Effect of senescence on human mesenchymal stromal cells' anti-fibrotic function

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2 Abstract

Background: Age-associated fibrotic diseases significantly contribute globally to elevated morbidity, mortality, and financial burdens. Fibrosis, a pathological response mirroring the wound healing process, is characterized by the aberrant accumulation of extracellular matrix components (most notably collagen type I), which can affect single or multiple organs. Transforming growth factor-beta is the master regulator of fibrosis, which orchestrates the activation of fibroblasts into myofibroblasts, α-smooth muscle actin positive contractile non-smooth muscle cells, and key cellular effectors in the fibrotic process. TGF-β also confers myofibroblast apoptosis resistance, amplifying extracellular matrix components synthesis and deposition. Eliminating the cause of injury, deactivating myofibroblasts, and extracellular matrix remodeling would be required for fibrosis reversal. Notably, current treatments fail to reverse fibrosis. Mesenchymal stromal cells (MSC) have potential therapeutic value in fibrosis reversal due to their anti-inflammatory, pro-angiogenic, and anti-fibrotic properties. The MSC therapeutic effects are predominantly mediated through secreted soluble molecules and extracellular vesicles constituting the MSC secretome.

Aging is coupled with a progressive decline and impairment of function at the molecular, cellular, tissue, and organismal levels, explaining the increased risk of disease and death. Senescent cells accumulate in age-associated diseases, including prevalent age-associated fibrotic diseases (i.e., pulmonary fibrosis). Senescence is a cellular response or program that limits the expansion of aged or damaged cells. Senescent cells acquire an inflammatory senescent-associated secretory phenotype; therefore, aging is associated with chronic low-grade inflammation, which fosters the fibrotic process.

Knowing that senescent MSC have reduced immunomodulatory and pro-angiogenic properties and that senescence is implicated in fibrosis, we hypothesized that replicative senescence changes the MSC secretome, impairing its anti-fibrotic effects and contributes to fibrotic diseases. To test this, we optimized in vitro anti-fibrotic assays that evaluated different phases of the fibrotic process, characterized non-senescent (NS-) and senescent (S-) MSC and their secretome, and compared the *in vitro* anti-fibrotic effects of NS- and S-MSC secretome.

Methods: Adipose-derived MSC were isolated from 6 adult donors undergoing programmed surgery and characterized according to the criteria proposed by the International Society for Cellular Therapy. Characterizing S-MSC was a prerequisite for collecting MSC-conditioned media for subsequent experiments. Replicative senescence was confirmed with a multi-marker approach: percentage of senescence-associated beta-galactosidase positive cells, doubling time, side scatter, autofluorescence, density of surface CD26, and p16 expression. To evaluate the secretome, conditioned media from NS-MSC and S-MSC collected, and their capacity to modulate fibrosis was assessed in four in vitro assays: (1) inhibition of transforming growth factor beta-induced fibroblast activation; (2) myofibroblast deactivation; (3) myofibroblast apoptosis sensitization, and (4) areal contraction assay. Readouts of these assays included protein quantification by western blotting (α-smooth muscle actin and procollagen I), flow cytometry (Annexin V and DRAQ7), and percent of contraction.

Results: Replicative senescence impaired MSC immunomodulatory capacity; however, it did not affect *the anti-fibrotic effects of MSC-conditioned media in vitro*. S-MSC conditioned media inhibited transforming growth factor beta fibroblast activation and

deactivated myofibroblasts without inducing fibroblasts' cell death. MSC-conditioned media (NS- and S-) did not restore myofibroblast apoptosis sensitivity, and both enhanced fibroblasts' contraction similarly.

Conclusions: *In vitro* assays allow for assessing the MSC secretome anti-fibrotic effects. The S-MSC secretome has preserved in vitro anti-fibrotic properties. These findings suggest potential compensatory mechanisms in S-MSC exist and that while senescence impairs the MSC immunomodulatory properties, it does not influence MSC anti-fibrotic effects *in vitro*.

3 Résumé

Contexte: Les maladies fibrotiques associées à l'âge apparaissent de plus en plus comme un contributeur important à une morbidité, une mortalité et un fardeau financier élevés à l'échelle mondiale. La fibrose, une réponse pathologique reflétant le processus de cicatrisation des plaies, est caractérisée par l'accumulation aberrante de composants de la matrice extracellulaire (notamment le collagène de type I), qui peuvent affecter un ou plusieurs organes. Dans ce contexte, le facteur de croissance transformant bêta est le principal régulateur de la fibrose, qui orchestre l'activation des fibroblastes en myofibroblastes, l'actine des muscles lisses positive des cellules musculaires contractiles non lisses, sont des effecteurs cellulaires clés dans le processus fibrotique. Le facteur de croissance transformant bêta confère également une résistance à l'apoptose des myofibroblastes, amplifiant la synthèse et le dépôt de le composants de la matrice extracellulaire. L'élimination de la cause des blessures, la désactivation des myofibroblastes et le remodelage de la composants de la matrice extracellulaire sont essentiels à l'inversion de la fibrose, mais les traitements actuels ne parviennent pas à inverser la fibrose. Cependant, les cellules stromales mésenchymateuses multipotentes humaines (CSM) sont apparues comme une thérapie cellulaire prometteuse, possédant des propriétés anti-inflammatoires, pro-angiogéniques et antifibrotiques. Ces effets thérapeutiques sont principalement médiés par la sécrétion de molécules solubles et de vésicules extracellulaires constituant le sécrétome des CSM.

Le vieillissement est le déclin progressif et l'altération des fonctions aux niveaux moléculaire, cellulaire, tissulaire et organisme, associés à un risque accru de maladie et de décès. Les cellules sénescentes s'accumulent dans les maladies liées à l'âge, y

compris les maladies fibrotiques prévalentes associées à l'âge (c'est-à-dire la fibrose pulmonaire). La sénescence est une réponse ou un programme cellulaire qui limite l'expansion des cellules âgées ou endommagées. Les cellules sénescentes acquièrent un phénotype sécrétoire associé à la sénescence inflammatoire; par conséquent, le vieillissement est associé à une inflammation chronique de faible intensité, qui favorise le processus fibrotique. Sachant que les CSM sénescentes ont des propriétés immunomodulatrices et pro-angiogéniques réduites et que la sénescence est impliquée dans la fibrose, nous avons émis l'hypothèse que la sénescence réplicative modifie le sécrétome des CSM, altérant ses effets anti-fibrotiques et contribuant aux maladies fibrotique. Pour tester cela, nous avons optimisé les tests antifibrotiques in vitro qui ont évalué différentes phases du processus fibrotique, caractérisé les CSM non sénescentes (NS-) et sénescentes (S-) et leur sécrétome, et comparé les effets antifibrotiques in vitro de le sécrétome des NS- et S-CSM.

Méthodes: Les CSM d'origine adipeuse ont été isolées chez 6 donneurs adultes subissant une intervention chirurgicale programmée et caractérisées selon les critères proposés par la Société internationale de thérapie cellulaire. La caractérisation des S-CSM était une condition préalable à la collecte les sécrétome des CSM afin de mener toutes les expériences ultérieures. La sénescence réplicative a été confirmée par la présence de marqueurs: pourcentage de cellules positives à la bêta-galactosidase associée à la sénescence, temps de dédoublement, diffusion latérale, autofluorescence, densité de surface CD26 et expression de p16. Les sécrétome des CSM provenant des NS-CSM et S-CSM ont été collectés, et leur capacité à moduler la fibrose a été évaluée dans quatre tests *in vitro:* (1) inhibition de l'activation des fibroblastes induite par le facteur

de croissance transformant bêta; (2) désactivation des myofibroblastes; (3) sensibilisation à l'apoptose des myofibroblastes et (4) areal contraction des fibroblastes. Les résultats de ces tests étaient les suivants: (1, 2) analyse par western blot (l'actine des muscles lisses et procollagène I); (3) cytométrie en flux (Annexine V et DRAQ7) ; et (5) pourcentage de contraction.

Résultats: Bien que la sénescence réplicative ait altéré la capacité immunomodulatrice des CSM, elle n'a pas altéré les effets antifibrotiques des sécrétome des CSM in vitro. Les sécrétome des S-CSM ont inhibé l'activation des fibroblastes le facteur de croissance transformant bêta et désactivé les myofibroblastes, sans induire la mort des cellules des fibroblastes. Les sécrétome des CSM (NS- et S-) n'ont pas restauré la sensibilité à l'apoptose des myofibroblastes et ont également amélioré la contraction des fibroblastes.

Conclusions: Les tests in vitro permettent d'évaluer les effets antifibrotiques du sécrétome des CSM. In vitro, les propriétés anti-fibrotiques du sécrétome des S-CSM sont préservées. Ces résultats suggèrent qu'il existe des mécanismes compensatoires potentiels dans les S-CSM, et même si la sénescence altère les propriétés immunomodulatrices des CSM, elle n'influence pas leurs effets antifibrotiques *in vitro.*

4 Acknowledgements

I want to extend my heartfelt gratitude to the individuals who had pivotal roles in helping me complete this thesis. This endeavor would not have been possible without their support, guidance, and encouragement.

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Last but not least, I am grateful to my family, boyfriend, and friends for their unwavering support, understanding, and encouragement throughout this program. I dedicate this thesis to my family (Teresa Morales, Martin Anaya, Alan Anaya Morales) and my boyfriend (Marc-Olivier Van Dorpe), whose love, support, and trust have been my driving force and constant motivation throughout this academic journey.

5 Preface and Contribution of Authors

All the samples used in the experiments for this project were acquired under the Ethics Review Board-approved protocol (GEN-10-107: 'The effects of aging on human mesenchymal stem cells'). Adipose tissue-derived Mesenchymal Stem Cells (MSC) were obtained by a cardiovascular surgeon -Dr. Shum-Tim from patients undergoing programmed cardiac surgery at the Royal Victoria Hospital (RVH).

All experiments in this project were conducted by Ingrid Anaya, with the supervision of Dr. Colmegna and Dr. Moraes, except for:

- Dr. Marianela Brizio performed isolation and phenotype characterization of MSC (Figure 16) at Dr. Colmegna's laboratory.
- Flow cytometry characterization was performed under the supervision of Dr. Maximilien Lora and MSc. Marie-Claude Moisan at the RI-MUHC immunophenotyping core facility.

The writing of this thesis was directed by Dr. Colmegna and reviewed by Dr. Moraes.

6 Abbreviations

AKT: Protein kinase B

AMPK: 5' adenosine monophosphate-activated protein kinase

ANG-1: Angiopoietin-1

AT-MSC: Adipose tissue-derived MSC

BM-MSC: Bone marrow-derived MSC

BMP: Bone morphogenetic protein

CFSE: Carboxyfluorescein succinimidyl ester

CCN1/CYR61: Cysteine-rich angiogenic inducer

CDKIs: Cyclin-dependent kinase inhibitors

CK19: Cytokeratin 19

CM: Conditioned Media

CTGF: Connective tissue growth factor

CXCL: Chemokines

DAPI: Diamidino-2- phenylindole

DDR: DNA damage response

Dkk-1: Dickkopf protein I

DMEM: Dulbecco's modified eagle's medium

E2F: E2 transcription factor

ECM: Extracellular matrix

ECs: Endothelial cells

EDA-fibronectin: Fibronectin extra domain A

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinase

ET1: Endothelin-1

EVs: Extracellular vesicles

FAK: Focal adhesion kinase

FBS: Fetal bovine serum

FGF: Fibroblast growth factor

FN: Fibronectin

FSC: Forward scatter

FZD6: Frizzled Class Receptor 6

GMFI: Geometric mean fluorescence intensity

HA: Hyaluronan

HGF: Hepatocyte growth factor

HLA: Human leukocyte antigen

HS: Hypertrophic scar

HSCs: Hepatic stellate cells

hTERT: Human telomerase reverse transcriptase

IDO: indoleamine 2,3-dioxygenase

IFN-γ: Interferon-

IL: Interleukin

IPA: Immunopotency assays

IPF: Idiopathic pulmonary fibrosis

ISCT: International Society for Cellular Therapy

JNK: c-Jun terminal kinase

LAP: Latency-associated peptide

LTBP: Latent TGF-β binding protein

MAPKs: Mitogen-activated protein kinases

MCP-1: Monocyte chemotactic protein-1

MEKKs: MAPK kinases

MFGE8: Milk fat globule-EGF factor 8

MI: Myocardial infarction

MiDAS: Mitochondrial dysfunction-associated senescence

MMPs: Matrix metalloproteinases

MOMP: mitochondrial outer membrane permeabilization

MSC: Mesenchymal stromal cells

mtDNA: Mitochondrial DNA

mTOR1: Mechanistic target of rapamycin 1

MyoD: Myogenic differentiation

NK: Natural killer cells

NOXs: NADPH oxidases

Nrf2: nuclear factor erythroid 2-related factor 2

NS: Non-senescent

PAI-1: Plasminogen activator inhibitor-1

PFA: Paraformaldehyde

PBMCs: Peripheral blood mononuclear cells

PCNA: Proliferating cell nuclear antigen

PDGF: Platelet-derived growth factor

PDT: Population doubling time

PEG: Poly-ethylene glycol

PGC-1α/β: Proliferator-activated receptor-gamma coactivator-1α/β

PGE2: Prostaglandin E2

PBS: Phosphate-buffered saline

PI3K: Phosphatidylinositol-3-kinase

PIGF: Placental growth factor

PPARγ: Peroxisome proliferator-activated receptor gamma

pRB: Phosphorylation of retinoblastoma

ROS: Reactive oxygen species

RS: Replicative senescence

S: Senescent

Smad: Small mothers against decapentaplegic

SAHF: Senescence-associated heterochromatic foci

SASP: Senescence-associated secretory phenotype

SA-β-gal: Senescence-associated beta-galactosidase activity

SFN: Sulforaphane

SSc: Systemic sclerosis

SSC: Side scatter

TIMPs: Tissue inhibitors of metalloproteinases

TGF-β: Transforming growth factor-β

TNF-α: Tumor necrosis factor-α

TSG-6: Tumor necrosis factor-α-induced protein 6

TβR: Membrane receptor serine/threonine kinase family

UC-MSC: Umbilical cord-derived mesenchymal stromal cells

VEGF: Vascular endothelial growth factor

α-SMA: α-smooth muscle actin

7 Introduction

In the following sections, I review key concepts that support the relevance of my project. Section 1 defines fibrosis as the 'process of interest,' which underlies frequent diseases associated with high morbidity and mortality and discusses mechanisms that promote fibrosis resolution. Section 2 defines multipotent mesenchymal stromal cells (MSC), highlights the relevance of their paracrine effects, and expands on MSC anti-fibrotic function. Section 3 focuses on aging/senescence as a 'biological problem' that impacts MSC function and is recognized as a pro-fibrotic state. At the end of each sub-section, I explained how the presented information related to my project (sentences in *italics*).

7.1 Section 1: Fibrosis

7.1.1 Definition and Burden

Fibrosis is the excessive accumulation of extracellular matrix (ECM) in response to chronic tissue injury that can lead to the disruption of organ architecture and organ dysfunction, which ultimately may result in death [1]. Hallmarks of fibrosis are the persistent myofibroblasts' activity, the primary source of the fibrotic ECM, and the absence of ECM degradation and remodeling [2]. Transforming growth factor-β (TGF-β) via activation of both canonical (Smad-based) and non-canonical (non-Smad-based) signaling pathways is the key mediator of myofibroblast activation [3]. Further, TGF-β can regulate cell proliferation, senescence, apoptosis, inflammatory response, and age-associated fibrosis [4].

Fibrosis is preceded by persistent inflammatory responses triggered by several factors, such as aging, chronic infections, autoimmune/allergic reactions, chemical/drug

insults, radiation exposure, and/or tissue injury [5,6]. Fibrosis can manifest either as an organ-specific or a multisystemic process. Organ-specific fibrosis encompasses myelofibrosis, cirrhosis, and fibrosis of the skin, kidney, pancreas, heart, or lungs [6]. On the other hand, systemic sclerosis (SSc or scleroderma), graft versus host disease, and nephrogenic systemic fibrosis are examples of systemic fibrotic disorders [7]. Overall, fibrotic conditions cause significant global morbidity and mortality, accounting for up to 45% of all deaths in the developed world [5]. Furthermore, the annual combined incidence of major fibrosis-associated diseases is estimated at 5000 per 100,000 person-years [8]. Fibrosis plays a significant role in chronic autoimmune and inflammatory disorders, such as SSc, rheumatoid arthritis, Crohn's disease, ulcerative colitis, chronic graft rejection, myelofibrosis, myopathies, and systemic lupus erythematosus. Fibrosis is also implicated in tumor invasion, metastasis, and prevalent diseases, including chronic kidney disease and atherosclerosis [9]. Therefore, fibrosis represents a significant economic and financial burden for healthcare and society [10], and thus *is the focus of my work*.

7.1.2 The Fibrotic Process

Fibrotic tissue remodeling is an abnormal and prolonged wound-healing response [9]. Thus, wound healing and fibrotic diseases have shared phases and mechanisms. Those include sequential hemostasis, inflammation, proliferation, and remodeling phases **(Figure 1)** [11]**.** Hemostasis is achieved after epithelial and endothelial damage by forming a platelet clot and a fibrin extracellular matrix. The local release of growth factors, cytokines, and chemokines recruits immune cells [12]. During the inflammatory phase, infiltration of neutrophils and macrophages removes tissue and cell debris [13]. In conjunction with inflammation, fibroblasts migrate and proliferate into the tissue injury site

[14]. Tissue cells undergo apoptosis, and the immune cells promote tissue repair by producing pro-inflammatory and pro-fibrotic effectors, including TGF-β [15]. TGF-β mediates fibroblasts to differentiate into myofibroblasts [16]. Finally, the provisional ECM is degraded and remodeled to rebuild the previous tissue architecture [17].

If the underlying etiology of fibrosis is eliminated, myofibroblasts deactivate and return to the low activity state of fibroblasts in homeostatic tissues (section 7.1.6.2 Myofibroblasts deactivation and reprogramming). Alternatively, they undergo apoptosis (programmed cell death) or senescence (section 7.1.6.3 Myofibroblasts apoptosis, and section 7.1.6.1 Myofibroblasts senescence). The build-up of senescent cells constitutes a significant characteristic of organismal aging [18]. Aging can lead to fibrosis in various organs through chronic inflammation, fostering fibrosis and ultimately reducing organ function [19, 20]. Furthermore, fibrosis is a marker of tissue aging associated with telomere shortening, mitochondrial dysfunction, and increased oxidative stress [21]. Dysregulation of myofibroblast deactivation or chronic injury promotes myofibroblasts to continue remodeling tissue beyond what is required for repair, resulting in pathological scarring [2]. *The link between senescence and fibrosis is at the core of my project*.

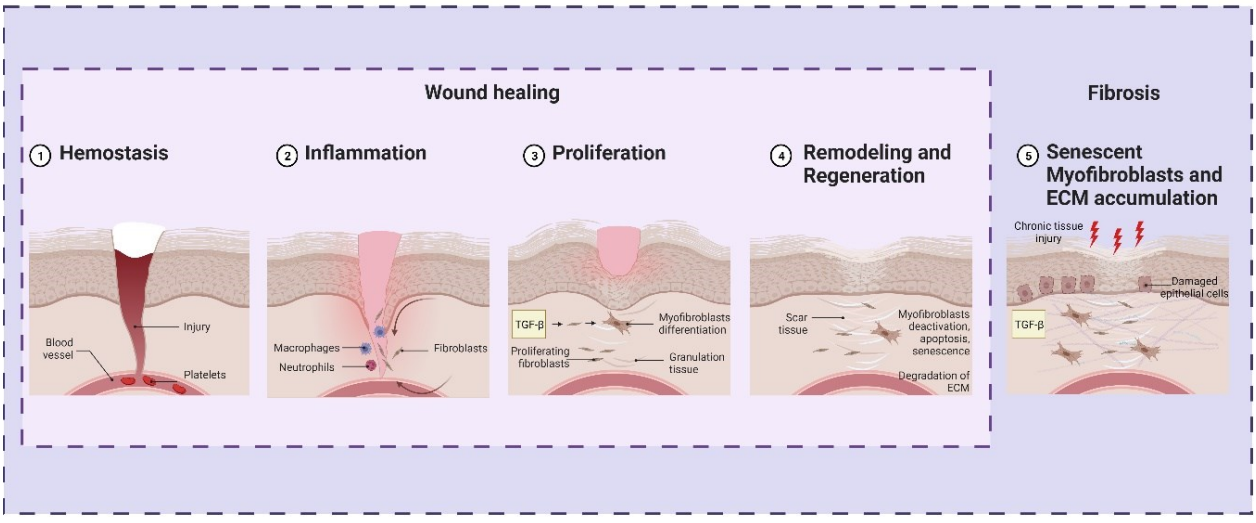


Figure 1. Phases of wound healing and fibrosis. A schematic showing the shared phases of wound healing and fibrosis: hemostasis, inflammation, proliferation, remodeling, and regeneration. Dysregulation of myofibroblast deactivation or chronic injury promotes senescent myofibroblasts to continue remodeling tissue beyond what is required for repair, resulting in fibrosis.

7.1.3 Myofibroblasts: Main Cellular Fibrosis Effectors

Myofibroblasts are heterogeneous contractile non-smooth muscle cells activated in response to tissue injury, remodeling lost or damaged ECM [22, 23]. They are characterized by the expression of α-smooth muscle actin (α-SMA), incorporated into stress fibers-like microfilament bundles that confer a highly contractile phenotype [24]. Myofibroblasts are vital in secreting fibrillar ECM, predominantly composed of collagen type I and III, along with fibronectin extra domain A (EDA-fibronectin) [25]. In fibrotic diseases, collagen production is significantly increased. In contrast, novel anti-fibrotic therapies act by reducing collagen biosynthesis, cross-linking, and deposition [6].

Myofibroblasts derive from heterogeneous sources of precursor cells with functional diversity [2, 26]. Whereas in lung fibrosis, most myofibroblasts derive from resident fibroblasts, myofibroblasts can arise from fibrocytes, pericytes, epithelial cells, endothelial cells, adipocytes, and hepatic stellate cells (**Figure 2)** [27]. Targeting specific myofibroblast precursor cells can suppress the early progression of fibrosis without affecting regenerative fibroblast populations [28]. Understanding the mechanisms that inhibit myofibroblast activation and survival may allow additional ways to antagonize fibrosis [2].

Myofibroblasts, vital effectors in fibrosis, are the effector cells in some in vitro assays in my work. Myofibroblasts fulfilled three minimal requirements in those assays:

expression of α-SMA, collagen I synthesis, and high contractile activity **(Figure 2)** [29]. *Moreover, we confirmed that TGF-β treatment confers myofibroblasts' apoptosis resistance.*

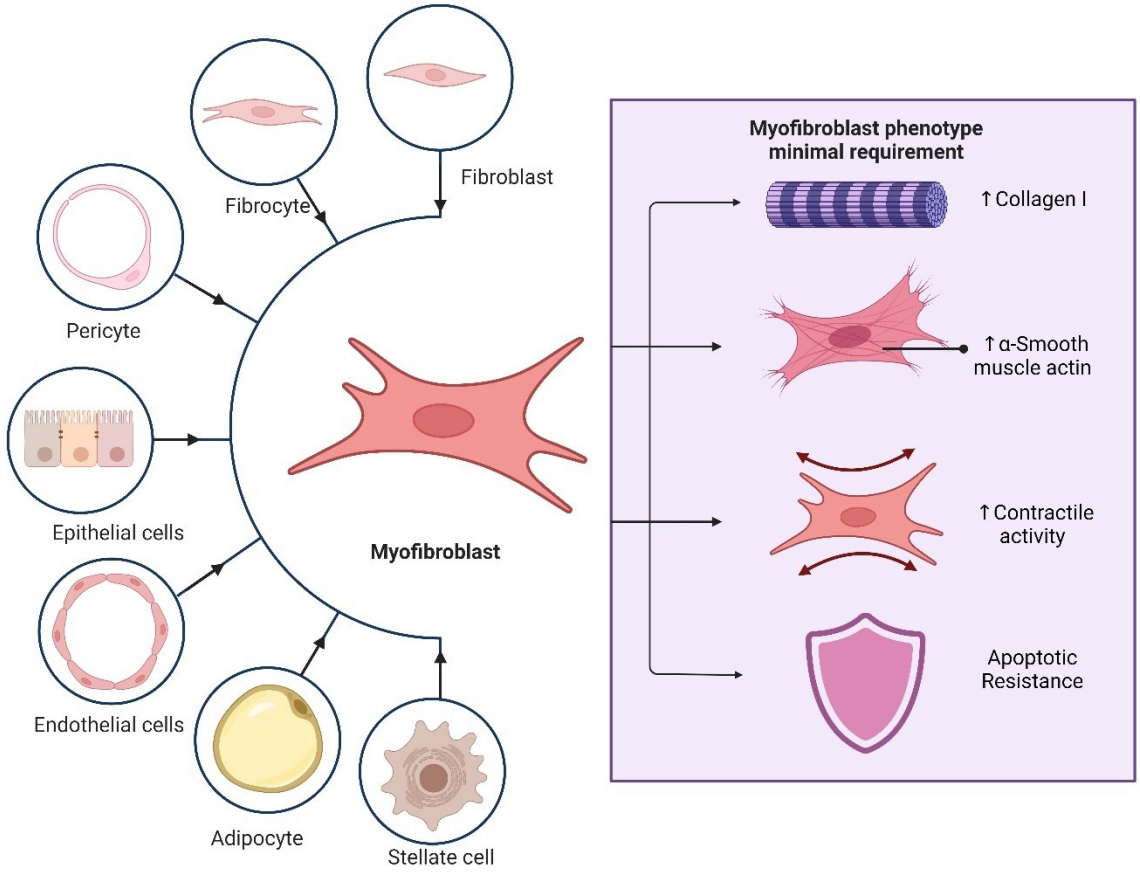


Figure 2. Myofibroblast sources and definition criteria. Myofibroblasts derive from resident fibroblasts, fibrocytes, pericytes, epithelial cells, endothelial cells, adipocytes, and hepatic stellate cells. Myofibroblasts minimal requirement: expression of α-SMA, collagen I synthesis, high contractile activity, and TGF-β treatment confers myofibroblasts' apoptosis resistance.

7.1.4 TGF-β Key Mediator of Fibrosis

TGF-β is the primary driver of fibrosis in most, if not all, fibrotic tissues regardless of the etiology of the initial tissue injury [16]. Additional factors directly cooperate with

TGF-β signaling in regulating fibrosis. For instance, Angiotensin II is produced locally by activated macrophages and myofibroblasts at the injury site, promoting fibrosis by enhancing TGF-β secretion and activation through shared intracellular signals with the TGF-β/Smad3 pathway [30].

