Human Multipotent Mesenchymal Stromal Cells Pro-angiogenic Properties: Effects of Replicative Senescence, Aging, and Cell Source

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Abstract

Background: Angiogenesis is a tightly regulated physiological process of new blood vessel formation from pre-existing vessels. Multipotent mesenchymal stromal cells (MSCs) promote angiogenesis via paracrine effects (e.g., secretion of pro-angiogenic factors). We demonstrated that MSCs from older donors have a senescent associated secretory phenotype (SASP), which reduced their immunomodulatory capacity. It is unknown whether MSC's from elderly donors or *in vitro* replicative senescence similarly impact the angiogenic properties of MSCs. My work optimized *in vitro* angiogenesis assays and evaluated the effects of replicative senescence, donor's age, and MSC's source on their angiogenic properties.

Methods: The angiogenic effects of MSCs on human umbilical cord vein endothelial cells (HUVEC) were assessed in three *in vitro* assays: (i) MSC:green fluorescent protein transfected HUVEC (HUVEC-GFP) co-culture tube formation assay, (ii) MSC conditioned medium (CM):HUVEC-GFP *in vitro* Matrigel tube formation assay, and (iii) trans-well HUVEC migration assay towards MSC-CM. Images of HUVEC migration and tube formation were analyzed with Wimasis image system. The concentration of various pro-angiogenic factors in MSC-CM was determined by multiplex assays and ELISAs. Adipose tissue-derived MSCs (AT-MSCs) were isolated from eight healthy pediatric/young (mean age: 16.5 ± 2.83 years) and eight healthy adult donors (mean age: 66.6 ± 10.0 years) and tested at passage 4 [P4, early passage MSC (EP-AT-MSC)]. Wharton's Jelly-derived MSC (WJ-MSCs) were also tested at P4. Replicative senescent AT-MSC (>P15, late passage MSC: LP-AT-MSC) was confirmed by the presence of traditional senescence markers.

Results: Replicative senescence impaired the pro-angiogenic effects of AT-MSCs. In MSC:HUVEC-GFP co-culture assays, EP-AT-MSCs induced significantly greater tube formation than LP-AT-MSCs at day 6 (D6) and D12. This was confirmed by *in vitro* Matrigel tube formation and trans-well migration assays where EP-AT-MSC-CM were more efficient than LP-AT-MSC-CM in promoting tube formation and migration of HUVEC. Ang-1 concentration in LP-AT-MSC-CM was significantly lower than that of EP-AT-MSC-CM. In contrast, MSC donor's age did not recapitulate the effects of replicative senescence on angiogenesis. Instead, adult AT-MSC stimulated more extensive HUVEC tube formation in MSC:HUVEC-GFP co-culture assays than pediatric/young AT-MSCs at D6 and D12. Adult AT-MSC-CM was also more efficient than pediatric/young AT-MSC-CM in inducing HUVEC migration and tube formation. Unlike adult and pediatric/young AT-MSCs, WJ-MSC did not induce tube formation of HUVECs in the MSC:HUVEC-GFP co-culture assay. Furthermore, WJ-MSC-CM induced significantly less HUVEC tube formation and migration than AT-MSC-CM. Assessment of soluble factors in WJ-MSC-CM showed lower VEGF-A and higher Ang-1 concentrations compared to AT-MSC-CM.

Conclusion: Replicative senescence decreased AT-MSC's pro-angiogenic function and was associated with a reduction of Ang-1 in the MSC secretome. In contrast, donor's age had opposite effects on MSCs, with adult AT-MSCs being more efficient in promoting angiogenesis than pediatric/young AT-MSCs, and WJ-MSCs exhibiting the lower pro-angiogenic capacity. These findings suggest that senescence is associated with a decline in the pro-angiogenic function of AT-MSCs and that potential compensatory mechanisms exist in adult AT-MSCs.

Résumé

Contexte: L'angiogenèse est un processus physiologique étroitement régulé de formation de nouveaux vaisseaux sanguins à partir de vaisseaux préexistants. Les cellules stromales mésenchymateuses multipotentes (CSM) favorisent l'angiogenèse via des effets paracrines (par exemple, la sécrétion de facteurs pro-angiogéniques). Nous avons démontré que les CSM de donneurs plus âgés ont un phénotype sécrétoire associé à la sénescence qui déterminent la capacité immunomodulatrice réduite des CSM. On ne sait pas si les CSM de donneurs âgés ou la sénescence réplicative in vitro ont un impact similaire sur les propriétés angiogéniques. Mon travail a optimisé les tests d'angiogenèse in vitro pour évalué les effets de la sénescence réplicative, de l'âge du donneur et de la source de CSM sur leurs propriétés angiogéniques.

Méthodes: Les effets angiogéniques des CSM sur les cellules endothéliales de la veine du cordon ombilical humain (HUVEC) ont été évalués dans trois tests in vitro : (i) test de formation de tube avec co-culture CSM : HUVEC protéine fluorescente verte (HUVEC-GFP), (ii) Milieu conditionné MSC (MC) : essai de formation de tube sur Matrigel in vitro avec HUVEC-GFP et (iii) essai de migration trans-puits HUVEC vers CSM-MC. Les images de la migration HUVEC et de la formation du tube ont été analysées avec le système d'imagerie Wimasis. La concentration de divers facteurs pro-angiogéniques dans CSM-MC a été déterminée par des tests multiplex et des ELISA. Les CSM dérivées du tissu adipeux (CSM-TA) ont été isolées chez huit donneurs pédiatriques/jeunes sains (âge moyen : 16,5 ± 2,83 ans) et huit donneurs adultes sains (âge moyen : 66,6 ± 10,0 ans) et testés au passage 4 [P4, passage tôt MSC (EP-AT-CSM)]. Les MSC dérivés de la gelée de Wharton (CSM-GW) ont également été testés au P4. Les CSM-TA de

sénescence réplicative (> P15, passage tardif MSC : LP-CSM-TA) a été confirmée par la présence de marqueurs de sénescence traditionnels.

Résultats: La sénescence réplicative a altéré les effets pro-angiogéniques des CSM-TA. Dans les tests de co-culture CSM:HUVEC-GFP, les EP-CSM-TA ont induit une formation de tubes significativement plus importante que les LP-CSM-TA au jour 6 (J6) et J12. Cela a été confirmé par la formation de tubes sur Matrigel in vitro et les essais de migration trans-puits où EP-MC-CSM-TA étaient plus efficaces que LP-MC-CSM-TA pour favoriser la formation de tubes et la migration des HUVEC. La concentration d'Ang-1 dans LP-MC-CSM-TA était significativement inférieure à celle de EP-MC-CSM-TA. En revanche, l'âge du donneur CSM n'a pas récapitulé les effets de la sénescence réplicative sur l'angiogenèse. Au lieu de cela, les CSM-TA adultes ont stimulé la formation de tubes plus étendue dans les tests de co-culture CSM:HUVEC-GFP que les CSM-TA pédiatriques/jeunes à J6 et J12. Les CSM-TA-MC adulte était également plus efficace que les CSM-TA-MC pédiatrique/jeune pour induire la migration des HUVEC et la formation de tubes. Contrairement aux CSM-TA adultes et pédiatriques/jeunes, CSM-GW n'a pas induit la formation de tubes dans le test de co-culture CSM:HUVEC-GFP. De plus, MC-CSM-GW a induit significativement moins de formation de tubes et de migration que MC-CSM-TA. L'évaluation des facteurs solubles dans MC-CSM-WG a montré des concentrations de VEGF-A plus faibles et d'Ang-1 plus élevées par rapport à MC-CSM-TA.

Conclusion: La sénescence réplicative a diminué la fonction pro-angiogénique des MSC-TA et a été associée à une réduction d'Ang-1 dans le sécrétome de CSM. En revanche, l'âge du donneur a eu des effets opposés sur les CSM, les CSM-TA adultes

étant plus efficaces pour promouvoir l'angiogenèse que les CSM-TA pédiatriques/jeunes, et les CSM-GW présentant la plus faible capacité pro-angiogénique. Ces résultats confirment que la sénescence est associée à un déclin fonctionnel de leur capacité pro-angiogénique et suggèrent l'existence de mécanismes compensatoires potentiels dans les CSM-TA adultes.

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This thesis is dedicated to my parents and my sister who have never doubted of my progress and development over the years. I also owe a big thank you to my friends who always support and encourage me.

Preface and Contribution of Authors

All samples included in this research were obtained following ERB approved protocols (GEN-10-107 and A01-M05-12A 'The effects of aging on human mesenchymal stem cells'). Samples were obtained from patients at the (1) Jewish General Hospital (JGH), (2) Shriners Hospital for Children (SHC), and (3) Royal Victoria Hospital (RVH).

All work was performed by me, Peter Hoseok Jeon, under the supervision of Dr. Colmegna except:

(1) Isolation of AT-MSCs: performed by previous members of Dr. Colmegna's laboratory (Dr. Lora, Dr. Kizilay Mancini, Natalia de França Shimabukuro, and Anastasia Cheng)

(2) Preparation of WJ-MSCs: performed at St-Louis Hospital, Paris-France (n=1) and the Polski Bank Komórek Macierzystych S.A, Warsaw-Poland (n=5). These clinical-grade samples were cryopreserved at the end of passage 3 and sent to our laboratory following a material transfer agreement. These cells were stored in liquid nitrogen until they were used for this research.

(3) Characterization of EP- and LP-AT-MSCs: performed by Pavitra Upadhyaya and Rose Psaroudis, former undergraduate students in honors immunology and interdepartmental honours program in immunology (IHI), respectively, in our laboratory.

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Abbreviations

Adipose tissue-derived MSCs	AT-MSCs
Allophycocyanin	APC
Angiopoietin	Ang-1
Bone marrow	BM
Bone marrow-Derived Mesenchymal Stromal Cells	BM-MSCs
Bone Morphogenetic Protein-9	BMP-9
Carboxyfluorescein Diacetate Succinimidyl Ester	CFSE
Colony Forming Unit-Fibroblast	CFU-F
Conditioned Medium	CM
Delta-like Ligand 4	DLL-4
Dulbecco's Modified Eagle Medium	DMEM
Early passage	EP
Endothelial Basal Medium 2	EBM-2
Endothelial Cells	ECs
Expansion Index	E.I
Extracellular Matrix	ECM
Extracellular-signal-regulated kinase 1/2	ERK 1/2
Extracellular Vesicles	EVs
Fetal Bovine Serum	FBS
Fluorescein Isothiocyanate	FitC
Focal Adhesion Kinase	FAK
Forward Scatter	FSC

Green Fluorescent Protein	GFP
Growth Factor-reduced Matrigel	GFR-Matrigel
GFP-transfected Human Umbilical Vein Endothelial Cells	HUVEC-GFP
Hematopoietic Stem and Progenitor Cells	HSPC
Human Umbilical Vein Endothelial Cells	HUVEC
Human Platelet Lysate	hPL
Human Endothelial Growth Factor	hEGF
International Society for Cellular Transplantation	ISCT
Interferon-Gamma	IFN-γ
Interleukin	IL-
Immunopotency Assay	IPA
Late Passage	LP
Matrix Metalloproteinase	MMP
Mesenchymal Stromal Cells	MSCs
Mesenchymal Stromal Cell-secreted Extracellular Vesicles	MSC-EVs
Micro RNAs	miRNAs
Mitogen-activated Protein Kinase	MAPK
Monocyte Chemoattractant Protein-1	MCP-1
Neuropilin	Nrp-
Peripheral Blood Mononuclear Cells	PBMCs
Phosphate Buffered Saline	PBS
Phycoerythrin	PE
Placental Growth Factor	PLGF
Platelet Derived Growth Factor B	PDGFB

Platelet Derived Growth Factor Receptor-Beta	PBGFR-β
Population Doubling Time	PDT
R3-Insulin-Like Growth Factor-1	R3-IGF-1
Rosewell Park Memorial Institute Medium	RPMI-1640
Side Scatter	SSC
Senescence Associated Beta-Galactosidase	SA-β-gal
Senescence Associated Secretory Phenotype	SASP
Senescence Messaging Secretome	SMS
Small Extracellular Vesicles	sEVs
Transforming Growth Factor-Beta	TGF-β
Tyrosine kinase with immunoglobulin-like and EGF-like domains 2	Tie-2
Tumor Necrosis Factor-Alpha	TNF-α
Vascular Endothelial Growth Factor	VEGF-
Vascular Endothelial Growth Factor Receptor	VEGFR-
Wharton's Jelly	WJ
Wharton's Jelly-derived Mesenchymal Stromal Cells	WJ-MSC
4',6- diamidino-2-phenylindole	DAPI
7-aminoactinomycin D	7-AAD

Chapter 1: Introduction

Section 1: Multipotent Mesenchymal Stromal Cells

1.1.1 Discovery, Nomenclature, and Definition

Two groups contemporaneously pioneered the concept of mesenchymal stromal cells (MSCs). In 1968, Tavassoli and Crosby first established that the bone marrow (BM) has osteogenic potential. They performed autologous transplantation of BM to extramedullary sites (i.e., spleen, kidney, liver, and muscles in rats, rabbits, and dogs) which led to the formation of osteoid tissue, followed by subsequent establishment of sinusoidal microcirculation and hematopoietic repopulation [1]. At the same time, Friendenstein and his colleagues showed that the BM, in postnatal life, is a reservoir of stem cells for mesenchymal tissues proving that osteogenic and hematopoietic cells derive from distinct precursors [2]. These cells were named “osteogenic stem cells”, which later become MSCs. Friedenstein expanded stromal cells obtained from BM cell suspensions *in vitro* and identified colonies of plastic adherent cells (unlike most of the hematopoietic cells) with fibroblast-like morphology. Each colony was named a colony-forming unit fibroblast (CFU-F), and their clonogenicity was confirmed *in vivo* as transplantation of these cells beneath the renal capsule of mice generated a structure replicating the histology and architecture of a miniature bone (i.e., ossicles). The ossicles were chimeric structures because the resulting bone tissue was genetically identical to the parental strain; however, the hematopoietic tissue that colonized the bone was of host origin [3].

Lack of consistency in MSC nomenclature is one of the factors that slowed-down progress in this field. The term “stromal stem cells” was proposed in 1988 by Maureen Owen, Ph.D. to indicate that the cells reside in the stromal region rather than the hematopoietic compartment [3]. In 1991 Arnold Caplan, Ph.D. named these cells ‘mesenchymal stem cells’ to emphasize their mesenchymal origin and their *in vitro* capacity to differentiate into cells of mesodermal lineage including bone, cartilage, and fat [4]. This nomenclature was challenged by James Dennis, Ph.D. who suggested that the cells may be progenitors rather than stem cells. As a result, the term “mesenchymal progenitor cells” was proposed [5]. In 2000, Paolo Bianco, M.D. and Pamela Gehron Robey, Ph.D. coined the term “skeletal stem cell” to specify that the cells give rise to components of the skeletal system [6]. Two years later, the term “multipotent adult progenitor cells” was proposed by Yuehua Jiang, M.D. to describe the multipotent nature and potential progenitor status of MSCs [7]. Since no direct evidence demonstrated the ability of MSCs to self-renew and differentiate *in vivo*, in 2006, the International Society for Cellular Therapy (ISCT) proposed the term “multipotent mesenchymal stromal cells”, while keeping the acronym MSC, as the cells are often observed in the stromal location of residing tissues [8]. This new nomenclature for MSCs was not universally adopted by the field, resulting in the use of several different terms to describe the same cell type. More recently, in 2010, Dr Caplan suggested that the acronym MSC stand for “medicinal signaling cells” to reflect that the primary therapeutic benefit of MSCs relates to the secretion of bioactive molecules rather than tissue regeneration [9, 10].

Another factor that slowed down the understanding of MSC biology is the lack of uniformity in their definition. There is no specific identification marker for MSCs. MSCs

are often isolated based on their plastic adherence; however, this method results in a heterogeneous population of cells that have different growth kinetics and differentiation capacities. MSCs heterogeneity is also influenced by the lack of standardization in the methods used to isolate and expand them, as well as by MSC-donor characteristics [11, 12]. MSC heterogeneity contributes to experimental variability, makes it difficult to compare data from different labs, and explains the difficulties to reproduce some pre-clinical studies [13]. In an attempt to reduce variability in reports, the ISCT proposed minimal criteria to define MSCs. Those include: (1) plastic adherence in standard culture conditions; (2) surface expression of positive markers that are absent from most hematopoietic cells, specifically: CD73 (5'-nucleotiodase), CD90 (Thy1), CD105 (Endoglin); and lack of negative markers expressed by hematopoietic cells, specifically: CD45, CD34, CD14 or 11b, CD19 or 79, and HLA-DR; and (3) ability to differentiate *in vitro* under specific stimuli into osteoblasts, adipocytes, and chondrocytes, as a proof of multipotency [14].

