 and/or Smurf2 do not affect TβRI protein levels [92]. Overall, the critical role of TGF-β in wound healing is reflected by the complex and multi-step regulation of its synthesis, secretion, activation and de-activation.

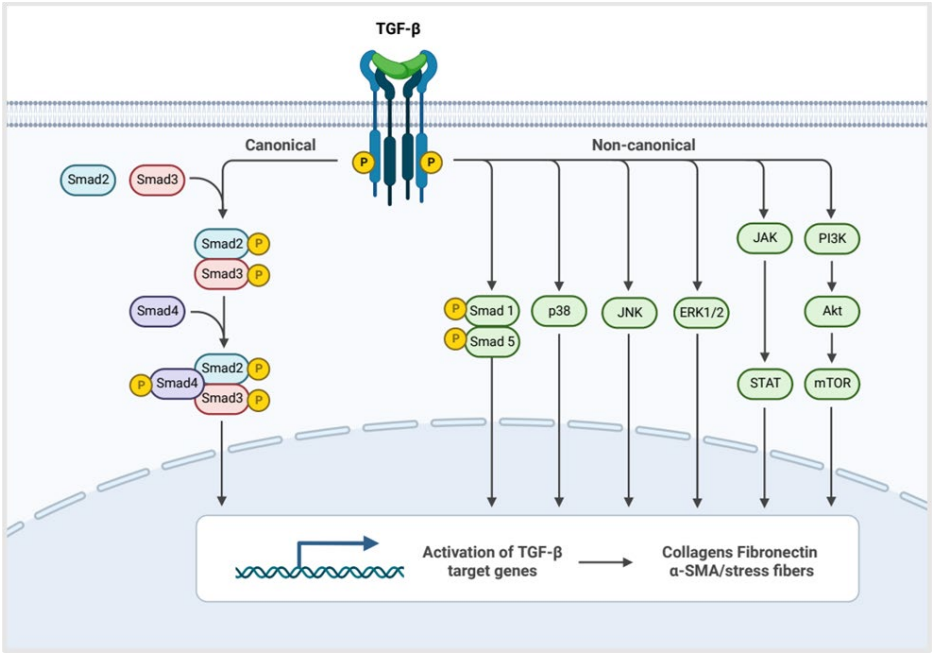


Figure 2. TGF-β canonical and non-canonical pathways

In vitro, TGF-β1 stimulation of healthy skin fibroblasts is sufficient to induce a profibrotic phenotype resembling that of SSc fibroblasts [93]. TGF-β1 activated fibroblasts express higher levels of *ACTA2, COL1A1, COL3A1* and *IL-6* and have lower protein levels of MMP1/TIMP1, MMP3/TIMP2 and COX2 [93]. When ECM profiles induced by TGF-β, PDGF and IL-6 in dermal fibroblasts were compared in vitro, TGF-β was the most prominent factor increasing the expression of *ACTA2* and the synthesis of fibronectin and collagen I [94]. PDGF promoted the synthesis of other types of collagens such as collagen V and VI. IL-6 increased type I collagen at both the gene and protein levels and, although it did not alter fibronectin genes, it decreased fibronectin protein levels. *This justifies why we treated skin fibroblasts with TGF-β1 (referred as TGF-β throughout this thesis) to generate myofibroblasts and to modulate ECM components.*

1.2.4.3. Extracellular matrix

The ECM is a three-dimensional, non-cellular structure that is present in all tissues. It is essential for the parenchyma, i.e., the functional cells of an organ, providing physical support for tissue integrity and elasticity, and mediating biochemical and biomechanical signaling implicated in morphogenesis, differentiation and homeostasis [95, 96].

The importance of ECM is reflected by diseases where gene expression of ECM components is altered. Altered collagen expression occurs in osteogenesis imperfecta, chondrodysplasias, Ehlers-Danlos syndrome, Alport syndrome, certain subtypes of epidermolysis bullosa, Knobloch syndrome and some cases of osteoporosis, arterial aneurysms, osteoarthrosis, and intervertebral disc disease [97]. Marfan's syndrome is attributed to a mutation in fibrillin 1 (*FBN1*). Even in the absence of altered gene expression of ECM components, an abnormal ECM such as that in interstitial pulmonary fibrosis, can induce myofibroblast activation [98] and downregulate microRNA-29 (miR-29), a negative regulator of stromal genes [99, 100]. In fibrotic diseases ECM accumulation and stiffness is a diagnostic biomarker associated with altered organ function [100].

The ECM is composed of two main classes of macromolecules: proteoglycans and fibrous proteins [95]. Proteoglycans fill most of the extracellular interstitial space in the form of a hydrated gel and have a wide variety of functions that reflect their unique buffering, hydration, binding, and force-resistance properties [95]. Proteoglycans are composed of glycosaminoglycan (GAG) chains covalently linked to a specific protein core. Proteoglycans are classified according to their core proteins, localization and GAG composition in three main families: small leucine-rich proteoglycans (SLRPs), modular

proteoglycans and cell-surface proteoglycans. SLRPs are involved in multiple signaling pathways, including regulation of inflammatory responses, as well as, binding and modulating TGF-β activation [101].

Fibrous proteins include collagens, elastins, fibronectins, fibrillins, and laminins [95]. Three of these fibrous proteins are relevant to our work. *Collagen* is the most abundant constituent of both normal and fibrotic ECM [102]. Collagens are transcribed and secreted by fibroblasts, and contribute to the tensile strength, regulate cell adhesion, support chemotaxis and migration, and direct tissue development [95]. Fibroblasts are capable of sensing matrix tension and to organizing and aligning collagen fibrils [103]. Most collagen molecules form a triple-stranded helix that later can assemble into supramolecular complexes, such as fibrils and networks, depending on the type of collagen [104]. Synthesis of collagen type I involves several enzymatic post-translational modifications, mainly the hydroxylation of proline and lysine residues, glycosylation of lysine, and cleavage of N- and C-terminal pro-peptides. Following their cleavage, collagen fibrils are strengthened by the covalent crosslinking between lysine residues of the constituent collagen molecules by lysyl oxidases (LOX) [104]. Excessive covalent cross-linking of collagen fibers by the LOX and tissue transglutaminase may further impair collagen turnover in fibrosis. Therapeutic strategies targeting LOX enzymes have shown reductions in tissue fibrosis in a rodent model of SSc [105]. Another fibrous protein relevant to our work is *fibronectin*, a “master organizer” in matrix assembly. Fibronectin forms bridges between cell surface receptors (e.g., integrins) and compounds such collagen, proteoglycans, and other focal adhesion molecules mediating cell attachment and function. Fibronectin is key in directing the organization of the interstitial ECM: its

deposits precedes collagen accumulation [106]. Cellular traction can stretch fibronectin multiple times, unfolding the protein and exposing integrin binding sites, inducing TGF-β activation as well as the activation of other growth factors. The last fibrous protein to highlight is *fibrillin-1*. These microfibrils regulate TGF-β bioavailability: fibrillin-1 and TGF-β interact through the LTBP protein [107]. Autoantibodies to a fibrillin-1 proline-rich region induce fibroblast activation possibly by releasing sequestered TGF-β1 from microfibrils [107].

Excessive accumulation of ECM is not only attributed to overproduction, but also to impaired degradation of fibrinous proteins due to an imbalance between proteolytic enzymes and their inhibitors [14]. TGF-β represses MMP-1 [108] and induces TIMP-1 and plasminogen activator inhibitor-1 (PAI-1), suppressing matrix-degradation. PAI-1 is a serine protease inhibitor that promotes fibrinolysis by inhibiting tissue plasminogen activator (tPA) and urokinase. Besides protease-mediated degradation by MMPs and PAI-1, cellular re-uptake of the ECM can also limit fibrosis. Milk fat globule-EGF factor 8 protein (MFGE8) inhibits pulmonary fibrosis by binding collagen and promoting its uptake by macrophages [109]. Collagen can then be degraded by lysosomal pathways [96].

ECM and cellular components form a complex and tightly regulated system that, when distorted, can lead to fibrosis. Modulating ECM is key to restoring normal tissue architecture. *We used collagen I, fibronectin and fibrillin-1 as readouts in our in vitro assays. In addition, we evaluated MMPs and TIMPs in the MSC secretome.*

Section 3. Fibrosis resolution and antifibrotic agents

1.3.1 Fibrosis resolution

Although fibrosis was initially thought to be an irreversible process, current evidence indicates that when the underlying causes of injury are eradicated, fibrosis can resolve [14, 110]. Proof of concept examples are the resolution of schistosomiasis-induced liver fibrosis upon treatment of schistosomiasis, and the improvement of diabetic nephropathy in pancreas transplant recipients [111]. However, even when the cause of injury is identified and eliminated, fibrosis may persist and progress [112]. Furthermore, for conditions in which the etiology of fibrosis is unclear (e.g., idiopathic pulmonary fibrosis) or for which effective treatments for the underlying cause are not available, altering the fibrotic process can result in fibrosis amelioration.

Fibrosis resolution, and the extent of the process, varies depending on the organ involved, the nature and chronicity of the injury, and host-specific factors including age, immunocompetence, and genetic predisposition [18]. However, common mechanisms of fibrosis resolution across various organs include the degradation of the ECM; the elimination of fibrogenic myofibroblasts through apoptosis, senescence, dedifferentiation, and/or reprogramming; and the restitution of functional tissue architecture (Figure 3) [14]. The exact molecular mechanisms underlying these overlapping events and their regulation are incompletely defined.

Collagen, the most abundant constituent of normal and fibrotic ECM, is degraded by MMPs and by macrophage internalization. The relevance of collagen degradation in fibrosis resolution is evidenced by the impaired collagenolytic activity of tissues in fibrotic diseases [14, 113]. In addition, given that activated myofibroblasts are the primary source

of the fibrotic ECM, their elimination is a prerequisite for fibrosis resolution. Increased expression of pro-fibrotic BCL2 family proteins was identified in apoptosis-resistant fibroblasts isolated from patients with non-resolving acute respiratory distress syndrome [114]. Therefore, the interruption of pro-survival signaling pathways enhances the myofibroblast susceptibility to apoptosis and promotes fibrosis resolution [112]. Myofibroblast apoptosis is required in murine models for the resolution of established liver, lung, and skin fibrosis [60, 115, 116]. Besides induction of apoptosis, pharmacological approaches that deactivate or reprogram myofibroblasts can limit fibrosis.

Fibrosis is a complex and redundant process. This highlights the importance of testing the effect of 'antifibrotic candidates' in different mechanisms implicated in fibrosis resolution. We took this approach for the assessment of the antifibrotic effects of the MSC-secretome by using in vitro readouts of ECM degradation, myofibroblast deactivation, and myofibroblast apoptosis.

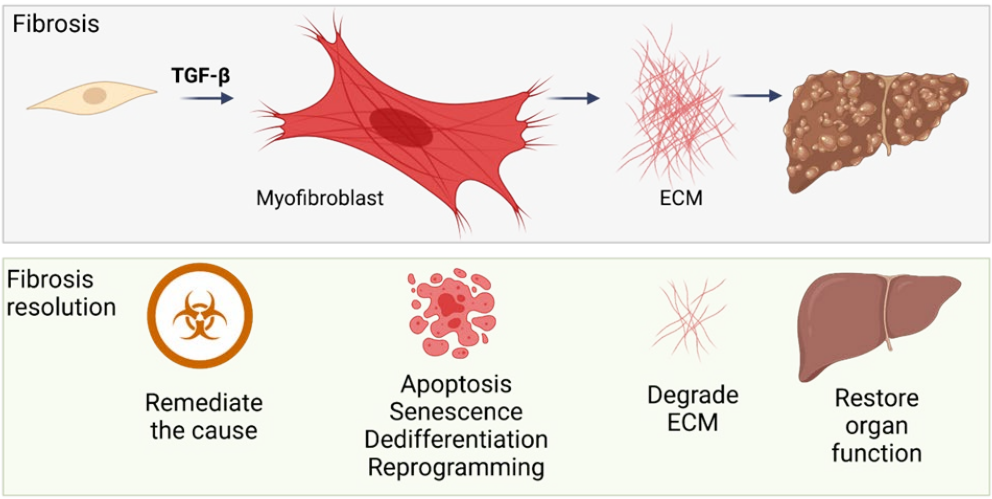


Figure 3. Fibrosis and fibrosis resolution

1.3.2. Antifibrotic agents

Several drug candidates targeting fibrosis, such as TGF-β inhibitors, endothelin inhibitors, relaxin, and others have failed clinical trials [117-119]. On the other hand, new promising targets including the long noncoding RNA (lncRNA) and noncoding RNAs (e.g., miRNA-21, miR-208a, and Meg 3) have emerged [120]. Therapeutic targets closer to the clinic tested by recent phase 2/3 clinical trials include epigallocatechin gallate (EGCG), an inhibitor of LOXL2 and TGF-β 1/2 (NCT03928847); pamrevlumab, a monoclonal antibody that targets CTGF [121]; recombinant human pentraxin-2 that inhibits differentiation of monocytes into proinflammatory macrophages and profibrotic fibroblasts [122]; and BI 1015550 an oral preferential inhibitor of phosphodiesterase (PDE) 4B [123]. BI 1015550 has combined antifibrotic and anti-inflammatory effects and prevented lung function decline in patients with IPF [123, 124]. PDE4 inhibitors block the proliferation and differentiation of fibroblasts, and their ability to produce ECM in the presence of an endogenous or exogenous cAMP trigger. In contrast to nintedanib (Section 1.3.3.), BI 1015550 inhibits TGF-β1– induced myofibroblast differentiation and ECM expression, a core fibrogenic pathway in multiple fibrotic conditions [125]. In animal models of fibrosis, PDE4 inhibition is antifibrotic across organ systems. The preferential targeting of PDE4B by BI 1015550 was developed to overcome the gastrointestinal side effects associated with broad PDE4 inhibition [123]. Phase 3 studies with this agent are ongoing.

In the next section, we will *review in vitro experiments that led to the approval of current antifibrotic therapies. These findings informed our experimental approach in the study of the antifibrotic effects of MSC-CM.*

1.3.3. Approved antifibrotics

Two antifibrotics, pirfenidone and nintedanib, were approved in 2014 by the Food and Drug Administration and Health Canada for the treatment of idiopathic pulmonary fibrosis (IPF). Subsequently those drugs were approved for use in progressive pulmonary fibrosis and SSc-associated interstitial lung disease (nintedanib).

Pirfenidone is an antifibrotic with anti-inflammatory properties that delay the decline of forced vital capacity in IPF [126]. Pirfenidone was initially developed as an anti-inflammatory agent and then found to modulate fibrogenic growth factors attenuating fibroblast proliferation, myofibroblast differentiation, collagen and fibronectin synthesis, and deposition of ECM [127]. These effects are mediated by the suppression of TGF-β, downstream TGF-β -associated mechanisms and other growth factors (e.g., PDGF and FGF-2). Specifically, pirfenidone reduces TGF-β protein production, suppresses TGF-β–mediated fibroblast proliferation and fibroblast differentiation into myofibroblasts through the attenuation of TGF-β/ Smad3-induced signaling; inhibits the expression of TGF-β1–induced genes such as tenascin-c, fibronectin, and collagen I, II, and III, and reduces TGF-β–induced expression of α-SMA [128, 129]. Pirfenidone also inhibits redox reactions and regulates oxidative stress–related genes and enzymes [127].

Nintedanib is a tyrosine kinase inhibitor that binds to the intracellular ATP binding pocket of the FGF-, PDGF- and VEGF- receptors, resulting in blockage of their autophosphorylation and the downstream signalling cascades. Nintedanib effects include reduced proliferation and migration of fibroblasts, and attenuation of pulmonary angiogenesis [130]. Nintedanib also inhibits non-receptor tyrosine kinases of the Src family which mediate the antifibrotic effects in IPF human lung fibroblasts and in the

bleomycin-induced lung fibrosis mice model [131]. Nintedanib prevents TGF-β-induced fibroblast to myofibroblast activation of primary human IPF lung fibroblasts, as determined by α-SMA mRNA expression [132]. Nintedanib also enhances the expression of MMP-2 and inhibits the expression of TIMP-2 [130]. It induces apoptosis and inhibits proliferation of human umbilical vascular endothelial cells, human umbilical artery smooth muscle cells and bovine retinal pericytes [133]. The contribution of the antiangiogenic effects of nintedanib to its antifibrotic activity in IPF is unclear.

Overall, there is a disconnection between the burden of fibrosis and the limited number of approved antifibrotic agents. The ultimate mechanisms of action responsible for the antifibrotic effect of nintedanib and pirfenidone are unknown. *The evidence we present suggests that in vitro modulation of fibrogenesis at different levels is required. This justifies the need to develop in vitro assays to test potential antifibrotic candidates (i.e., mesenchymal stromal cells).*

Section 4. Multipotent mesenchymal stromal cells: a therapy for fibrosis?

1.4.1. Definitions

Multipotent mesenchymal stromal cells (MSC) are heterogeneous multipotent non-hematopoietic plastic-adherent cells that were initially isolated in the bone marrow [134]. Subsequently, MSC were recognized as perivascular cells in the stroma of every adult and embryonic tissue, where they contribute to tissue homeostasis [135]. For therapeutic purposes, MSC are most frequently isolated from adipose tissue (AT), umbilical cord (Wharton-Jelly) (UC), and bone marrow (BM).

The International Society for Cellular Therapy (ISCT) established minimal requirements to define MSC. They should be (1) a plastic-adherent polyclonal population

with fibroblast-like morphology; (2) positive for CD73, CD90 and CD105 and negative for hematopoietic and endothelial surface markers; and (3) able to differentiate into osteoblasts, adipocytes, and chondroblasts provided specific in vitro conditions [136].

MSC have proangiogenic, immunosuppressive, and less well characterized antifibrotic effects that are mediated by cell contact-dependent and -independent mechanisms [137]. Paracrine effects are the predominant mechanism of action of MSC. Those are mediated by secreted soluble molecules and small vesicles which together constitute the 'MSC secretome' [138]. The composition of the MSC secretome is modulated by several factors including the local milieu (i.e., proinflammatory cytokines, hypoxia), MSC passage (i.e., early versus replicative senescence), and culture conditions.

1.4.2. MSC cytokine priming

Interferon gamma (IFN-γ) with or without tumor necrosis factor alpha (TNF-α) are recommended by the ISCT for MSC priming. These conditions resemble the microenvironment that MSC are exposed to in patients with dysregulated immune responses / systemic inflammation [135, 139].

IFN-γ priming enhances the immunomodulatory effects of MSC, by inducing the activation of the enzyme indoleamine 2,3-dioxygenase 1 (IDO) in human MSC [135], which catalyzes the conversion of L-tryptophan into L-kynurenine. This mediates the immunomodulatory effects of MSC suppressing lymphocytes activation and proliferation [135]. Primed MSC secrete multiple immunomodulatory molecules, such as PGE2, hepatic growth factor (HGF), TGF-β, and IL-10, increase their expression of class I and

class II histocompatibility leucocyte antigen (HLA) molecules and of co-stimulatory molecules [139].

TNF-α, another pro-inflammatory cytokine, influences the immune suppressive ability of MSC, increasing the release of TNF-α-induced protein 6 (TSG-6) [140]. This secreted glycoprotein induces anti-inflammatory M2 macrophages and reduces T cell proliferation [141]. TNF-α also increases the expression of chemokine receptors on MSC, resulting in enhanced migration towards chemokine gradients [142]. Although IFN-γ and TNF-α priming enhances the MSC immunomodulatory properties, it is unknown whether it has a similar effect on their antifibrotic effects.

In this project, we used MSC culture conditions recommended by the ISCT (IFN-γ and TNF-α) to prime MSC [135]. We compared the effect of primed versus non-primed ('resting') MSC secretome in in vitro antifibrotic assays.