TGF-β is a pleiotropic growth factor involved in tissue repair and maintenance of immune system homeostasis. However, dysregulated TGF-β signaling promotes fibrogenesis. Three distinct isoforms of TGF-β have been identified in mammals: TGF-β1, TGF-β2, and TGF-β3 [31]. TGF-β1 is the main pathogenic factor in fibrosis [3]. TGF-β1 is produced by multiple cells, including fibroblasts, macrophages, platelets, T cells, and mast cells. TGF-β1 is synthesized in a precursor form, the latency-associated peptide (LAP), which binds to the inactive TGF-β1 homodimer and facilitates its attachment to the latent TGF-β binding protein (LTBP) [32]. In the ECM, TGF-β is stored as a latent complex that can be activated by reactive oxygen species (ROS), cell contractile forces transmitted by αv integrins [23, 32] or can be cleaved by a wide range of proteases, including matrix metalloproteinases (MMPs) and thrombospondin-1 [32]. After TGF-β activation, it binds to a membrane receptor serine/threonine kinase family of type I (TβRI) and type II (TβRII) receptors. It activates canonical and non-canonical signaling pathways **(Figure 3)** [34]**.**

The TGF-β1 canonical pathway follows the phosphorylation of TβRI and activation of Smad2 and Smad3, which subsequently binds to Smad4, forming a complex that translocates to the nucleus and initiates the transcription of pro-fibrotic molecules, including collagens and fibronectin (FN) [34, 35]. Smad7, a negative feedback inhibitor, can compete with Smad2 and Smad3 for binding to activated TβRI and thus decrease TGF-β/Smad signaling [37]. Unlike Smad2 and Smad4, which lack DNA binding domains

and primarily function as regulators of Smad3-mediated gene transcription, Smad3 directly binds to Smad-binding elements within gene promoters, thereby facilitating transcriptional enhancement [3]. The activation of TβRI by TGF-β1 can also trigger non-canonical signaling or Smad-independent pathways.

The non-canonical signaling pathways exhibit crosstalk with TGF-β1 in regulating fibrosis, and their mechanisms are well-defined [3]. The family of mitogen-activated protein kinases (MAPKs) consists of three primary kinases: p38 MAPK, c-Jun terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) [38]. In response to a pathogenic stimulus such as angiotensin I, p38 MAP kinases upregulate the TGF-β/Smad signaling pathway by inducing activation of latent TGF-β via thrombospondin-1 [38, 39]. Blockade of the p38 MAP kinases can downregulate the activated TβRI, TβRII, and Smad3, thus reducing myofibroblasts accumulation and collagen deposition [3, 40]. TGF-β1 can directly induce ERK MAP activation needed to promote epithelial-mesenchymal transformation [42], phosphorylate receptor-activated Smads to regulate their nuclear translocation [43] and interact with Smads to regulate gene expression [44]. Furthermore, TβRI can induce the activation of p38 and JNK via a pathway involving TNF receptor-associated factor 6 [45]. Other signaling pathways that mediate TGF-β induced fibrosis include aberrant activation of epidermal growth factor receptor (EGFR), p53, and canonical Wnt pathways [45, 46]. TGF-β signaling decreases the expression of Dickkopf protein I (Dkk-1), a naturally secreted antagonist of Wnt signaling [48]. Dkk-1 inhibits glycogen synthase kinase-3β, which limits β-catenin stability and promotes Wnt activation [49]. Following Wnt activation, β-catenin target genes involved in the fibrotic response,

including Snail1, plasminogen activator inhibitor-1 (PAI-1), MMP7, and components of the renin–angiotensin system [50].

TGF-β activates myofibroblasts and is the critical mediator of fibrosis [3]*. In the in vitro assays I conducted, fibroblasts were treated with TGF-β1 (referred to as TGF-β throughout this thesis) to generate myofibroblasts.*

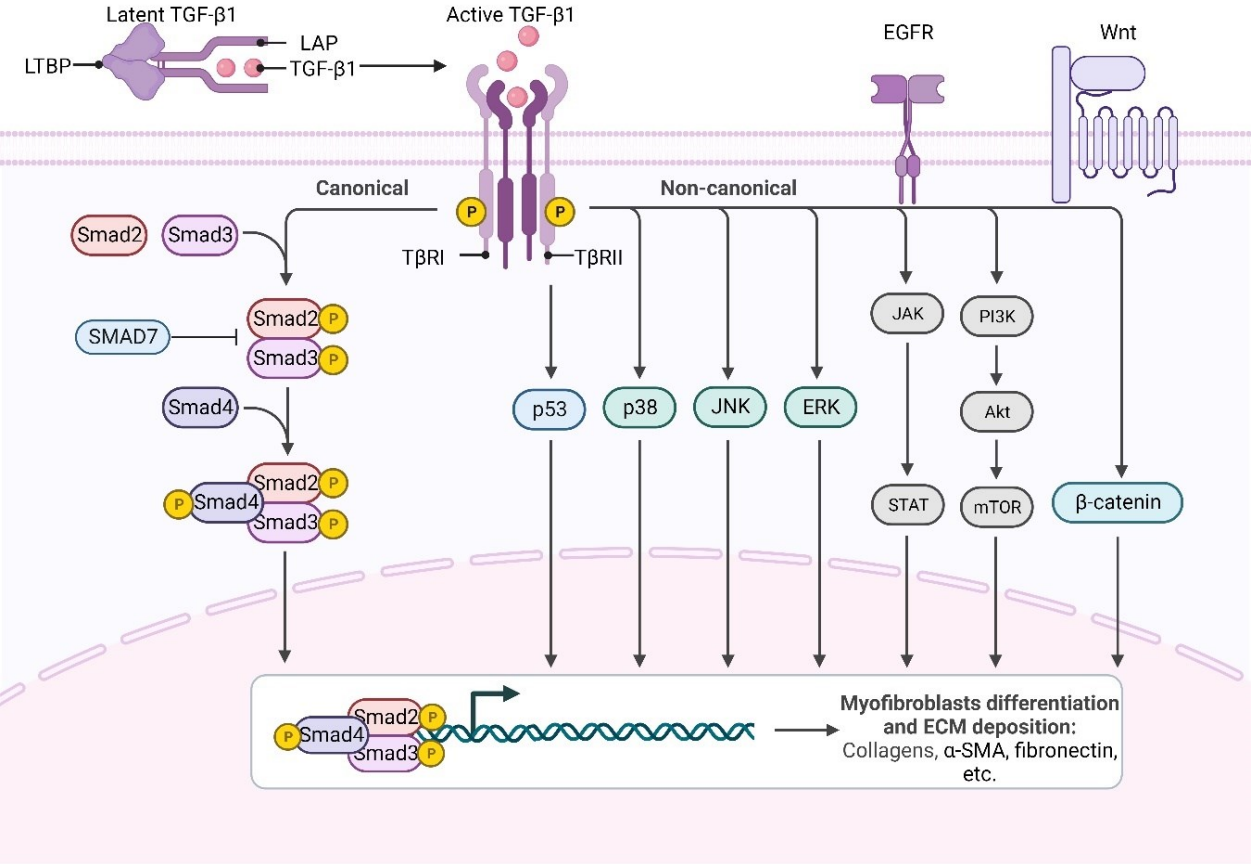


Figure 3. Canonical and non-canonical TGF-β signaling pathways in fibrosis. TGF-β1 is synthesized in a precursor form, the latency-associated peptide (LAP), which binds to the inactive TGF-β1 homodimer and facilitates its attachment to the latent TGF-β binding protein (LTBP). After TGF-β activation, it binds to a membrane receptor serine/threonine kinase family of type I (TβRI) and type II (TβRII) receptors. The TGF-β1 canonical pathway follows the phosphorylation of TβRI and activation of Smad2 and Smad3, which subsequently binds to Smad4, forming a complex that translocates to the

nucleus and initiates the transcription of pro-fibrotic molecules, including collagens and fibronectin (FN). Smad7, a negative feedback inhibitor, can compete with Smad2 and Smad3 for binding to activated TβRI and thus decrease TGF-β/Smad signaling. The activation of TβRI by TGF-β1 can also trigger non-canonical signaling or Smad-independent pathways. The family of mitogen-activated protein kinases (MAPKs): p38 MAPK, c-Jun terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Other signaling pathways that mediate TGF-β induced fibrosis include aberrant activation of epidermal growth factor receptor (EGFR), p53, and canonical Wnt pathways. Adapted from “TGF-β: the master regulator of fibrosis”, by Meng, X. M., 2016, Nature reviews Nephrology, 12(6), 325–338, Copyright © 2016 Springer Nature Limited, adapted with permission.

7.1.5 Targeting TGF-β for Fibrosis Resolution

Anti-fibrotic approaches include inhibiting TGF-β synthesis and activation, preventing TGF-β binding to its receptor, and blocking TGF-β receptors [3, 8]. There are five groups of anti-TGF-β drugs [8, 50]: (1) nucleic acid drugs blocking TGF-β synthesis, (2) inhibitors of TGF-β receptor kinases that hinder Smad2 and Smad3 activation, (3) monoclonal antibodies preventing TGF-β from binding its receptors, (4) ligand traps that stop TGF-β from binding to its receptor, and (5) molecules inhibiting TGF-β activation, such as drugs targeting αv/β integrins [8].

Pirfenidone is one of the two FDA-approved anti-fibrotic drugs that inhibit both the synthesis and activation of TGF-β [52]. Pirfenidone inhibits fibroblast proliferation and activation, reducing collagen synthesis, secretion, and fibril formation [53]. These effects are primarily mediated through inhibiting TGF-β downstream signalling pathways and other growth factors like platelet-derived growth factor (PDGF) and fibroblast growth factor-2 (FGF-2). Specifically, pirfenidone reduces TGF-β protein production and prevents fibroblasts' differentiation into myofibroblasts by attenuating TGF-β/Smad3-

induced signalling. It also hinders the expression of genes induced by TGF-β1, such as collagen types I, II, and III, fibronectin, and tenascin-c. Additionally, pirfenidone diminishes the TGF-β-induced expression of α-SMA [53, 54]; this effect is cell-line dependent [55, 56]. Moreover, in a gel contractility model (i.e., keloid fibroblasts + acid-soluble collagen solution), pirfenidone suppressed myofibroblasts-gel contraction in a TGF-β dose-dependent manner [58]. In clinical trials, pirfenidone reduced lung function decline and improved survival of patients with idiopathic pulmonary fibrosis (IPF) [59]. Furthermore, pirfenidone inhibits redox reactions and regulates genes and enzymes related to oxidative stress, providing an additional mechanism for its anti-fibrotic and anti-inflammatory actions [60].

Approved anti-fibrotic drugs modulate different steps of fibrogenesis, which can be tested in vitro. Similarly, we used in vitro assays to test the effects of senescent mesenchymal stromal cells conditioned media (S-MSC-CM).

7.1.6 Alternative Approaches to Promote Fibrosis Resolution

The notion that fibrosis is irreversible is challenged by evidence indicating that by eradicating the underlying etiology, fibrosis can be reversed [1]. Fibrosis resolution was demonstrated in the context of liver fibrosis due to viral hepatitis or schistosomiasis, where patients exhibited fibrosis regression or histological improvement, even in cases of cirrhosis, following the treatment of the infection [60–64]. However, as previously mentioned, human fibrotic diseases are often multifactorial, and in some cases, the etiology of fibrosis is unknown (e.g., IPF) [66]. Thus, eliminating the injurious triggers might not be feasible. The resolution of fibrosis and the degree of its progression strongly depend upon the affected organ, the type and duration of the injury, and individual-

specific factors such as age, immunocompetence, and genetic predisposition [67]. *New and more effective anti-fibrotic agents are needed to treat these conditions. My work evaluated the anti-fibrotic effects of the MSC-CM in the context of cellular senescence.*

Once the cause of injury is eliminated, fibrosis resolution involves three main mechanisms: the elimination of myofibroblasts, degradation of the fibrotic ECM, and the regeneration of standard tissue architecture [1, 67]. Myofibroblasts can be eliminated via deactivation, reprogramming, senescence, and apoptosis **(Figure 4)** [1]. These specific myofibroblasts' fates will be discussed in the following sections.

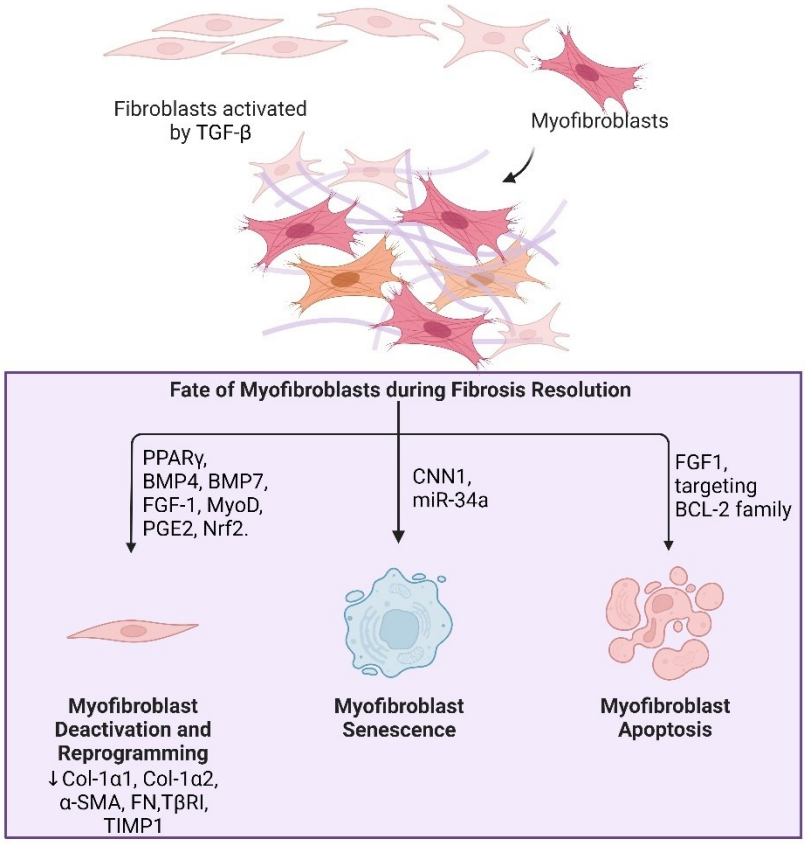


Figure 4. The fate of myofibroblasts during fibrosis resolution. Fibrogenic myofibroblasts can be eliminated during fibrosis resolution through one of several alternative cell fates: deactivation, reprogramming, senescence, and apoptosis. Adapted

from “Resolution of organ fibrosis”, by Jun, J. I., 2018, The Journal of clinical investigation, 128(1), 97–107, Copyright © 2018 American Society for Clinical Investigation, adapted with permission.

7.1.6.1 Clearance of Myofibroblasts Senescence

Human myofibroblasts from patients with various fibrotic diseases display a senescent phenotype [67–70]. In the late tissue remodeling phase, myofibroblasts switch from proliferating, ECM-producing pro-fibrotic cells to growth-arresting, with an ECM-degrading phenotype [71–77]. Senescent myofibroblasts are ultimately eliminated by NK cells, CD4+ T cells, and macrophages, which selectively target them for immunological clearance [77–79]. Impaired clearance of senescent myofibroblasts shifts their pro-repair activities to pro-fibrotic via persistent secretion of senescence-associated secretory phenotype (SASP) (see section 6.3 Senescence). The SASP contains pro-fibrotic cytokines (TGF-β, PDGF), pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, and IL-8), and ECM proteins (fibronectin, collagens) [80–82].

Myofibroblasts’ senescence can have different effects depending on when it occurs [84]. The matricellular cell adhesive protein Cysteine-rich angiogenic inducer 61 (CCN1/CYR61) is upregulated during injury, repair and regulates myofibroblast senescence through the p53 and p16 pathways [76, 84]. In mouse models of cardiac fibrosis, cardiac myofibroblasts enter senescence in a CCN1-dependent manner, decreasing fibrosis and ameliorating heart function [73, 75]. In contrast, in cardiac ischemia-reperfusion injury, a sustained oxidative stress-induced senescence response in cardiomyocytes promotes a pro-inflammatory SASP, leading to fibrosis and impaired heart function. Senolytic drugs that clear senescent cells by targeting pro-survival

pathways [86] attenuate multiple components of this response and improve clinically relevant parameters [72]. Indeed, Zhu et al. propose that while myofibroblasts' senescence reduces collagen deposition in the short-term post-myocardial infarction (MI), senescent myofibroblasts are also a source of chronic inflammation, contributing to long-term cardiac fibrosis [76].

Another example of the dual effects of myofibroblasts' senescence on fibrosis is miR-34a. This micro-RNA is upregulated in lung myofibroblasts from patients with IPF and mice with experimental pulmonary fibrosis. Mechanistically, miR-34a increases senescence-associated beta-galactosidase activity (SA-β-gal), enhances the expression of senescence markers (i.e., p21 and PAI-1), and promotes cell cycle arrest. Altogether, miR-34a induces a senescent phenotype in lung fibroblasts. Mice with miR-34a knockdown have a diminished senescent phenotype, enhanced apoptosis resistance, and developed more severe pulmonary fibrosis than wild-type animals [73]. These results suggest that miR-34a functions through a negative feedback mechanism to restrain pulmonary fibrotic responses by promoting the senescence of lung fibroblasts. In contrast to these anti-fibrotic effects, miR-34a ablation in old mice protected aged animals from developing experimental lung fibrosis. miR-34a is upregulated in alveolar epithelial cells in aged mice but not in lung fibroblasts [87]. The pro-fibrotic effects of senescence are further supported by a mouse model of IPF in which senescent epithelial cells and myofibroblasts accumulate exponentially over time, inducing myofibroblast differentiation and increasing the fibrotic response. In this model, eliminating senescent epithelial cells or myofibroblasts improved lung function [69].

Together, these results suggest that the senescence of other cell types may trigger a fibrotic response in myofibroblasts [73]. *These emphasize the importance of assessing the effects of senescent MSC-CM on myofibroblasts biology.*

7.1.6.2 Myofibroblasts Deactivation and Reprogramming

Myofibroblasts can deactivate into a low-activity phenotype characteristic of myofibroblasts precursor cells after injury resolution. This was shown by genetic lineage tracing analysis [87–89]. The processes underlying the deactivation of myofibroblasts are still not fully understood. However, numerous studies have highlighted the importance of peroxisome proliferator-activated receptor gamma (PPARγ) inhibiting the TGF-β pathway and mediating myofibroblast deactivation [80, 87, 89, 90]. Quiescent hepatic stellate cells (HSCs) are the primary source of myofibroblasts in the liver. Genetic labelling of myofibroblasts demonstrated that half of the myofibroblasts escape apoptosis during regression of liver fibrosis and downregulated fibrogenic genes (Col-1α1, Col-1α2, α-SMA, TβRI, and TIMP1). The deactivation of HSCs was associated with the upregulation of Hspa1a/b, which protects against stress-induced HSC apoptosis *in vitro* and in vivo [90].

Myofibroblast reversal is also observed in other organs, such as the lungs, heart, and skin [87, 91]. Lineage tracing showed that cardiac myofibroblasts are derived from resident fibroblasts of the Tcf21 lineage [93]. Periostin-traced myofibroblasts revert to a less active state upon acute MI, losing expression of myofibroblast-associated genes (e.g., α-SMA, collagen, fibronectin) and restoring the expression of fibroblasts-associated genes including Tcf21 and Pdgfa [89]. Accordingly, Tcf21 is a deactivation factor of fibrogenic HSCs and protects mice from liver fibrosis induced by carbon tetrachloride

(CCl4) [94]. In the lungs, myofibroblasts persist and undergo a lipogenic fibroblast phenotype switch during the resolution phase [88]. Activation of PPARγ signaling reinforced the lipogenic phenotype at the expense of TGFβ1-mediated fibrogenic response in primary human lung fibroblasts [88].

Anti-fibrotic mediators such as bone morphogenetic protein-4 and -7 (BMP7, BMP4), FGF-1, myogenic differentiation 1 (MyoD), prostaglandin E2 (PGE2), and nuclear factor erythroid 2-related factor 2 (Nrf2) can induce the reversal of myofibroblasts *in vitro* [94–99]. BMP7 prevented and reversed TGF-β1-driven myofibroblast differentiation through internalization of cell-surface hyaluronan (HA) matrix. HA accumulation promotes the persistence of myofibroblast phenotype into catalytic endosomes within the cytoplasm [95]. Similarly, BMP4 significantly decreases the expression of α-SMA and ECM components in skin myofibroblasts through the activation of PPARγ signaling [100]. Further, PGE2 is a potent anti-fibrotic factor that modulates fibroblast proliferation, differentiation, and collagen production [101]. Treatment with PGE2 attenuated in a dose-dependent manner fibroblast activation by inhibiting α-SMA and collagen I at the protein level [96, 101]. Moreover, PGE2 promoted the reversal of myofibroblast differentiation by inhibiting the focal adhesion kinase (FAK) signaling pathway [97]. Likewise, sulforaphane (SFN), an Nrf2 activator, induces IPF human lung myofibroblasts deactivation by decreasing α-SMA and collagen I mRNA expression [96]. Similarly, FGF-1 possesses anti-fibrotic effects by down-regulating type-I collagen, α-SMA, and Hsp47 chaperonin expression and upregulating MMPs [103]. FGF-1 reverts the EMT process mediated by the MAPK/ERK kinase pathway, resulting in ERK-1 phosphorylation and Smad2 dephosphorylation [99]. The activation of MyoD through the TGFβ1-TβRI (ALK5)

signaling pathway leads to the upregulation of α-SMA expression in human lung fibroblasts [98]. Mitogenic factors, such as PDGF, induced myofibroblasts deactivation via tyrosine kinase receptors, which activate the ERK1/2 MAPK and CDK pathways to inhibit MyoD.

In summary, these studies demonstrate the capacity of myofibroblasts to deactivate via reprogramming into resting cells or transitioning into other cell types, promoting fibrosis resolution. This justifies the importance of testing the effects of senescent MSC-CM in myofibroblast deactivation.

7.1.6.3 Induction of Myofibroblasts Apoptosis

Myofibroblasts often evade apoptosis in fibrotic tissues due to an altered balance between pro-apoptotic and anti-apoptotic mechanisms [2]. Apoptosis is a homeostatic mechanism coordinated by two interconnected molecular pathways: the intrinsic, mediated through the mitochondrial outer membrane permeabilization (MOMP), and the extrinsic, involving death receptors [104]. Myofibroblasts can undergo apoptosis after tissue remodeling, and macrophages and dendritic cells remove the cell debris [105], [106]. After tissue repair, pro-apoptotic cytokines are released and selectively induce myofibroblasts' apoptosis. For example, FGF1 induces caspase-3-mediated apoptosis in activated myofibroblasts from skin granulation tissue by inhibiting the phosphorylation of the FAK signaling pathway; in contrast, FGF1 does not induce apoptosis in fibroblasts [107]. Alternatively, the depletion of pro-survival growth factors during wound healing can induce myofibroblast apoptosis [108]. However, autocrine production of pro-survival proteins such as TGFβ1 and endothelin-1 (ET1) mediates resistance to apoptosis in cultured scleroderma and IPF fibroblasts via increased activation of the PI3K/AKT

signaling pathway [108, 109]. In addition, ECM stiffening promotes the upregulation of anti-apoptotic proteins, including the BCL-2 family, that bind and sequester pro-apoptotic BH3 proteins, thus preventing MOMP and enhancing the myofibroblast survival [110–112]. Furthermore, the ECM stiffness influences the myofibroblasts' apoptosis resistance via the Rho/ROCK mechanotransduction pathway [114]. Inducing myofibroblast apoptosis can reverse established fibrosis in mouse models [68, 112]. This approach also has the promise to reverse human fibrotic diseases.

This highlights the relevance of testing the effects of senescent MSC-CM in a myofibroblasts' apoptosis sensitivity assay.

7.2 Section 2: Multipotent Mesenchymal Stromal Cells (MSC)

7.2.1 Definition

Multipotent mesenchymal stromal cells (MSC) are nonhematopoietic perivascular cells with anti-fibrotic, immunomodulatory, and pro-angiogenic properties [115]. Paracrine factors mainly mediate MSC effects with some contribution of cell-contact-dependent mechanisms [116]. MSC secrete a wide range of bioactive trophic factors known as the ‘MSC secretome.’ The MSC secretome comprises growth factors, cytokines, chemokines, and extracellular vesicles (EVs) that contain a wide range of small molecules such as messenger RNA, peptides/proteins, and microRNAs [3]. The composition of the MSC secretome is modulated by several factors, including the local microenvironment (i.e., inflammatory cytokines and hypoxia factors), MSC passage (i.e., early versus late / senescence), and experimental culture conditions among others [117].

MSC were first isolated from bone marrow by Friedenstein et al. in the 1960–1970s [117, 118] and further renamed by Caplan [120]. MSC are present in almost every tissue;

however, the most common sources of MSC used in clinical trials are bone marrow, adipose tissue, and umbilical cord **(Figure 5)** [121]. In 1995, Lazarus pioneered the first human MSC trials [122]. Since then, MSC have been extensively studied to determine their mechanisms of action and to evaluate their therapeutic efficacy and safety in a diverse range of inflammatory and immune-mediated diseases [122–124].

In clinical trials, bone marrow-, adipose tissue- or umbilical cord-derived MSC are most frequently delivered intravenously. MSC directly and indirectly affects the immune system, vasculature and microenvironments [126]. MSC-based products are governed by specific regulatory frameworks [127]. In the United States of America, the Food and Drug Administration (FDA) classifies MSC as Human Cellular and Tissue-based Products (HCT/Ps) that are regulated as biological drugs under the Code of Federal Regulations (part 1271). In contrast, the European Medicine Agency (EMA) categorizes human MSC as Advanced Therapies Medicinal Products (ATMPs), and their regulation falls under the scope of Regulation No. 1394/2007 [128]. In Canada, MSC-based products are regulated by Health Canada, composed of acts, regulations, guidelines, and policies as biologic drugs [129].

To harmonize the definition of MSC, the International Society for Cellular Therapy (ISCT) proposed three minimal criteria: (a) adherence to plastic in standard culture conditions, (b) surface positivity for CD90, CD73, and CD105 and lack of hematopoietic markers CD45, CD34, CD14, CD19, and HLA-DR, and (c) *in vitro* trilineage differentiation ability (i.e. into osteoblasts, chondroblasts and adipocytes), as a proof of multipotency **(Figure 5)** [130]. *My project focuses on human-adipose-derived MSC. All samples used in my experiments were obtained with previously published* [130, 131]*, established*

methods and fulfilled the ISCT criteria [133]. *Given the relevance of the MSC paracrine properties, my studies focused on the anti-fibrotic effect of MSC-conditioned media (MSC-CM).*

7.2.2 Function

MSC have immunomodulatory, anti-inflammatory, pro-angiogenic, and anti-fibrotic properties. As a result, they promote tissue repair and homeostasis maintenance **(Figure 5)** [134].