Our work focuses on the study of human derived MSCs isolated based on currently standard methods and characterized according to the ISCT criteria. These aspects are fundamental as they ensure the reproducibility of our results despite potential inter-individual variations in MSC donors. In the next sections, I will review key concepts pertinent to human MSCs of relevance to my project.

1.1.2 Sources

MSCs can be isolated from almost every tissue in the body. According to their source, MSCs can derive from birth-associated (fetal/neonatal-derived tissues) or adult tissues. Among the MSCs from birth-associated tissues, the most commonly used

sources in clinical trials include cord blood, placenta, and Wharton's jelly (WJ-MSCs). Among the adult MSCs, bone marrow-derived (BM-MSCs) and adipose tissue-derived (AT-MSCs) are most often used [15, 16]. *My work compared MSCs from an adult adipose tissue (AT) and a birth-associated (WJ) sources.*

The MSC's source impacts their function, which may lead to differences in clinical efficacy [17-19]. AT- and WJ-MSCs have comparative advantages with respect to other MSC sources (Table 1). Compared to BM-MSCs, the isolation of AT-MSCs is less invasive [20] and yields a greater number of MSCs per gram of tissue [21]. The latter eliminates the need for extensive *in vitro* expansion of MSCs to get the dose required for clinical use (*in vitro* expansion of MSCs leads to replicative senescence which potentially reduces their function) [22]. Other advantages of AT-MSCs versus BM-MSCs are their higher proliferative capacity [23], lower immunogenicity (i.e., decreased upregulation of HLA-DR) [24], and increased capacity to suppress T cell proliferation [24]. Comparative studies of the effects of different MSC sources on their angiogenic and fibrosis remodeling capacities are limited. Some data suggest that AT-MSCs are more pro-angiogenic and anti-fibrotic than BM-MSCs. Specifically, AT-MSCs secrete higher levels of pro-angiogenic factors including vascular endothelial factor (VEGF-) A, insulin-like growth factor 1 (IGF-1), and interleukin (IL-) 8 [25-27] and promote greater endothelial cell (ECs) tube formation *(in vitro)* [27, 28] and neovascularization (hindlimb ischemia model) [28] than BM-MSCs. In a mice model of diffuse cutaneous systemic sclerosis, AT-MSCs were more efficient in reducing skin fibrosis than BM-MSCs [29]. Overall, these data suggest that AT-MSCs may have functional advantages compared to BM-MSCs.

Compared to BM- and AT-MSCs, the isolation of WJ-MSCs is non-invasive (WJ is a mucous connective tissue of the umbilical cord enclosing the umbilical vein and two umbilical arteries). Other advantages of WJ-MSCs over adult tissue MSCs include their (1) stemness (clonogenicity WJ- vs. BM-MSCs: 11.75% vs 1.13%) [30]; (2) homogeneity post-isolation (minimal contamination with other cell types) [31]; (3) rapid proliferation (population doubling time WJ- vs BM-MSCs: 40h vs 70h) [32], and (4) lower immunogenicity (% increase in HLA-DR WJ- vs. AT-MSCs: ~5% vs. ~50%) [33]. Comparative functional studies testing WJ-MSCs with adult tissue MSCs are not conclusive on the superiority of WJ-MSCs. In fact, some studies show greater effects of WJ-MSCs on inhibition of T-cell proliferation [17, 34] while others report the opposite [18, 19]. Similarly, there is not enough evidence to conclude on whether WJ-MSCs are superior to AT-MSCs in promoting angiogenesis or anti-fibrotic effects [26, 35].

Table 1. Comparison of basic properties of MSCs from different sources

MSC Type	BM-MSC	AT-MSC	WJ-MSC
Isolation	Invasive	Less Invasive	Non-invasive
Number of MSC	0.001-0.01% [21]	5 x 10^3 cells per 1g [21]	1-5 × 10^4 cells per cm [16]
Proliferation (relative)	Low	High [23]	High [31]
Functional Advantage		Higher immunopotency (T-cell inhibition, compared to BM-MSC) [24] Greater reduction of skin fibrosis (compared to BM-MSC) [28]	Greater stimulation of tube formation and migration of endothelial cells (compared to BM-MSC) [26]
Other		Lower-immunogenicity (relative to BM-MSC) [24]	Greater homogeneity of cell population [30] Rich stemness (increased clonogenicity relative to BM-MSC) [29] Low immunogenicity [32] (relative to AT-MSC)

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; AT-MSC, adipose tissue-derived mesenchymal stromal cells; WJ-MSC, Wharton's jelly-derived mesenchymal stromal cells.

The source of MSCs impact their function. However, currently there is not enough evidence to support the selection of MSCs from specific sources for specific clinical indications. In particular, there is limited knowledge to inform the superiority of an MSC source for ischemic and/or fibrotic processes. My work focused on comparing the pro-angiogenic properties of AT- and WJ-MSCs, the two commonly used sources of MSCs used in clinical trials.

1.1.3 Mechanism of Action and Function

MSC products are currently used in over 1,200 clinical trials (clinicaltrials.gov; March 31, 2021; keyword: mesenchymal stromal cells). This is explained by MSC multi-functionality which can be largely grouped in three areas: immunomodulation [12], pro-angiogenic activities [36], and anti-fibrotic effects [37].senes

The capacity of MSCs to interact and modulate each and every cell of the innate and adaptive immune systems is their best characterized function. Of relevance, MSCs are not intrinsically immunosuppressive. Instead, they acquire this property following their activation (i.e., priming) by external stimulus (e.g., sensing pro-inflammatory cytokines). *In vitro* MSC priming with interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) promotes their immunosuppressive function. The net effect of the MSC-immune cell interaction is the promotion of immune tolerance. Several readouts were proposed by the ISCT to characterize the immunomodulatory effects of MSCs. Among them, MSC/T-cell

suppression assays are reproducible and accepted as a standard *in vitro* assay to measure MSC immunopotency [38, 39]. Specific requirements for this assay include the use of monocyte depleted peripheral blood mononuclear cells (PBMCs) from a single donor to minimize inter-donor variability; and the use of a "physiologic" stimulus to activate PBMCs [40]. *In my work, MSC immunopotency was tested in a potency assay previously standardized in our laboratory (i.e., MSC inhibition of CD3/CD28 activated T-cells proliferation)* [11, 12, 41-43].

The immunomodulatory functions of MSCs are mainly mediated by their secretome that is composed of soluble factors and extracellular vesicles (EVs), with a lesser contribution of cell-contact dependent mechanisms. MSC priming increases the expression and secretion of those factors [40]. MSC-secreted EVs (MSC-EVs) contain a wide range of proteins, lipids, and nucleic acids. Our laboratory recently demonstrated that AT-MSCs cytokine priming increases the secretion of exosome-like small EVs (sEVs) containing two key mediators of immunopotency: A20 and TSG-6. These EVs inhibited T cell proliferation in a dose-dependent manner. Moreover, we showed that AT-MSCs isolated from pediatric donors secreted more sEVs than adult MSCs which may contribute to their increased immunopotency [41].

Another key functional property of MSCs is their capacity to modulate fibrosis. Fibrosis results from an imbalanced wound healing response to injuries, leading to an abnormal tissue architecture and potentially multi-organ dysfunction [44]. Fibrosis occurs when fibroblasts are overactivated, reprogrammed, and lose their normal homeostatic properties, leading to persistence of myofibroblasts with ongoing matrix remodeling and increased extracellular matrix (ECM) deposition [45]. Transforming growth factor- β (TGF-

β) is a master regulator of fibrosis as it mediates myofibroblast differentiation, and augments matrix protein synthesis by modulating the balance between matrix metalloproteinase (MMPs) and tissue inhibitor of metalloproteinases [46]. In preclinical studies, MSCs appear to be effective in attenuating fibrosis. This results from an indirect effect of MSCs on immune cells reducing inflammation and consequently ameliorating fibrosis. In addition, MSCs exert direct anti-fibrotic effects through several mechanisms mediated by their secretome. Well characterized anti-fibrotic soluble factors secreted by MSCs include HGF, VEGF, and prostaglandin E2 [47, 48]. In addition, MSCs release EVs that mediate anti-fibrotic effects and regulate TGF-β/Wnt/SMAD signaling pathways [49]. There is currently no consensus on which assays should be used to assess anti-fibrotic activity of MSCs *in vitro*, which is crucial for the selection of MSC products to be used in clinical trials of fibrotic conditions.

In addition to the modulation of immune responses and fibrotic processes, MSCs promote angiogenesis. This is the focus of my work which I will describe in depth in the next section.

Section 2: Angiogenesis

1.2.1 Definition

Angiogenesis is the physiological process through which, by sprouting or splitting, new blood vessels form from pre-existing ones. Angiogenesis should be differentiated from vasculogenesis, which is the embryonic formation of ECs from mesoderm cell precursors [50].

Angiogenesis is a complex and essential process in growth and development, wound healing, and tumor biology. There are two forms of angiogenesis that occur *in utero* and adulthood: sprouting and intussusceptive [51, 52]. Sprouting angiogenesis was the first identified and is best characterized. Its initial signal comes from parenchymal cells from hypoxic tissue areas that secrete VEGF-A, a pro-angiogenic molecule which activates receptors on ECs present in pre-existing blood vessels [51, 53]. Then, activated ECs, known as tip cells, release proteases (i.e., MMPs) that degrade the basement membrane allowing ECs to be liberated from the original vessel walls [54]. ECs proliferate into the surrounding matrix and form solid sprouts connecting neighboring vessels. The ECs that are proliferating located behind the tip cells are known as stalk cells. The proliferation of these cells allows the capillary sprouts to grow in length. Tip and stalk cells are transient phenotypes and not stable cell fates. ECs undergo iterative cycles of sprouting, branching, and tubulogenesis, requiring dynamic transitions between tip and stalk cell phenotypes. In contrast, intussusceptive (i.e., splitting) angiogenesis is the formation of a new blood vessel by splitting an existing vessel into two. As such, it requires the reorganization of existing cells, leading to more rapid generation of blood vessels which is especially important in embryonic development [52]. *Our work specifically relates*

to sprouting angiogenesis, and the in vitro angiogenesis assays I established evaluate that process.

1.2.2 Regulation of Sprouting Angiogenesis

The dynamic process of tip and stalk cell specification required for sprouting angiogenesis is regulated by delta-like ligand 4 (DLL4)-Notch lateral inhibition. This is mediated by VEGF, a master regulator of angiogenesis. This family of heparin-binding growth factors consists of VEGF-A, -B, -C, -D, and placental growth factor (PLGF). In particular, VEGF-A is extensively characterized for its role in survival [55], proliferation [56], migration [56], and tube formation [57] of ECs as well as degradation of the ECM [58]. VEGF-A binds to VEGF receptor (VEGFR-) 2 on ECs with a high affinity [59]. Upon binding, the expression of DLL4 is upregulated in tip cells [60, 61]. In turn, DLL4 binds to Notch 1 receptor in neighboring stalk cells, leading to downregulation of VEGFR-2 and upregulation of VEGFR-1 [62]. Unlike VEGFR-2, VEGFR-1 sequesters VEGF-A and limits tip cell behavior in the stalk cells. In addition, the stalk cells express a high level of Jagged 1, another ligand of Notch 1 receptor. Jagged 1 inhibits DLL4-Notch signaling in the tip cell [63]. Thus, the tip cell demonstrates increased expression of VEGFR-2 and co-receptors, neuropilin 1 and 2 (Nrp1 and Nrp2) [64]. This complex DLL4/Notch lateral inhibition mechanism between tip and stalk cells is a fundamental process driving angiogenesis (Figure 1).

Once blood vessels are formed, the vessels are stabilized by pericytes. The tip cell expresses a high level of platelet derived growth factor B (PDGFB), and this factor acts on its receptor (PDGF receptor β, PDGFRβ) expressed by pericytes [53]. The recruited pericytes then produce angiopoietin-1 (Ang-1). This activates their endothelial receptor

[tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (TIE2)] to stabilize vessels, promote adhesion of pericytes, and induce endothelial junctions [65]. Ultimately, this leads to decreased permeability of the newly formed blood vessels. *Given the relevance of VEGF-A and Ang-1 in promoting angiogenesis, in my project, I have assessed their role in the context of MSC-induced angiogenesis.*

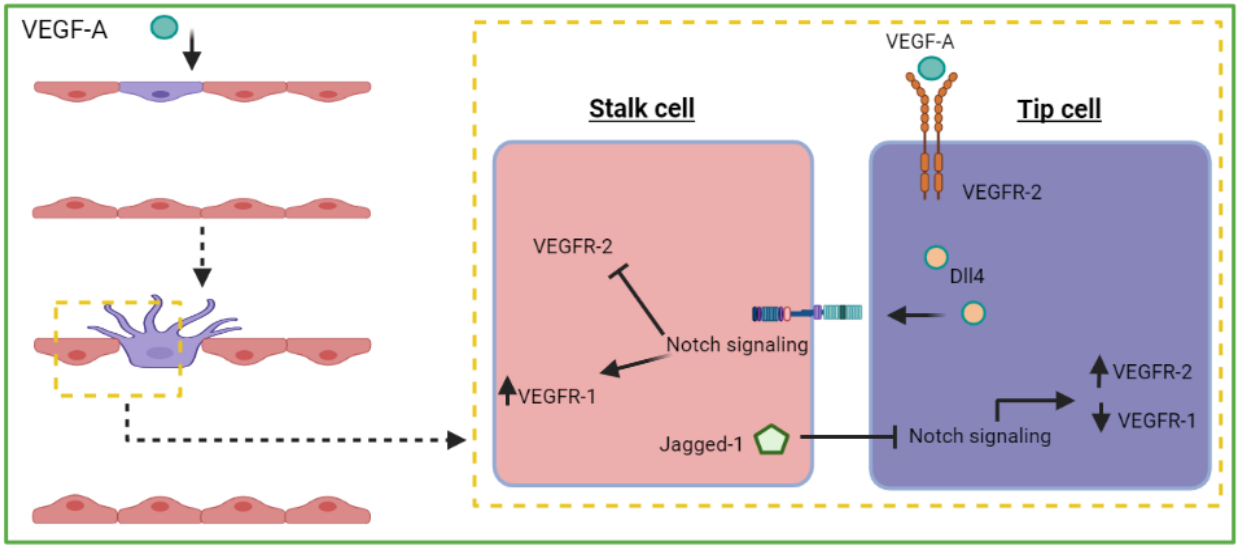


Figure 1. Schematic diagram of tip/stalk endothelial cell selection process. Once VEGF-A binds to VEGFR-2 on the tip cell, delta-like ligand 4 (DLL4) expression is increased. DLL4 then activates Notch signaling pathway in the neighboring stalk cells. Notch signaling suppresses the expression of VEGFR-2 and induces VEGFR-1 expression. Stalk cells secrete Jagged-1 ligand which act as an antagonist of Notch signaling in the tip cell. Inhibition of Notch signaling in the tip cell leads to increased expression of VEGFR-2 and reduced expression of VEGFR-1. This figure has been created with Biorender.com.

1.2.3 Role of MSCs in Angiogenesis

MSCs promote angiogenesis through the secretion of pro-angiogenic factors and other molecules. Key pro-angiogenic factors secreted by MSC are shown in Table 2. Most of these factors are VEGF-dependent and activate key pro-angiogenic signaling pathways, including extracellular-signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase (MAPK), and focal adhesion kinase (FAK). This leads to the survival, migration, and tube formation of ECs [66-69]. Interestingly, pro-inflammatory cytokines in the MSC secretome [i.e., IL-6 and monocyte chemoattractant protein-1 (MCP-1)] also have a role in angiogenesis as they stimulate migration and tube formation of ECs [70-73]. In addition to soluble factors, recent evidence suggests that MSC-EVs mediate angiogenesis. These EVs release cargos upon being internalized by ECs, leading to activation of intracellular signaling pathways to stimulate angiogenesis [74]. In particular, microRNAs (miRNAs) in MSC-EVs help regulate the tip and stalk cell specification process and inhibit anti-angiogenic regulators [75-79]. MSCs can also directly modulate angiogenesis through a contact-dependent mechanism. In co-cultures of MSCs and ECs, MSCs behave like pericytes and co-align with the tube structures of ECs [80, 81]. This has been shown both *in vitro* and *in vivo*, supporting the current concept that MSCs are mural cells (i.e., pericytes) [82, 83]. Studies of preclinical animal models further confirm the *in vivo* pro-angiogenic function of MSCs. MSCs 1) increase the expression of VEGF in tissues [84], 2) stimulate neovascularization [84], 3) increase blood perfusion [85], and 4) are cardioprotective (increased ejection fraction and left ventricular end-diastolic and systolic diameter) [86]. Thus, MSCs promote angiogenesis both *in vitro* and *in vivo*, which support their use in clinical trials for the treatment of vasculopathies.