1.4.3. Therapeutic applications

MSC are tested as cellular therapy due to their immunomodulatory capacity and proposed antifibrotic effects [137]. The first phase 1 clinical trial was reported in 1995 [143]. Thereafter, MSC's were tested as therapeutics for a wide variety of inflammatory and immune mediated diseases [e.g., osteoarthritis, rheumatoid arthritis, diabetic foot ulcers, cardiovascular disease, neurodegenerative diseases, graft versus host disease (GvHD), Crohn's disease, SSc and Lupus] [144] . Despite the encouraging results of pre-clinical animal studies, the findings of human clinical trials were less impressive. Several factors contributed to this discrepancy, including differences between patient populations, MSC -donors, -sources, and -preparations [145]. As a consequence, there are currently only 11 approved indications for MSC therapeutic use in humans (Table 2) [146]. Of

relevance, MSC therapy has been approved for two fibrotic conditions: GvHD and Crohn's disease. There are no s*tandardized in vitro assays to inform the selection of the optimal MSC product to be used in fibrotic human diseases [135]. This is the primary focus of my work.*

Table 2. Approved MSC therapeutic agents

MSC product	Country/year of approval	Indication	Type of MSC
Queencell	South Corea/ 2010	Subcutaneous tissue defects	Autologous hMSC(AT)
Cellgram-AMI	South Corea/ 2011	Acute myocardial infarction	Autologous hMSC(BM)
Cartistem	South Corea/ 2012	Knee articular cartilage defects	Allogenic hMSC(UC)
Cupistem	South Corea/ 2012	Crohn's perianal fistula	Autologous hMSC(BM)
Prochymal, remestemcel-L	Canada/ 2012	GvHD	Allogenic hMSC(BM)
Prochymal, remestemcel-L	New Zealand/ 2012	GvHD	Allogenic hMSC(BM)
Neuronata-R	South Corea/2014	Amyotrophic lateral sclerosis	Autologous hMSC(BM)
Temcell HS	Japan/ 2015	GvHD	Allogenic hMSC(BM)
Stempeucel	India/ 2016	Critical limb ischemia	Allogenic hMSC(BM)
Alofisel	Europe/ 2018	Crohn's complex perianal fistula	Allogenic hMSC(BM)
Stemirac	Japan/ 2018	Spinal cord injury	Autologous hMSC(BM)

AT: adipose tissue, BM: Bone marrow, GvHD: graft versus host disease, UC: Umbilical cord

1.4.4. Antifibrotic effects

Three approaches have been used to examine the antifibrotic effects of MSC: in vitro experiments, in vivo experiments in animals, and clinical trials. In fibroproliferative diseases, chronic inflammation plays an important role in perpetuating fibrosis [147]. Since MSC modulate immune responses, they indirectly have an antifibrotic effect [137, 148]. However, it is unclear whether MSC have a direct antifibrotic effect on

myofibroblasts, and whether that is an additional mechanism by which MSC modulate fibroproliferative diseases [137].

In vitro studies are inconsistent in reporting the antifibrotic effects of MSC-CM. This could be explained by differences in experimental designs. While most studies show that MSC-CM modulates different aspects of fibrosis including migration, α-SMA levels, and other fibrotic proteins, others do not show in vitro fibrosis modulation [148, 149]. MSC(AT)-CM treatment of fibroblasts from hypertrophic scars and keloids inhibits the synthesis of collagen I and Smad2/3 phosphorylation [150, 151], and decreases the expression of *COL1A1, TGFβ1, TβRII, and α-SMA [93]*. Similar results are reported following treatment of SSc fibroblasts [93]. In addition, MSC-CM treatment of cardiac fibroblasts up-regulates Smad7, an inhibitory Smad [152]. The MSC secretome also modulates the balance between MMPs and TIMPs, increasing MMP-9, MMP-1 [93], MMP-13, MMP-14 [153] and decreasing TIMP-1 [93, 154]. The CM from MSC isolated from lung inhibits the proliferation of lung fibroblasts and improves the regeneration of injured lung epithelium [155]. Two studies demonstrate the limited capacity of MSC to inhibit TGF-β-induced activation of fibroblasts [148, 149] and showed that MSC do not induce apoptosis of myofibroblasts [156-158]. *The discordance between these studies reflects the lack of standardization of in vitro assays evaluating antifibrotic effects*. Table 3 summarizes in vitro studies in which MSC and the MSC secretome antifibrotic effects were investigated. The potential mediators of the MSC-CM antifibrotic effects are discussed in Section 1.4.4.

Animal models of organ specific or systemic fibrosis (e.g., chronic kidney disease, wound healing, SSc and liver fibrosis models) were used to administer MSC or MSC

subproducts (i.e., MSC-CM or extracellular vesicles) from a variety of sources (e.g., BM and UC). Readouts in these models included prevention of α-SMA, TGF-β1 and caspase-3 gene expression, and histological improvement of fibrosis [159-163]. These studies support the potential of MSC to ameliorate and/or revert fibrosis in organs with regenerative capacity. An important limitation of several of these studies is that they did not describe what mediates the MSC's antifibrotic effects.

A number of clinical trials reported the benefit of MSC treatment for systemic fibrotic diseases. A search in clinicaltrials.gov using the terms "Mesenchymal cells" and "fibrosis" done on Dec 16, 2022 rendered 79 studies. Two examples in systemic fibrotic diseases are discussed. In refractory GvHD, a phase 3 trial of 244 patients compared standard of care ± MSC treatment or placebo. This trial showed superiority of MSC treatment in patients with skin, liver and gut involvement, and reduction in the progression of liver dysfunction [164]. A recent phase 1/2 study in SSc suggested that a single infusion of MSC(BM) is associated with a decrease in skin fibrosis and stability in forced vital capacity at one-year post-infusion, without significant adverse effects [48]. Of relevance, this study contributed to identify predictive biomarkers of therapeutic activity related to the effects of MSC. Specifically, to explore the functional heterogeneity of different bone marrow derived MSC, the expression of soluble and membrane factors, were quantified and related to the capacity of MSC to inhibit T cells in vitro. Low IDO activity, low CXC motif ligand 2 (CCL2) production, and low HLA-DR expression following IFN-γ stimulation were associated with a clinical non-response. Of concern, clinical non-responders compared with clinical responders, presented significantly elevated plasma TGF-β concentrations.

Larger controlled studies are required to confirm the antifibrotic effects of MSC and to define their underlying mechanisms.

1.4.5. Antifibrotic mediators in the MSC secretome

In the MSC secretome, the following soluble factors were associated with reduction of in vitro fibrotic readouts (Table 3): prostaglandin E2 (PGE2), interleukin-10 (IL-10), hepatocyte growth factor (HGF) and MFGE8 [156, 158, 162, 165] . Extracellular vesicles (EVs) were also described as contributors of antifibrotic effects through miRNAs (Figure 4) [166].

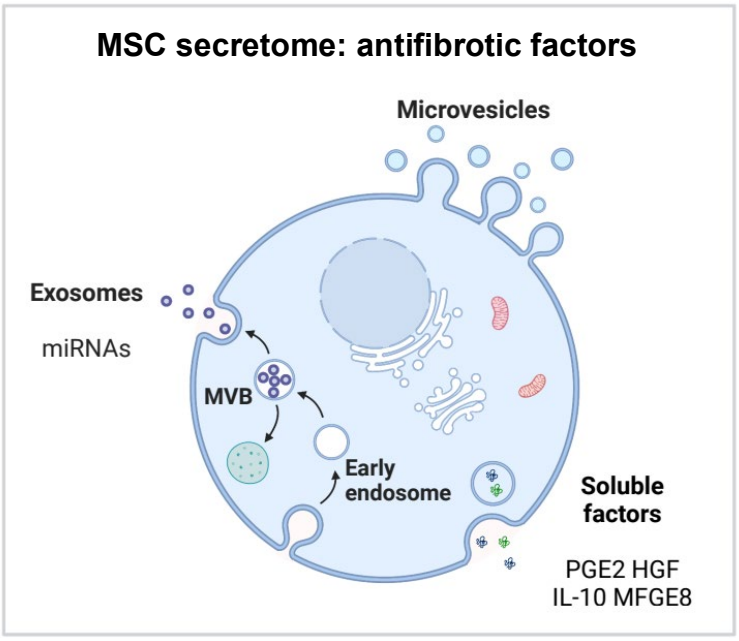


Figure 4. MSC secretome composition: antifibrotic factors

PGE2 mediates indirect antifibrotic effects by interacting with immune cells. In response to PGE2, T cells [167] and macrophages [156] were reprogrammed and secreted high levels of IL-10, an important anti-inflammatory cytokine [168]. IL-10 is a powerful inhibitor of neutrophil invasion into the wound and prevents oxidative tissue damage by inhibiting the release of reactive oxygen species (ROS) [169]. IL-10 and PGE2

may also have direct effects on fibrosis. IL-10 down-regulates the expression of TGF-β and reprograms fibroblasts to favor ECM remodeling by up-regulating the expression of MMPs and down-regulating collagens [170]. PGE2 treatment in vitro deactivates myofibroblasts and increases apoptosis sensitivity [171].

HGF is present in the MSC-CM and has been recognized for its antifibrotic properties [152, 162]. HGF down-regulates TGF-β, collagen I and collagen III expression in fibroblasts [172, 173]. Mechanistically, HGF works as an antagonist of the canonical pathway, excluding Smad3 from the nucleus [173, 174]. HGF also up-regulates MMP-1, MMP-3, and MMP-13 in fibroblasts [175], thereby contributing to the ECM modulation. In addition, HGF stimulates keratinocyte migration, proliferation [176], and up-regulates the expression of VEGF-A [177]. Therefore, the secretion of HGF by MSC may contribute to the proper regeneration of injured tissue and enhance wound re-epithelialization [176].

MFGE8 was identified by mass spectrometry in the MSC secretome from UC, teeth and BM MSC. MFGE8 downregulated the expression of *TGFBR1* decreasing α-SMA and Smad 2 phosphorylation in hepatic stellate cells in a model of hepatic fibrosis treated with MSC(UC)-CM [158].

EVs are defined by the International Society of Extracellular Vesicles (ISEV) as “particles naturally released from any cell that are delimited by a lipid bilayer and cannot replicate” [178], excluding erythrocytes and platelets. EVs can be characterized according to their origin, size, content, and function in micro-vesicles (MVs), exosomes, and apoptotic bodies. EVs are known as a cell mechanism of communication. EV’s cargo and membrane components are determined by the cell of origin, their nucleic acid content (e.g., mRNA, miRNA, DNA), and their proteins (e.g., membrane receptors, hormones and

growth factors), lipid and amino acid composition [179, 180]. miRNAs are a class of endogenous noncoding single-stranded RNA molecules with a length of ~ 22 nucleotides that regulate gene expression. miRNAs act as repressors decreasing the expression of messenger RNAs that contain part of their complementary sequences [181]. MSC-EVs contain several miRNAs implicated in profibrotic-gene regulation. MSC(BM)-exosomes have miR-21a-5p that repress phosphofructokinase (PFKM) inhibiting glycolysis in tubular epithelial cells impairing renal fibrosis [182].

MSC-EVs deliver miR-223-3p to attenuate lipid accumulation and fibrosis in a murine model of NAFLD inhibiting the transcription factor E2F1 [183]. E2F1 promotes the expression of ADAM12 [184]. Another study describes an antifibrotic effect of MSv-EVs through miR-29c, preventing the increase of α-SMA gene expression and protein levels in myofibroblasts [166]. The nuclear factor (NF)-κB signaling pathway promotes the expression of ADAM12 by inhibiting the expression of miR-29c [184].

Together, these results confirm that the MSC-CM contains several factors that modulate fibrosis. The relative relevance of those factors to fibrosis resolution is unknown.

Table 3. Antifibrotic effects of MSC: in vitro studies

Ref.	MSC/ Secretome component	Fibroblast source	Fibroblast activation	Readout	Results	MoA
[166]	hAT ASC52tel/ hTERT MSC-CM/ EVs	Human dermal fibroblasts	TGF-β 5ng/mL 4days	Prevention assay IH qRT-PCR WB	MSC-CM, EV and soluble factors prevented the increase of α-SMA gene expression and protein levels	miRNA-29c from EV
[152]	hAT	Rat cardiac fibroblasts	Stiff surface	ELISA IH qRT-PCR	MSC-CM downregulated AT1R, TGF-β, COL1, COL 3 and upregulated Smad 7	Secreted HGF upregulated Smad7
[93]	hAT MSC-CM/ EVs	SSc and HC	TGF-β 5ng/mL for 24h	Prevention assay: qRT-PCR	MSC, MSC-CM and EVs downregulated αSMA, COL1A1, and upregulated MMP1/TIMP1, COX2	-
[150]	hAT EVs	Keloid fibroblasts	-	qRT-PCR WB	EVs inhibited gene and protein expression of COL1 COL3 α-SMA and FN	Protein inhibition of Smad-3 and Notch-1
[151]	hAT EVs	Keloid fibroblasts	-	IH qRT-PCR Scratch wound assay WB	Decreased α-SMA mRNA level and IH intensity. Decreased COL1 COL3 and α-SMA protein level. Reduction fibroblast migration	miR-192 5p target IL-17RA and inhibited Smad axis
[185]	hAT MSC-CM	Hypertrophic / normal skin fibroblasts	-	Scratch assay WB qRT-PCR	Reduction in COL1, COL 3 and α-SMA gene and protein expression in a dose dependent effect. Reduction in fibroblast migration	Inhibition of p-p38/MAPK pathway
[162]	hBM	hTERT hepatic stellate cells	TGF-β 1ng/mL for 24h	Prevention assay IH Cell viability and apoptosis Cytokines levels by ELISA	In co-culture, BM-MSC reduced α-SMA ,TGF-β1 and IL-6, increased IL-10, and HGF in supernatant. Reduced HSC viability	Secreted HGF + IL-10
[182]	hBM Exosomes	Tubular epithelial cells TCMK-1 cells	TGF-β 10ng/mL for 48hs	Prevention assay IH WB	α-SMA, Collagen I and Collagen III decreased by MSC-exo	miR-21a-5p repressed PFKM inhibiting glycolysis in tubular epithelial cells

[186]	hWJ	None (mouse myoblast cell line)	H_2O_2	ELISA RayBio Label-Based Human Antibody Array 507 WB	Myoblasts + MSC coculture: lower FN levels. Higher concentration of MMP-1, INHBA, IGFBP7, PDGFA, and THBS1 in the co-cultured supernatant	Secreted MMP-1 lowers FN levels.
[158]	hUC MSC-CM	hTERT hepatic stellate cells	TGF-β 1ng/mL for 24hs	Prevention assay qRT-PCR WB IH ELISA Nano-Chip-LC/QTOF-MS	MSC-CM suppressed TGF-β-mediated HSC activation: decreased α-SMA protein levels, inhibited p-Smad 2. MSC-CM did not induce apoptosis or senescence. MFGE8 reduced α-SMA, and hydroxyproline	Secreted MFGE8
[157]	hAmniotic membrane/ CM	LX2 cell line Hypertrophic skin fibroblasts	-	WB IH Flow cytometry Antibody array: cytokines si-RNA	MSC-CM no effect on apoptosis or proliferation on HSC. IGFBP-3, Dkk3, and DKK-1 secretion inhibited HSC activation, (WB) reduction of α-SMA, P-GSK3β and β-catenin.	IGFBP-3, Dkk3, and DKK-1 Inhibited Wnt/β-Catenin pathway
[155]	h Lung MSC-CM	Lung fibroblasts		Proliferation Scratch wound assay	MSC-CM reduced fibroblast proliferation and migration.	-
[183]	Mouse AT EVs	NCTC1469 cell line: mouse hepatocyte	Palmitic acid	IH qRT-PCR WB	MSC-EVs reduced α-SMA, COL1A1 and TGF-β1 of NCTC1469	MSC-EVs deliver miR-223-3p to attenuate lipid accumulation and fibrosis
[165]	Rat/ hBM resting vs. INF-ɣ primed	Human Kidney -2 cell line	TGF-β 10ng/mL for 30 min or 24h	Prevention assay IH WB	Primed hMSC-CM decreased α-SMA, p-Smad2 in HK 2 cells. rMSC transfected with PTGES siRNA was less antifibrotic.	PGE2

AT1R: angiotensin II type 1 receptor; EV: extracellular vesicles; FN: fibronectin; HSC: hepatic stellate cells; HS: hypertrophic scar; hAT: human adipose tissue; hBM: human bone marrow; hUC: human umbilical cord; hWJ: human Wharton jelly; IGFBP7: insulin like growth factor binding protein 7;IH: immunohistochemistry; INHBA: inhibin, beta A; INF-ɣ: interferon gamma; MMP: matrix metalloproteinase; MoA: mechanism of action; Nano-Chip-LC/QTOF-MS: nanochip-liquid chromatography/quadrupole time-of-flight mass spectrometry; PFKM: phosphofructokinase muscle isoform ; PDGFA: platelet derived growth factor A; qRT-PCR: quantitative reverse transcription PCR;THBS1: thrombospondin 1; WB: Western blot.

1.4.6. MSC dysregulation in SSc

Little is known about the homeostatic in vivo effects of MSC in SSc. Specifically, the MSC contribution to SSc pathogenesis is not defined. Few studies, most with human MSC-(BM) or -(AT), assessed the characteristics of MSC derived from SSc patients (Table 4). Although SSc MSC per definition fulfill the ISCT minimal definition criteria [187-189], SSc serum impairs the adipogenic differentiation of SSc MSC [190]. This was evidenced by a reduction of FABP4 and PPARɣ protein expression, two markers of mature adipocytes but not by histology [191]. Further characterization of SSc MSC showed signs of premature senescence, stress, and reduced proliferative capacity, with components of the senescence associated secretory phenotype involved in promoting fibrosis [192, 193]. Increased α-SMA and collagen I in SSc MSC(AT) were associated with lower protein levels of caveolin-1 at early passages [191]. This fibrotic phenotype was reverted by treating MSC with caveolin-1. TGβRII was increased in SSc MSC(BM) [194], and a profibrotic profile was present in miRNAs from both SSc MSC(AT) and MSC(BM) [195]. The overexpression of ADAM12 also support the profibrotic phenotype of SSc MSC [64].

In addition to cell intrinsic defects, studies reported that in the SSc microenvironment, healthy MSC differentiate into myofibroblast-like cells characterized by an increase in α-SMA stress fibers and collagen I with the ability to contract collagen gels [190, 196].

An additional mechanism that supports the concept of an impaired antifibrotic effect in SSc MSC relate to their reduced proangiogenic capacity due to an altered crosstalk with endothelial cells [197]. This results in a profibrotic phenotype in MSC with

an increase in α-SMA, COL1A1 and COL1A2 mRNA levels. Lastly, the immunosuppressive effects of SSc MSC in in vitro lymphocyte proliferation suppression assays seem to be preserved [188, 193].

Although limited, these data suggest that dysfunctional SSc MSC may have reduced antifibrotic effects and contribute to SSc progression.

Table 4. SSc MSC characteristics

Ref.	MSC source	Samples (n)	Readout	Results from SSc
[189]	Adipose tissue	6 SSc 6 HC	Differentiation (Osteocytes/Adipocytes) Cell surface markers Proliferation (growth kinetics) Migration	Same phenotype Lower proliferation/ migration
[187]	Bone marrow	12 SSc 13 HC	Differentiation (Osteocytes/Adipocytes) Cell surface markers CFU-F/ stemness Immunosuppression	No differences
[188]	Adipose tissue	10 SSc 8 HC	Differentiation (Osteocytes/Adipocytes) Proliferation (growth kinetics) Matrigel assay- Angiogenesis Immunosuppression	No differences
[193]	Bone marrow	10 SSc 10 HC	Senescence Immunosuppression T cell profile	Higher β-Gal and p21 positivity Similar suppression of PBMC proliferation Similar regulatory T cells
[195]	Adipose tissue Bone marrow	3 SSc AT 3 SSc BM	miRNA Prediction KEGG signaling pathways	Senescent and profibrotic profile (do not share common miRNAs)
[198]	Adipose tissue	7 SSc 7 HC	Matrigel assay- Angiogenesis Co-culture SSc dermal fibroblasts with AT-MSC Senescence (SA-β-Gal, p21, p16, p53) WB: α-SMA, collagen I	MSC antifibrotic and proangiogenic effect preserved

[194]	Bone marrow	9 SSc 9 HC	Expression of TβRI TβRII in BM-MSC Smad2, Smad3, Smad4 Smad7 mRNA	TβRII up regulation: collagen I P-Smad3
[199]	Bone marrow	5 SSc 5 HC	Matrigel assay- Angiogenesis Characterize BM-MSC + VEGF (50 ng/ml), or TGFβ (1 ng/ml) RT-PCR, WB: CXCR4, VEGF, TGFβ1/TβRs	Proangiogenic properties preserved CXCR4 /TGFRII up regulation
[190]	Adipose tissue	Serum 6 SSc 6 HC TGF-β 10ng/ml	Differentiation (Osteocytes/Adipocytes) Collagen contraction assay Myofibroblast-like phenotype: RT-PCR /immunofluorescence staining α-SMA+ stress fibers S100A4 and collagen I	SSc serum inhibit HC and SSc MSC adipogenic differentiation. SSc serum up-regulate: ACTA2 S100A4 COL1A1 COL1A2 in HC and SSc MSC
[197]	Bone marrow EC	10 SSc 10 HC	Matrigel assay- Angiogenesis EC influence MSC into a pathogenic phenotype. PCR: VEGF2 TGF-β PDGF	EC SSc: Impair tube formation despite higher VEGF2 Induction of TGF-β in MSC
[191]	Adipose tissue	4 SSc 4 HC	Differentiation (Adipocytes) Immunohistochemistry WB	SSc MSC had low caveolin-1, high α-SMA, high HSP47, low pAKT, decreased expression of FABP4 and PPARγ.
[64]	Skin MSC	20 SSc 10 HC	Immunohistochemistry WB qRT-PCR	ADAM12 induced p-Smad and synthesis of α-SMA

In red ISCT minimal criteria, in blue functional assays, in green profibrotic characteristics of SSc MSC. ADAM12: ADAM Metallopeptidase Domain 12; AT: adipose tissue; BM: bone marrow; CFU-F: colony formation unit- fibroblasts; EC: endothelial cells; FABP4: Fatty acid binding protein 4; HC: healthy control; PBMC: peripheral blood mononuclear cells; PPARγ: Peroxisome proliferator- activated receptor gamma; p-Smad: phosphorylated Smad; qRT-PCR; quantitative reverse transcription PCR; SSc: Systemic sclerosis; WB: western blot; α-SMA: alpha smooth muscle actin.