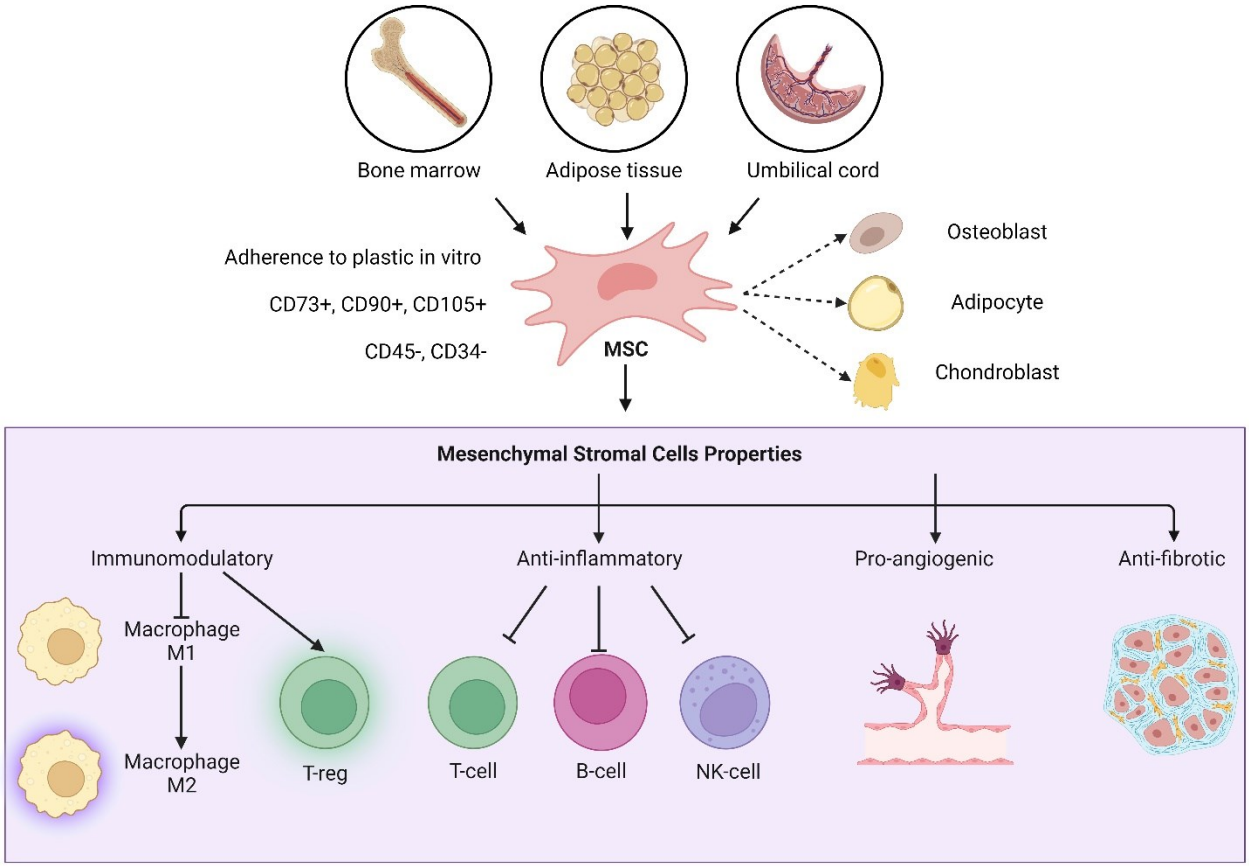


Figure 5. MSC most common sources, minimal definition criteria, and properties. The International Society for Cellular Therapy (ISCT) proposed three MSC minimal criteria: (a) adherence to plastic in standard culture conditions, (b) surface positivity for CD90, CD73, and CD105 and lack of hematopoietic markers CD45, CD34, CD14, CD19,

and HLA-DR, and (c) in vitro trilineage differentiation ability (i.e., into osteoblasts, chondroblasts and adipocytes). MSC have immunomodulatory, anti-inflammatory, pro-angiogenic, and anti-fibrotic properties.

7.2.2.1 MSC immunoregulatory properties

MSC interact and modulate all innate and adaptive immune cells, including T lymphocytes, macrophages, natural killer cells, neutrophils, and dendritic cells [135]. Pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), activate/prime MSC enhancing their anti-inflammatory or immunosuppressive effects [136]. MSC activation leads to the secretion of various soluble factors such as indoleamine 2,3-dioxygenase (IDO), PGE2, interleukin-10 (IL-10), TGF-β, human leukocyte antigen-G (HLA-G), and chemokines (e.g., CXCL9, CXCL10, CXCL11, and CXL12) [133, 134]. MSC priming also enhances EV production [139]. Our laboratory reported that cytokine priming of adipose tissue-derived MSC (AT-MSC) leads to increased secretion of small exosome-like EVs (sEVs) containing two vital regulators of immunopotency: A20 and TNF-α-induced protein 6 (TSG-6). These AT-MSC-EVs effectively suppress T-cell proliferation in a dose-dependent manner. We also showed that AT-MSC obtained from pediatric donors produce more sEVs than adult MSC, potentially contributing to their heightened immunopotency [132].

MSC induce cell cycle arrest in T-cells after the downregulation of cyclin D2 and upregulation of p27kip1 *in vitro* [140]. Furthermore, MSC exert their immunoregulatory capacity by inducing T-regs and inhibiting B lymphocyte-related humoral immune response by blocking B-cells' proliferation, differentiation, and chemotactic cytokine production [141]. In pro-inflammatory microenvironments, the conversion of tryptophan

into kynurenine through an IDO-dependent pathway leads to the suppression of T-cell proliferation at mid-G1 phase, the inhibition of activated T-effector cells, and the induction of T-, B-, and natural killer (NK) cell apoptosis [127, 139]. MSC with high IDO activity promote the generation of anti-inflammatory M2 macrophages to block T-cell activation; thus, T-cell inhibition further amplifies the immunosuppressive effects of MSC [143]. MSC enhance the phagocytic activity of macrophages by altering their differentiation from monocytes into inflammatory M1 macrophages, measured as an increase in CD68, CD14, and CD11b. In addition, MSC modulate the bioenergetic status driving macrophage's polarization [144]. They can induce a metabolic-associated anti-inflammatory phenotype to improve several biological processes, including wound healing, immune tolerance, and regeneration [145]. Additionally, MSC can suppress the cytotoxic activity of NK cells [146]. MSC decrease IL-2-induced proliferation, cytotoxicity, and cytokine secretion (IFN-γ, IL-10 and TNF-α) in activated NK cells in an IDO- and PGE2-dependent manner [147]. MSC also inhibit the generation and antigen presentation of peripheral blood monocyte-derived DCs after inhibiting cytokine release, differentiation, and maturation of DCs [148]. Altogether, the outcome of MSC-immune cell interaction is the promotion of immune tolerance.

The ISCT has proposed several readouts to assess the immunomodulatory effects of MSC [132, 146, 147]. Notably, MSC: T-cell suppression assays are highly reproducible and widely accepted as the standard *in vitro* method for evaluating the immunopotency of MSC [146, 147]. *In my project, MSC immunomodulation was tested in a potency assay previously standardized in our laboratory (i.e., MSC inhibition of CD3/CD28 activated T-cell proliferation)* [136, 148, 149].

7.2.2.2 MSC Pro-angiogenic Properties

In addition to the modulation of immune responses, MSC promote angiogenesis through the secretion of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), TGF-β, FGF-2, hepatocyte growth factor (HGF), PDGF, angiopoietin-1 (ANG-1), placental growth factor (PIGF), IL-6, and monocyte chemotactic protein-1 (MCP-1), and SDF-1α, as well as EVs carrying microRNAs (miRNAs) with angiogenic function *in vitro* and in vivo [150–152].

Most of these factors rely on VEGF and trigger crucial pro-angiogenic signaling pathways, such as MAPK, ERK, and FAK [156]. Consequently, these pathways promote endothelial cells (ECs) survival, migration, and ECs' tube formation [157]. MSC can also influence angiogenesis directly through contact-dependent mechanisms. When MSC and ECs are co-cultured, MSC align themselves with tube structures formed by ECs [158], supporting the current concept that MSC are mural cells (i.e., pericytes) [156, 157]. Furthermore, pre-clinical animal models confirmed in vivo the pro-angiogenic capabilities of MSC as they: 1) enhance the expression of VEGF within tissues [161], 2) stimulate the formation of new blood vessels (neovascularization) [161], 3) augment blood perfusion [162], and 4) provide cardioprotective effects [163].

7.2.2.3 MSC Anti-fibrotic Properties

Considering the close interplay between chronic inflammatory responses and fibrosis, and given that MSC modulate immune responses, MSC could ameliorate fibrotic diseases [161, 162]. Multiple investigations support that MSC exert direct anti-fibrotic effects, to some extent, by regulating the TGF-β and Wnt signaling pathways [163–166]; however, the mechanisms are not fully elucidated.

In vitro studies showed that MSC-CM downregulates collagen I, collagen III, α-SMA, connective tissue growth factor (CTGF), fibronectin, and TGF-β1 and upregulates Smad7 in myofibroblasts and activated smooth muscle cells [167–172]. Furthermore, MSC-CM inhibits fibroblast proliferation and mediates lung epithelium wound healing in different lung tissues from patients with IPF and emphysema compared to healthy controls [176]. Moreover, some studies implied that MSC-CM limit the activation of myofibroblasts partially by releasing paracrine factors such as HGF and TSG-6 [177] and regulating MMPs and TIMPs; increasing MMP-9, MMP-1 [175, 176], MMP-13, MMP-14 [180]; and decreasing TIMP-1, and TIMP-2 [178, 179]. **Table 1** summarizes the *in vitro* anti-fibrotic effects of MSC and MSC-CM.

Animal models of organ-specific or systemic fibrosis were used to test the anti-fibrotic effects of MSC-CM. Human bone marrow-derived (BM-) MSC-CM reduced collagen I, III, and FN in silica rat-induced pulmonary fibrosis [183]. In a similar model, hBM-MSC-CM increased the expression of epithelial markers, including E-cadherin and cytokeratin 19 (CK19), and decreased the expression of fibrosis mesenchymal markers, including vimentin and α-SMA after exposure to silica suspension [184]. Moreover, the Wnt/β-catenin signaling pathway is abnormally activated in silica-induced pulmonary fibrosis, suggesting that BM-MSC-CM reduced fibrosis via inhibition of the EMT program [184]. In a more recent study, hypertrophic scar (HS) formation in a rabbit ear model treated with MSC-CM demonstrated a lower scar elevation index, a method of measurement of the collagen fiber arrangement by immunohistochemical staining, compared to control samples [185]. These studies provide evidence of the MSC-CM

potential to improve and potentially reverse fibrosis in organs that possess regenerative capabilities.

Table 1. Anti-fibrotic *in vitro* effects of MSC and MSC-CM.

	MSC source/ secretome component	Fibroblasts Source	Fibroblasts Activation/ Treatment	Readout	Outcome	MoA
[170]	hBM-MSC-CM/EVs	Human bladder fibroblasts	Irradiation Inhibition assay: MSC-EVs and MSC-CM for 72 h	qRT-PCR, Proteome Profiler Human XL Cytokine Array	↓α-SMA, collagen I, and CTGF mRNA levels ↓ IGFBP2, IL1β, IL6, IL18, PDGF, TNFα, and HGF ↑IFNγ, IL10, and IL27	MSC-EVs mediated anti-fibrotic effect.
[186]	hAT-MSC EVs	Keloid fibroblasts	Stiff surface	qRT-PCR, WB	↓ collagen I, collagen III, α-SMA, and FN	Inhibition of Smad-3 and Notch-1.
[171]	hA-MSC and hA-MSC-CM	LX2 cell line Hypertrophic skin fibroblasts	Inhibition assay: hAMSC or hAMSC-CM for 48 h	WB, IH, FC, antibody array: cytokines si-RNA	hAMSC or hAMSC-CM: ↓ Collagen I, collagen III, TGF-β and α-SMA protein level hAMSC-CM does not affect apoptosis or proliferation of HSC	IGFBP-3, DKK-3, and DKK-1 inhibited the Wnt signalling pathway.
[187]	Mouse-AT-MSC-EVs: miR-223-3p	NCTC1469 cell line: mouse hepatocyte	Palmitic acid	WB, qRT-PCR, IH	↓α-SMA, collagen I, and TGF-β1	MSC-EVs deliver miR-223-3p to attenuate lipid accumulation and fibrosis.
[188]	hBM-MSC Exosomes	Tubular epithelial cells	TGF-β (10ng/mL)	WB, IH	↓α-SMA, collagen I	miR-21a-5p repressed

			Inhibition assay: MSC Exosomes for 48hs		and collagen III	the expression of PFKM, limiting glycolysis.
[189]	hAT-MSC-EVs	Hypertrophic scar-derived Fibroblasts	Stiff surface	WB, qRT-PCR, IH Scratch wound assay	↓ collagen I and collagen III protein level ↓α-SMA mRNA level and IH intensity ↓proliferation and migration	miR-192 5p targets IL-17RA and inhibits the Smad axis.
[177]	hAT-MSC-CM: HGF and TSG-6	Intestinal fibroblasts and smooth muscle cells	Irradiation Deactivation assay: MSC co-culture for 12 h (without cell-cell contact)	qRT-PCR	hIFs: ↓TGF-β1, α-SMA, and collagen III expression hCoSMCs: ↓ TGF-β1, collagen I, and FN expression	Silencing of TSG-6 in MSC may counteract the effect on α-SMA, collagen III, and fibronectin expression.
[172]	hAT-MSC-CM: Decorin	Myoblast cell line	TGFβ1 (2.5-10 ng/mL) for 72 hours Deactivation assay: MSC-CM for 8 h – 24 h	qRT-PCR, WB	↓αSMA protein and mRNA levels	Suggested Decorin, an inhibitor of TGFβ1, an anti-fibrotic mediator.
[190]	hBM-MSC-EVs	LX-2 cell line	TGFβ1 (10 ng/mL) for 6 h Deactivation assay: 3 doses of MSC-EVs (50 k particles per cell) for 72 h.	qRT-PCR, WB	mRNA level (24 h): ↓α-SMA and collagen I mRNA level (72 h): No effect Protein level (72 h): ↑α-SMA and collagen I	MSC-EVs induce only a transient attenuating effect at the mRNA level, with a loss of effect at 72 h.
[178]	hAT-MSC Membranes particles	Epithelial cells A549 and IPF fibroblasts with short telomeres	TGFβ (5 ng/mL) Inhibition assay:	qRT-PCR,	A549: ↓ collagen I, collagen III, FN, and PAI-1	FS could not inhibit the TGFβ increase in α-SMA,

		and normal telomeres	48h with MPs from MSC		FN and FS: ↓ collagen I, Tenascin-c, PAI-1, and ↑MMP-1 gene expression	PDL1, and FN when fibroblasts were treated with MSC-MPs.
[179]	hAT-MSC-CM/ EVs	Fibroblasts derived Systemic sclerosis	TGF-β (5ng/mL) Inhibition assay: hAT-MSC-CM/ EVs for 24h	qRT-PCR,	↓αSMA and collagen I ↑MMP1/TIMP1 and COX2	-
[191]	Rat/hBM-MSC-CM resting vs. INF-ɣ primed	Human Kidney-2 cell line	TGF-β (10ng/mL) Inhibition assay: MSC-CM for 30 min or 24h	WB, IH	Primed hMSC-CM ↓α-SMA, p-Smad2	Secreted PGE2
[192]	hAT-MSC-CM/EVs	Human dermal fibroblasts	TGF-β (5ng/mL) Inhibition assay: MSC-CM/EVs for 4 days	qRT-PCR, WB, IH	↓α-SMA gene and protein levels	MSC-EVs mediated anti-fibrotic effect: miRNA-29c and miRNA-21
[173]	hAT-MSC-CM	α-SMA+ human hypertrophic scar-derived myofibroblast	Stiff surface Deactivation assay: MSC-CM for 24 h	qRT-PCR, WB, Scratch assay	↓α-SMA, collagen I, and collagen III protein levels and expression are dose-dependent. ↓migration of HSC	Inhibition of p38/MAPK pathway
[174]	hAM-MSC-CM	αSMA+ human limbal myofibroblast	Stiff surface Deactivation assay: MSC-CM for 12 h	WB	↓αSMA protein levels	-
[193]	hUC-MSC	Human hepatic stellate cell lines (hTert and LX2)	TGF-β (1ng/mL)	WB IH	↓α-SMA and pSMAD2 protein levels	MFGE8 downregulates TβRI.

		and human primary HSCs	Inhibition assay: hUC-MSC for 24h			
[176]	hLung-MSC-CM	Lung fibroblasts	Stiff surface	Proliferation, Scratch wound assay	↓Fibroblast proliferation and migration	-
[175]	hAT-MSC: HGF	Rat cardiac fibroblasts	Stiff surface	ELISA, IH, qRT-PCR	↓Angiotensin II type 1 receptor (AT1R), TGF-β, collagen I, and collagen III ↑HGF and Smad 7	Secreted HGF upregulated Smad7.
[194]	hBM-MSC: HGF and IL-10	Hepatic stellate cells	TGF-β (1ng/mL) Inhibition assay: hBM-MSC for 24h	IH, Cell viability, ELISA	↓α-SMA, TGF-β1 and IL-6, ↑ IL-10, and HGF ↓HSC viability	Secreted HGF and IL-10.
[195]	hBM-MSC-CM	Human kidney proximal tubular epithelial cells	TGF-β (10 ng/ml) for 72 h	WB	↓a-SMA and ↑E-cadherin protein level	-
[182]	Rat-BM-MSC-CM	Rat cardiac fibroblasts	Stiff surface	WB, qRT-PCR, IH	↓α-SMA, TIMP-2 expression ↓collagen I and collagen III content ↑ MT1-MMP expression ↑ MMP-2/MMP-9 activity	Regulation of MMPs and TIMPs.

MoA: mechanism of action; MSC: mesenchymal stromal cells; hBM: human bone marrow; hAT: human adipose tissue; hAM: human amniotic; hUC: human umbilical cord; EVs: extracellular vesicles; HSC: hepatic stellate cells; LX-2: human hepatic stellate cells; hIFs: intestinal fibroblasts; hCoSMCs: smooth muscle cells; WB: western blot; qRT-PCR: quantitative reverse transcription PCR; IH: immunohistochemistry; FC: flow cytometry; TGF-β1: transforming growth factor beta 1; α-SMA: alpha-smooth muscle actin, CTGF: connective tissue growth factor; IGFBP-: insulin like growth factor binding protein-; IL-: interleukin-; PDGF: platelet-derived growth factor, TNFα: tumor necrosis factor alpha, HGF: hepatocyte growth factor; IFNγ:

interferon-gamma; FN: fibronectin; Notch-1: Neurogenic locus notch homolog protein 1; DKK-3: Dickkopf-3; DKK-1: Dickkopf-1; miR: microRNAs; PFKM: phosphofructokinase muscle isoform; TSG-6: tumor necrosis factor-stimulated gene 6; FS: short telomeres; FN: normal telomeres; Membranes particles: MPs; PAI-1: Plasminogen activator inhibitor-1; MMP1: matrix metalloproteinase 1; TIMP1: tissue inhibitor of metalloproteinase-1; COX2: cyclooxygenase-2; PDL1: programmed death-ligand 1; p-SMAD2: phospho-SMAD2; PGE2: prostaglandin E2; p38/MAPK: p38 mitogen-activated protein kinases; MFGE8: secreted milk fat globule-EGF factor 8; TβR1: TGFβ type I receptor; AT1R:Angiotensin II type 1 receptor.

7.2.2.3.1 MSC-CM Anti-fibrotic Factors

The MSC secretome contains growth factors and cytokines, such as HGF, PGE2, secreted milk fat globule-EGF factor 8 (MFGE8), TGF-β3, TNF-α, Cadherin 2, VEGF, and IL-10 that attenuate fibrosis **(Figure 6)** [188, 190, 191, 193–199]**.**

PGE2 mediates anti-fibrotic effects directly by modulating fibroblasts' activation and indirectly by interacting with immune cells [96, 188]. Knockdown of PGE2 synthase weakens the anti-fibrotic effect of MSC treated with IFN-γ in rats with ischemia–reperfusion injury. Further, MSC treated with IFN-γ exert more potent anti-fibrotic effects by directly inhibiting the TGF-β/Smad signalling pathway, documented by reduced α-SMA, Col-I, and Col-III protein levels [191].

HGF exerts an anti-fibrotic effect on decreasing TGF-β and collagen I [203] and increasing MMP-1, MMP-3, and MMP-13 expression [204] within fibroblasts. It functions as an antagonist to the canonical pathway, effectively preventing the nuclear entry of Smad3 [205]. Gazdhar et al. transfected BM-MSC to express in higher amounts HGF, hydroxyproline collagen content measurements showed an enhanced reduction of bleomycin-induced pulmonary fibrosis after treatment with HGF-modified BMSC-CM in comparison with unmodified BMSC-CM [199].

IL-10 downregulates collagen type I and upregulates MMPs gene expression in fibroblasts [206]. Transfecting IL-10 mRNA enhanced MSC therapeutic potential and inhibited T cells' proliferation in the spleen in a mouse model for GvHD [197].

MFGE8 is a potent inhibitor of the activation of human primary HSCs by inhibiting α-SMA and reducing SMAD2 phosphorylation. MFGE8 down-regulates the expression of TβRI by binding to $\alpha_v\beta_3$ integrin on HSCs and is secreted by MSC from the umbilical cord, teeth, and bone marrow [193, p. 8].

Cadherin 2 and VEGF mediate the anti-fibrotic efficacy of human umbilical cord-derived mesenchymal stromal cells (UC-MSC) in a rat MI model. UC-MSC displayed elevated levels of Cadherin 2 and subsequently heightened expression of VEGF. UC-MSC were effective in reducing cardiac fibrosis and treating MI [201]. Furthermore, pre-treatment of UC-MSC with ET1 induced the upregulation of Cadherin 2 and VEGF through the involvement of transcription factors GATA2 and MZF1. As a result, ET1-treated UC-MSC enhanced their anti-fibrotic functions in a rat model of MI [202].

In addition, MSC-EVs have anti-fibrotic effects, as demonstrated in several *in vitro* models of fibrosis [167, 185, 184, 187, 186, 189]. The mechanisms underlying the anti-fibrotic effects of MSC-EVs include their miRNA cargo. miR-21/-23/-29/-let7 inhibited the TGFβ/Smad signaling pathway, a key pro-fibrotic mechanism [207]. In IPF, miR-29b-3p suppresses fibroblasts proliferation by down-regulating Frizzled Class Receptor 6 (FZD6) [208], while miR-186 suppresses the expression of SOX4 and DKK1 blocking activated fibroblasts [209]. *In vitro*, MSC-EVs downregulated myofibroblasts' secretion of ECM proteins and suppressed their contractility [192]. *These results confirm that multiple factors in the MSC-CM can modulate fibrosis.*

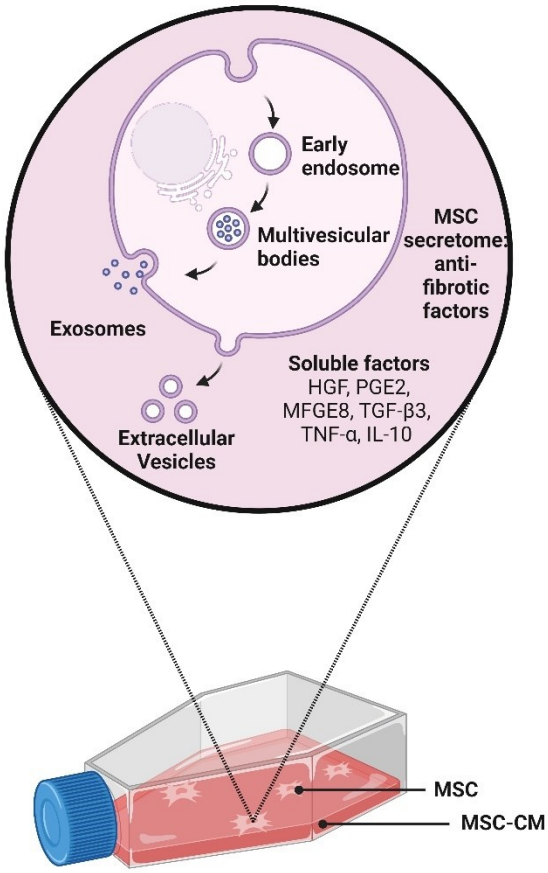


Figure 6. Anti-fibrotic factors of MSC secretome. The MSC therapeutic effects are predominantly mediated through secreted soluble molecules and extracellular vesicles constituting the MSC secretome (i.e., MSC-CM). The MSC secretome contains growth factors and cytokines, such as HGF, PGE2, secreted milk fat globule-EGF factor 8 (MFGE8), TGF-β3, TNF-α, Cadherin 2, VEGF, and IL-10 that attenuate fibrosis.

7.2.3 Therapeutical Applications

Clinical trials, most of which are early phase, used autologous or allogenic BM-MSC, AD-MSC, UC-MSC, and MSC-EVs in a broad spectrum of diseases [e.g., osteoarthritis, rheumatoid arthritis, diabetic foot ulcers, cardiovascular diseases, neurodegenerative diseases, graft-versus-host disease (GvHD), Crohn's disease (CD),

amyotrophic lateral sclerosis (ALS), MI, and acute respiratory distress syndrome (ARDS), lupus, and SSc] [122–124]. These trials support the safety and tolerability of MSC, but there are limited efficacy data [207, 208]. Eleven MSC therapies are currently approved for two fibrotic conditions: GvHD and CD **(Table 2)** [212].

Table 2. Approved MSC therapeutic agents.

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

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

7.3 Section 3: Cellular Senescence

7.3.1 Definitions and Readouts

Cellular senescence is a cellular state of stable and long-term loss of proliferative capacity [213]. Senescence is a cellular response or program that limits the expansion of

aged or damaged cells [214]. A cell can initiate the senescence program regardless of organismal age. However, senescent cells accumulate in aged tissues, and senescence is a hallmark of aging and a powerful mechanism of tumor suppression [212, 213]. Aging is the progressive decline and impairment of function at the molecular, cellular, tissue, and organismal levels associated with the increased risk of disease and death [215]. Besides senescence, the hallmarks of aging include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion, and altered intercellular communication [217]. Significantly, senescent cells acquire an inflammatory SASP; therefore, aging is associated with chronic low-grade inflammation termed "inflammaging" [19, 214], which in turn fosters the fibrotic process [20]. Senescent cells accumulate in age-associated diseases, including prevalent age-associated fibrotic conditions (i.e., pulmonary fibrosis, atherosclerosis) [18, 215, 216].

Different types of senescence have been described (i.e., replicative, oncogene-induced, stress-induced, and developmental), but all of them lead to a similar cellular phenotype. Senescent cells are characterized by distinct phenotypic alterations, including stable and generally irreversible growth arrest unresponsive to mitogenic stimuli; altered metabolic activity, apoptosis resistance, persistent DNA damage response (DDR), increased lysosomal activity, macromolecular damage, and a SASP [221]. The SASP factors include (IL-1, IL-6, IL8, MMP1), TNF-α and VEGF [222]. Senescent cells also develop morphological and structural changes, including enlarged and flattened, with large vacuoles, altered plasma membrane composition, and nuclear changes [223]. A distinctive feature of senescent cells is the increased expression of cell cycle-inhibitory

proteins, known as cyclin-dependent kinase inhibitors. The cyclin-dependent kinase inhibitor with the most prominent role in senescent cell accumulation during aging is p16, which is crucial for maintaining the state of proliferative arrest [224]. The most frequently used marker for cellular senescence is SA-β-gal. However, other authors suggest that combining the proliferation markers Ki-67, proliferating cell nuclear antigen (PCNA), and the DNA damage marker γH2AX is optimal [225].