Table 2. Examples of pro-angiogenic factors secreted by MSCs

MSC Source	Factor	Function	Reference
BM, AT	VEGF	Master regulator of angiogenesis (proliferation, migration, and tube formation of ECs)	[25, 33, 36]
BM, AT, WJ	Ang-1	Stabilization of blood vessels (induction of cell-cell junction)	[25, 36]
BM, AT, WJ	HGF	Promotes migration and tube formation of ECs in a VEGF-dependent manner	[25, 33, 36]
BM, AT, WJ	FGF	Promotes migration and tube formation of ECs in a VEGF-dependent manner	[25, 33, 36]
BM, AT, WJ	IL-6	Induces migration and tube formation of ECs	[25, 33, 36]
BM, AT, WJ	MCP-1	Increases the expression of VEGF and stimulates migration of ECs	[25]

Abbreviations: BM, bone marrow; AT, adipose tissue; WJ, Wharton's jelly; VEGF, vascular endothelial growth factor; Ang-1, angiopoietin-1; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; ECs, endothelial cells.

Most studies that assessed the pro-angiogenic properties of MSCs used BM and AT as sources for those cells. Only two head-to-head studies compared the angiogenic potency of MSCs from different sources and reported opposite findings on the results of *in vitro* Matrigel tube formation assay comparing BM- and WJ-MSCs. Du et al. suggested the superior effects of BM-MSCs to stimulate tube formation [26] while Hsieh et al. demonstrated enhanced pro-angiogenic activity of WJ-MSCs [35]. The lack of consistency in the results of these two studies may be attributed to differences in technical aspects of the tested assay.

In summary, MSCs regulate angiogenesis through secreted factors (i.e., soluble molecules and EVs) and cell-cell contact dependent mechanisms both *in vitro* and *in vivo. An unresolved issue is the lack of well standardized angiogenic assays to allow the comparison of pro-angiogenic effects between MSCs from different sources. Optimizing a set of assays to assess different steps of sprouting angiogenesis in a reproducible way, was one of the objectives of my work.*

1.2.4 Angiogenesis assays

Consensus guidelines for the use and interpretation of angiogenesis assays were recently issued [87]. In that paper, *in vivo*, *ex vivo*, and *in vitro* bioassays available for the evaluation of angiogenesis are described and critical aspects relevant for their execution and interpretation are highlighted. However, there is currently no clear consensus on the "gold standard" method to evaluate MSC-mediated angiogenesis. Assays are chosen depending on the aspect of angiogenesis to be evaluated (proliferation, migration/invasion, or tube formation) and experimental condition (i.e., cell-cell contact dependent or independent). The two assays most commonly used to assess MSC-angiogenesis that we standardized to test the angiogenic properties of MSCs are:

EC migration *(in vitro):* a) Cell culture wound closure assay, also known as wound healing assay, scratch assay, or lateral migration assay. It evaluates random 2-dimensional lateral migration of ECs to the wound. ECs are grown to confluency in a cell culture dish and then scraped with a razor blade/pipette tip, allowing the ECs at the wound edge to migrate into the scraped area [88]. In this method, a chemogradient of pro-angiogenic signals is not established, thus it does not allow the determination of EC directed migration toward or away from a compound [87].

The lack of reproducibility of uniform wounds across different wells leads to inter-well and inter-experiment variability. Automated devices to induce uniform wounds across different wells and obtain real-time images are preferable (i.e., Incucyte Live-cell Analysis). b) Boyden chamber assay, also known as trans-well cell migration assay. The assay requires a chamber of two medium-filled compartments separated by a microporous membrane of a defined pore size. ECs are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment were the chemotactic agent of interest or cell-secreting chemotactic agents are present. The membrane between the fluid-filled compartments is harvested, fixed, and stained, and the number of cells that migrated to the bottom side of the membrane are counted. In contrast to the cell culture wound closure assay, the trans-well cell migration assay allows the assessment of directional migration. In general, migration assays should not exceed 24 hours to prevent EC proliferation masking the effects of EC migration. *In my work*, *trans-well migration assay was standardized to assess directional migration of ECs towards MSC conditioned medium (MSC-CM). This closely resembles the migration of sprouts towards angiogenic signals in vivo.*

Tube formation *(in vitro):* The 'cell culture tube formation assay' first described in 1988 by Kubota [89] involves plating ECs onto a basement-membrane-like substrate on which they form tubules within six to 20 hours. ECs develop tight cell-cell and cell-matrix contacts and assemble into tubules that mostly contain a lumen, [90-92]. Quantification can be performed by measuring the tube area (most common) or the length and/or number of branching points [93]. This

semiquantitative assay has several advantages including that it is reproducible, fast and easy to perform and can be scaled up for high-throughput analysis. Additionally, factors can be added exogenously to the medium, transfected into ECs, or knocked down. For the tube formation assay, the basement membrane can be established using tissues, tumors, or a gel matrix layer of fibrin, collagen or Matrigel/ECM. The type of matrix used is important, as different matrices result in different rates of tube formation. The Matrigel tube formation assay [90] involves seeding ECs on plates coated with ECM proteins. Matrigel contains high levels of pro-angiogenic factors and is a potent stimulator of sprouting. Thus, in my work, *growth-factor reduced Matrigel (GFR-Matrigel) was used to prevent Matrigel overriding the pro-angiogenic effects of MSCs.* However, coating the plates with Matrigel and other ECM materials may lead to formation of menisci near the walls of the wells. This leads to unevenly focused images that may interfere with analysis. *To overcome this limitation, in my work I used specialized 15-well ibidi µ-slide angiogenesis plates that are specifically designed to avoid meniscus formation.* Several types of endothelial cells can be used for the tube formation assay including both primary cells and immortalized cell lines [91]. Due to their large availability, most studies use human umbilical vein ECs (HUVECs). *In my work, we used a pooled sample of HUVECs from multiple donors.*

Several other angiogenic assays are available including three-dimensional models of vascular morphogenesis (i.e., fibrin bead assay, collagen lumen assay, retina explant assay, and vascularized micro-organ) and the aortic ring assay, as well as specific tests to evaluate angiogenesis in the context of cancer and other diseases. Three-dimensional

models in general require the use of freshly isolated ECs which makes standardization challenging as patient-to-patient variations between different EC isolations can lead to inconsistent assay results [94]. The aortic ring assay reproduces *ex vivo* cellular and molecular mechanisms that are essential for the regulation of the angiogenic process; however, the source of angiogenic ECs is the aorta whereas *in vivo*, angiogenic outgrowth occurs from postcapillary veins [95].

In summary, to characterize the angiogenic properties of MSCs from different sources, I used a trans-well cell migration assay and an in vitro Matrigel tube formation assay, which mimics capillary tube formation as it occurs in vivo. It is important to acknowledge that, although these assays evaluate fundamental processes of angiogenesis, they are two-dimensional assays. In vivo, angiogenesis involves complex systems and interactions between different cells and molecules in a three-dimensional environment. Therefore, the findings from my work should be confirmed in three-dimensional systems or in in vivo models (e.g., hindlimb ischemic models, angiogenesis plug assay).

Section 3: Replicative Cellular Senescence and Aging

1.3.1 Definition and Readouts

Cellular senescence is a cell state triggered by stressful insults and certain physiological processes, characterized by a prolonged and generally irreversible cell-cycle arrest with secretory features, macromolecular damage, and altered metabolism [96]. Stress factors that can promote senescence include both environmental/cell extrinsic (e.g., irradiation, genotoxic drugs, epigenetic modifiers, high-fat diet) and intrinsic (e.g., shortening of telomeres, hyperproliferation, oxidative stress, autophagy, lamin B1 silencing) events that lead to accumulation of DNA damage and activate oncogenes such as p53/p21 and p16 which are important for establishing senescent cell-cycle arrest [97]. Normal human fetal fibroblasts in culture reach a maximum of approximately 50 cell population doublings before reaching the 'Hayflick limit' and becoming 'replicative senescent' [98]. Senescent cells accumulate in aged tissues and in aging-associated diseases, contributing to aging [99, 100]. Aging is defined as the progressive and generalized impairment of function that results in an increased vulnerability to environmental challenges and growing risk of disease and death [101]. Aging represents the intersection of a gradually failing system selected for early growth and reproductive fitness with the cumulative effects of growth suppressive mechanisms and acquired somatic insults.

Similar to fibroblasts, MSCs undergo senescence (e.g., within 43–77 days of expansion human bone marrow-derived MSCs undergo senescence) [102]. Late passage MSCs have genetic and epigenetic changes similar to those MSCs harvested from older individuals [103, 104]. This suggests that replicative senescent MSCs and those from

elderly individuals share similar molecular mechanisms leading to a decline in their immunomodulatory and anti-inflammatory properties and to a reduction of their ability to repair tissues [43, 105]. Senescence leads to distinct MSC changes including altered morphology with increased size and granularity, and a flat and irregular shape (i.e. loss of spindle-like aspect) [102]. The increase in the size of senescent MSCs translates into an increase in forward scatter (FSC), while the increase in their granularity is reflected by an increase in the side scatter (SSC). Autofluorescence, a novel marker of MSC senescence, is related to the accumulation of lipofuscin-related proteins and correlates with the increased expression of SA-β-Gal [102, 106]. The decrease in proliferative potential that defines senescence results in prolonged population doubling time (PDT) [102]. Other markers of cellular senescence that have been described in MSCs include senescence-associated beta-galactosidase (SA-β-gal) activity, detectable at pH 6.0 [107, 108], telomeric shortening [109], increased genomic instability (impaired DNA repair response) [110], and increased production of reactive oxygen species (ROS) [42, 111]. Senescent MSCs also show an altered secretome profile with increased levels of pro-inflammatory cytokines (i.e., IL-6, IL-8, and MCP-1) [112, 113]. This unique feature is shared by MSCs from elderly people as well as patients with diabetes and coronary artery disease [11]. Besides pro-inflammatory cytokines and chemokines, senescent cells secrete growth modulators, angiogenic factors, and MMPs, collectively termed as senescent associated secretory phenotype (SASP) or senescence messaging secretome (SMS). The SASP constitutes a hallmark of senescent cells and mediates many of their pathophysiological effects [113].

There is no specific marker of the senescent cell-cycle arrest; thus, a multi-marker approach quantifying multiple factors and features [96] is recommended to define senescence. *In my work, markers including increased β-gal positive population of cells, FSC, SSC, and autofluorescence were assessed to define the replicative senescent state of AT-MSCs.*

1.3.2 Effects of Cellular Senescence and Aging on MSC Function

Senescent MSCs have reduced immunomodulatory effects with decreased capacity to suppress T-cell proliferation [22] and polarize M2 macrophages *in vitro* [114]. This is mediated by pro-inflammatory SASP components. Factors secreted by senescent MSCs, specifically insulin-like growth factor binding proteins 4 and 7, also induce senescence of early passage non-senescent MSCs [115] and decrease the clonogenicity of hematopoietic stem and progenitor cells (HSPCs) by activating inflammatory genes [22]. Similarly, MSCs from older donors are less immunosuppressive than MSCs from younger donors; and even the least immunosuppressive early-passage MSC lines are more effective than the most immunosuppressive late-passage cell lines [116]. Findings from our laboratory support these findings [12]. Specifically, we showed that AT-MSCs isolated from elderly patients (older than 65 years) with atherosclerosis have a proinflammatory secretome with increased levels of IL-6, IL-8, and MCP-1, and a diminished capacity to suppress T cell proliferation [11]. Furthermore, the neutralization of such pro-inflammatory cytokines with monoclonal antibodies enhanced the immunosuppressive function of the MSCs. Overall, cellular senescence and aging deteriorate the immunopotency of MSCs through an increased secretion of pro-inflammatory molecules.

Few studies assessed the effects of replicative senescence and aging on the anti-fibrotic effects of MSCs. MSCs from patients with idiopathic pulmonary fibrosis have markers of senescence, show reduced migratory capacity, and are less able to prevent fibrosis in bleomycin-treated mice. They also induced senescence in normal healthy fibroblasts [117], suggesting that senescence may impact the anti-fibrotic function of MSCs.

1.3.3 Effects of Cellular Senescence and Aging on Angiogenesis

Systemic and intrinsic changes during aging limit vascular homeostasis and angiogenesis in the elderly. Elderly patients have reduced capillary density and reduced angiogenesis in response to ischemia [118-120]. At the tissue level, aging reduces the response to angiogenic growth factors [121] and expression of VEGF [122]. This ultimately leads to decreased proliferation, migration, and tube formation of ECs, which are fundamental processes of sprouting angiogenesis [123, 124]. Clinically, vascular aging translates into increased risk and prevalence of coronary artery disease and peripheral vascular disease as well as their complications (e.g., myocardial infarction, lower limb amputation) [125, 126].

Few studies have assessed how aging and senescence impact MSCs' angiogenic properties. Those studies reported contrasting findings. On one hand, AT-MSCs from pre-eclampsia patients, a condition associated with senescence [127], were shown to have an increased β-gal expression and impaired capacity to stimulate angiogenesis based in their reduced capacity to induce EC tube formation [128]. Treatment of those MSCs with senolytic drugs improved their pro-angiogenic function, confirming that decreased pro-angiogenic activity of MSCs in pre-eclampsia is associated at least in part to senescence

[128]. Another study tested AT- MSC samples that underwent replicative senescence. In that study, CM from senescent MSCs (passage number, P19) had impaired capacity to induce EC tube formation compared to non-senescent ones (P3-6) [129]. In contrast, a limited number of studies report opposite effects of aging. AT-MSCs from younger donors (mean age: 29 ± 5 years) showed enhanced capacity to promote angiogenesis in a mice model of hindlimb ischemia [130]. This was attributed to the higher level of ROS production and decreased VEGF-A secretion by MSCs from older donors (mean age: 61 ± 7 years). Similar findings were observed in a different study, in which aging impaired the pro-angiogenic function of MSCs to stimulate EC tube formation *in vitro.* This was also attributed to the decreased secretion of VEGF-A [131]. Finally, another study that evaluated the angiogenic potential of MSCs showed lack of association with donor's age. Specifically, that study evaluated MSC-CM from 5 young (<26 years-old) and 5 old donors (>60 years-old). Both groups induced similar tube formation of ECs *in vitro* [132]. Altogether, given the scarcity and inconsistency of data, there is a lack of consensus on how aging and senescence affect the pro-angiogenic function of MSCs.

In summary, the effects of source, replicative senescence, and donor's age on MSCs' angiogenic properties are ill-defined. MSCs are key regulators of vascular homeostasis and are used in a number of clinical trials for the treatment of vasculopathies. Understanding how source- and donor-related factors impact the pro-angiogenic properties of MSCs is a key factor to optimize the selection of MSC for specific clinical trials.

We **hypothesize** that, *similar to the age-associated functional decline in MSCs immunopotency, the pro-angiogenic function of MSCs is impaired in the context of*

senescence, and aging. Furthermore, we posit that given the more 'immature state', WJ-MSCs are more pro-angiogenic than MSCs derived from adult sources (i.e., AT-MSCs).

The specific aims of my work* are to:

1- Characterize the pro-angiogenic functions of early (non-senescent) and late (senescent) passage AT-MSCs;
2- Compare the pro-angiogenic properties of AT-MSC from pediatric/young and adult donors;
3- Describe the angiogenic properties of MSCs from pre-natal tissues (WJ-MSC) and adult tissues (AT-MSC)

*All MSCs used for my work were human samples, and all assays I performed were *in vitro*.

Chapter 2: Methods

2.1.1 Human AT-MSCs: Procurement, Isolation, and Culture

This study was approved by the McGill University Health Center Ethics Review Board (10-107GEN). A written informed consent was obtained from every participant. Subcutaneous adipose tissue was isolated from a total of 16 patients, including eight healthy pediatric/young donors (mean age 16.5 ± 2.83 years) undergoing elective orthopedic surgery, and eight adult donors undergoing orthopedic or aortic valve replacement surgery (mean age 66.6 ± 10.0 years). The demographics of the donors are shown in Table 3.

Table 3. Demographics of EP-, LP-, pediatric/young, and adult AT-MSC donors

	EP- and LP-AT-MSC (n=8, paired)	Pediatric/Young AT-MSC (n=8)	Adult AT-MSC (n=8)
Age (yr, mean ± SD)	41.5 ± 25.0	16.5 ± 2.83	66.6 ± 10.0
Sex (F)	4	5	6
Smoking	3	0	0
Chronic Inflammatory Diseases	3	0	0
Diabetes	0	0	0
Cancer	0	0	0
Hypertension	4	0	3
Hypercholesterolemia	3	0	0
Cardiovascular Disease	4	0	2

Abbreviations: EP-, early passage; LP, late passage; AT-MSC, adipose tissue-derived mesenchymal stromal cells.

