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**Effects of secretomes from early and late passages of jaw
periosteum derived stem cells on neuronal differentiation**

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List of abbreviations

Central nervous system (CNS)
Peripheral nervous system (PNS)
Nerve growth factor (NGF)
Brain-derived neurotrophic factor (BDNF)
Glial cell line–derived neurotrophic factor (GDNF)
Extracellular matrix (ECM)
Mesenchymal stem/stromal cells (MSCs)
International Society for Cellular Therapy (ISCT)
Extracellular vesicles (EVs)
Jaw periosteum-derived mesenchymal stem cells (JPCs)
DNA damage response (DDR)
Reactive oxygen species (ROS)
Senescence-associated secretory phenotype (SASP)
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
C-C motif chemokine ligand 2 (CCL2)
C-C motif chemokine ligand 20 (CCL20)
Vascular endothelial growth factor (VEGF)
Transforming growth factor beta 1 (TGF- β 1)
Senescence-associated β -galactosidase (SA- β -gal)
Dulbecco's phosphate-buffered saline (DPBS)
Dimethyl sulfoxide (DMSO)
Induced pluripotent stem cells (iPSCs)
Essential 8™ Flex medium (E8 Flex)
Human platelet lysate (hPL)
Neural induction medium (NIM)

Neuronal basal medium (NBM)
Fetal bovine serum (FBS)
Cell counting kit-8 (CCK-8)
Senescence-associated β -galactosidase (SA- β -gal)
Enzyme-linked immunosorbent assay (ELISA)
Horseradish peroxidase (HRP)
Absolute log2 fold change ($|\log_2FC|$)
Differentially expressed proteins (DEPs)
Principal component analysis (PCA)
Gene Ontology (GO)
Kyoto Encyclopedia of Genes and Genomes (KEGG)
Bovine serum albumin (BSA)
Doublecortin (DCX)
Microtubule-associated protein 2 (MAP2)
Neurotrophin-3 (NT-3)
Transforming growth factor- β (TGF- β)
Bone morphogenetic protein (BMP)
Rho-associated protein kinase (ROCK)
Neurogenin-2 (NGN2)

1. Introduction

1.1 Neural regeneration and its clinical challenges

Injuries to the nervous system are a frequent and severe clinical problem and occur across diverse contexts, including traumatic brain injury, spinal cord injury, peripheral nerve transection, and complex orthopedic trauma[1-3]. In orthopedic practice, high-energy fractures, open fractures, and severe limb injuries are accompanied by damage to adjacent neural structures in up to 2.3% of cases[4]. While current therapeutic approaches for bone injury, such as internal fixation, autologous or allogeneic bone grafting, and biomaterial scaffolds with or without growth factors, are highly effective in restoring mechanical stability and osseous healing, they primarily emphasize structural reconstruction[2, 5, 6]. Consequently, concomitant neural damage is often inadequately addressed, leading to sensory loss, motor dysfunction, chronic pain, or irreversible neurological impairment[1, 2, 5].

Specific clinical strategies for neural injury depend heavily on the lesion's location and severity. Peripheral nerve injuries are commonly treated with microsurgical neurorrhaphy, autologous nerve grafting, or artificial nerve conduits[7-10], whereas central nervous system (CNS) injuries are mainly managed by decompression surgery, pharmacological therapy, and rehabilitation[11-14]. Although these approaches can partially restore function, their ability to promote consistent axonal regeneration and long-term functional recovery remains insufficient[7, 9-14]. Surgical reconstruction may be limited by donor-site morbidity, restricted graft availability, and incomplete integration with host tissues[7-10, 15]. Furthermore, large nerve gaps, extensive tissue damage, and severe trauma are frequently associated with incomplete and variable outcomes,

reflecting the inherently restricted regenerative capacity of the nervous system[7-10, 15, 16]. In the CNS, injured neurons exhibit minimal axonal regrowth and poor functional reconstruction due to intrinsic growth constraints and inhibitory extracellular signals [16-19]. Even in the peripheral nervous system (PNS), which possesses greater regenerative potential, functional restoration after large-segment defects or severe trauma is frequently insufficient[7-10, 15].

Neural regeneration is a complex process requiring neuronal survival, neurite extension, cellular differentiation, and the restoration of functional circuits[20-22]. At the molecular level, intrinsic regeneration relies on neurotrophic factors, such as NGF, BDNF, NT-3, and GDNF, which promote neurite elongation and Schwann cell proliferation primarily via intracellular cascades like the Trk-PI3K/Akt-mTOR and Trk-Ras/MAPK/ERK signaling pathways[20-23]. However, these intrinsic programs are frequently insufficient because of an inhibitory local microenvironment post-injury[24-28]. Persistent inflammatory cytokine signaling, growth-inhibitory extracellular matrix (ECM) remodeling, and the activation of stress-associated pathways collectively suppress regenerative capacity and sustain neurotoxic M1 macrophage polarization[24-28].

These extrinsic constraints directly impair regeneration at the cellular level by disrupting the cytoskeletal machinery responsible for neurite outgrowth[29-31]. Neurons extend neurites through dynamic growth cones at their leading edges, whose directional motility depends on coordinated actin and microtubule remodeling regulated by adhesion-dependent signaling[29-32]. Growth cones sense and translate ECM biochemical and biomechanical signals into cytoskeletal responses primarily through integrin-mediated adhesion signaling[30-33]. This signaling links ECM ligands (like laminin and fibronectin) to pathways driving actin polymerization[31-34]. Following injury, however, the

enrichment of inhibitory ECM components such as chondroitin sulfate proteoglycans (CSPGs) and tenascin-C, combined with persistent inflammatory signaling, disrupts integrin function[17, 18, 25, 31]. This triggers growth cone collapse or stalling, critically halting target reinnervation. Therefore, therapies that neutralize inflammatory cytokines and inhibitory ECM remodeling while enhancing integrin-dependent growth cone motility is essential for enhancing clinical neural repair[25, 31, 33, 35, 36].

1.2 Mesenchymal stem cells and their secretome in regenerative medicine

Mesenchymal stem/stromal cells (MSCs) are adult multipotent progenitor cells of mesodermal origin. According to the International Society for Cellular Therapy (ISCT), MSCs are characterized by plastic adherence under standard culture conditions, expression of CD73, CD90, and CD105, minimal expression of hematopoietic markers including CD45 and CD34, and the capacity for osteogenic, adipogenic, and chondrogenic differentiation in vitro[37]. Although initially identified in bone marrow, MSC-like populations have been isolated from multiple tissues, including adipose tissue, umbilical cord, periosteum[38, 39]. Owing to their immunomodulatory properties, regenerative potential, and relatively low immunogenicity, MSCs have been explored as a candidate cell source for tissue repair[38].

In preclinical models of tissue injury, such as bone, cartilage, tendon, and nerve injury, MSCs have been reported to support structural repair and functional recovery[40-43]. Increasing evidence suggests that these benefits are mediated largely by MSC-driven regulation of the injury microenvironment via paracrine mechanisms, rather than solely by their direct engraftment and differentiation into target cell types[44]. Mechanistically, MSC can modulate inflammatory responses,

reduce apoptosis and fibrosis, promote angiogenesis, and support activation of endogenous progenitor cells, thereby establishing conditions permissive for regeneration (**Figure 1**)[45-47]. This paracrine function is executed through the MSC secretome[45-47]. This secretome consists of both extracellular vesicles (such as exosomes) and a diverse array of soluble molecules, including cytokines, growth factors, and ECM proteins[48, 49]. In neural injury models specifically, MSC-derived neurotrophic and anti-inflammatory mediators have been closely associated with improved neuronal survival, enhanced neurite outgrowth, and functional recovery[20-23]. Consequently, the MSC secretome is increasingly recognized as a key modulator of the regenerative microenvironment in neural repair.

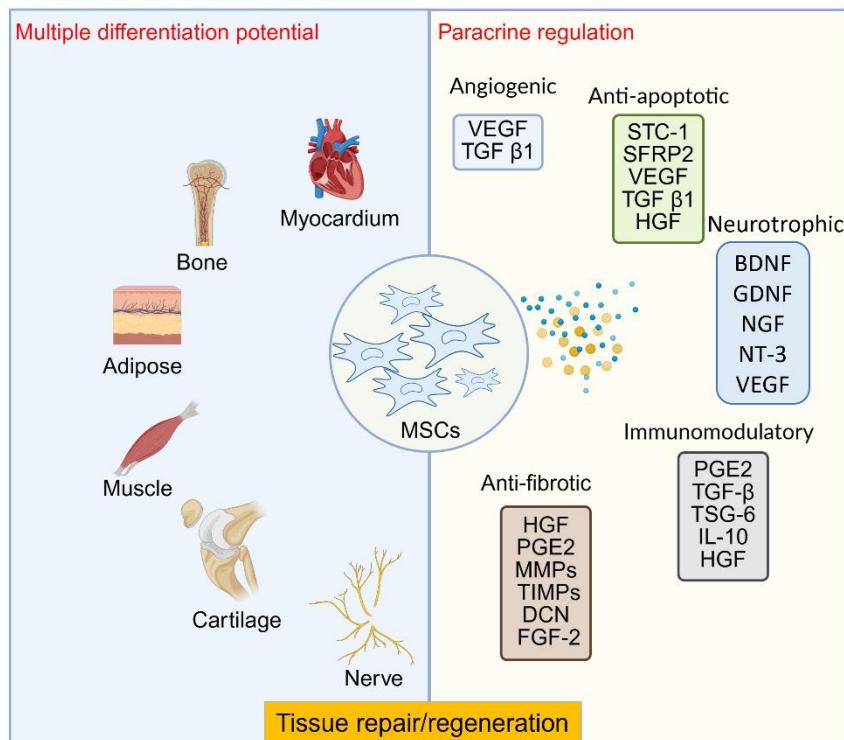


Figure 1. Mesenchymal stem cells (MSCs) in tissue repair and regeneration. MSCs contribute to regeneration through multipotent differentiation into multiple lineages, including osteogenic, adipogenic, myogenic, chondrogenic,

cardiomyogenic, and neurogenic fates, and through paracrine signaling that promotes angiogenesis, cell survival and proliferation, immunomodulation, and anti-fibrotic responses. Created with BioRender.com.

Compared with cell transplantation, secretome-based cell-free approaches offer significant translational advantages. They help mitigate risks linked to immune incompatibility, uncontrolled differentiation, and tumorigenicity, while allowing for concentration and standardization to improve therapeutic consistency and reproducibility[50]. However, the composition of the secretome is highly dynamic and inherently dependent on the tissue source, culture conditions, and cellular state[51, 52]. For instance, prolonged *in vitro* expansion or organismal aging can induce cellular senescence, profoundly reshaping the secretory profile and potentially compromising its regenerative efficacy[53, 54]. Therefore, defining how MSC states influence their secretome composition and its impact on neural regeneration is essential for optimizing these cell-free therapeutic strategies.

1.3 Jaw periosteum-derived mesenchymal stem cells and their secretome in regenerative medicine

Jaw periosteum-derived mesenchymal stem cells (JPCs) are a highly accessible population of adult mesenchymal stromal-like cells obtained from the jaw periosteum in the maxillofacial region[55]. Unlike MSCs derived from bone marrow or adipose tissue, JPCs can be easily harvested during routine oral surgeries. This anatomical advantage minimizes donor site morbidity and facilitates standardized cellular procurement[56].

Functionally, JPCs have been reported to display strong *in vitro* proliferative capacity and high osteogenic capacity[57-59]. Beyond bone formation, they exert potent immunomodulatory effects mediated primarily by their secretome. Specifically, JPC-derived extracellular factors inhibit dendritic cell maturation,

polarize macrophages toward an anti-inflammatory M2 phenotype, and suppress both CD4⁺ T cell activation and osteoclastogenesis[60-63]. Consequently, JPCs have gained significant attraction in bone tissue engineering. Despite their established benefits in bone healing, the specific composition of the JPC secretome and its potential to promote neural regeneration remain unexplored.

1.4 Cellular senescence and secretome remodeling

Cellular senescence was originally described as a stable, long-term proliferation arrest after repeated cell divisions in vitro, a phenomenon termed replicative senescence[64]. It is now recognized as a broader stress response triggered by telomere attrition, genotoxic and oxidative insults, oncogenic signaling, and mitochondrial dysfunction[65, 66]. Molecularly, this senescent state is driven by the sustained activation of cell-cycle regulatory pathways, particularly the p53/p21 and p16/Rb axes[52, 53, 66]. Furthermore, the activation of the DNA damage response (DDR) and elevated intracellular reactive oxygen species (ROS) can also contribute to the establishment and maintenance of the senescent phenotype (**Figure 2**)[67, 68].

Beyond intrinsic growth arrest, a defining hallmark of senescent cells is the alteration of their secretory profile. This transformation is known as the senescence-associated secretory phenotype (SASP). The SASP involves the increased release of inflammatory cytokines, chemokines, growth factors, and matrix-remodeling proteases[66, 69]. Although specific SASP profiles vary depending on the cellular context, commonly upregulated components include interleukin-6 (IL-6), interleukin-8 (IL-8), C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 20 (CCL20), vascular endothelial growth factor (VEGF), and transforming growth factor beta 1 (TGF- β 1)[70].

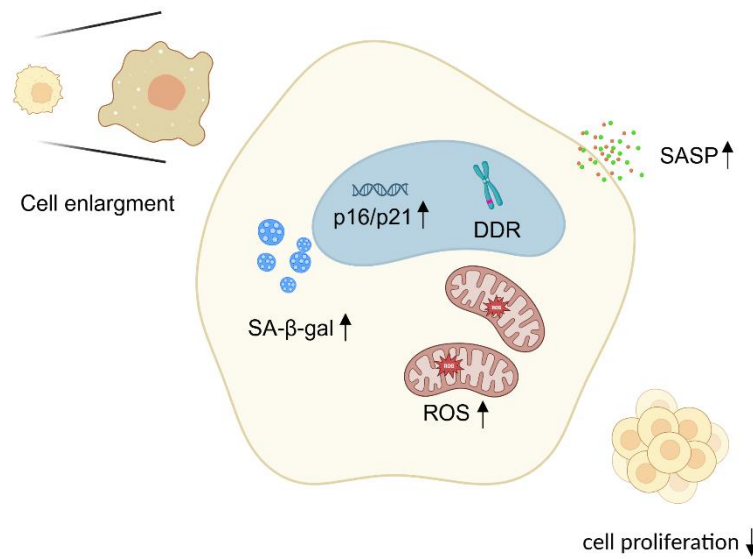


Figure 2. Key hallmarks of cellular senescence. Senescent cells exhibit enlarged morphology, persistent DNA damage (DDR) signaling with elevated p16/p21 leading to stable growth arrest, organelle dysfunction marked by increased mitochondrial reactive oxygen species (ROS) and lysosomal senescence-associated β -galactosidase (SA- β -gal) activity, and a senescence-associated secretory phenotype (SASP) that releases pro-inflammatory and tissue-remodeling factors into the microenvironment. Created with BioRender.com.

Through potent paracrine signaling, these SASP factors profoundly shape neighboring cell behaviors, modulate local inflammatory responses, and alter extracellular matrix dynamics[53, 66, 70]. In certain settings, they can even propagate senescence to adjacent healthy cells. Consequently, the progressive accumulation of senescent cells impairs tissue repair by driving persistent inflammatory signaling and hostile ECM remodeling within the local microenvironment[53, 69]. Ultimately, senescence is not merely a static state of cellular exhaustion but a dynamic process that reshapes the tissue microenvironment, potentially transforming a regenerative MSC secretome into an inhibitory one.

1.5 Knowledge gap and research aim

Although MSCs and their secretome have attracted considerable attention for neural repair, prior studies have largely focused on differences among tissue sources or the effects of exogenous conditioning on secretome composition[71-74]. For JPCs, which are increasingly considered for regenerative applications in bone tissue engineering, in vitro expansion is typically required to obtain sufficient cell numbers[59, 61]. This process inevitably promotes replicative senescence, accompanied by reduced proliferative kinetics, stable cell-cycle arrest, and upregulation of canonical markers such as p16 and p21[66, 68, 70]. Although senescent JPCs lose proliferative potential, they remain metabolically active and may exert substantial paracrine influence via altered secretome composition[70].

Given that neuronal differentiation and neurite extension are highly sensitive to ECM composition and integrin-mediated cytoskeletal regulation[25, 26, 30, 31], senescence-related secretome remodeling may alter ECM-associated signaling, inflammatory mediators, and neurotrophic support, thereby influencing growth cone dynamics and the stability of the regenerative microenvironment.