7.3.2 MSC Replicative Senescence

After extended culture, a prerequisite to generate adequate MSC numbers for cell therapy, MSC undergo replicative senescence (RS). Similar to other senescent cells, senescent MSC display morphological changes, including a flat, enlarged, and more granular morphology with loss of spindle-like shape, as well as changes to nuclear morphology with the formation of a distinct chromatin structure, called senescence-associated heterochromatic foci (SAHF) [226]. Senescent MSC have deficient proliferative and differentiation capacity (i.e. more likely to differentiate toward adipogenesis), stain positive for SA-β-Gal, and have a SASP [227].

MSC senescence has been reported as early as passage 5, with a 4.8-fold larger size in passages 6-9 compared to passage 1 [228]. The increase in size in senescent MSC is proportional to forward scatter (FSC) (i.e., the bigger the cell, the more light is scattered, the higher the detected signal). In contrast, increased MSC granularity correlates with side scatter (SSC) [227]. Autofluorescence, a novel marker of MSC senescence, relates to the build-up of lipofuscin-related proteins and correlates with the increased SA-β-gal activity [229]. For instance, our group demonstrated that late-passage senescent AT-MSC have elevated CD26 surface expression levels and increased total

protein abundance compared to early-passage AT-MSC. The levels of CD26 correlate with established senescence markers, and senescent AT-MSC with high CD26 expression have reduced immunopotency compared to those with lower CD26 levels [53]. Moreover, MSC from older donors have reduced clonogenicity (i.e. reduced number of colony forming units -CFU), prolonged cell population doubling time, display a reduced maximum lifespan in contrast to those from younger donors (24 ± 11 population doubling time (PDT) versus 41 ± 10 PDT), and have a lower PDT rate than MSC from young donors (0.05 ± 0.02 PDT per day versus 0.09 ± 0.02 PDT per day) [230]. Overall, the lifespan of MSC ranges from 30 to 40 PDT *in vitro* [231]. In addition, the expression of stemness-associated genes, such as Oct4, Nanog and Tert, decreases during MSC senescence [232]. Since there is no single biomarker to characterize senescent cells, a multi-marker approach is recommended [233].

To define MSC senescence, my project used SA-β-gal, PDT, CD26, SSC, and autofluorescence.

7.3.3 Mechanisms of MSC Senescence

Cellular senescence is triggered by stressful insults and developmental signals, and the mechanisms implicated in senescence are interrelated and interact among them. Stress factors encompass both environmental/cell-extrinsic (i.e., irradiation, genotoxic drugs, epigenetic modifiers, high-fat diet) and intrinsic (i.e., DDR, oxidative stress, mitochondrial dysfunction, impaired autophagy, telomere attrition, and oncogene activation) all of which culminate in the accumulation of DNA damage and activation of critical oncogenes like p53/p21 and p16, establishing senescence [213]. In the

subsequent sections, I summarize key concepts of mechanisms leading to MSC senescence.

7.3.3.1 DNA Damage Response Mediates MSC Senescence

DNA damage, which occurs daily due to exposure to internal and external DNA damage agents, is a driver of senescence [234]. In response to genotoxic insults, the DDR orchestrates DNA damage checkpoint activation and facilitates the removal of DNA lesions. The DDR involves a complex network of genes responsible for sensing and repairing specific types of DNA damage and encompasses specific types of machinery mediating DNA repair, cell cycle regulation, replication stress responses and apoptosis [235]. Persistent DDR triggers senescent cells to acquire a SASP.

DDR is the master regulator of cell cycle arrest, a key characteristic of MSC senescence, and is primarily regulated by the p21 and p16 signalling pathways [236]. Mutagens such as oncogene activation, irradiation, telomere shortening, and reactive oxygen species/ mitochondrial dysfunction are sources of DNA damage; consequently, p53/p21 and/or p16 are activated **(Figure 7)** [228]. They sustain the state of senescence primarily by modulating transcriptional factors such as p53 and pRB, and as p21/p16 are cyclin-dependent kinase inhibitors (CDKIs) that tightly regulate pRB activity they act as negative regulators of cell cycle progression. Sustained overexpression of p53, pRB, p16, and p21 is enough to trigger senescence [237]. p21 is transcriptionally activated by p53. p21 reduces the phosphorylation of retinoblastoma (pRB), decreasing the levels of pRB through the inhibition of CDK2 [238]. pRB maintains its functionality and continues to inhibit the E2 transcription factor (E2F), a critical regulator of essential genes in controlling cell growth and proliferation [234, 235]. Similarly, p16 can induce senescence by blocking

CDK4/CDK6 and keeping active pRB [223, 236]. Ultimately, these processes promote senescence and prevent re-entry into the cell cycle [30].

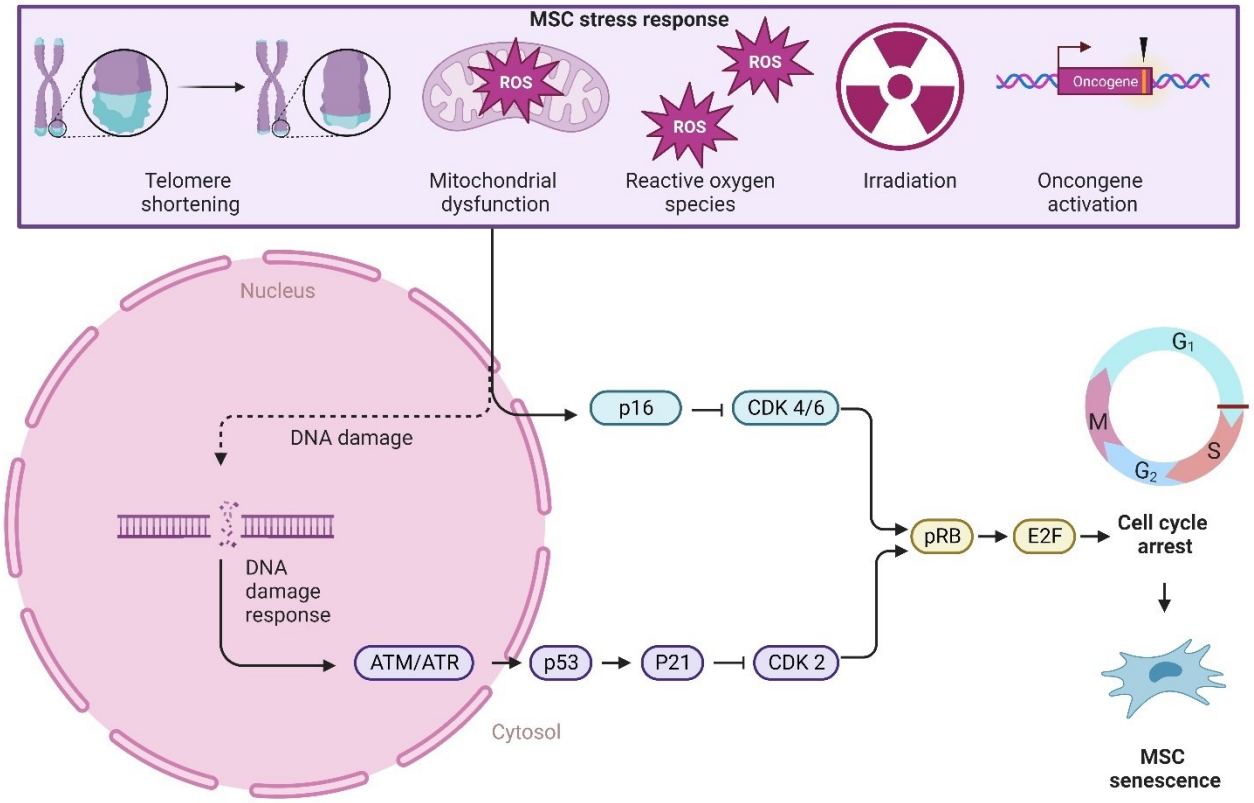


Figure 7. MSC cell cycle arrest is induced by DNA damage. Mutagens such as oncogene activation, irradiation, telomere shortening, and reactive oxygen species/ mitochondrial dysfunction are sources of DNA damage; consequently, p53/p21 and/or p16 are activated, leading to cell cycle arrest and MSC senescence. They sustain the state of senescence primarily by modulating p21/p16, cyclin-dependent kinase inhibitors (CDKIs) that tightly regulate pRB activity. They act as negative regulators of cell cycle progression. Adapted from “Mesenchymal Stem/Stromal Cell Senescence: Hallmarks, Mechanisms, and Combating Strategies”, by Weng, Z., 2022, Stem cells translational medicine, 11(4), 356–371, Copyright © 2022 Published by Oxford University Press, adapted with permission.

7.3.3.2 Telomere Attrition Mediates MSC Senescence

As MSC undergo divisions, their telomeres, tandem repetitive DNA sequences which form a protective loop structure against chromosome fusion and degradation [242], progressively shorten until 30-40 PDT **(Figure 8)**, as previously mentioned [231]. In comparison, human fibroblasts reach senescence at approximately 50 PDT, the 'Hayflick limit' [243]. Senescence occurs when telomeres reach a length at which the preservation of chromosomal stability can no longer be ensured [244]. The protection of telomere length is primarily upheld by telomerase, which continually replenishes 5'-TTAGGG repeats [245]. These repeats have been suggested to engage with the p53 and TGF-β1 signalling pathways [246]. Human telomerase reverse transcriptase (hTERT) is critical in regulating cellular senescence. hTERT overexpression triggers telomerase activation, elongating telomere and enhancing MSC ability to withstand oxidative stress [247]. The substantial variability in telomere length among donors makes it unreliable to predict or monitor MSC senescence solely based on telomere length [243, 244].

7.3.3.3 Reactive Oxygen Species Mediate MSC Senescence

The accumulation of reactive oxygen species (ROS) promotes MSC senescence, activates the DDR, and enhances mitochondrial dysfunction, inhibition of autophagy, telomere attrition, and protein degradation **(Figure 9)** [250]. AT-MSC from older individuals exhibits higher ROS from mitochondrial sources than MSC from young people [251]. The phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) signalling pathways play a role in ROS-triggered MSC senescence. ROS directly activates PI3K, initiating the production of phosphatidylinositol 3,4,5-triphosphate, amplifying downstream signalling, such as AKT. Subsequently, this process promotes the

transcription of AKT target genes, including the mechanistic target of rapamycin 1 (mTOR1) and p53. Ultimately, this promotes MSC senescence. p38 MAPK is key in orchestrating ROS-induced senescence [252]. ROS influences ERK/MAPK kinase 1/2/3/4 (MEKK1/2/3/4) or mixed lineage kinase 3. This stimulation results in the activation of MKK3 and MKK6, which subsequently phosphorylate p38, thus initiating the activation of the p38 MAPK pathway [253], leading to MSC cycle arrest through the p53/p21 pathway [254].

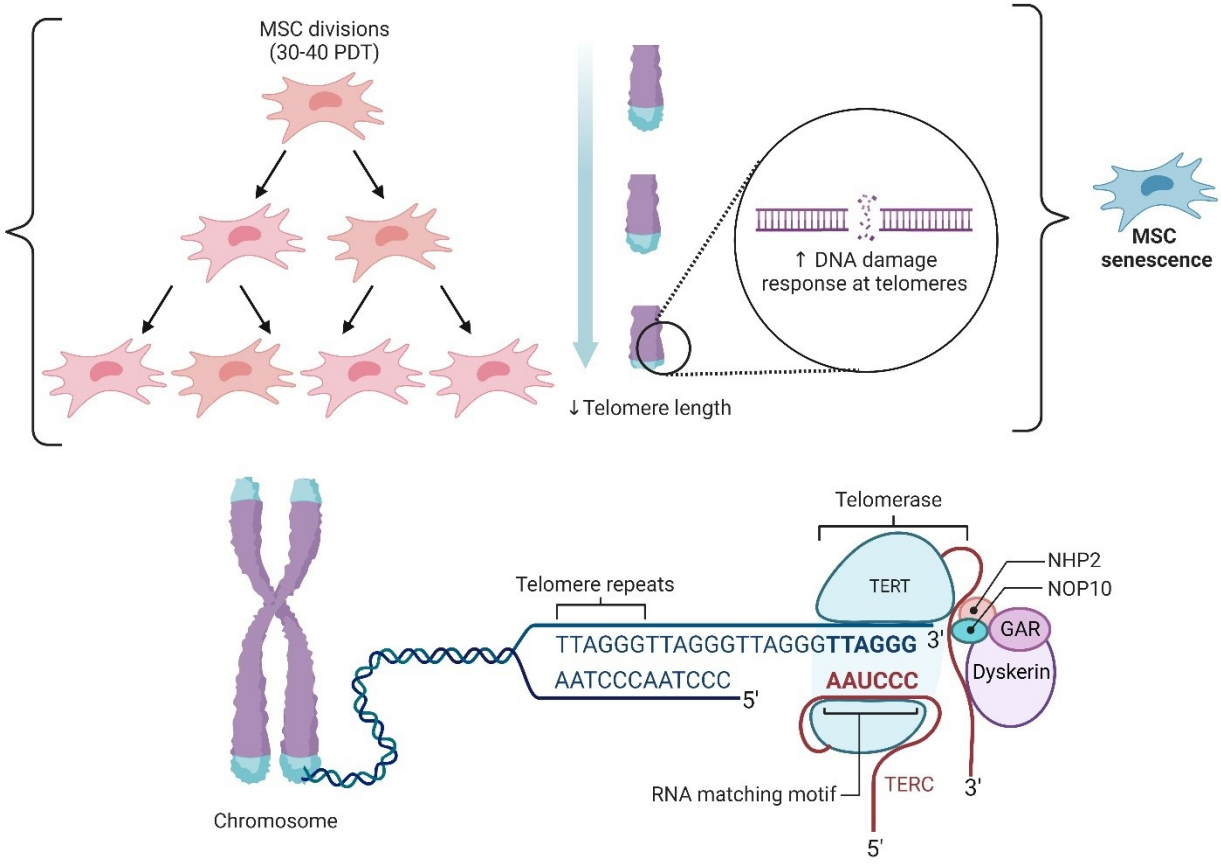


Figure 8. Telomere attrition mediates MSC senescence. As MSC undergo divisions, their telomeres progressively shorten until 30-40 PDT. The protection of telomere length is primarily upheld by telomerase, which continually replenishes 5'-TTAGGG repeats.

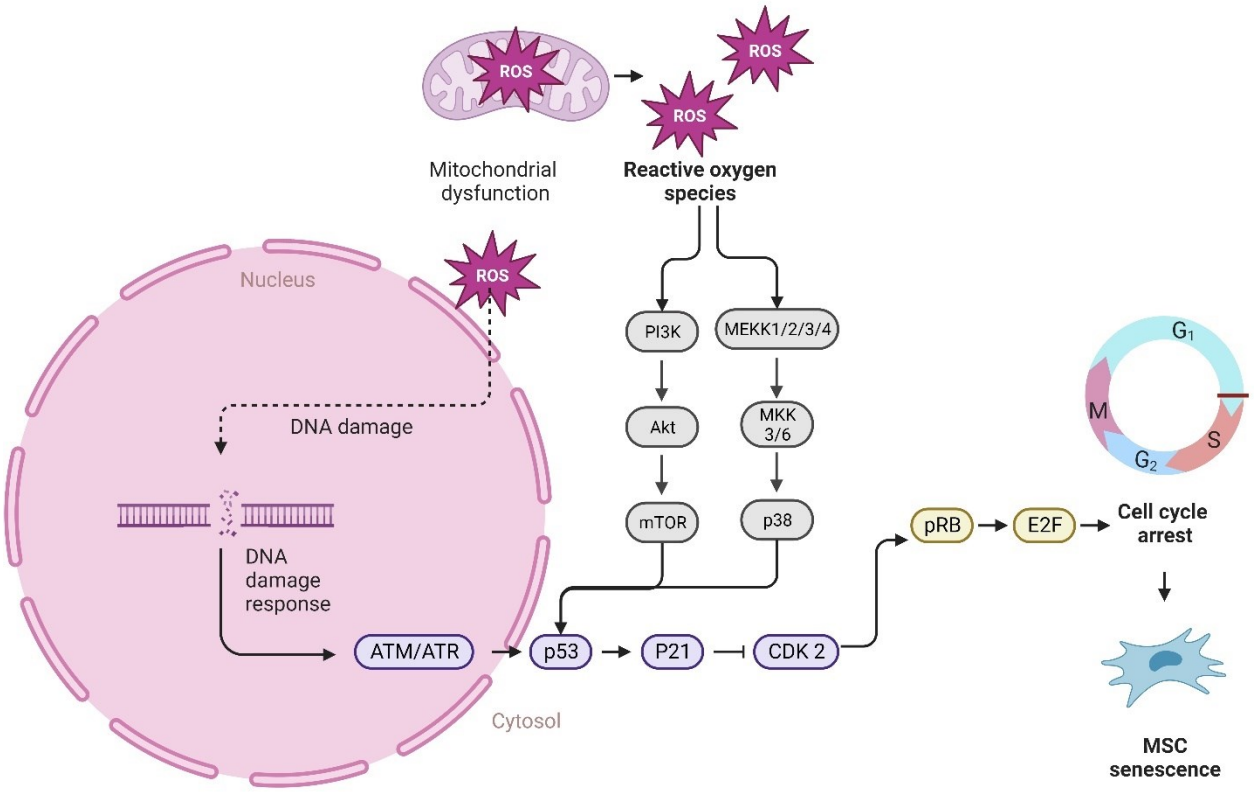


Figure 9. ROS promotes MSC cell cycle arrest. The accumulation of reactive oxygen species (ROS) activates the DNA damage response (DDR) and enhances mitochondrial dysfunction. ROS directly activates PI3K and amplifies downstream signalling such as AKT. Subsequently, this process promotes the transcription of AKT target genes, including the mechanistic target of rapamycin 1 (mTOR1) and p53. Ultimately, this promotes MSC senescence. ROS influences ERK/MAPK kinase 1/2/3/4 (MEKK1/2/3/4) or mixed lineage kinase 3. This stimulation results in the activation of MKK3 and MKK6, which subsequently phosphorylate p38, thus initiating the activation of the p38 MAPK pathway, leading to MSC cycle arrest through the p53/p21 pathway. Adapted from "Mesenchymal Stem/Stromal Cell Senescence: Hallmarks, Mechanisms, and Combating Strategies", by Weng, Z., 2022, Stem cells translational medicine, 11(4), 356–371, Copyright © 2022 Published by Oxford University Press, adapted with permission.

7.3.3.4 Mitochondrial Dysfunction Mediates MSC Senescence

Mitochondrial dysfunction leads to oxidative stress and can increase stress-induced apoptosis in MSC [255]–[258]. Mitochondria dysfunction plays a crucial role in reinforcing the positive feedback loop of ROS-induced senescence because elevated ROS leads to mitochondrial DNA (mtDNA) damage **(Figure 10)**. Due to the limited repair mechanisms, mtDNA is more vulnerable to mutations than nuclear DNA [259]. ROS-triggered DNA damage promotes mitochondrial biogenesis via the mTOR/proliferator-activated receptor-gamma coactivator-1α/β (PGC-1α/β) axis. Consequently, this increases mitochondria and ROS production [260].

Furthermore, mitochondria dysfunction is linked to respiratory chain anomalies, including NAD+/NADH and ATP/ADP ratios associated with MSC senescence [261]. The disrupted ATP/ADP ratio may activate AMP-activated protein kinase (AMPK), ultimately contributing to senescence through the p53/p21 pathway [262]. On the other hand, an abnormal NAD+/NADH ratio, which is linked to a distinct form of senescence known as mitochondrial dysfunction-associated senescence (MiDAS), promotes senescence with an IL-1-deficient SASP via the AMPK-induced p53 pathway [83].

In senescence-associated mitochondrial dysfunction, the balance between fusion and fission events is disrupted [263]. Mitochondria adopt a pro-fusion state during the senescence process, creating an elongated and highly interconnected mitochondrial network. This state hinders mitophagy, which is essential for clearing MSC damaged or malfunctioning mitochondria [253, 262]. Specifically, mitophagy is induced by the PINK1 (PTEN-induced putative kinase 1)/Parkin pathway [265], while p53 upregulation inhibits mitophagy by suppressing the translocation of PINK1 [266]. In BM-MSC, P53

downregulation can effectively enhance mitophagy and protect them from senescence [258].

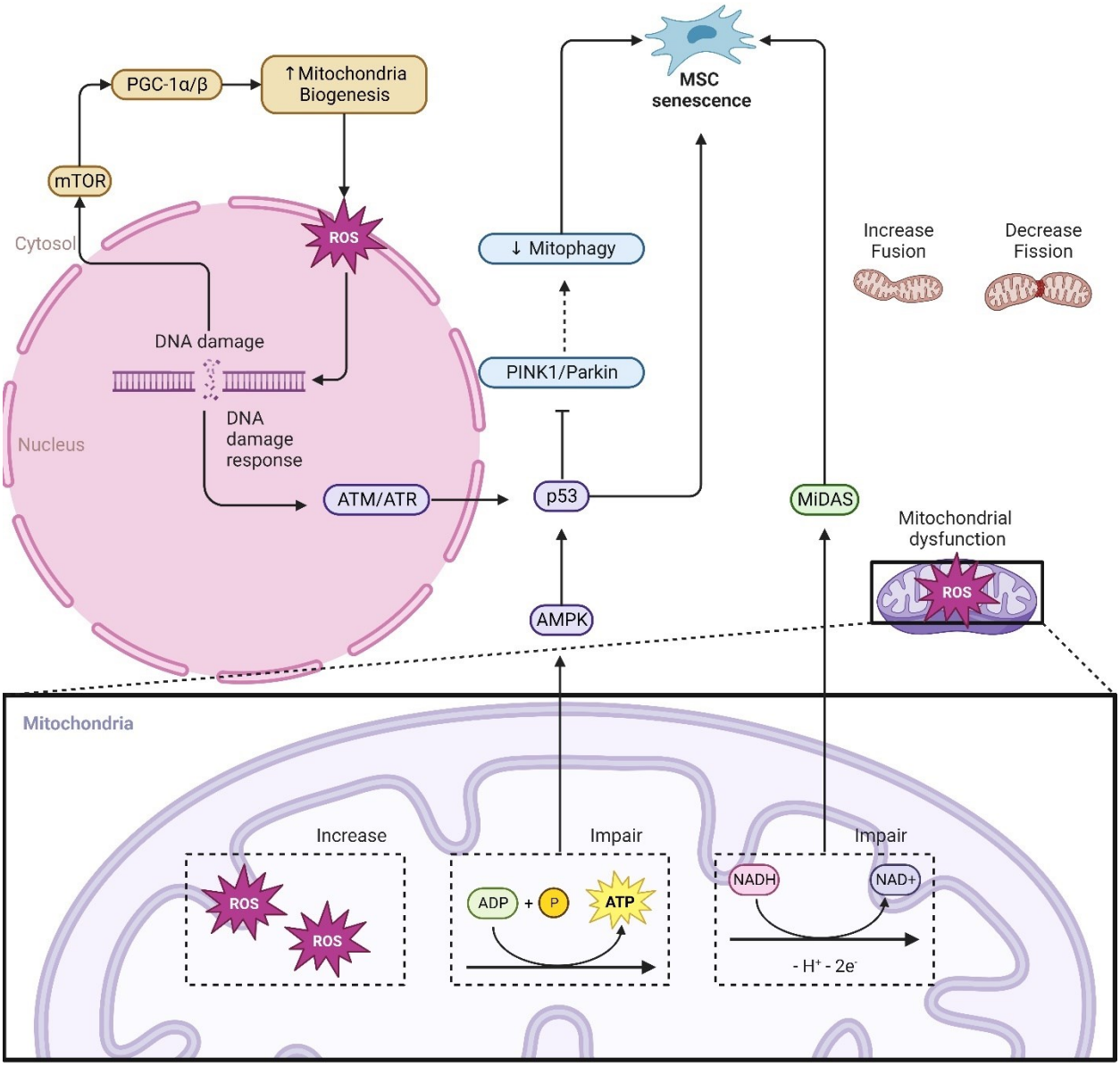


Figure 10. Mitochondrial dysfunction mediates MSC senescence. Senescence-associated mitochondria include dysregulated mitochondrial biogenesis, decreased mitophagy, and hyper-fused mitochondrial networks. ROS-triggered DNA damage promotes mitochondrial biogenesis via the mTOR/proliferator-activated receptor-gamma coactivator-1α/β (PGC-1α/β) axis. Consequently, this increases mitochondria and ROS production, forming a positive feedback loop. AMP/ATP and NAD+/NADH ratios are

metabolically disturbed during aging, initiating downstream signaling cascades. The disrupted ATP/ADP ratio may activate AMP-activated protein kinase (AMPK), ultimately contributing to senescence through the p53/p21 pathway. At the same time, p53 upregulation inhibits mitophagy by suppressing the translocation of PINK. An abnormal NAD+/NADH ratio is linked to a distinct form of senescence known as mitochondrial dysfunction-associated senescence (MiDAS). Mitochondria adopt a pro-fusion state during the senescence process, creating an elongated and highly interconnected mitochondrial network. Adapted from “Mesenchymal Stem/Stromal Cell Senescence: Hallmarks, Mechanisms, and Combating Strategies”, by Weng, Z., 2022, Stem cells translational medicine, 11(4), 356–371, Copyright © 2022 Published by Oxford University Press, adapted with permission.

7.3.3.5 TGF-β as a Mediator of MSC Senescence

TGF-β is one of the predominant pathways regulating multiple aspects of aging-associated fibrosis, including senescence **(Figure 11)** [211, 259]. In BM-MSC, it has been reported that TGF-β increases the expression of markers associated with aging: p16, 4-Hydroxynonenal (4-HNE) subunits, SA-β-gal activity, and the generation of mitochondrial reactive oxygen species (ROS) in a dose-dependent manner [268]. The linkage between ROS and TGF-β activity was acknowledged to different NADPH oxidases (NOXs). NOXs represent a distinctive category of enzymes dedicated solely to producing ROS [269]. Increased ROS-dependent mechanisms initiated by TGF-β1 are key upstream mediators of the p53 pathway, the most extensively studied mediator of senescence [238]. p53 tumour suppressor is known to intersect with the TGF-β pathway by interacting directly with Smad2 and Smad3, which activates encoding p21 [270] and increases PAI-1 and CTGF expression [47]. In addition, increased ROS levels enhance the activities of p38 MAPK, c-JNK, and ERK [271] and cause DNA damage leading to senescence [267], [272]. Moreover, TGF-β induces senescence by prompting the expression of other CDKIs,

such as p15 and p21, and by hindering the activity of various proliferation factors, including c-Myc [270]. The oncogene c-Myc controls hTERT gene transcription, inhibiting senescence variations linked to telomeres' shortening [267]. Thus, TGF-β indirectly modulates hTERT expression by suppressing c-Myc expression. Furthermore, TGF-β/Smad3 can directly engage with the hTERT promoter, resulting in direct suppression [273]. TGF-β signalling triggers senescence via miR-29a and miR-29c accumulation, which directly hinder Suv4-20h, ultimately diminishing the levels of H4K20me3 through a Smad-dependent mechanism. This reduction compromises DNA damage repair and genomic stability, contributing to aging in vivo [274].