This technique was previously established in our laboratory [11, 12, 41, 42]. Briefly, subcutaneous adipose tissue obtained from human donors was washed with phosphate-buffered saline (PBS), containing 1% penicillin/streptomycin (10,000 units/mL penicillin, 10,000 mg/mL streptomycin, WISENT Inc., St. Bruno, QC) for 45 minutes, minced surgically, and digested with 0.5% collagenase type 1 (Millipore Sigma, Etobicoke, ON), dissolved in Hank's balanced salt solution (Invitrogen, Carlsbad, CA) for 2 hours while vortexing the mix every 15 minutes. Collagenase was subsequently neutralized, and the digested sample was centrifuged. The supernatant was discarded, and the AT pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, WISETN Inc., St. Bruno, QC), containing 10% MSC qualified fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA) and 1% penicillin/streptomycin (10,000 units/mL penicillin, 10,000 mg/mL streptomycin, WISENT Inc., St. Bruno, QC). One gram of tissue was cultured in a 75-cm^2 tissue culture flask. Two days later, non-adherent cells were washed away. At 80% confluency, AT-MSC were detached with 0.25% Trypsin-EDTA (37°C, 5 minutes) to be sub-cultured at a density of 3,333 cells per cm^2. P3-P5 MSC were used for the experiments in which we compared pediatric/young and adult AT-samples. All samples tested negative for mycoplasma after isolation and at subsequent passages.

2.1.2 AT-MSCs: Induction and Assessment of Replicative Senescence

AT-MSC were allowed to replicate *in vitro* and passed when they reached 80% confluency. Early passage AT-MSC (EP) were defined as those in P3-4 and late passage (LP) as the same samples at P>15. AT-MSC were stained for senescence-associated (β-

gal) activity according the manufacturer's protocol (Cell Signaling Technology, Whitby, ON). For quantification, AT-MSC were counterstained with 0.3μM 4',6- diamidino-2-phenylindole (DAPI) to visualize the nuclei. Bright-field and DAPI images were obtained. The percentage of senescent AT-MSC was calculated as the total number of positive β-gal AT-MSC divided by the total number of AT-MSC counted (>100 AT-MSC counted) using the ImageJ software (U.S. National Institute of Health, Bethesda, MD). In addition, the following senescence markers were tested in EP- and LP-AT-MSCs by FACS: FSC, SSC, and autofluorescence.

2.1.3 Human WJ-MSCs: Procurement

A total of 6 clinical grade WJ-MSC samples were tested. One of the samples was obtained through a collaboration agreement between Saint-Louis Hospital, APHP, and the Research Institute of the McGill University Health Centre (RI-MUHC). That sample was from WJ-MSC and was produced for the validation tests prior to starting the MSC-SLE clinical trial (NCT03562065), coordinated by Dr Dominique Farge, St-Louis Hospital, AP-HP Paris-France. The other five samples were obtained through a collaboration agreement between the Polski Bank Komórek Macierzystych S.A, Warsaw-Poland and the RI-MUHC.

2.1.4 Human WJ-MSCs: Culture Conditions

Frozen vials of WJ-MSC were thawed and plated at 1 million cells per T-75 flask pre-coated with MSC attachment solution 1X. The following day, living cells were counted and re-plated also in pre-coated plates at a density of 3,333 cells per cm^2. WJ-MSC were cultured in alpha-Minimum Essential Medium (α-MEM, LONZA, Basel, SW) with 5%

human platelet lysate (hPL), 1% penicillin/streptomycin, and 1% GlutaMAX™ supplement. At 80% confluency, WJ-MSC were trypsinized and replated at the same initial density. WJ-MSC were subsequently characterized and tested for angiogenic assays at the end of P4.

2.1.5 MSC Immunophenotyping

EP-MSCs (P3-4) were treated with Fc receptor blocker and stained with fluorochrome-conjugated monoclonal antibodies against CD73 (phycoerythrin, PE), CD90 (fluorescein isothiocyanate, FITC), CD105 (allophycocyanin, APC), CD44 (APC), CD34 (APC), CD45 (FITC), CD20 (FITC), HLA-DR (APC), and CD14 (peridinin chlorophyll protein complex: CY5.5, PerCP-Cy5.5) for 45 minutes, after which they were analyzed in a BD LSRFortessa™ Flow Cytometer. MSC samples with over 95% surface expression of CD73, CD90, CD105 and CD44, and with less than 5% surface expression of CD14, CD20, CD34, CD45 and HLA-DR were functionally characterized in angiogenesis assays.

2.1.6 MSC Tri-lineage Differentiation

MSCs were plated in 24-wells at a density of 25,000 cells per cm^2 and incubated in the differentiation medium for osteogenesis, adipogenesis, and chondrogenesis for two-three weeks as recommended by the manufacturer [133-135]. Cells were fixed with 4% formaldehyde before staining with Alizarin Red S (osteogenesis) or Oil Red O (adipogenesis). Chondrogenic differentiation was induced in MSC micro mass (250,000 cell pellet), which were then sectioned and stained with Alcian Blue and Safranin O at the Histopathology Platform of the RI-MUHC. Tri-lineage differentiation was tested in a) pediatric/young and adult AT-MSCs and WJ-MSCs at P3; and b) EP- and LP-AT-MSCs.

2.1.7 MSC Conditioned Medium (CM) Preparation

MSCs were plated at $1x10^5$ cells/cm^2 per well in 6-well plates overnight, washed with PBS and cultured in Endothelial Growth Basal Medium-2 (EBM-2)/ 2% FBS for 72 hours. CM was collected, centrifuged to remove cell debris, and frozen in aliquots at -80˚C for further studies. After collection of MSC-CM, the number of MSCs were counted to ensure the lack of differential cell death between conditions (Figure 2A & B).

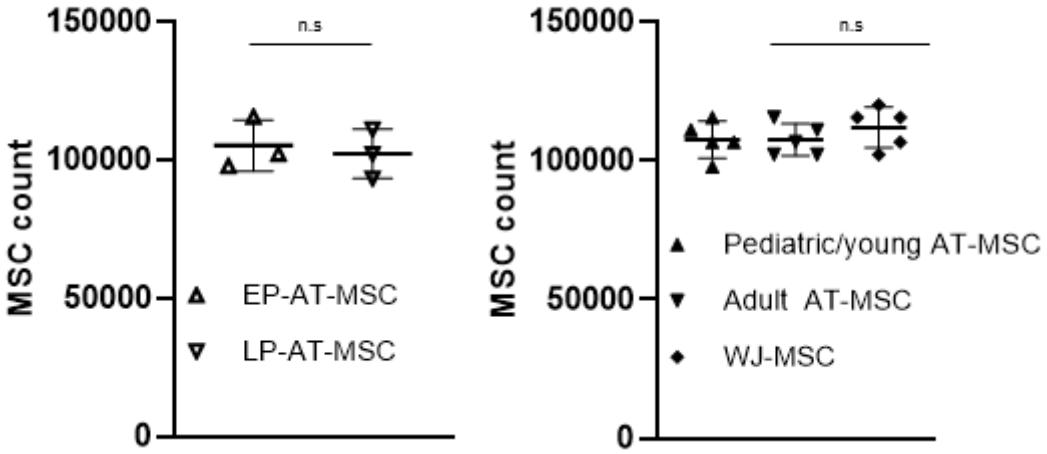


Figure 2. MSC count after collection of CM. No differential cell death was observed in (**A)** EP- and LP-AT-MSCs and **(B)** pediatric/young AT-MSC, adult AT-MSC, and WJ-MSC after 72 hours of generating CM. Abbreviations: CM, conditioned medium.

2.1.8 Immunopotency Assay (IPA)

IPA was performed as previously described in our lab [12]. Briefly, PBMCs were isolated from a single donor (23-year-old non-smoking healthy male) using Lymphocyte Separation Medium through density gradient centrifugation (Mediatech, Inc., Corning, Manassas, VA). The isolated PBMCs were cultured in Rosewell Park Memorial Institute medium (RPMI-1640, WISENT Inc., St. Bruno, QC) supplemented with 10% FBS and 1%

Penicillin Streptomycin in a T-75 flask overnight to deplete monocytes which are plastic adherent. On the same day, MSC were plated at 25,000 cells, 12,500 cells, and 6,250 cells/well in a flat bottom 96-well plate and left overnight.

Next day, the monocyte depleted PBMCs were stained with 0.145 uM of 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). Then, 200,000 activated and CFSE-stained PBMCs were added to 25,000 MSCs in cell-cell contact dependent or independent (trans-well) conditions, 12,500 MSCs, and 6,250 MSCs (1:8, 1:16, and 1:32 MSC:PBMC ratio respectively). For controls, activated and non-activated CFSE-stained PBMCs were cultured alone in a 96-well (maximal and minimal proliferation, respectively). After 72 hours, PBMCs were collected and stained with 7-aminoactinomycin D (7-AAD) (PE-Cy5.5) and anti-CD4 antibodies (APC) and analyzed by flow cytometry. The expansion index (E.I) of 7-AAD^{neg} CD4^{+} cells was determined with FlowJo software v9.7.2. The immunopotency of MSCs (% of CD4^{+} T-cell inhibition) was calculated using the formula below:

$$CD4\ T-cell\ inhibition\ (\%) = 100 - \left(\frac{E.I\ of\ interest - E.I\ of\ minimal\ proliferation)}{E.I\ of\ maximal\ proliferation - E.I\ of\ minimal\ proliferation)} \times 100\%\right)$$

2.1.9 Human Umbilical Cord Vein Endothelial Cells (HUVEC) Culture

HUVECs were purchased from Lonza at P2, and green fluorescent protein (GFP) transfected HUVEC (HUVEC-GFP) were purchased from Sartorius at P3. Before plating, a 75-cm^2 tissue culture flask was coated with 0.1% gelatin from porcine skin. HUVEC ± GFP were cultured in EBM-2, supplemented with EGM-2 Endothelial SingleQuots Kit [hydrocortisone, VEGF, human endothelial growth factor (hEGF), human fibroblast

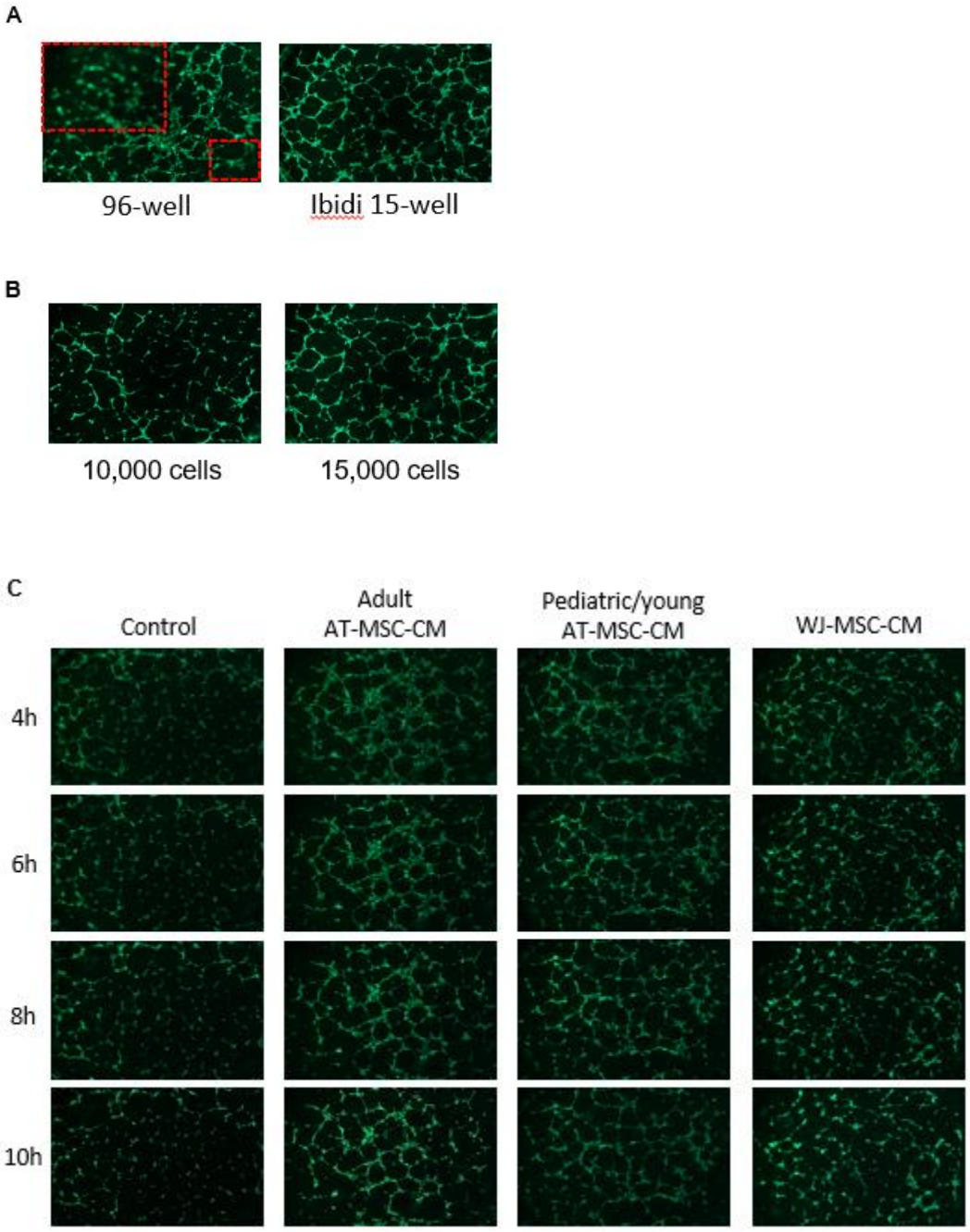
growth factor (hFGF), R3- IGF-1, ascorbic acid, gentamicin sulfate-Amphotericin (GA-1000), and heparin] (Lonza) and 2% FBS at a density of 2,500 cells per cm^2 [136].

2.1.10 MSC:HUVEC-GFP Co-culture Tube Formation Assay

This assay was modified from a study by Sarkanen et al. [137]. MSCs were plated at a density of $4x10^4$ cells per well in 24-well plates in EGM-2/ 2% FBS, together with HUVEC-GFP at a density of $2x10^4$ cells per well (2:1 ratio MSC:HUVEC). Images were obtained with an inverted fluorescent microscope every 72 hours until day 18 and analyzed with the Wimasis Image Analysis program. Viability [7-AAD (PE-Cy5.5)] of MSCs and HUVECs in co-culture was assessed at day 6 and 12 by flow cytometry [11].

2.1.11 *In vitro* Matrigel Tube Formation Assay

Prior to starting the experiment, ibidi u-slide 15 well angiogenesis plates were cooled to 4˚C. Phenol-red free GFR-Matrigel was used to avoid Matrigel, which is enriched with pro-angiogenic growth factors, overriding the effects of MSC-CM. GFR-Matrigel was thawed overnight on ice at 4˚C. An ibidi u-slide 15 well angiogenesis plate was used to improve the quality of the images (Figure 3A). The plate was coated using 10µl/well of GFR-Matrigel and incubated at 37˚C for 30 minutes [138]. HUVEC-GFP were re-suspended in EBM-2/2% FBS, plated at $1.5x10^4$ cells per 10µl per well (Figure 3B), and co-cultured with 40µl of MSC-CM per well. HUVEC-GFP were allowed to form tubes for 10 hours. The time of tube length assessment was optimized (Figure 3C), and images of wells were taken with an inverted fluorescent microscope. The images were analyzed using the Wimasis Image Analysis program [139].


A
96-well
Ibidi 15-well
B
10,000 cells
15,000 cells
C
Control
Adult AT-MSC-CM
Pediatric/young AT-MSC-CM
WJ-MSC-CM
4h
6h
8h
10h

D

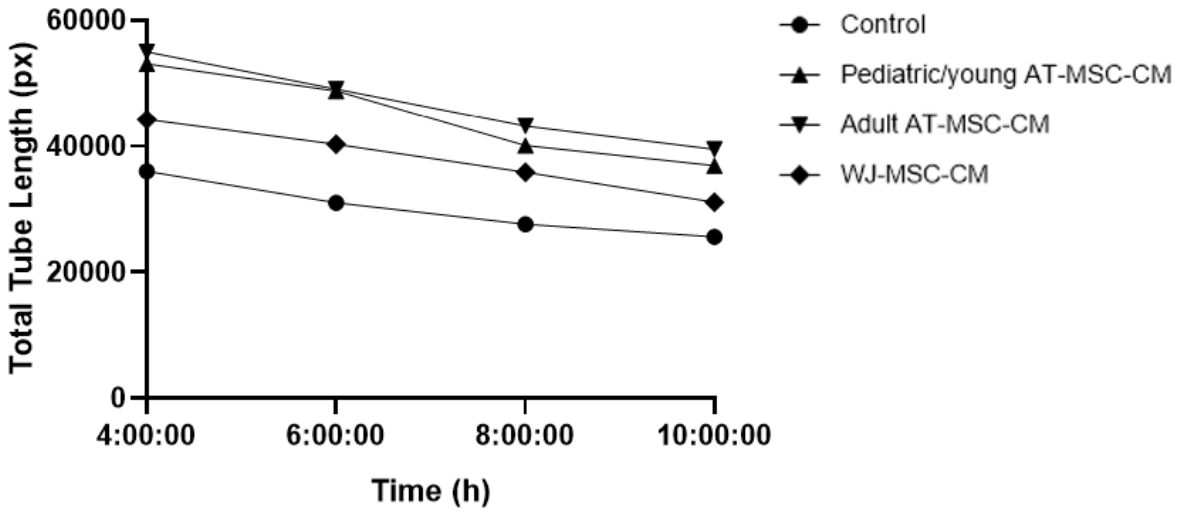


Figure 3. Optimization of *in vitro* Matrigel assay. (A) Meniscus of Matrigel in a standard 96-well plate was solved with a 15-well u-slide ibidi angiogenesis plate**. (B)** HUVEC number per well and **(C)** the time point of image acquisition (n=2) were optimized. **(D)** Summary data of time-point kinetics for total tube length promoted by AT- and WJ-MSC.