Section 5. Summary of study rationale

Fibrosis is a complex pathogenic mechanism involved in a wide array of both organ specific and systemic diseases. Reversing fibrosis is a necessary goal to limit morbidity and mortality associated outcomes of fibroproliferative diseases. Fibrosis reversal requires the elimination of myofibroblasts, modulation of the ECM, and regeneration of

functional tissue. MSC have anti-inflammatory and less well characterized antifibrotic properties. The MSC secretome is the key mediator of these effects. The lack of standardized in vitro and in vivo assays that predict in human antifibrotic effects is a barrier for the advancement of this field. Moreover, the characterization of MSC antifibrotic effects under homeostatic and pathological conditions is fundamental to optimize MSC donor selection (i.e., autologous vs. allogeneic).

We hypothesize that the MSC secretome (MSC-CM) promotes fibrosis resolution in vitro and that this function is impaired in MSC secretome from SSc patients.

We propose to test this hypothesis with two primary objectives:

(1) to establish in vitro assays to assess the direct antifibrotic effects of MSC,

(2) to compare the antifibrotic potential of MSC-CM from SSc patients and healthy controls (HC).

CHAPTER 2. METHODS

2.1. Study subjects

This study was approved by the McGill University Health Centre Ethics Review Board (Protocols: 10-107 GEN and MEO-05-2022-8285). Under protocol 10-107 GEN, MSC were isolated from subcutaneous adipose tissue obtained from a total of eight patients (i.e., four young healthy individuals undergoing elective orthopedic surgery and four adults undergoing programmed cardiovascular surgery). The demographic characteristics of the study participants are summarized in Table 5. Under protocol MEO-05-2022-8285, forearm skin punch biopsies were obtained from five patients with diffuse scleroderma and five age-/sex-matched non-scleroderma controls attending the Scleroderma Clinic at the Jewish General Hospital (JGH). The adipose tissue contained within skin biopsies was used to derive MSC which were subsequently characterized. The demographic features of the SSc and control samples are presented in Table 6.

Table 5. Study donor demographics: subcutaneous adipose tissue

Table 5.		
Adipose Tissue Donors	Adult	Pediatric
N	4	4
Age (mean ± SD)	70.2±7.7	15.5±1.5
Sex (Females/Males)	2/2	4/0
Race		
Caucasian	3	3
Indian	1	1
Weight (mean ± SD, lb)	186.5±20.5	141.8±62.5
Smoke (Yes)	1	-
Comorbidities		
Hypertension	3	-
Hypercholesterolemia	2	-
Heart disease	3	-
Treatment		
Statins	1	-
ASA	2	-
Diuretics	1	-
ACEI/ARB	3	-

ASA: Acetylsalicylic acid; ACEI: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker

Table 6. Study donor demographics: skin biopsies

Table 6.		
Skin Biopsy Donors	Scleroderma	Control
N	5	5
Age (mean ± SD)	42.7±15	41.2±13.9
Sex (Female/Male)	4/1	4/1
mRSS	19.5±3.35	-
Treatment		
None	2	-
Prednisone	1	-
HCQ	1	-
MMF	2	
Tacrolimus	1	-
Tocilizumab	1	-

HCQ: Hydroxychloroquine, MMF: Mycophenolate mofetil, mRSS: modified Rodnan skin score

2.2. Human adipose derived multipotent mesenchymal stromal cells - hMSC(AT)

2.2.1. Isolation, characterization and functional assessment

For MSC isolation a previously described protocol was used [145]. Briefly, subcutaneous adipose tissue samples were incubated for 40 min in phosphate-buffered saline (D-PBS, Wisent Inc, St Bruno, QC) containing 1% of penicillin/streptomycin (10,000mg/mL penicillin 10,000mg/mL streptomycin, Wisent Inc. St Bruno, QC). Adipose tissue was minced and digested with 0.05% collagenase (Millipore Sigma, Etobicoke, ON) in Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA). After 2hs, the collagenase activity was inhibited with 5% MSC Fetal Bovine Serum Qualified (Gibco FBS Thermo Fisher Scientific, Waltham, MA). Samples were centrifuged (4˚C, 800 x g for 10 min), the supernatant was discarded, and the cells were resuspended in complete medium: Dulbecco's modified Eagle's medium with 1.0g/L glucose, with L-glutamine &

sodium pyruvate (DMEM, Wisent Inc.) supplemented with 10% FBS and 1% penicillin/streptomycin. The cell culture medium was changed every two or three days. When MSC reached 80% confluency, they were detached with trypsin (Wisent Inc.) and seeded at a density of 5000 cells/cm^2. The number of passages of each sample was recorded and only early passage (P3-P5) cells were used for experiments.

MSC from SSc patients and controls were isolated from the adipose tissue fraction contained in two 4mm punch skin biopsies from the forearm. All biopsies were performed by a rheumatologist (Dr Marie Hudson, Jewish General Hospital), following a standard procedure [200]. Biopsies were placed in complete DMEM 20% FBS medium on ice and immediately transferred to our laboratory at the RI-MUHC. The subcutaneous adipose tissue was identified, mechanically detached from the dermis, weighed, and processed using the same methods as described above for subcutaneous adipose tissue.

Passage 3 MSC were characterized according to the ISCT criteria (i.e., plastic adherence, surface markers, and tri-lineage differentiation) as previously described [145]. Surface markers were determined by flow cytometry using the BD LSRFortessa^{TM} cell analyzer. The following fluorochrome-conjugated monoclonal antibodies from BD Biosciences were used: fluorescein isothiocyanate (FITC)-conjugated anti-CD90 (Catalog #: 555595), anti-CD20 (555622) and anti-CD45 (555482); phycoerythrin (PE)-conjugated anti-CD73 (550257); allophycocyanin (APC)-conjugated anti-CD34 (555824), anti-CD105 (562408) and anti-HLA-DR (559866); and peridinin chlorophyll (PerCP) conjugated anti-CD14 (562692). Data analysis was done using the FlowJo software version 10.8.1. To assess osteogenic and adipogenic differentiation, MSC were seeded in 24-well plates at a density of 5000 cells/cm^2. After 24h, the medium was replaced with

differentiation medium (Gibco™ StemPro® Adipogenesis or Osteogenesis Differentiation Kit, Thermo Fisher Scientific, Waltham, MA) or complete medium, and was replenished every 3-4 days for 20 days. MSC were fixed with 4% formaldehyde and stained with Alizarin Red S (osteocytes) or Oil Red O (adipocytes). For chondrogenic differentiation, a pellet of 250,000 MSC was expanded in a 24-well plate for 20 days with Gibco™ StemPro® Chondrogenesis Differentiation Kit medium (Thermo Fisher Scientific), fixed, sectioned (1 µm), and stained with Alcian Blue by the Histopathology Platform at the RI-MUHC.

We *analyzed three functional MSC* properties: proliferation, stemness and immunopotency.

MSC proliferation was estimated by calculating the doubling time, which is the time it takes a cell population to double in number, according to the formula:

$$Doubling\ time = \frac{time * \log(2)}{\log cells\ harvest - \log cells\ seeded}$$

Time was defined as the number of days between initial plating and harvest for the respective passage.

MSC stemness refers to the ability of MSC to generate colonies after they are plated at low density [201]. We assessed clonogenicity of the MSC using with the CFU-F assay. Briefly, MSC were seeded in a 96-well plate at four different cell-densities: 1, 3, 10, and 30 MSC per well (24 replicates each) in complete DMEM low glucose medium. After 2 weeks of culture, without any change in medium, the plate was washed with PBS and stained with 0.1% crystal violet solution. Finally, we identified the number of wells

positive for colonies for each dilution and calculated the stem cell frequency with ELDA software https://bioinf.wehi.edu.au/software/elda/. A low stem cell frequency (1 / 50 MSC) is associated with a senescent state, while a high stem cell frequency (1 / 4 MSC) indicates a cell population with a high clonogenicity and a higher cumulative population doubling limit.

An immunopotency assay to evaluate the capacity of MSC to inhibit proliferating T-cells [145] was used to characterize MSC from SSc and controls. For all of these assays, peripheral blood mononuclear cells (PBMCs) were isolated from one unrelated donor (34-year-old non-smoking healthy female) with Lymphocyte Separation Medium through density gradient centrifugation (Wisent Inc.). For monocyte depletion, PBMCs were cultured overnight in Rosewell Park Memorial Institute medium (RPMI 16-40) (Wisent Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE, Millipore Sigma, Etobicoke, ON) and stimulated with CD3/CD28 antibody coated beads (1 bead/cell) (Dynabeads® Human T-Activator CD3/CD28, Thermo Fisher Scientific). $2x10^6$ activated CFSE-stained PBMCs (100µl) were added to $2.5x10^4$ MSC in cell-cell contact dependent conditions. CFSE-stained, activated PBMCs were cultured in complete medium (maximal proliferation), and CFSE-stained non-activated PBMCs served as controls. After 72h, the PBMCs were collected and stained with Annexin V, 7- Aminoactinomycin D (7-AAD) (559925), and CD4-APC (555349) (BD Biosciences, San Jose, CA). The Expansion Index (EI) of Annexin V- /7AAD- /CD4+ cells (viable CD4) was determined with FlowJo software. The immunopotency (i.e., proportion of non-proliferating CD4+ T cells in the presence of MSC) of the MSC was calculated using the following formula:

$$Proliferation\ (\%) = \frac{Expansion\ index\ of\ sample - 1}{Expansion\ index\ of\ control - 1}.100$$

2.2.2. Resting and primed MSC(AT) conditioned medium (MSC-CM)

MSC ($8x10^3$ cells/cm^2) were stimulated ('primed') or not ('resting') for 72h with IFN-γ and TNF-α (10 ng/mL and 15 ng/mL, respectively, R&D Systems, Minneapolis, MN), following ISCT recommendations [135]. MSC were washed with PBS three times and fresh medium was added (phenol red-free DMEM high glucose containing 1% penicillin-streptomycin and no FBS). Following 72h, CM was collected, centrifuged (13,000g for 20 minutes at 4°C) to remove cell debris, aliquoted, and stored at -80°C.

Cytokine-induced MSC activation was confirmed by comparing the concentration of kynurenine in resting and primed MSC-CM. Kynurenine, a surrogate of indolamine dioxygenase enzyme activity, increases following MSC priming [135]. Kynurenine was determined with an absorbance-based assay [202]. In brief, MSC-CM (150 µL) was incubated with an equal volume of 30% (w/v) trichloroacetic acid (BioShop Canada Inc, ON) for 15 minutes at 50°C, centrifuged (10,000 x g for 5 minutes) and 75 µL of the solution was added to a 96 well-plate with an equal volume of Ehrlich's reagent (2% w/v p-dimethylaminobenzaldehyde in glacial acetic acid, Millipore Sigma, ON). After 10 minutes, the kynurenine absorbance was measured at a 492 nm wavelength and compared to a commercially- available standard curve (Millipore Sigma, ON).

2.3. Fibroblasts

2.3.1. Tissue source and in vitro culture conditions

Immortalized HCA2 human foreskin fibroblasts expressing the telomerase catalytic subunit (hTERT) were provided by Dr Francis Rodier (University of Montreal). This

immortalized cell line was used to establish the assays to assess the antifibrotic effect of MSC-CM. Dr Deiter Reinhardt (McGill University) provided primary human foreskin fibroblasts to validate the hTERT cell-line results. Environmental factors, including culture conditions and fibroblast passage need to be considered to avoid confounders [203]. In these experiments, fibroblasts were grown in 2D on a plastic surface at 37°C in a 5% CO2 atmosphere in complete fibroblast culture medium [high glucose DMEM supplemented with 10% fetal bovine serum (FBS, Wisent, Inc.) and 1% penicillin-streptomycin]. Although this is a standard formulation for in vitro fibroblast culture, FBS contains proteins and growth factors that can modulate the response of fibroblasts through activation of intracellular signal transduction pathways [204]. To avoid these confounders, we conducted experiments using serum-free conditions. We used early passage fibroblasts (passage <10) for all experiments to prevent replicative senescence.

2.3.2. Activation

Fibroblasts $5x10^3/cm^2$ were seeded in 6-well cell culture plates in complete media. Once attached, media was replaced by DMEM with 1% penicillin/ streptomycin, without FBS (serum-free DMEM). Fibroblasts were activated with 5ng/mL of TGF-β /well (R&D Systems, Minneapolis, MN) for 72h. This is the optimal concentration to activate fibroblasts [205, 206]. Activation was confirmed by determining *α-SMA* and *procollagen I* gene expression and protein levels, visualizing stress fibers and collagen I by immunofluorescence, and evaluating acquired apoptosis resistance by flow cytometry.

2.4. In vitro assessment of MSC-CM antifibrotic effect

2.4.1. MSC-CM modulation of fibroblasts migration

The in vitro IncuCyte® Scratch Wound assay was used to determine changes in human fibroblast migration by MSC-CM (Figure 5). Fibroblasts were seeded at two different densities that formed a confluent cell monolayer: 2 x 10^4 and 3 x 10^4 cells/well (96-well plate in DMEM 10% FBS). Following overnight attachment, a linear scratch was performed by the IncuCyte® 96-Well WoundMakerTM. Plated fibroblasts were washed twice with PBS and incubated with MSC-CM at 37°C, 5% CO2 in the IncuCyte® Live-Cell Analysis System. Images were taken every 3 h for 72h and were analyzed with the IncuCyte® Scratch Wound Cell Migration Software. Serum-free DMEM was used as positive control and 10 ng/ml TNF-α-treated fibroblasts were used as negative control. The readout of this assay was the Relative Wound Density (RWD), which is the percent density of the wound region relative to the density of the cell region. RWD is defined by the equation:

$$\%RWD(t) = 100 . \frac{(w(t) - w(0)}{(c(t) - w(0))}$$

W(t)= density of wound region at time t

C(t)= density of cell region at time t

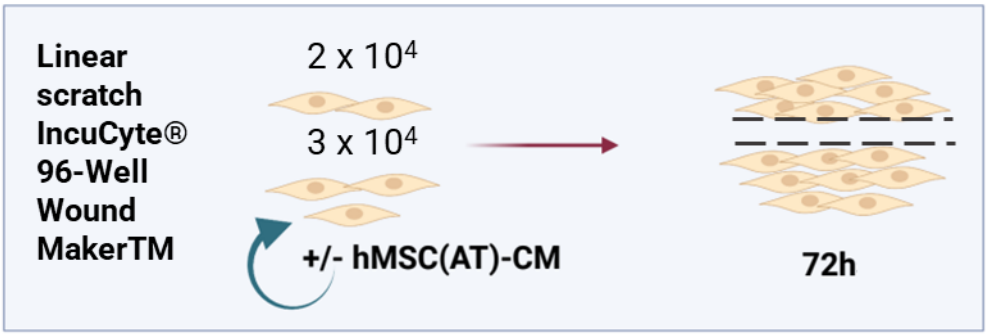


Figure 5. Fibroblast migration assay

2.4.2. MSC-CM prevention of TGF-β induced fibroblasts activation

To investigate the ability of MSC-CM in preventing fibroblasts activation, fibroblasts were seeded at a density of 5 x 10^3 cells/cm^2 in a 6-well plate in complete fibroblast culture medium and incubated overnight at 37°C. After attachment, complete medium was replaced by simultaneously adding TGF-β (5ng/ml) and CM from either resting or primed MSC for 72h. Fibroblasts and myofibroblasts cultured in serum-free DMEM were used as negative and positive controls, respectively. After 72h, proteins were collected and α-SMA and procollagen I were analyzed by Western blot. Collagen I and stress fibers were visualized by immunofluorescence (Section 2.5) (Figure 6).

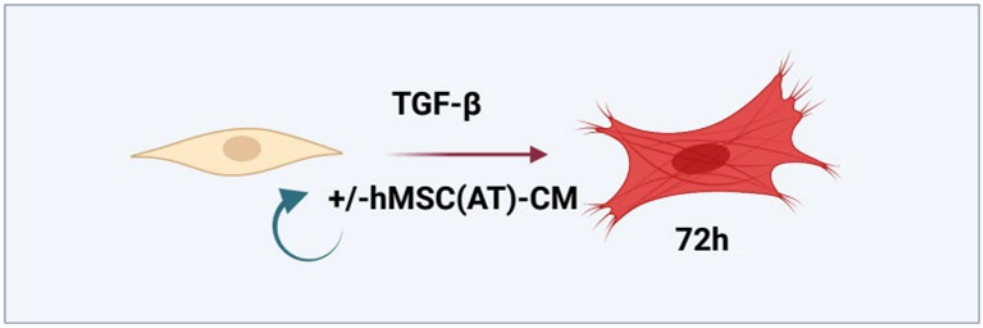


Figure 6. Prevention assay

2.4.3. MSC-CM induction of myofibroblasts' deactivation

To evaluate the capacity of MSC-CM to modulate/revert the myofibroblast phenotype, $5x10^3$ fibroblasts/cm^2 were seeded in 6 well plates and treated with TGF-β (5ng/mL) for 72h. Following activation, myofibroblasts were washed with PBS, and medium was replaced by MSC-CM for 72h. Serum-free DMEM was used as a control. The readouts for these experiments included gene expression and protein levels of *α-SMA* and *procollagen I*.

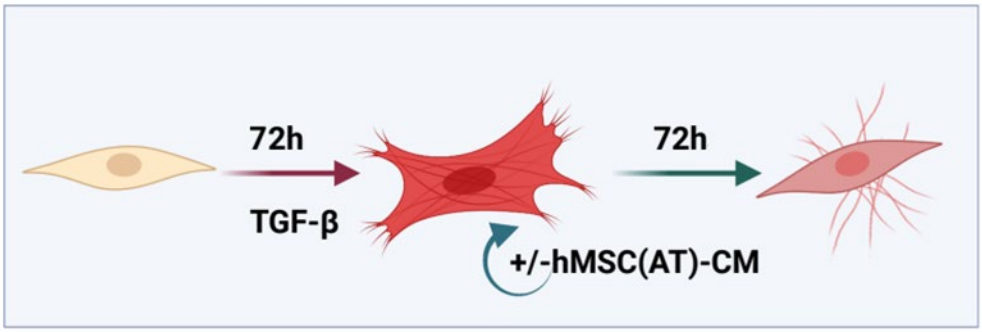


Figure 7. Deactivation assay

2.4.4. MSC-CM modulation of myofibroblast apoptosis resistance

Apoptosis resistance characterizes myofibroblasts and differentiates them from fibroblasts [9]. To assess the ability of MSC-CM to revert apoptosis resistance of myofibroblasts, $1x10^4$ fibroblasts/cm^2 were seeded and activated in a 12 well-plate as described in Section 2.3.2. Myofibroblasts were treated with MSC-CM for 72h. Serum-free DMEM was added to fibroblasts (positive control) and to myofibroblasts (negative control). During the last 24h, apoptosis was induced with staurosporine (40nM, S6942 Sigma-Aldrich, St Louis, MO). After 24h, the supernatant was collected, myofibroblasts were trypsinized and both were processed to assess cell viability. Data was analyzed by FlowJo software 10.8.1.