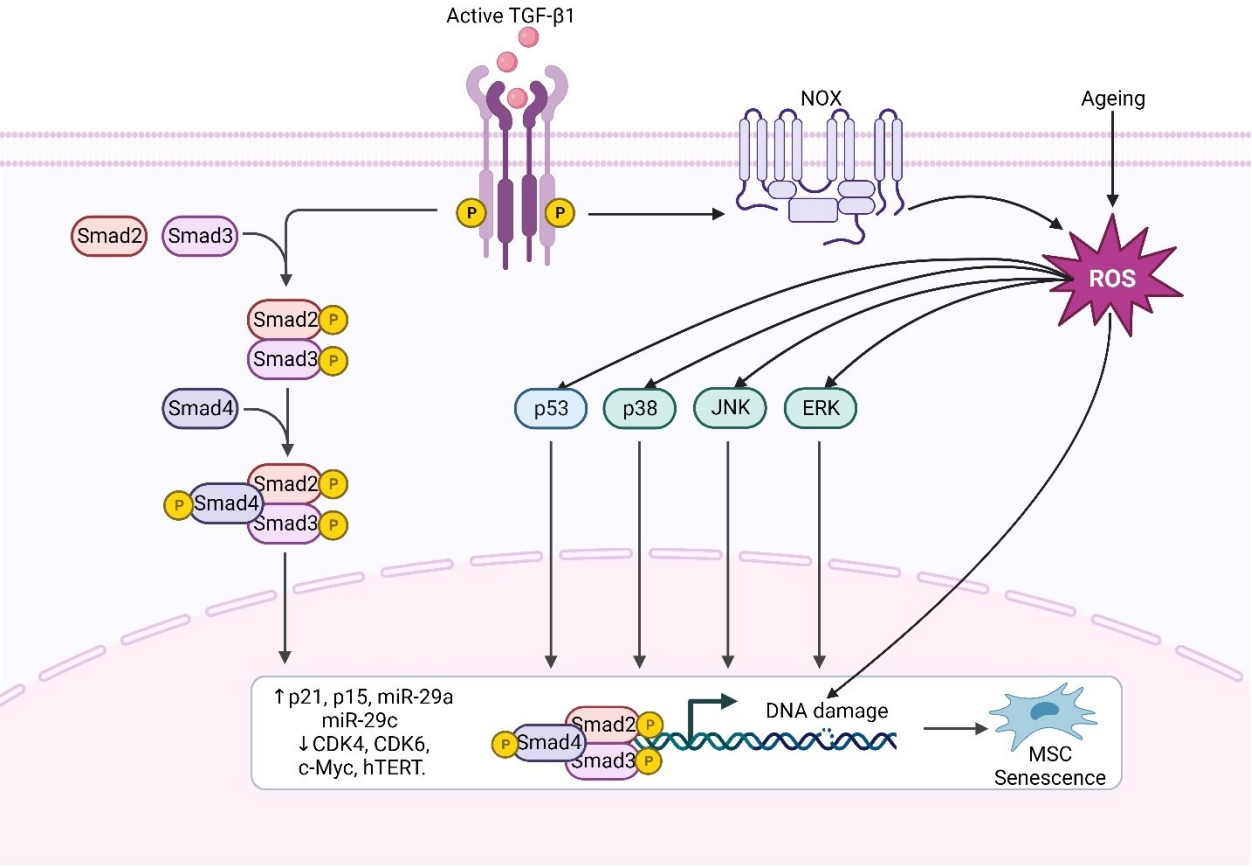


Figure 11. TGF-β key mediator of aging-associated fibrosis and senescence. The linkage between ROS and TGF-β activity was acknowledged to different NADPH

oxidases (NOXs). NOXs represent a distinctive category of enzymes dedicated solely to producing ROS. Increased ROS-dependent mechanisms initiated by TGF-β1 are key upstream mediators of the p53 pathway. In addition, increased ROS levels enhance the activities of p38 MAPK, c-JNK, and ERK, causing DNA damage and leading to senescence. Adapted from “TGF-β as A Master Regulator of Aging-Associated Tissue Fibrosis”, by Ren, L. L., 2023, Aging and disease, 14(5), 1633–1650, Copyright © 2023 Creative Commons Attribution License (CC BY), adapted with permission.

7.3.4 Functional Consequences of MSC Senescence

MSC from elderly donors exhibit lower immunosuppressive capabilities than young MSC. Remarkably, even the least immunosuppressive early-passage MSC are more effective than the most immunosuppressive late-passage MSC [275]. Similarly, AT-MSC from atherosclerosis patients aged 65 and older exhibit a pro-inflammatory secretome characterized by elevated levels of IL-6, IL-8, and MCP-1 and reduced ability to inhibit T cell proliferation. Blocking these pro-inflammatory cytokines restores the MSC immunosuppressive properties [276].

Senescence compromises MSC ability to promote angiogenesis. AT-MSC from preeclampsia patients, a condition linked to senescence [277], have a high frequency of SA-β-gal positivity and reduced capacity to promote ECs’ tube formation. Treatment of these MSC with senolytics enhanced their pro-angiogenic function, supporting the link between senescence and the diminished pro-angiogenic activity of MSC in preeclampsia [278]. In another study, AT-MSC-CM (at P19) exhibited an impaired ability to stimulate the formation of ECs’ tubes in contrast to non-senescent MSC (at P3-6) [279].

Further, MSC from older donors exhibit impaired proliferation and differentiation abilities compared to younger ones [127, 136]. This includes a reduced adipogenic

differentiation capacity and loss of osteogenic differentiation [140]. Accordingly, the transcriptome of replicative senescent MSC shows downregulation of genes associated with cell differentiation [141].

MSC immunosuppressive, *pro-angiogenic and differentiation abilities decline with aging* [142]*. The effect of senescence on MSC anti-fibrotic effects is unknown. Understanding the effects of MSC senescence on their anti-fibrotic function is critical to optimize the therapeutic efficacy of MSC. My work addresses this issue by comparing the anti-fibrotic potency of non-senescent (NS-) and senescent (S-) MSC-CM in vitro.*

7.3.5 Link Between Cellular Senescence and Fibrosis

Several human studies support a pathogenic link between senescence and fibrosis. In IPF, there is an increase in fibroblasts and epithelial cells senescence markers [e.g., p16, p21, and SA- β -gal] [69] and patients with IPF have improved physical function when senescent fibroblasts are eliminated by senolytics (i.e., Dasatinib plus Quercetin) or when fibroblast to myofibroblast differentiation and fibroblast migration is abrogated with rapamycin, a SASP inhibitor, in addition to pirfenidone [54] In addition, a subset of IPF patients have hTERT mutations [280]. Animal models corroborate the pathogenic link between senescence and fibrosis, showing that senescent fibroblasts evade immune clearance and apoptosis and accumulate over time impairing fibrosis resolution [2, 267, 268]; that components of the SASP from senescent fibroblasts are pro-fibrotic [69]; that senescent epithelial cells and fibroblasts induce myofibroblast differentiation exacerbating the fibrotic response [68, 71]; and that senolytic treatment, improves pulmonary function and enhances physical health [68, 71].

Knowing that MSC have established anti-fibrotic properties, that MSC undergo cellular senescence, and that senescence is implicated in fibrosis, we postulate that MSC senescence promotes fibrosis.

Hypothesis

Senescence impairs the anti-fibrotic properties of the adipose tissue (AT) derived MSC secretome.

Aims

1. To optimize *in vitro* assays to test the anti-fibrotic properties of AT-MSC.

2. To characterize non-senescent (NS-) and senescent (S-) MSC.

3. To compare the *in vitro* anti-fibrotic effects of NS- and S-MSC secretome.

8 Methods

8.1 Study Subjects

Adults undergoing elective orthopedic or cardiovascular surgery provided samples from subcutaneous adipose tissue. MSC were isolated from those samples. The demographic characteristics of the study donors are summarized in **Table 3.**

Table 3. Demographic characteristics of adipose tissue (AT)-MSC donors.

Demographic Table	
No. of subjects	6
Age (mean ± SD, years)	68.5 ± 7.2
Sex (Females/Males)	4/2
Comorbidities	
Hypertension	4
Hypercholesterolemia	1
Heart disease	6
Diabetes	-
Chronic inflammatory diseases	-
Cancer	-
Smoking	2

8.2 Human Adipose-derived Multipotent MSC

8.2.1 MSC Characterization

MSC were characterized according to the ISCT criteria (i.e., plastic adherence, surface markers, and tri-lineage differentiation). We used flow cytometry (BD LSR Fortessa™ cell analyzer) to determine MSC surface markers. 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 cyanine dye (PerCP-Cy5.5) conjugated anti-CD14 (562692). Data analysis was done with FlowJo software version 10.8.1. MSC samples with over 95% surface presence of CD73, CD90, and CD105 and less than 5% surface presence of CD14, CD20, CD34, CD45 and HLA-DR were used for subsequent experiments.

8.2.2 MSC Functional Assessment

We analyzed two functional MSC properties: proliferation 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:

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

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

To evaluate immunopotency, we assessed the capacity of NS- and S-MSC to inhibit activated-proliferating T cells. For the immunopotency assays (IPA), peripheral blood mononuclear cells (PBMCs) were isolated from a single donor (34-year-old non-smoking healthy female) with Lymphocyte Separation Medium by Ficoll density gradient centrifugation (Mediatech, Inc., Corning, Manassas, VA). For monocyte depletion, PBMCs were cultured overnight in Rosewell Park Memorial Institute medium (RPMI-1640, Wisent Inc., St. Bruno, QC) supplemented with 10% fetal bovine serum (FBS) (Wisent Inc) and 1% penicillin-streptomycin. To assess the effect of AT-MSC-CM on PBMCs proliferation, 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, Life Technologies, CA). Two million activated CFSE-stained PBMCs (100µl) were added to $2.5x10^4$ MSC in direct cell-cell contact conditions and cultured for 72 hours. The IPA measures the percentage of suppression of CD4+ T cell proliferation exerted by MSC. CFSE-stained, activated PBMCs cultured in a complete medium were used as negative controls (i.e., maximal proliferation), and CFSE-stained non-activated PBMCs as positive controls (lack of proliferation). Flow cytometry gating on CD4+ populations and estimating CFSE dilutions were used to assess proliferation. The Expansion Index (EI) of 7AAD-/CD4+ cells (viable CD4) was determined with FlowJo software. The immunopotency (i.e., the proportion of non-proliferating CD4+ T cells in the presence of MSC was calculated using the following formula:

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

8.2.3 Characterization of Replicative Senescent MSC

MSC were expanded *in vitro* and passed at 80% confluency until they reached RS. Senescent (S-) MSC were defined as those that fulfilled 3 out of the 5 criteria indicated in **Table 4**. (1) >20 days PDT [249], (2) >50% SA-β-gal [283], (3) SSC >100,000 a.u. [284], (4) autofluorescence >1000 GMFI [229], and (5) CD26 > 15,000 geometric mean fluorescence intensity (GMFI) [131]. In contrast, NS- MSC had: (1) population doubling time (PDT) <10 days, (2) <25% SA-β-gal, (3) SSC <50,000 a.u., (4) autofluorescence <500 GMFI, and (5) CD26 < 7,500 GMFI.

MSC were stained with SA-β-gal according to the manufacturer's protocol (Cell Signalling Technology, Whitby, ON). For quantification, MSC were counterstained with 0.3μM 4',6- diamidino-2- phenylindole (DAPI) for nuclei visualization. Bright-field and DAPI images were obtained. The percentage of S-MSC was calculated as the total number of positive SA-β-gal MSC divided by the total number of MSC counted using the ImageJ software (U.S. National Institute of Health, Bethesda, MD). Flow cytometry determined surface markers using the BD LSR Fortessa TM cell analyzer.

Table 4. Replicative senescent MSC criteria.

MSC	NS- MSC	S-MSC	Reference
Population Doubling time (days)	<10	>20	[249]
Beta-Gal (%)	<25	>50	[283]
SS-A (a.u.)	<50,000	>100,000	[284]
CD26 (gMFI)	<7,500	>15,000	[131]
Autofluorescence (gMFI)	<500	>1000	[229]

8.2.4 Replicative Non-senescent (NS-) and Senescent (S-) MSC-CM

MSC were seeded at a density of ∽5,000 cells/cm^2 density in T75 flasks with complete Dulbecco's modified eagle's medium (DMEM) [with 1.0g/L glucose, with L-glutamine, 10% FBS (Gibco MSC certified) and 1% penicillin-streptomycin] and incubated overnight. MSC were washed with phosphate-buffered saline (PBS) (Wisent Inc, St Bruno, QC) three times, and fresh medium was added (phenol red-free DMEM high glucose containing 1% penicillin-streptomycin and without FBS). Following 72 hours, CM was collected, centrifuged (4°C, 1000 rpm for 20 minutes) to remove cell debris, aliquoted, and stored at -80°C.

8.3 Fibroblasts

8.3.1 Source and Culture Conditions

Immortalized HCA2 human foreskin fibroblasts expressing the telomerase catalytic subunit (hTERT) were used to establish the *in vitro* assays to evaluate the anti-fibrotic effect of MSC-CM. Additionally, primary human foreskin fibroblasts were used for validation. Both cell lines were cultured in complete fibroblast culture media (complete media) consisting of High DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. To eliminate any potential effects of FBS proteins and growth factors that could modulate the response of fibroblasts through the activation of intracellular signal transduction pathways, we conducted experiments in serum-free conditions [285]. Early passage fibroblasts (passage <12) were utilized in this study.

8.3.2 Fibroblasts Activation (Characterization of Myofibroblasts)

Fibroblasts were seeded in 6-well cell culture plates at $5x10^3$ cells/cm^2 density in complete media. After overnight incubation (37°C, 5% CO2), media was replaced by DMEM with 1% penicillin/streptomycin, without FBS. Fibroblasts' activation was induced by adding 5ng/mL of TGF-β/well (R&D Systems, Minneapolis, MN) for 72 hours, the optimal concentration to activate fibroblasts [286]. Confirmation of activation was done by assessing α-SMA and procollagen I gene expression and protein levels, as well as evaluating acquired apoptosis resistance by flow cytometry.

To visualize stress fibers and collagen I, fibroblasts were seeded at a 4x10^^{3} cells/cm^2 density in 8-chamber slides in complete media and were incubated overnight. After 72 hours of TGF-β-induced fibroblasts' activation, the cells were fixed using a 4% paraformaldehyde (PFA) solution for 12 minutes. Following fixation, permeabilization was

carried out by treating the cells with 0.1% Triton-X in PBS for 10 minutes. To prevent non-specific binding, the samples were subjected to a 40-minute incubation with a blocking solution [22.52 mg/ml glycine in 0.1% Tween 20 in PBS supplemented with 5% fetal bovine serum (FBS)]. The fibroblasts were then incubated overnight at 4°C with the following primary antibodies: sheep anti-human Procollagen I antibody (AF6220, R&D systems) at a 1:750 dilution, and Phalloidin-iFluor 647 reagent (176759, Abcam, Boston, MA) at a 1:1000 dilution, which binds to fibrillar actin (F-actin) and highlights stress fibers. Alexa Fluor 488-conjugated donkey anti-sheep immunoglobulin G (Abcam) was employed for the secondary antibody staining at a 1:250 dilution. The fibroblasts' nuclei were stained with 0.3 μM DAPI. The visualization of α-SMA, collagen I, and stress fibers was achieved using a Zeiss LSM780 Laser Scanning Confocal Microscope.

8.4 *In Vitro* Assessment of NS- and S-MSC-CM Anti-fibrotic Effect.

8.4.1 MSC-CM Inhibition of TGF-β-induced Fibroblasts' Activation.

To evaluate the ability of MSC-CM to inhibit fibroblasts' activation, fibroblasts were seeded at a density of 5 x 10^3 cells/cm^2 in a 6-well plate with complete media. After overnight incubation (37°C, 5% CO2), the whole medium was washed with D-PBS and replaced by adding both TGF-β (5ng/ml, as described in Section 8.3.2) and CM from either NS- or S-MSC simultaneously for 72 h. Fibroblasts and myofibroblasts cultured in serum-free DMEM were utilized as negative and positive controls, respectively. Following 72 hours, proteins were collected, and α-SMA and procollagen I readouts were analyzed by western blot **(Figure 12).**

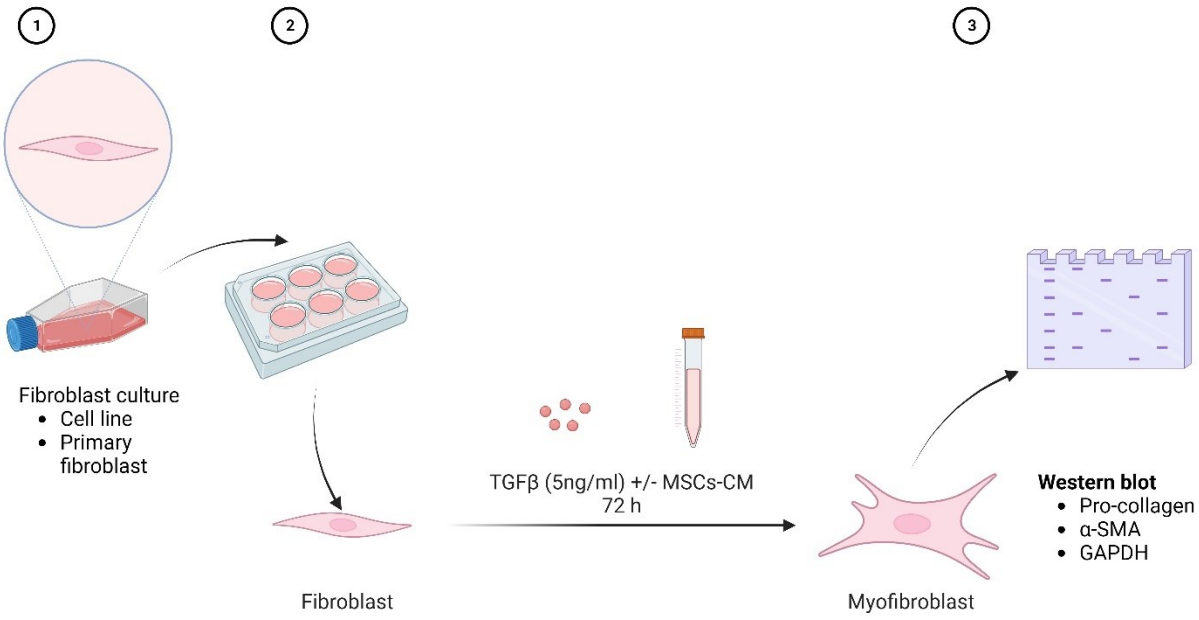


Figure 12. Inhibition of TGF-β induced fibroblasts' activation. Fibroblasts were seeded in a 6-well plate with complete media. After overnight incubation, the whole medium was washed with D-PBS and replaced by adding TGF-β (5ng/ml) and CM from either NS- or S-MSC simultaneously for 72 h. Following, proteins were collected and α-SMA and procollagen I readouts were analyzed by western blot.

Moreover, the expression of TGF-β-induced activation of the ACTA2 gene was analyzed in fibroblasts (activated or not with TGF-β) treated with either NS- or S-MSC-CM **(Table 5).** Fibroblasts not treated with MSC-CM served as a negative control. The RNA samples were protected using RNAase Protect Reagent (Qiagen, Germany). Subsequently, RNA purification was carried out with the RNeasy® Micro Kit (Qiagen). The RNA concentration was determined using a BioDrop µlite spectrophotometer (Harvard Bioscience, Holliston, MA). For reverse transcription (RT), 1 µg of purified RNA was employed, and the QuantiTect reverse transcription kit (Qiagen) was used per the manufacturer's instructions. Following RT, the resulting complementary deoxyribonucleic acid (cDNA) was mixed with RT^2 SYBR Green qPCR Master Mix (Qiagen). A total of 20

µl of this mixture was loaded into each well of a custom RT^2 Profiler PCR Array. Quantitative real-time PCR was conducted using the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific). The PCR protocol involved an initial activation step at 95°C for 10 minutes, followed by a two-step cycling process for 40 cycles (15 seconds at 95°C and 1 minute at 60°C), and concluded with a final melting curve analysis (95°C for 15 seconds and 60°C for 60 seconds). The fold change in mRNA expression was calculated using the ΔΔCt method, with GAPDH as the housekeeping gene.

Table 5. Primer sequences of ACTA2 gene induced by TGF-β.

Primer	Direction	Sequence 5' to 3'
α-SMA/ACTA 2	Forward	CTTTCTACAATGAGCTTCGTG
	Reverse	ATTTGAGTCATTTTCTCCCG
GAPDH	Forward	GAGTCAACGGATTTGGTCGT
	Reverse	TTGATTTTGGAGGGATCTCG

8.4.2 MSC-CM Induction of Myofibroblasts' Deactivation

To assess the potential of MSC-CM in modulating or reversing the myofibroblast phenotype, fibroblasts were seeded at 5 x 10^3 cells/cm^2 density in 6-well plates and treated with TGF-β (5ng/mL) for 72 hours to activate the fibroblasts to myofibroblasts' differentiation as described in Section 8.3.2. After activation, the complete medium was replaced with NS- or S-MSC-CM for 72 hours. Fibroblasts and myofibroblasts cultured in serum-free DMEM were utilized as negative and positive controls, respectively. The readouts of these experiments were α-SMA and procollagen I protein quantification **(Figure 13)**.

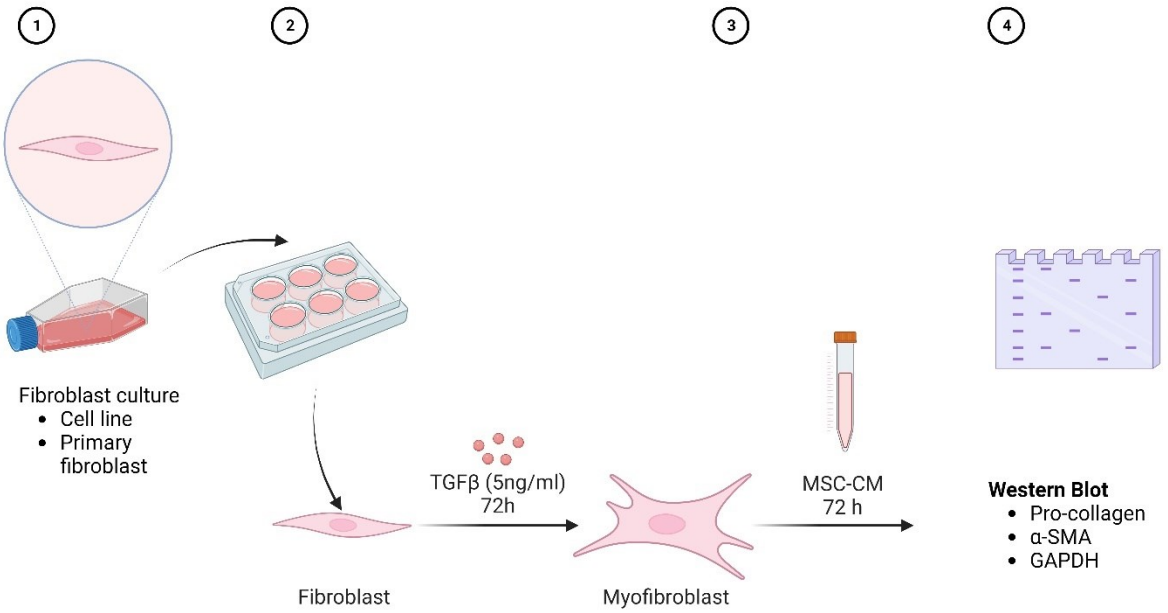


Figure 13. Induction of myofibroblasts' deactivation. Fibroblasts were seeded in a 6-well plate with complete media. After overnight incubation, the whole medium was washed with D-PBS and replaced by adding both TGF-β (5ng/ml) for 72h. After activation, the complete medium was replaced with NS- or S-MSC-CM for 72 hours. Following, proteins were collected and α-SMA and procollagen I readouts were analyzed by western blot.

8.4.3 MSC-CM Modulation of Myofibroblasts' Apoptosis Resistance

To evaluate the ability of MSC-CM to reverse the apoptosis resistance characteristic of myofibroblasts and rescue them from the staurosporine-induced apoptosis, fibroblasts were seeded at $1x10^4$ cells/cm^2 density in a 12-well plate and activated as described in Section 8.3.2. After activation, myofibroblasts were treated with MSC-CM or serum-free DMEM for 72 hours. During the last 24 hours, staurosporine (40nM, S6942 Sigma-Aldrich, St Louis, MO) was added to induce apoptosis. Finally, the supernatant was collected, myofibroblasts were trypsinized, and both were processed to

assess cell viability by flow cytometry **(Figure 14)**. The data were analyzed using FlowJo software 10.8.1.

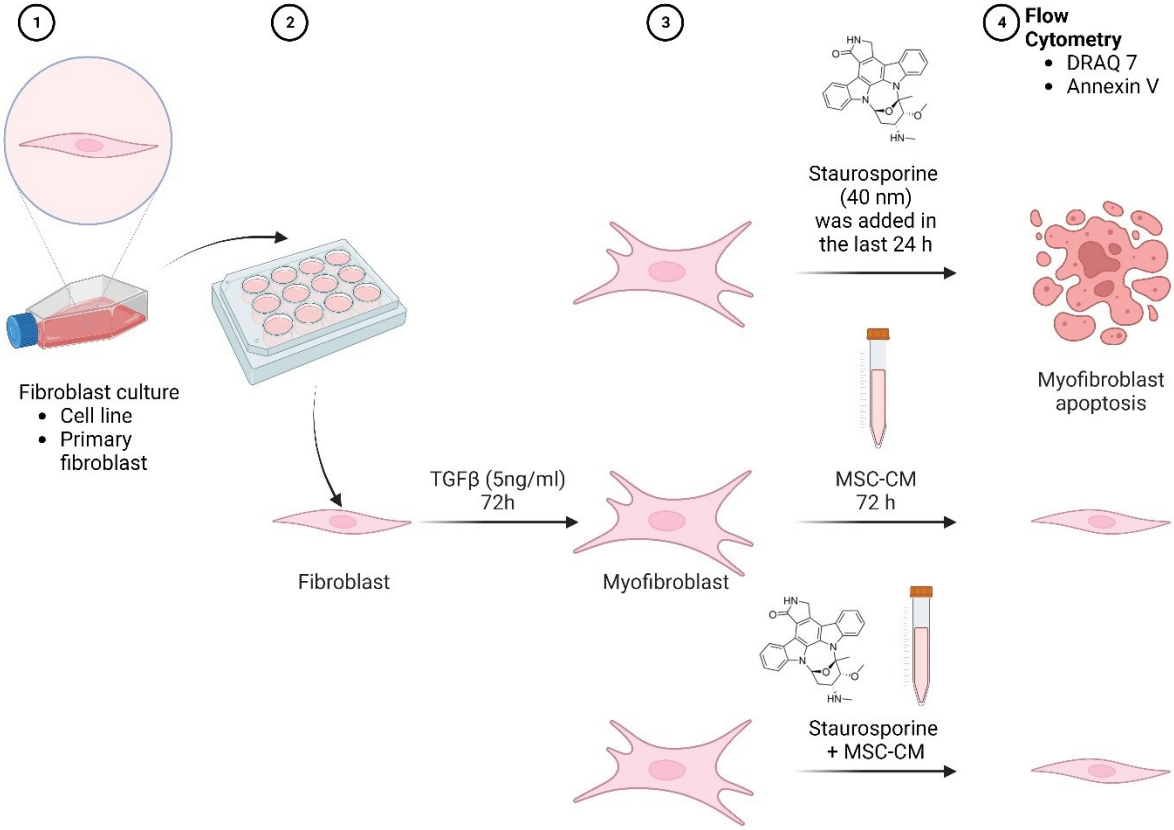


Figure 14. Modulation of myofibroblasts' apoptosis resistance. Fibroblasts were seeded in a 12-well plate with complete media. After overnight incubation, the whole medium was washed with D-PBS and replaced by adding both TGF-β (5ng/ml) for 72h. After activation, the complete medium was replaced with MSC-CM or serum-free DMEM for 72 hours. During the last 24 hours, staurosporine (40nM) was added to induce apoptosis. Finally, the supernatant was collected, myofibroblasts were trypsinized, and both were processed to assess cell viability by flow cytometry.