However, the specific characteristics of these secretome alterations during JPC senescence and how they impact neural differentiation-related pathways has not yet been studied. Addressing this gap is clinically relevant because a better understanding of how JPC senescence modifies their paracrine effects may support the development of cell-free therapeutic strategies for nerve regeneration, particularly in oral and maxillofacial surgery and orthopedic surgery, where peripheral nerve injury can occur after trauma surgery, reconstructive procedures, or tumor resection[4, 75]. Therefore, the present study aims to systematically compare the secretome of early-passage (non-senescent) and late-passage

(senescent) JPCs to evaluate their effects on neural regeneration. The specific objectives are:

1. To characterize the senescence phenotype of JPCs across progressive passage stages.
2. To perform comparative proteomic profiling of secretomes derived from non-senescent and senescent JPCs, identifying differentially expressed proteins (DEPs) and enriched pathways.
3. To correlate secretome remodeling with senescence-related signaling, linking proteomic alterations to specific senescence-associated molecular programs.
4. To generate induced neurons from iPSCs using a Neurogenin-2 (NGN2) induction protocol.
5. To assess functional effects of JPC secretomes from distinct senescence states on neural survival, neurite extension and neuronal differentiation.

A schematic overview of the experimental workflow for assessing JPC senescence and the effects of the JPC secretome on neuronal regeneration is provided in **Figure 3**.

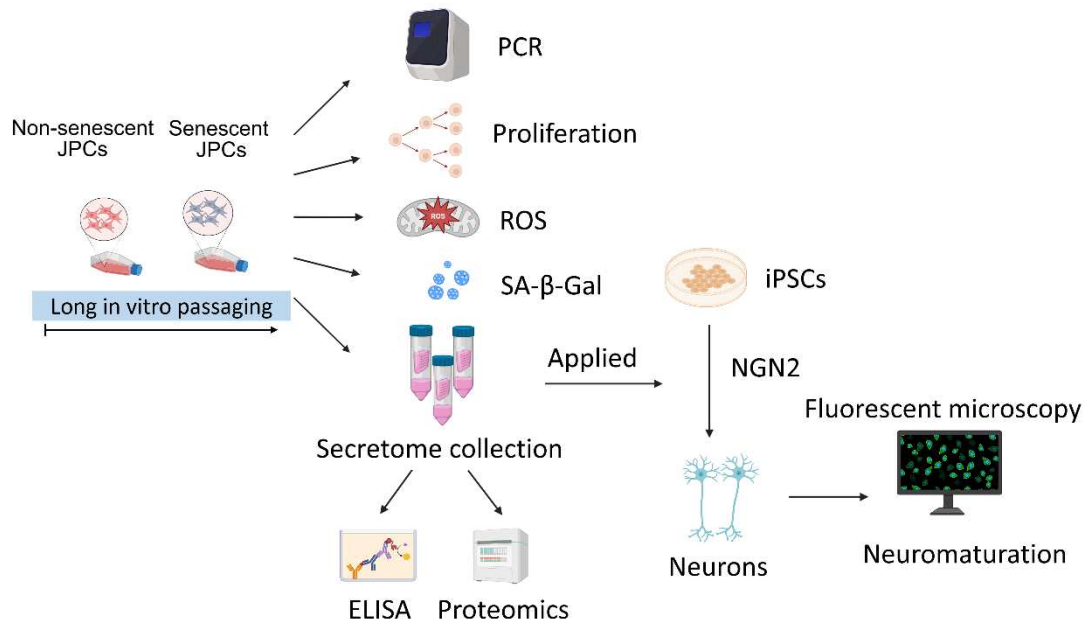


Figure 3. Experimental workflow for assessing JPC senescence and the effects of the JPC secretome on neuronal regeneration [76]. Non-senescent and senescent JPCs were obtained by short-/long-term in vitro culturing. Senescence-associated phenotypes were evaluated by PCR-based gene expression analysis, proliferation, reactive oxygen species (ROS) measurement, and senescence-associated β -galactosidase (SA- β -gal) staining. The non-senescent/senescent secretome was collected and characterized by ELISA and proteomic analysis. Subsequently, iPSCs were induced to differentiate into neurons using a Neurogenin-2 (NGN2)-based protocol. JPC-derived secretomes were then applied during neuronal differentiation, followed by fluorescence imaging as an endpoint assessment to determine the differential effects of non-senescent and senescent JPC secretomes on neuronal differentiation-related outcomes. Created with BioRender.com. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

2. Materials and methods (This section published in “Wang et al., Cells 2026, 15(17), 1526”)

2.1 Reagents and equipments.

Table 1. Reagents used in this project

Reagents	Company
DMEM/F12 medium	Gibco
Human platelet lysate	PL-Bioscience
Penicillin and Streptomycin	Gibco
Amphotericin B	Neofroxx
TrypLE™ Express	Gibco
Dimethyl sulfoxide	Sigma-Aldrich
DPBS	Gibco
Essential 8™ Flex medium	Gibco
Accutase	Gibco
Y-27632	Selleck Chemicals
mTeSR™ Plus medium	STEMCELL Technologies
Matrigel	Corning
KnockOut™ DMEM	Gibco
KnockOut™ Serum Replacement	Gibco
NEAA	Gibco
GlutaMAX	Gibco
2-Mercaptoethanol	Sigma-Aldrich
Heparin	STEMCELL Technologies
Glucose solution	Gibco
Neurobasal medium	Gibco
N2 supplement	Gibco
B-27™ Plus Supplement	Gibco
SB431542	Selleck Chemicals
XAV939	Selleck Chemicals
Doxycycline	Sigma-Aldrich
Puromycin	Sigma-Aldrich
BDNF	PeproTech
LDN-193189	STEMCELL Technologies
Cell Counting Kit-8 (CCK-8)	Selleck Chemicals
DCFDA / H2DCFDA - Cellular ROS Assay Kit	Abcam

Materials and methods

SPiDER-βGal	Dojindo Technologies	Molecular
Hank's Balanced Salt Solution	Gibco	
HEPES	Gibco	
Vivaspin 20 5kD	Sartorius	
Pierce™ BCA Protein Assay Kit	Thermo Scientific	
ELISA kits	R&D Systems	
16% paraformaldehyde	Electron Microscopy Sciences	
Triton 100	AppliChem	
Tween-20	AppliChem	
Bovine Serum Albumin	Sigma-Aldrich	
Recombinant Anti-Doublecortin antibody	Abcam	
Recombinant Anti-MAP2 antibody	Abcam	
Recombinant Anti-Ki67 antibody	Abcam	
Alexa Fluor 488 goat anti mouse IgG	Abcam	
Alexa Fluor 594 goat anti Rabbit IgG	Abcam	
DAPI	BioLegend	
NucleoSpin RNA kit	MACHEREY-NAGEL	
Ethanol	VWR Chemicals	
LunaScript RT SuperMix Kit	New England Biolabs	
PowerTrack™ SYBR Green Mastermix	Applied Biosystems™	
Dexamethason	Sigma-Aldrich	
β-Glycerophosphate	PanReac AppliChem	
Ascorbic acid 2-phosphate	Sigma-Aldrich	
Formalin solution 4%	Otto Fischer GmbH	
Gelatin	Sigma-Aldrich	
Alizarin Red S	Carl Roth	
Fetal bovine serum	Sigma-Aldrich	
Indomethacin	Sigma-Aldrich	
3-isobutyl-1-methylxanthine	Sigma-Aldrich	
Insulin	Gibco	
Oil Red O	Sigma-Aldrich	
Isopropanol	VWR Chemicals	
ITS+ Premix	Corning Inc.	
Recombinant human TGF-β1	PeproTech	
Toluidine Blue	Waldeck GmbH & Co. KG	
DePeX	SERVA GmbH	Electrophoresis

Table 2. Equipments used in this project

Equipment	Company
TC20 Automated Cell Counter	Bio-Rad Laboratories
Guava EasyCyte 6HT-2L flow cytometer	Merck Millipore
NanoDrop One instrument	Thermo Fisher Scientific
Mastercycler Nexus thermal cycler	Eppendorf
QuantStudio 3 Real-Time PCR instrument	Thermo Fisher Scientific
Axio Observer Z1 fluorescence microscope	Zeiss
Microplate reader	BioTek Instruments
Inverted light microscope	Leica

2.2 Donor and cell information

Table 3. Donor and cell information

Cell No.	Gender	Age	Cell information
JPC 114	Male	31	Mandibular periosteum
JPC 116	Female	29	Mandibular periosteum
JPC 117	Female	23	Mandibular periosteum
JPC 122	Male	20	Mandibular periosteum

2.3 Cell culture and characterization

2.3.1 Isolation and culture of JPCs

The study was approved by the Ethics Committee of the University of Tübingen (Approval No. 618/2017BO2 and 358/2025BO2), and written informed consent was obtained from all donors prior to sample collection. Jaw periosteal biopsies were transferred into Dulbecco's phosphate-buffered saline (DPBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA; without Ca^{2+} and Mg^{2+}) and gently agitated for 5 s to remove residual preservation solution. Tissue was placed on a Petri dish lid, and adherent blood was removed using a knife and tweezers, followed by an additional DPBS wash. The tissue was then cut into small fragments and evenly distributed in a T75 culture flask. After allowing fragments to adhere for 5 min, 10 mL of MSC medium was added.

MSC medium consisted of DMEM/F-12 (Gibco) supplemented with 10% (v/v) human platelet lysate (hPL; PL BioScience, Aachen, Germany), penicillin–streptomycin (final concentration: 100 U/mL penicillin and 100 µg/mL streptomycin; Gibco), and amphotericin B (2.5 µg/mL; neoFroxx, Einhausen, Germany). Cultures were maintained at 37 °C, 5% CO₂, and 20% O₂ for 7 days without medium change to promote cell outgrowth and prevent detachment of tissue fragments.

After 7 days, the medium was removed and cells were washed once with DPBS prior to enzymatic detachment using 2 mL TrypLE™ Express (Gibco) for 5–8 min. Detachment was stopped by adding 3 mL MSC medium, and the suspension was filtered through a 40-µm cell strainer into a 15-mL tube. Cells were counted using a TC20 Automated Cell Counter (Bio-Rad Laboratories, Hercules, CA, USA), centrifuged at 300 × g for 7 min at 8 °C, and cell pellet was resuspended in fresh MSC medium. For expansion approximately 1 × 10⁶ cells were seeded into each T175 culture flask. At passage 1, cells were cryopreserved in MSC medium containing 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Cells at passages 5 and 10 were used for subsequent experiments.

2.3.2 Culture and maintenance of iPSCs

Induced pluripotent stem cells (iPSCs) were generated from JPCs by Felix Umrath as previously described[56]. The study was approved by the Ethics Committee of the University of Tübingen (Approval No. 074/2016BO2). iPSCs were maintained on 6-well plates coated with 1% (v/v) Matrigel (Corning Inc., Corning, NY, USA) diluted in DMEM/F-12 and cultured in Essential 8™ Flex medium (E8 Flex; Gibco; 2 mL per well). Medium was changed every 2 days. Cells were passaged when reaching 80% confluence (typically every 5-7 days).

For passaging, cultures were washed once with DPBS and incubated with 0.5 mL Accutase (Gibco) at 37 °C for 3 min. Dissociation was terminated with 1 mL E8 Flex supplemented with 10 μ M Y-27632 (Selleck Chemicals, Houston, TX, USA), a Rho-associated protein kinase (ROCK) inhibitor used to improve cell survival after dissociation. Cells were collected and centrifuged at 200 $\times$ g for 5 min at room temperature, then resuspended in E8 Flex containing 10 μ M Y-27632. Cells from one well were split into 6-10 newly coated wells (2 mL per well) of a 6-well plate. After 24 h, the medium was replaced with E8 Flex without Y-27632.

For cryopreservation, iPS cells were resuspended in E8 Flex containing 10 μ M Y-27632 and 10% DMSO. Cells collected from approximately one well of a 6-well plate was aliquoted into three cryovials and frozen using a controlled-rate freezing container prior to long-term storage in liquid nitrogen. Cells between passages 10 and 30 were used for subsequent experiments.

2.3.3 Induction of NGN2 neurons

NGN2 neurons were generated from human iPSCs using a protocol (**Figure 4**) adapted from Zhang et al. (2013)[77]. NGN2, also known as NEUROG2, is a proneural transcription factor commonly used to rapidly induce neuronal differentiation of human iPSCs[77]. In this protocol, NGN2 expression was induced by doxycycline through a tetracycline-inducible lentiviral system. Lentiviral vectors (**Table 4**) were kindly provided by PD Dr. Julia Fitzgerald (Research Group Leader Mitochondrial Biology, Hertie Institute for Clinical Brain Research, University Hospital Tübingen). On day 0, Matrigel-coated 6-well plates were pre-equilibrated with 800 μ L mTeSR™ Plus medium (STEMCELL Technologies, Vancouver, Canada) containing 10 μ M Y-27632. For transduction, pLV-TetO-hNGN2-GFP-Puro (0.25 μ L/mL) and FUDeltaGW-rtTA (0.25 μ L/mL)

lentiviral stocks were diluted in 1.2 mL mTeSR™ Plus supplemented with 10 μ M Y-27632 and kept on ice until use.

iPSCs cultured in 10-cm dishes were dissociated with Accutase and stopped with 1 mL mTeSR™ Plus containing 10 μ M Y-27632. Cells were collected, counted, and centrifuged at $200 \times g$ for 5 min at room temperature. Approximately 6×10^6 iPSCs was resuspended in the viral suspension and incubated at room temperature for 5 min. Subsequently, 200 μ L of the transduction mixture was added per well of the pre-equilibrated 6-well plate. Cultures reached approximately 70–80% confluence on day 1.

On day 1, the medium was changed to KOSR medium composed of KnockOut™ DMEM (Gibco) supplemented with 15% KnockOut™ Serum Replacement, GlutaMAX (1 $\times$), and non-essential amino acids (NEAA, 1 $\times$) (all from Gibco), together with the transforming growth factor- β (TGF- β) inhibitor SB431542 (10 μ M; Selleck Chemicals), the Wnt signaling inhibitor XAV939 (2 μ M; Selleck Chemicals), the bone morphogenetic protein (BMP) inhibitor LDN-193189 (100 nM; STEMCELL Technologies) and 0.1% 2-Mercaptoethanol (Sigma-Aldrich). Doxycycline (1 μ g/mL; Sigma-Aldrich) was added to induce NGN2 expression.

On day 2, the medium was changed to a 1:1 mixture of KOSR medium and neural induction medium (NIM), supplemented with doxycycline (1 μ g/mL) and puromycin (1 μ g/mL; Sigma-Aldrich). NIM consisted of DMEM/F-12 medium supplemented with GlutaMAX (1 $\times$), NEAA (1 $\times$), N2 supplement (1 $\times$; Gibco), glucose (final concentration, 3 g/L; Gibco), and heparin (2 μ g/mL; STEMCELL Technologies). On day 3, cultures were maintained in NIM containing doxycycline (1 μ g/mL) and puromycin (1 μ g/mL) to continue selection.

On day 4, cells were transferred to neuronal basal medium (NBM) consisting of neurobasal medium (Gibco) supplemented with GlutaMAX (1×), NEAA (1×), N2 supplement (1×), and B-27™ Supplement (1×; Gibco), a serum-free supplement used to support neuronal survival and maturation in Neurobasal™-based culture systems. The medium was further supplemented with brain-derived neurotrophic factor (BDNF, 10 ng/mL; PeproTech, Cranbury, NJ, USA) and doxycycline (1 µg/mL). At this stage, NGN2 neurons were either used for downstream applications or cryopreserved in NBM containing 10 µM Y-27632 and 10% DMSO for future use.

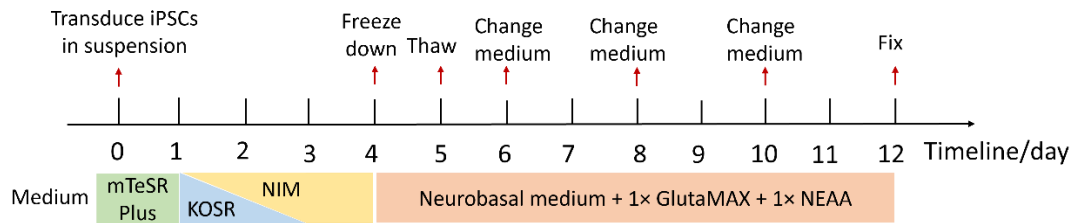


Figure 4. Timeline for NGN2-induced neuronal induction [76]. Schematic overview of the stepwise differentiation protocol, indicating sequential culture media conditions. Red arrows denote key intervention time points during the induction process. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

Table 4. Virus information

Lentiviral stocks	Virus titer ($\times 10^{10}$ lentiviral particles/mL)
pLV-TetO-hNGN2-GFP-Puro	48.8
FUdeltaGW-rtTA	69.9

2.3.4 Trilineage Differentiation of JPCs

2.3.4.1 Osteogenic differentiation

For osteogenic differentiation, 4×10^4 passage 5 JPCs were seeded into 6-well plate pre-coated with 0.1% bovine skin-derived gelatin (Sigma-Aldrich) to

promote cell attachment and reduce detachment during prolonged culture. After 24 h, the culture medium was replaced with osteogenic induction medium consisting of DMEM/F12 medium supplemented with 10% hPL, L-ascorbic acid 2-phosphate (100 μ M; Sigma-Aldrich), β -glycerophosphate (10 mM; AppliChem, Darmstadt, Germany), and dexamethasone (4 μ M; Sigma-Aldrich)[78]. Cells were cultured for 21 days, with medium changes every 2-3 days.