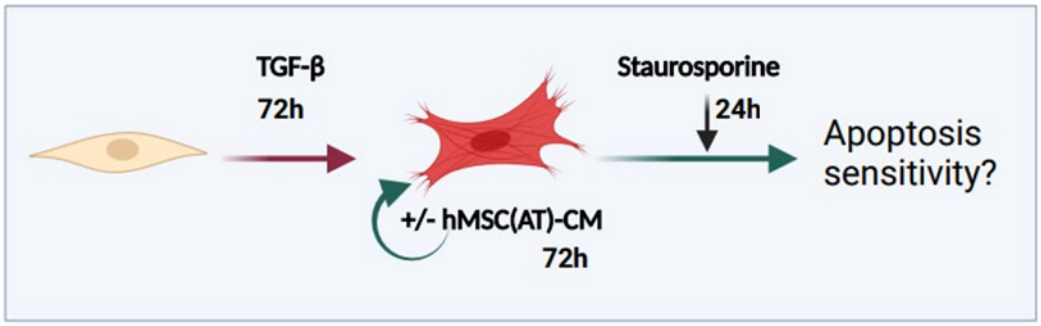


Figure 8. MSC-CM modulation of myofibroblasts' apoptosis resistance

2.4.5. MSC-CM modulation of extracellular fibrillin-1 and fibronectin

To study the effect of MSC-CM on extracellular matrix deposition of fibroblasts, 15 x 10^4 fibroblasts/ well were seeded in an 8-chamber slide. After attachment, fibroblasts were activated by TGF-β (5 ng/ml) in serum-free DMEM. After 48h, medium was replaced by 300 µl of MSC-CM obtained from 50,000/ml of MSC. Following five days of incubation, cells were washed with PBS and fixed with 4% paraformaldehyde. Fibrillin-1 and fibronectin were evaluated by immunofluorescence as previously described with some modifications (Section 2.5.3) [207].

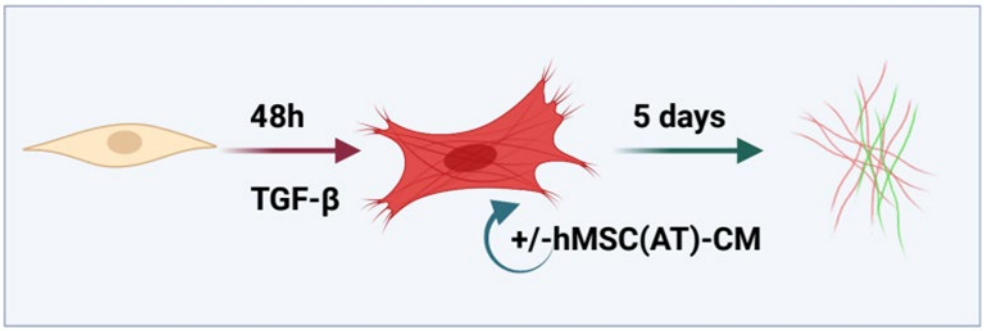


Figure 9. MSC-CM modulation of extracellular fibrillin-1 and fibronectin

2.5. Readouts of in vitro antifibrotic assays

2.5.1. Procollagen I and α-SMA (Western blot)

Cell lysates were prepared in RIPA buffer (89900, Thermo Fisher Scientific, Rockford, USA) and 1X Protein Arrest (KP14001-2 EMD Millipore Corp, Oakville, ON). Proteins were quantified with a bicinchoninic acid assay kit Thermo Fisher Scientific), and 8- 10 µg of cell lysates was loaded into a Mini-protean TGX™ precast gel 4-15% SDS-PAGE (BioRad, USA), transferred to a PVDF membrane, and incubated with 1:3000 rabbit anti-human α-SMA antibody (ab5694, Abcam, Boston, MA) or 1:3000 sheep anti-human Procollagen I antibody (AF6220, R&D systems, USA). Secondary antibodies were 1:3000 HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch labs, West Grove, PA) or HRP-conjugated donkey anti-sheep IgG (HAF016 R&D systems). Mouse anti-human GAPDH antibody (Santa Cruz Biotechnology, Dallas, TX) was used as a loading control. Page Ruler™ Plus Prestained Protein Ladder (26619 Thermo Fisher Scientific) was used as molecular weight standard. Immunoreactive proteins were visualized with Clarity Western ECL Substrate (BioRad) and using a Omega Lum™ C Imaging System (Aplegen®, San Francisco, CA) and analysed with the ImageJ software.

2.5.2. TGF-β activated profibrotic genes (quantitative Real-Time-PCR)

The expression of TGF-β activated genes (Table 7) was analyzed in fibroblasts (activated or not with TGF-β) treated with either resting or primed MSC-CM (n=4 per group for each sample from a different MSC donor). Fibroblasts not treated with MSC-CM served as a negative control.

RNAase protect reagent (Qiagen, Germany) was used to preserve the samples once collected. RNeasy® Micro Kit (Qiagen) was used to purify RNA which was then

quantified with the BioDrop µlite spectrophotometer (Harvard Bioscience, Holliston, MA). Reverse-transcription (RT) was performed using 1 µg of purified RNA and QuantiTect reverse transcription kit (Qiagen) according to manufacturer's instructions. Following RT, the complementary deoxyribonucleic acid (cDNA) was combined with RT2 SYBR Green qPCR Master Mix (Qiagen), and 20 µl of this mix was loaded per well of the custom RT2 Profiler PCR Array. Quantitative real-time PCR was performed using StepOne plus Real-Time PCR system (Thermo Fisher Scientific) with a 10-min initial activation step (95°C), a two-step cycling for 40 cycles (15 s at 95°C and 1 min at 60°C), and a final melting curve analysis (95°C 15s, 60°C 60s). The fold change in mRNA expression was calculated by the ΔΔCt method. GAPDH was used as a housekeeping gene.

Table 7. Primer sequences of profibrotic genes induced by TGF-β

PRIMER	DIRECTION	SEQUENCE 5' TO 3'
α-SMA/ACTA 2	Forward	CTTTCTACAATGAGCTTCGTG
	Reverse	ATTTGAGTCATTTTCTCCCG
COL1A1	Forward	CAGGCTGGTGTGATGGGATT
	Reverse	CTCCATCTTTGCCAGCAGGA
CTGF/CCN2	Forward	ATTCTGTCACTTCGGCTCCC
	Reverse	CTGCTACTTGCAGCTGCTCT
PAI-1/SERPINE-1	Forward	CTCATCAGCCACTGGAAAGGCA
	Reverse	GACTCGTGAAGTCAGCCTGAAAC
GLS-1	Forward	AGTTGCTGGGGGCATTCTTTTAGTT
	Reverse	CCTTTGATCACCACCTTCTCTTCGA
ADAM 12	Forward	GCAGTTTCACGGAAACCCAC
	Reverse	ACACGTGCTGAGACTGACTG
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
	Reverse	TTGATTTTGGAGGGATCTCG

2.5.3. α-SMA, stress fibers, collagen I and ECM proteins (Immunofluorescence)

To visualize α-SMA, stress fibers and collagen I, fibroblasts were seeded at a density of 4 x 10^3 cells/cm^2 in 8-chamber slides in complete fibroblast medium and

incubated overnight at 37°C. After 72h of activation, cells were fixed with 4% paraformaldehyde (PFA) for 12 min, and permeabilized with 0.1% Triton-X in PBS for 10 min. To prevent non-specific binding, samples were treated for 40 min with blocking solution (22.52 mg/ml glycine in PBST -0.1% Tween 20 in PBS- supplemented with 5% FBS), and incubated overnight at 4°C with 1:750 rabbit anti-human α-SMA antibody (ab5694, Abcam, Boston, MA), 1:750 sheep anti-human Procollagen I antibody (AF6220, R&D systems), and 1:1000 Phalloidin-iFluor 647 reagent (Abcam) which binds fibrillar actin highlighting stress fibers. Secondary antibodies were a CY3-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:250) and Alexa fluor 488 donkey anti-sheep immunoglobulin G (Abcam). The nucleus was stained with 0.3 μM DAPI. α-SMA, collagen I, and stress fibers were visualized using a Zeiss LSM780 Laser Scanning Confocal Microscope.

To evaluate fibrillin-1 and fibronectin, myofibroblasts were washed three times with PBS (200 μl/ well) and fixed with a solution of 70% methanol and 30% acetone. Cells were not permeabilized so as to visualize only the secreted extracellular fibers. After washing with PBS, 150 μl of blocking buffer, (1:10 of normal donkey serum/PBS, Jackson Immuno Research labs, 005-555121) was added to each well and incubated for 30 min at 25°C. Cells were incubated for 90 min with 1:1000 mouse anti-C-terminal-fibrillin 1 antibody (Reinhardt Laboratory, McGill University) and 1:1000 rabbit anti-fibronectin antibody (IST-9 ab6328, Abcam). Cells were washed with PBS as previously described and incubated with the secondary antibodies for 1 h: Alexa Fluor 400 conjugated goat anti-rabbit IgG (Life technologies, A11008) and Cy3-conjugated affiniPure goat anti-mouse IgG (Jackson Immuno Research Lab. 111-166-003). After washing, nuclei were

stained with 0.3 μM DAPI. The chamber-slide was removed and mounted with Vectashield mounting medium. Fibrillin-1 and fibronectin were visualized using the Zen 2012 software (Zeiss) with an Axio Imager M2 microscope (Zeiss) equipped with an ORCA-flash4.0 camera (Hamamatsu). For quantification we followed a standardized protocol and measured the Total Specific Intensity of the ECM fibers normalized to the number of cells [208]. ImageJ was used for quantification of the Total Specific Intensity of each fiber and normalized to the number of cells.

2.5.4. Cell viability (Flow cytometry)

Fibroblast, myofibroblast and MSC viability was assessed by flow cytometry. Both cells and supernatant were collected and centrifuged at 1000 x g, for 10 min at 4°C. The cell pellet was resuspended in Annexin V Binding Buffer 1X (BD Biosciences) with PE-conjugated Annexin V (AB_286907, BD Biosciences) and DRAQ7 (far-red fluorescent DNA dye, Abcam UK), and incubated for 15 min at 25 °C in the dark. Cell viability (i.e. viability defined as annexin V negative and DRAQ7 negative) was assessed by flow cytometry using BD LSRFortessa cell analyzer, and the data was analyzed by FlowJo software 10.8.1.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (Graph-Pad, San Diego, CA). Results from experiments evaluating the effect of CM obtained from different MSC donors are exhibited as individual points in graphs and means ± standard deviation are shown. Non-parametric analyses were used for all comparisons. The Wilcoxon signed-rank test was used to compare paired samples (e.g., resting and primed MSC-CM from same donor). The Kruskal-Wallis test was used for comparisons between

groups (e.g., primed MSC-CM versus resting MSC-CM versus control medium). All tests were 2-sided. A p-value of <0.05 was considered statistically significant. In each figure, asterisks indicate the statistical significance.

CHAPTER 3. RESULTS

3.1. MSC(AT) fulfill ISCT minimal definition criteria

The MSC used in this study fulfilled the minimal definition criteria proposed by the ISCT [136]. Specifically, MSC had spindle-cell morphology and adhered to plastic under standard culture conditions (Figure 10 A). Under appropriate conditions, they differentiated in vitro into three lineages: adipocytes recognized by Oil Red O stain, osteoblasts that generated extracellular calcium deposits detected by Alizarin Red, and chondrocytes with sulfated proteoglycans deposits stained by Alcian Blue (Figure 10 B). MSC also had the characteristic surface antigens as determined by flow cytometry. They were positive for CD90, CD105, CD73 and negative for CD45, CD34, CD20, CD14 and HLA-DR (Figure 10 C).

IFN-γ licensing leads to massive transcriptional induction of Indoleamine 2,3-dioxygenase 1 enzyme (IDO) in human MSC [135], catalyzing the conversion of L-tryptophan into L-kynurenine. In turn, IDO activity becomes a central immunomodulatory effector in MSC, promoting lymphocytes inhibition [135]. To confirm the effect of cytokine priming on MSC, kynurenine concentrations were measured as a surrogate of IDO metabolism in five MSC-CM obtained from independent donors. Kynurenine was detected in primed, but not in resting MSC-CM (n=5, primed vs. resting MSC, mean ±SD: 3.74±0.31 vs. -0.26±0.02, $p<0.0001$) (Figure 10 D).

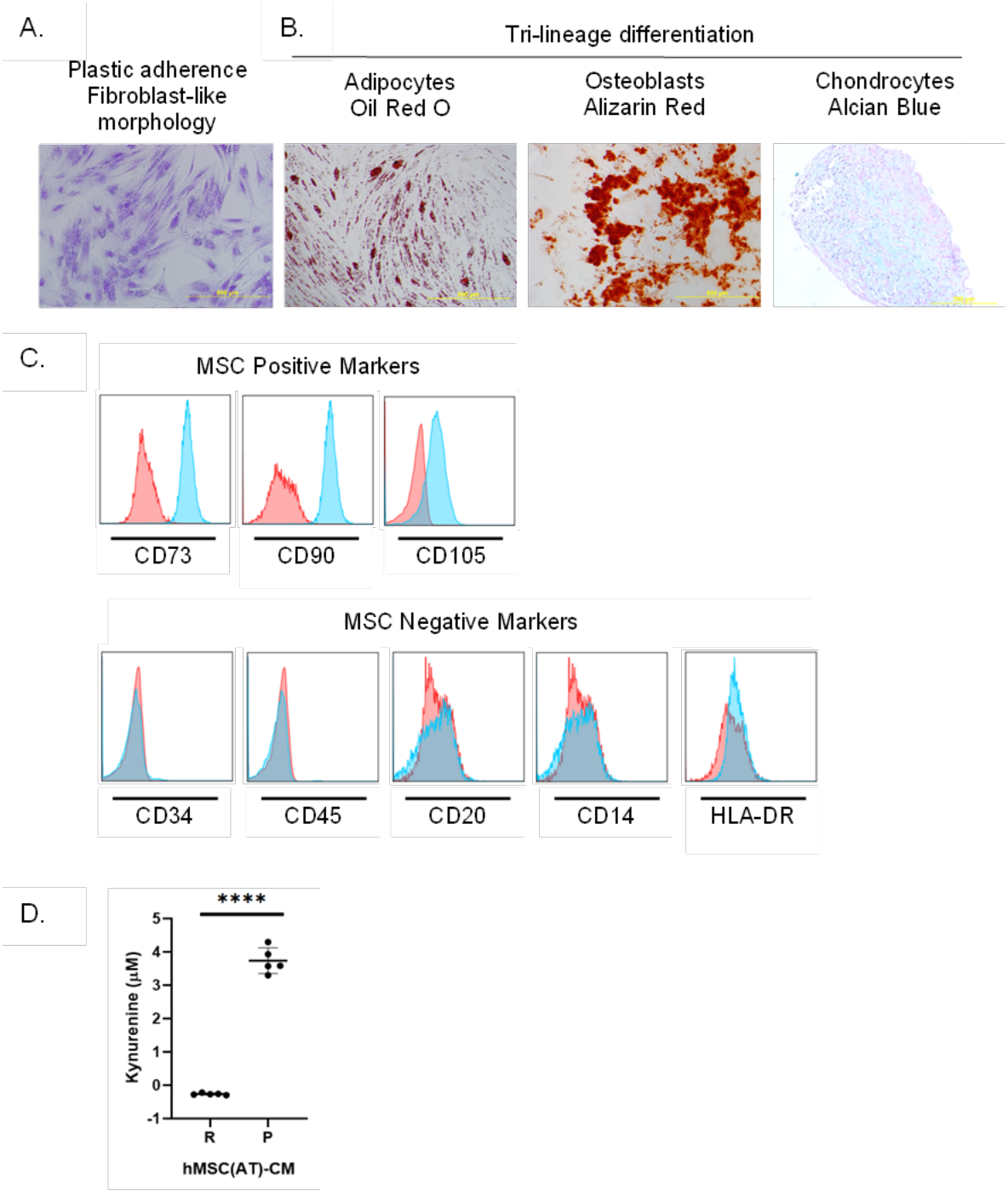
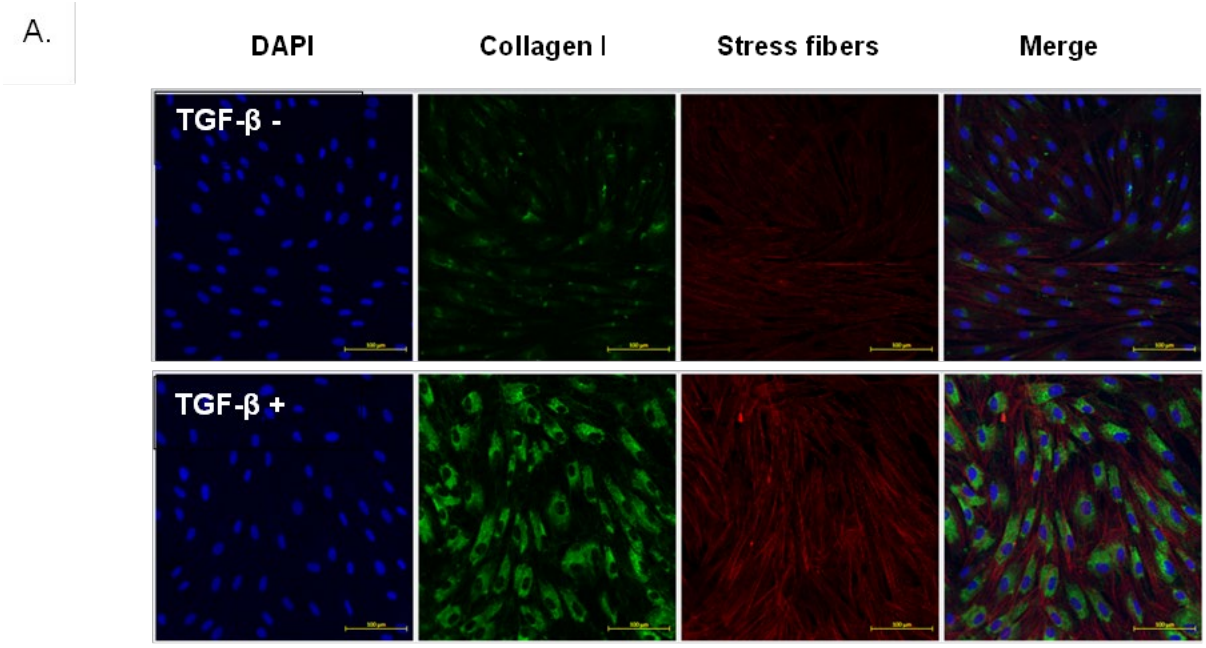


Figure 10**. Characterization of hMSC(AT)**
Human adipose tissue-derived MSC: **A**. are adherent to plastic and have spindle-shaped morphology in standard culture conditions; **B.** differenciate into osteoblasts, adipocytes and chondroblasts when provided appropriate culture conditions; and **C**. are positive for the following surface markers: CD73, CD90 and CD105, and negative for CD34, CD45, CD20, CD14 and HLA-DR. **D.** The concentration of kynurenine, an indicator of indoleamine-2,3-dioxygenase (IDO) activity, increased in CM following MSC activation (resting-R vs. primed-P hMSC(AT)-CM, n=5, ****p<0.0001).