8.4.4 Effect of MSC-CM on Fibroblasts' Contraction

To assess the ability of MSC to contribute biomechanically to wound contraction and closure, we tested how MSC-CM contracted three-dimensional collagen microgels.

The effect of MSC-CM was compared to that of myofibroblasts. Using an aqueous two-phase droplet printing technique, we used an automated liquid handler to fabricate free-floating collagen microdroplets. The two aqueous phases, polyethylene glycol (PEG) and dextran, were prepared as follows. 35 kDa PEG (Sigma-Aldrich, 94646) at a concentration of 6% w/v in supplemented DMEM was sterile filtered through a 0.22 μm pore size sterile filter cup. 500 kDa dextran (www.dextran.ca) at a concentration of 20% w/v in sterile RO water was sterilized under UV light (45 min).

Briefly, Type I bovine collagen (Advanced Biomatrix, 3 mg/mL) was diluted to 1.5 mg/mL in PBS and 10× DMEM solution to obtain a final 1× DMEM concentration and kept on ice. The acidic solution was neutralized with 1M NaOH by titration based on the color of the phenolphthalein indicator in DMEM. Fibroblasts were passaged at 80% confluency, centrifuged (4°C, 1000 rpm for 20 minutes), and re-suspended at a final concentration of 2.7 x 10^6 cells/mL in collagen and supplemented DMEM containing 15% v/v dextran solution. 100 μL of PEG solution was robotically dispensed into each well of a round bottom 96-well plate by an automated liquid handler (Pipetmax, Gilson, Middleton, Wisconsin). 2 μL of cell-laden dextran-collagen solution was robotically dispensed into 96 well plates. The plate was incubated (37 °C, 45 min) for collagen gelation. After gelation, PEG-rich DMEM was removed and replaced by adding 100 μL of both TGF-β (5ng/ml) and CM from either NS- or S-MSC simultaneously and incubated (37 °C, 5% CO2) over one day to assess contraction **(Figure 15).** TGF-β (5ng/ml) was evaluated alone as a positive control, and negative control media without FBS was used.

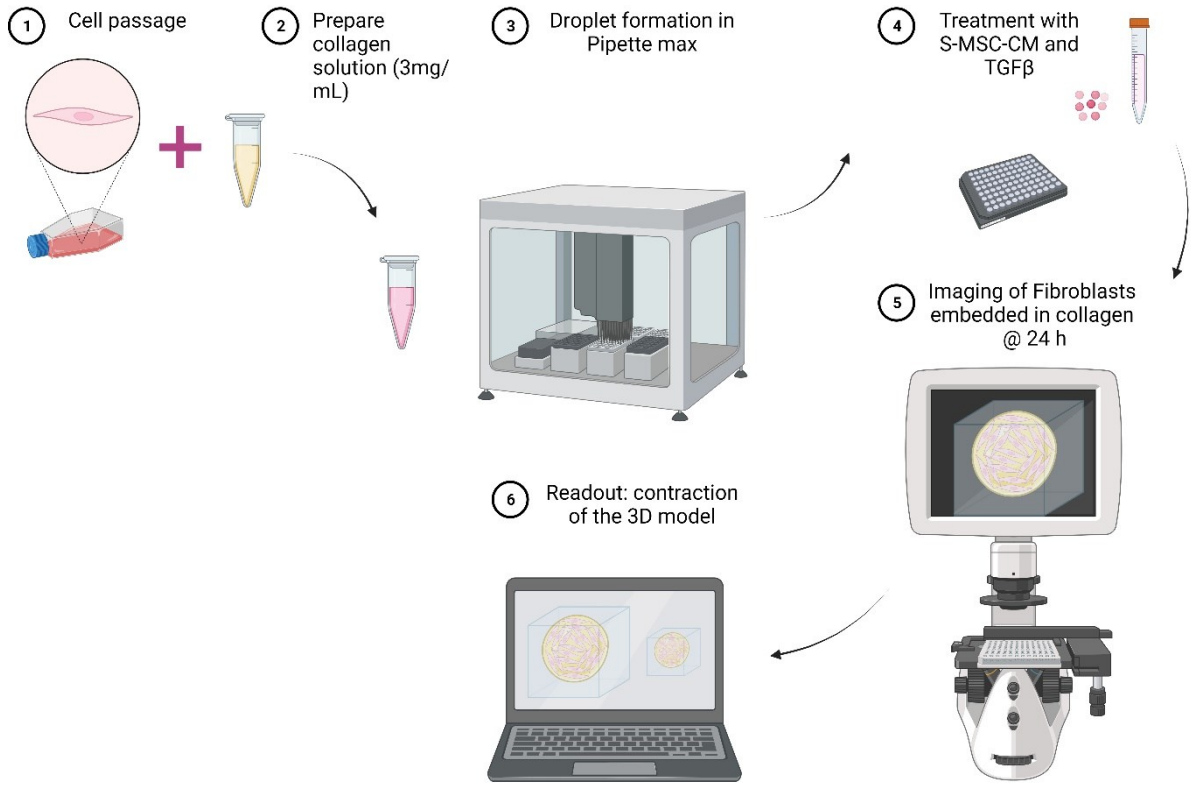


Figure 15. Fibroblasts' contraction assay. Fibroblasts were re-suspended at a final concentration of 2.7 x 10^6 cells/mL in collagen and supplemented with DMEM containing 15% v/v dextran solution. PEG solution was robotically dispensed into each well of a round bottom 96-well plate by an automated liquid handler (Pipetmax). 2 µL of cell-laden dextran-collagen solution was robotically dispensed into 96 well plates. After gelation, PEG-rich DMEM was removed and replaced by adding 100 µL of TGF-β (5ng/ml) and CM from either NS- or S-MSC simultaneously and incubated over one day to assess contraction.

8.5 Readouts of *In Vitro* Anti-fibrotic Assays

8.5.1 Procollagen I and α-SMA by western blot.

To conduct western blot analysis for Procollagen I and α-SMA, cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (89900, Thermo Fisher Scientific, Rockford, USA) and 1X Protein Arrest (KP14001-2 EMD Millipore Corp,

Oakville, ON), and total protein concentration was measured using a bicinchoninic acid assay kit (BCA, Thermo Fisher Scientific). Ten µg of cell lysates were then loaded into a Mini-protean TGXTM precast gel 4-15% SDS PAGE (BioRad, USA), transferred onto a PVDF membrane, and incubated with either a rabbit anti-human α-SMA antibody (ab5694, Abcam, Boston, MA) (1:3000) or a sheep anti-human Procollagen I antibody (AF6220, R&D systems, USA) (1:3000). The secondary antibodies used were HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch labs, West Grove, PA) (1:3000) 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 RulerTM Plus Prestained Protein Ladder (26619 Thermo Fisher Scientific) was used as a molecular weight standard. The immunoreactive proteins were visualized with Clarity Western ECL Substrate (BioRad) and an Omega LumTM C Imaging System (Aplegen®, San Francisco, CA) and analyzed using ImageJ software.

8.5.2 Cell Viability

Fibroblasts, myofibroblasts, MSC and supernatants were collected and centrifuged (4°C, 1000 rpm for 10 min). The resulting cell pellet was washed, re-suspended in Annexin V Binding Buffer 1X (BD Biosciences) containing PE-conjugated Annexin V (AB_286907, BD Biosciences) and DRAQ7 (a far-red fluorescent DNA dye, Abcam UK), and incubated in the dark (25°C, 15 minutes). Viability was evaluated by flow cytometry in a BD LSRFortessa and analyzed with FlowJo software version 10.8.1.

8.5.3 Microgels Contraction

Live collagen microgels were imaged at selected time points using the EVOS FL and a 4X objective. The collagen droplet area was measured by ImageJ (NIH). The change in droplet size (% areal contraction) was calculated as follows:

$$\%A\ real\ contraction\ = \frac{Af}{Ao} * 100$$

9 Results

9.1 Characterization of Replicative Senescent MSC-CM and Senescence-Associated Secretory Phenotype (SASP).

Before assessing senescence in MSC, we confirmed that MSC fulfilled the ISCT minimal definition criteria **(Figure 16)**. There is currently no single biomarker to identify senescent cells [287]; thus, testing for several biomarkers is recommended [288]. S-MSC are larger and more granular, with a higher percentage of SA-β-gal positive cells, doubling time, and autofluorescence, and have a higher density of surface CD26 and increased expression of p16 than NS-MSC **(Figure 17)**. Characterizing S-MSC (n=6 per group for each sample from a different MSC donor) was a pre-requisite to collecting MSC-CM to conduct all subsequent experiments.

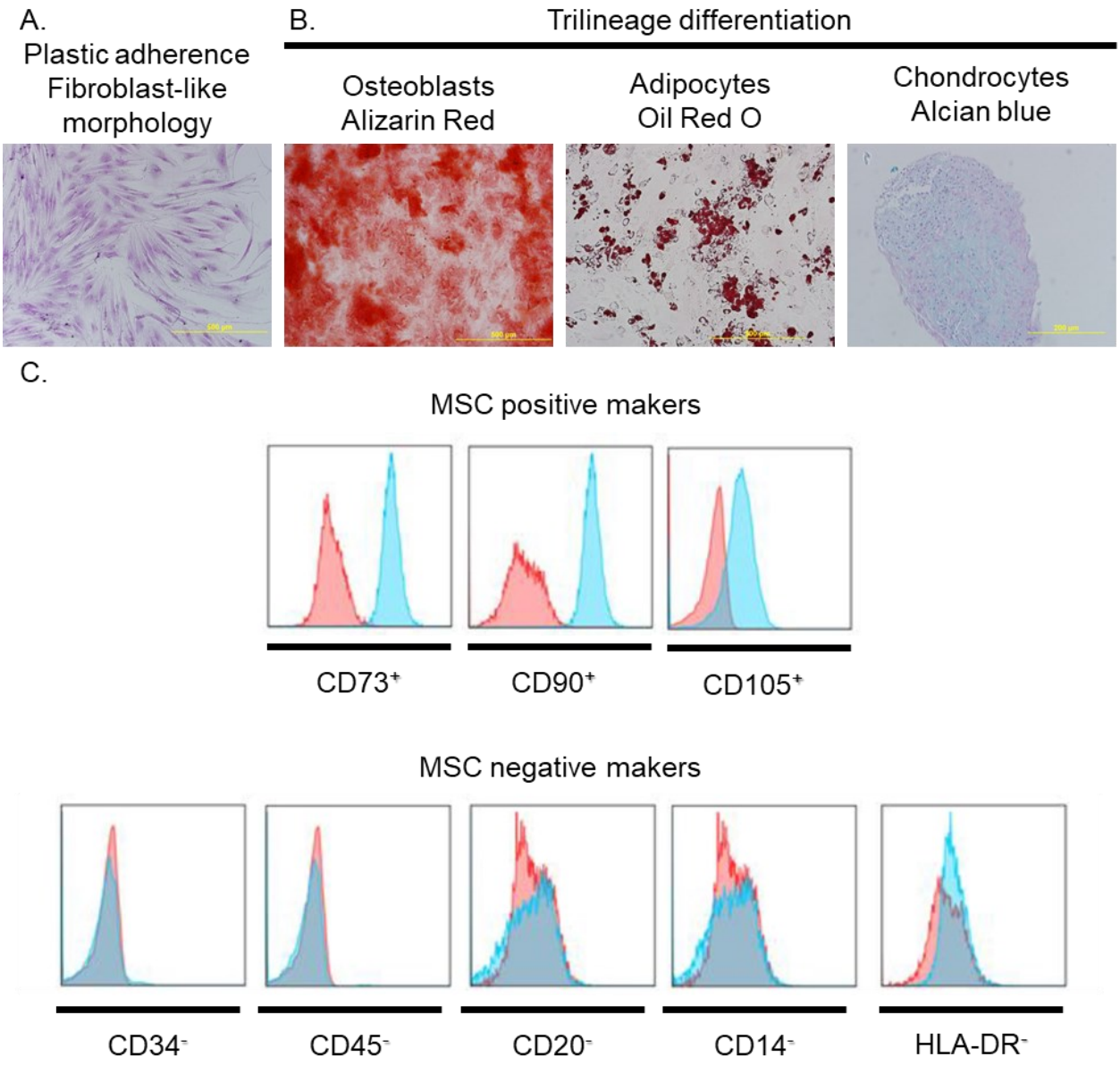


Figure 16. Characterization of human adipose tissue-derived MSC. A. MSC were adherent to plastic and had spindle-shaped morphology in standard culture conditions; **B.** MSC differentiated into osteoblasts, adipocytes and chondroblasts when provided appropriate culture conditions; and **C**. MSC were positive for the following surface markers: CD73, CD90 and CD105, and negative for CD34, CD45, CD20, CD14 and HLA-DR.

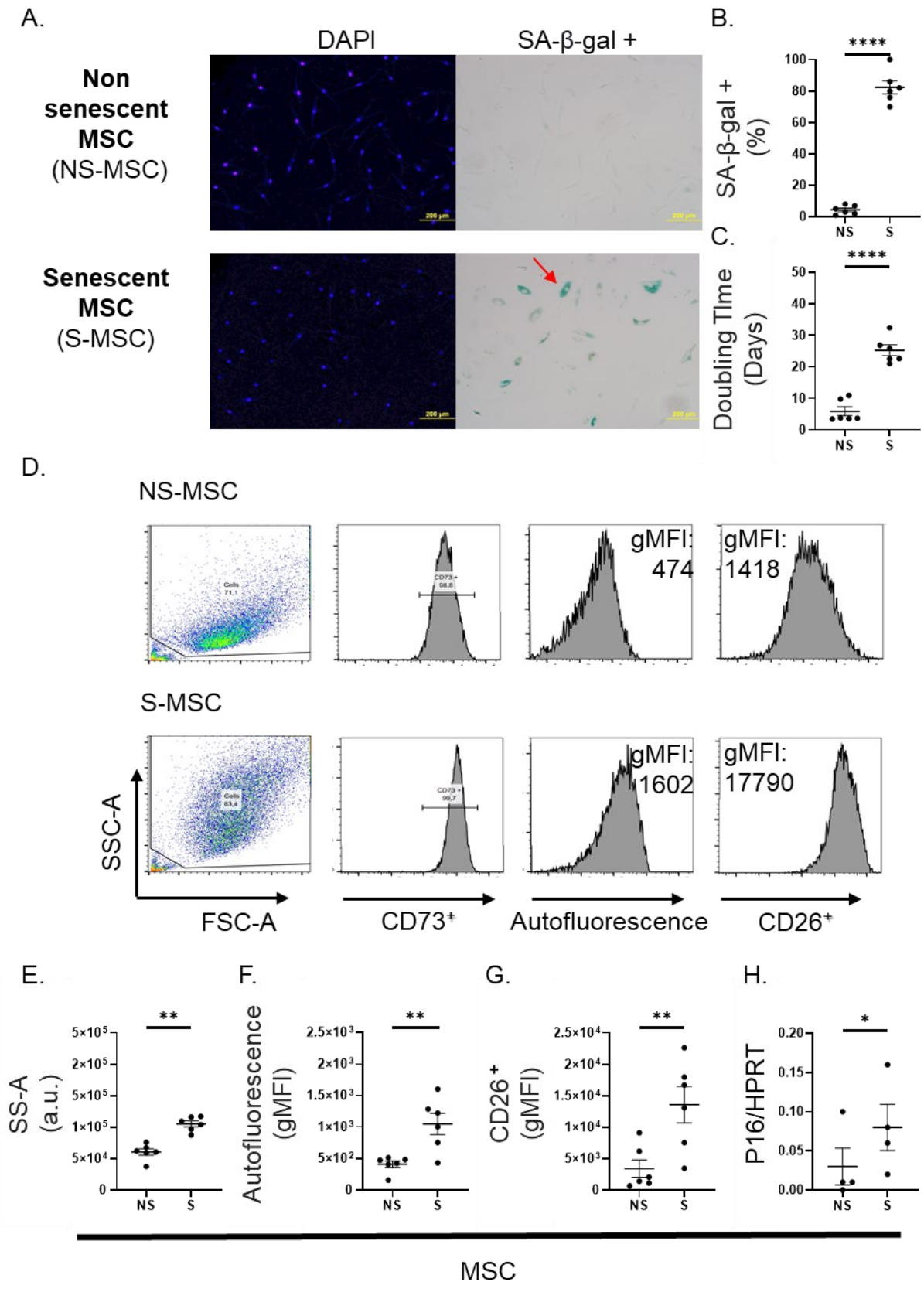

A.
DAPI
SA-β-gal +
Non senescent MSC (NS-MSC)
Senescent MSC (S-MSC)
B.
SA-β-gal + (%)
****
NS
S
C.
Doubling TIme (Days)
****
D.
NS-MSC
gMFI: 474
gMFI: 1418
S-MSC
gMFI: 1602
gMFI: 17790
SSC-A
FSC-A
CD73+
Autofluorescence
CD26+
E.
SS-A (a.u.)
**
F.
Autofluorescence (gMFI)
**
G.
CD26+ (gMFI)
**
H.
P16/HPRT
*
MSC

Figure 17. Characterization of senescent MSC. MSC increased in senescence markers following replicative senescence: **A.** Representative example of MSC positive for SA-β-gal, **B.** Percentage SA-β-gal (NS- vs. S- MSC-CM, n=6, **** p<0.0001), **C.** Doubling Time (NS- vs. S- MSC-CM, n=6, **** p<0.0001), **D.** Representative example of MSC gating strategy positive for the following surface markers: CD73, side scatter (SSC-A), CD26, and autofluorescence, **E.** SSC-A (NS- vs. S- MSC-CM, n=6, ** p<0.01), **F.** Autofluorescence (NS- vs. S- MSC-CM, n=6, ** p<0.01), **G.** CD26 (NS- vs. S- MSC-CM, n=6, ** p<0.01), and **H.** p16 (NS- vs. S- MSC-CM, n=4, * p<0.05). Abbreviations: gMFI, geometric mean fluorescence intensity; a.u., arbitrary units; HPRT, Hypoxanthine-guanine phosphoribosyltransferase.

9.2 Senescence Reduces the Immunopotency (IPA) of MSC

Before assessing the anti-fibrotic effect of S-MSC, we compared the immunopotency of NS- and S-MSC. As previously described, S-MSC have a reduced ability to suppress T-cell proliferation than NS-MSC (MSC: PBMCs ratio 1:8) (**Figure 18**).

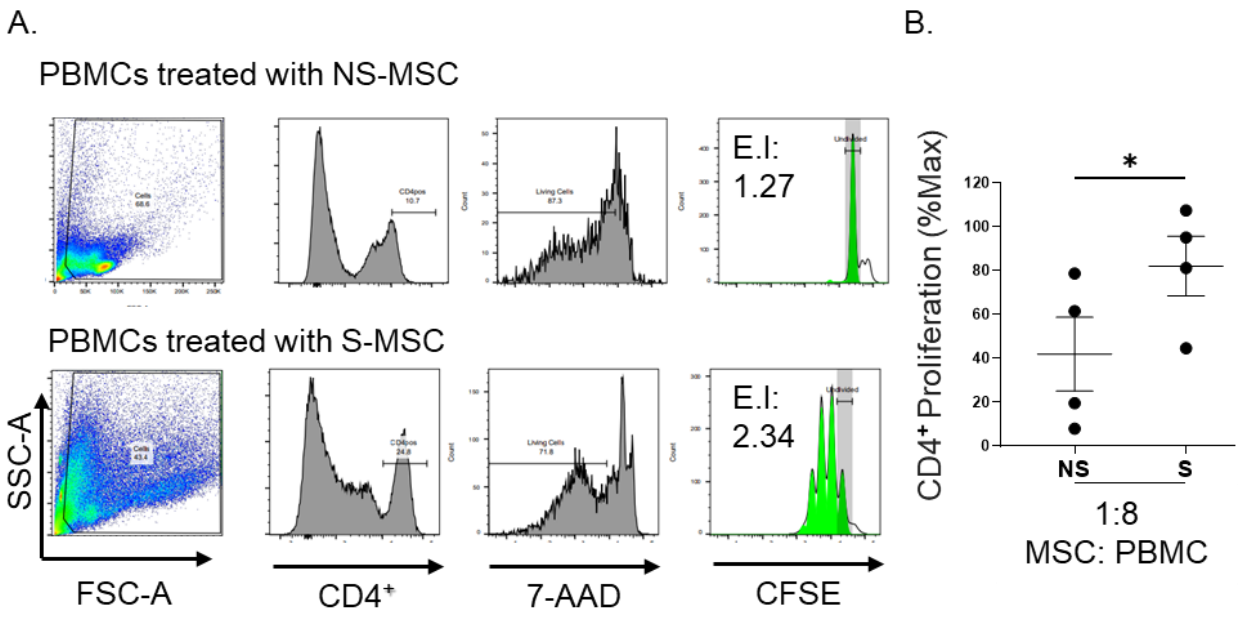
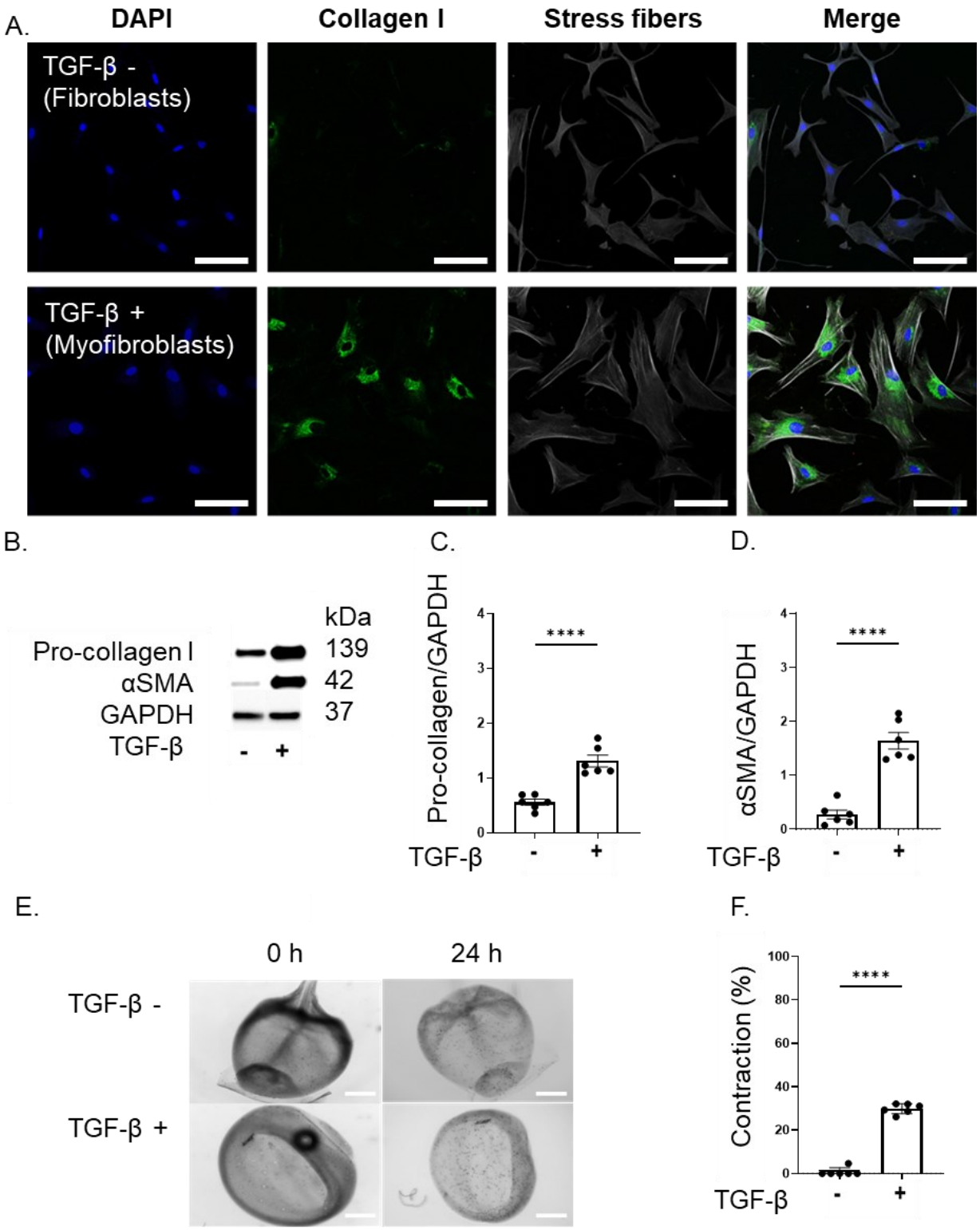


Figure 18. Senescence impairs the immunosuppressive properties of MSC. The MSC immunopotency assay (IPA) assessed the capacity of MSC to inhibit activated $CD4^+$ T-cell proliferation. PBMCs were co-cultured with NS- or S-MSC. **A)** Representative example of MSC IPA gating strategy and expansion index (E.I), and **(B)** summary graph of IPA in cell-cell contact-dependent and MSC: PBMC ratio 1:8 *p<.05. Abbreviations: PBMCs, peripheral blood mononuclear cells; 7-AAD,7-Aminoactinomycin D.