After 21 days, mineralized matrix deposition was assessed by Alizarin Red S staining. Cells were rinsed with DPBS, fixed with 4% formalin (Otto Fischer GmbH, Saarbrücken, Germany) for 15 min at room temperature, and washed twice with DPBS. Fixed cultures were incubated with 40 mM Alizarin Red S solution (pH 4.2; Carl Roth, Karlsruhe, Germany; 1 mL per well) for 20 min under gentle agitation. Excess dye was removed by four washes with distilled water (3 mL per wash). Mineralized nodules were visualized by adding distilled water to each well and imaging using an inverted light microscope (Leica, Wetzlar, Germany).

2.3.4.2 Adipogenic differentiation

For adipogenic differentiation, JPCs at passage 5 were seeded at 2×10^4 cells/cm² onto culture dishes pre-coated with 0.1% bovine skin-derived gelatin. Upon reaching full confluence (approximately 48 h post-seeding), the culture medium was replaced with adipogenic induction medium A. Cells were cultured in induction medium A for 3 days followed by maintenance medium B for 1 day; this 3-day induction/1-day maintenance cycle was repeated until day 21.

Adipogenic induction medium A consisted of DMEM/F-12 supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), penicillin–streptomycin (100 U/mL penicillin and 100 μ g/mL streptomycin), amphotericin B (2.5 μ g/mL), dexamethasone (1 μ M), indomethacin (0.2 mM; Sigma-Aldrich), insulin (10 μ g/mL;

Gibco), and 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM; Sigma-Aldrich). Maintenance medium B contained DMEM/F-12 supplemented with 10% FBS, penicillin–streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin), amphotericin B (2.5 µg/mL), and insulin (10 µg/mL)[79].

After 21 days, adipogenic differentiation was evaluated by Oil Red O staining. Cells were washed twice with DPBS and fixed with 4% formalin for 15 min at room temperature. Following fixation, cultures were rinsed with distilled water, incubated with 60% isopropanol (VWR Chemicals, Radnor, PA, USA) for 5 min, and stained with freshly prepared Oil Red O working solution (Sigma-Aldrich) for 15 min. Excess stain was removed by washing four times with distilled water, and lipid droplets were visualized using an inverted light microscope.

2.3.4.3 Chondrogenic differentiation

For chondrogenic differentiation, JPCs at passage 5 were harvested, centrifuged, and resuspended at 1.25×10^6 cells/mL in chondrogenic differentiation medium composed of high-glucose DMEM (4.5 g/L glucose, with L-glutamine and 1 mM sodium pyruvate; Gibco) supplemented with penicillin–streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin), amphotericin B (2.5 µg/mL), ITS+ Premix (1×; Corning Inc), L-ascorbic acid 2-phosphate (1 µM), dexamethasone (0.1 µM), and recombinant human TGF-β1 (10 ng/mL; PeproTech)[80]. Aliquots of 200 µL cell suspension (2.5×10^5 cells) were dispensed into individual wells of 96-well V-bottom polypropylene plates and centrifuged at $350 \times g$ for 5 min to promote pellet formation. Pellets were cultured at 37 °C, 5% CO₂ and 20% O₂. After 24 h, pellet formation was confirmed by visual inspection. Medium was replaced every 3 days with minimal disturbance of the aggregates, and chondrogenic induction was continued for 21 days.

At day 21, chondrogenic differentiation was evaluated by Toluidine Blue staining. Pellets were collected, washed with DPBS, and fixed in 4% formalin for 30 min at room temperature. Aggregates were then dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin using standard histological procedures. Paraffin blocks were sectioned at 5 μ m, mounted on glass slides, deparaffinized, and stained with 0.04% Toluidine Blue (Waldeck GmbH & Co. KG, Münster, Germany). Sections were rinsed, dehydrated, mounted with DePeX (SERVA Electrophoresis GmbH, Heidelberg, Germany), and examined using an inverted light microscope.

2.3.5 Assessment of cell proliferation

Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) assay (Selleck Chemicals). JPCs (2,000 cells per well) were seeded into 96-well plates in 200 μ L culture medium. At 24, 48, 72, and 96 h of incubation, 20 μ L CCK-8 reagent was added to each well and plates were incubated at 37 °C for 2 h. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA), and metabolic activity was used as an indicator of proliferation.

2.3.6 Quantification of cellular reactive oxygen species

Intracellular ROS levels were quantified using a DCFDA-based assay according to the manufacturer's instructions (Abcam, Cambridge, UK). JPCs (1×10^4 cells per well) were seeded in 24-well plates and cultured in DMEM/F12 medium containing 10% hPL for 5 days with medium changes every 2–3 days. On day 5, cells were detached, collected, and centrifuged. The pellet was resuspended in ROS buffer containing 20 μ M DCFDA and incubated for 30 min at 37 °C. Cells were then washed once with ROS buffer, centrifuged, and resuspended in 200

μL ROS buffer for acquisition. Fluorescence activity was measured using a Guava easyCyte 6HT-2L (Merck Millipore, Billerica, MA, USA) with 488-nm excitation and detection in the FITC channel. Mean fluorescence intensity (MFI) was quantified using the guavaSoft 2.2.3 software (InCyte 2.2.2, Luminex Corporation, Chicago, IL, USA).

2.3.7 Measurement of cellular senescence

Senescence-associated β-galactosidase (SA-β-gal) activity was assessed using SPiDER-βGal (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. JPCs (1×10^4 cells per well) were seeded in 24-well plates and cultured in DMEM/F12 medium containing 10% hPL for 5 days. On day 5, cells were detached, collected, and centrifuged, followed by two washes with Hank's HEPES buffer. Hank's HEPES buffer was prepared by supplementing Hank's Balanced Salt Solution (HBSS; Gibco) with HEPES (20 mM; Gibco). The cell pellet was resuspended in SPiDER-βGal (1 μM) prepared in Hank's HEPES buffer and incubated at 37 °C for 15 min. Cells were then washed twice with Hank's HEPES buffer, centrifuged, and resuspended in 200 μL Hank's HEPES buffer for acquisition. Fluorescence was measured using a Guava EasyCyte flow cytometer with 488-nm excitation and detection in the FITC channel.

2.4 Secretome collection and analysis

2.4.1 Secretome collection

JPCs at passages 5 and 10 were seeded into T175 flasks (2×10^6 cells per flask) and cultured in MSC medium for 4 days until reaching 80–90% confluence, with medium changes every 2–3 days. On day 4, the medium was removed and cells

were washed three times with 10 mL DPBS, followed by one wash with 10 mL DMEM/F-12 basal medium (DMEM/F-12 supplemented with penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) and amphotericin B (2.5 µg/mL)). Subsequently, 37 mL fresh DMEM/F-12 basal medium was added per flask and cells were incubated for an additional 24 h. Conditioned medium (secretome) was collected in 50-mL conical tubes and centrifuged at $600 \times g$ for 7 min at 4 °C. The supernatant (34 mL) was carefully transferred to fresh tubes without disturbing the pellet and shock-frozen in liquid nitrogen for 5 min. Samples were stored at -80 °C until secretome enrichment.

2.4.2 Secretome enrichment

Secretome enrichment was performed according to the manufacturer's instructions using Vivaspin 20 centrifugal concentrators (50 kDa MWCO; Sartorius, Goettingen, Germany). Briefly, 15 mL of secretome was loaded per concentrator and centrifuged at $6,000 \times g$ at 4 °C until the volume was reduced. The remaining secretome was subsequently added, and centrifugation was continued until an approximately 100-fold concentration was achieved. Concentrated secretomes from four donors were pooled to reduce individual differences. The concentrated flow was collected, aliquoted to avoid repeated freeze-thaw cycles, and stored at -80 °C until further use.

2.4.3 Detection of total protein concentration in secretomes

Total protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Secretome samples were diluted 1:10 in DPBS. The BCA working reagent was prepared by mixing Reagent A and Reagent B at a 50:1 ratio, and standards were prepared as recommended by the manufacturer. Diluted samples

(25 μ L) and standards were dispensed into a 96-well plate, with DPBS (25 μ L) used as the blank. Subsequently, 200 μ L of BCA working reagent was added to each well, and the plate was incubated at 37 °C for 30 min. Absorbance was measured at a wavelength of 570 nm using a microplate reader. All measurements were performed in technical replicates.

2.4.4 Detection of secreted SASP-factors by ELISA

Concentrations of IL-6, IL8, CCL2, CCL20, TGF- β 1, VEGF in secretome samples were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Bio-Techne, Wiesbaden, Germany) according to the manufacturers' instructions for each analyte. Frozen samples were thawed on ice and diluted as required to ensure that measured values fell within the linear range of the respective standard curves. Standards were prepared according to the kit manuals. Standards, samples, and blanks were loaded onto antibody-precoated 96-well plates using the specified sample volumes, incubation times, and temperatures, with washing steps performed as instructed. Biotinylated detection antibodies and horseradish peroxidase (HRP)-conjugated streptavidin were added sequentially, followed by the recommended incubation and wash steps. Color development was initiated by adding tetramethylbenzidine (TMB) substrate and stopped after the recommended incubation time using stop solution. Absorbance was measured at 450 nm using a microplate reader. Measurements were performed in technical replicates and repeated in 3 independent experiments. Concentrations of each target protein were normalized to the total protein concentration of the corresponding sample.

2.4.5 Determination of secretome proteome profiles by proteomics

Secretome samples were submitted to the core facility of the University Hospital

Tübingen for proteomic analysis and measured in four biological replicates. Each fraction was analyzed independently. Protein intensity data were processed and differential abundance was assessed using Perseus (v2.1.3.0; Max Planck Institute of Biochemistry, Martinsried, Germany). Intensities were log₂-transformed and quantile-normalized to reduce global intensity differences across samples. Differentially expressed proteins (DEPs) were defined using an adjusted *p* value < 0.05 and an absolute log₂ fold change (log₂FC) > 1. The resulting differential expression table (including log₂FC and adjusted *p* values) was exported and used for downstream analyses in R (v4.4.1; R Foundation for Statistical Computing, Vienna, Austria). In R, exploratory analyses included principal component analysis (PCA) and hierarchical clustering to evaluate sample relationships, and heatmaps were generated to visualize protein abundance patterns. Functional annotation was performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

2.5 Treatment of induced neurons with JPC-derived secretomes

NGN2 neurons were thawed, counted, and seeded at a density of 5×10^4 cells per well in 24-well plates pre-coated with 1% (v/v) Matrigel diluted in DMEM/F-12 medium. Cells were plated in Neurobasal medium supplemented with GlutaMAX (1×), NEAA (1×), and Y-27632 (10 μM). After 24 h, the medium was replaced and cultures were assigned to one of four conditions: (i) negative control, Neurobasal base medium supplemented with GlutaMAX (1×) and NEAA (1×); (ii) positive control, Neurobasal medium supplemented with GlutaMAX (1×), NEAA (1×), N2 supplement (1×), B-27™ Supplement (1×), and BDNF (10 ng/mL); (iii) JPC P5, Neurobasal base medium supplemented with GlutaMAX (1×) and NEAA (1×) supplemented with JPC passage 5 secretome (500 μg/mL total protein); and (iv) JPC P10, Neurobasal base medium supplemented with GlutaMAX (1×) and

NEAA (1×) supplemented with JPC passage 10 secretome (500 µg/mL total protein). Half of the respective medium was replaced every 2 days, and cultures were cultivated for 6 days.

2.6 Immunofluorescent analyses of neurons

2.6.1 Immunofluorescence staining of cells

Immunocytochemistry was performed to assess neuronal differentiation and visualize neurite outgrowth. Cells were rinsed with DPBS (1 mL per well) for 5 min and fixed with 4% paraformaldehyde (300 µL per well; prepared by diluting 16% paraformaldehyde stock solution in DPBS; Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min at room temperature. After fixation, cells were washed once with DPBS (1 mL per well) for 5 min. Permeabilization and blocking were carried out for 1 h at room temperature in DPBS containing Triton X-100 (0.1% v/v; AppliChem) and bovine serum albumin (BSA; 1% w/v; Sigma-Aldrich).

Primary antibodies were diluted in antibody solution (200 µL per well) and incubated overnight at 4 °C, including mouse anti-doublecortin (DCX; 1:1,000; Abcam) and rabbit anti-microtubule-associated protein 2 (MAP2; 1:200; Abcam). The next day, primary antibody solution was removed and cells were washed for 10 min with washing buffer consisting of DPBS supplemented with Tween-20 (0.1% v/v; AppliChem) and BSA (0.1% w/v).

Fluorescent secondary antibodies were then applied (200 µL per well) for 1 h at room temperature, including goat anti-mouse IgG Alexa Fluor 488 (1:1,000; Abcam) and goat anti-rabbit IgG Alexa Fluor 594 (1:1,000; Abcam) depending on the primary antibody used. Nuclei were counterstained with DAPI (1 µg/mL; BioLegend, San Diego, CA, USA) for 5 min at room temperature in the dark,

followed by a 10-min wash with washing buffer. Finally, cells were maintained in DPBS (200 μ L per well) and imaged using an Axio Observer Z1 fluorescence microscope (Zeiss, Jena, Germany).

2.6.2 Quantitative analysis of neuronal differentiation

Immunofluorescence images were analyzed using ImageJ (NIH, Bethesda, MD, USA). For each experimental condition, 9-10 wells were analyzed and nine images per well were acquired using identical microscope settings. MAP2-positive cell counts per image were used as the quantitative marker for neuronal differentiation. DAPI-stained nuclei per image were counted to estimate overall cell survival across culture conditions. Neurite outgrowth was assessed in DCX-stained cells using the Ridge Detection plugin in ImageJ. Neurites were automatically traced and total neurite length per image was extracted based on the plugin output. All image-processing steps including background subtraction, thresholding, and filtering were applied using identical settings across all conditions to ensure comparability. Quantification results were exported for downstream statistical analysis.

2.7 Quantitative gene expression analysis of JPC

2.7.1 Isolation of RNA from JPC

Total RNA was isolated from adherent JPCs using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Cells were cultured in 6-well plates until reaching 80–90% confluence and washed once with DPBS to remove residual medium. After removal of DPBS, cells were lysed directly in the wells by adding 350 μ L RA1 lysis buffer supplemented with 7 μ L TCEP, followed by gentle pipetting to ensure complete

lysis and dissociation of nucleoprotein complexes. Lysates were mixed with 350 μL 70% (v/v) ethanol (VWR Chemicals) and loaded onto NucleoSpin RNA silica columns. Columns were processed according to the kit protocol, including on-column DNase digestion to remove genomic DNA. After the final wash, RNA was eluted in 40 μL RNase-free water. RNA concentration and purity were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific), and samples were stored at $-80\text{ }^{\circ}\text{C}$ until cDNA synthesis.

2.7.2 cDNA synthesis

For reverse transcription, 1,000 ng total RNA per sample was used. cDNA was generated using the LunaScript™ RT SuperMix Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Briefly, RNA was combined with LunaScript RT SuperMix in a total reaction volume of 20 μL (**Table 5**) and reverse transcription was performed in an Eppendorf Mastercycler Nexus thermal cycler (Eppendorf, Hamburg, Germany) using the supplier-recommended program (**Table 6**). The resulting cDNA was diluted in nuclease-free water to a final concentration of 5 ng/ μL and used as template for subsequent quantitative real-time PCR (qPCR).

Table 5. Composition of the 20 μL reverse transcription reaction mix

Component	20 μL reaction	Final concentration
Lunascript RT supermix (5x)	4 μL	1x
RNA sample	1 μg	1 μg
Nuclease-free water	to 20 μL	

Table 6. Thermal cycling program for reverse transcription

Cycle step	Temperature	Time	Cycles
Primer annealing	25 $^{\circ}\text{C}$	2 minutes	1

Materials and methods

cDNA synthesis	55 °C	10 minutes	
Heat inactivation	95 °C	1 minute	
Hold	4 °C	∞	

2.7.3 Quantitative real-time PCR

mRNA expression was quantified using a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were prepared using PowerTrack™ SYBR Green Master Mix (Applied Biosystems™, Thermo Fisher Scientific, Carlsbad, CA, USA) and gene-specific primer pairs targeting human GAPDH, IL6, IL8, CCL2, CCL20, VEGFA, TGFB1, CDKN2A (p16), CDKN1A (p21), TP53 (p53), NGF (β-NGF) and GDNF. Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA), and sequences are provided in **Table 7**. Reaction setup followed the manufacturer's recommendations (**Table 8**), and amplification was performed using the supplier-recommended fast cycling program (**Table 9**). Relative expression levels were calculated using the $\Delta\Delta C_t$ method with GAPDH as the reference gene and are presented as $2^{-\Delta\Delta C_t}$.