3.2. TGF-β activates fibroblasts into myofibroblasts

TGF-β is a key fibrogenic cytokine central to SSc pathogenesis [209]. TGF-β mediates fibroblast-to-myofibroblast conversion and promotes the secretion of ECM proteins [12]. In our in vitro experiments, we activated fibroblasts with TGF-β to mimic fibrogenic conditions relevant to SSc. Following TGF-β activation, the spindle-shape fibroblast morphology was lost and instead cells had a prominent cytoplasm and high collagen I content (i.e. myofibroblasts) (Figure 11 A). Confirming the phenotype of myofibroblasts was essential for their subsequent in vitro modulation. One of the earliest cellular responses to TGF-β signaling is the reorganization of the actin cytoskeleton [210]. Phalloidin is a bicyclic peptide that selectively labels actin filaments (F-actin). We used fluorescent phalloidin conjugates for actin staining and confirmed that myofibroblasts contained more actin fibers than normal fibroblasts (Figure 11 A). Myofibroblast activation culminates with the expression of α-SMA and an associated increase in contractile forces. In fact, neo-expression of α-SMA in stress fibers is the most widely used molecular marker for myofibroblasts [56] which, in turn, are the predominant source of collagen I. We determined the expression of *ACTA2* and *COL1A1,* and measured α-SMA and procollagen I protein content in cell lysates from fibroblasts and myofibroblasts. Fibroblast-to-myofibroblast conversion was characterized by increased mRNA and protein levels of α-SMA and procollagen I [fibroblasts vs. myofibroblasts fold increase (mRNA n=4; protein n=5). mRNA: *ACTA2*: 8.24± 6.92, $p<0.05$; *COL1A1:* 3.67±1.27, $p<0.05$. Protein: α-SMA: 1.708 ± 0.37, $p=0.0016$; procollagen 1.17±0.22, $p=0.0003$]. We also evaluated the expression of downstream genes that mediate the profibrotic effects of TGF-β. Specifically, we evaluated connective tissue growth factor (*CCN2*),

plasminogen activator inhibitor-1 (*PAI-1*), ADAM Metallopeptidase Domain 12 (*ADAM12*), and glutaminase 1 (*GLS1*). We confirmed that following fibroblast induced-TGF-β activation these pro-fibrotic genes were up-regulated (fibroblasts vs. myofibroblasts fold increase mRNA n=4, *CCN2: 5.17±3.03, p=0.04; PAI-1:2.49±0.82, p=0.009; ADAM12: 13.29 ± 4.79, p=0.03; GLS1:* 2.92±1.34,p=0.02) (Figure 11 B-C-D). TGF-β was shown to promote myofibroblast resistance to apoptosis [9]. In order to test this, we exposed fibroblasts and myofibroblasts for 24h to two doses of staurosporine, a well-known inducer of apoptosis [211]. Irrespective of the dose, staurosporine-induced apoptosis was reduced in myofibroblasts (difference of means myofibroblasts vs. fibroblasts, n=4, survival rate following 20nM: 30.43 ±6.17, p<0.01 and 40 nM of staurosporine: 35.01±8.26 p<0.001) (Figure 11 E). In summary, these data confirm the expected cellular phenotypes following TGF-β-induced fibroblast-to-myofibroblast activation.



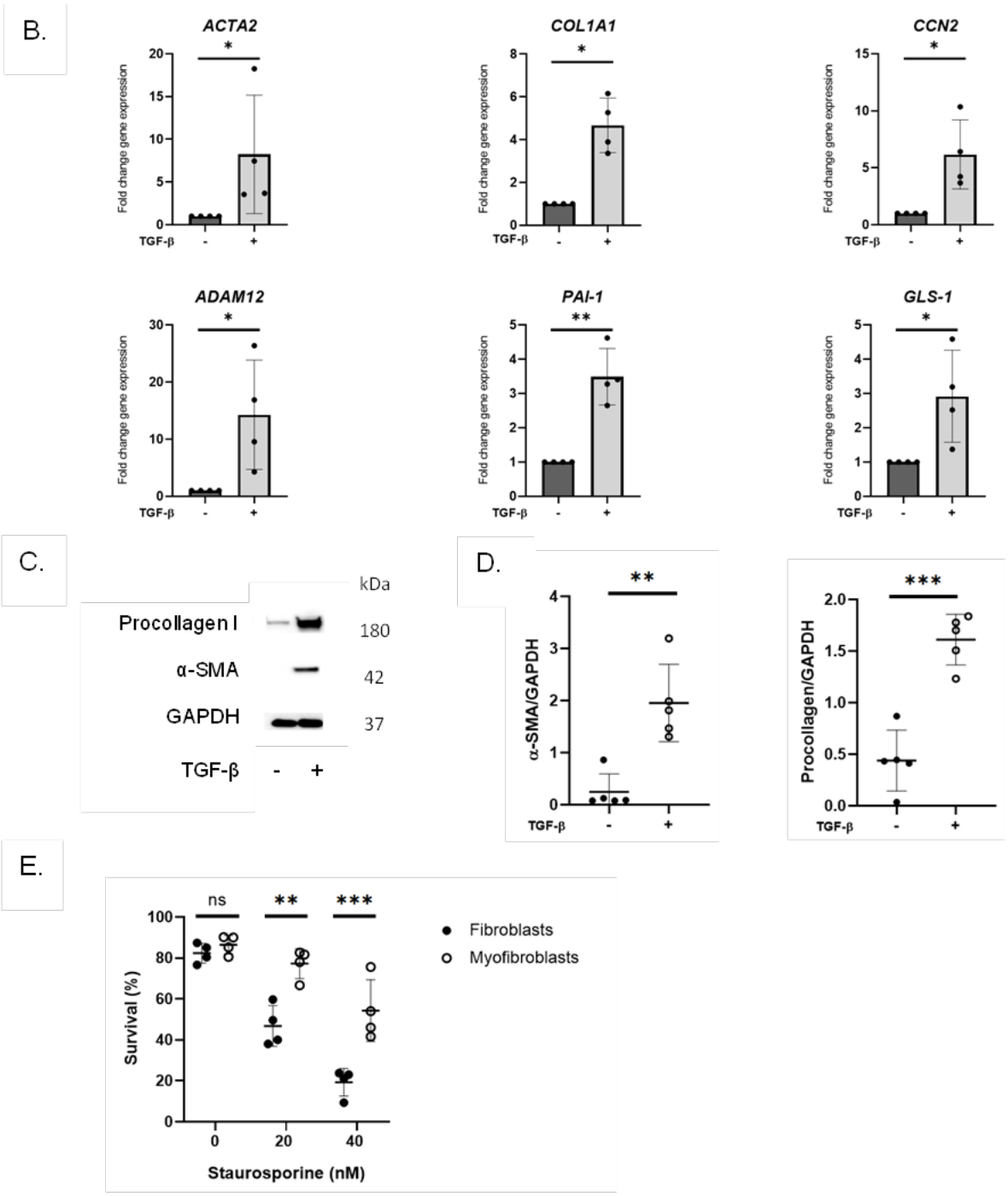
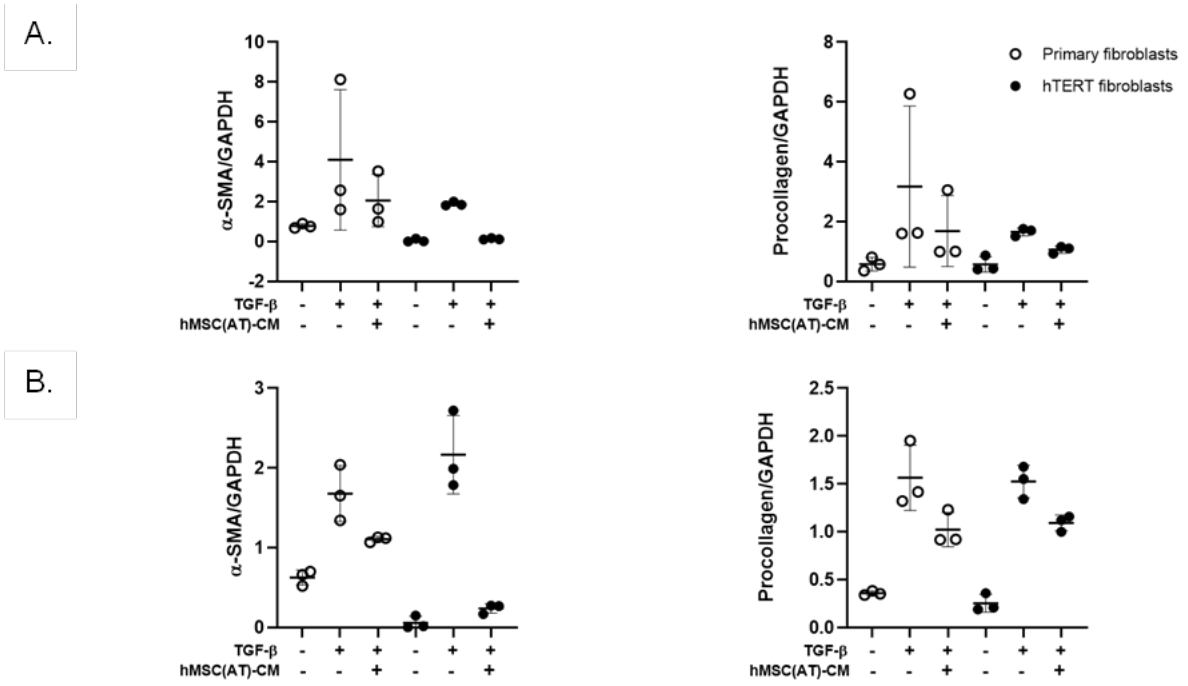


Figure 11**. TGF-β induces fibroblast activation**
Graphs represent data from fibroblasts (TGF-β-) and myofibroblasts (TGF-β+). **A**. Representative example of staining of collagen I and stress fibers by immunofluorescence (Scale: 100μm), **B**. qRT-PCR of TGF-β regulated profibrotic genes. Data are presented as mean ± SD of fibroblasts vs. myofibroblasts, n = 4: ACTA2:*p = 0.03, COL1A1: *p = 0.01, CCN2: *p = 0.04, ADAM12: *p = 0.03, PAI-1:**p = 0.009, GLS1: *p = 0.03. **C**. Representative example of α-SMA and Procollagen type I by Western blot. **D**. Summary data of α-SMA and Procollagen I protein levels normalized to GAPDH, fibroblasts vs. myofibroblasts (n=5): α-SMA **p<0.01, and procollagen I ***p<0.001. **(E)** Fibroblasts vs. myofibroblasts survival after staurosporine apoptosis induction (staurosporine dose 20nM **p<0.01 and 40 nM ***p<0.001).

3.3. MSC-CM exert antifibrotic effects in vitro

3.3.1. Optimization of in vitro antifibrotic assays

We performed proof-of-concept experiments for assay optimization. First, we compared the variability of prevention and deactivation assays using primary versus hTERT fibroblasts (Figure 12 A-B). Although the effect of MSC-CM was evident regardless of the type of fibroblasts used, the inter-assay variability was reduced with hTERT fibroblasts. Second, we performed dose response curves using different dilutions of MSC-CM (Figure 12 C-D). The demonstration of a dose-response relationship in the prevention and deactivation assay provides strong evidence for a causal relationship between the exposure (i.e. MSC-CM) and the outcome (i.e. fibroblast modulation). Finally, we evaluated the intra-assay reproducibility (testing MSC-CM obtained from the same donor at subsequent MSC passages (Figure 12 E). Altogether these results support the reliability of using the in vitro antifibrotic tests proposed.



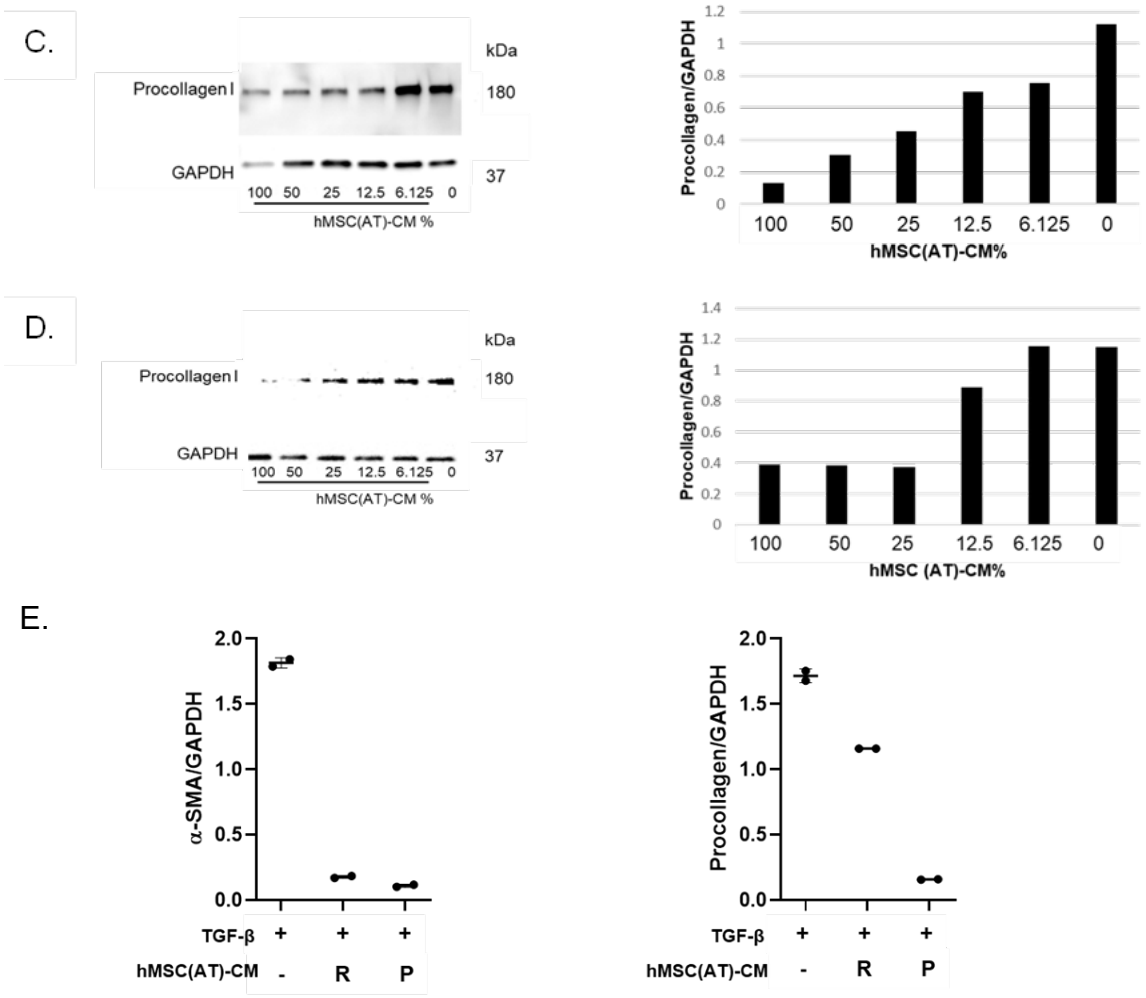


Figure 12. Validation of in vitro antifibrotic assays
A. Prevention and **B.** deactivation assays were performed with either primary fibroblasts or hTERT fibroblasts. The effect of MSC-CM titration experiments on the **C**. prevention and **D**. deactivation assays was tested. **E.** Example of the consistency of CM from same MSC to promote myofibroblast deactivation (i.e., CM from the same MSC-donor were obtained at two different timepoints).

3.3.2. MSC-CM inhibit fibroblast migration

During the physiological wound healing process, fibroblasts are stimulated to migrate to the injury site. In pathologic conditions like SSc, there is an increased number of myofibroblasts in skin that correlates with the severity of clinical manifestations [212]. We tested the capacity of MSC-CM to limit fibroblast migration, using the IncuCyte® Scratch Wound assay, which is a standardized and reproducible method. MSC-CM

inhibited the migratory capacity of fibroblasts. This effect was evidenced by a lower RWD at two different fibroblasts' densities (serum-free DMEM vs. MSC-CM, mean ± SD at 36 h, 2 x 10^4 fibroblasts/well: 76.69±4.28 vs. 33.01±4.22, n=5, p<0.0001, and 3 x 10^4 fibroblasts/well: 73.22±11.28 vs. 43.00±8.28 n=7, p<0.01) (Figure 13 A-C). These results suggest that MSC-CM may modulate an early step of the fibrosis process: the migration of fibroblasts.

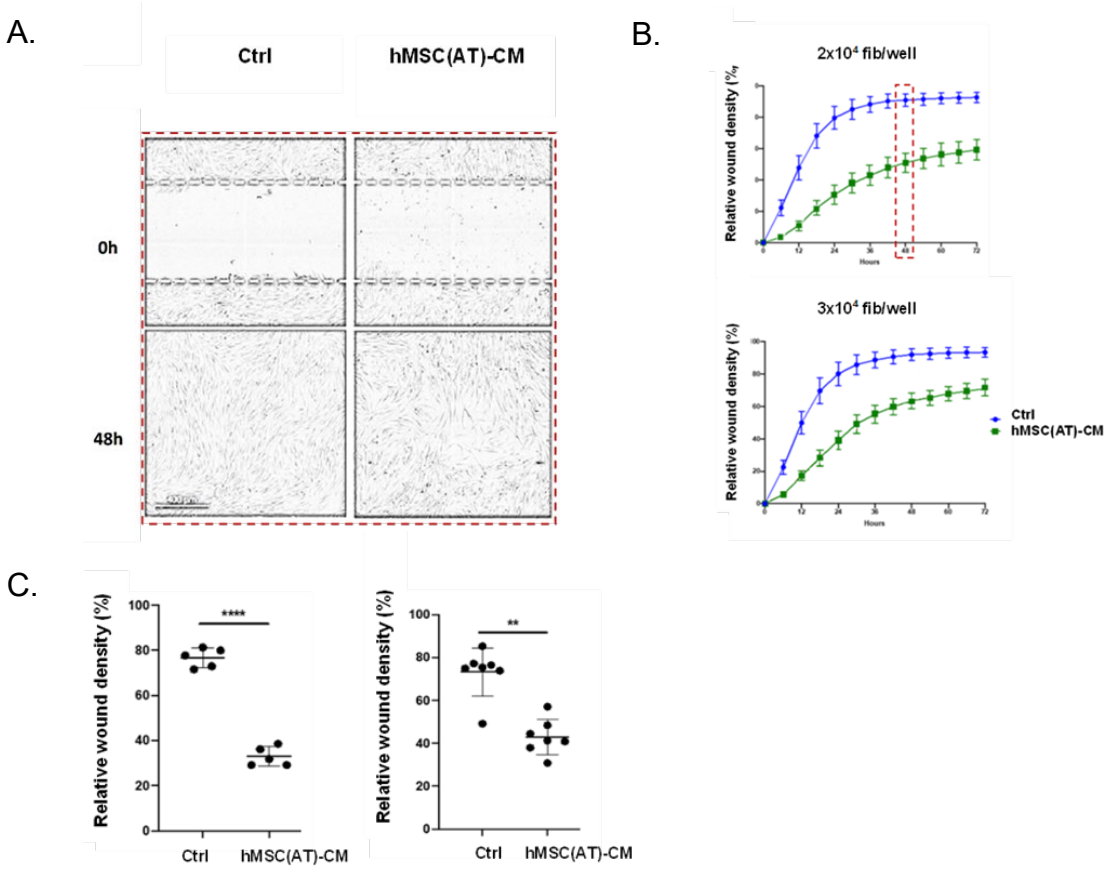


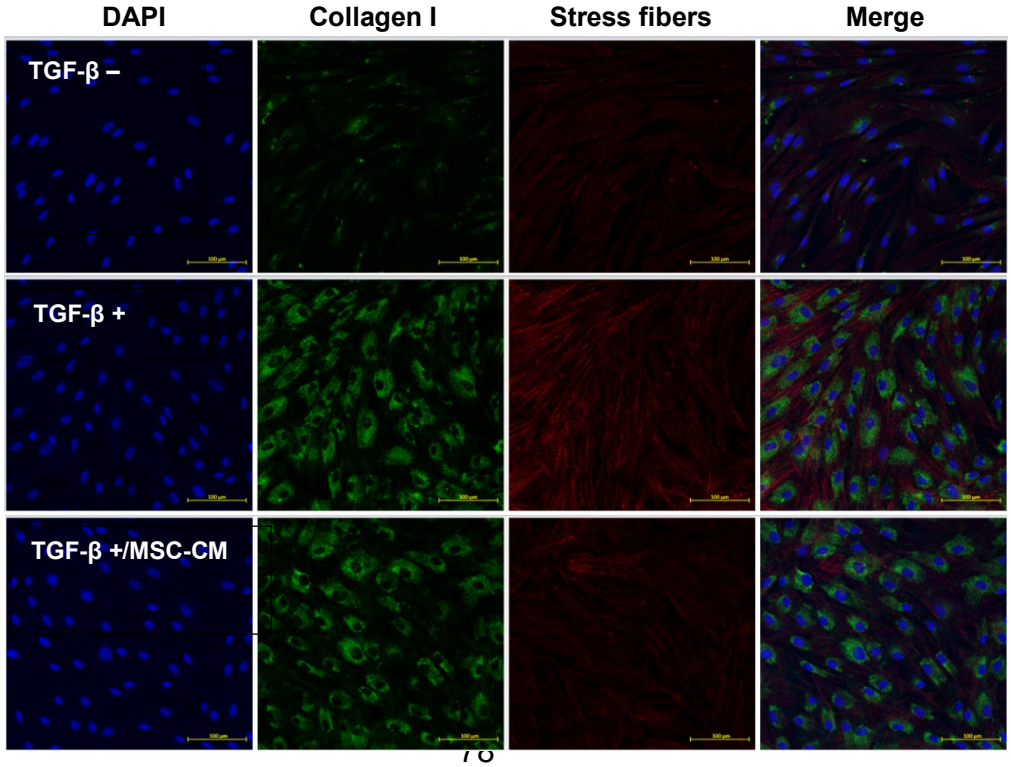
Figure 13**. MSC-CM inhibit random fibroblasts migration in vitro.**
MSC-CM modulation of fibroblast migration was evaluated in the IncuCyte® Scratch Wound assay. **A**. Representative images of fibroblast migration (2x10^4 cells/well) immediately after (0h) and 48h after scratching (red line) under control conditions (Ctrl) and MSC-CM treated conditions [hMSC(AT)-CM]. **B**. Summary graphs with means ± SEM of the relative wound density overtime (12 h intervals between 0 and 72h). Two different initial fibroblast seeding densities (2 and 3 x 10^4) are depicted in the upper and lower graphs. **C**. Summary data of the relative wound density at 36h. Mean ± SD of each experimental condition: 2x10^4 fibroblasts/well (n=5, ****p<0.0001) and 3x10^4 fibroblasts/well (n=7, p**<0.01).