2.1.12 HUVEC Trans-well Migration Assay

This assay was performed following the methods described in a study by Potapova et al. [140] with minor modifications. HUVECs were synchronized in EBM-2/ 2% FBS for 14 hours. Then, $5x10^4$ HUVECs were plated on the top chamber of an 8.0µM pore sized cell culture Insert trans-well. The trans-well was placed in a well of a 24-well plate, filled with 750µl of MSC-CM. HUVECs were allowed to migrate for 18 hours. The trans-well insert membrane was fixed with 4% paraformaldehyde for 30 minutes and stained with 0.3µM DAPI solution. Images (10X magnification) of the migrated HUVECs were taken with an inverted fluorescent microscope (3 images per well, duplicate). The number of HUVECs that migrated was counted with the ImageJ software (U.S. National Institute of

Health, Bethesda, MD). The number of HUVECs after 18-hour incubation with MSC-CM was assessed using CountBright™ Absolute Counting Beads (Thermofisher) [141].

2.1.13 MSC-CM Angiogenic and Inflammatory Factors: Quantification

Aliquots of MSC-CM were tested in the Human Angiogenesis Array & Growth Factor 17-plex Array by Eve Technologies which assesses: Ang-2, bone morphogenetic protein-9 (BMP-9), EGF, Endoglin, Endothelin-1, FGF-1, FGF-2, Follistatin, granulocyte colony stimulating factor (G-CSF), heparin-binding EGF-like growth factor (HB-EGF), HGF, IL-8, Leptin, PLGF, VEGF-A, VEGF-C, and VEGF-D. Ang-1 was quantified by ELISA (R&D Systems).

2.1.14 Statistical Analysis

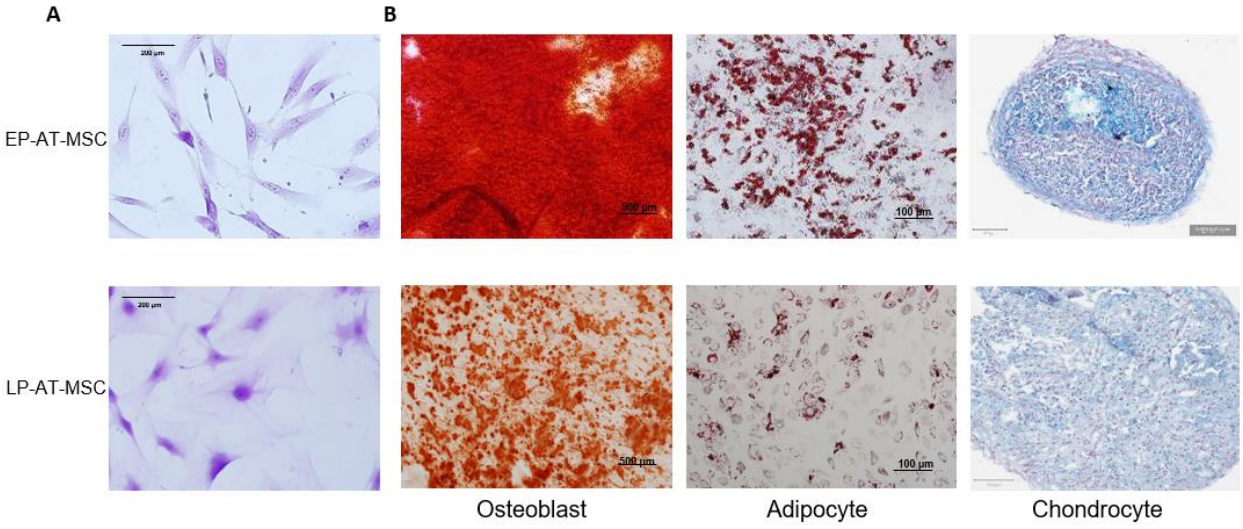
All statistical analyses were performed with GraphPad Prism software (Graph-Pad, San Diego, USA). Non-parametric analyses were used for all comparisons. Wilcoxon-signed-rank test and Mann-Whitney test were performed for paired (EP- vs. LP-AT-MSCs from the same donor) and unpaired (pediatric/young AT-MSCs vs. adult AT-MSCs vs. WJ-MSCs) analyses, respectively. A p-value of less than .05 was considered statistically significant.

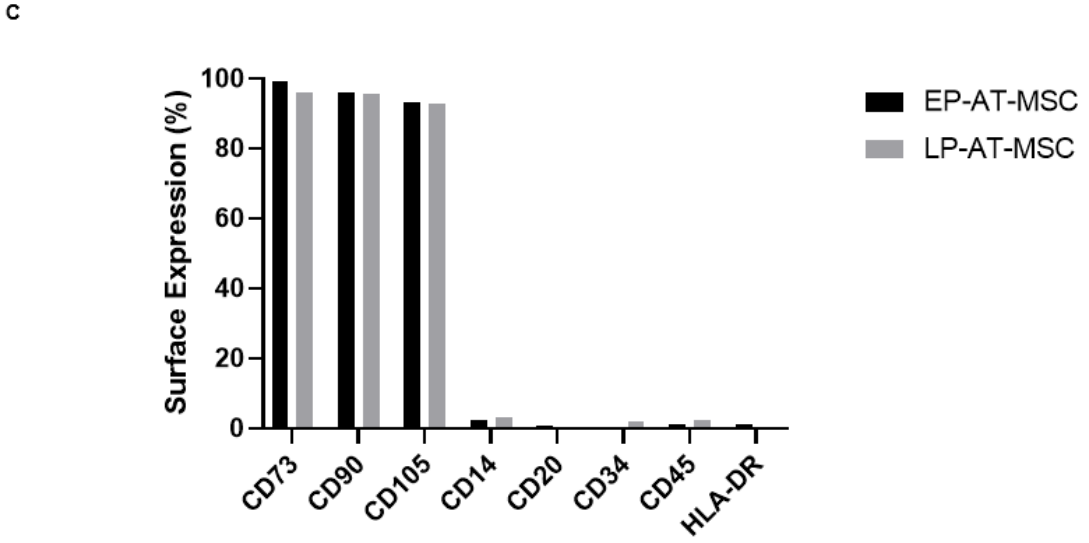
Chapter 3: Results

3.1.1 EP- vs. LP-AT-MSCs: Characterization

All EP- and LP-AT-MSCs tested fulfilled the ISCT minimal criteria for MSC [14]. MSCs adhered to plastic in standard culture conditions. Unlike EP-AT-MSCs that had a spindle-like shape, LP-AT-MSCs were larger and flat (Figure 4A). All MSCs differentiated *in vitro* into osteoblasts, adipocytes, and chondrocytes [positive for with Alizarin Red (calcium deposits), Oil Red O (lipid droplets), and Alcian Blue (sulfated proteoglycans), respectively] (Figure 4B). Surface expression of markers by flow cytometry confirmed that more than 95% EP- and LP-AT-MSCs were positive for CD73, CD90, and CD105, and that less than 3% were positive for CD14, CD20, CD34, CD45, and HLA-DR (Figure 4C). This proves that the induction of replicative senescence did not change the MSC phenotype as defined by the ISCT.

To confirm the senescent phenotype of LP-AT-MSCs, established senescence markers were assessed. Compared to EP-AT-MSCs, LP-AT-MSCs had higher FSC-A [geometric mean fluorescence intensity (gMFI) EP-AT-MSCs vs. LP-AT-MSCs; mean ± SD; 155750 ± 11650 vs. 183875 ± 25643, $p<0.01$] (Figure 4D) and SSC-A (mean ± SD; 78125 ± 11983 vs. 141188 ± 23499, $p<0.01$) (Figure 4E). Additionally, LP-AT-MSCs were more autofluorescent (gMFI EP-AT-MSCs vs. LP-AT-MSCs; mean ± SDL 254.6 ± 99.20 vs. 1356 ± 872.5, $p<0.01$) and had a higher frequency of β-gal positivity (percent of β-gal positive EP-AT-MSCs vs. LP-AT-MSCs; mean ± SD; 11.26 ± 12.06 vs. 47.29 ± 29.05, $p<0.01$) than EP-AT-MSCs (Figure 4F & 4G). All these features confirmed the presence of a senescent phenotype in LP-AT-MSCs.


A
B
EP-AT-MSC
LP-AT-MSC
Osteoblast
Adipocyte
Chondrocyte


C
Surface Expression (%)
EP-AT-MSC
LP-AT-MSC
CD73
CD90
CD105
CD14
CD20
CD34
CD45
HLA-DR

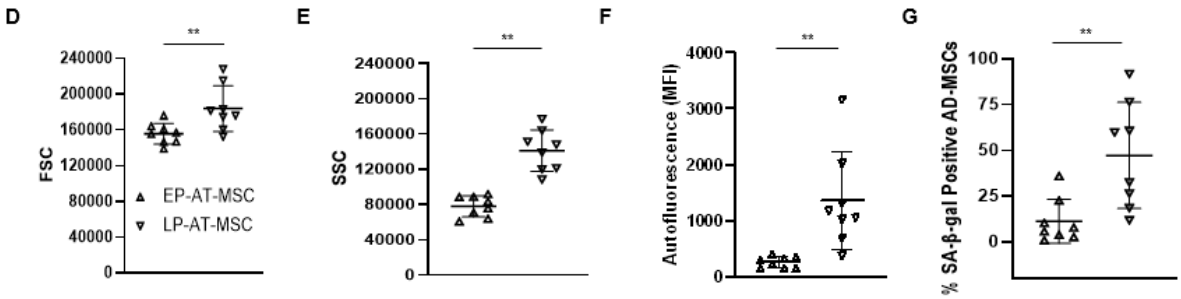

D
E
F
G
FSC
SSC
Autofluorescence (MFI)
% SA-β-gal Positive AD-MSCs
EP-AT-MSC
LP-AT-MSC

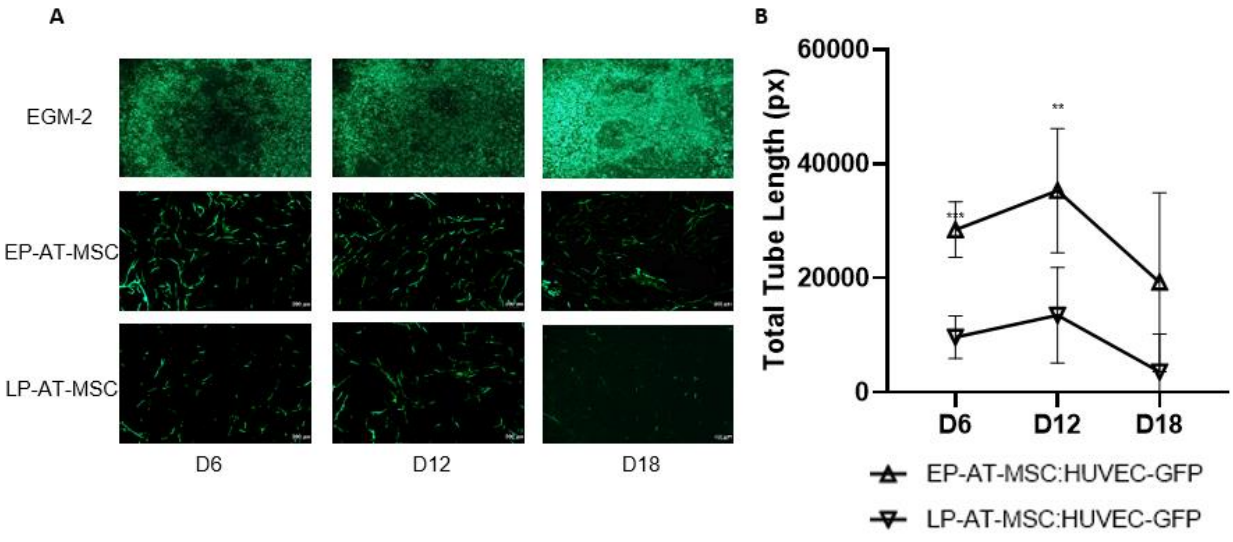
Figure 4. **MSC minimal criteria and senescence markers.** Adipose-tissue derived MSCs **(A)** adhere to plastic under standard culture conditions, **(B)** differentiate into osteoblasts (10x), adipocytes (10x), and chondrocytes (40x) *in vitro*, and **(C)** express CD73, CD90, and CD105 and lack expression of CD14, CD20, CD34, CD45, and HLA-DR. Traditional senescence markers in LP MSC included increased **(D)** forward scatter (FSC), **(E)** side scatter (SSC), **(F)** autofluorescence, and **(G)** percentage of positive β-gal cells. Abbreviations: EP-AT-MSC, early passage mesenchymal stromal cells; LP-AT-MSC, late passage mesenchymal stromal cells; FSC, forward scatter; SSC, side scatter; β-gal, β-galactosidase.

3.1.2 Replicative Senescent MSCs: *in vitro* Angiogenesis Assays

Three assays were optimized to test the effects of MSCs' replicative senescence on angiogenesis. First, we developed a long-term co-culture of MSC and HUVEC-GFP and assessed tube formation at day 6 (D6), D12, and D18. In HUVEC monocultures, tube formation was not induced (Figure 5A). In both EP- and LP-AT-MSC:HUVEC-GFP co-cultures, HUVEC tube formation (i.e., total tube length) increased up to D12 and decreased afterwards. However, the abundance and length of the tubes were greater in EP-AT-MSC:HUVEC-GFP co-cultures than in LP-AT-MSC:HUVEC-GFP co-cultures at D6 and D12 (EP- vs. LP-AT-MSC:HUVEC-GFP total tube length in pixels; mean ± SD; D6: 28496 ± 4869 vs. 9660 ± 3725, $p<.001$; D12: 35323 ± 10872 vs. 13527 ± 8345, $p<.01$) (Figure 5B).

We next simplified the system to determine if the differential effects of EP- and LP-AT-MSCs on tube formation and migration of HUVECs were due to differences in their secretome. In *in vitro* Matrigel tube formation assay, EP-AT-MSC-CM, but not LP-AT-

MSC-CM, promoted more HUVEC tube formation than the EBM-2 control. The total tube length induced by EP-AT-MSC-CM was greater than that induced by LP-AT-MSC-CM (EP- vs. LP-AT-MSC-CM total tube length percent increase compared to control; mean ± SD: 150 ± 15.80 vs. 119.1 ± 24.64, p<.05) (Figure 5C & 5D). These results were consistent with the findings of the MSC:HUVEC-GFP co-culture assay. Next, we assessed in a trans-well migration assay, the directional migration of HUVEC induced by MSC-CM. EP-AT-MSC-CM promoted more HUVEC migration than LP-AT-MSC-CM (EP- vs. LP-AT-MSC-CM percent increase compared to negative control; mean ± SD: 155.7 ± 22.11 vs. 110.5 ± 9.73, p<.001) (Figure 5E & 5F). These results were not explained by a differential death rate of HUVEC induced by MSC-CM (HUVEC cell count after incubation with EP- vs. LP-AT-MSC-CM; mean ± SD: 30465 ± 2316 vs. 29960 ±1579, p=ns) (Figure 6). Overall, the results of the three *in vitro* angiogenic assays performed, suggest that replicative senescence reduces the pro-angiogenic capacity of MSCs.