Table 7. Primer sequences [76]. (Table published in “Wang et al., Cells 2026, 15(17), 1526”)

Gene	Sequence P1	Sequence P2
GAPDH	ACA TCG CTC AGA CAC CATG	TGT AGT TGA GGT CAA TGA AGGG
IL6	ATT CGT TCT GAA GAG GTG AGTG	CCT TCC CTG CCC CAG TA
IL8	GAG ACA GCA GAG CAC ACA AG	CTT CAC ACA GAG CTG CAG AA
CCL2	AGC AGC CAC CTT CAT TCC	GCC TCT GCA CTG AGA TCT TC
CCL20	CCA TGT GCT GTA CCA AGA GT	TTA GGA TGA AGA ATA CGG TCT GTG
VEGF-A	CCA TGA ACT TTC TGC TGT CTTG	GCG CTG ATA GAC ATC CAT GA

Materials and methods

TGFβ1	CCG ACT ACT ACG CCA AGGA	GTT CAG GTA CCG CTT CTCG
p16	AGC TGT CGA CTT CAT GAC AAG	TGA GCT TTG GTT CTG CCA TT
p21	GCA GAC CAG CAT GAC AGAT	GAG ACT AAG GCA GAA GAT GTA GAG
p53	TGTGACTTGACGTACTCCC	ACCATCGCTATCTGAGCAGC
βNGF	CGG ACC CAA TAA CAG TTT TACC	TGT GAT CAG AGT GTA GAA CAA CA
GDNF	TCA TAG TAA CAA GAA GCA GCA GT	GAG CAG AAA GGA CAG AGA AGG

Table 8. Composition of the 10 µl qPCR reaction mix

Component	10 µl reaction	Final concentration
PowerTrack™ SYBR Green Master Mix for qPCR (2x)	5 µl	1x
primer/probe (20x)	0.5	1x
Template DNA	1 µl	-
Nuclease-free Water	3.5 µl	-

Table 9. Thermal cycling program for qPCR reaction

	Temperature	Time	cycles
Initial Denaturation	95 °C	2 mins	1
Denaturation	95 °C	5 s	40
Extension	60 °C	30 s	

2.8 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (v27.0; IBM Corp., Armonk, NY, USA). Data are presented as mean ± standard deviation (SD). Comparisons of ROS levels, SA-β-gal activity, ELISA measurements, and qPCR results were performed using Student's *t*-test. CCK-8 proliferation data were analyzed by two-way analysis of variance (ANOVA) with the factors group (P5 vs P10) and time (24, 48, 72, and 96 h); where appropriate, simple main effects at

96 h were examined. Quantification of immunofluorescence imaging data was analyzed using Welch's one-way ANOVA followed by the Games–Howell post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results (This section published in “Wang et al., Cells 2026, 15(17), 1526”)

3.1 Trilineage Differentiation of JPCs

To confirm the mesenchymal characteristics of JPCs, trilineage differentiation potential was assessed using passage 5 JPCs under osteogenic, adipogenic, and chondrogenic induction conditions. After 21 days of osteogenic induction, JPCs showed robust mineralized matrix deposition, as indicated by the strong Alizarin Red S staining (**Figure 5a**). Under adipogenic induction condition, the Oil Red O staining revealed prominent intracellular lipid droplet accumulation, confirming adipogenic differentiation (**Figure 5b**). In pellet-based chondrogenic cultures, Toluidine Blue staining demonstrated abundant proteoglycan-rich extracellular matrix deposition, consistent with chondrogenic maturation (**Figure 5c**). These findings demonstrate that JPCs possess characteristic trilineage differentiation potential, confirming their mesenchymal stromal-like phenotype.

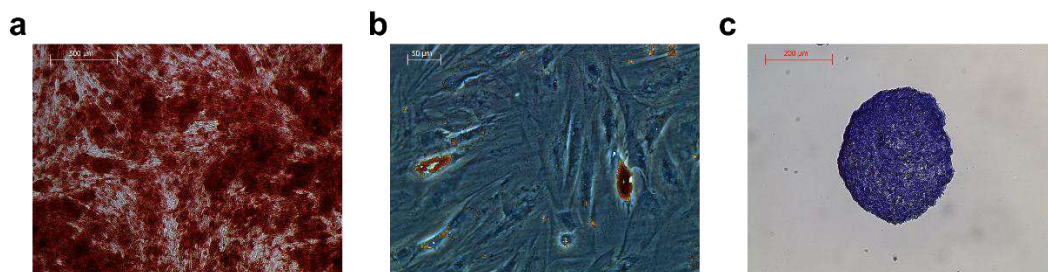


Figure 5. Trilineage differentiation potential of JPCs [76]. Representative images of osteogenic, adipogenic, and chondrogenic differentiation of JPCs after 21 days of induction. (a) Osteogenic differentiation assessed by Alizarin Red S staining showing mineralized matrix deposition (scale bar = 500 μm). (b) Adipogenic differentiation evaluated by Oil Red O staining showing intracellular lipid droplets (scale bar = 50 μm). (c) Chondrogenic differentiation analyzed by Toluidine Blue staining demonstrating proteoglycan-rich extracellular matrix deposition (scale bar = 200 μm). (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.2 Characterization of Senescent and Non-Senescent Cells

3.2.1 Early-passage JPCs show higher proliferation activity than late-passage JPCs

To investigate the effect of passage number on the proliferative capacity of JPCs, a CCK-8 assay was performed on passage 5 (P5) and passage 10 (P10) cells. As shown in Figure 1a, both groups exhibited comparable proliferation at early time points; however, from 72 h onward, P5 cells consistently displayed higher OD450 values than P10 cells. At 96 h, this difference reached statistical significance (two-way ANOVA simple main effect at 96 h: $p = 0.0352$) (**Figure 6b**). Given that proliferative capacity is closely associated with cellular aging, these findings indicate that late-passage JPCs exhibit reduced growth potential and a more pronounced senescent phenotype compared with early-passage cells.

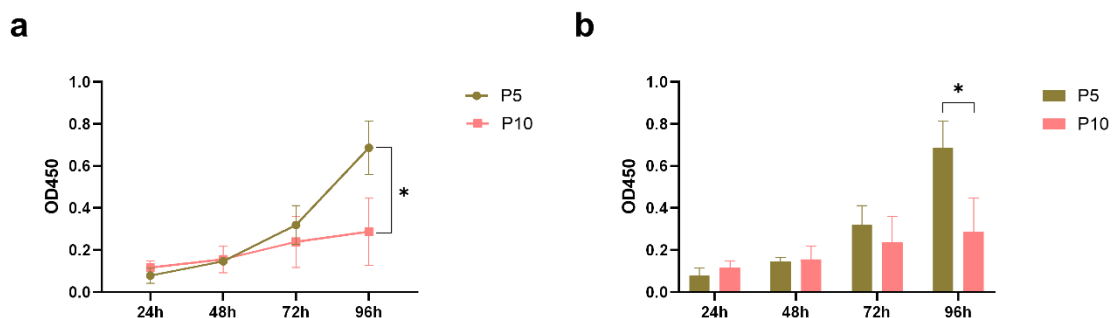


Figure 6. Proliferative activity of early- and late-passage JPCs [76]. (a) Growth curves of JPCs at passage 5 (P5) and passage 10 (P10) over 24, 48, 72, and 96 hours measured using the CCK-8 assay. (b) Quantification of OD450 values demonstrating consistently higher proliferation in P5 compared with P10 at later time points. A statistically significant difference between the two groups was observed at 96 h (two-way ANOVA simple main effect at 96 h: $p = 0.0352$). Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis was performed using two-way ANOVA with simple main effects analysis at individual time points. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.2.2 β -galactosidase staining reveals increased senescence in P10 JPCs

Flow cytometric analysis of senescence-associated β -galactosidase (SA- β -Gal) activity showed that passage 10 (P10) JPCs exhibited markedly higher β -Gal levels compared with passage 5 (P5) cells. Quantitative analysis confirmed this difference, with P10 cells displaying significantly elevated SA- β -Gal mean fluorescence intensity (P5: 3.070 ± 0.9940 ; P10: 8.257 ± 1.261 ; $p = 0.0007$) (**Figure 7**). These findings indicate that late-passage JPCs exhibit a more pronounced senescent phenotype compared with early-passage cells.

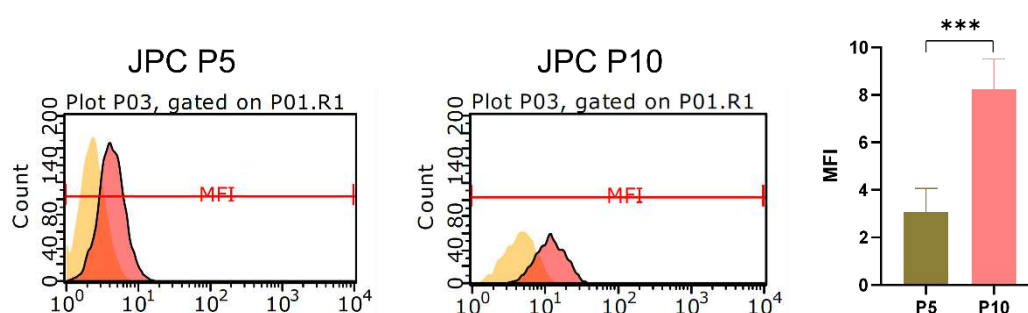


Figure 7. Flow cytometric analysis of SA- β -Gal mean fluorescence intensity (MFI) in JPCs at different passages [76]. Representative flow cytometry histograms (left) and quantified MFI values (right) showing senescence-associated β -galactosidase (SA- β -Gal) levels in passage 5 (P5) and passage 10 (P10) JPCs. P10 cells exhibited a significantly higher SA- β -Gal MFI compared with P5 cells ($p = 0.0007$), indicating increased senescence in late-passage JPCs. Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis was performed using an unpaired two-tailed Student's t -test. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.2.3 ROS analysis indicates similar oxidative status in early- and late-passage JPCs

Flow cytometric analysis of intracellular reactive oxygen species (ROS) levels revealed no significant difference between passage 5 (P5) and passage 10 (P10)

JPCs (**Figure 8**). Although P10 cells exhibited a slightly higher mean ROS intensity compared with P5 cells, this increase did not reach statistical significance. As ROS accumulation is a commonly used marker of cellular senescence, these findings indicate that oxidative stress levels do not substantially differ between early- and late-passage JPCs under the conditions tested.

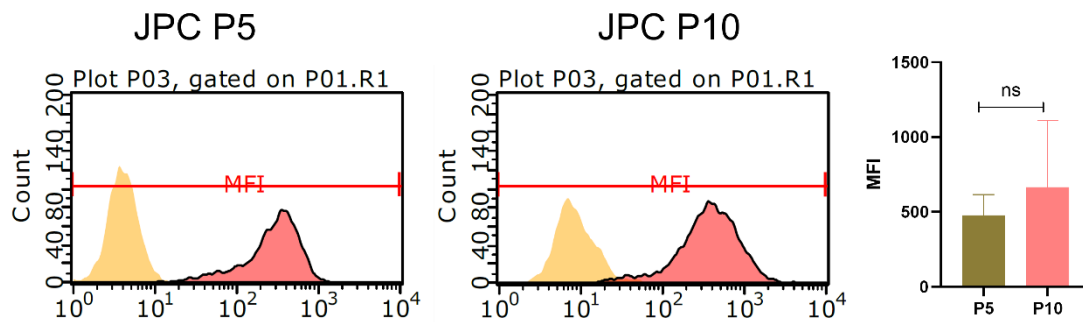


Figure 8. Flow cytometric analysis of intracellular ROS levels in JPCs at different passages [76]. Representative flow cytometry histograms (left) and quantified mean fluorescence intensity (MFI) values (right) showing intracellular reactive oxygen species (ROS) levels in passage 5 (P5) and passage 10 (P10) JPCs. Although P10 cells exhibited a slightly higher ROS MFI compared with P5 cells, the difference was not statistically significant (ns). Data are presented as mean $\pm$ SD ($n = 4$). Statistical analysis was performed using an unpaired two-tailed Student's t-test. (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.3 Differential Gene Expression Between Early- and Late-Passage JPCs

3.3.1 Expression of SASP-related genes in JPCs

To further assess the senescence-associated secretory phenotype (SASP) at the transcriptional level, qPCR was performed to evaluate the mRNA expression of representative SASP-related genes in early- (P5) and late-passage (P10) JPCs. As shown in **Figure 9**, the expression levels of pro-inflammatory cytokines and chemokines, including IL8, CCL2, CCL20, VEGFa, and TGFB1, were slightly

increased in P10 cells compared with P5 cells; however, no statistical difference was calculated.

Although not statistically significant at the mRNA level, these transcriptional trends are in line with the enhanced secretion of SASP-related proteins observed in the secretome analysis. Together, these findings support the notion that late-passage JPCs exhibit a more pronounced senescence-associated secretory phenotype at the protein level, with relatively subtle changes at the transcriptional level.

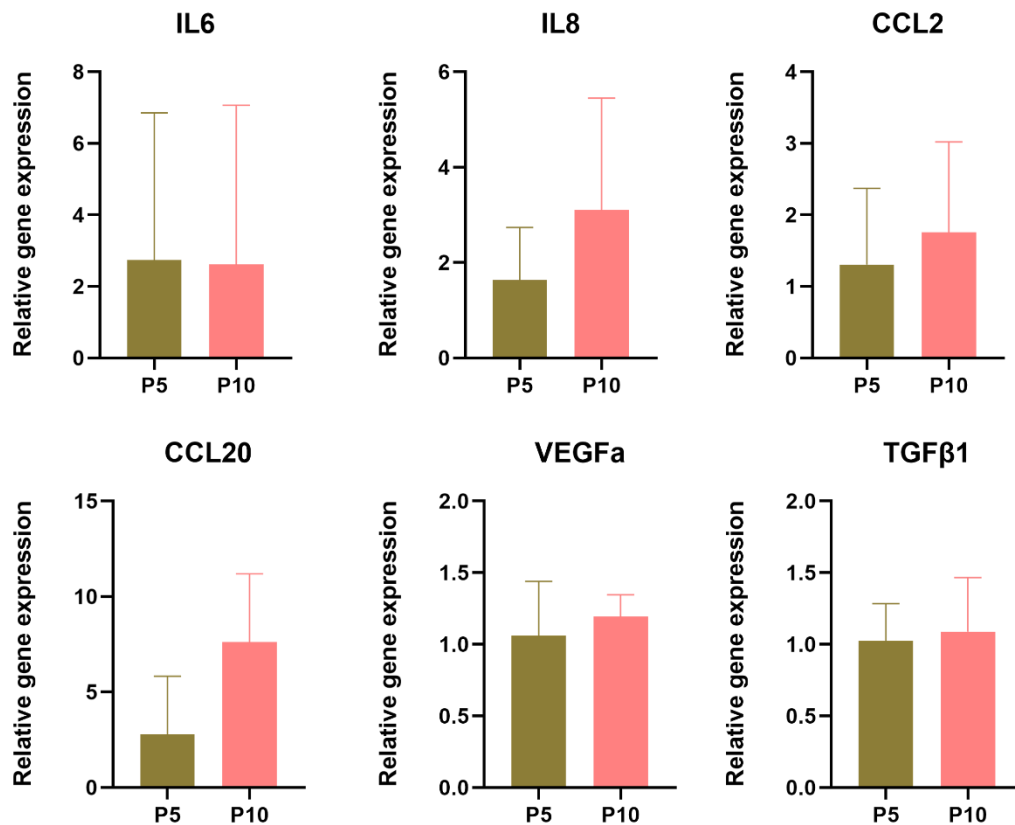


Figure 9. Expression of SASP genes in early- and late-passage JPCs [76]. Quantitative real-time PCR (qPCR) analysis of IL6, IL8, CCL2, CCL20, VEGFA, and TGFB1 mRNA expression in passage 5 (P5) and passage 10 (P10) JPCs. Relative expression levels were normalized to the housekeeping gene GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SD (n

= 4). Statistical significance was determined using an unpaired two-tailed Student's t-test. (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.3.2 Expression of senescence-related genes in JPCs

To further assess the senescence status of JPCs at the transcriptional level, the mRNA expression of key senescence-associated regulators, including CDKN2A (p16), CDKN1A (p21), and TP53 (p53), was analyzed by quantitative real-time PCR. As shown in **Figure 10**, p16 expression was significantly elevated in P10 cells compared with P5 cells ($p = 0.0012$), indicating enhanced activation of cell cycle arrest mechanisms in late-passage JPCs. And p53 expression exhibited a slight increase in P10 cells; however, this difference did not reach statistical significance. These results further support the idea that long-term passaging promotes the acquisition of a senescent phenotype in JPCs, primarily characterized by activation of the p16-mediated cell cycle arrest pathway.