3.3.3. MSC-CM prevent TGF-β induced fibroblast activation

In vitro, TGF-β promotes fibroblasts differentiation, which resembles the SSc myofibroblast profile [213]. Moreover, constitutive activation of TGF-β signaling in fibroblastic cells of mice recapitulates the fibrotic phenotype characteristic of SSc [214]. TGF-β signaling is increased in SSc [215]. We evaluated if MSC-CM interfered with the TGF-β effect on fibroblasts.

The addition of MSC-CM to TGF-β treated fibroblasts reduced the contractile fibers and the intracellular deposition of collagen I, as visualized by immunofluorescence microscopy (Figure 14 A). This was confirmed by α-SMA and procollagen I protein quantification. MSC-CM prevented TGF-β induced increase of α-SMA (no MSC-CM vs. MSC-CM, n=8: 2.15±0.98 vs. 0.37±0.26, $p \leq 0.01$) and procollagen I proteins (no MSC-CM vs. MSC-CM, n=8: 1.70±0.85 vs. 0.77±0.39 $p<0.01$) (Figure 14 B-C). These results demonstrate that MSC-CM reduces fibroblast-myofibroblast activation in vitro.

A.



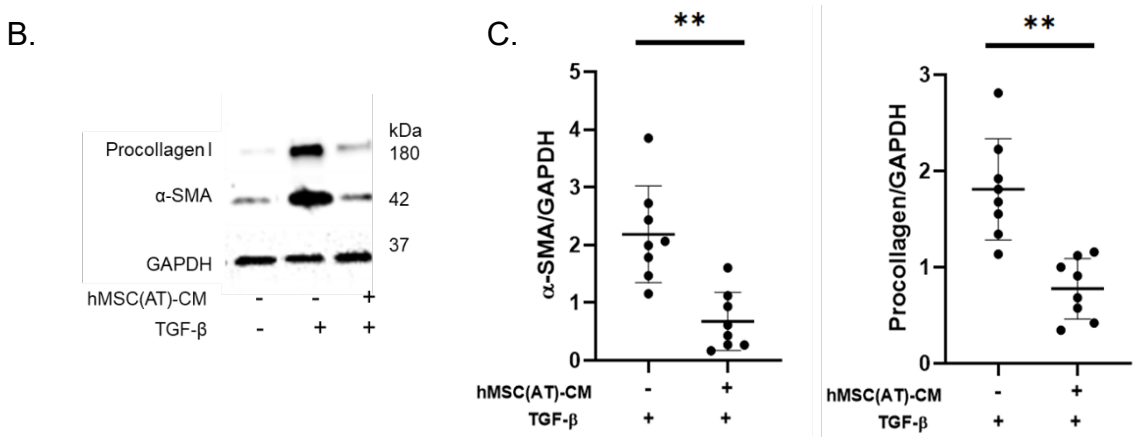


Figure 14**. MSC-CM prevent TGF-β induced fibroblasts-to-myofibroblasts activation.**
A. Representative immunofluorescence images of collagen I and stress fibers in non-activated fibroblasts, fibroblasts activated with TGF-β, and fibroblasts simultaneously treated with TGF-β and MSC-CM. **B** Representative Western Blot image for α-SMA and procollagen I.**C.** Summary data of α-SMA and type I procollagen protein levels from prevention assays. The levels of those proteins are reduced in fibroblasts treated simultaneously with TGF-β and MSC-CM indicating lower activation compared to controls. α-SMA: Control vs. MSC-CM, n=8: **p<0.01; type I procollagen: Control vs. MSC-CM, n=8: **p<0.01, normalized to GAPDH.

3.3.4. MSC-CM deactivate myofibroblasts

In a normal wound healing response myofibroblasts are a transient phenotype adopted by a heterogeneous number of cells. Myofibroblast persistence leads to excessive ECM production. Therefore, the deactivation or elimination of myofibroblasts is key for fibrosis resolution [112]. We tested the capacity of the MSC-CM to deactivate myofibroblasts. MSC-CM treatment resulted in reduced myofibroblast RNA expression of the following key TGF-β-induced pro-fibrotic genes: *ACTA2* (fold change, n=4: 2.8±0.5)*, COL1A1* (fold change, n=4:1.18±0.3)*, CCN2* (fold change, n=4: 0.9±0.2)*, ADAM12* (fold change, n=4: 4.97±1.5), *PAI-1* (fold change, n=4:0.7±0.46)*,* and *GLS1* (fold change, n=4: 0.74±0.2) (Figure 15 A)*.* The MSC-CM effect on deactivating myofibroblasts was also documented at a protein level, with a reduction of α-SMA (no MSC-CM vs. MSC-CM, n=8: 2.18±0.83 vs. 0.67±0.50, p<0.01) and procollagen I (no MSC-CM vs. MSC-CM, n=8:

1.81±0.52 vs. 0.77±0.31, p<0.01). This effect was not confounded by cell death, as myofibroblast viability prior to protein quantification was similar in myofibroblasts treated or not treated with MSC-CM (% myofibroblast survival, control vs. MSC-CM, n=6: 90.18±3.22 vs.93.48±1.79, p>0.05) (Figure 15 B-C). In summary, these data show that MSC-CM deactivates myofibroblasts, an outcome of most significance for fibrosis reversal.

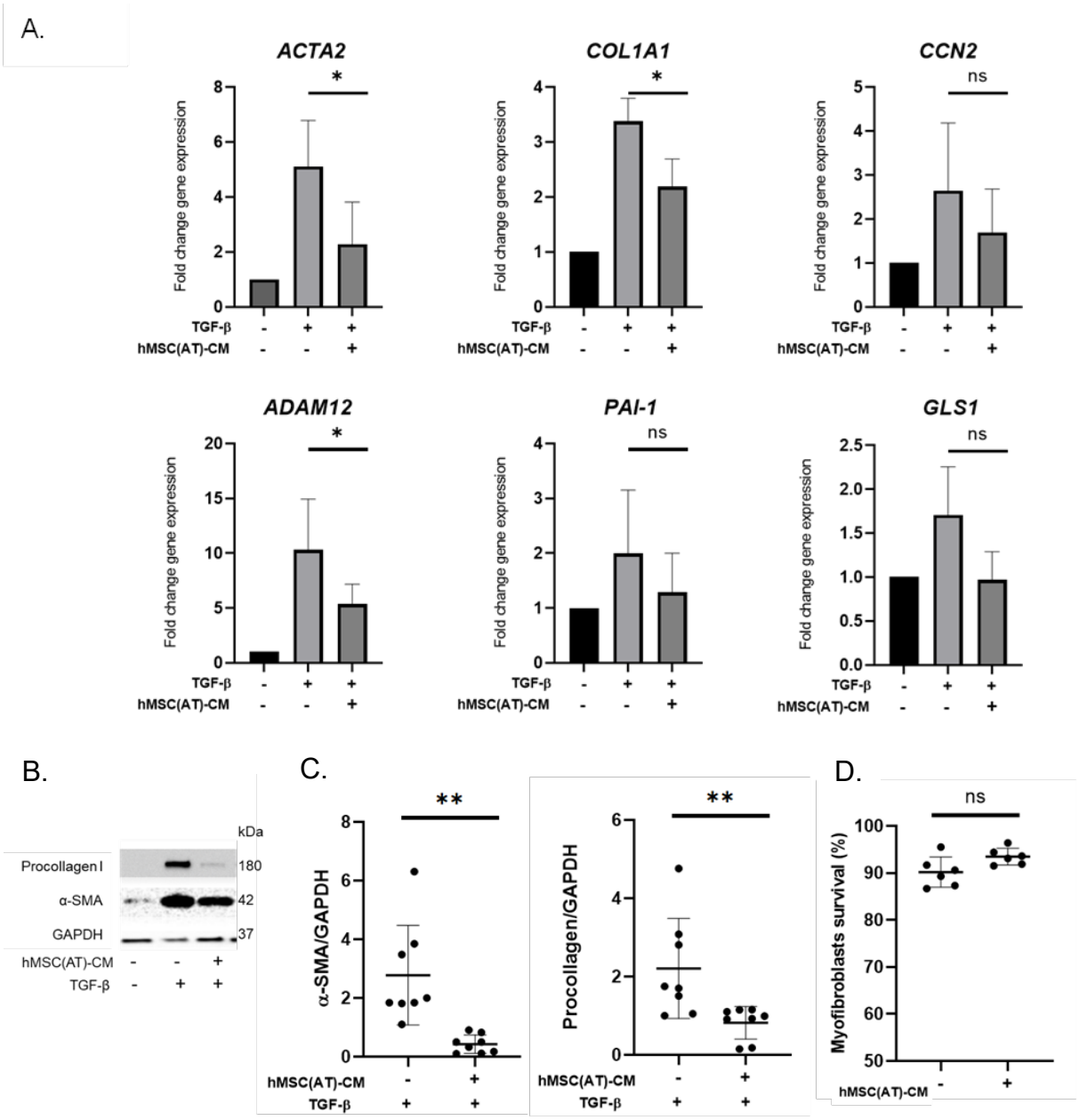


Figure 15. **MSC-CM promote myofibroblast deactivation**.
A. Preliminary data suggesting that MSC-CM may reduce the expression of TGF-β regulated profibrotic genes (myofibroblasts vs. myofibroblast treated with MSC-CM n=4, *p<0.05). α-SMA and collagen I protein levels were determined: **B.** representative Western blot images, **C.** summary data of α-SMA (Control vs. MSC(AT)-CM **p<0.01, n=8), and procollagen I (Control vs. MSC(AT)-CM **p<0.01, n=8) normalized to GAPDH. **D.** Myofibroblast viability at the time of protein quantification was similar in myofibroblasts and myofibroblasts treated with MSC-CM (ns=p>0.05).

3.3.5. MSC-CM do not restore apoptosis sensitivity in myofibroblasts

After wound closure, myofibroblasts undergo apoptosis leading to the last phase of the wound healing process called regeneration [216]. Myofibroblasts elimination allows the functional tissue cells to proliferate and restore organ function [217]. In fibrosis, a disbalance between proapoptotic and anti-apoptotic factors promotes myofibroblasts survival leading to an excessive deposition of ECM [59]. MSC are known for their capacity to promote apoptosis of activated T cells [218]. We aimed to evaluate if MSC-CM restored the myofibroblasts apoptosis sensitivity. In section 3.3.4, we demonstrated that MSC-CM did not promote myofibroblast apoptosis. In section 3.2, we presented evidence indicating that, compared to fibroblasts, myofibroblasts were more resistant to staurosporine apoptosis induction. Here we assessed if MSC-CM restored apoptosis sensitivity to staurosporine. Contrary to our expectations, following staurosporine treatment, myofibroblasts had similar viability (% myofibroblast viability, control vs. MSC-CM, n=5: 63.40±12.62 vs. 76.50±13.72, p>0.05) whether treated with MSC-CM or not (Figure 16). From this experiment, we concluded that MSC-CM were not able to restore myofibroblast apoptosis sensitivity.

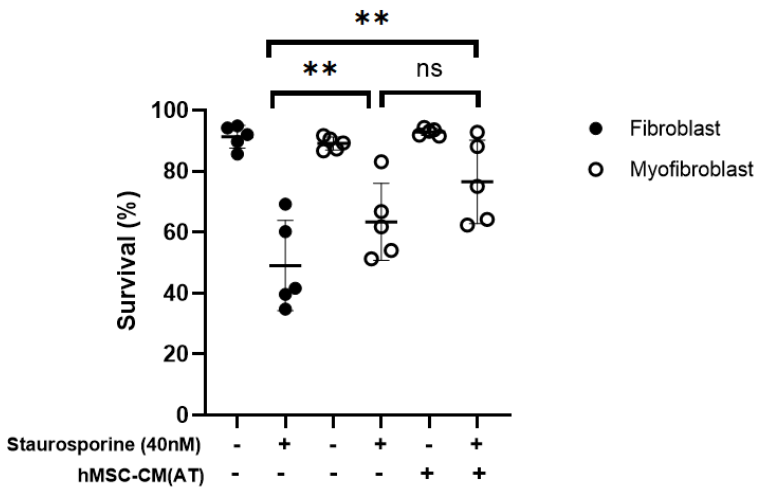


Figure 16**. MSC-CM do not restore myofibroblasts' apoptosis sensitivity**
Fibroblasts, myofibroblasts and myofibroblasts treated with hMSC-CM(AT) were induced to undergo apoptosis with 40 nM of staurosporine. Compared to fibroblasts, myofibroblasts were more resistant to staurosporine induced-apoptosis. This effect was not modulated by MSC-CM treatment. Survival of fibroblasts vs. myofibroblasts after treatment with staurosporine n=5: **p<0.01, myofibroblasts vs. myofibroblasts treated with MSC-CM ns p>0.05.

3.3.6. MSC-CM modulate extracellular matrix components

Fibrosis is characterized by the excessive production and accumulation of ECM and a dysregulation between proteolytic enzymes and their inhibitors. Eliminating excessive extracellular matrix is another key factor to reversing fibrosis [112]. Fibronectin and fibrillin-1 are two key ECM proteins. Fibronectin is a "master organizer" in ECM assembly [106], while fibrillin-1 is a microfilament that modulates TGF-β activation [101]. Of relevance, mutations in fibrillin-1 cause an autosomal dominant form of SSc [219]. We tested the MSC-CM capacity to modulate fibronectin and fibrillin-1. Following MSC-CM treatment of myofibroblasts, the fibronectin and fibrillin-1 fibers appeared less mature and thinner compared to fibers secreted by myofibroblasts not treated with MSC-CM. The quantification of fibers (i.e., *Total Specific Intensity* of the ECM fibers normalized to the number of myofibroblasts) confirmed that MSC-CM treatment reduced the amount of

fibrillin -1 (fibrillin-1 control vs. MSC-CM, n=4: 2524±1070 vs. 1227±329.0, p<0.05. Fibronectin control vs. MSC-CM, n=4: 6856±4396 vs. 4724±1876 p=0.40). These results provide evidence for the ability of MSC to reduce the accumulation of ECM.

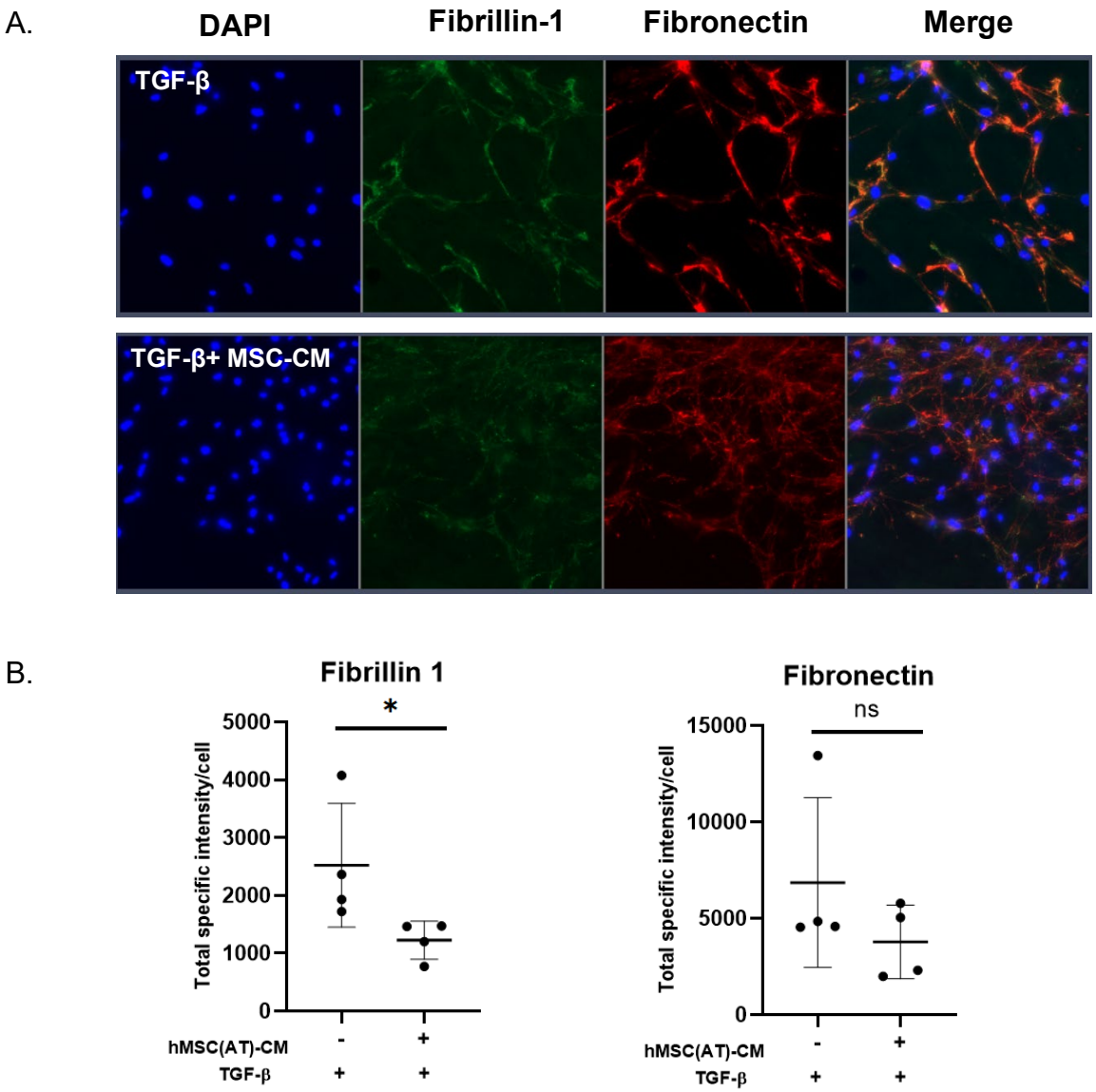


Figure 17**. MSC-CM modulate ECM components.**
A. Representative immunofluorescence of myofibroblasts and myofibroblasts treated with MSC-CM. In MSC-CM treated myofibroblasts, fibronectin and fibrillin-1 fibers appear less mature and thinner compared to fibers secreted by myofibroblasts not treated with MSC-CM. **B.** Summary data of Fibrillin-1 and Fibronectin quantification in myofibroblasts treated or not with MSC-CM. (Myofibroblasts vs. Myofibroblasts treated with MCS-CM, n=4, Fibrillin-1: *p<0.05; Fibronectin: ns p>0.05).