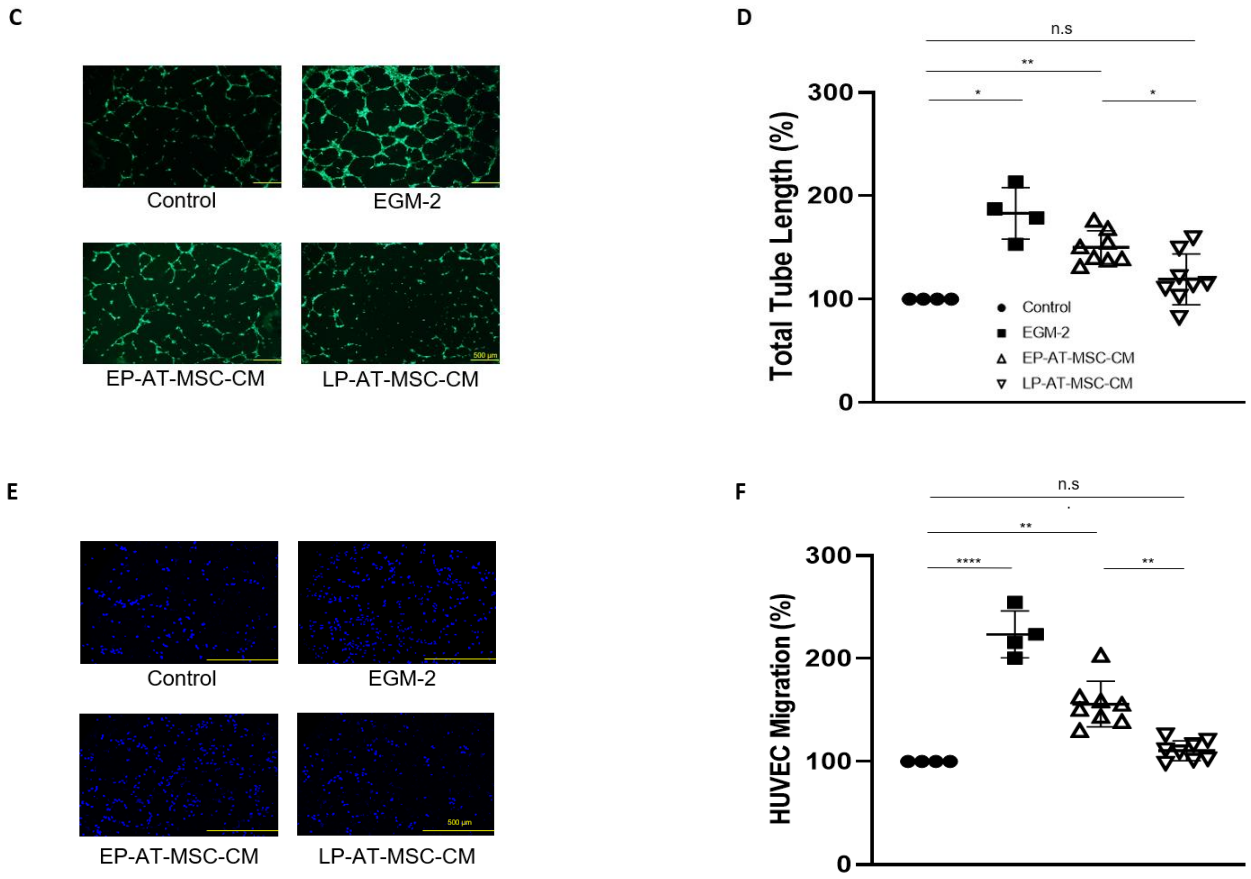


Figure 5. **Effect of replicative senescence on the angiogenic potential of AT-MSCs.** **(A)** Representative images (4x) of HUVEC-GFP monoculture and MSC:HUVEC-GFP co-culture at day 6 (D6), D12, and D18, and **(B)** summary graph of total tube length per field of view at D6, D12, and D18 (4 fields/experiment/time point). **(C)** Representative images (4x) and **(D)** summary graph of *in vitro* Matrigel tube formation assay. **(E)** Representative images (10x) and **(F)** summary graph of HUVEC migration assay (n=8 for both EP- and LP-AT-MSC). *p<.05, **p<.01, ***p<.001. Abbreviations: MSC, mesenchymal stromal cells; HUVEC, human umbilical vein endothelial cells; GFP, green fluorescent protein; CM, conditioned medium.

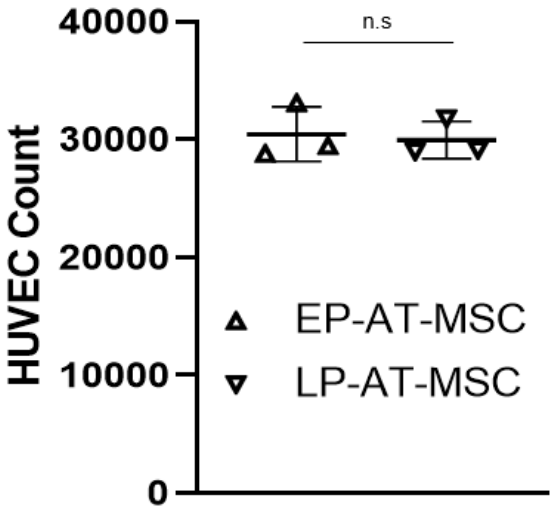


Figure 6. HUVEC count after incubation with EP- and LP-AT-MSC-CM. No differential cell death observed in HUVEC after exposure to AT- and WJ-MSC-CM for 18 hours.

3.1.3 EP- vs. LP-AT-MSC: Conditioned Medium

To explore mechanisms associated with the reduced pro-angiogenic effects of senescent MSCs, we determined the concentrations of pro-angiogenic factors in MSC-CM. BMP-9, endothelin, FGF-2, G-CSF, VEGF-D, HB-EGF, and leptin were below the threshold of detection. The levels of Ang-2, endoglin, EGF, FGF-1, HGF, IL-8, follistatin, VEGF-A, and VEGF-C were similar in EP- and LP-AT-MSC-CM. However, Ang-1 in EP-AT-MSC-CM was significantly higher than that in LP-AT-MSC-CM (EP- vs. LP-AT-MSC-CM concentration of Ang-1; mean ± SD: 331.8 ± 146 vs. 114.3 ± 37.49 pg/mL, $p<.05$) (Figure 7). These findings indicate that the impaired secretion of Ang-1 by replicative senescent MSCs may underlie their reduced pro-angiogenic potential.

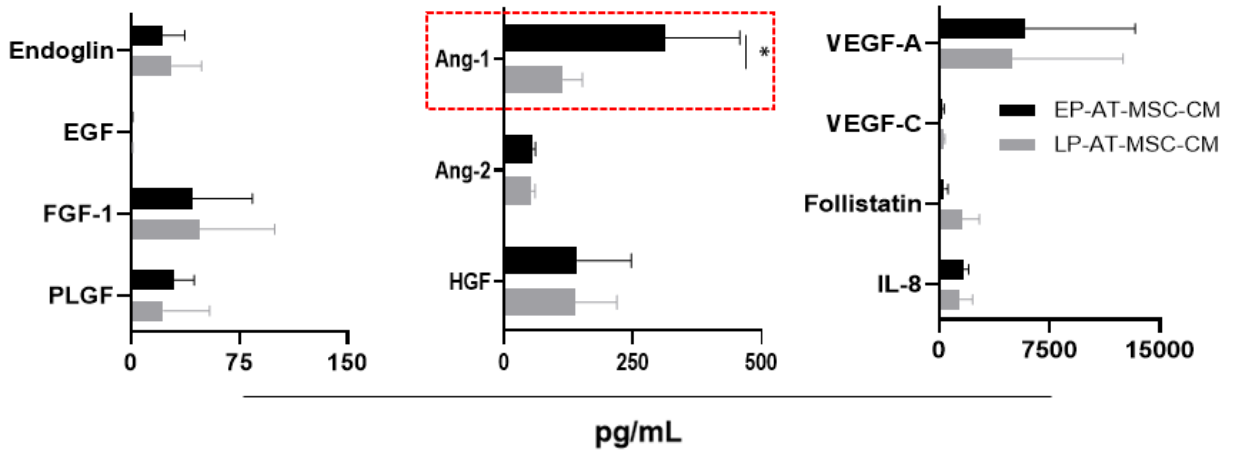


Figure 7. Comparison of CM from EP- and LP-AT-MSC. **(A)** Pro-angiogenic factors in EP- and LP-AT-MSC-CM were quantified by a multiplex assay. BMP-9, FGF-2, G-CSF, HB-EGF, Leptin, and VEGF-D were investigated, but were below the threshold of detection. *p<.05. Abbreviations: EGF, endothelial growth factor; FGF-1, fibroblast growth factor-1; PLGF, placental growth factor; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; HGF, hepatocyte growth factor; VEGF-A, vascular endothelial growth factor A; VEGF-C, vascular endothelial factor C; IL-8, interleukin-8.

3.1.4 WJ-MSC: Definition Criteria and Immunopotency

Prior to assessing the pro-angiogenic function of WJ-MSCs, we first confirmed that WJ-MSCs fulfilled the ISCT minimal criteria [14]. Under standard culture conditions, like AT-MSCs, WJ-MSCs adhered to plastic (Figure 8A), underwent tri-lineage differentiation (Figure 8B), and fulfilled the required surface phenotype (Figure 8C). In addition, WJ-MSCs lacked senescence markers and had similar FSC, SSC, and autofluorescence compared to EP-AT-MSC (Figure 8D-F). Moreover, following IFN-γ and TNF-α priming, WJ-MSC HLA-DR upregulation was lower than pediatric/young AT-MSC (WJ-MSC vs.

pediatric/young AT-MSC HLA-DR gMFI fold increase; mean ± SD: 4.44 ± 1.27 vs. 9.71 ± 2.44, p<.01) (Figure 9A & 9B). This was in contrast to AT-MSC from adult donors which expressed higher HLA-DR in their surface than pediatric/young ones (adult vs. pediatric/young AT-MSC HLA-DR gMFI fold increase; mean ± SD: 24.60 ± 6.25 vs. 9.71 ± 2.44, p<.01). These findings suggest the existence of differences in the immunogenicity of MSC according to their source and donor's age.

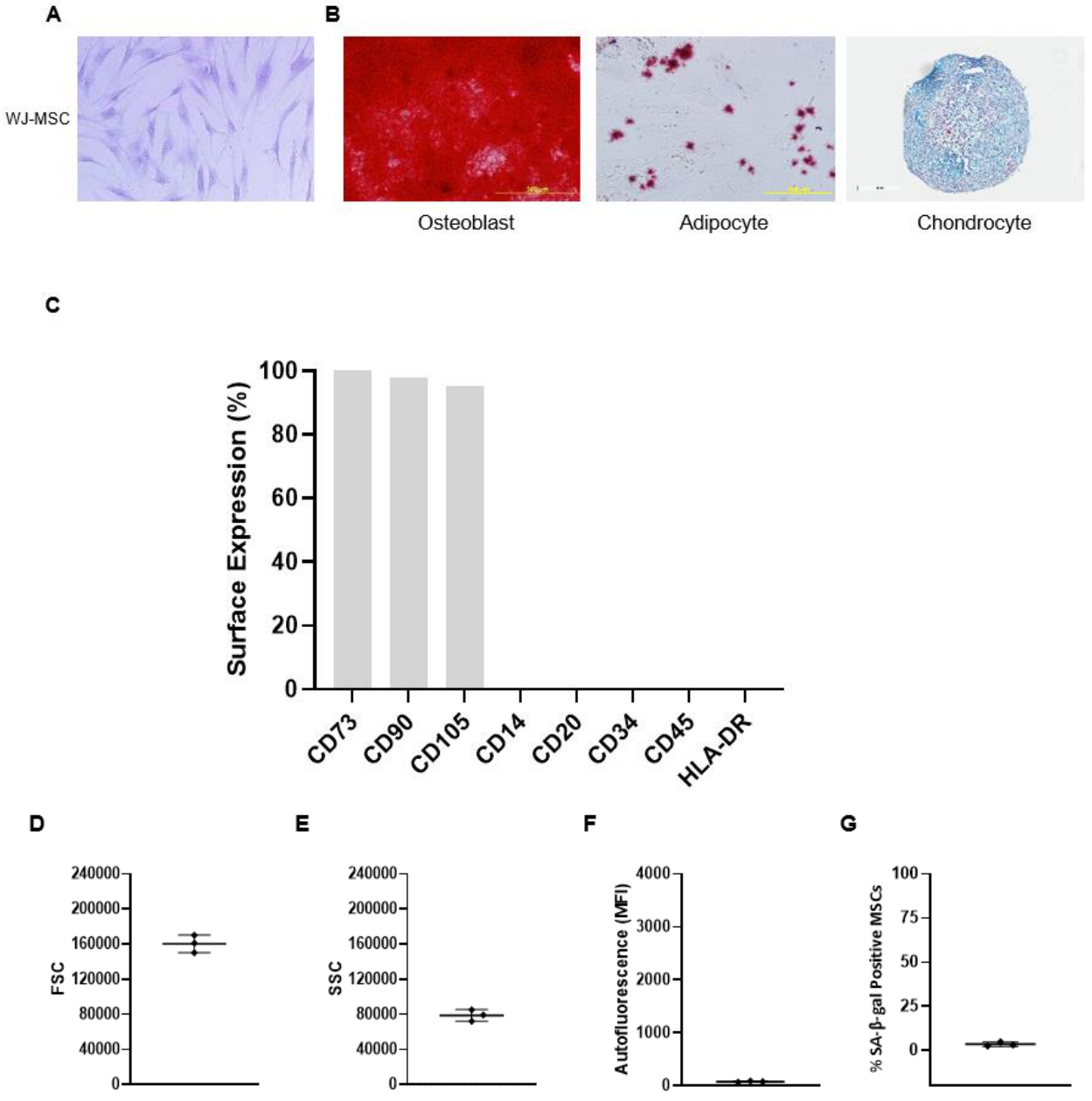


Figure 8. Characterization of WJ-MSCs. WJ-MSCs **(A)** adhere to plastic under standard culture conditions, **(B)** differentiate into osteoblasts, adipocyte, and chondrocytes *in vitro*, and **(C)** express CD73, CD90, and CD105 and lack the expression of CD14, CD20, CD34, CD45, and HLA-DR. Traditional senescence markers **(D)** FSC, **(E)** SSC, **(F)** autofluorescence, and **(G)** positive β-gal cells are not observed in WJ-MSCs. Abbreviations: WJ-MSC, Wharton's Jelly-derived MSC.

Next, we compared the immunopotency of MSCs from adult and pediatric/young AT-MSCs and WJ-MSCs. Both pediatric/young and adult AT-MSC and WJ-MSCs suppressed T-cell proliferation in a dose-dependent manner. There were no differences in immunopotency related to the MSC source (Figure 9D). To assess the relative importance of the MSC secretome versus cell-cell contact dependent mechanisms in mediating immunopotency, we performed IPA in cell-cell contact independent conditions using a trans-well system (MSC:PBMC ratio 1:8). We observed a reduction in the immunopotency in the trans-well system for all MSC subgroups. This suggests the relevance of cell-cell contact dependent mechanisms in this system. There were no differences in the immunopotency of AT- versus WJ-MSCs when tested in trans-well (Figure 9D). These findings support that under optimized culture conditions (i.e., using hPL in the media as well as coated plates) AT- and WJ-MSCs have similar immunomodulatory capacity primarily mediated by secreted factors.