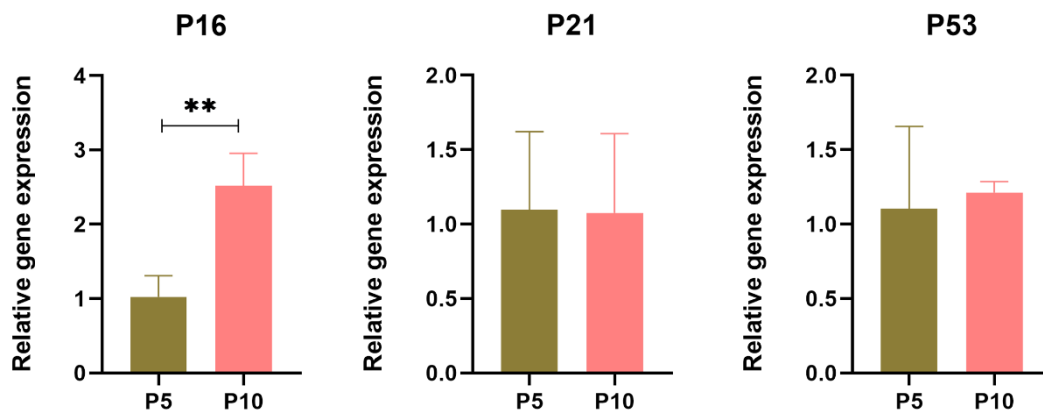


Figure 10. Expression of senescence-associated genes in early- and late-passage JPCs [76]. Quantitative real-time PCR analysis of CDKN2A (p16), CDKN1A (p21), and TP53 (p53) mRNA expression in passage 5 (P5) and passage 10 (P10) JPCs. Relative gene expression levels were normalized to the housekeeping gene GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. P10 cells exhibited a significant increase in p16 expression compared with P5 cells ($p = 0.0012$), whereas p53 expression showed a slight, but non-significant upregulation in P10 cells. Data are presented as mean $\pm$ SD ($n = 4$). Statistical

analysis was performed using an unpaired two-tailed Student's t-test. (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.3.3 Expression of neurotrophic factor genes in JPCs

To assess the neurotrophic potential of JPCs at the mRNA level, the expression of key neurotrophic factors, including nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF), was quantified by real-time quantitative PCR (RT-qPCR). As shown in **Figure 11**, NGF and GDNF mRNA levels tended to be lower in P10 cells than in P5 cells; however, the differences were not statistically significant.

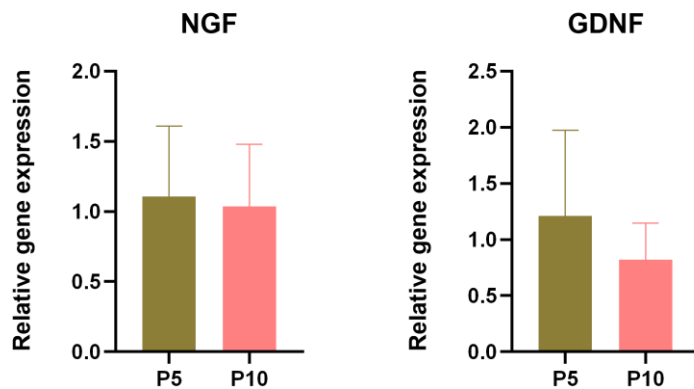


Figure 11. Expression of neurotrophic factor genes in early- and late-passage JPCs [76]. Quantitative real-time PCR (qPCR) analysis of NGF and GDNF mRNA expression in passage 5 (P5) and passage 10 (P10) JPCs. Relative expression levels were normalized to the housekeeping gene GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method. Data are presented as mean $\pm$ SD (n = 4). Statistical significance was determined using an unpaired two-tailed Student's t-test. (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.4 Composition of Secretomes

3.4.1 Increased SASP factors in P10 JPC secretome

To further elucidate the secretory profile of early- and late-passage JPCs, ELISA assays were performed to quantify key components of the senescence-associated secretory phenotype (SASP), including the factors IL-6, IL-8, CCL-2, CCL-20, VEGF, and TGF- β 1. All measurements were normalized to the total protein content of the secretome. As showed in **Figure 12**, IL-6 levels in the P10 secretome (847.5 ± 17.23) were substantially elevated compared with those detected in P5 (508.1 ± 19.56). A significant increase was also observed for IL-8, with P10 JPCs secreting more than fivefold higher IL-8 levels relative to P5 (P5: 732.7 ± 44.15 ; P10: 3914 ± 55.63). Similar patterns were seen for the chemokines CCL-2 and CCL-20: P10 cells produced nearly fourfold higher CCL-2 (9822 ± 426.9) than P5 cells (2603 ± 73.83), and CCL-20 secretion in P10 cells (549.2 ± 21.52) was markedly increased compared with P5 counterparts (38.72 ± 1.899). In addition, VEGF levels were substantially higher in P10 secretome (4791 ± 429.9) than in P5 (509.0 ± 35.41). TGF- β 1 followed the same trend, with P10 cells secreting markedly higher amounts (6130 ± 102.3) relative to P5 (654.1 ± 17.90) (Figure 9). In summary, these findings reveal a strong and consistent upregulation of SASP-related factors in the secretome of P10 JPCs ($P < 0.0001$), indicating that late-passage cells display a markedly more pronounced senescence-associated secretory phenotype compared with early-passage cells.

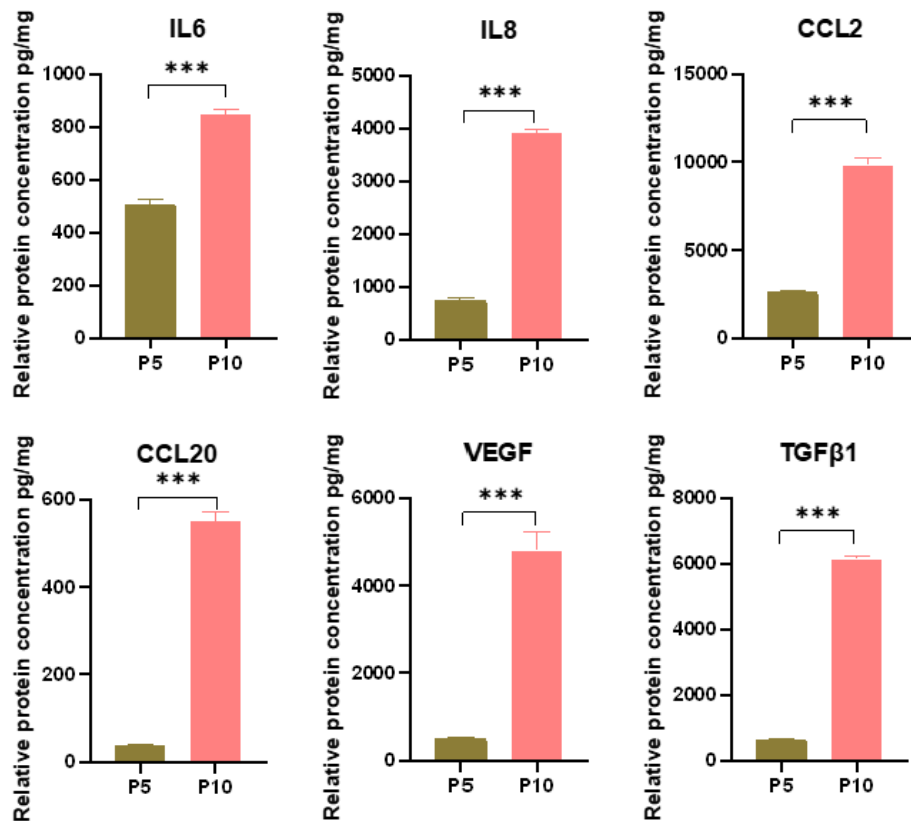


Figure 12. Quantification of senescence-associated secretory phenotype (SASP) factors in the secretome of early- and late-passage JPCs [76]. All examined SASP components were markedly elevated in the P10 secretome compared with P5, showing highly significant differences (for all analysed factors $p < 0.0001$). Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using an unpaired two-tailed Student's t-test. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.4.2 Proteomic profiling reveals distinct secretory signatures between P5 and P10 JPCs

To characterize global protein expression differences between early- and late-passage JPCs, label-free quantitative proteomic profiling was performed. In total, 9,365 proteins were identified and quantified across all samples. Quality control analyses indicated high reproducibility among biological replicates. Principal

component analysis (PCA) revealed clear separation between the P5 and P10 groups, with 95% confidence ellipses indicating distinct proteomic signatures (**Figure 13a, b**). Using thresholds of $|\log_2FC| \geq 1$ and adjusted $p < 0.05$, volcano plot analysis identified 901 differentially expressed proteins (DEPs), including 474 proteins upregulated and 427 proteins downregulated in P5 compared with P10 JPCs (**Figure 13c**).

Results

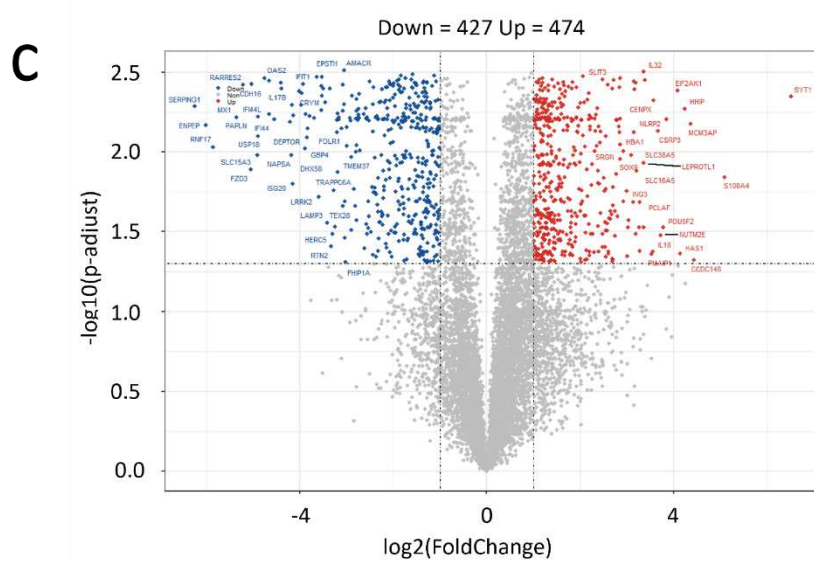
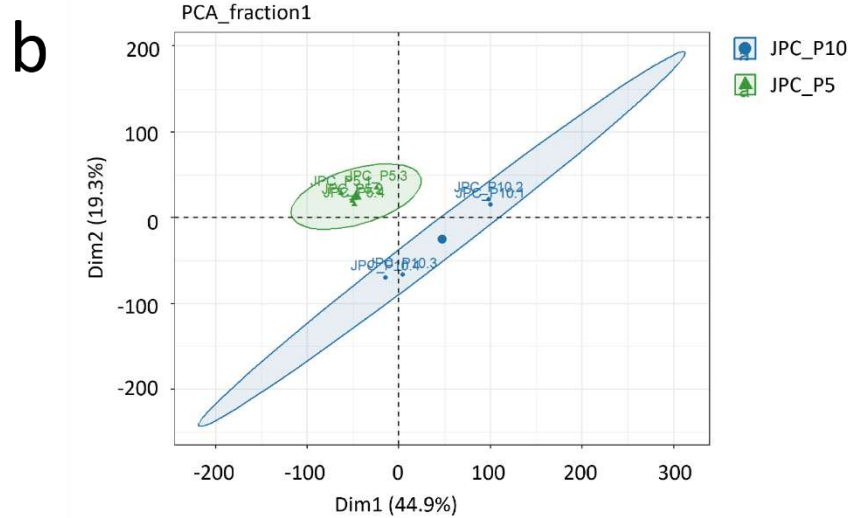
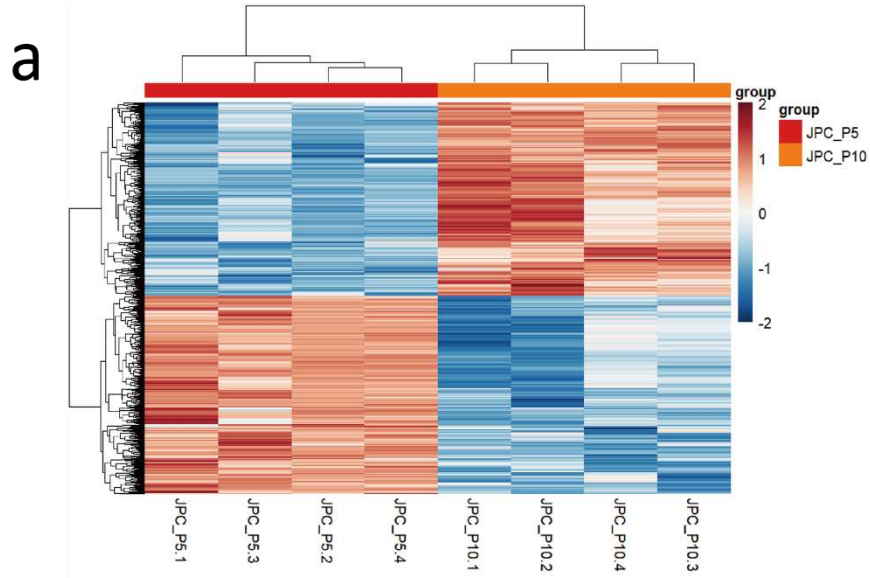


Figure 13. Proteomic profiling reveals distinct molecular signatures between early- and late-passage JPCs [76]. (a) Heatmap showing hierarchical clustering of differentially expressed proteins (DEPs) between passage 5 (P5) and passage 10 (P10) JPCs. Protein expression levels are scaled by Z-score, with red indicating higher expression and blue indicating lower expression. (b) Principal component analysis (PCA) of proteomic data demonstrating clear separation between P5 and P10 samples, indicating distinct proteomic profiles between early- and late-passage JPCs. Ellipses represent the 95% confidence interval. (c) Volcano plot displaying differentially expressed proteins between P5 and P10 JPCs. Proteins with an absolute $\log_2$ fold change ≥ 1 and adjusted p value < 0.05 are highlighted, with upregulated proteins shown in red and downregulated proteins shown in blue. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.4.3 KEGG pathway enrichment analysis of differentially expressed proteins

KEGG pathway enrichment analysis was performed to investigate functional pathways associated with DEPs in the secretomes of late-passage and early-passage JPCs. As shown in **Figure 14a**, a total of 901 DEPs identified between passage 10 (P10) and passage 5 (P5) JPC secretomes were mapped to 15 significantly enriched pathways, primarily related to cellular aging, inflammation and immune responses, and ECM interactions.

Pathways closely associated with cellular aging, including cell cycle, DNA replication, and ferroptosis, were significantly enriched among the DEPs identified in the P10 versus P5 comparison, suggesting passage-dependent alterations in proliferative regulation and stress-related signaling. In parallel, several immune- and inflammation-related pathways, such as complement and coagulation cascades and virus-associated pathways including herpes simplex virus type 1 infection and Epstein-Barr virus infection, were also significantly enriched among these DEPs. These findings are consistent with a senescence-associated secretory phenotype characterized by enhanced inflammatory signaling.

Results

Additionally, multiple pathways with established roles in neuronal differentiation, neurite outgrowth, and regeneration were also significantly enriched among the DEPs identified between P10 and P5 JPC secretomes, including ECM-receptor interaction, integrin signaling, and cytoskeleton-related pathways. The KEGG pathway-protein heatmap (**Figure 14b**) further demonstrated coordinated regulation of ECM-, integrin-, and cytoskeleton-associated proteins, indicating that passage-dependent remodeling of the JPC secretome simultaneously affects multiple functional modules.