9.3 TGF-β Activates Fibroblasts into Myofibroblasts.

TGF-β orchestrates the differentiation of fibroblasts into myofibroblasts, stimulating the production of ECM proteins [16]. We induced fibroblast activation with TGF-β to mimic fibrogenic conditions. Upon TGF-β stimulation, fibroblasts lose their spindle-shaped morphology, becoming cells with enlarged cytoplasm (i.e., myofibroblasts morphology) **(Figure 19 A).** One of the earliest responses triggered by TGF-β signaling is the reorganization of the actin cytoskeleton [289]. We confirmed a greater abundance of actin fibers following TGF-β treatment than fibroblasts **(Figure 19 A).** Notably, the expression of α-SMA within stress fibers is the most widely accepted molecular marker for identifying myofibroblasts [290], the primary contributors to the synthesis of collagen I in the extracellular matrix **(Figure 19 A)**. To confirm the myofibroblasts' phenotype, we measured α-SMA and pro-collagen I protein content in cell lysates **(Figure 19 B-D).** Following TGF-β activation, fibroblasts increased in α-SMA and pro-collagen I protein levels **(Figure 19 B-D)** and enhanced associated-contractile force **(Figure 19 E, F)** compared to baseline**.** Moreover, TGF-β confers myofibroblasts' apoptosis resistance, another feature associated with the perpetuation of fibrosis [291]. We tested this by treating fibroblasts and myofibroblasts with staurosporine, a well-established apoptosis inducer [292]. Accordingly, myofibroblasts are more resistant to staurosporine-apoptosis induction than fibroblasts **(Figure 19 G, H).** Altogether, these findings validate the

phenotype of fibroblasts and myofibroblasts, which was crucial for subsequent *in vitro* assays.



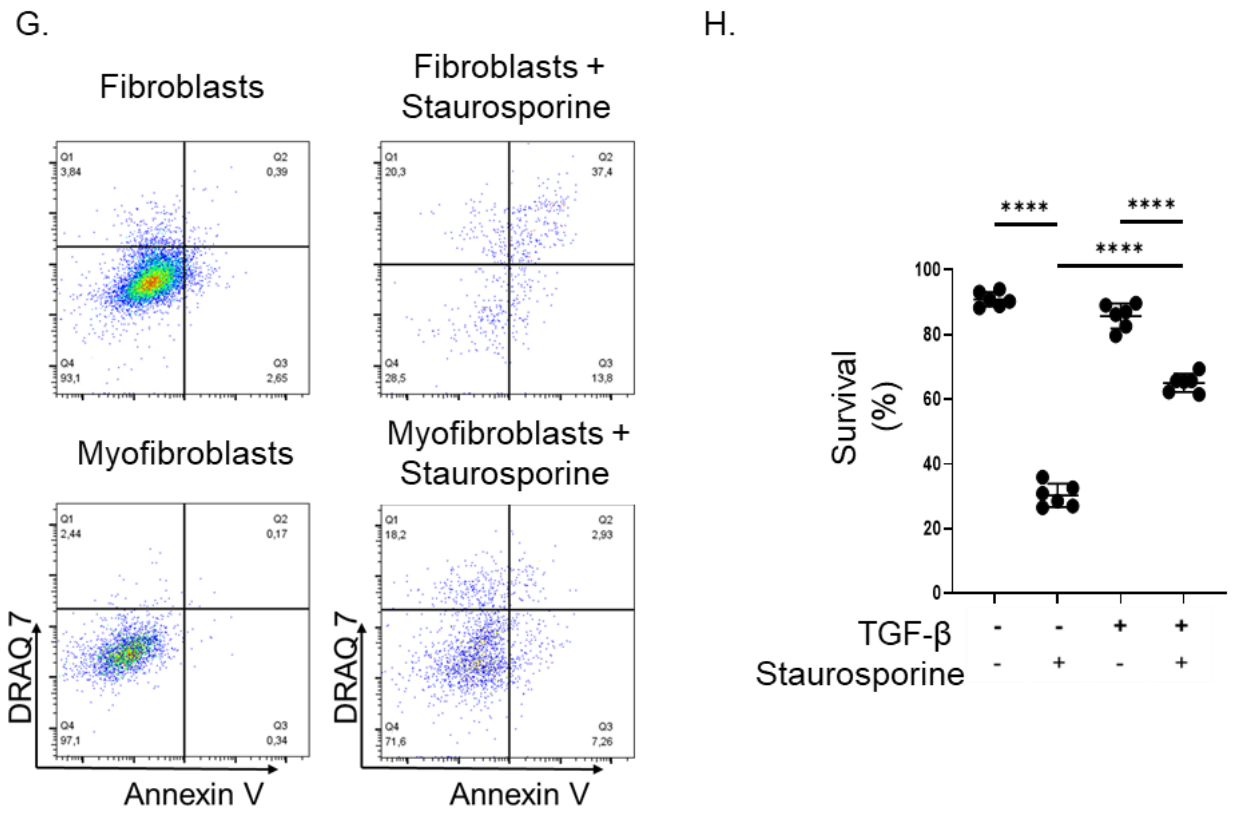


Figure 19. TGF-β activates fibroblasts into myofibroblasts. Characterization of fibroblasts (TGF-β-) and myofibroblasts (TGF-β+): **A.** Representative example of staining of collagen I and stress fibers by immunofluorescence (Scale: 100 μm), **B.** Representative example of pro-collagen I and α-SMA by western blot, summary data of **C.** pro-collagen I and **D.** α-SMA protein levels normalized to GAPDH (fibroblasts vs. myofibroblasts, n=6; pro-collagen I, **** p<0.0001; α-SMA, **** p<0.0001). **E.** Representative example of myofibroblasts' contraction, and **F.** Summary data of myofibroblasts' contraction (fibroblasts vs. myofibroblasts, n=6; contraction, **** p<0.0001). **G.** Representative example of the gating strategy of myofibroblasts staurosporine-induced apoptosis and **H.** Fibroblasts vs myofibroblasts survival (staurosporine dose 40 nm, **** p<0.0001).

9.4 S-MSC-CM Inhibits TGF-β-induced Fibroblasts' Activation.

The persistence of activated fibroblasts is considered the result of constitutive TGF-β signaling [3]. We evaluated if S-MSC-CM promotes the TGF-β activation of immortalized HCA2 human foreskin fibroblasts (hTERT). Contrary to our expectations, treatment of TGF-β activated immortalized human fibroblasts with S-MSC-CM reduced the production of pro-collagen and αSMA to a greater extent than NS-MSC-CM **(Figure 20).** NS- and S-MSC-CM did not differ in their anti-fibrotic properties, which were maintained even when low volumes of CM were used (i.e., high CM dilution). Undiluted CM and a 50% dilution were used in subsequent experiments, as they had maximal effects and lower variability of results **(Figure 21)**. These experiments were repeated using primary fibroblasts, confirming that S-MSC-CM was as effective at inhibiting TGF-β induced fibroblast activation as NS-MSC-CM. Further, S-MSC-CM was more potent at decreasing αSMA than NS-MSC-CM **(Figure 22).** In addition, we investigated the anti-fibrotic effect of the S-MSC-CM compared to pirfenidone, an approved anti-fibrotic agent for IPF. Based on a dose-response curve, we tested one mM pirfenidone, which had the maximal effect in reducing αSMA protein level. The effect of S-MSC-CM in reducing ACTA2 expression was comparable to that of pirfenidone. These results suggest that MSC senescence does not impair the *in vitro* ability of MSC-CM to inhibit fibroblasts to myofibroblasts' activation.

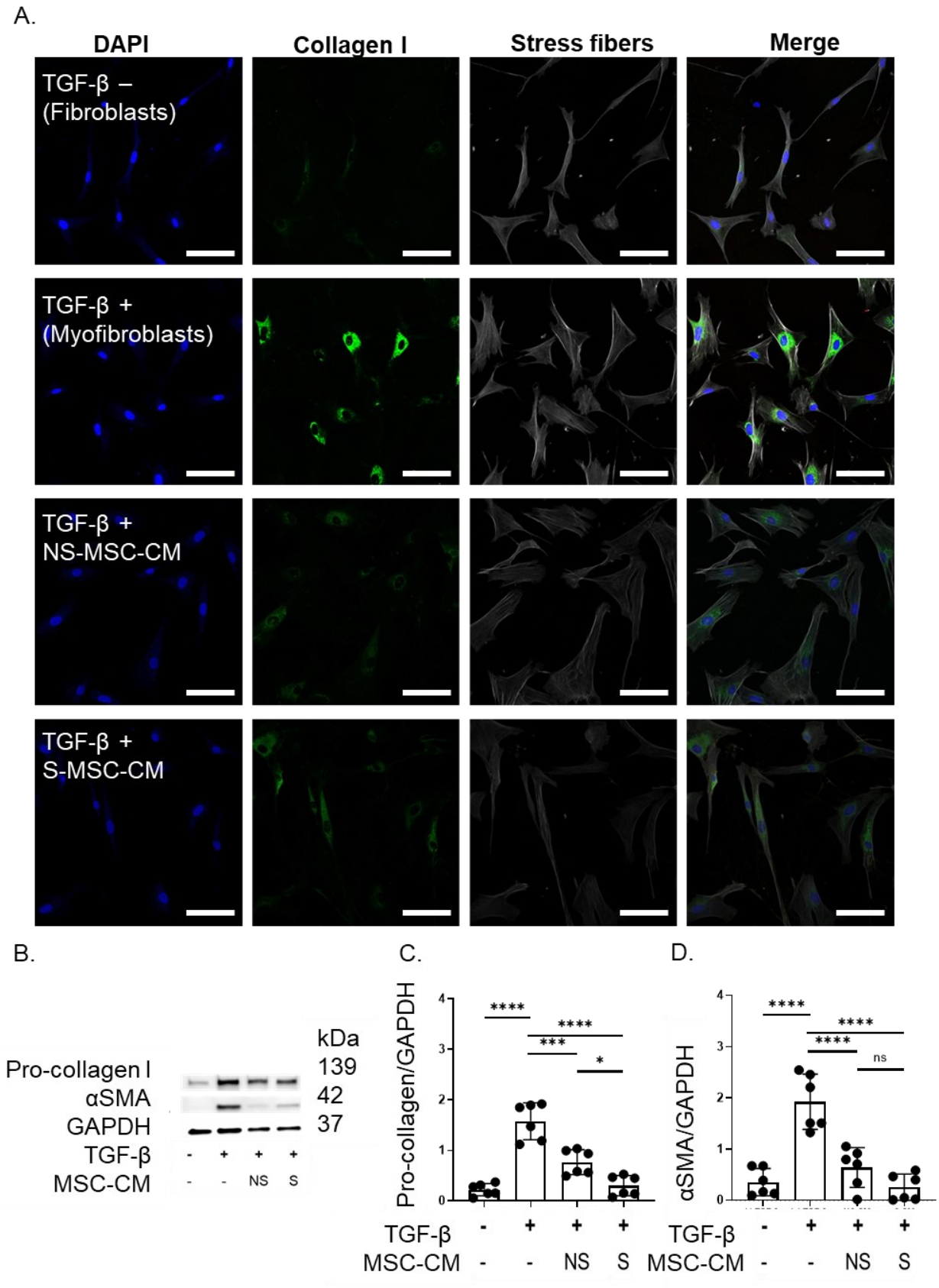
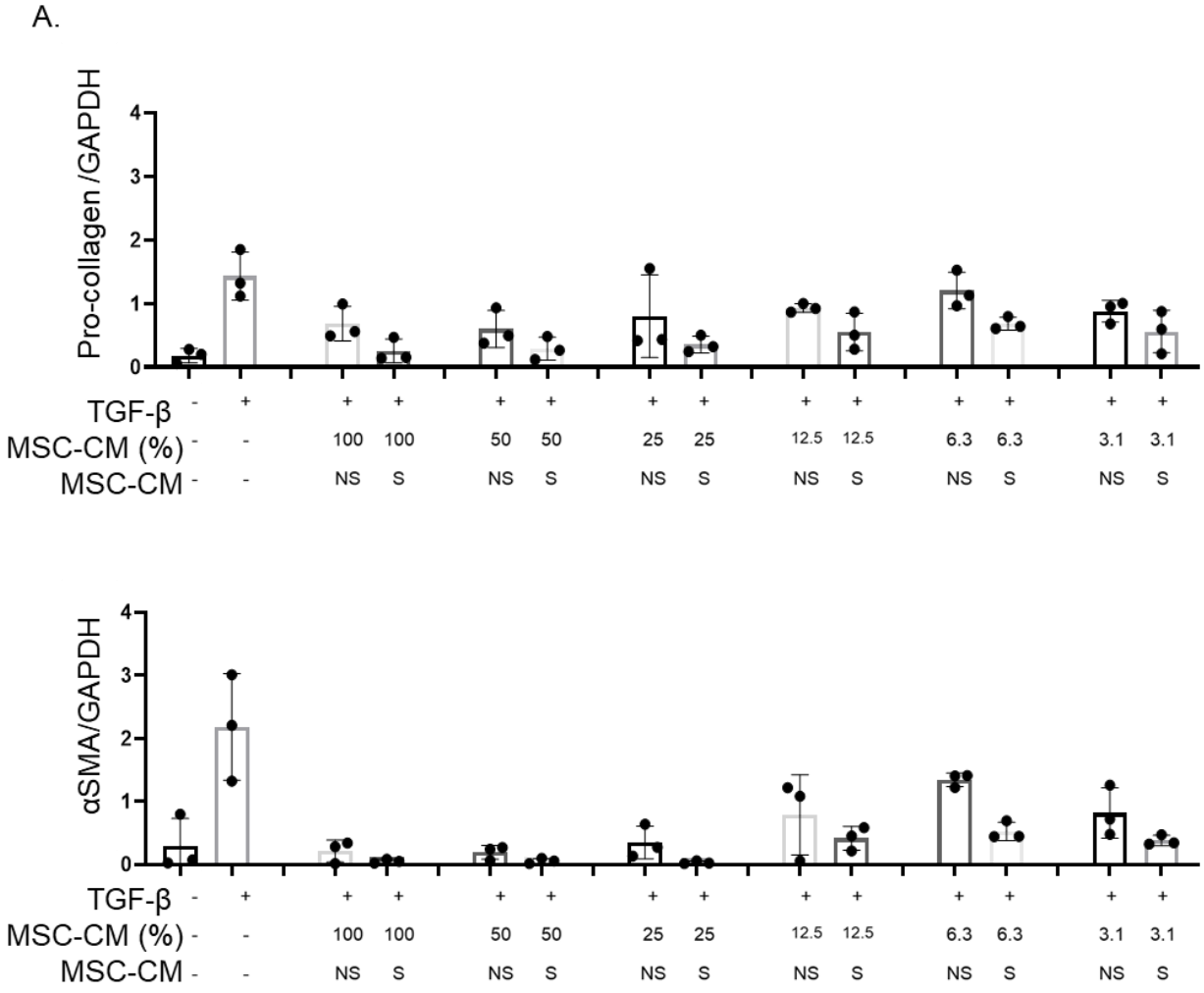

A.
DAPI
Collagen I
Stress fibers
Merge
TGF-β –
(Fibroblasts)
TGF-β +
(Myofibroblasts)
TGF-β +
NS-MSC-CM
TGF-β +
S-MSC-CM
B.
kDa
Pro-collagen I
αSMA
GAPDH
139
42
37
TGF-β
MSC-CM
NS
S
C.
Pro-collagen/GAPDH
****
***
*
D.
αSMA/GAPDH
ns

Figure 20. S-MSC-CM prevents TGF-β induced fibroblasts' activation in immortalized HCA2 human foreskin fibroblasts (hTERT). A. Representative immunofluorescence images depicting collagen I and stress fiber patterns in three conditions: non-activated fibroblasts, fibroblasts activated with TGF-β, and fibroblasts co-treated with TGF-β and MSC-CM. **B.** Representative example of pro-collagen I and α-SMA by western blot. Summary data of **C.** pro-collagen I and **D.** α-SMA protein levels from inhibition assays. The levels of pro-collagen I were lower in myofibroblasts treated with S-MSC-CM than those treated with NS-MSC-CM. **C.** Pro-collagen I: S-MSC-CM vs NS-MSC-CM, n=6, *p=0.02, **D.** α-SMA: S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.33, normalized to GAPDH.



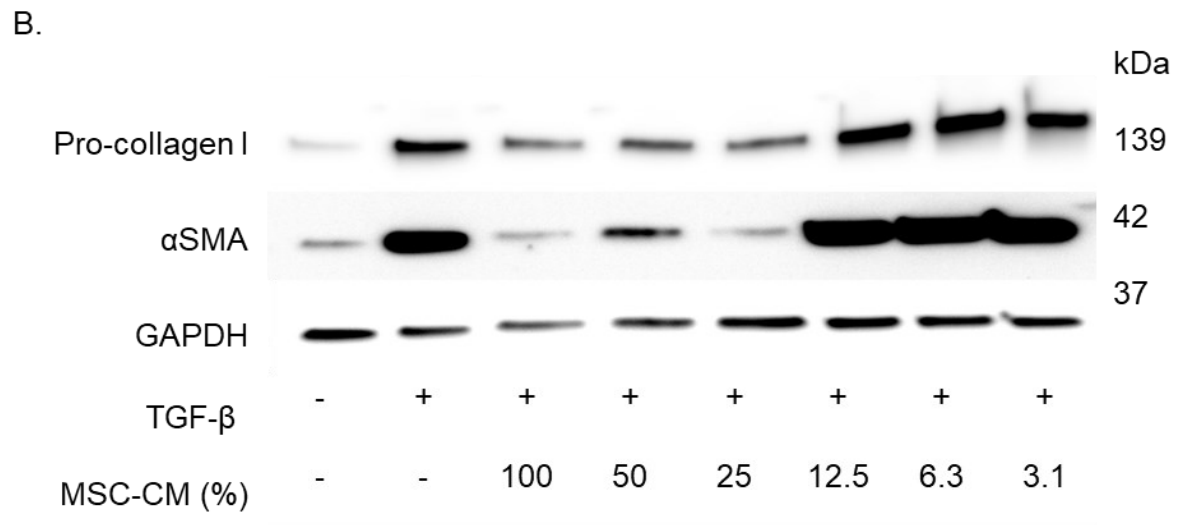
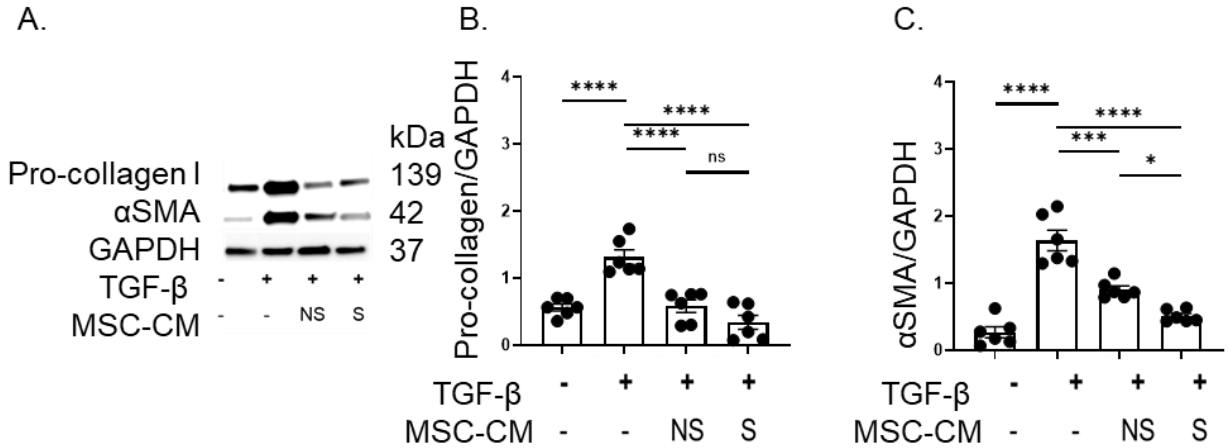


Figure 21. S-MSC-CM prevents TGF-β induced fibroblasts' activation in HCA2 human fibroblasts (hTERT). Non-activated fibroblasts, TGF-β activated fibroblasts, and fibroblasts were co-exposed to TGF-β and MSC-CM at different dilutions. **A.** Summary data of pro-collagen I and α-SMA protein levels from inhibition assays. In this assay, the reduction of pro-collagen by S-MSC-CM was consistently higher than NS-MSC-CM at all concentrations tested. The effect of MSC-CM on α-SMA dose-response relationship in the prevention assay suggests a causal relationship between the exposure (i.e., MSC-CM) and the outcome (i.e., fibroblast modulation). **B.** Representative example of pro-collagen I and α-SMA by western blot.



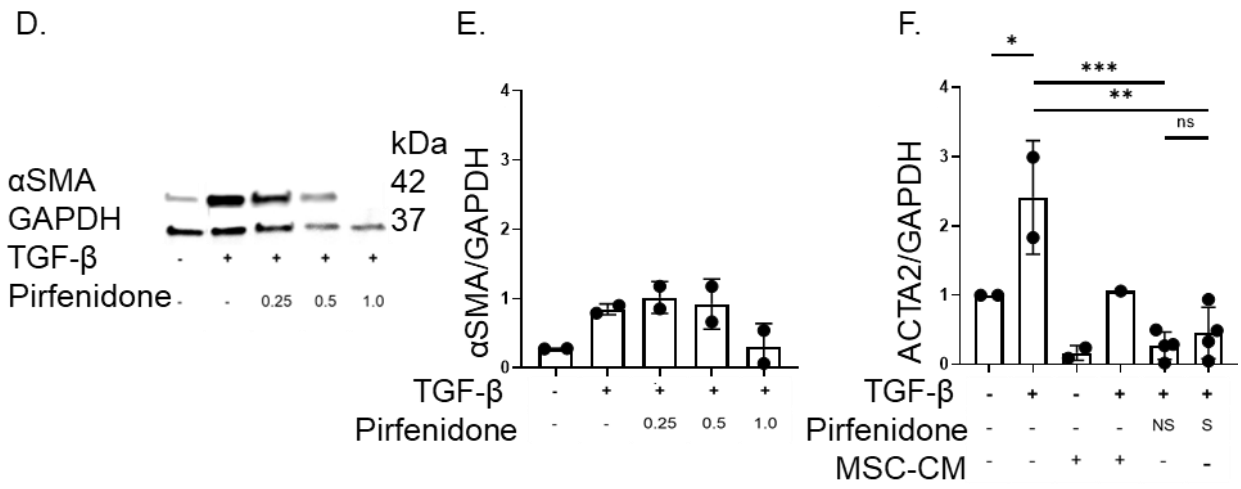


Figure 22. S-MSC-CM prevents TGF-β induced fibroblasts' activation in primary human fibroblasts. Fibroblasts were treated with TGF-β or co-treated with TGF-β and MSC-CM. **A.** Representative example of pro-collagen I and α-SMA by western blot and summary data of **B.** pro-collagen I and **C.** α-SMA protein levels from inhibition assays. The levels of those proteins were reduced in myofibroblasts treated with S-MSC-CM, indicating a more potent reduction in α-SMA compared to NS-MSC-CM. **B.** Pro-collagen I: S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.28, **C.** α-SMA: S-MSC-CM vs NS-MSC-CM, n=6, *p=0.03, normalized to GAPDH. Fibroblasts received TGF-β treatment, while another group of fibroblasts were subjected to co-treatment with TGF-β and Pirfenidone. **D.** Representative example of α-SMA by western blot and **E.** Summary data of α-SMA protein levels from inhibition assays. The levels of α-SMA protein were reduced in myofibroblasts treated with one mM Pirfenidone; thus, further, this concentration was used as an internal control for qRT-PCR experiments. **F.** Preliminary data suggesting that S-MSC-CM may reduce the expression of TGF-β up-regulated ACTA2 profibrotic gene (myofibroblasts vs. myofibroblast treated with S-MSC-CM, n=4, ** p<0.01; S-MSC-CM vs NS-MSC-CM treatment, n=4, ns=p=0.98, normalized to GAPDH).

9.5 S-MSC-CM Deactivates Myofibroblasts

The deactivation of myofibroblasts is essential for fibrosis reversal [1]. We tested the capacity of the S-MSC-CM to impair the myofibroblasts' deactivation using as a

readout the reduction of α-SMA and pro-collagen I in a h-TERT transfected and primary fibroblasts **(Figure 23, 25).** Cell death did not confound this effect, as myofibroblast viability before protein quantification was similar in myofibroblasts treated or not with MSC-CM **(Figure 23 D, E).** To sensitize this assay, we tested serial dilutions of MSC-CM. NS- and S-MSC-CM maintained their anti-fibrotic effects even at lower concentrations **(Figure 24).** In summary, these data support that senescence does not impact the ability of MSC-CM to deactivate myofibroblasts, an outcome of most significance for fibrosis resolution.