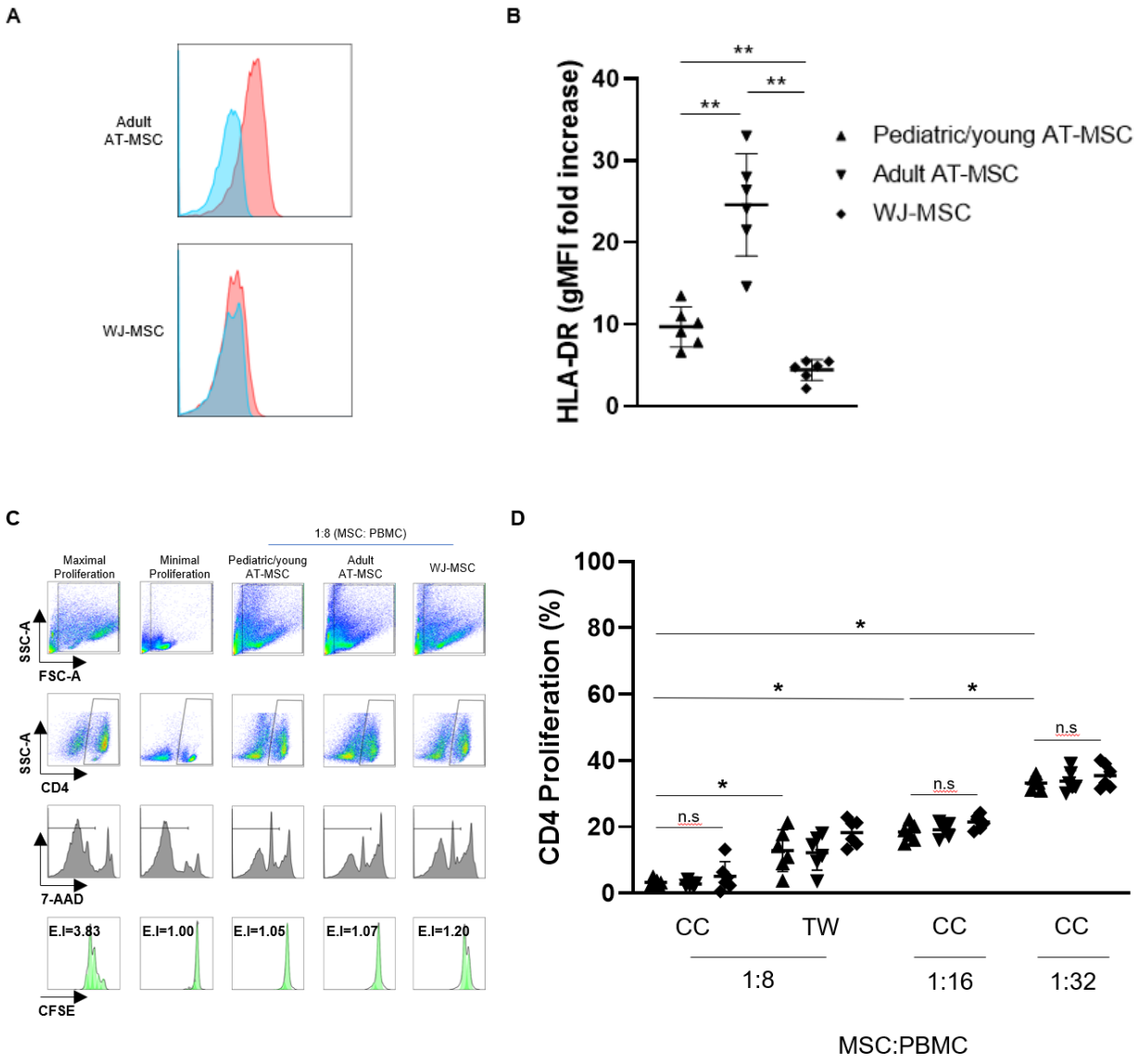


Figure 9. HLA-DR surface expression after cytokine priming and immunopotency of AT- vs. WJ-MSC. (A) Representative example of HLA-DR expression in resting (blue) and primed (red) MSCs and **(B)** summary graph of fold increase in HLA-DR gMFI (n=6 for all MSC groups). **(C)** Representative example of MSC IPA gating strategy and expansion index (E.I), and **(D)** summary graph of IPA in cell-cell contact dependent and independent conditions and different ratios of MSC:PBMC. *p<.05, **p<.01, ***p<.001, ****p<.0001. Abbreviations: gMFI, geometric mean fluorescence intensity; 7-AAD, 7-

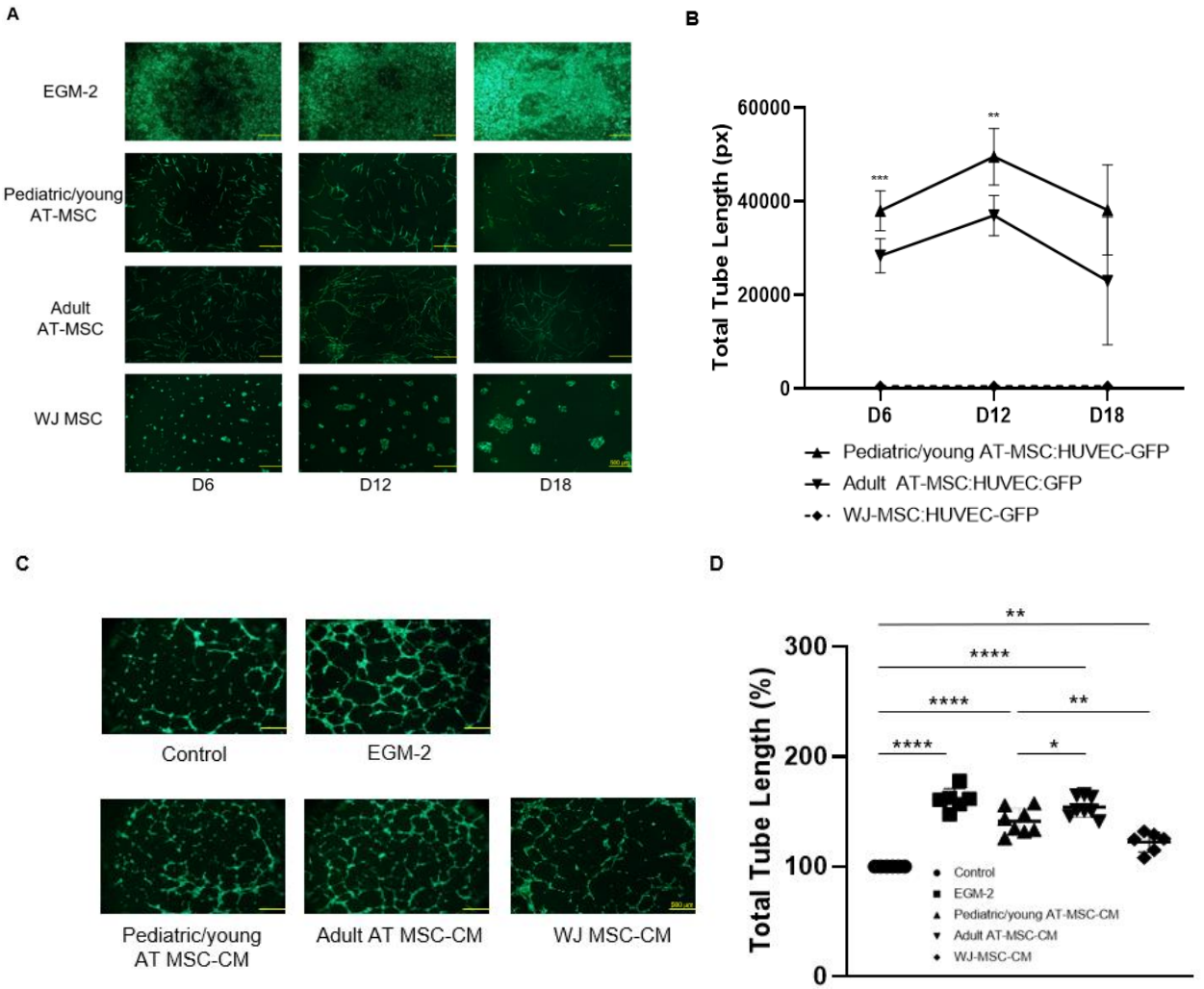
aminoactinomycin D; CFSE, carboxyfluorescein succinimidyl ester; E.I., expansion index; CC, cell-cell contact condition; TW, trans-well condition.

3.1.5 Pediatric/Young and Adult AT-MSC: *in vitro* Angiogenesis Assays

In MSC:HUVEC-GFP co-culture assays, both pediatric/young and adult AT-MSCs induced HUVEC-GFP tube formation in contrast to WJ-MSCs. In the presence of WJ-MSCs, HUVECs aggregated into 'small islands' without forming tubes (Figure 10A). The abundance and length of the tubes were greater in adult MSC:HUVEC-GFP co-cultures than in pediatric/young ones at D6 and D12 (adult vs. pediatric/young AT-MSC:HUVEC-GFP total tube length in pixels mean ± SD; D6: 38019 ± 4267 vs. 28420 ± 3629, $p<.001$, & D12: 49558 ± 6023 vs. 36616 ± 4311, $p<.01$) (Figure 10A & 10B). Similar to EP- and LP-AT-MSC:HUVEC-GFP co-cultures, the total tube length in AT-MSC:HUVEC-GFP co-cultures increased until D12 and regressed afterwards (Figure 10B). The viability of MSCs and HUVECs in co-cultures did not differ at D6 and D12, ensuring that the differences in HUVEC tube formation was not confounded by differential cell death (Figure 11).

In the *in vitro* Matrigel tube formation assay, pediatric/young and adult AT-MSC-CM and WJ-MSC-CM induced tube formation of HUVECs. However, both pediatric/young and adult AT-MSC-CM were more effective at inducing tubes than WJ-MSC-CM. Additionally, adult AT-MSC-CM generated more tubes than pediatric/young AT-MSC-CM (adult vs. pediatric/young AT-MSC-CM total tube length percent increase compared to control; mean ± SD: 154.2 ± 0.13 vs. 141.3 ± 11.7, $p<.05$). This was consistent with the results of the AT-and WJ-MSC:HUVEC-GFP co-cultures (Figure 10C & 10D). Similarly, in trans-well HUVEC migration assay, pediatric/young AT-MSC-CM and WJ-MSC-CM induced the same extent of HUVEC migration which was lower than adult AT-MSC-CM

(adult vs. pediatric/young AT-MSC-CM & adult AT-MSC-CM vs. WJ-MSC-CM percent increase compared to control; mean ± SD: 177.1 ± 37.07 vs. 120.2 ± 27.49, p<.05, & 177.1 ± 37.07 vs. 120.0 ± 15.70, p<.01) (Figure 10E & 10F). This suggests that CM from adult AT-MSCs have increased pro-angiogenic effects compared to those of pediatric/young AT-MSCs and WJ-MSCs. Pediatric/young and adult AT-MSCs and WJ-MSCs also did not induce differential cell death of HUVECs after 18 hours of incubation (Figure 12), confirming that the differential pro-angiogenic effects of MSC-CM was not confounded by differential HUVEC cell death. Overall, these results suggest that increased donor's age augments the pro-angiogenic function of MSCs.



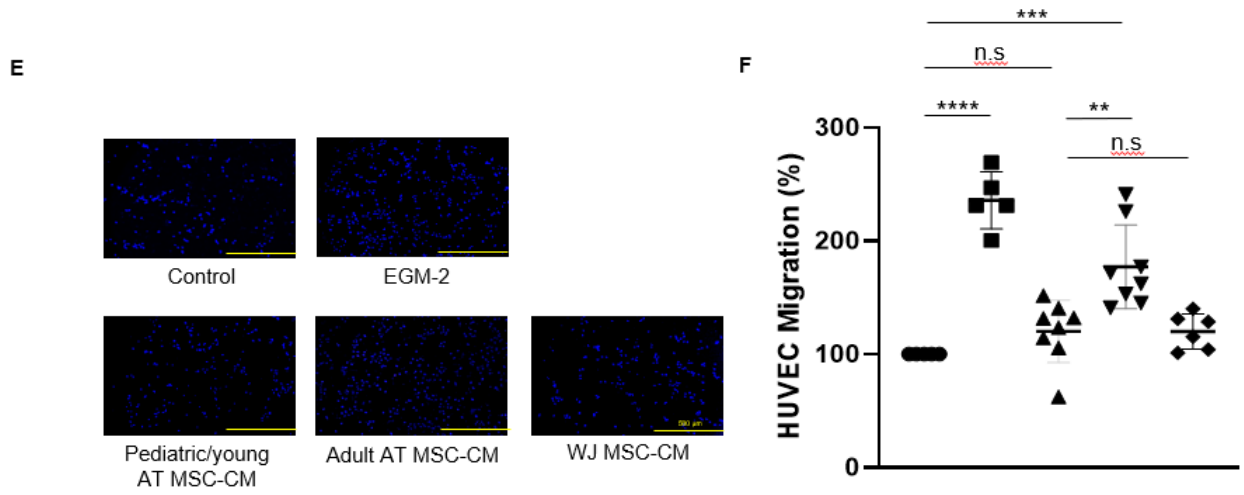


Figure 10. **Comparison of the angiogenic properties of AT- and WJ-MSC. (A)** Representative images (4x) of HUVEC-GFP monoculture and MSC:HUVEC-GFP co-culture at day 6 (D6), D12, and D18, and **(B)** summary graph of total tube length per field of view at D6, D12, and D18 (4 fields/experiment/time point). **(C)** Representative images (4x) and **(D)** summary graph of *in vitro* Matrigel tube formation assay. **(E)** Representative images (10x) and **(F)** summary graph of HUVEC migration assay (n=8 for both pediatric/young and adult AT-MSC and n=6 for WJ-MSC). *p<.05, **p<.01, ***p<.001, ****p<.0001

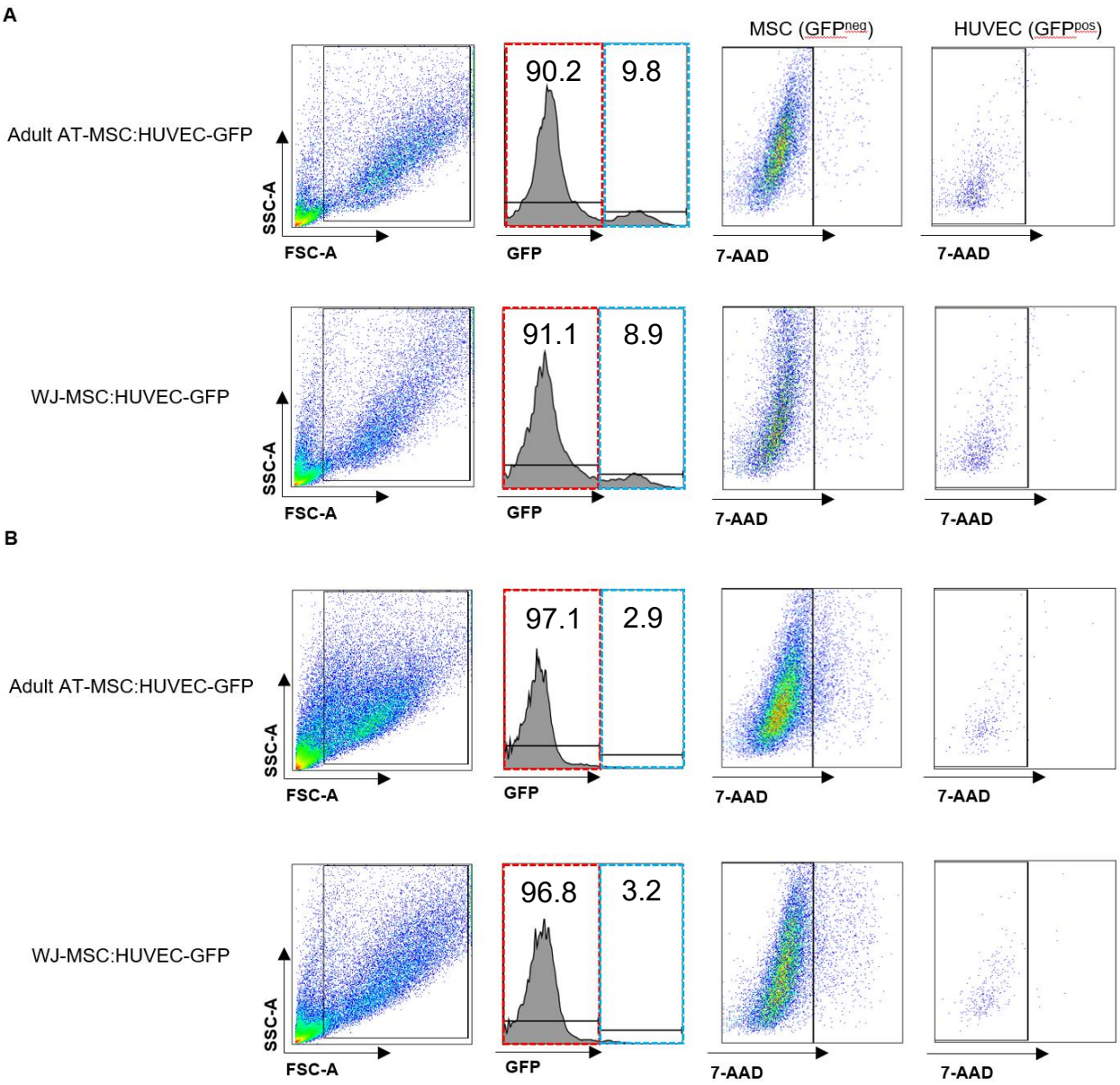


Figure 11. **Viability of MSCs and HUVEC in MSC:HUVEC co-culture**. Assessment of MSC and HUVEC viability at **(A**) day 6 (D6) and **(B)** D12 of MSC:HUVEC-GFP co-cultures. n=1.

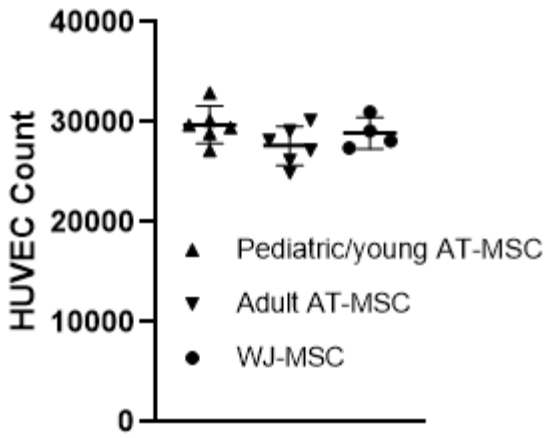


Figure 12. HUVEC count after incubation with AT- and WJ-MSC-CM. No differential cell death observed in HUVEC after exposure to AT- and WJ-MSC-CM for 18 hours.