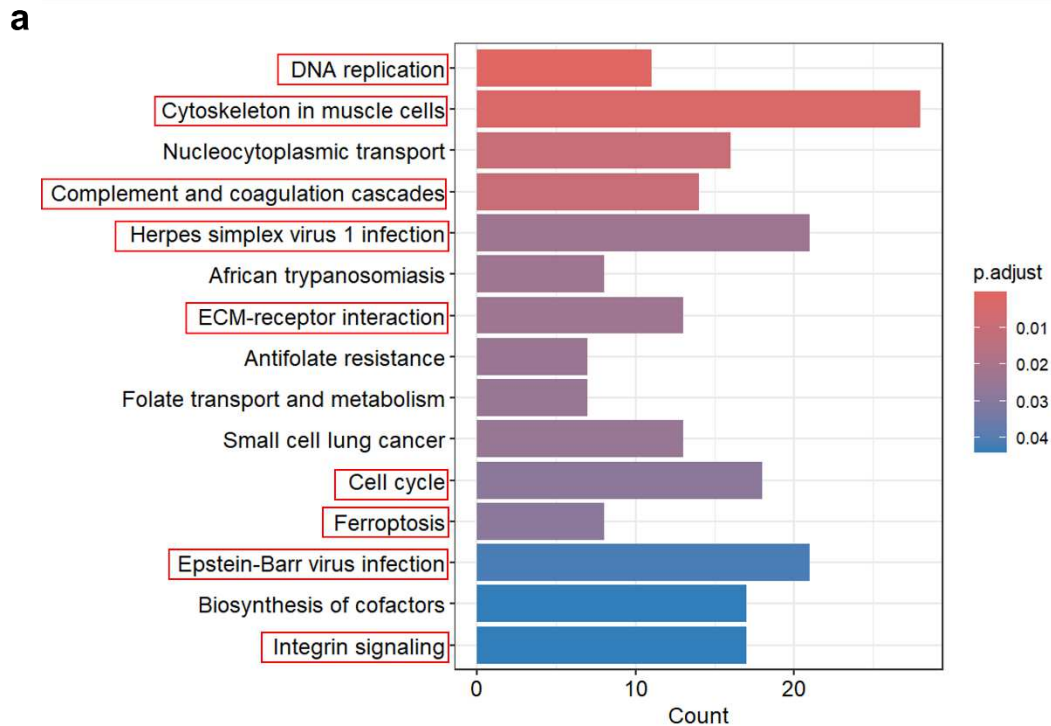




Figure 14. KEGG pathway enrichment analysis of differentially expressed proteins between early- and late-passage JPC secretomes [76]. (a) Bar plot showing significantly enriched KEGG pathways based on differentially expressed proteins between early- and late-passage JPC secretomes. The color of the bar indicates the adjusted p value, and pathways are arranged by adjusted p value. (b) Heatmap illustrating the $\log_2$ fold change ($\log_2FC$) of individual proteins associated with the enriched KEGG pathways. Each column represents a differentially expressed protein, and each row corresponds to a KEGG pathway. Red and blue colors indicate upregulation and downregulation, respectively, in P5 JPC secretome relative to P10 secretome. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

3.4.4 Gene Ontology enrichment analysis of differentially expressed proteins

GO pathway enrichment analysis was performed to investigate the functional pathways associated with DEPs in the secretome of early- and late-passage JPCs. Comparative GO analysis revealed significant alterations in biological processes related to immune responses, cell cycle regulation, and neuronal development (**Figure 15**).

Proteins enriched in the P10 secretome were significantly associated with antiviral and innate immune-related processes, including response to virus, defense response to virus, antiviral innate immune response, and positive regulation of type I interferon production. In addition, multiple cell cycle- and genome maintenance-related pathways were significantly enriched, including DNA replication, DNA replication initiation, positive regulation of chromosome separation, as well as cell growth and negative regulation of cell growth. These findings indicate altered regulation of proliferative activity and genomic integrity in late-passage cells, which are hallmark features of replicative senescence.

Furthermore, multiple biological processes associated with neurodevelopment and neuroplasticity were significantly enriched, including regulation of neuron

Results

projection development, dendrite development, dendrite morphogenesis, regulation of dendritic spine morphogenesis, synapse organization, and response to axon injury. These findings indicate that the JPC secretome contains numerous proteins capable of modulating neuronal structure and plasticity, and that this composition changes with increasing passage number and cellular aging.

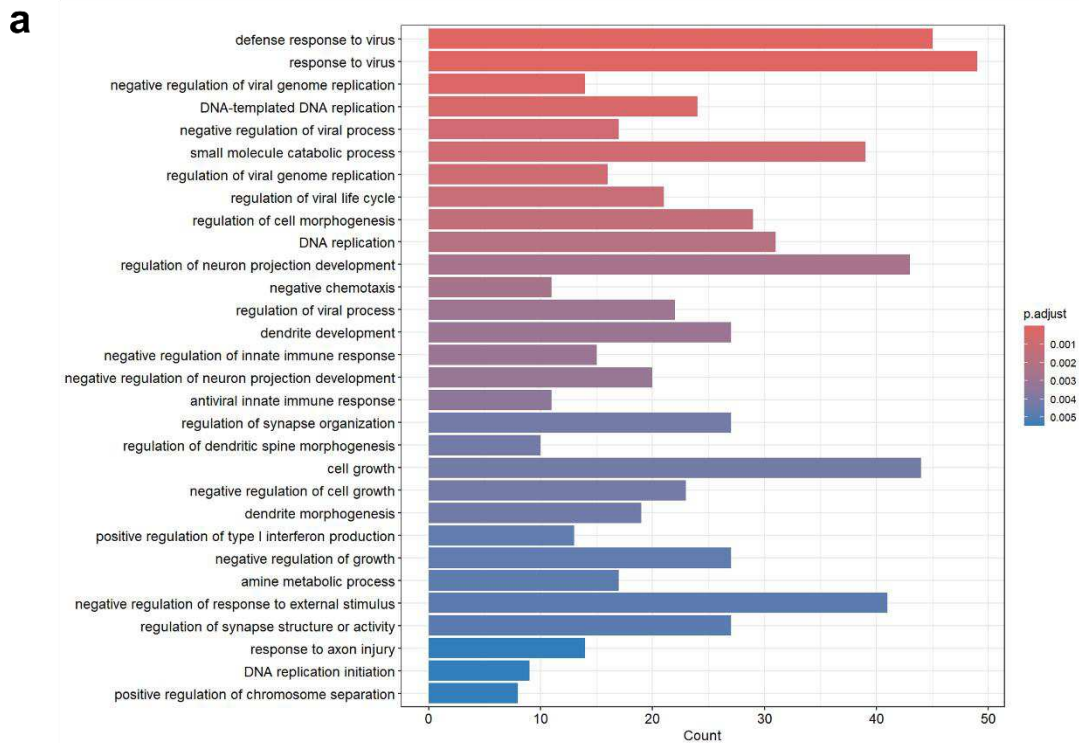




Figure 15. Gene Ontology (GO) enrichment analysis of differentially expressed proteins between early- and late-passage JPC secretomes [76].

(a) Bar plot showing the top 30 significantly enriched GO pathways based on differentially expressed proteins. Pathways are ranked according to adjusted p value, and bar color represents the adjusted p value. (b) Heatmap displaying the $\log_2$ fold change ($\log_2FC$) of individual proteins associated with the enriched GO pathways. Each column corresponds to a differentially expressed protein and each row represents a GO pathway. Red indicates upregulation and blue indicates downregulation in the P5 secretome relative to P10. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

Following the criteria of an adjusted p value < 0.05 and $|\log_2FC| > 4$, we identified 29 significantly differentially expressed proteins (**Table 10, Table 11**). The detailed quantitative data are presented in Table 10. Among these proteins, SYT1, S100A4, CCDC148, MCM3AP, H1-3, HHIP, HAS1, UBE2E1, and EIF2AK1 were more abundant in the P5 secretome than in the P10 secretome. In contrast, SOD3, LAMA1, ISG20, IL17B, NAPSA, CLYBL, HOGA1, TNFAIP6, IFI44, IFI44L, ALDH1A1, OAS2, USP18, PAPLN, SLC15A3, CDH16, FZD3, RARRES2, MX1, RNF17, ENPEP, and SERPING1 were more abundant in the P10 secretome.

Table 10. Significantly differentially secreted proteins in secretomes from JPC P5 and P10 cells [76]. (adjusted P value < 0.05 and $|\log_2 FC| > 4$) (Table published in “Wang et al., Cells 2026, 15(17), 1526”)

Protein/Gene	Mean protein abundance		Adjusted P value
	JPC P5	JPC P10	
SYT1	15.67 $\pm$ 0.35	9.15 $\pm$ 0.79	0.0045
S100A4	15.25 $\pm$ 1.44	10.15 $\pm$ 1.70	0.0143645
CCDC148	15.05 $\pm$ 1.21	10.61 $\pm$ 2.53	0.0475592
MCM3AP	13.62 $\pm$ 0.35	9.25 $\pm$ 0.36	0.00666667
HHIP	14.05 $\pm$ 0.18	9.80 $\pm$ 1.11	0.0053494
HAS1	13.45 $\pm$ 2.52	9.31 $\pm$ 0.19	0.0435553
EIF2AK1	13.58 $\pm$ 0.26	9.49 $\pm$ 0.70	0.00411429
SOD3	10.06 $\pm$ 0.40	14.07 $\pm$ 0.36	0.00415385

Results

LAMA1	9.66 ± 0.57	13.80 ± 0.06	0.00586667
ISG20	9.93 ± 0.49	14.08 ± 1.79	0.0158018
IL17B	9.88 ± 0.69	14.05 ± 0.83	0.00504641
NAPSA	9.84 ± 0.58	14.02 ± 1.51	0.0104516
CLYBL	10.17 ± 1.21	14.38 ± 0.20	0.00644648
HOGA1	10.84 ± 0.38	15.23 ± 0.63	0.004
TNFAIP6	9.60 ± 0.45	14.00 ± 0.54	0.00366667
IFI44	10.79 ± 1.38	15.33 ± 0.45	0.00624113
IFI44L	10.25 ± 1.23	14.90 ± 0.36	0.00576056
ALDH1A1	10.06 ± 0.88	14.71 ± 0.51	0.00357746
OAS2	9.91 ± 0.94	14.66 ± 0.47	0.00343243
USP18	9.40 ± 0.61	14.28 ± 1.65	0.00793594
PAPLN	9.48 ± 0.52	14.37 ± 0.23	0.006
SLC15A3	10.19 ± 0.45	15.10 ± 1.86	0.0104916
CDH16	9.67 ± 0.79	14.70 ± 0.79	0.00373333
FZD3	9.76 ± 1.24	14.80 ± 1.75	0.0128416
RARRES2	9.43 ± 0.61	14.65 ± 0.86	0.00376923
MX1	14.38 ± 1.65	19.74 ± 0.35	0.00602941
RNF17	12.27 ± 1.71	18.13 ± 1.36	0.00930706
ENPEP	8.94 ± 0.68	14.95 ± 0.33	0.00675
SERPING1	9.35 ± 0.14	15.60 ± 0.88	0.00517647

Table 11. GO biological process terms associated with selected differentially secreted proteins in secretomes from JPC P5 and P10 cells [76]. (Table published in “Wang et al., Cells 2026, 15(17), 1526”)

Gene symbol	Full name	Number of enriched GO BP terms	GO BP terms
SYT1	Synaptotagmin 1	6	regulation of cell morphogenesis; cell growth; response to metal ion; regulation of cell growth; developmental growth involved in morphogenesis; developmental cell growth

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S100A4	S100 calcium binding protein A4	4	mesenchymal cell differentiation; mesenchyme development; chemotaxis; taxis
CCDC148	Coiled-coil domain containing 148	0	
MCM3AP	Minichromosome maintenance complex component 3 associated protein	10	nucleocytoplasmic transport; nuclear transport; RNA export from nucleus; mRNA transport; nuclear export; mRNA export from nucleus; nucleic acid transport; RNA transport; establishment of RNA localization; nucleobase-containing compound transport
HHIP	Hedgehog interacting protein	7	lung development; respiratory tube development; respiratory system development; morphogenesis of a branching structure; branching morphogenesis of an epithelial tube; morphogenesis of a branching epithelium; epithelial tube branching involved in lung morphogenesis
HAS1	Hyaluronan synthase 1	4	ameboidal-type cell migration; extracellular matrix organization; extracellular structure organization; external encapsulating structure organization
EIF2AK1	Eukaryotic translation initiation factor 2 alpha kinase 1	1	multicellular organismal-level chemical homeostasis

Results

SOD3	Superoxide dismutase 3	8	response to metal ion; reactive oxygen species metabolic process; response to oxygen levels; response to decreased oxygen levels; response to hypoxia; response to copper ion; cellular response to oxidative stress; response to oxidative stress
LAMA1	Laminin subunit alpha 1	12	positive regulation of cell adhesion; lung development; respiratory tube development; respiratory system development; morphogenesis of a branching structure; branching morphogenesis of an epithelial tube; morphogenesis of a branching epithelium; extracellular matrix organization; extracellular structure organization; external encapsulating structure organization; gland morphogenesis; epithelial tube branching involved in lung morphogenesis
ISG20	Interferon stimulated exonuclease gene 20	10	defense response to virus; response to virus; negative regulation of viral genome replication; negative regulation of viral process; regulation of viral genome replication; regulation of viral life cycle; regulation of viral process; viral genome replication; viral process; viral life cycle
IL17B	Interleukin 17B	1	positive regulation of cytokine production

Results

NAPSA	Napsin A aspartic peptidase	2	surfactant homeostasis; multicellular organismal-level chemical homeostasis
CLYBL	Citrate lyase beta like	2	vitamin metabolic process; water-soluble vitamin metabolic process
HOGA1	4-hydroxy-2- oxoglutarate aldolase 1	10	small molecule catabolic process; cellular modified amino acid metabolic process; organic acid catabolic process; carboxylic acid catabolic process; dicarboxylic acid metabolic process; carboxylic acid biosynthetic process; organic acid biosynthetic process; alpha-amino acid catabolic process; carbohydrate derivative catabolic process; amino acid catabolic process
TNFAIP6	Tumor necrosis factor alpha induced protein 6	7	negative regulation of response to external stimulus; ossification; regulation of chemotaxis; negative regulation of chemotaxis; chemotaxis; taxis; osteoblast differentiation
IFI44	Interferon induced protein 44	1	response to virus
IFI44L	Interferon induced protein 44 like	2	defense response to virus; response to virus
ALDH1A1	Aldehyde dehydrogenase 1 family member A1	3	carboxylic acid biosynthetic process; organic acid biosynthetic process; carbohydrate derivative catabolic process

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OAS2	2'-5'-oligoadenylate synthetase 2	21	defense response to virus; response to virus; negative regulation of viral genome replication; negative regulation of viral process; regulation of viral genome replication; regulation of viral life cycle; regulation of viral process; positive regulation of type I interferon production; regulation of type I interferon production; type I interferon production; response to type I interferon; positive regulation of interferon-beta production; viral genome replication; cellular response to type I interferon; interferon-mediated signaling pathway; interferon-beta production; regulation of interferon-beta production; type I interferon-mediated signaling pathway; viral process; viral life cycle; positive regulation of cytokine production
USP18	Ubiquitin specific peptidase 18	14	defense response to virus; response to virus; negative regulation of innate immune response; antiviral innate immune response; negative regulation of response to external stimulus; response to type I interferon; regulation of innate immune response; negative regulation of response to biotic stimulus; cellular response to type I interferon; interferon-mediated signaling pathway; type I interferon-mediated

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			signaling pathway; negative regulation of type I interferon-mediated signaling pathway; regulation of type I interferon-mediated signaling pathway; regulation of response to cytokine stimulus
PAPLN	Papilin, proteoglycan like sulfated glycoprotein	3	extracellular matrix organization; extracellular structure organization; external encapsulating structure organization
SLC15A3	Solute carrier family 15 member 3	5	regulation of innate immune response; cytoplasmic pattern recognition receptor signaling pathway; pattern recognition receptor signaling pathway; positive regulation of innate immune response; innate immune response-activating signaling pathway
CDH16	Cadherin 16	0	
FZD3	Frizzled class receptor 3	6	response to xenobiotic stimulus; regulation of nervous system development; regulation of neurogenesis; positive regulation of nervous system development; axon guidance; neuron projection guidance
RARRES2	Retinoic acid receptor responder 2	3	regulation of chemotaxis; chemotaxis; taxis
MX1	MX dynamin like GTPase 1	12	defense response to virus; response to virus; negative regulation of viral genome replication; negative regulation of viral process; regulation of viral genome replication; regulation of viral life cycle; regulation of viral

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			process; antiviral innate immune response; response to type I interferon; viral genome replication; viral process; viral life cycle
RNF17	Ring finger protein 17	0	
ENPEP	Glutamyl aminopeptidase	0	
SERPING1	Serpin family G member 1	6	negative regulation of innate immune response; negative regulation of response to external stimulus; regulation of innate immune response; negative regulation of response to biotic stimulus; regulation of endopeptidase activity; wound healing

Note: The listed genes were selected from the significantly differentially secreted proteins shown in Table 10. Gene full names and associated GO biological process terms were retrieved from the Gene Ontology database based on their gene symbols.

3.5 Effects of JPC secretomes on NGN2 neurons

3.5.1 Effects of JPC secretomes on NGN2 neuron survival

To evaluate the functional effects of JPC secretomes at different passages, NGN2 neurons were treated with secretomes derived from JPCs at passage 5 (JPC P5) or passage 10 (JPC P10) in addition to the neuronal differentiation cocktail. Neuronal survival/retention was evaluated by quantifying DAPI-positive nuclei in DCX/DAPI double-stained images. DCX staining was observed in all remaining DAPI-positive cells across all experimental groups, indicating that the surviving cells had acquired an immature neuronal phenotype. Therefore, the number of DCX-positive cells per image was identical to the number of DAPI-positive nuclei per image shown in **Figure 16**, and an additional quantitative graph for DCX-

positive cells was not included. Quantitative analysis demonstrated that cell survival/retention in the group additionally treated with secretome from JPC P5 cells was comparable to that obtained in the positive control group (JPC P5: 188.2 ± 33.35 vs. Po: 183.6 ± 55.40), with no significant difference between the two groups (Figure 16). In contrast, cell survival in the JPC P5 group was significantly higher than that observed in the negative control (Ne: 100.8 ± 62.32 ; $P = 0.005$) and that obtained in the JPC P10 group (80.28 ± 36.06 ; $p < 0.001$).