3.4. MSC priming enhances the antifibrotic activity of MSC-CM without restoring myofibroblasts apoptosis sensitivity

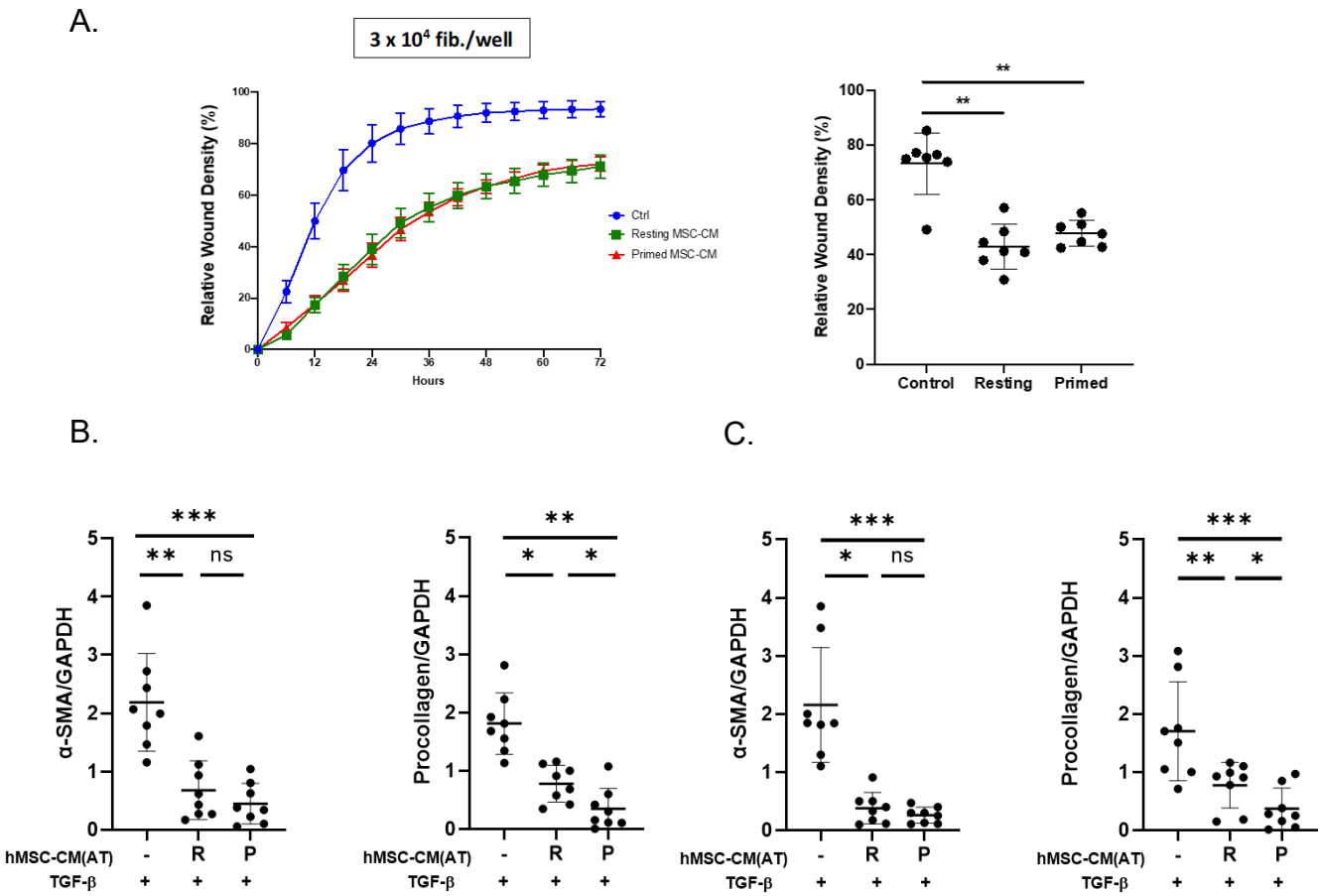
Cytokine priming modifies the composition of the MSC secretome [220], enhancing MSC immunopotency [221]. We investigated the effect of priming on MSC's antifibrotic activity using the in vitro assays that we have established. Priming did not affect the inhibition of fibroblast migration (resting vs. primed MSC-CM: 33.01 ± 4.225 vs. 39.95 ± 5.618 n=5, p=ns) (Figure 18 A). In contrast, primed MSC-CM had a stronger effect in modulating procollagen I levels in both, prevention and deactivation assays. (Prevention assay: procollagen I, resting vs. primed MSC-CM, n=8: 0.77±0.39 vs.0.37±0.35; $p<0.05$ -Figure 18 B; Myofibroblast deactivation assay: procollagen I, resting vs. primed MSC-CM, n=8: 0.78±0.31 vs. 0.34±0.35; $p<0.05$ -Figure 18 C).

To determine whether primed MSC-CM restore myofibroblasts apoptosis sensitivity to staurosporine, we compared the viability of staurosporine-treated myofibroblasts with or without primed MSC-CM. Primed MSC-CM did not affect myofibroblast viability (% myofibroblast viability, control vs. primed MSC-CM, n=4: 66.43±12.3 vs. 72.68±17.22, $p>0.05$), which was similar to that of MSC-CM not exposed myofibroblasts. Thus, we concluded that neither resting (section 3.3.5) nor primed MSC-CM restore myofibroblast apoptosis sensitivity to staurosporine (Figure 18 D).

The post-translational modulation of collagen depends on the balance between its synthesis and degradation [222]. Metalloproteinases (MMP) and their antagonists [tissue inhibitors of metalloproteinases (TIMPs)] regulate ECM deposition. As an exploratory experiment, we evaluated the effect of MSC priming on the CM quantity of MMPs and TIMPS (Human MMP 9-Plex and TIMP 4-Plex Eve technology, Calgary, AB). The

concentrations of MMP-1, MMP-3, and MMP-10 were higher in primed MSC-CM, while those of TIMP-2 were reduced (Figure 18 E) (Resting vs. primed, mean ± SD, n=3: MMP-1: 421.5± 579.6 vs. 3259±1700; MMP-3: 3527.1±40.87 vs. 8992 ±5587; MMP-10: 174.3±112.1vs. 624.4±296.8; TIMP-2 8368 ±951.3 vs. 4780 ±1528).

In conclusion, MSC-cytokine priming 'selectively' enhances the antifibrotic effects of MSC-CM. Specifically, MSC priming increases the effect of CM in the fibroblast prevention and myofibroblast deactivation assays (readout procollagen I), but not in the fibroblast migration assay. An effect of priming on the ECM remodeling is suggested by the post-cytokine activation increase of MMPs in MSC-CM.



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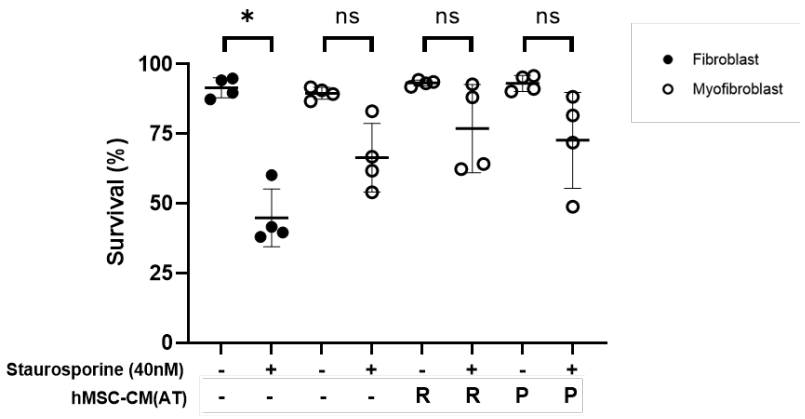

E.

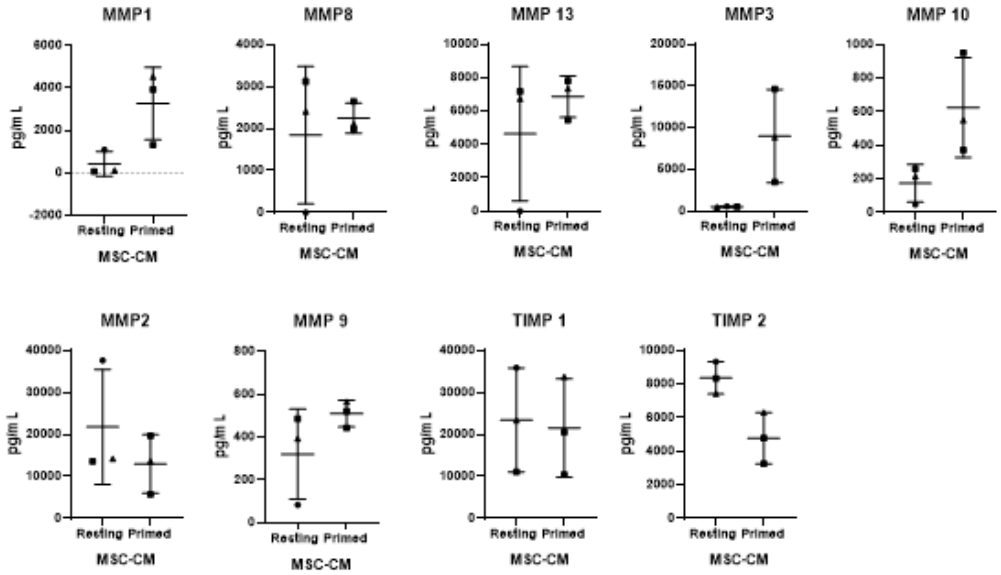

Figure 18. **Cytokine priming increases MSC-CM antifibrotic effects.**
A. Priming did not increase the wound healing ability of MSC-CM in the scratch assay. Fibroblasts were seeded at a density of 3 x 10^4; the relative wound density at 36 h (n=7) of resting versus primed MSC-CM was ns ($p>0.05$). In contrast, in the **B.** prevention of fibroblast activation, and **C.** myofibroblasts deactivation assays priming resulted in a reduction of Procollagen type I. (Procollagen I, prevention assay, resting vs. primed MSC-CM, n=8: *$p<0.05$; deactivation assay, resting vs. primed SMC-CM, n=7: *$p<0.05$). There were no differences in the levels of α-SMA in the prevention and deactivation assays following treatment with resting or primed MSC-CM (α-SMA, prevention assay, resting vs. primed MSC-CM, n=8: ns $p>0.05$: deactivation assay: resting vs. primed MSC-CM, n=7: ns $p>0.05$). **D.** Primed MSC-CM did not restore myofibroblasts' apoptosis sensitivity. Myofibroblasts were more resistant to staurosporine induced-apoptosis than fibroblasts. This effect was not modulated by neither resting nor primed MSC-CM. Survival of fibroblasts pre- vs. post-staurosporine treatment n=4: *$p<0.05$; survival of myofibroblasts versus

myofibroblasts treated with either primed or resting MSC-CM n=4: p>0.05 (ns = non-significant) **E.** Concentrations of specific MMPs and TIMPs were measured in resting and primed MSC-CM.

3.5. SSc MSC(AT): clonogenicity, immunopotency and in vitro antifibrotic effects

The MSC functional properties vary according to multiple determinants including the MSC donor [145]. There is limited evidence suggesting that SSc MSC have a senescent phenotype and that components of their secretome could be involved in promoting fibrosis [193]. We characterized MSC from SSc patients and controls (n=5). MSC from both groups fulfilled ISCT criteria (Figure 19 A-B), and did not differ in their proliferation capacity (doubling time in days: HC vs. SSc MSC: 2.76±0.42 vs. 3.67±0.99; p=0.14), clonogenicity (1/stem cell frequency: HC vs. SSc MSC: 7.47±3.72 vs. 8.54±4.19; p=0.46) or immunopotency (HC vs. SSc MSC: 25.26±13.5 vs. 25.4±9.86; p=0.97) (Figure 19 C-F). Similar to controls, SSc MSC-CM did not promote fibroblasts activation (α-SMA, DMEM vs. HC vs. SSc MSC-CM, n=5: 0.02± 0.01 vs. 0.014±0.01 vs. 0.023±0.027, p=0.520. Procollagen I, DMEM vs. HC vs. SSc MSC-CM: 0.11±0.01 vs. 0.04±0.02 vs. 0.10±0.09, p>0.05). Moreover, SSc MSC-CM prevented TGF-β induced fibroblasts activation and deactivated myofibroblasts to the same extent as MSC-CM from controls (Prevention assay: α-SMA, DMEM vs. HC vs. SSc MSC-CM: 4.93±5.23 vs. 0.52±0.66 vs. 0.47±0.45 p>0.05; and procollagen I, 2.11±0.97 vs. 0.93±0.50 vs. 1.05±0.21 p>0.05) (Figure 20 A). (Myofibroblast deactivation assay: α-SMA, HC MSC-CM vs. SSc MSC-CM: 0.76±0.69 vs. 0.79±0.55, p= 0.99; and procollagen I, HC MSC-CM vs. SSc MSC-CM: 1.23±0.94 vs.1.38 ±0.89 p= 0.99 -Figure 20 B). However, lower concentrations of MMP-1, MMP-2, MMP-3, MMP-10 and MMP-9 were measured in SSc MSC-CM compared to HC MSC-CM (Figure 20 C). (Ctrl vs. SSc, mean ± SD :MMP-1: 16641±10385 vs.

1810±3033; MMP-2: 810681 ±5956 vs. 10590±4320; MMP-3: 2311±2055 vs. 173.5±160.4; MMP-10: 113.4±28.69 vs. 5.82±6.72 and MMP-9: 157.0±78.35 vs. 75.58±6.51).

These data do not suggest an in vitro profibrotic effect of SSc MSC-CM. In contrast, SSc MSC-CM maintained antifibrotic properties by modulating TGF-β effects on myofibroblasts. However, the lower concentrations of MMPs in SSc MSC-CM may have implications in ECM remodeling, but these results require confirmation.

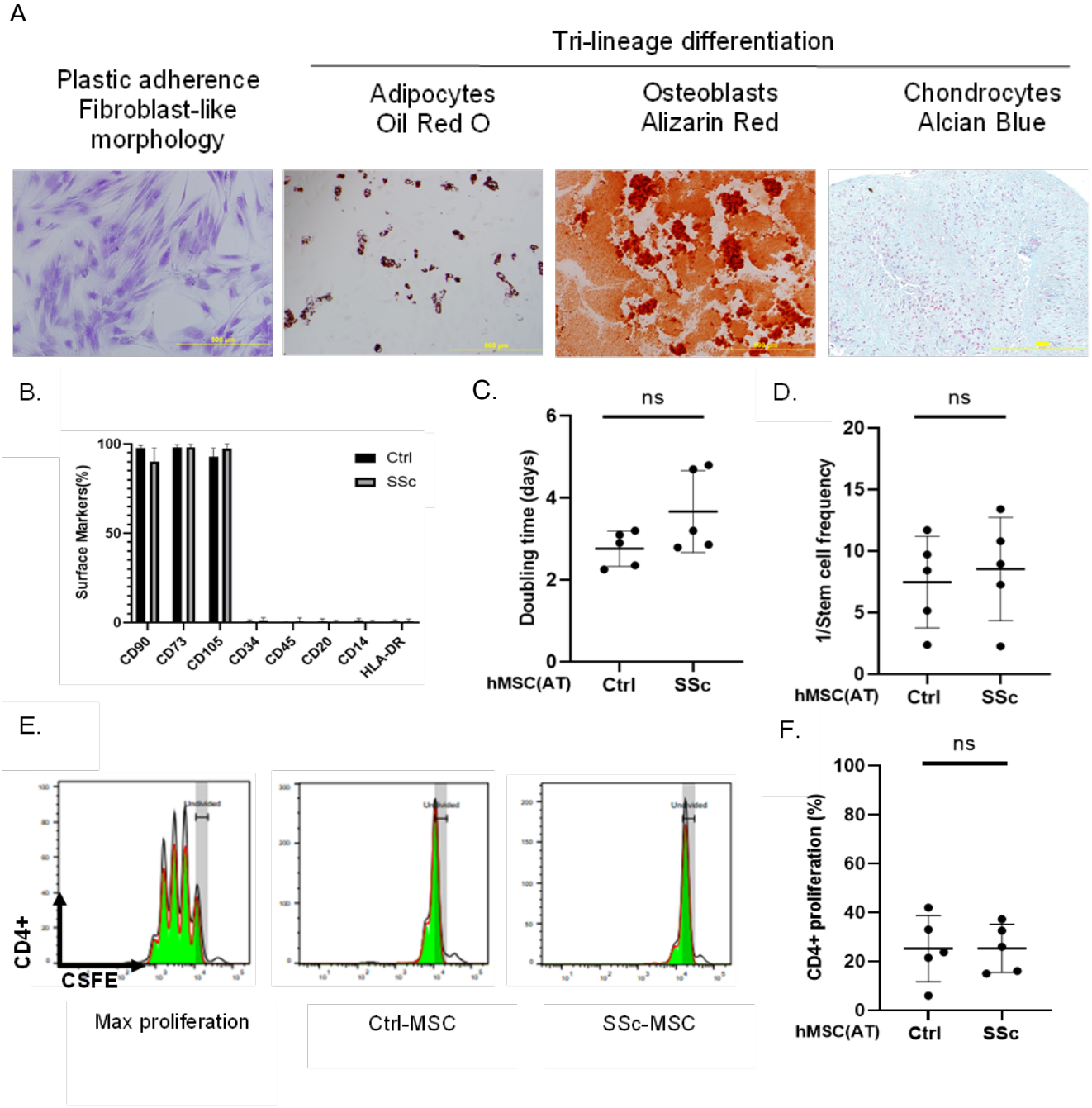

Figure 19**. Characterization of MSC(AT) from SSc patients.**
SSc MSC **A.** are plastic adherent, have spindle shape morphology, and differentiate into adipocytes, osteoblasts, and chondrocytes. Their **B.** surface markers, **C.** proliferation (doubling time), and clonogenicity capacities are similar to MSC(AT) from healthy controls. **D.** MSC Immunopotency assay (i.e., MSC inhibition of activated T-cell proliferation), **E.** representative images of maximal proliferation and effect of healthy versus SSc MSC. **F.** Summary data of immunopotency assay, n=5, Ctrl MSC vs. SSc MSC, ns p>0.05.

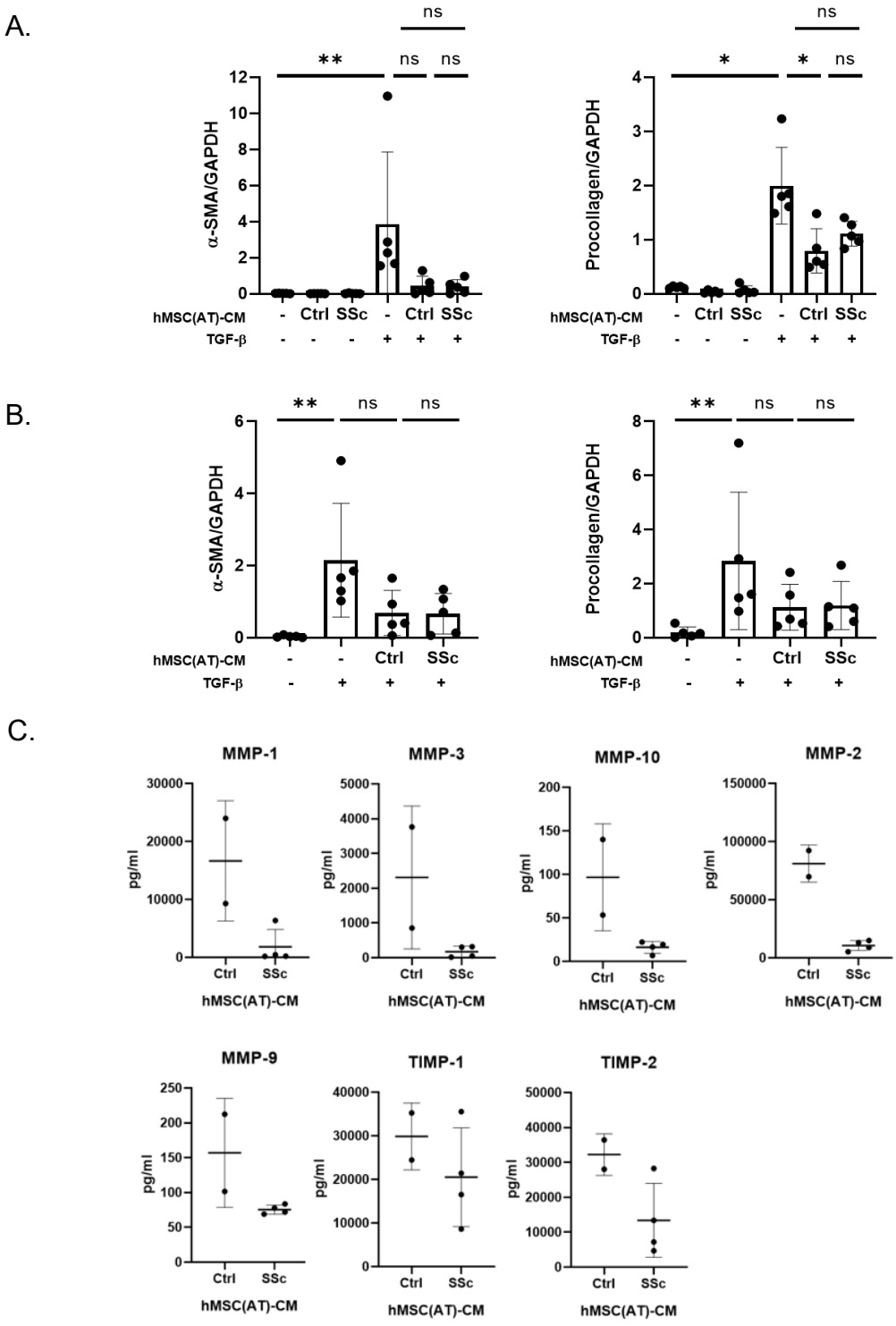

Figure 20. **Similar in vitro antifibrotic effects of SSc and healthy MSC-CM.**
A. SSc MSC-CM did not activate fibroblasts, and similar to healthy MSC-CM, SSc MSC-CM prevented TGF-β activation of fibroblasts, and **B.** deactivated myofibroblasts. **(C)** The concentrations of several MMPs in SSc MSC-CM are reduced compared to controls.