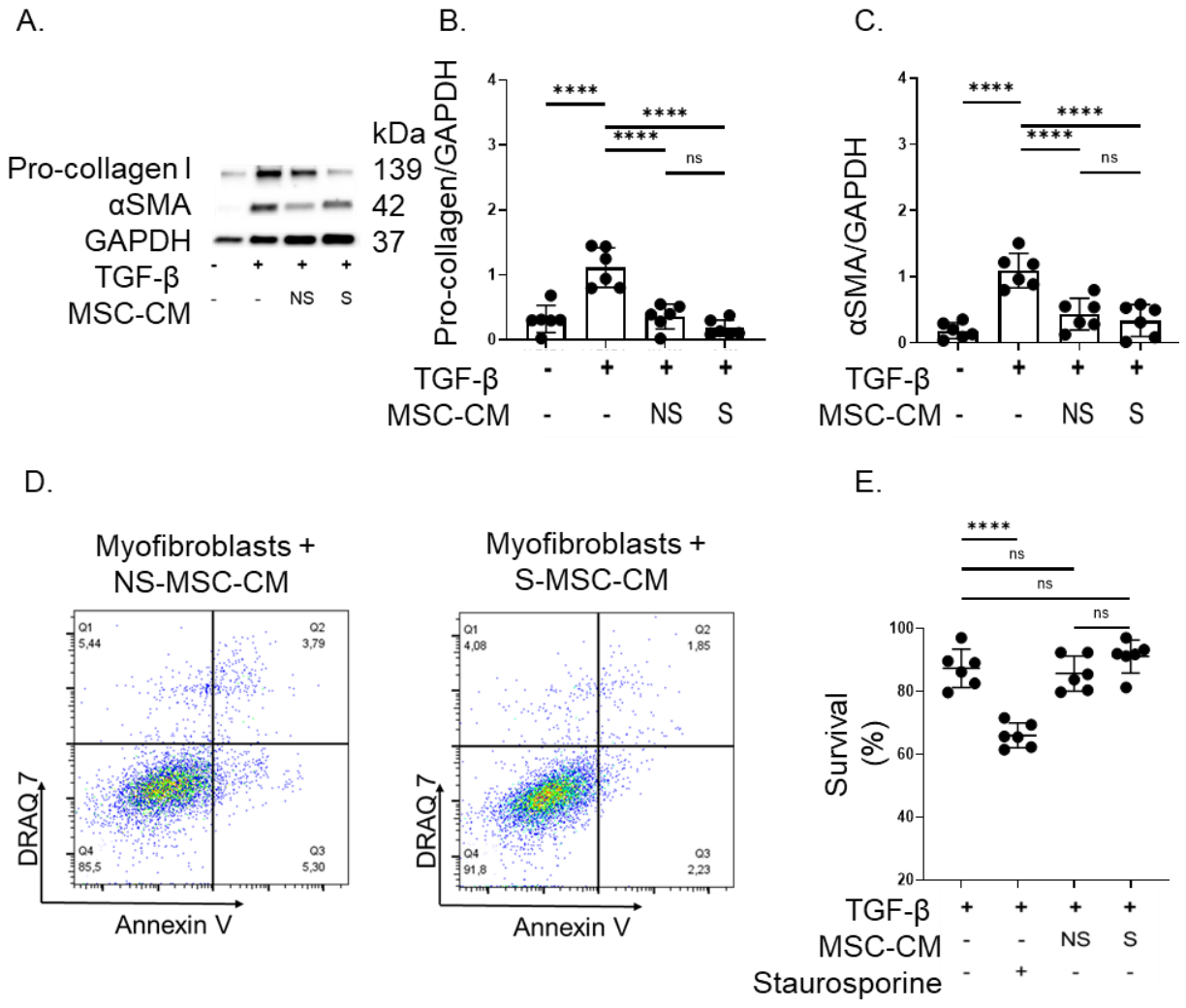
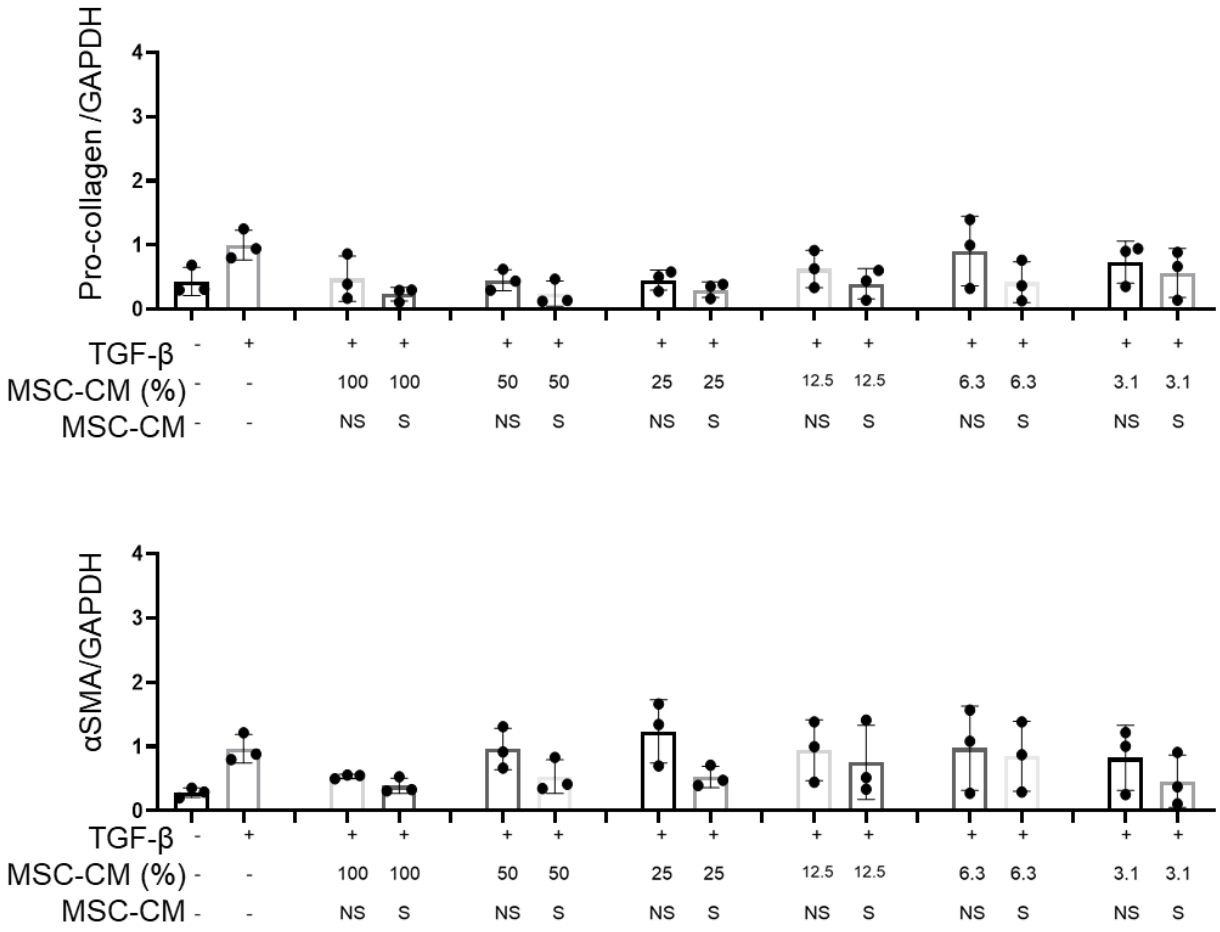


Figure 23. S-MSC-CM promotes myofibroblasts' deactivation in immortalized HCA2 human foreskin fibroblasts (hTERT). After activating TGF-β fibroblasts, myofibroblasts were treated with MSC-CM. A. Representative example of pro-collagen I and α-SMA by western blot—summary data of **B.** pro-collagen I and **C.** α-SMA protein levels from deactivation assays. The levels of those proteins were reduced in myofibroblasts treated with S-MSC-CM; the anti-fibrotic effect was similar to NS-MSC-CM. **B.** Pro-collagen I: S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.57, **C.** α-SMA: S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.87, normalized to GAPDH. **E.** Representative example of viability gating strategy of myofibroblasts treated with MSC-CM. **F.** Neither NS- nor S-MSC-CM stimulate myofibroblasts' apoptosis.

A.



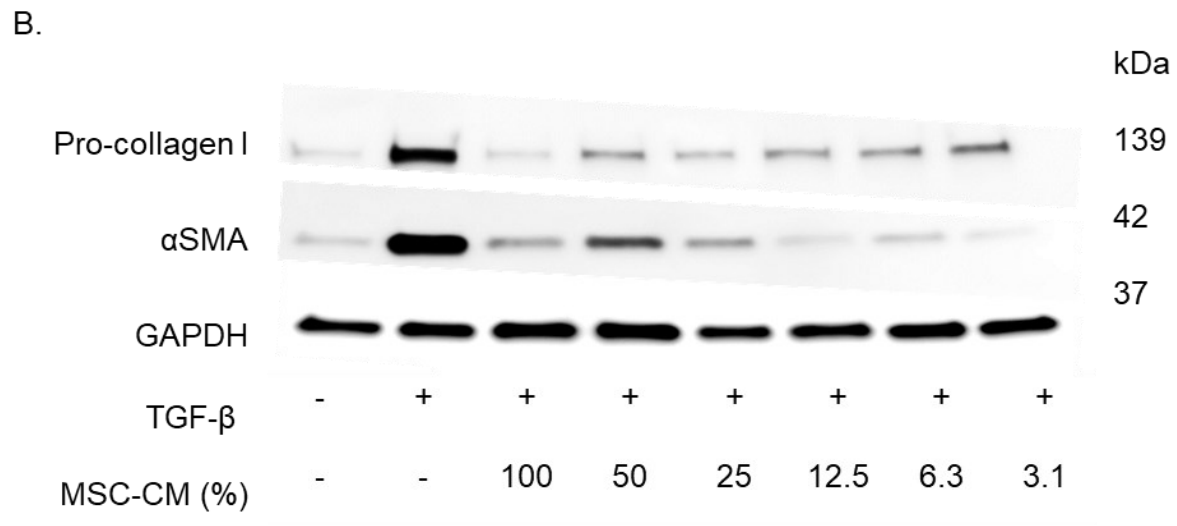


Figure 24. S-MSC-CM promotes myofibroblasts' deactivation in HCA2 human fibroblasts (hTERT). Following TGF-β activation of fibroblasts, myofibroblasts were treated with MSC-CM. **A.** Summary data of pro-collagen I and α-SMA protein levels from deactivation assays indicating that consistently CM titration from the same MSC promoted myofibroblast deactivation and **B.** Representative example of pro-collagen I and α-SMA western blot.

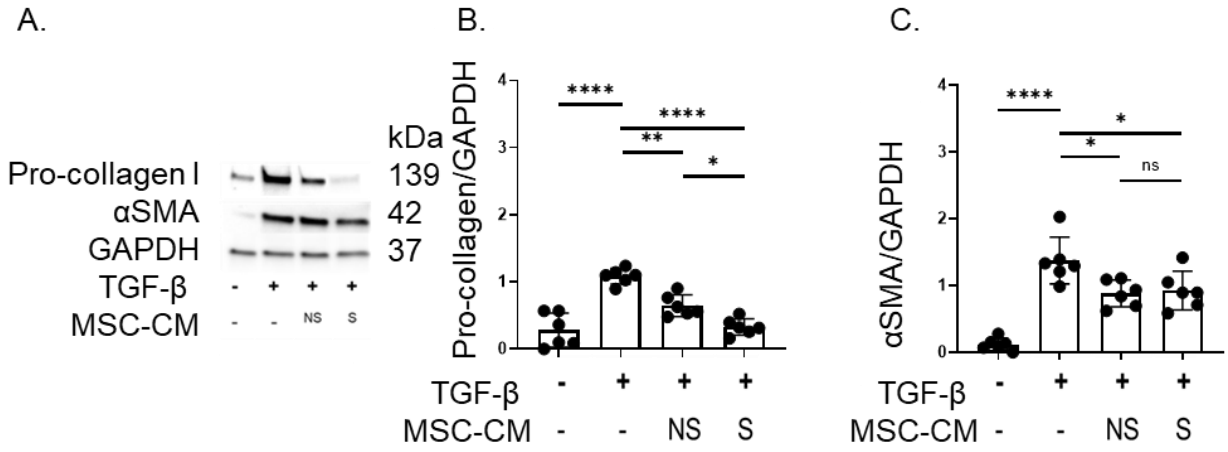


Figure 25. S-MSC-CM does not impair myofibroblast deactivation in primary human foreskin fibroblasts. After TGF-β activation of fibroblasts, myofibroblasts were treated with MSC-CM. A. Representative example of pro-collagen I and α-SMA by western blot

and summary data of B. pro-collagen I and C. α-SMA protein levels from deactivation assays. The levels of those proteins were reduced in myofibroblasts treated with S-MSC-CM, indicating a more potent effect to inhibit pro-collagen I compared to NS-MSC-CM. B. Pro-collagen I: S-MSC-CM vs NS-MSC-CM, n=6: *p=0.02, C. α-SMA: S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.10, normalized to GAPDH.

9.6 S-MSC-CM Reduces Staurosporine-induced Myofibroblasts' Apoptosis

The elimination of myofibroblasts plays a crucial role in allowing functional tissue cells to proliferate and restore organ function [293]. MSC are known for promoting apoptosis in activated T cells [294]. Here, we tested if senescence altered the effects of MSC-CM on a staurosporine induced-myofibroblasts' apoptosis assay. There were no numerical differences in the effect of NS- and S-MSC-CM and reduced staurosporine-induced-myofibroblasts' apoptosis. Some of the S-MSC-CM more effectively antagonized the effect of staurosporine than their NS-counterparts **(Figure 26).**

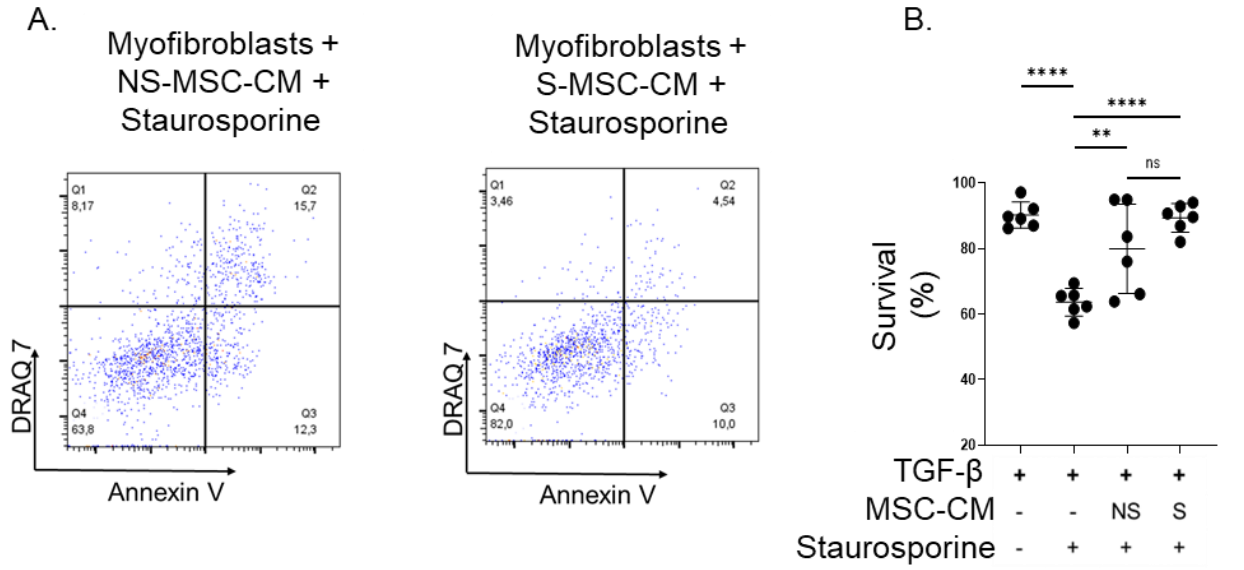


Figure 26. S-MSC-CM reduces staurosporine-induced myofibroblasts' apoptosis. Myofibroblasts were induced to undergo apoptosis with 40 nM of staurosporine in the presence or absence of S- or NS-MSC-CM **A.** Representative example of gating strategy.

B. NS- and S-MSC-CM had a similar effect in reducing staurosporine-induced myofibroblast apoptosis (percentage of myofibroblast viability, control vs. S-MSC-CM, n=6, ****p<0.0001; S-MSC-CM vs NS-MSC-CM, n=6, ns=p=0.18)

9.7 S-MSC-CM Promotes TGF-β-induced Fibroblasts Contraction in Collagen Microdroplets

The contraction assay with fibroblasts embedded in collagen microdroplets is a three-dimensional method to determine how MSC-CM affects activated fibroblasts' contractile behaviour [295]. After the migration of fibroblasts into the wound area, they interact with the ECM and generate biomechanical contractile forces by linking the cytoskeleton to integrin receptors on the cell membrane [296]. TGF-β induced-fibroblasts contraction in collagen microdroplets. Both NS-and S-MSC-CM enhanced the contraction of the TGF-β induced fibroblast microdroplets. No significant differences in contraction were seen between NS and S-MSC-CM **(Figure 27).**

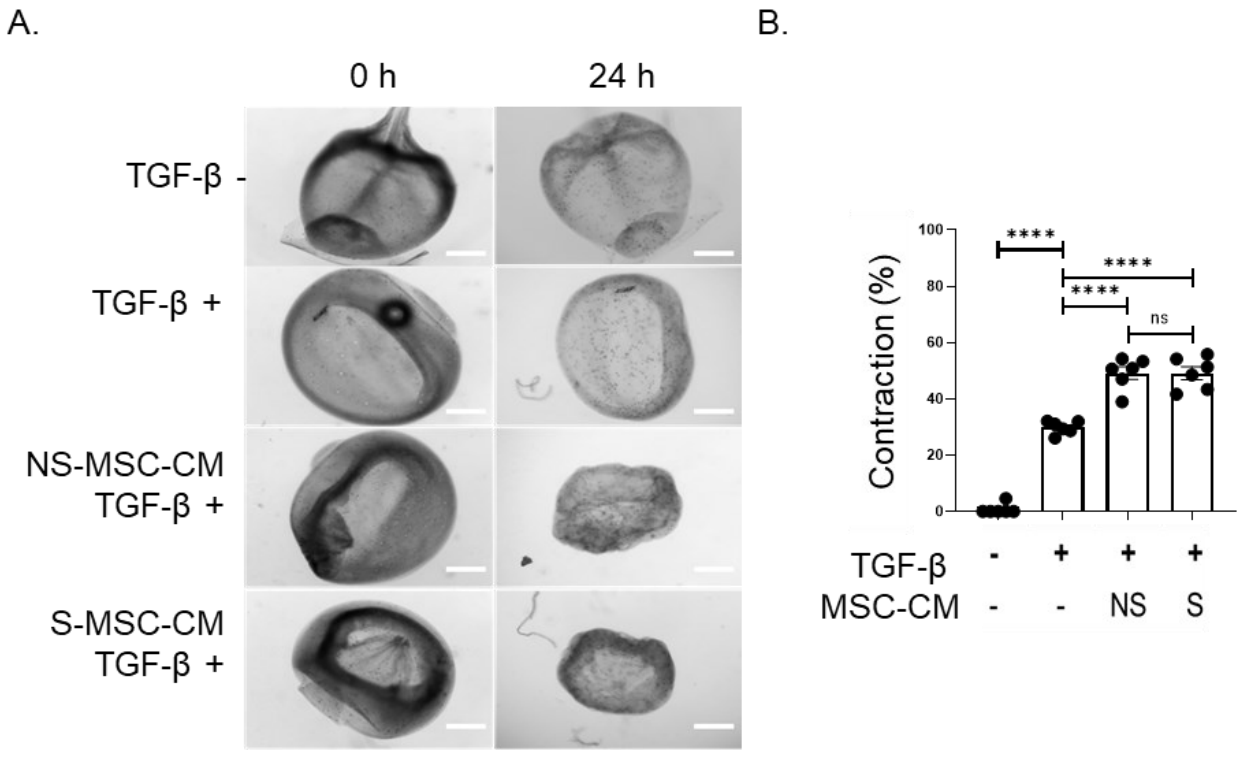


Figure 27. S-MSC-CM promotes TGF-β induced fibroblasts' contraction in primary human foreskin fibroblasts (24h). Cell-dense collagen type I microdroplets were printed, TGF-β-induced contraction, microdroplets were treated with MSC-CM and assayed after 24 hours. **A.** Representative example of imaging before and after 24 hours. **B.** Collagen droplet contraction after 24 hours normalized to the original size of the microdroplet.

10 Discussion

Fibrosis is a complex, multistep, chronic cellular and molecular process implicated in the pathogenesis of various diseases. The burden of fibrotic-related diseases is significant [1, 8]. Although several 'anti-fibrotic agents' are approved for specific indications, they slow down the progression of fibrosis but do not reverse it. MSC anti-fibrotic effects justify their therapeutic assessment in clinical trials. Several 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 November 11, 2023, rendered 83 studies. For instance, a phase 1/2 study conducted on SSc patients showed that a single infusion of BM-MSC yielded favorable outcomes. This treatment approach was linked to reduced skin fibrosis and the maintenance of forced vital capacity at the one-year post-infusion. Notably, no significant adverse effects were reported [297]. However, MSC require in vitro expansion for their clinical use, a process associated with replicative senescence. It is unknown whether replicative senescence impacts the anti-fibrotic properties of MSC. Knowing that senescent MSC have reduced immunomodulatory and pro-angiogenic properties [132] and that senescence is implicated in fibrosis [69], we tested if senescence impacted in vitro the anti-fibrotic effects of MSC. Specifically, using *in vitro* assays, we assessed the effect of senescence on MSC-CM anti-fibrotic. The *in vitro* assays used evaluated independent and fundamental steps in the fibrotic process: modulation of the TGF-β effect (i.e., inhibition of fibroblasts' activation, myofibroblasts' deactivation, ECM contraction) and restoration of myofibroblasts' apoptosis threshold.

Consistent with previous reports [131, 276], we confirmed that S-MSC had impaired immunopotency. Accordingly, S-MSC had higher $CD26^{+}$ surface levels and reduced immunopotency [131]. Differences between the NS- and S-MSC secretome have been previously shown to mediate the impaired immunopotency and in contrast to young MSC, MSC from elderly patients with atherosclerosis had a SASP with elevated IL-6, IL-8/CXCL8, and monocyte chemoattractant protein-1 (MCP-1/CCL2) levels. The immunomodulatory capacity of elderly MSC was improved by neutralizing these factors [276]. A decline in the S-MSC immunomodulatory capacity and secretion of pro-inflammatory cytokines may disrupt tissue homeostasis, resulting in a persistent inflammatory stimulus crucial for triggering the activation of the wound-healing program that if not reversed leads to fibrosis [9]. However, contrary to our initial hypothesis, our results showed that S-MSC-CM retained the ability to inhibit in vitro the differentiation of fibroblasts into myofibroblasts and to reverse the fibrotic phenotype. S-MSC-CM inhibited the differentiation of fibroblasts into myofibroblasts *in vitro.* In the TGF-β-induced fibroblasts to myofibroblasts' activation assay, we tested if S-MSC-CM antagonized the pro-fibrotic effect of TGF-β. The readouts of these experiments were the decrease in the synthesis of procollagen I and α-SMA that was similar in S- and NS- MSC-CM. Moreover, we confirmed that S-MSC-CM deactivates myofibroblasts, an effect independent of the fibroblasts' source and unrelated to apoptosis induction. We also showed that S-MSC-CM reduced staurosporine-induced myofibroblasts' apoptosis instead of restoring the myofibroblasts' apoptosis sensitivity threshold in vitro. Finally, our results indicate that S-MSC-CM promoted myofibroblast contraction in collagen microdroplets to the same extent as NS-MSC-CM. Altogether, these results suggest that although senescence

impacts the MSC phenotype and the composition of the MSC-CM, it does not alter the anti-fibrotic properties of MSC-CM in vitro.

Our results indicating that senescence does not impair the anti-fibrotic effect of MSC *in vitro* conflict with those of in vivo studies showing that the secretome of senescent IPF fibroblasts is pro-fibrotic and that the persistent accumulation of senescent cells impairs fibrosis resolution [69]. Several aspects may account for this discrepancy (see study limitation paragraph). A critical concept in senescence is the relevance of time in senescence's detrimental effects. With persistent senescence, senescent cells contribute to chronic age-related diseases (e.g., cancer development and fibrosis) and organismal aging [18]. S-MSC-CM reduces staurosporine-induced myofibroblasts' apoptosis in vitro. Apoptosis resistance characterizes myofibroblasts and differentiates them from fibroblasts [81]. Since activated myofibroblasts are the primary sources of the fibrotic ECM, eliminating these cells is a prerequisite for sustained fibrosis resolution [1]. 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 pro-apoptotic factor. MSC-CM did not restore the apoptosis sensitivity of the myofibroblasts; instead, they reinforced it. However, our findings corroborate previous research; MSC exert anti-apoptotic effects partially by paracrine action suppressing the activation of pro-apoptotic genes, leading to a reduction in the population of apoptotic cells within the injured tissue [302, 303]. S-MSC-CM promoted the myofibroblast contraction in collagen microdroplets. Our findings corroborate previous studies, which showed that MSC-CM enhanced the contraction of their fibroblasts–collagen gels, which could contribute to a wound healing context [300].

As a process, fibrosis implicates several effectors and is difficult to recapitulate *in vitro*. As a consequence, our work has several limitations. First, the *in vitro* assays we use are appropriate for assessing the modulation of specific aspects of fibrosis but not the whole process. Moreover, most fibrosis modulation assays are performed in a simplified 2D system, with one pro-fibrotic agent (TGF-β1) acting on a single cellular effector (i.e., fibroblasts or myofibroblasts). As such, these assays do not inform and may not reflect how senescence impacts the in vivo anti-fibrotic effects of MSC-CM. For example, our systems did not allow evaluation of the effect of TGF-β1 on MSC. This is relevant as TGF-β1 plays a crucial role in directing fate decisions in MSC and modulating their regenerative function [257], [301], [302]. Our results need to be confirmed in more advanced multicellular systems that allow assessing the interplay of multiple cellular effectors of inflammation and fibrosis. Alternative in vitro senescent systems (i.e., bleomycin, irradiation, and oncogene activation) will also be relevant to confirm that the effect we report is not unique to replicative senescence. Second, our studies focused on assessing MSC-CM and not MSC themselves in the context of fibrosis resolution. This, in part, is explained by the fact that no current surface molecule is differentiating MSC from fibroblasts in co-cultures. Organoids, on-chip systems and in vivo models that allow the use of NS- and S-MSC will provide valuable insights and allow the evaluation of cell-cell and cell-ECM interactions [295 - 297]. Third, it was reported that the secretome of S-MSC can induce senescence in normal-aged fibroblasts [257]. Future experiments could assess this concept.

Despite their limitations, *in vitro* anti-fibrotic assays are required by regulatory agencies as evidence of an effect. Moreover, by simplifying the experimental model, *in*

vitro assays may contribute to identifying specific cellular and molecular mechanisms activated during fibrosis, which is essential for understanding crucial events that trigger progressive tissue scaring and abnormal tissue repair [306]. We used established *in vitro* anti-fibrotic assays that supported the FDA approval of anti-fibrotic drugs (i.e., Pirfenidone and Nintedanib) testing their effect on the modulation of α-SMA, fibronectin, procollagen I and III on lung fibroblasts from IPF patients. Those assays used PDGF, FGF-2, VEGF and lower doses of TGF-β to stimulate fibroblasts [53, 299]. Using IPF lung fibroblasts, pirfenidone and nintedanib reduced collagen I and α-SMA I protein levels [53, 54, 56, 300]. In those *in vitro* assays, pirfenidone and nintedanib have anti-fibrotic effects; however, they did not revert fibrosis in clinical trials. Optimization of *in vitro* assays may increase their value in predicting *in vivo* fibrosis resolution; moreover, the sensitivity of the in vitro assays to detect differences between samples is a relevant consideration. However, even if our in vitro tests do not predict senescence outcomes, these assays remain appropriate for investigating mechanisms underlying fibrosis.

In summary, this is the first study to report on the anti-fibrotic activity of S-MSC-CM. Our results suggest that, when tested in vitro, the anti-fibrotic properties of S-MSC-CM are preserved. On one hand, senescence has long been linked with aging. On the other hand, senescence has beneficial roles in various physiological processes, from embryonic development to cellular reprogramming and tissue regeneration [309]. As such, senescence contributes to wound healing and tissue repair in organs such as the liver [78], skin [84], and heart [74]. It was postulated that the MSC secretome exhibits variations depending on the specific triggers prompting senescence and can dynamically

adapt in response to the microenvironment that hosts the senescent cells [310]. The ‘true in vivo’ contribution of MSC senescence and its regulation remains to be established.

11 Conclusions

Through standardized in vitro assays, my work evaluated the effects of replicative senescence on the anti-fibrotic effect of human adipose tissue-derived MSC. These in vitro assays targeted independent and fundamental steps in the fibrotic process: modulation of the TGF-β effect (i.e., inhibition of fibroblast activation, myofibroblasts deactivation, ECM contraction), and restoration of myofibroblasts apoptosis threshold. In vitro, the anti-fibrotic properties of the S-MSC secretome are preserved. These findings suggest that potential compensatory mechanisms in S-MSC exist and remain to be defined. While senescence impairs the MSC immunomodulatory properties, it does not influence MSC anti-fibrotic effects in vitro. Further studies should evaluate the effect of senescence on the anti-fibrotic properties of MSC-CM in animal models.

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