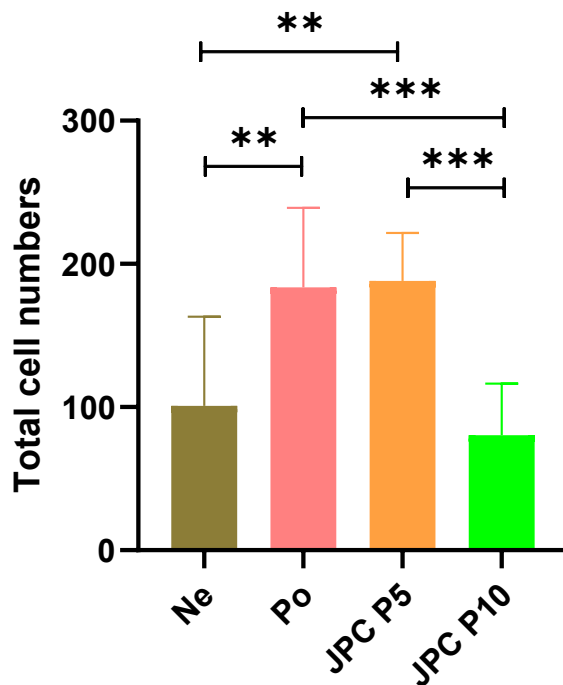


Figure 16. Effects of JPC secretomes of different passages on the survival/retention of NGN2 neurons [76]. Cells were stained for DCX and counterstained with DAPI. The number of DAPI-positive nuclei per image was quantified as a measure of surviving/retained cells in DCX/DAPI double-stained images. Since DCX staining was detected in all remaining DAPI-positive cells across all experimental groups, the number of DAPI-positive nuclei corresponded to the number of DCX-positive cells, and DCX-positive cell counts were not shown as a separate graph. Quantitative data are presented as mean $\pm$ SD. Cell survival/retention in the JPC P5 group was comparable to that obtained in the positive control and significantly higher than that in the negative control and the

JPC P10 group (Ne, negative control: n = 10; Po, positive control: n = 10; JPC P5: n = 10; JPC P10: n = 9). Statistical analysis was performed using Welch's one-way ANOVA followed by the Games-Howell post hoc test (* p < 0.05; ** p < 0.01; *** p < 0.001). (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.5.2 Effects of JPC secretomes on neurite outgrowth

Neurite outgrowth was quantified in doublecortin (DCX) stained cells by measuring the total neurite length per image using ImageJ. Quantitative analysis demonstrated that treatment with JPC secretomes significantly modulated neurite extension, as reflected by differences in total neurite length among groups (**Figure 17**). Cells cultured under negative control conditions (without differentiation cocktail) exhibited limited neurite extension ($7098 \pm 4187 \mu\text{m}$), whereas the positive control group showed robust neurite outgrowth ($45661 \pm 34472 \mu\text{m}$, p < 0.05). Treatment with the JPC P5 secretome significantly increased neurite length compared with both the negative control and the JPC P10 group (both p < 0.001). Neurite length in the JPC P5 group reached $17134 \pm 4497 \mu\text{m}$, compared with $7924 \pm 2432 \mu\text{m}$ in the JPC P10 group. These findings indicate that secretome derived from early-passage JPCs promotes neurite extension more effectively than that from late-passage cells.

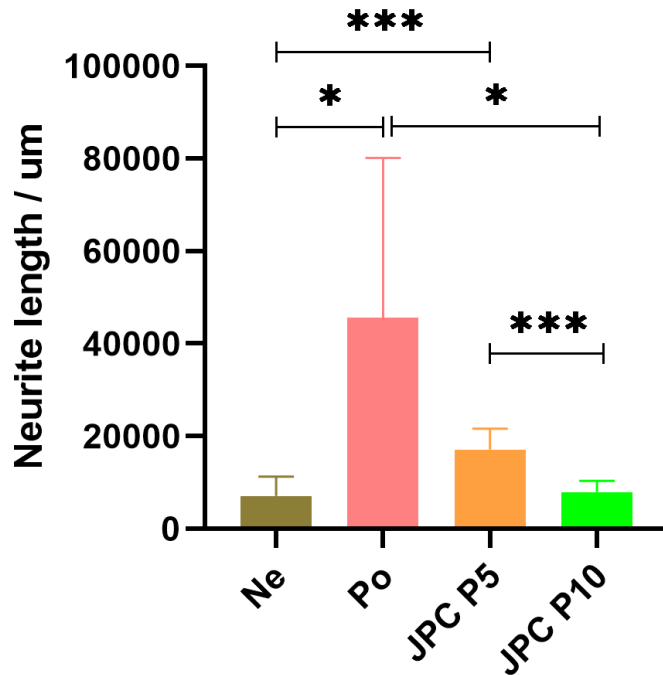


Figure 17. Effects of JPC secretomes on neurite outgrowth in induced neuronal progenitor cells [76]. Neurite outgrowth was quantified as total neurite length per image. Quantitative analysis revealed significantly increased neurite length in the JPC P5 group compared with both the negative control and JPC P10 groups. Data are presented as mean $\pm$ SD (Ne: $n = 10$; Po: $n = 10$; JPC P5: $n = 10$; JPC P10: $n = 9$). Statistical analysis was performed using Statistical analysis was performed using Welch's one-way ANOVA followed by the Games-Howell post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). (Image published in "Wang et al., Cells 2026, 15(17), 1526")

3.5.3 Effects of JPC secretomes on neuron differentiation

Neuronal differentiation was evaluated by immunofluorescence staining for microtubule-associated protein-2 (MAP2), a marker associated with neuronal differentiation and maturation, together with doublecortin (DCX), which labels immature neurons (**Figure 18**). As described in Section 3.5.1, DCX staining was detected in all remaining DAPI-positive cells across all experimental groups; therefore, DCX-positive cell numbers were not quantified separately. Further

neuronal differentiation was assessed by quantifying MAP2-positive cells per image (**Figure 19**). Cells cultured under negative control conditions exhibited low numbers of MAP2-positive cells (2.435 ± 1.159), whereas the positive control group showed markedly higher values (13.30 ± 8.895 ; Ne vs. Po, $p = 0.017$). Treatment with the JPC P5 secretome significantly increased the number of MAP2-positive cells compared with the negative control (5.103 ± 1.484 ; JPC P5 vs. Ne, $p = 0.002$). The JPC P10 group displayed intermediate and highly variable values (6.535 ± 8.048). No statistically significant difference was observed between the JPC P5 and JPC P10 groups. Overall, these findings indicate that JPC-derived secretomes promote neuronal differentiation compared with the negative control, although no significant difference was detected between early- and late-passage JPC secretomes under the present conditions.

Results

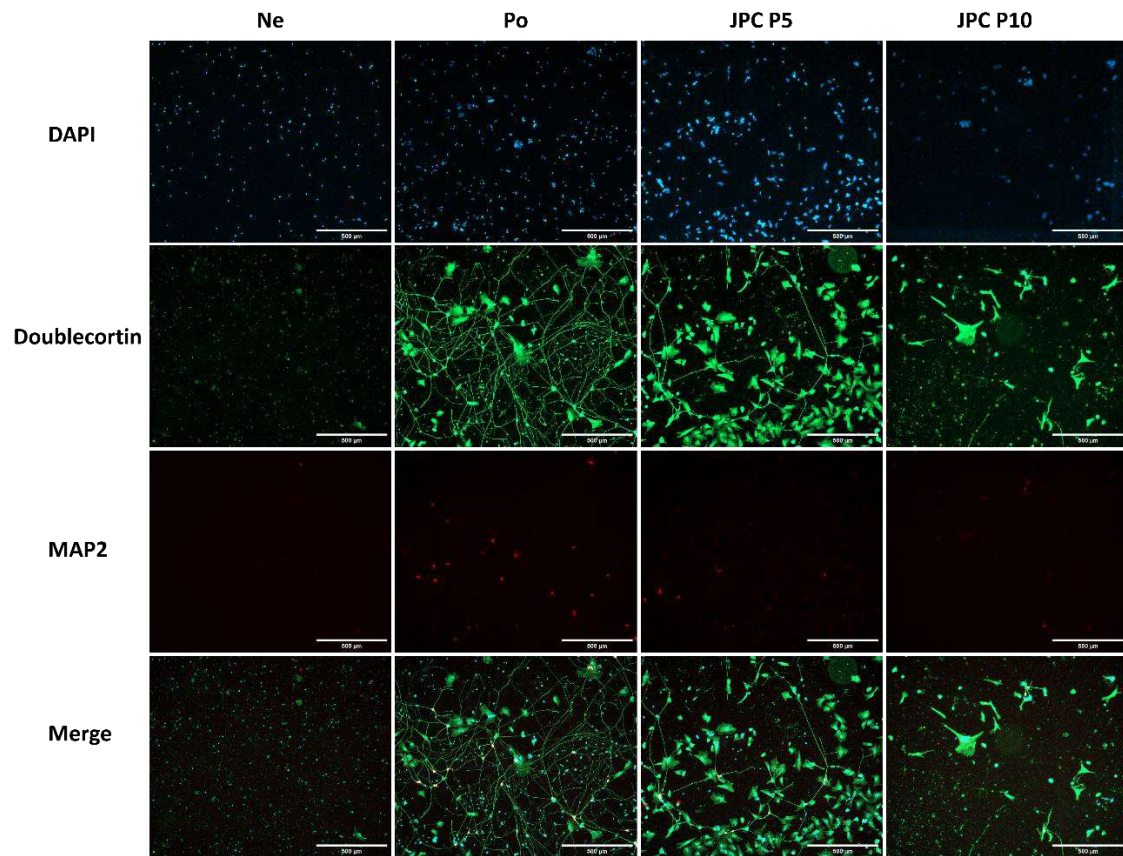


Figure 18. Immunofluorescence images of neural differentiation under different conditions [76]. Representative fluorescence micrographs from four groups (Ne – negative control, Po – positive control, addition of secretome from JPC P5/P10) showing nuclei (DAPI, blue), a marker for immature neurons (Doublecortin, green), a marker for mature neurons (MAP2, red), and the merged images (Merge). Scale bar = 500 µm. (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

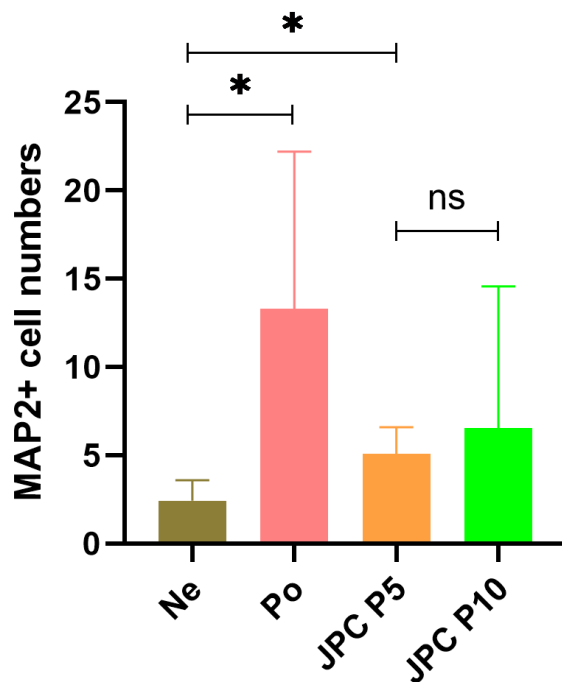


Figure 19. Effects of JPC secretomes on neuronal differentiation [76]. Neuronal differentiation was quantified by counting the number of MAP2-positive cells per image. The positive control group exhibited the highest number of MAP2-positive cells, whereas the negative control group showed minimal neuronal differentiation. Treatment with JPC-derived secretomes resulted in increased numbers of MAP2-positive cells compared with the negative control group. Data are presented as mean $\pm$ SD (Ne: $n = 10$; Po: $n = 10$; JPC P5: $n = 10$; JPC P10: $n = 9$). Statistical analysis was performed using Welch's one-way ANOVA followed by the Games–Howell post hoc test ($*p < 0.05$). (Image published in “Wang et al., Cells 2026, 15(17), 1526”)

4. Discussion

4.1 Replicative senescence phenotype in late-passage JPCs

Peripheral nerve regeneration is of particular relevance in oral and maxillofacial as well as orthopedic surgery, where nerve injury may occur in the context of trauma, reconstructive procedures, or tumor resection[4, 75]. Rather than revisiting general regenerative strategies, the present study focused specifically on whether prolonged in vitro expansion and senescence-associated changes in JPCs modify their paracrine effects on neuronal regeneration-related processes. Therefore, this study investigated senescence-associated alterations in JPCs and evaluated whether secretomes derived from early- and late-passage JPCs differentially affect neuronal viability, neurite outgrowth, and neuronal differentiation.

In the present study, we could demonstrate that late-passage JPCs (P10) displayed characteristic features of cellular senescence when compared with early-passage cells (P5). CCK-8 assays performed here indicated a significant decline in proliferative capacity of P10 cells, consistent with the onset of replicative growth arrest during prolonged in vitro expansion. This reduction in proliferative potential represents a hallmark of replicative senescence and indicates diminished regenerative capacity at advanced passage stages[81].

At the molecular level, P10 JPCs demonstrated significantly increased SA- β -gal activity, confirming the accumulation of senescent cells[82]. In parallel, gene expression analysis revealed marked upregulation of *CDKN2A* (p16), a key cyclin-dependent kinase inhibitor. In human derived MSCs, the p16-Rb pathway is recognized as the "final gatekeeper" of senescence. By inhibiting CDK4/6, p16 maintains the retinoblastoma protein in its hypophosphorylated state, thereby

permanently silencing E2F (transcription factor regulating cell cycle progression) -target genes required for the S-phase entry[83-86].

Interestingly, although p16 mRNA expression was significantly elevated in P10 compared to P5 JPCs, no significant changes were observed in *CDKN1A* (p21) or *TP53* transcript levels. The observed combination of increased p16 expression and unchanged p21 levels aligns with the molecular characteristics of late-stage or stabilized senescence[70, 87]. In many primary human cell models, the p53/p21 axis functions as an early and often transient mediator of growth arrest in response to telomeric DNA damage or acute stress[88, 89]. As senescence becomes stabilized and irreversible, the p16/Rb pathway frequently assumes a dominant role in maintaining long-term cell cycle arrest[90, 91]. The absence of sustained p53 or p21 elevation in P10 JPCs suggests that these cells may have progressed beyond the initial DNA damage response (DDR) phase and entered a consolidated senescent state primarily maintained by p16-dependent signaling. Indeed, the lack of concurrent p21 upregulation further corroborates the absence of functional p53 activation in this late-stage senescent phenotype.

Additionally, the upregulation of SASP factors at transcriptional level showing only a slight tendency and at protein level showing a highly significant elevation in cells of late passage, indicates substantial remodeling of the JPC secretory profile during long-term culture. Such changes suggest that senescent JPCs may exert altered paracrine effects on the surrounding microenvironment, potentially influencing the behavior of neighboring cells[92-94]. Given that SASP components include inflammatory cytokines, chemokines, and ECM-modulating factors, their elevation supports the establishment of a stable senescent state with functional consequences beyond cell-autonomous growth arrest.

Notably, no significant but only a slight tendency of accumulation of intracellular ROS was detected in late-passage JPCs. This observation suggests that the senescence phenotype observed here is consistent with culture-induced replicative exhaustion, rather than stress-induced premature senescence (SIPS) driven by acute oxidative damage[95, 96]. While oxidative stress is a well-established trigger of SIPS in many contexts, our findings indicate that the onset and stabilization of replicative senescence in JPCs can occur independently of sustained ROS elevation.

In summary, prolonged in vitro expansion of JPCs appears to induce a replicative senescence program characterized by p16-dominant cell cycle arrest and SASP activation, without evidence of persistent p53/p21 signaling or elevated oxidative stress. These findings identify p16 as a central regulator of JPC aging during culture expansion and provide a mechanistic framework for understanding subsequent secretome remodeling.

4.2 Secretome remodeling elucidated by proteomic enrichment analysis

The proteomic landscape of the JPC secretome exhibited marked functional reprogramming during replicative senescence. KEGG and GO enrichment analyses, together with differential protein abundance profiles, suggest that senescence did not merely alter individual secreted proteins but reshaped interconnected functional modules related to extracellular matrix organization, neuronal structural development, immune activation, antiviral response, complement regulation, and stress-associated signaling. These pathway-level changes provide a mechanistic framework for understanding why early- and late-passage JPC secretomes exerted different effects on neuronal survival and neurite outgrowth.