CHAPTER 4. DISCUSSION

Fibrosis is a complex, multistep, redundant cellular and molecular process implicated in various diseases [10, 20]. Furthermore, the burden of fibrotic-related diseases is significant and is estimated to increase [15, 223]. This and the lack of interventions that effectively promote fibrosis resolution highlight both the relevance and need for innovative strategies and provide the rationale for my work [16]. The in vitro effect of approved antifibrotic agents and MSC-CM are summarized in Figure 21. Our results support the following concepts (in bold novel findings):

1- Reliable in vitro assays confirm the existence of a direct antifibrotic effect of MSC-CM. We provide evidence that **MSC-CM deactivate myofibroblasts**.

2- Cytokine priming enhances the in vitro antifibrotic effects of MSC-CM. Specifically, **primed MSC-CM are more efficient than resting ones in deactivating myofibroblasts**.

3- The **antifibrotic effects of SSc MSC-CM in vitro are preserved**.

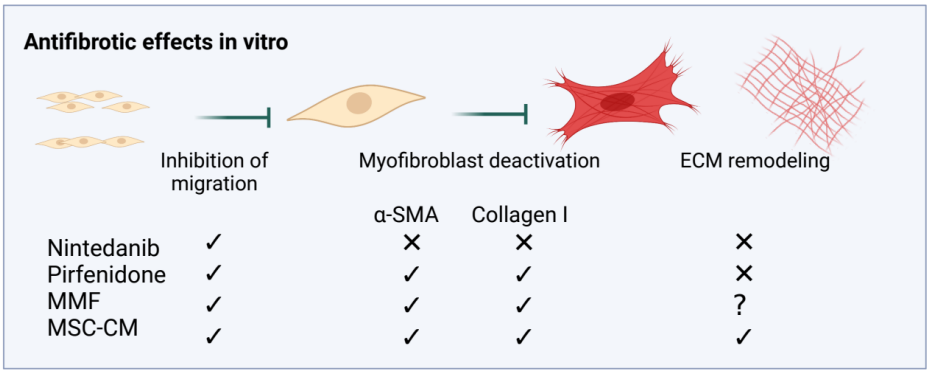

Figure 21. In vitro effects of antifibrotic drugs and MSC-CM

Fibrosis, as a process, is difficult to recapitulate in vitro. We established reliable in vitro assays that confirm the existence of an antifibrotic effect of MSC-CM. The relevance of this part of this project relies on the fact that the lack of standardized and predictive assays with sufficient throughput for drug discovery limited the identification of antifibrotic agents [224]. An 'ideal fibrosis assay' should build on physiologically relevant cells and needs to incorporate applicable readouts. We used fibroblasts as effector cells activated by TGF-β, the major pathway implicated in fibrosis [225]. Moreover, TGF-β is sufficient to induce a myofibroblast phenotype similar to that seen in skin biopsies of SSc patients [93, 213]. In vivo, TGF-β generates a clinical histological and biochemical profile similar to SSc in mice [214]. Fibrosis readouts include the activation of fibroblasts, the modulation of myofibroblasts, the activation of multiple canonical and noncanonical signaling pathways, the induction of fibrogenic genes, and the production, secretion, processing, and maturation of collagen and other ECM proteins. We have established assays to evaluate the continuum of processes that lead to fibrosis. Our approach was modeled in cardiac fibrosis, where fibrosis assays are divided into four groups according to their readout: (a) fibroblast proliferation or migration, (b) TGF-β pathway activation/TGF-β-dependent gene expression, (c) α-SMA expression and (d) mature ECM detection [226]. In addition to relevant readouts, in vitro antifibrotic assays should be robust, cost- and time-effective, and high-throughput. We provide evidence of the reproducibility of the in vitro assays established, which have the potential to be modified for high-throughput testing. However, given that less than 10% of drugs in phase I progress to clinical approval [195], a fundamental question concerns the role of in vitro assays in informing the clinical approval of antifibrotic agents.

Pirfenidone and nintedanib are the two antifibrotic drugs approved for treating idiopathic pulmonary fibrosis. The in vitro antifibrotic assays that supported the approval of these drugs tested their effect on migration and proliferation of fibroblasts, and the modulation of α-SMA, fibronectin, procollagen I and III on lung fibroblasts from ILD patients. Those assays used PDGF, FGF-2, VEGF and lower doses of TGF-β to stimulate fibroblasts. Both, nintedanib and pirfenidone inhibit fibroblasts migration in a similar assay to the one we established with the advantage that our assay was better standardized (see below) [128, 133]. Nintedanib was also tested in a prevention of activation assay using fibroblasts from controls and IPF patients. Only the highest dose of nintedanib tested (1μM) was effective in partially reducing collagen I protein levels [130]. In contrast, another study that used lower doses of TGF-β (4ng/mL) did not show an effect on reducing α-SMA [123]. In a similar assay using lung fibroblasts, pirfenidone reduced collagen I and α-SMA I but did not reduce fibronectin protein levels [128]. Despite not being classified as an antifibrotic, mycophenolate mofetil (MMF) impacts fibroblast biology. MMF is commonly used as an immunosuppressive to prevent organ rejection after transplantation, and it is an established treatment for patients with SSc ILD [227, 228]. MMF reduces the de novo synthesis of purines in fibroblasts decreasing their proliferation, similar effects are seen in lymphocytes [229, 230]. In in vitro studies MMF reduces fibroblast migration as well as α-SMA and collagen I transcription and protein levels; while promoting MMP-1 synthesis [231]. These effects were reported on confluent lung fibroblasts; however it remains to be determined whether MMF has a similar effect to MSC-CM on TGF-β activated fibroblasts.

In summary, despite their limitations, in vitro antifibrotic assays are required by regulatory agencies as evidence of a drug effect. In those in vitro assays, pirfenidone and nintedanib have antifibrotic effects however, in clinical trials, they did not revert fibrosis. In contrast, patients treated with MMF showed variable anti-fibrotic responses with stabilization of lung function and skin involvement [232]. At least two processes are required to reverse fibrosis: myofibroblasts deactivation or apoptosis, and ECM degradation [9, 112]. Optimization of in vitro assays and the development of assays to test these processes may increase their value in predicting *in vivo* fibrosis resolution.

A fundamental aspect in optimizing in vitro assays is the in-depth characterization of fibroblasts and their response to TGF-β treatment (i.e., fibroblast activation = myofibroblasts). We documented that fibroblasts following TGF-β activation increased *ACTA2, COL1A1* mRNA and protein levels; upregulated expression of *CCN2*, *PAI-1, ADAM12*, and *GLS-1*. Morphologically, fibroblasts experienced changes in the cytoskeleton with prominent stress fibers and collagen accumulation. This characterization ensured the development of cells with a fibrotic phenotype: myofibroblasts. Next, we established five in vitro assays to assess the direct antifibrotic effect of MSC-CM (i.e., the effect of MSC-CM on fibroblasts independent of immune cells). These assays targeted three independent and fundamental steps in the fibrotic process: fibroblast migration, modulation of TGF-β effect (i.e., prevention of fibroblast activation, myofibroblasts deactivation, restoration of myofibroblasts apoptosis threshold), and ECM modulation. The additive value of each of these assays to establish the direct antifibrotic effects of MSC-CM will be discussed in the following paragraphs.

MSC-CM inhibited the migratory capacity of fibroblasts. The wound scratch assay was performed with the IncuCyte® Live-Cell Analysis System. This automated system allows to make consistent, precise, and reproducible wounds simultaneously in a 96-well plate and to continuously monitor and analyze cell migration with no interference in the cell environment while the experiment is done. Our results replicate previous findings, where MSC(AT)-CM inhibited the migration of hypertrophic skin and keloid fibroblasts [151, 185]. For this assay we used fibroblasts and not myofibroblasts as the latter lose the capacity to migrate because their cytoskeleton is reorganized to contract the injured tissue [7]. As a confirmatory approach, we tested two different fibroblasts' densities and showed that MSC-CM had an inhibitory effect even at the higher density. These results imply that MSC-CM limit fibroblasts migration, reducing the number of the main cellular effectors of fibrosis in target organs. This mechanism could prevent fibrosis perpetuation.

MSC-CM antagonized the effect of TGF-β, decreasing the synthesis of procollagen I and α-SMA. Previous studies using a similar in vitro assay to test MSC-CM showed contradictory results, some supporting an antifibrotic effect [166] and others not [149]. Differences in the preparation of the MSC-CM (e.g., MSC density, culture medium, and time of incubation prior to the collection of MSC-CM) may account for the discrepant results of this assay. MSC-CM deactivates myofibroblasts. Physiologically, myofibroblasts deactivation occurs in the resolution phase of wound healing where part of the myofibroblasts change their role to remodel the ECM. We demonstrated this effect at a gene expression and protein level with a reduction of α-SMA and procollagen I. We confirmed that this effect was dose-dependent and unrelated to cell death. The lack of MSC-CM induction of myofibroblast apoptosis in our assay contrasts with reports showing

that MSC(BM)-CM induce apoptosis of hepatic stellate cells preventing liver fibrosis [162]. These results confirm that MSC-CM change the profibrotic phenotype of myofibroblasts. This is further supported by our results confirming the downregulation of TGF-β1-dependent profibrotic genes *CCN2/CTGF, ADAM12, PAI-1* and *GLS-1* in the presence of MSC-CM. *CCN2* is one of the most TGF-β up-regulated genes and encodes the extracellular matrix protein CTGF which promotes fibroblast proliferation, migration, adhesion, and ECM formation through non-canonical Smad1 ERK1/2 pathway [233, 234]. Increased plasma levels of CTGF are reported in fibrotic conditions like cardiac failure and fibrosis, IPF and liver fibrosis, thus CTGF is a proposed fibrogenesis biomarker [235-237]. ADAM12 is a marker of profibrotic myofibroblasts [238]. ADAM12 knockdown limits myofibroblasts generation and reduces ECM [65]. PAI-1 is a serine protease inhibitor (serpin) gene family member. It inhibits urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), enzymes that degrade ECM. PAI-1 is elevated in SSc and together with CTGF, predicts skin progression in this disease [239]. The reduction of PAI-1 would lead to ECM degradation. GLS-1 is an enzyme that converts glutamine to glutamate (i.e., glutaminolysis) that is upregulated by myofibroblasts during fibrosis. In vivo, inhibition of GLS1 diminishes TGF-β1–induced collagen production in mouse lung fibroblasts [10, 68, 240]. Overall, MSC-CM targets multiple profibrotic pathways including myofibroblasts structural genes, genes related to ECM degradation and genes implicated in the metabolism of myofibroblasts. The downregulation of *CCN2/CTGF, ADAM12, PAI-1* and *GLS-1* by MSC-CM support the concept that MSC-CM deactivates myofibroblasts by antagonizing the effect TGF-β. MSC-CM did not modulate myofibroblast apoptosis threshold. Apoptosis resistance characterizes

myofibroblasts and differentiate them from fibroblasts [9]. To assess the ability of MSC-CM to revert apoptosis resistance we treated myofibroblasts with MSC-CM in the presence or absence of staurosporine, a proapoptotic factor. The apoptotic rate of myofibroblasts did not change after 24h of staurosporine apoptosis induction, independently of MSC-CM pre-treatment.

The effect of MSC-CM antagonizing multiple aspects of the fibrotic response highlights their potential value as a therapeutic strategy. Moreover, MSC-CM modulates established fibrosis, promoting fibrosis resolution.

The concept of fibrosis reversal is novel and requires the remodeling of ECM. The fibrotic stroma has altered vascularization and excessive ECM crosslinking with altered orientation and distribution of the fibrous proteins [95, 100]. We showed that MSC-CM modulates ECM components by evaluating fibronectin and fibrillin-1 fibers by immunohistochemistry. Myofibroblasts treated with MSC-CM secrete fibronectin and fibrillin-1 fibers that are less mature and thinner than those secreted by untreated myofibroblasts. The relevance of this finding relates to the function of the ECM. Specifically, the ECM promotes myofibroblasts activation though different mechanisms (i.e. biomechanical traction, activation of pro-fibrotic growth factors, and promoting the interaction between fibroblasts and immune cells such as macrophages) [7]. Within the ECM, fibronectin is key for matrix assembly [106] and in addition to fibrillar collagen, is used as a diagnostic marker of fibrosis [7]. In altered scarring, fibronectin biosynthesis is four times higher compared to normal wound healing [241, 242]. Fibrillin-1 is a microfilament that initiates elastic fiber assembly and when cleaved promotes TGF-β activation [101]. Disorganized deposits of fibrillin-1 were reported in SSc skin [243].

Moreover, some SSc patients present anti-fibrillin-1 autoantibodies that promote the release of TGF-β and fibroblast activation [244]. Mutations in fibrillin-1 cause an autosomal dominant form of SSc due to altered cell-matrix interactions, excessive microfibrillar deposition, impaired elastogenesis, and increased TGF-β concentration and signaling in the skin [219]. Our results suggest that MSC-CM not only inhibit fibrosis, a process that is shared with currently approved antifibrotic agents, but more importantly promote ECM remodeling and fibrosis reversal.

MSC cytokine priming (i.e., TNF-α and INF-ɣ added to the culture medium of MSC prior the generation of the MSC-CM) enhances the in vitro antifibrotic effects of MSC-CM in the prevention and deactivation assays. Priming also increases the MSC-CM fibrosis resolution effects (i.e., ECM remodeling assay). In animal models of renal fibrosis [165], SSc [245], and Crohn's disease [246], INF-ɣ primed MSC were more effective than resting MSC in reducing the deposition of collagen I and α-SMA in target organs (histology). In these studies, fibroblasts were treated with MSC prior to TGF-β exposure. The novelty of our work is that we simultaneously treated fibroblasts with TGF-β and MSC-CM; as well as myofibroblasts with MSC-CM. In this setting, we prove that primed MSC-CM decreased TGF-β-induced collagen protein levels. This which is important as collagen I represents approximately 70% of the ECM proteins. We also provide preliminary data suggesting that priming increases MMPs in the MSC-CM. We have previously described changes in the composition of the MSC secretome following priming which are linked to enhanced immunomodulatory effects [247]. Now we demonstrate that priming also increases the direct in vitro antifibrotic effects of the MSC-CM. The relevance of this

finding relates to the possibility of modulating MSC culture conditions as a mean to optimize the effects of the MSC-CM.

Finally, we explored the in vitro antifibrotic effects of MSC-CM from SSc patients. SSc is a prototypic systemic fibrotic disease affecting middle-aged women more than men (F:M: 3:1 - 14:1, age of onset: 20 - 50 years). In SSc, the clinical manifestations derive from three major mechanisms: (a) vasculopathy (Raynaud's, digital ulcers, pulmonary arterial hypertension, renal crisis), (b) chronic inflammation (inflammatory cell activation, production of autoantibodies and growth factors), and (c) progressive fibrosis (skin most frequent but may involve any organ). Current SSc treatments are limited and MSC are tested as a possible cellular therapy [48]. We hypothesized that the in vitro antifibrotic effects of MSC would be impaired in the context of SSc. Consistent with previous reports, SSc MSC have preserved clonogenicity and immunopotency [188, 189]. However, in contrast to our hypothesis, our preliminary results based on the study of five samples suggest that SSc MSC-CM prevent fibroblast activation and promote myofibroblast deactivation to a similar extent to MSC-CM from healthy controls. Previous reports showed that healthy MSC exposed to a SSc microenvironment developed a profibrotic profile [190]. Based on this, we speculate that although SSc MSC are not primarily dysfunctional their antifibrotic capacity is impaired due to the profibrotic milieu. This could account for the different composition of MMPs in the SSc versus control MSC-CM. The disbalance between MMPs and TIMPs in skin samples and serum was previously reported in SSc and other fibrotic diseases [248-250]. Only two SSc-MSC samples included in this study were from treatment-naïve patients. Although immunosuppressants such as glucocorticoids, MMF, and rapamycin can alter the MSC metabolism and

function, they do not appear to impact in vitro MSCs' immunopotency assays [251-253]. However, the influence of immunosuppressives on the antifibrotic effects of MSCs in vitro remains unknown.

Our work has several limitations. First, the in vitro assays we established are well suited to capture the respective parts of the fibrotic process and to evaluate their modulation by MSC-CM but do not mimic the complex physiology of fibrosis as a whole. Therefore, results of individual in vitro assays may not necessarily translate into clinical applications. However, the use of multiparametric approaches combining early (e.g., α-SMA) and late (e.g., mature collagen) hallmarks of fibrosis, adds additional information and reduces the intrinsic bias of screening assays [254]. Second, the in vitro assays are based on two-dimensional (2D) monocultures that do not recapitulate the 3D structure, dynamics, and microenvironment of tissues. This limitation could be improved with the use of organoids generated from patient-derived primary cells or from induced pluripotent stem cells that could better predict antifibrotic drug efficacy [255]. Also, since MSC mainly function via paracrine mechanisms, we tested the antifibrotic effects of the secretome. Future studies should evaluate the contribution of cell-cell contact-dependent mechanisms to the antifibrotic effects of MSC. Third, there are a number of limitations related to the evaluation of the SSc MSC-CM antifibrotic effects. The sensitivity of the in vitro assays to detect differences is a relevant consideration. The fact that the test allowed detecting differences related to cytokine priming does not rule out that variations between SSc and controls are below the detection threshold of the test. Moreover, in SSc skin, clinical manifestations are diverse and reflect multiple histological manifestations [62, 256]. A higher number of SSc patients would be required to better characterize the

antifibrotic effects of MSC-CM. Recruitment of additional samples is ongoing. Furthermore, multiple cell types beyond fibroblasts are involved in SSc pathogenesis (e.g., immune, endothelial, and epithelial cells). Our system was designed to evaluate the direct effect of MSC-CM on a fibroblast cell line. Whether similar results would be obtained if SSc-derived fibroblasts were used remains to be tested. This is important as SSc fibroblasts have a profibrotic profile and altered epigenetic signatures [257]. In vitro assays standardized in our laboratory allow testing the MSC effect on other cell types (i.e., endothelial cells, T cells). Characterizing the indirect antifibrotic effects of MSC-CM would require the addition of immune cells to the in vitro assays.

Our work resulted in significant contributions to the field. First, we standardized a number of in vitro assays that allow to evaluate the complex process of fibrosis. If these assays are proven to predict *in vivo*/in human responses, they would be relevant to optimize the selection of MSC products. If the predictive value of the proposed in vitro tests is not demonstrated, these assays are still fundamental for analyzing mechanisms implicated in fibrosis. We demonstrated that MSC-CM has the unique capacity to revert the myofibroblast phenotype and to remodel ECM. We are currently conducting studies (i.e., proteomic analysis of the MSC-CM) that will allow the identification of the specific factors in the MSC-CM that are responsible for their antifibrotic effects. Additionally, we plan to perform comparative studies using MSC-CM and approved antifibrotic drugs (i.e., pirfenidone/nintedanib) in order to establish their relative antifibrotic effects. Even when we acknowledge the limitations of the assays done using SSc MSC-CM (e.g., limited sample size), this is the first study to report on the antifibrotic activity of primary SSc MSC-CM. Our results suggest that ex-vivo SSc MSC have preserved antifibrotic properties.

CHAPTER 5. CONCLUSION

The data presented in this thesis supports that the dysregulated repair response that occurs in the context of fibrosis characterized by persistent epithelial injury, death and failed regeneration, coupled with the accumulation of activated myofibroblasts and an abnormal ECM turnover can be modeled in a series of in vitro assays. Those assays allow detecting differences between resting and cytokine-primed MSC-CM. The in vitro assays confirmed the antifibrotic effects of MSC-CM, allow for further characterization of their mechanistic basis, and facilitate the comparison of MSC from different sources, states and individuals. Ultimately, those assays should be further tested in their ability to perform as a relevant pre-clinical tool to select for optimal subcellular MSC therapeutic products.

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