3.1.6 AT- vs. WJ-MSCs: Conditioned Medium

To further characterize the differential pro-angiogenic effects of AT- and WJ-MSCs, we determined the concentration of pro-angiogenic factors in MSC-CM. BMP-9, endothelin, FGF-2, G-CSF, VEGF-D, HB-EGF, and leptin were below the detection threshold. However, the level of VEGF-A was higher while the level of Ang-1 lower in both pediatric/young and adult AT-MSC-CM than in WJ-MSC-CM (pediatric/young AT-MSC-CM vs. WJ-MSC-CM & adult AT-MSC-CM vs. WJ-MSC-CM; VEGF-A: 3383 ± 5797 vs. 3.65 ± 0.31 pg/ml, $p<.001$, & 5121 ± 6811 vs. 3.65 ± 0.31 pg/ml, $p<.001$; Ang-1: 255.5 ± 140.5 vs. 1058±670.5 pg/ml, $p<.01$, & 184.3 ± 213.4 vs. 1058±670.5 pg/ml, $p<.01$) (Figure 13A). This suggests an association between the lower angiogenic potential of WJ-MSCs, and their decreased secretion of VEGF-A.

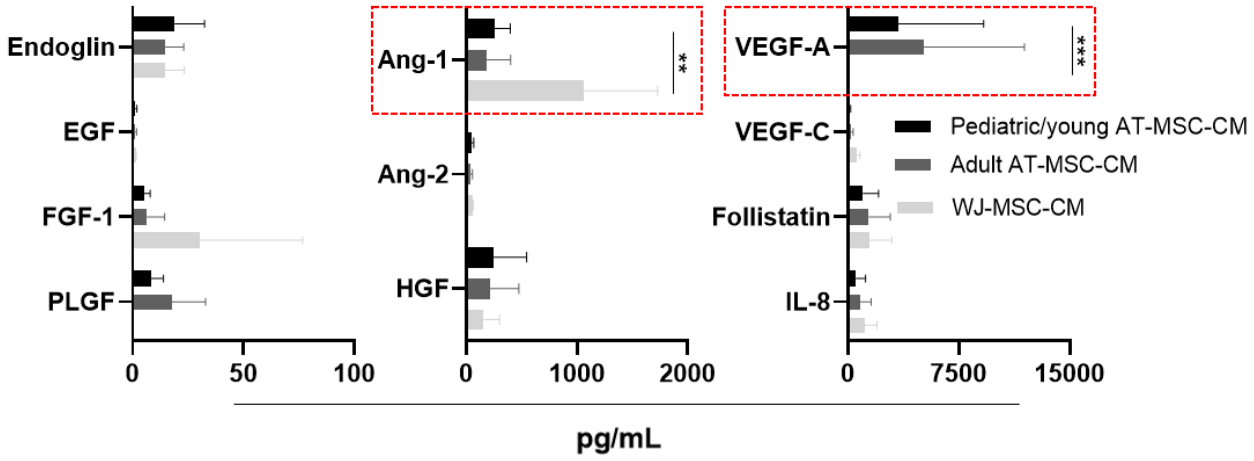


Figure 13. Characterization of AT- and WJ-MSC-CM. Pro-angiogenic factors in AT-MSC and WJ-MSC-CM were quantified by a multiplex assay. Other factors including BMP-9, FGF-2, G-CSF, HB-EGF, Leptin, and VEGF-D were investigated, but were below the threshold of detection (n=8 for both pediatric/young and adult AT-MSC and n=6 for WJ-MSC).**p<.01, ***p<.001.

Chapter 4: Discussion

The pro-angiogenic effects of MSCs provide the rationale for their use in clinical trials as therapeutic agents for vasculopathies. However, it is unknown whether replicative senescence, MSC donor's age, and MSC source impact the pro-angiogenic properties of MSCs. This is in part due to the lack of consensus on the optimal angiogenic functional assays to assess MSCs, which contrasts with the specific recommendations from the ISCT on assays to characterize MSC immunomodulatory properties [39, 40]. Informed by a recent consensus paper [87], we optimized three *in vitro* angiogenesis assays. We next tested the impact of replicative senescence, MSC donor's age, and MSC source on MSC angiogenic properties (Table 4). While replicative senescence decreased the pro-angiogenic function of MSCs, MSCs from older donors had an increased angiogenic potential. Furthermore, we demonstrated that MSCs from birth-associated tissue (WJ-MSCs) were less pro-angiogenic than those from adult tissue (AT-MSCs).

		Replicative Senescence (AT-MSC)	Aging (AT-MSC)	Source (AT-MSC vs. WJ-MSC)
Immunomodulation	IPA		=	=
Angiogenesis Assays	Migration	↓	↑	↑
	Tube Formation (*In* vitro Matrigel assay)	↓	↑	↑
	Tube Formation (co-culture assay)	↓	↑	↑

Table 4. Comparison of MSC's angiogenic properties: effect of replicative senescence, MSC donor's age, and MSC source.

Cellular senescence is implicated in physiological processes and promotes a wide spectrum of age-related diseases. Senescent cells are involved from embryogenesis, where they contribute to tissue development, to adulthood, where they promote tissue repair and tumor suppression [96]. Specifically, senescence in ECs impairs angiogenesis and is pathogenically related to prevalent vasculopathies [142, 143]. Whether senescence reduces the angiogenic capacity of MSCs is not well established. We showed that in a MSC-HUVEC co-culture system, LP-AT-MSCs are less efficient at promoting tube formation (i.e., reduced tube number and length) than EP-AT-MSCs. These findings were replicated using MSC-CM, suggesting that senescence impairs the pro-angiogenic properties of MSCs and that this is mediated by MSC-secreted soluble factors. These data contribute to and are consistent with the limited evidence on the effects of senescence on the angiogenic properties of human MSCs. A previous study compared AT-MSCs (n=10) from pre-eclampsia patients to those of healthy controls (n=12). In pre-eclampsia, a condition associated with cellular senescence [127], MSCs had hallmarks of senescence, and in MSC:Fibroblast:HUVEC-GFP co-cultures, MSCs had reduced tube formation capacity [128]. A more recent study, using an *ex-vivo* angiogenesis assay (i.e., chorioallantoic membrane assay), confirmed that replicative senescent AT-MSCs (n=5 female donors) had decreased capacity to promote EC tube formation [129]. This study did not show differential pro-angiogenic effects in *in vitro* migration (scratch wound healing assay) and Matrigel tube formation assays between senescent and non-senescent MSCs. Multiple factors may account for the differences in the results between these studies and ours including technical issues (e.g., number of ECs, time of image acquisition, dilution of MSC-CM) and the type of assay selected (i.e.,

scratch assay versus trans-well migration assay). These differences further highlight the importance of standardized angiogenesis assays for comparison of results across studies.

Changes in the composition of the secretome are a hallmark of senescence and mediate functional transformations of senescent cells. Specifically, the SASP of senescent AT-MSCs facilitates their reduced immunopotency [144]. In our work, MSC-CM recapitulated the differences in the angiogenic properties of senescent MSCs, suggesting a differential composition of EP- and LP-AT-MSC-CM. We documented a reduction in secreted Ang-1 by senescent MSCs which may contribute to their impaired pro-angiogenic function. Ang-1 is a key pro-angiogenic molecule required for stabilization of vessels. It also promotes survival and proliferation of ECs and is actively secreted by healthy MSCs from different sources [25]. Although our findings are consistent with previous studies [128, 129], they are in disagreement with a recent report that did not find differences in Ang-1 levels in CM from senescent and non-senescent MSCs [129]. The number of MSCs used and conditions to generate CM were not specified in that paper. In addition, differences may derive from the type of assay used to detect Ang-1, while the multiplex assay that we used is a quantitative test, the proteome profiler kit used by Ratushnyy et al. is semiquantitative [129]. In summary, our data suggest that senescence impairs the pro-angiogenic properties of MSCs and that the reduced secretion of Ang-1 may mediate the impaired pro-angiogenic function of senescent MSCs.

MSCs can be isolated from birth-associated and adult tissues; however, the resulting MSCs have functional differences. For example, AT-MSCs have a greater proliferative capacity [23] and lower immunogenicity [24] compared to BM-MSCs. Other studies also describe differences in MSCs according to their source and MSC donor's

age [11, 12, 19, 24, 32]. We observed that the upregulation of surface HLA-DR post-priming is higher in AT-MSCs than in WJ-MSCs; and in adult AT-MSCs compared to pediatric/young AT-MSCs. The lower surface HLA-DR in WJ-MSCs post-IFN-γ stimulation was previously described [33]. This could result in a reduced risk of allogenic immune responses after repeated administration of WJ-MSCs. However, these differences in HLA-DR expression after exposure to pro-inflammatory signals were not associated with a reduction in the inhibition of T-cell proliferation when co-cultured with WJ-MSCs. The increased HLA-DR expression in adult AT-MSCs compared to pediatric/young AT-MSCs was also not associated with differences in the immunopotency of MSCs. In a previous report from our laboratory, impaired T-cell suppression was observed in MSCs from elderly donors [12]. This discrepancy is explained by differences in culture conditions. In my work, I used medium supplemented with hPL to co-culture MSCs and activated PBMCs unlike in the experiments previously reported, where media with FBS was used [12]. The switch to a non-FBS media was done in order to accelerate the translation of results into clinic (i.e., the use of FBS in media is not allowed when MSC will be used clinically). hPL is a potent stimulator of MSC proliferation due to its enriched content of growth factors, cytokines, and EVs [145, 146]. The addition of hPL to the media provided an ‘ideal’ environment enhancing the immunosuppressive function of all MSCs, which masked functional differences in their immunomodulatory effects. This key finding reinforces the concept that the optimization of culture conditions (i.e., use of hPL) can overcome/mask *in vitro* functional variations between different MSC sources or MSC donor groups.

Aging and senescence are not synonymous [147]. Cells can undergo senescence, regardless of organismal age. However, accumulation of senescent cell populations in aged tissues and aging-associated diseases suggest that aging and senescence are closely related processes [99, 100]. The SASP composition, a key marker of cellular senescence, varies depending on the duration of senescence, senescence stimulus, and cell type [147]. Defining the senescent secretome in each biological context helps identify of replicative senescence, MSCs from adult donors had higher pro-angiogenic activity (i.e., EC migration and tube formation) than those MSCs from pediatric/young donors. Only few studies compared the angiogenic properties of MSCs from young and old donors. In one study, AT-MSCs from old donors secreted lower VEGF-A and higher ROS and were less efficient in treating mice with hindlimb ischemia than MSCs from young donors [130]. In another study by Nakamura et al., AT-MSC donor's age did not affect their angiogenic potential [132]. Specifically, in a mixed tube formation assay of HUVEC and fibroblasts, MSC-CM from young and old donors had similar effects in inducing tube formation. This may again be explained by technical differences in the assays. Nakamura's study evaluated the effects of MSC-CM on a tube formation assay, containing an additional cell population (i.e., fibroblasts). In contrast, we used a direct MSC:HUVEC co-culture system. In summary, technical differences, lack of standardization in angiogenesis assays, and limited previous data are potential factors that account for 'contradictory/insufficient' evidence and lack of agreement on the effects of aging on the pro-angiogenic function of MSCs. Upon further assessment, we did not observe differences in the concentration of pro-angiogenic factors between pediatric/young and adult AT-MSC-CM. Other molecules not assessed in my work [e.g.,

anti-angiogenic factors (thrombospondin-1 and plasminogen activator inhibitor-1)] may account for the higher *in vitro* pro-angiogenic effects of adult AT-MSCs. The enhanced angiogenic effects of adult AT-MSCs compared to pediatric/young ones can reflect an age-associated compensatory mechanism. In the elderly, decreased expression of VEGF [148] and injured tissues require increased angiogenesis, and in turn, MSCs may need to enhance their pro-angiogenic activity as key regulators of vascular homeostasis. In other cases, this compensatory mechanism may be maladaptive (e.g., promoting tumor growth). Overall, my results suggest that increased donor's age is associated with enhanced *in vitro* pro-angiogenic effects of MSCs.

WJ of the umbilical cord is a source of MSCs increasingly used in clinical studies [31], as MSCs from birth-associated tissues contain more clonogenic cell subpopulations than MSCs derived from adult tissues [149]. Supporting the reduced pro-angiogenic capacity of MSCs associated with earlier age (i.e., pediatric/young samples), WJ-MSCs were less efficient than AT-MSCs in promoting angiogenesis. Using a mouse model of dermal regeneration, Edwards et al. observed that WJ-MSCs were more efficient than AT-MSCs at promoting angiogenesis [36]. These findings apparently contradict ours; however, it is important to highlight that *in vivo* findings do not always recapitulate the results of *in vitro* experiments or even those seen in *in human* studies. What is the best surrogate of the *in human* MSC function remains to be determined. This will allow the appropriate selection of MSCs for specific clinical scenarios. The differential pro-angiogenic effects of AT- and WJ-MSCs in my work could be related to the concentrations of VEGF-A and Ang-1 in the secretome. Similar to our results, previous studies described low/undetectable VEGF-A levels in WJ-MSC secretome [25, 150, 151]. VEGF-A is a key

member of VEGF family that stimulates proliferation, migration, and tube formation of ECs. In my work, WJ-MSCs, that secreted very low levels of VEGF-A, induced less migration and tube formation compared to AT-MSCs in *in vitro* angiogenesis assays. The increased concentration of Ang-1 in WJ-MSCs secretome in comparison to that of AT-MSCs may be an unsuccessful attempt to compensate for the reduction in VEGF-A [152, 153]. A recent study suggests that the Sonic Hedgehog signaling pathway enhances the angiogenic potential of WJ-MSCs by upregulating the expression of Ang-1 [154]. Differences in the Sonic Hedgehog signaling pathway in AT- and WJ-MSCs may underlie the distinct pro-angiogenic potential of both MSC sources.

Several limitations should be considered in the interpretation of our results. First, pediatric/young and adult AT-MSCs samples were pooled to study the effects of replicative senescence on angiogenesis. These two sources of AT-MSCs show different proliferative capacities as adult AT-MSCs proliferate at a slower rate and reach senescence faster. To overcome this issue, we defined replicative senescence based on the presence of multiple senescence markers (i.e., FSC, SSC, autofluorescence, and SA-β-gal positivity) [102, 106] instead of relying only on *in vitro* population doubling and passage number of cells. Another aspect to consider is that the levels of pro-angiogenic factors in MSC-CM were measured using commercial multiplex arrays and ELISAs. Not every factor involved in angiogenesis were tested in those assays. Anti-angiogenic factors including thrombospondin-1 and plasminogen activator inhibitor-1 may also be differentially secreted, affecting the outcome of the *in vitro* assays performed. Moreover, recent studies suggest that EVs mediate the pro-angiogenic function of MSCs through the delivery of pro-angiogenic miRNA [75-79]. For example, miRNA-31 in EVs-secreted

by MSCs targets and suppresses factor-inhibiting HIF-1 in ECs which in turn express higher levels of VEGF [76]. Therefore, a differential vesiculation or micro-RNA content in vesicles from AT- and WJ-MSC can contribute to their differential angiogenic properties. Further work is required to characterize other components of the MSC secretome that are implicated in angiogenesis. For the experiments I performed, CM from resting MSCs was used. Previous studies showed that the immunosuppressive function of MSCs is greatly enhanced, following IFN-γ and TNF-α priming [40, 41, 155]. Future studies are required to assess the effects of cytokine priming on the angiogenic potential of MSCs. Lastly, two-dimensional *in vitro* assays do not completely recapitulate the complex three-dimensional *in vivo* environment. Our results need to be confirmed using relevant animal models and in multi-dimensional *in vitro* assays.

Chapter 5: Conclusion

Through standardized *in vitro* assays, my work evaluated the effects of replicative senescence, donor's age, and tissue source on the angiogenic potential of human MSCs. The results presented indicate that replicative senescence reduced the pro-angiogenic function of MSCs. This was associated with a reduction in the secretion of Ang-1, a key pro-angiogenic factor, by LP-AT-MSCs. In contrast, MSCs from adult donors did not have an impaired angiogenic potential. Instead, increased donor's age enhanced the pro-angiogenic capacity of AT-MSCs *in vitro*. Moreover, WJ-MSCs, which derive from a birth-associated tissue, showed a lower pro-angiogenic potential and secreted less VEGF-A and higher Ang-1 compared to AT-MSCs. Future studies will explore whether those differences relate to distinct Shh signaling levels in AT- and WJ-MSCs. Taken together, my work emphasizes that instead of expanding MSCs *in vitro* until late passages, it is key to select early passage MSCs for the treatment of conditions that would benefit from the pro-angiogenic effects of MSCs. The discrepancy between the effects of senescence and chronological aging on the *in vitro* pro-angiogenic effects of MSCs are of interest. Whether the enhanced angiogenic properties *in vitro* of pediatric/young and adult AT-MSCs and WJ-MSCs represent a compensatory mechanism remains to be defined. Furthermore, the assessment on the effects of donor's age and MSC tissue source on angiogenesis remain to be evaluated *in vivo.*

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