Proteins more abundant in the P5 secretome may indicate a more neuro-supportive and structurally permissive microenvironment. For example, SYT1 was associated with synapse-related biological processes, suggesting that the P5 secretome may be more closely linked to neuronal maturation and synaptic organization[97, 98]. S100A4 is involved in cytoskeletal regulation, cell motility, and tissue remodeling, and may therefore reflect a secretory profile associated with structural plasticity[99, 100]. In addition, HHIP and HAS1 may contribute to developmental signaling and hyaluronan-dependent matrix organization, respectively[101-103]. Importantly, the stronger neuronal effect of the P5 secretome is unlikely to be explained only by highly differentially secreted proteins. Several ECM- and adhesion-related proteins, including ITGA2, ITGA6, COL6A3, and LAMC2, were also higher in P5 than in P10, although they did not meet the stricter threshold of absolute log2 fold change > 4. These moderate but coordinated changes may be functionally relevant because integrin- and ECM-associated components regulate cell adhesion, mechanotransduction, cytoskeletal organization, and growth cone behavior[31, 32, 34].

In contrast, the P10 secretome showed an immune- and stress-associated shift rather than a simple loss of secretory activity. This shift was mainly reflected by interferon- and antiviral-response proteins, including ISG20, IFI44, IFI44L, OAS2, MX1, and USP18[104-108]; inflammatory or immune-regulatory proteins, including IL17B and RARRES2[109, 110]; the complement-related protein SERPING1[111]; and the matrix-remodeling protein TNFAIP6[112, 113]. Together, these proteins suggest that late-passage JPCs acquired an innate immune-like, inflammatory, complement-associated, and matrix-remodeling secretory phenotype during replicative senescence. Such a profile is consistent with a SASP-like secretory context.

The interpretation of ECM-related proteins requires particular caution. Senescence did not simply reduce ECM-associated secretion; instead, it induced qualitative ECM remodeling. HAS1 was higher in P5, whereas LAMA1, PAPLN, and TNFAIP6 were higher in P10. Therefore, the presence of individual ECM-associated proteins in the P10 secretome does not necessarily indicate a more neuro-supportive matrix environment. ECM function depends on the balance, organization, and receptor compatibility of multiple matrix components[35, 103, 114, 115]. In the P10 secretome, potentially supportive matrix-associated proteins were embedded within a broader inflammatory, interferon-associated, complement-related, and stress-responsive context, which may limit or override their beneficial effects[25, 112, 115].

Several P10-enriched proteins, including FZD3, ALDH1A1, SOD3, LAMA1, and PAPLN, may also be associated with neuronal development, retinoid metabolism, antioxidant activity, or matrix organization[31, 116-120]. However, their presence did not translate into superior neuronal outcomes in the functional assays. This supports the interpretation that neuronal responses are determined by the integrated secretome context rather than by single proteins[25, 115]. However, their presence should not be interpreted as evidence of a globally neuro-supportive P10 secretome. Instead, these findings indicate that neuronal or ECM-associated proteins can coexist with inflammatory, antiviral, complement-related, and stress-associated signatures in the senescent secretome. Thus, replicative senescence appears to shift the JPC secretome from a structurally permissive and adhesion-supportive profile toward a remodeled but potentially less regenerative secretory phenotype.

4.3 Functional superiority of early-passage JPC secretome in supporting neural differentiation

To assess whether senescence-associated secretome remodeling has functional consequences for neuronal regeneration-related processes, NGN2-induced neurons were exposed to secretomes derived from early- and late-passage JPCs. The NGN2-induced neuronal model has been widely used as a reproducible platform for generating human iPSC-derived neurons[77]. The functional assays demonstrated that both early- and late-passage JPC secretomes supported neuronal differentiation relative to the negative control, as indicated by MAP2 expression. However, only the early-passage P5 secretome clearly enhanced neuronal survival and neurite extension. This indicates that senescence-associated remodeling of the JPC secretome does not completely abolish its neuro-supportive activity, but selectively reduces its capacity to support more demanding regenerative processes such as neuronal viability and neurite elongation.

The differential effects observed across the functional readouts may reflect distinct biological requirements. MAP2-associated neuronal differentiation may depend on a basic level of trophic or permissive support, which appears to be provided by both P5 and P10 secretomes. In contrast, neuronal viability and neurite extension are likely more sensitive to the quality of ECM-associated cues, integrin-mediated adhesion, cytoskeletal regulation, and inflammatory or stress-related signals in the extracellular environment[31, 32, 34, 115].

This interpretation is consistent with the proteomic findings described above. The superior effect of the P5 secretome may reflect a more favorable balance of matrix-supportive, adhesion-related, and neuro-supportive components. P5-

enriched or moderately increased proteins associated with cytoskeletal regulation, hyaluronan-dependent matrix organization, ECM-receptor interaction, and integrin signaling may collectively support cell-matrix communication, growth cone dynamics, and neurite extension[31, 32, 34, 115, 121]. In contrast, the weaker effect of the P10 secretome may result from its broader immune-, interferon-, complement-, and stress-associated secretory context, which may have counteracted the activity of individual ECM- or neurodevelopment-associated proteins. Thus, the neuronal outcomes observed in the functional assays appear to depend on the integrated secretome context rather than on single supportive proteins[25, 115].

These findings are in line with previous studies showing that MSC-derived secretomes or extracellular vesicles from bone marrow, adipose tissue, and umbilical cord sources can promote neuronal survival, Schwann cell activity, neurite outgrowth, axonal regeneration, and functional recovery through paracrine mechanisms involving neurotrophic factors, ECM-associated proteins, extracellular vesicles, and immunomodulatory mediators[46, 121-123]. At the same time, studies on MSC aging have shown that donor age, prolonged in vitro expansion, and cellular senescence can reduce MSC regenerative potential and alter secretory activity[124-126]. The present findings extend these observations to JPC-derived secretomes and indicate that passage number and senescence status are critical determinants of their neuroregenerative capacity.

Taken together, the functional superiority of the early-passage JPC secretome in promoting neuronal survival and neurite extension appears to result from a more favorable balance between trophic, ECM-related, adhesion-supportive, and low-inflammatory signals. Conversely, replicative senescence shifts the JPC secretome toward an immune-activated and stress-associated phenotype that

remains partially neuro-supportive but is less effective in promoting neuronal regeneration-related outcomes. These results highlight the importance of cellular age and passage number when considering JPC-derived secretomes for neural repair and support the potential utility of early-passage JPC secretomes as a cell-free strategy for enhancing nerve regeneration in clinically relevant contexts.

4.4 Limitations and Future Directions

Despite the mechanistic and functional insights provided in this study, several limitations should be acknowledged. First, although proteomic analysis revealed significant pathway-level remodeling of the JPC secretome during replicative senescence, we did not directly validate which specific proteins or pathways were responsible for the observed neuronal phenotypes. Future functional blockade or supplementation experiments targeting individual ECM components, integrin-related proteins, complement factors, interferon-associated proteins, or inflammatory mediators will help identify the key drivers of neurite extension and neuronal survival.

Second, the present study relied primarily on in vitro assays. While these systems provide controlled conditions to evaluate secretome-mediated effects, they do not fully capture the complexity of the in vivo neural microenvironment, including vascular, immune, and glial interactions. Future studies using in vivo nerve injury models are needed to confirm the therapeutic potential of JPC-derived secretomes under physiologically relevant conditions.

Third, although the NGN2-inducible system is a mature and efficient method for generating neurons from iPSCs, the resulting neurons primarily resemble glutamatergic cortical excitatory neurons[77, 127]. In clinical contexts such as oral and maxillofacial surgery or orthopedic nerve injury, however, the affected

neurons are predominantly sensory and motor neurons[4, 75, 128]. Therefore, the current neuronal model may not fully recapitulate the functional requirements of craniofacial or peripheral nerve regeneration. Future studies employing iPSC-derived sensory neurons, motor neurons, or primary neurons relevant to craniofacial and peripheral nerve repair will be essential to validate the neuroregenerative potential of JPC-derived secretomes in clinically relevant neuronal populations.

Fourth, replicative senescence in this study was established based on passage-dependent changes and classical molecular markers, including p16 upregulation and SASP activation. While these markers support the senescent state of the overall cell population, future studies using single-cell senescence profiling, telomere length measurement, and more detailed DNA damage response analysis would help elucidate the aging process in greater detail and identify potential subpopulation heterogeneity within JPC cultures[129]. It would also be valuable to investigate whether senescence-associated secretome changes can be prevented or reversed using targeted interventions, such as senolytic or senomorphic treatments[130].

Finally, while this study demonstrates that cellular aging reduces the neuro-supportive capacity of JPC secretome, donor-to-donor variability remains a challenge for clinical translation. Standardizing culture conditions, setting strict passage limits, defining potency assays, and establishing quality control metrics based on senescence markers and secretome composition will be critical for developing reliable and reproducible JPC secretome-based therapies.

In summary, addressing these limitations will not only strengthen the mechanistic understanding of JPC secretome function, but also help optimize JPC-derived

secretomes for neural regeneration. Future investigations incorporating sensory and motor neuron models, targeted protein validation, and in vivo nerve injury studies will be crucial to confirm the therapeutic potential of JPC secretomes in clinically relevant neuronal populations.

5. Summary

This study systematically investigated how prolonged in vitro culturing finally inducing replicative senescence influences the biological properties and neuroregenerative potential of jaw periosteum cell (JPCs)-derived secretome. By integrating senescence characterization, proteomic profiling, and functional neuronal assays, we demonstrate that cellular aging profoundly reshapes the paracrine landscape of JPCs and alters their capacity to support neural repair.

First, prolonged in vitro expansion induced a replicative senescence phenotype in JPCs, characterized by reduced proliferative capacity, increased SA- β -gal activity, elevated p16 expression, and activation of senescence-associated secretory phenotype (SASP) factors. Notably, this senescent state appeared predominantly p16-driven and was not accompanied by sustained p53/p21 activation or significant ROS accumulation, consistent with stabilized replicative senescence rather than acute stress-induced aging.

Second, proteomic analysis revealed extensive remodeling of the JPC secretome during senescence. Early-passage JPCs exhibited enrichment of pathways associated with extracellular matrix organization, integrin signaling, and cytoskeletal regulation, whereas late-passage JPCs showed increased representation of inflammatory, immune-related, and stress-responsive pathways. These findings indicate a functional shift from a matrix-supportive and growth-permissive secretory profile toward an immune- and stress-associated phenotype during replicative aging.

Third, functional assays demonstrated that this secretome remodeling has direct biological consequences. While both early- and late-passage secretomes supported neuronal differentiation relative to baseline conditions, only early-

passage JPC secretome robustly promoted neuronal survival and neurite extension. These results suggest that replicative senescence does not abolish neuro-supportive activity but selectively compromises structural and trophic support functions that are critical for effective neural regeneration.

All in all, this study establishes a mechanistic link between replicative senescence of JPCs, secretome reprogramming, and altered neuroregenerative efficacy. Since passage-dependent replicative senescence represents an *in vitro* correlate of increasing biological age, our findings highlight cellular biological age as a critical determinant of JPC secretome quality. These results underscore the importance of controlling passage number and senescence status in the development of secretome-based regenerative therapies. By delineating how senescence reshapes the extracellular signaling environment, this work provides a conceptual framework for optimizing periosteum-derived paracrine strategies in neural repair applications.

6. Zusammenfassung

In der vorliegenden Arbeit wurde systematisch untersucht, wie eine lange Zellkultivierung, die in der Induktion der replikativen Seneszenz resultiert, die biologischen Eigenschaften und das neuroregenerative Potenzial von Sekretomen aus Kieferperiostzellen (Jaw Periosteum Cells, JPCs) beeinflusst. Durch die Kombination von Seneszenzcharakterisierung, proteomischer Analyse und funktionellen neuronalen Assays konnte gezeigt werden, dass zelluläre Alterung die parakrine Signalumgebung von JPCs grundlegend verändert und ihre Fähigkeit zur Unterstützung neuronaler Regeneration moduliert.

Erstens führte eine verlängerte in-vitro Expansion zur Ausbildung eines replikativen Seneszenzphänotyps in JPCs, der durch eine verminderte Proliferationsfähigkeit, erhöhte SA- β -Gal-Aktivität, gesteigerte p16-Expression sowie die Aktivierung von Faktoren des seneszenzassoziierten sekretorischen Phänotyps (SASP) gekennzeichnet war. Bemerkenswerterweise schien dieser seneszente Zustand überwiegend p16-dominiert zu sein und ging nicht mit einer anhaltenden Aktivierung der p53/p21-Achse oder einer signifikanten Akkumulation reaktiver Sauerstoffspezies (ROS) einher. Dies spricht für eine stabilisierte replikative Seneszenz und nicht für eine akut stressinduzierte vorzeitige Alterung.

Zweitens zeigte die proteomische Analyse eine ausgeprägte Umgestaltung des JPC-Sekretoms im Verlauf der Seneszenz. Frühe Passagen der JPCs wiesen eine Anreicherung von Signalwegen auf, die mit der Organisation der extrazellulären Matrix, Integrin-Signalübertragung und Zytoskelettregulation assoziiert sind. Demgegenüber war das Sekretom spätpassagierter JPCs durch eine verstärkte Repräsentation entzündungsassoziiierter, immunologischer und

stressbezogener Signalwege charakterisiert. Diese Ergebnisse weisen auf einen funktionellen Wandel von einem matrixunterstützenden und wachstumsfördernden sekretorischen Profil hin zu einem entzündungs- und stressassoziierten Phänotyp im Rahmen der replikativen Alterung.

Drittens belegten funktionelle Analysen, dass diese Umstrukturierung des Sekretoms direkte biologische Konsequenzen hat. Während sowohl früh- als auch spätpassagierte Sekretome im Vergleich zu Kontrollbedingungen die neuronale Differenzierung unterstützten, förderte ausschließlich das Sekretom frühpassasierter JPCs signifikant das neuronale Überleben und das Neuritenauswachsen. Diese Befunde legen nahe, dass replikative Seneszenz die neuroprotektive Aktivität nicht vollständig aufhebt, jedoch selektiv strukturelle und trophische Unterstützungsfunktionen beeinträchtigt, die für eine effektive neuronale Regeneration essenziell sind.

Zusammenfassend etabliert diese Arbeit einen mechanistischen Zusammenhang zwischen replikativer Seneszenz, Sekretom-Umprogrammierung und veränderter neuroregenerativer Wirksamkeit. Da passageabhängige replikative Seneszenz ein in-vitro Korrelat zunehmenden biologischen Alters darstellt, unterstreichen die Ergebnisse die Bedeutung des zellulären biologischen Alters als entscheidenden Faktor für die Qualität des JPC-Sekretoms. Die vorliegenden Befunde verdeutlichen die Notwendigkeit, Passagenzahl und Seneszenzstatus bei der Entwicklung sekretombasierter regenerativer Therapien gezielt zu kontrollieren. Durch die Aufklärung der seneszenzbedingten Veränderungen der extrazellulären Signalumgebung liefert diese Arbeit eine konzeptionelle Grundlage für die Optimierung Periostzell-basierter parakriner Strategien in der neuronalen Regeneration.

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Appendix

Declaration of Contribution

The dissertation work was carried out at the Department of Oral and Maxillofacial Surgery, University Hospital Tübingen, under the supervision of Prof. Dr. Dorothea Alexander-Friedrich.

The study concept, hypotheses, and experimental design were developed in collaboration with Prof. Dr. Dorothea Alexander-Friedrich. The experimental methods were selected in discussion with Prof. Dr. Dorothea Alexander-Friedrich, Dr. Felix Umrath, PD Dr. Julia Fitzgerald, and me. The lentiviral vectors for the generation of NGN2-induced neurons were kindly provided by PD Dr. Julia Fitzgerald. Prof. Dr. Dr. Bernd Lethaus contributed to patient recruitment, and the experimental resources were provided with the support of Prof. Dr. Dorothea Alexander-Friedrich and Prof. Dr. Dr. Bernd Lethaus.

After training by Dr. Felix Umrath and PD Dr. Julia Fitzgerald, I carried out the experiments independently and performed the statistical analysis and data interpretation myself. I wrote the manuscript under the supervision of Prof. Dr. Dorothea Alexander-Friedrich, who subsequently corrected and reviewed it. All additional sources of information have been duly cited.

ChatGPT was used only for language editing and grammatical correction. I carefully reviewed and adapted the output before incorporating any changes into the manuscript.

I therefore certify that, unless otherwise stated, this thesis represents my own independent scientific work.

Signature: _____

Appendix

List of Publications

Parts of the present dissertation have been published in the following article:

Wang, Y., et al. *Prolonged In Vitro Expansion Shapes the Neuro-Supportive Potential of Jaw Periosteum Secretomes: Implications for Secretome Product Quality*. Cells, 2026. **15**, 1526 DOI: 10.3390/cells15171526.

Appendix

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I am very grateful to Dr. Felix Umrath, PD Dr. Julia Fitzgerald, Ms. Wanjing Cen, Ms. Liuran Wang, and Ms. Inka Schumacher for their technical assistance, practical advice, and generous help during my experimental work.

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